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Constitutive Activation of a Single Effector Pathway: Evidence for Multiple Activation States of a G Protein-Coupled Receptor

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SUMMARY

A cysteine-to-phenylalanine mutation in the third transmembrane domain of the α_{1B}-adrenergic receptor constitutively activates the receptor, resulting in G protein coupling in the absence of agonist and activation of only a single effector pathway (phospholipase C but not phospholipase A_2). This mutant receptor displays a higher affinity for the catecholamines, norepinephrine, and epinephrine, as well as for other phenethylamines, but not for imidazolines, a class of structurally distinct α agonists. Dose-response studies demonstrate a higher potency and intrinsic activity of phenethylamines for polyphosphoinositide turnover but not for arachidonic acid release. Imidazolines have wild-type potencies and intrinsic activities for both pathways. These data indicate that a single receptor subtype forms multiple conformations (i.e., exhibits induced conformational pleiotropy) for G protein interactions (high affinity states) that are specific for a particular G protein/effector pathway and that multiple binding sites exist for agonists, which promote or induce these specific interactions. Pharmacological diversity may, thus, be achieved through a single receptor by the development of compounds that induce a single activated conformer. This has major ramifications for the eventual development of signaling-specific therapeutics.

The processes by which agonist binding by membrane receptors activates cellular responses are central to understanding of biological regulation by hormonal and neurotransmitter stimuli. Classic theories of receptor activation (1) hold that agonist binding by receptors leads to a conformation that is productive for effector activation. Antagonists, on the other hand, are considered to be able to bond effectively with the receptor-protein without requiring an induced conformational change and, thus, to inhibit rather than stimulate receptor signaling.

The ternary complex model, which is the most widely accepted model describing activation of G protein-coupled receptor, relates the active form of the receptor to a ternary complex that involves the interaction of the hormone (agonist), the receptor and the G protein (2). However, based on recent analyses of G protein-coupled receptor ligand binding and activation, this ternary complex model of receptor activation has been revised (3). Central to this revised model is the finding that mutated receptors can exist in a constitutively active state, that is, a state that can initiate productive signaling in the absence of agonist. In support of this revised model is the finding that overexpression of wild-type receptors can also initiate biochemical responses in the absence of agonist. Accordingly, it has been proposed that similar to channels that spontaneously isomerize between a closed (or resting) and open (or active) conformation, receptors resonate between a basal state, R, and an active state, R*, and that only the R* state can productively interact with G protein to allow effector activation by the R*G complex. As a corollary, with wild-type receptors the R state predominates, whereas with constitutively active receptors the resident time in the R* state is increased. Furthermore, agonists bind with higher affinity to the active state and "trap" the receptor in the R* conformation. Antagonists, on the other hand, can bind with similar affinity to both R and R*. In this situation, G protein coupling and effector activation are unaltered, but further activation by the addition of an agonist is blocked. Such antagonists are referred to as neutral antagonists. In contradistinction, other compounds have been suggested to bind with higher affinity to R than to R* and to trap the basal R conformation. These compounds actively inhibit G protein coupling and effector activation by R* and are referred to as negative agonists.

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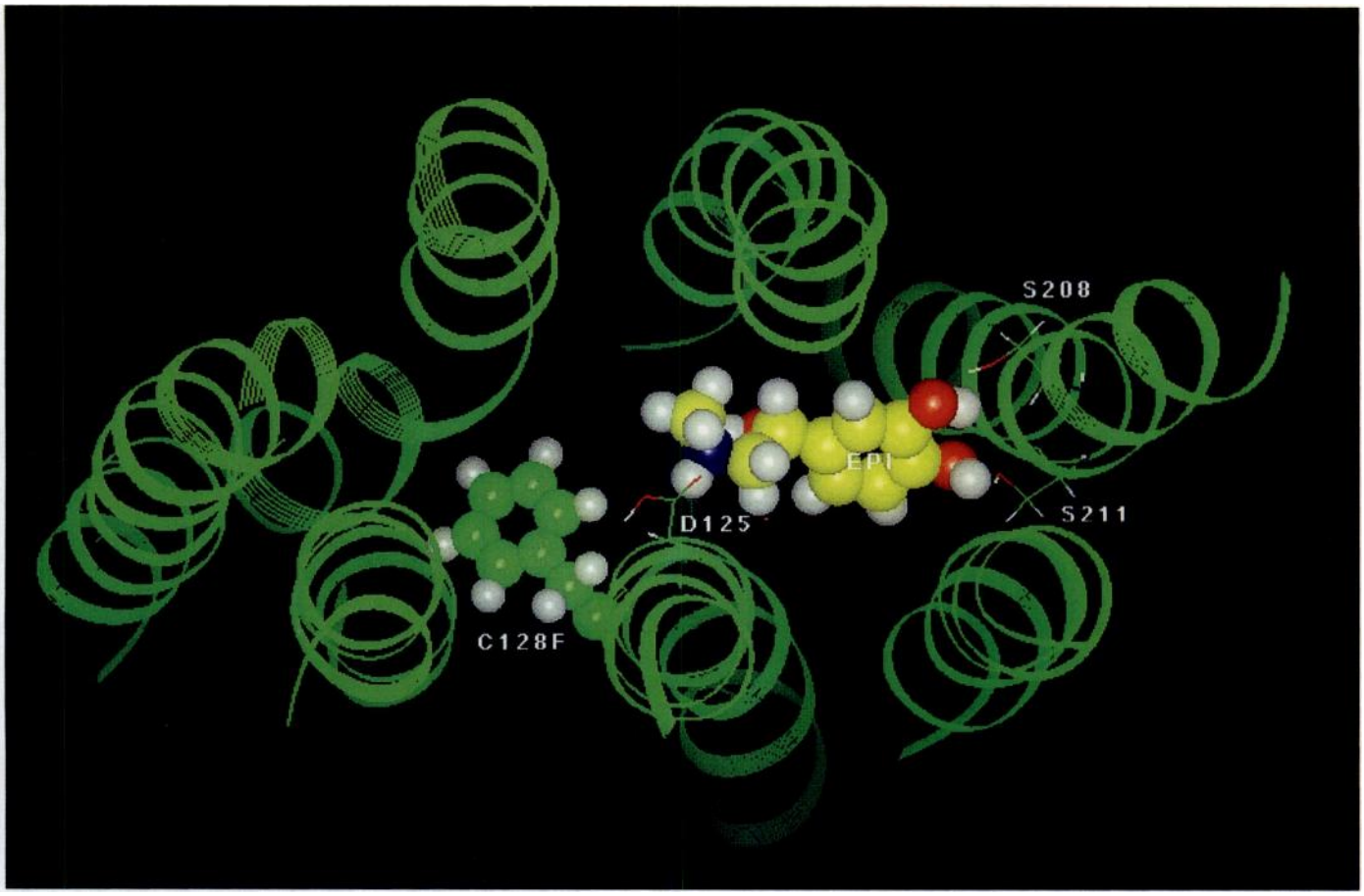
ABBREVIATIONS: AR, adrenergic receptor; [^{125}I]HEAT, 2-[β-(4-hydroxyl-3-[I^{125}]iodophenyl)ethylaminomethyl]tetralone; PI, polyphosphoinositide; PLC, phospholipase C; PLA_2, phospholipase A_2; bp, base-pairs; DMEM, Dulbecco's modified Eagle's medium; PTX, pertussis toxin; Gpp(NH)p, guanyly imidodiphosphate; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

