

Constitutive Activation of the α_{1B} -Adrenergic Receptor by All Amino Acid Substitutions at a Single Site

EVIDENCE FOR A REGION WHICH CONSTRAINS RECEPTOR ACTIVATION*

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Mutations in an intracellular region of the α_{1B} -adrenergic receptor constitutively activate the receptor, resulting in G protein coupling in the absence of agonist, as evidenced by elevated levels of polyphosphoinositide hydrolysis. Remarkably, all 19 possible amino acid substitutions at a single site in this region (alanine 293) confer constitutive activity. This set of mutated receptors exhibits a graded range of elevated biological activities, apparently representing a spectrum of receptor conformations which mimic the "active" state of the wild type receptor. In addition to their constitutive activities, these mutated receptors all demonstrate a higher affinity for agonists, another primary characteristic of the "active" conformation of G protein-coupled receptors. The fact that all possible mutations at this particular site result in increased activity suggests that this region may function to constrain the G protein coupling of the receptor, a constraint which is normally relieved by agonist occupancy.

The receptors for many hormones and neurotransmitters are members of the superfamily of receptors coupled to guanyl nucleotide-binding regulatory proteins (G proteins)¹ (1). These receptors share a number of conserved structural motifs, including the presence of seven putative membrane-spanning domains joined by extracellular and intracellular loops (Fig. 1) (2). Molecular biological and biochemical approaches have indicated that the transmembrane domains cooperate in forming a ligand-binding pocket within the plane of the plasma membrane (2, 3). On the other hand, the

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¹ The abbreviations used are: G protein, guanyl nucleotide-binding regulatory protein; α_{1B} AR, α_{1B} -adrenergic receptor; PI, polyphosphoinositide; [¹²⁵I]HEAT, 2-[β -(4-hydroxy-3-[¹²⁵I]iodophenyl)ethylamino-methyl]-tetralone; **R**, inactive receptor conformation; **R***, active receptor conformation; **G**, G protein; **H**, hormone or agonist; Gpp(NH)p, guanyl-5'-yl imidodiphosphate.

coupling to specific G proteins appears to be mediated by the intracellular loops, especially those regions in close proximity to the inner surface of the plasma membrane (4-11). An essential paradigm is that G protein-coupled receptors exist in an equilibrium between "inactive" and "active" conformational states: **R** \leftrightarrow **R***, respectively (12-14). Activating ligands, generally termed agonists, stabilize the "active" conformation by virtue of a preferentially higher affinity for **R*** (14), thereby shifting the equilibrium to the right and resulting in productive receptor-G protein coupling.

Recently, we observed that, in the α_{1B} -adrenergic receptor (α_{1B} AR), a conservative substitution in the carboxyl end of the third intracellular loop (a region involved in receptor-G protein coupling) leads to constitutive activation of the receptor, i.e. activity even in the absence of agonist (9). Reasoning that this domain might play an important role in the equilibrium between the "active" and "inactive" forms of the receptor, we have systematically mutated a key residue in this region (alanine 293, Fig. 1) by substituting each of the 19 other amino acids and subsequently determined the biological activities of these mutated receptors when expressed in mammalian cell lines. Our expectation was that we might identify a small number of specific residues responsible for receptor activation. Rather, we report here the surprising result that all amino acid substitutions at this site result in some degree of constitutive activity, demonstrating that the specificity of the wild type sequence in this region is in uniquely constraining the activity of the receptor in the unliganded state.

EXPERIMENTAL PROCEDURES

Construction of Mutated Receptor cDNAs—Mutated receptor cDNAs were each constructed by a modified site-specific polymerase chain reaction-mediated mutagenesis technique (15) using Replisase (Du Pont). The final mutated DNA fragment obtained was digested with *Bam*HI and *Bss*HII (New England Biolabs) and cloned into the expression vector pBC12BI (16) containing the α_{1B} AR cDNA. Recombinant clones were isolated, and the DNA fragments between *Bam*HI and *Bss*HII were sequenced by dideoxy sequencing of double-stranded DNA with Sequenase (U. S. Biochemical Corp.).