The studies contributing to this revised model of receptor activation have been based largely on the evaluation of receptor signaling via only a single effector pathway. However, α_1ARs, like other members of the G protein-coupled receptor family, can activate multiple effectors via coupling to distinct G proteins. For α_1ARs, these effectors include PLC and PLA_2. In the present study, α_1AR signaling via both of these pathways was evaluated for a constitutively active α_{1B} receptor that results from mutation of a single residue, Cys^{128}, in the third transmembrane-spanning domain. This residue is situated approximately one helical turn below Asp^{125} in the third transmembrane segment (Fig 1). Because Asp^{125} is the putative counter ion that binds the protonated amine of adrenergic ligands, one can postulate that an agonist-induced conformational change of the third transmembrane segment is involved in receptor signaling. Perturbation, therefore, of this critical agonist interaction might induce changes in the conformation of this helix. Interestingly, this point mutation (Cys^{128}Phe) in the third transmembrane segment of the α_{1B}AR results in a conformation that partially mimics the activated state of the receptor but only for a single effector pathway. This contrasts with another constitutively activating α_{1B}AR mutation (Ala^{293}Glu) that promiscuously activates both effector pathways. Because the selective activation of a single effector pathway by the Cys^{128}Phe mutation is not the result merely of the unmasking of an enhanced receptor efficacy for signaling via this pathway, we suggest that the current model of receptor activation be extended to incorporate isomerization of R, not to only one active state but rather to at least two active states, R^*_1 and R^*_2, each of which interacts with a distinct G protein and effector pathway. Furthermore, this implies that it should be possible to



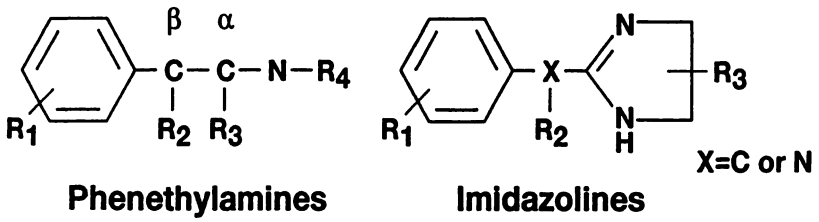


Fig. 1. A, Molecular model of the α_{1B}AR showing the putative amino acid position of the mutated C128F residue in the third transmembrane-spanning domain. D125 is the conserved counterion residue postulated to bind the protonated amine of agonists (epinephrine shown here) and is conserved in all ARs. III and V, third and fifth transmembrane-spanning domains, respectively. Transmembrane-spanning domains are shown as helical ribbons as seen looking down onto the plasma membrane. B, Chemical structures of phenethylamines and imidazolines with respective sites of substitutional R groups.

develop agonists that selectively activate only a single effector pathway by trapping either R^*_1 or R^*_2 or inverse agonists that selectively inhibit signaling by either the R^*_1 or R^*_2 conformations. Development of such signaling-specific agents could have major therapeutic potential.

Materials and Methods

Site-directed mutagenesis. The construct used was the hamster α_{1B}AR (4), which included a manufactured *Eco*RI restriction site at the 5′ end and a region encoding an octapeptide tag (ID4) at the end of the coding region that was used to evaluate membrane expression using a monoclonal antibody (anti-ID4). The attachment of this epitope after the coding region does not affect protein expression or the functional nature of the receptor. The construct ends with a stop codon and a *Not*I restriction site. The α_{1B}AR cDNA was divided into two 800-base pair fragments by restriction with *Bam*HI endonuclease, and each fragment was inserted individually into M13 mp19; this was done to reduce the potential incidence of spurious modifications in the DNA due to the M13 system as reported with large constructs (5). Site-directed mutagenesis was performed as previously described using the oligonucleotide-mediated double-primer method (5). This uses a 21-base mutagenic primer (Cys^{128}Phe: 5′-GACGTCCTGTTCTGTACGGCC-3′; Ala^{293}Glu: 5′-AAGAAAGCAGAGAAAACCTTG-3′) encoding the codon mismatches to achieve the point mutation and the universal primer for efficient extension on single-stranded M13 templates. After transformation of the extended products into DH5αF′ cells, plaques were screened for the mutation on nitrocellulose lifts and probed with the ^{32}P end-labeled mutagenic primer 5° below the calculated T_m. The efficiency of mutagenic incorporation was 5% of total plaques. Positive plaques were purified, and the DNA was isolated and sequenced by the dideoxy method (Sequenase, Amersham) to verify the mutation. The replicative form of the DNA was isolated from the M13, and the insert was removed and subcloned into the expression vector pMT2′. The full-length plasmid DNA was again sequenced to verify the mutation.

Cell culture and transfection. COS-1 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% fetal bovine serum. Cells (1×10^6) were plated into 60-mm dishes for transfection. cDNAs encoding the wild-type α_{1B}AR, and various mutants were subcloned into the mammalian expression vector pMT2′, as previously described (6). Plasmid DNA, purified by CsCl gradient centrifugation and Biogel A-50 m (Bio-Rad) column chromatography, was used to transfect cells. Transient expression in COS-1 cells was accomplished by the DEAE-dextran method (5). DNA levels (1–8 μg/1×10^6 cells) were varied to achieve similar receptor expression among the constructs for the dose-response studies. Transfection efficiency ranged from 10% to 20% of total cells used. Cells were harvested or assayed 60 hr after transfection.

Membrane preparation. COS-1 membranes were prepared as previously described (6). Membranes were prepared by washing the culture plates twice with warm Hanks' balanced salt solution. One milliliter of Hanks' balanced salt solution was added, and the plates were scraped and transferred to a 50-ml centrifuge tube. The intact cells were centrifuged at $1000 \times g$ in a Sorvall RT6000B rotor for 5 min, and the pellet was resuspended in 5 ml of 0.25 M sucrose. The cell suspension was centrifuged again at $1000 \times g$ for 5 min, and the pellet was resuspended in 10 ml of 0.25 M sucrose containing the following protease inhibitors: 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml bacitracin, 20 μg/ml benzamidine, and 17 μg/ml phenylmethylsulfonyl fluoride. The cells were disrupted by N_2 cavitation and then homogenized in a Dounce homogenizer by 10 strokes with a loose-fitting B-type pestle. The mixture was then centrifuged at $1260 \times g$ for 5 min. Buffer consisting of 50 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 5 mM EGTA was added to the supernatant fraction, which was then centrifuged at $30,000 \times g$ for 15 min. The resulting pellet was resuspended in 50 ml of buffer and recentrifuged for 15 min. The resulting pellet was resuspended in 1 ml of buffer containing 10% glycerol and stored in aliquots at −70°. The protein concentration was measured according to the method of Bradford (7).

Radioligand binding. The ligand-binding characteristics of the expressed receptors were determined in a series of radioligand binding studies using the α_1-antagonist radioligand [^{125}I]HEAT or [^{3}H]prazosin as previously described (6). Competition reactions (total volume, 0.25 ml) contained 20 mM HEPES, pH 7.5, 1.4 mM EGTA, 12.5 mM $MgCl_2$, 200 pM [^{125}I]HEAT, COS-1 membranes, and increasing amounts of unlabeled ligands known to interact with ARs. Nonspecific binding was determined in the presence of 10^{-4} M phentolamine. Reactions were stopped after 1 hr by the addition of cold HEPES buffer and were filtered onto Whatman GF/C glass-fiber filters with a Brandel cell harvester. Filters were washed five times with HEPES buffer, and bound radioactivity was determined with the use of a Packard Auto-gamma 500 counter. Binding data were analyzed with the iterative curve-fitting program LIGAND. Hill coefficients were determined by using the slope of the log-logit curve. For saturation binding studies, we used [^{125}I]HEAT concentrations ranging from 25 to 2000 pM and [^{3}H]prazosin concentrations ranging from 0.5 to 13 pM. Saturation curves were obtained by incubating cell membranes with increasing amounts of [^{125}I]HEAT or [^{3}H]prazosin in the same buffer system used for the competition studies. To reduce interassay variation, all binding assays were performed simultaneously with all three constructs. Statistical testing was performed using an analysis of variance and Student's t test to determine significant differences ($p < 0.05$) for both ligand binding and functional assays.

PI hydrolysis. Inositol phosphate determination were performed as described (8). Cells expressing the receptor constructs (grown in 60-mm dishes; 3×10^6 cells/dish) were labeled for 16–24 hr with [^{3}H]inositol at 1 μCi/ml in DMEM supplemented with 5% fetal bovine serum. After 24 hr, cells were washed three times with DMEM (no serum) and incubated in the serum-free media for 30 min, followed by a 30-min incubation in DMEM containing 10 mM LiCl. Agonists were then added for 30 min, the media were removed, and the cells were lysed with 1 ml of ice-cold 0.4 M perchloric acid. One-half volume of 0.72 N KOH/0.6 M $KHCO_3$ was added, and the sample was centrifuged to settle the precipitate. The supernatant was applied to 0.8-ml packed AG1-X8 (Bio-Rad) columns (100–200 mesh, formate form), and total inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid after the column was washed with 8 ml of 0.1 M formic acid. For basal measurements performed in separate studies, Ins(1,4,5)P_3 production was determined with the use of an [^{3}H]/Ins(1,4,5)P_3 radioreceptor assay kit according to the manufacturer's specifications. This kit contains known Ins(1,4,5)P_3 standards to generate curves for quantification. Scatchard analyses of equilibrium binding studies performed on parallel plates were used to determined receptor density per milligram of membrane protein. Bradford assays were used to determine protein concentrations. To reduce interassay variability, functional studies were always done for all three constructs in a single assay with a particular drug. To block receptor signaling, prazosin (1 μM) was added to the media 16–24 hr before the assay.

Arachidonic acid assay. For arachidonic acid release, transfected cells in 60-mm dishes (3×10^6 cells/dish) were incubated overnight with [^{3}H]arachidonic acid (1 μCi/ml media or 40 nmol total). The cells were washed three times with DMEM (no serum). After the final wash, the cells were incubated with DMEM for 30 min followed by the addition of the dual lipoxygenase and cyclooxygenase pathway inhibitor phenidone (100 μM final) for 30 min to allow free arachidonic acid to accumulate. Agonists were then added in the media and incubated for 30 min. The media were removed, and the cells were lysed with 0.4 M perchloric acid and extracted with a methanol/chloroform//HCl mixture (40:40:0.5). The top phase was removed and put into vials, 20 ml of scintillation cocktail (Ecoscint A; National Diagnostic) was added, and the samples were counted after

dark-adaptation. Here and in previous studies (8), we verified the identity of the radioactive extracts as released arachidonic acid by thin layer chromatography. The extracts were evaporated to dryness, resuspended in 50 μl of chloroform, and applied to silica gel thin layer chromatography plates (LK5D; Whatman). The plates were developed in a heptane/diethyl ether/acetic acid (75:25:4, v/v/v) solvent system. Nonradioactive standards (2 μg) were run in each lane as carriers. Carriers were visualized with iodine vapor, and radioactivity was quantified by scraping the plates and adding 10 ml scintillation cocktail to the resin. Samples were dark-adapted overnight before being counted. To reduce interassay variability, functional studies were always done for all three constructs in a single assay with a particular drug. PTX (0.5 μg/ml) was added directly to the media 24 hr before assay. In previous studies (7), we determined that 0.5 μg/ml PTX is sufficient to totally block [^{32}P]NAD incorporation into a 41-kDa protein in the COS-1 cell system.