Cell Culture and Transfections—COS-7 cells (gift of Brian Cullen, Howard Hughes Medical Institute, Duke University) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μ g/ml gentamicin (Gibco). Cells (5×10^6) were plated in 30-mm dishes and transfected by the DEAE-dextran method (16) using 2 μ g of DNA/dish. Cells were harvested 48 h after transfection.

Ligand Binding—Membranes were prepared from COS-7 cells expressing the mutated receptors by washing and homogenizing cells in 5 mM Tris-HCl (pH 7.4), 5 mM EDTA (Sigma). After centrifugation ($40,000 \times g$ for 10 min), the pellet was resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA. Ligand binding of 2-[β -(4-hydroxy-3-[¹²⁵I]iodophenyl)ethylaminomethyl]-tetralone ([¹²⁵I]HEAT) was measured at 25 °C for 1 h using 10^{-6} M prazosin (Pfizer) to determine nonspecific binding. For saturation binding analysis, cell membranes were incubated with [¹²⁵I]HEAT concentrations ranging from 10 to 500 pM. For competition binding, cell membranes were incubated with 50 pM [¹²⁵I]HEAT and epinephrine concentrations ranging from 10^{-9} to 10^{-3} M. Data were analyzed by nonlinear least square regression (17).

Inositol Phosphate Determination—COS-7 cells expressing the mutated receptors were labeled for 18-24 h with [³H]inositol (Du Pont-New England Nuclear) at 4 μ Ci/ml in Dulbecco's modified Eagle's medium supplemented with 3% fetal bovine serum. After labeling, cells were washed for 30 min with phosphate-buffered saline (no calcium) and preincubated for 30 min in phosphate-buffered saline

containing 20 mM LiCl. After 40 min of stimulation with (-)epinephrine (Sigma) concentrations ranging from 10^{-10} to 10^{-3} M, inositol phosphates were extracted (18) and separated on Dowex AG1-X8 (Bio-Rad) columns (19). Total inositol phosphates were eluted with 1 M ammonium formate, 0.1 M formic acid.

RESULTS AND DISCUSSION

All 19 possible amino acid substitutions at alanine 293 (Fig. 1) resulted in mutated receptors which possessed at least some degree of constitutive activity (Fig. 2). In the absence of agonist, inositol phosphate levels in cells expressing the wild type α_{1B} AR were similar to control levels in cells transfected with the expression vector alone. In contrast, cells expressing the mutated receptors all exhibited agonist-independent increases in PI hydrolysis, ranging from 21 to 211% above control levels (Table I). These increased basal levels of inositol phosphates were up to 51% of those observed in epinephrine-stimulated cells expressing the wild type α_{1B} AR.

However, the mutated receptors were impaired in their ability to mediate an agonist-induced increase in biological response. After exposure to epinephrine, the levels of inositol phosphates in cells expressing the mutated receptors were, in nearly every case, lower than in cells expressing the wild type receptor (Table I). This impairment is even more pronounced (and present in all 19 mutants) when the stimulated inositol phosphate levels are considered as relative increases above the elevated basal levels.

Interestingly, these mutated receptors all exhibited affinities for agonists substantially higher than those observed for the wild type receptor, without significant differences in antagonist binding properties. The mutants bound the radiolabeled α_1 -specific antagonist [125 I]HEAT with affinities similar to that of the wild type α_{1B} AR (Table I). The K_i values of both the antagonists prazosin and WB4101 were also similar

for the mutated and wild type receptors (data not shown). A great deal of variation, however, was seen in the affinities of the mutated receptors for the agonist epinephrine (Fig. 3, Table I). The K_i values of epinephrine for the mutated receptors were 3–172-fold higher than for the wild type α_{1B} AR. Similarly, the potency of epinephrine to activate receptor-mediated PI hydrolysis was increased for all of the mutants, as compared with the wild type (Fig. 4, Table I). Changes in EC_{50} and K_i values were of similar magnitude, and the two parameters correlated well for the individual mutants ($r = 0.99$). Similar increases in both affinity and potency were also observed for the agonists norepinephrine and phenylephrine (data not shown).