Molecular modeling. The coordinates of the α-carbon positions were determined by an overlay of the putative α_1AR transmembrane residues with the transmembrane coordinates of bacteriorhodopsin (9) with data files generated using the Insight II molecular modeling software from Biosym Technologies. The boundaries of the putative transmembrane domains were determined by an algorithm based on the weighted pairwise comparisons of adjacent residues (10). The α_1AR model was then minimized, and conflicts were adjusted as previously described (11). Assumptions of key amino acids involved in ligand binding, such as the Asp^{125}, are based on previous mutagenesis work and proposed models for the βAR (12).

Materials. Drugs were obtained from the following manufacturers: WB4101 and 5-methylurapidil (Research Biochemicals); (−)-epinephrine, (−)-norepinephrine, prazosin, oxymetazoline, clonidine, methoxamine, phenylephrine, phentolamine (Sigma Chemical Co.); rauwolscine (Roth); and cirazoline and (−)-dobutamine (a gift from Pfizer). SKF 35886, 43315, and 89748 were the generous gift of Robert Ruffolo, Jr. and Paul Hieble of SmithKline Beecham. [^{125}I]HEAT, [^{3}H]inositol, [^{3}H]arachidonic acid, and the [^{3}H]/Ins(1,4,5)P_3 radioreceptor kit were from Dupont-NEN. AG1-X8 and Biogel A-50 m resin were from Bio-Rad.

Results

Saturation binding of [^{125}I]HEAT and [^{3}H]prazosin. COS-1 cells transfected with cDNAs encoding the wild-type hamster α_{1B}AR or the mutants Cys^{128}Phe and Ala^{293}Glu were analyzed for receptor density and for their affinity for the radioligands [^{125}I]HEAT and [^{3}H]prazosin. [^{125}I]HEAT labeled an apparently homogeneous populations of binding sites, with similar affinities, in membranes prepared from each of the three transfected constructs. Binding of [^{125}I]HEAT was statistically best fit to a one-site model. The mean K_D values for the binding of [^{125}I]HEAT by the wild-type, Cys^{128}Phe, and Ala^{293}Glu receptors were 96, 157, and 147 pM, respectively. [^{3}H]Prazosin also labeled an apparently homogeneous population of binding sites in membranes prepared from all three constructs, and the binding data statistically fit best to a one-site model. The mean K_D values for the binding of [^{3}H]prazosin were 2.8, 1.7, and 2.3 nM, respectively. The mean density of α_1AR binding sites from these transfected constructs using [^{125}I]HEAT was 1.9, 2.8, and 1.4 pmol/mg, respectively, using 8 μg of plasmid DNA/60-mm plate in the transfection protocol.

Agonist and antagonist inhibition of specific [^{125}I]HEAT binding. The potencies of a series of agonists and antagonists in inhibiting specific [^{125}I]HEAT binding in membrane preparations from each of the transfected constructs is shown in Table 1. Phenethylamine agonists displayed a 5–20-fold higher affinity at the Cys^{128}Phe and Ala^{293}Glu mutant receptors than at the wild-type construct. An exception, SKF 89748, a phenethylamine with a constrained amine, showed similar binding affinities for all three receptors. Imidazoline agonists had similar affinities for all three constructs. SKF 43315, an imidazoline with a catechol moiety, had a slightly higher affinity (2–2.5-fold) at the two mutant receptors than at the wild-type α_{1B}AR, but in functional studies it did not have a phenethylamine phenotype. The α_1AR antagonists prazosin, 5-methylurapidil, and WB4101 and the α_2AR antagonists rauwolscine and yohimbine also had similar affinities at all three constructs. The agonist and antagonist inhibition curves were best fit to a one-site model, reflected in Hill coefficients between 0.7 and 1.0, as determined from the slope of the log-logit curve (linear regression).

The possibility that differences in the apparent affinity of agonists might be enhanced by G protein coupling was tested by performing competition studies in the absence and presence of 0.1 mM Gpp(NH)p. The apparent K_i and Hill coefficient for epinephrine binding were not significantly altered with Gpp(NH)p, even with the mutants displaying a higher affinity for epinephrine (data not shown).

Stimulation of inositol phosphate formation. The ability of each construct to activate the phospholipase C pathway was determined by prelabeling the transfected COS-1 cells in 60-mm plates with *myo*-[^{3}H]inositol (1 μCi) for 16–24 hr and measuring agonist-stimulated [^{3}H]inositol

TABLE 1

Pharmacological characterization of wild-type and mutant α_{1B}-ARs

COS-1 cell membranes transfected with the pMT2′ expression vector containing either the wild-type hamster α_{1B} cDNA or the mutated Cys^{128}Phe or Ala^{293}Glu cDNAs were incubated with the α_1-AR antagonist [^{125}I]HEAT in the absence or presence of increasing concentrations of various agonists or antagonists. Each point represents the mean value of at least two to five individual experiments in duplicate. Ten concentrations of each ligand were treated, and the points were chosen to be on the linear portion of the displacement curve. K_i values were generated using the iterative curve-fitting program LIGAND. Values in parentheses are the ratio of wild-type to mutant K_i values. All phenethylamine affinities for Cys^{128}Phe and Ala^{293}Glu were significant ($p < 0.01$) from the wild-type receptor except for SKF 89748.