Because of the elevated agonist affinities and potencies observed in the mutated receptors, we entertained the idea that their apparent constitutive activity might, in fact, be simply due to their extraordinarily high affinity for endogenous levels of catecholamines present in the serum-containing culture media. However, several findings refuted this notion. First, the correlation between the parameters of increased agonist affinity and elevation in basal activity was poor ($r = 0.31$), suggesting a dissociation between these two phenomena in the mutants. Specifically, some mutants (e.g. cysteine) demonstrated a high constitutive activity but a small increase in affinity for agonist. Second, in cells deprived of serum for 6 days prior to inositol phosphate measurement, expression of the mutated receptors resulted in basal PI hydrolysis elevations similar to those seen in the presence of serum (data not shown).

The G protein coupling (reflected as constitutive activity) and increased affinity for agonists exhibited by all of the mutated receptors are the two primary characteristics of the "active" form of a G protein-coupled receptor (R^*). Although stabilization of R^* is generally thought to require both G protein (G) interaction and agonist (H) binding, thus forming a high affinity "ternary" complex HR^*G (14), this appeared not to be the case for the mutated receptors. In fact, the high affinity agonist binding was observed in the absence of Mg^{2+} (normally required for HR^*G formation) (12) and was relatively unaffected by GTP or Gpp(NH)p (data not shown). This suggests that the high agonist affinity of these receptor mutants does not require interaction with the G protein but is rather an intrinsic property of the receptors themselves. This characteristic, together with the phenomenon of agonist-independent G protein coupling, suggests that the mutated receptors mimic the "active" conformation of the wild type receptor (R^*) to various degrees, perhaps due to conformational differences similar to those induced by agonist when it binds to the wild type α_{1B} AR.

Notably, the elevated basal levels of inositol phosphates in cells expressing the mutated receptors were reverted by the antagonists prazosin (Table I) or phentolamine (data not shown). This suggests that these antagonists may shift the equilibrium away from the "active" conformation (R^*) by stabilizing the "inactive" form (R) of the receptor (20, 21).

The unexpected observation that all 19 possible amino acid substitutions at alanine 293 constitutively activate the α_{1B} AR demonstrates that any alteration at this site leads to conformational deviations toward R^* . The degree of constitutive activity conferred by the different mutations suggests no clear pattern when viewed in the framework of traditionally cited amino acid characteristics such as residue size, charge, or hydrophobicity. Thus, rather than delineating a specific mutated sequence resulting in receptor activation, this series of mutated receptors signifies the importance of the wild type sequence in maintaining the "inactive" conformation of the

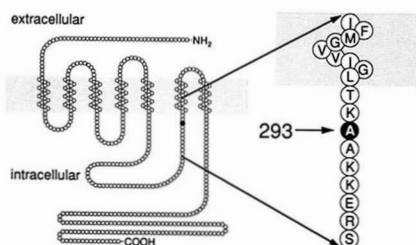


FIG. 1. Putative membrane topography of the α_{1B} AR showing the amino acid sequence and position of residue 293 in the carboxyl end of the third intracellular loop.

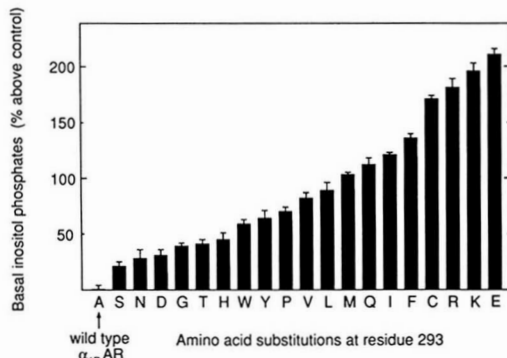


FIG. 2. Constitutive activation of the α_{1B} AR resulting from amino acid substitutions at position 293. Basal levels of inositol phosphates in COS-7 cells expressing the wild type and mutated α_{1B} ARs are represented relative to levels in cells transfected with expression vector alone (control). Results are expressed as means \pm S.E. of three independent experiments each performed in triplicate and are listed in Table I.

TABLE I

Ligand binding and PI hydrolysis parameters observed in COS-7 cells expressing the mutated α_{1B} ARs

Each mutant is represented by the single-letter abbreviation of the amino acid substitution at position 293 and listed in order of relative constitutive activity. Epinephrine (Epi) and [125 I]HEAT binding were determined by competition and saturation binding analyses, respectively, as described. Total inositol phosphates were measured as described under "Experimental Procedures" and are reported as a percentage increase above basal levels in cells transfected with expression vector alone: R_{bas} indicates basal levels, R_{praz} indicates levels in cells labeled in the presence of 5 μ M prazosin, R_{max} indicates levels after stimulation with 100 μ M epinephrine. Results are the means of three independent experiments \pm S.E., each performed in triplicate (except for K_d and R_{praz} values which each represent one or two independent experiments performed in triplicate).

Residue 293	Ligand binding			PI hydrolysis			
	Epi	[125 I]HEAT		Basal		Epi	
	K_i	K_d	B_{max}	R_{bas}	R_{praz}	R_{max}	Epi EC ₅₀
	nM	pM	pmol/mg	%	%	%	nM
A	4300 \pm 220	55	2.3 \pm 0.11	2 \pm 3.8	0.4	410 \pm 23	68 \pm 2.8
(α_{1B} AR)							
S	1400 \pm 65	53	2.0 \pm 0.14	21 \pm 4.1	-0.5	223 \pm 9.1	20 \pm 0.62
N	77 \pm 6.8	48	1.6 \pm 0.15	28 \pm 7.6	3.5	168 \pm 7.1	1.7 \pm 0.08
D	130 \pm 6.9	36	1.6 \pm 0.19	31 \pm 4.9	1.7	234 \pm 9.5	1.7 \pm 0.09
G	1000 \pm 49	45	1.7 \pm 0.23	39 \pm 3.2	1.9	242 \pm 9.7	13 \pm 0.69
T	330 \pm 12	44	1.8 \pm 0.15	41 \pm 3.8	-0.4	388 \pm 14	4.2 \pm 0.17
H	110 \pm 3.4	36	1.9 \pm 0.19	45 \pm 6.3	-3.7	213 \pm 9.3	2.2 \pm 0.10
W	83 \pm 3.8	52	1.6 \pm 0.15	59 \pm 3.9	1.2	198 \pm 5.5	2.0 \pm 0.09
Y	67 \pm 2.9	36	1.2 \pm 0.21	64 \pm 7.1	-2.0	215 \pm 5.8	1.0 \pm 0.04
P	43 \pm 2.1	38	1.4 \pm 0.15	70 \pm 3.6	-2.9	215 \pm 8.0	1.4 \pm 0.05
V	60 \pm 1.5	49	1.7 \pm 0.14	82 \pm 5.4	3.6	227 \pm 11	1.3 \pm 0.07
L	57 \pm 2.2	44	1.3 \pm 0.20	89 \pm 7.2	1.1	226 \pm 8.5	1.2 \pm 0.04
M	150 \pm 6.6	49	1.4 \pm 0.21	103 \pm 2.9	0.7	303 \pm 14	3.2 \pm 0.11
Q	56 \pm 3.0	47	1.6 \pm 0.17	112 \pm 6.0	3.7	332 \pm 13	0.80 \pm 0.03
I	61 \pm 2.0	42	1.2 \pm 0.17	121 \pm 2.1	4.6	257 \pm 8.2	1.5 \pm 0.05
F	70 \pm 2.8	41	1.2 \pm 0.23	136 \pm 4.6	3.8	387 \pm 19	0.83 \pm 0.05
C	900 \pm 29	41	1.1 \pm 0.20	171 \pm 3.9	-2.1	438 \pm 14	18 \pm 0.94
R	56 \pm 3.1	47	1.0 \pm 0.16	181 \pm 8.0	-1.5	357 \pm 13	1.2 \pm 0.04
K	25 \pm 1.5	43	0.9 \pm 0.13	196 \pm 7.3	-1.7	404 \pm 8.7	0.52 \pm 0.03
E	50 \pm 2.7	48	1.3 \pm 0.18	211 \pm 5.9	2.6	566 \pm 13	0.44 \pm 0.02