Ligand	K_i				
	Wild-type	Cys^{128}Phe		Ala^{293}Glu	
	pM				
Agonist					
Phenethylamine					
(−)-Epinephrine	2,223	133	(16.7)	91	(24)
(−)-Norepinephrine	3,700	373	(9.9)	194	(19)
(+)-Norepinephrine	247,000	16,000	(15)	n.d.	
Methoxamine	450,000	47,000	(9.5)	66,000	(6.8)
Phenylephrine	10,360	1,913	(5.4)	933	(11.1)
(−)-Dobutamine	1230	550	(2.2)	603	(2)
SKF (−)-89748	2,399	2,042	(1.2)	3,715	(0.64)
Imidazoline					
Oxymetazoline	596	560	(1.1)	1,270	(0.5)
Cirazoline	1,337	1,066	(1.3)	1,160	(1.2)
Clonidine	1,506	1,113	(1.4)	n.d.	
SKF 35886	1,995	1,122	(1.7)	1,288	(1.5)
SKF 43315	15,850	7,943	(2.0)	6,310	(2.5)
Antagonist					
Prazosin	0.35	0.28	(1.3)	0.43	(0.8)
5-Methylurapidil	99	60	(1.7)	50	(1.9)
WB4101	15	12	(1.3)	7.7	(1.9)
Rauwolscine	2,993	1,660	(1.8)	n.d.	
Yohimbine	1,230	1,323	(0.9)	n.d.	

phosphate formation. Receptor expression was approximately equal for the three constructs and was accomplished by titering the amount of DNA used in the transfection. Mean receptor density was 3.0, 2.2, and 2.2 pmol/mg membrane protein for the wild-type, Cys^{128}Phe, and Ala^{293}Glu receptors, respectively. Composite concentration-response curves for various full and partial agonists are shown in Figs. 2 and 3. The full agonist epinephrine showed a greater potency in activating responses in the Cys^{128}Phe and Ala^{293}Glu mutant receptors. Thus, the EC_{50} values for epinephrine were 8- and 13-fold lower that for the wild-type receptor (Table 2). Cirazoline, an imidazoline with high intrinsic ac-

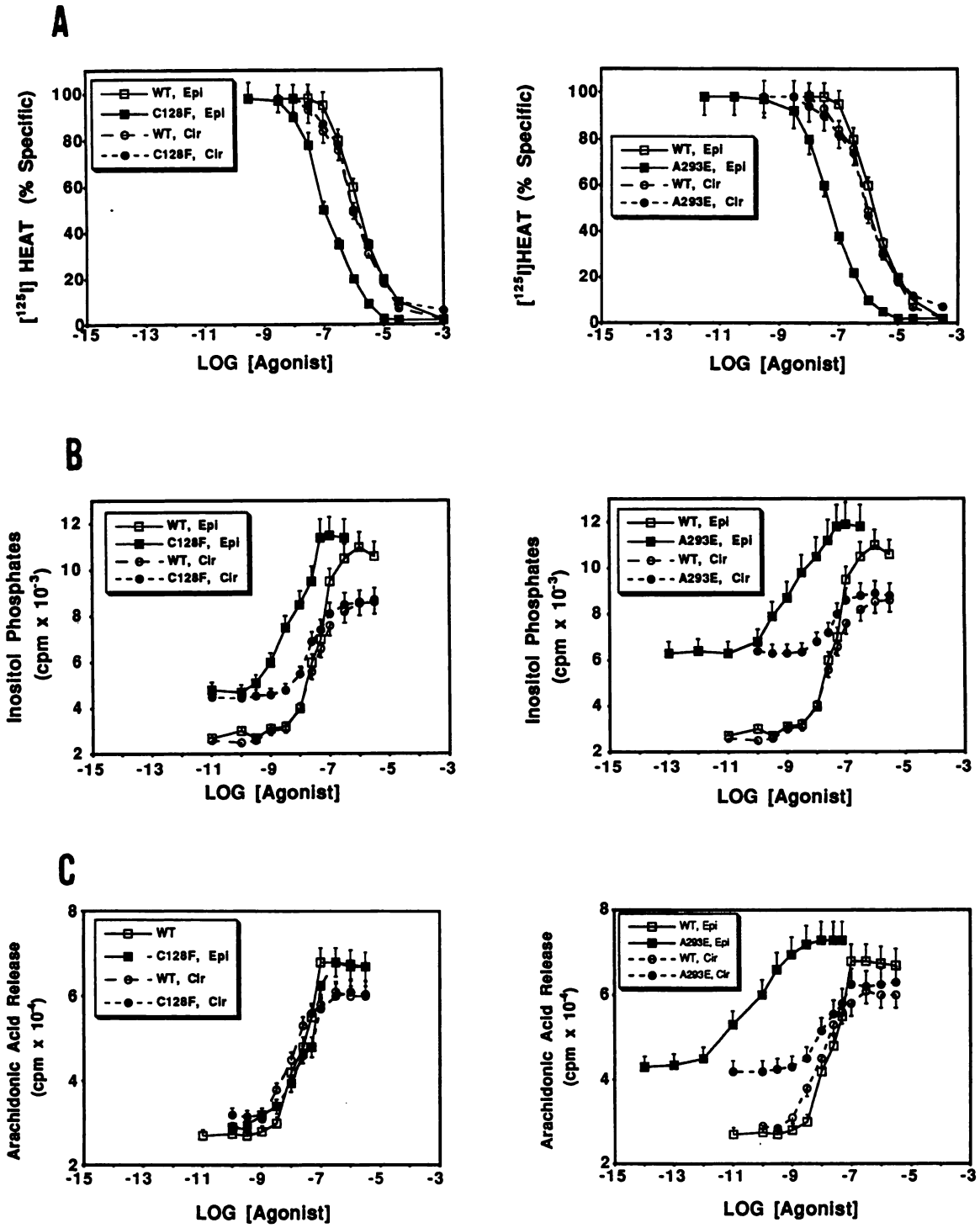
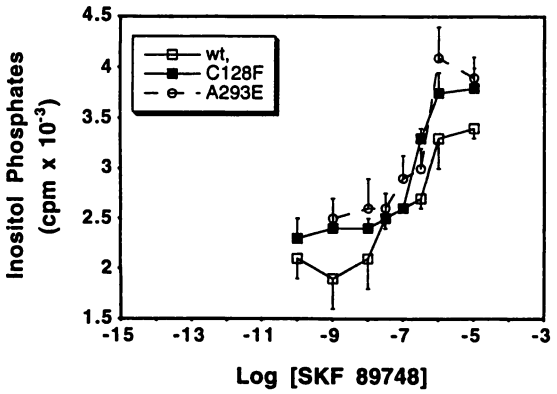
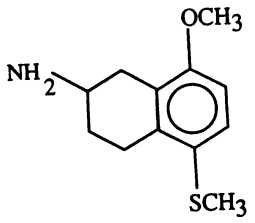


Fig. 2. Agonist binding and effector activation of wild-type and mutated α_1ARs. A, Epinephrine and cirazoline binding by membranes of COS-1 cells expressing the wild-type α_{1B}AR, the Cys^{128}Phe mutation, and the Ala^{293}Glu mutation. B, Epinephrine and cirazoline potencies for PI hydrolysis in COS-1 cells expressing wild-type α_{1B}AR, the Cys^{128}Phe mutation, and the Ala^{293}Glu mutation. C, Epinephrine and cirazoline potencies for arachidonic acid release in COS-1 cells expressing wild-type α_{1B}AR, the Cys^{128}Phe mutations, and the Ala^{293}Glu mutation. Each value shown is the mean ±standard error (*error bars*) for at least three individual experiments. EC_{50} values are given in Table 3. Mean receptor expressions in B and C were 3, 2.2, and 2.2 pmol/mg membrane protein for the wild-type, Cys^{128}Phe, and Ala^{293}Glu mutations, respectively.





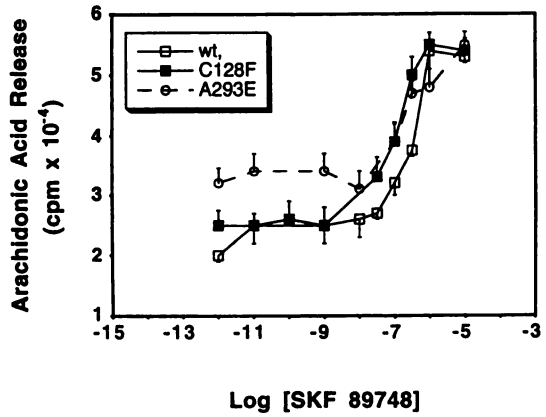


Fig. 3. The effect of SKF 89748 on effector activation of wild-type and mutated α_1ARs. *Top*, SKF 89748 potency for PI hydrolysis in COS-1 cells expressing the wild-type, $Cys^{128}Phe$, and $Ala^{293}Glu$ mutations. *Middle*, Chemical structure of SKF 89748. *Bottom*, SKF 89748 potency for arachidonic acid release in COS-1 cells expressing the wild-type, $Cys^{128}Phe$, and $Ala^{293}Glu$ mutations. Each value shown is the mean ± standard error of the mean, for at least three individual experiments. EC_{50} values are cited in Table 3. Mean receptor expression in *top* and *bottom* panels was 3, 2 and 1.8 pmol/mg membrane protein for the wild-type, $Cys^{128}Phe$ and $Ala^{293}Glu$ mutations, respectively.

tivity (Table 3), did not show a change in potency with any of the three constructs (Table 2). Various phenethylamine and imidazoline partial agonists were also tested for their ability to stimulate [^{3}H]inositol phosphate formation. The phenethylamines methoxamine and (−)-dobutamine are very poor partial agonists for the wild-type receptor, but the $Cys^{128}Phe$ and $Ala^{293}Glu$ mutants displayed greater intrinsic activity (Table 3) and greater potency with these two partial agonists (Table 2). EC_{50} values were 3- and 30-fold lower for methoxamine and 2- and 6-fold lower for (−)-dobutamine with the

TABLE 2

Potency of various agonists for activating PI turnover versus arachidonic acid release at wild-type and mutant receptors

Transfected COS-1 cells with the wild-type or mutated receptors were stimulated with increasing concentrations of different drugs and their IP or arachidonic acid release measured as described in Materials and Methods. The results are the mean ± standard error of three independent experiments based on the curves in Figs. 2–5.

Agonist	Potency (EC_{50})		
	Wild-type	$Cys^{128}Phe$	$Ala^{293}Glu$
		nM	
PLC pathway			
Phenethylamine			
(−)-Epinephrine	40 ± 8	5 ± 2[a]	3 ± 1[a]
Methoxamine	1500 ± 160	500 ± 75[a]	50 ± 8[a]
(−)-Dobutamine	1000 ± 140	300 ± 54[a]	100 ± 16[a]
SKF 89748	150 ± 25	150 ± 24	150 ± 23
Imidazoline			
Cirazoline	21 ± 5	12 ± 2	25 ± 5
SKF 35886	150 ± 35	150 ± 38	150 ± 17
SKF 43315	150 ± 30	500 ± 95[a]	500 ± 56[a]
PLA_2 pathway			
Phenethylamine			
(−)-Epinephrine	42 ± 7	37 ± 8	0.1 ± 0.1[a]
Methoxamine	1000 ± 130	700 ± 110	150 ± 40[a]
(−)-Dobutamine	300 ± 55	400 ± 85	100 ± 32[a]
SKF 89748	120 ± 25	80 ± 22	80 ± 24
Imidazoline			
Cirazoline	22 ± 5	35 ± 8	35 ± 9
SKF 35886	37 ± 30	32 ± 27	32 ± 26
SKF 43315	37 ± 31	16 ± 33	16 ± 15

[a] Significant difference from the wild-type receptor ($p < 0.01$).

$Cys^{128}Phe$ and $Ala^{293}Glu$ receptors, respectively, than for the wild-type receptor (Table 2). Imidazolines with less intrinsic activity than cirazoline, SKF 35886, and SKF 43315 also did not show any changes in potency for the three constructs (Table 2). SKF 89748, the phenethylamine with the constrained amine, showed no changes in potency (EC_{50} = 0.15 μM)(Fig. 3, Table 2)) with either mutant construct. Increased basal levels of inositol phosphate formation were observed for both the $Cys^{128}Phe$ and $Ala^{293}Glu$ mutant receptors even though both are expressed at slightly lower numbers than the wild-type receptor. At equal receptor densities, the $Ala^{293}Glu$ mutant always displayed greater basal activity and greater potency for activation of the PLC pathway than the $Cys^{128}Phe$ mutant.