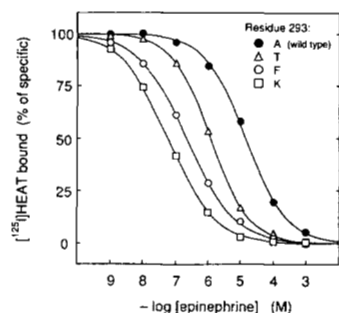


FIG. 3. Epinephrine binding affinity for membranes of COS-7 cells expressing the wild type and mutated α_{1B} ARs determined by competition binding analysis with the antagonist [125 I]HEAT as described. The K_i of epinephrine was 4300 nM for the wild type α_{1B} AR (alanine (A) at position 293), 320 nM for the threonine (T) mutant, 72 nM for the phenylalanine (F) mutant, and 24 nM for the lysine (K) mutant. The results are from a representative experiment performed in triplicate. K_i values for all mutants are provided in Table I.

receptor (R) in the absence of agonist. The carboxyl end of the third intracellular loop, therefore, may be a key determinant in mediating the functional equilibrium between R and R*, perhaps through an influence on conformational changes in the receptor which regulate ligand binding and G protein coupling.

In addition to inducing an overall conformational change, mutations in this region may also actually interfere with productive G protein coupling to some degree, as suggested by the somewhat impaired agonist-induced increase in PI hydrolysis mediated by the mutated α_{1B} ARs and by the lack of correlation between the degree of constitutive activity and

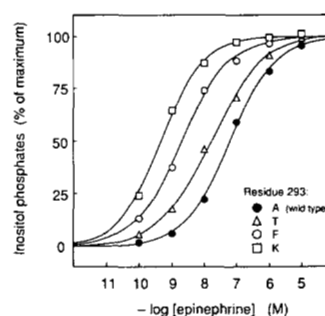


FIG. 4. Epinephrine potency to increase PI hydrolysis in COS-7 cells expressing the wild type and mutated α_{1B} ARs. Total inositol phosphates were measured as described. The EC₅₀ of epinephrine was 66 nM for the wild type α_{1B} AR (alanine (A) at position 293), 4.2 nM for the threonine (T) mutant, 0.85 nM for the phenylalanine (F) mutant, and 0.52 nM for the lysine (K) mutant. The results are from a representative experiment performed in triplicate. EC₅₀ values for all mutants are provided in Table I.

increased agonist affinity conferred by the different mutations. Some mutations (e.g. asparagine) may be particularly effective in altering receptor conformation (reflected as high agonist affinity) but might have a more direct adverse effect on G protein activation (resulting in relatively lower levels of constitutive and agonist-induced PI hydrolysis).

Recent studies have indicated that discrete amino acid sequences in the amino and carboxyl ends of the third intracellular loop in the α_{1B} AR (9) and in other G protein-coupled receptors (5, 7, 10, 11) are crucially involved in productive receptor-G protein coupling. Moreover, synthetic peptides of those regions are quite potent in activating isolated G proteins *in vitro* (22, 23). Why then does such activation not proceed

constitutively *in vivo*? One might speculate that receptor structures have evolved to constrain such activation, either by concealing the activating peptide or by forcing it into an inactive conformation. The constraint is then relieved on demand by the binding of specific agonists or, as shown here, by a variety of mutations if strategically placed. In our series of receptors, only the wild type α_{1B} AR was truly "silent" in the absence of agonist, a desirable characteristic for a signaling molecule which must switch between distinct "on" and "off" functional states.

Mutations in a constraining region such as we have described could conceivably serve to activate the proto-oncogene function recently documented for G protein-coupled receptors (24–26). Mutation of a single amino acid is one of the well known mechanisms which activate the transforming potential of several proto-oncogenes. In particular, the constitutive activation of the α_{1B} AR by any substitution at alanine 293 is reminiscent of p21^{ras}, whose transforming ability is activated by any amino acid substitution at glycine 12 other than proline (27). Because the diverse and numerous members of the G protein-coupled receptor family share various structural and functional motifs, mutations analogous to these reported for the α_{1B} AR might be predicted to have a wide range of pathogenic effects.

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