Basal Ins(1,4,5)P_3 production and sensitivity to prazosin and PTX. As a more accurate and sensitive method of measuring basal PLC activity, the amount of Ins(1,4,5)P_3 production was measured with the use of a radioreceptor assay. This assay quantifies the Ins(1,4,5)P_3 released and not just a proportion of a labeled phospholipid pool. As shown in Fig. 4, this method of measurement showed increases in Ins(1,4,5)P_3 production of 130% for the $Cys^{128}Phe$ mutant and 300% for the $Ala^{293}Glu$ mutant over that of the wild-type receptor. The increased basal activity of the mutants did not change with the addition of 500 μg/ml PTX 24 hr before the assay. In addition, the increased basal activity of the mutants can be blocked with the α_1-selective antagonist prazosin at 1 μM concentration added 16–24 hr before the assay (Fig. 4).

Blockade can also be accomplished with the α_1-specific antagonist phentolamine at 100 μM under the same conditions (data not shown).

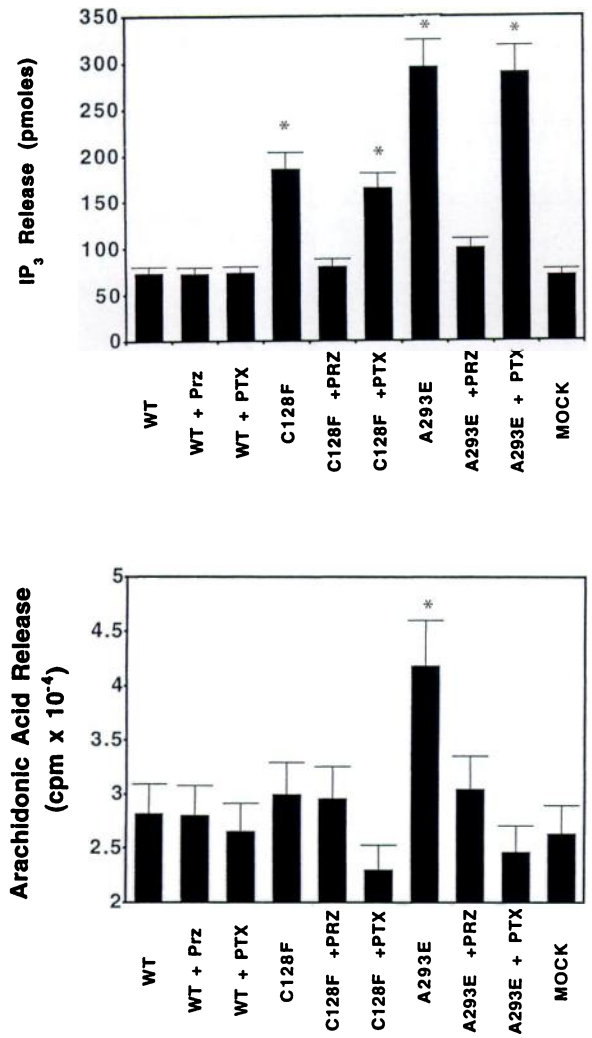


Fig. 4. Ins(1,4,5)P_3 production (*top*) or arachidonic acid release (*bottom*) in the absence of agonist by the $Cys^{128}Phe$ or $Ala^{293}Glu$ α_1AR mutations. Basal levels of Ins(1,4,5)P_3 and arachidonic acid release in COS-1 cells expressing the mutated α_{1B}ARs are represented relative to levels in cells transfected with the vector alone (*MOCK*). Expression levels of receptors were 1.9, 2.2, and 1.4 pmol/mg membrane protein for the wild-type, $Cys^{128}Phe$, and $Ala^{293}Glu$ mutations, respectively. PTX (0.5 μg/ml) was added directly to the medium 24 hr before assay. Prazosin concentration was at 1 μM final and was added 16–24 hr before assay. *, Statistical significance as evaluated with the Student's *t* test ($p < 0.001$). Values are the mean ± standard error (*error bars*) of three independent experiments determined in triplicate.

Stimulation of [^{3}H]arachidonic acid release. The ability of each construct to activate the PLA_2 pathway was determined by prelabeling the transfected COS-1 cells with [^{3}H]arachidonic acid (1 μCi/ml) for 16–24 hr and measuring agonist-stimulated [^{3}H]arachidonic acid release. Composite-concentration curves for both full and partial agonists are shown in Figs. 2 and 3. The phenethylamine full agonist (−)-epinephrine displayed greater potency (100-fold, Table 2) for the $Ala^{293}Glu$ mutant but for the $Cys^{128}Phe$ mutant compared with the wild-type receptor. The potency of the imidazoline cirazoline did not change with any of the three constructs (Table 2). The phenethylamine partial agonists methoxamine and (−)-dobutamine showed an increased potency only with the $Ala^{293}Glu$ mutant (10-fold, Table 2). Neither the imidazoline partial agonists SKF 35886 and SKF 43315 nor the phenethylamine SKF 89748 (Fig. 3) showed any changes in their potency with either of the mutant constructs (Table 2). The $Ala^{293}Glu$ receptor also had increased basal levels of arachidonic acid release compared with the wild-type or $Cys^{128}Phe$ mutant. As shown in Fig. 4, this increased basal activity was sensitive to pretreatment (24 hr) with 500 μg/ml PTX or 1 μM prazosin.

Effect of receptor number on $Ala^{293}Glu$ signaling. To test the hypothesis that the ability of the $Ala^{293}Glu$ receptor to couple to the PLA_2 pathway is not due merely to its greater constitutive activity compared with the $Cys^{128}Phe$ mutant, dose-response curves at various receptor number were performed by titering the amount of DNA (either 8, 2, or 1 μg/plate) added to the transfection cocktail. As seen in Fig. 5, the $Ala^{293}Glu$ receptor at densities of 1.4, 0.5, and 0.2 pmol/mg membrane protein still had increased basal levels of arachidonic acid release and increased potency for epinephrine compared with wild-type receptor densities at 2, 1.2, and 0.7 pmol/mg membrane protein.

Discussion

In the present study, we evaluated the ligand binding and signaling properties of a constitutively active α_{1B}AR. The constitutive activity of this mutant receptor was initially suggested by the finding that it binds the catecholamines epinephrine and norepinephrine and other phenethylamines with higher affinity than the wild-type receptor. Moreover, the ability of this mutant to bind a spectrum of chemically diverse antagonists was unaltered. As with other constitutively active mutants, this increase in agonist affinity was not due to enhanced G protein coupling because it was still observed in the presence of the nonhydrolyzable GTP analogue Gpp(NH)p, which promotes receptor/G protein dissociation.

Because it had been postulated that not only α_1ARs but also α_2ARs interact differentially with phenethylamines compared with the chemically distinct imidazoline agonists (13–15), we also evaluated the binding of imidazoline agonists by the $Cys^{128}Phe$ mutant. In addition, the $Cys^{128}Phe$ mutation was developed in an effort to delineate differences in the binding-pocket residues between α_1ARs and α_2ARs; the latter contains a phenylalanine at the position equivalent to Cys^{128} in the α_1AR. Interestingly, the affinity of the mutant for imidazolines was unaltered from that observed with the wild-type receptor. This difference between the binding of imidazoline and phenethylamine agonists cannot be attributed to the low intrinsic activity commonly observed for imidazolines because the binding of the phenethylamine partial agonists methoxamine and (−)-dobutamine was also of a higher affinity. Nevertheless, it is likely that the determinants of phenethylamine and imidazoline binding, although distinct, overlap. Previous studies of βARs and α_2ARs, for example, suggest conservation of key amino acids (e.g., Asp^{113}, Ser^{211}) for agonist binding (12), and both classes of agonists share common pharmacophores, such as a phenyl ring and a pronoated amine, located three bond lengths from the aromatic ring. Most likely, the overlapping determinants would be the phenyl ring, shared by both phenthylamines and imidazolines, and the distinguishing determinant would be the position of the protonated amine. Imidazolines have a constrained basic nitrogen as opposed to the freely rotating

TABLE 3

Intrinsic activities of full and partial agonists in wild-type and mutant α_{1B}-ARs

Transfected COS-1 cells with the wild-type or mutated receptors were stimulated with increasing concentrations of different drugs and their IP or arachidonic acid release measured as described in Materials and Methods. The intrinsic activity indicates the ratio between maximal IP or arachidonic acid accumulation elicited by each drug and that obtained with the full agonist (−)-epinephrine in the wild-type receptor. Values in parentheses are the percent increase or decrease in intrinsic activity from the wild-type receptor. The results are the mean ± standard error of three independent experiments based on the curves in Figs. 2–5.

Ligand	Wild-type	$Cys^{128}Phe$	$Ala^{293}Glu$
PLC pathway			
Phenethylamine			
(−)-Epinephrine	1.0 ± 0.25	1.1 ± 0.23 (10)	1.14 ± 0.26 (14)
Methoxamine	0.64 ± 0.1	0.82 ± 0.2[a] (28)	1.08 ± 0.2[a] (69)
Dobutamine	0.40 ± 0.07	0.71 ± 0.23[a] (77)	1.03 ± 0.2[a] (157)
SKF 89748	0.48 ± 0.07	0.53 ± 0.1 (10)	0.55 ± 0.1 (14)
Imidazoline			
Cirazoline	0.76 ± 0.2	0.76 ± 0.2 (0)	0.81 ± 0.2 (6)
SKF 35886	0.49 ± 0.1	0.53 ± 0.1 (8)	0.55 ± 0.1 (12)
SKF 43315	0.64 ± 0.15	0.71 ± 0.21 (10)	0.71 ± 0.2 (10)
PLA_2 pathway			
Phenethylamine			
(−)-Epinephrine	1.0 ± 0.2	1.0 ± 0.2 (0)	1.05 ± 0.2 (5)
Methoxamine	0.60 ± 0.15	0.65 ± 0.14 (8)	0.75 ± 0.15[a] (25)
Dobutamine	0.60 ± 0.15	0.61 ± 0.18 (0)	0.75 ± 0.2[a] (25)
SKF 89748	0.97 ± 0.2	0.97 ± 0.2 (0)	0.97 ± 0.2 (0)
Imidazoline			
Cirazoline	0.87 ± 0.15	0.87 ± 0.14 (0)	0.88 ± 0.15 (1)
SKF 35886	0.62 ± 0.18	0.62 ± 0.2 (0)	0.64 ± 0.2 (3)
SKF 43315	0.70 ± 0.2	0.63 ± 0.2 (−10)	0.63 ± 0.2 (−10)

[a] Significant difference from the wild-type receptor ($p < 0.01$).

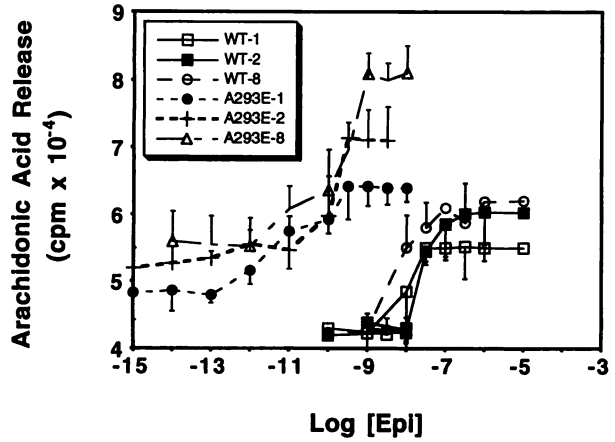


Fig. 5. Effect of receptor number on the coupling ability of $Ala^{293}Glu$ to arachidonic acid release. (−)-Epinephrine potency for arachidonic acid release measured at $Ala^{293}Glu$ receptor mean densities of 1.4 (Δ), 0.5 (+), and 0.2 (●) pmol/mg membrane protein. This corresponded to titering down the cDNA added to the transfection cocktail from 8, 2, and 1 μg/plate. Wild-type receptor mean densities are 2 (○), 1.2 (■), and 0.7 (□) pmol/mg membrane protein.

ethylamine side chain of phenethylamines. In support of this structural difference being a factor in both binding and receptor activation by agonists, SKF 89748, a phenethylamine with a constrained ethylamine side chain, essentially displayed the binding phenotype and functional properties of an imidazoline when tested with the mutant receptors (Tables 1 and 2, Fig. 3). In addition, SKF 43315, an imidazoline with a catechol moiety (Tables 1 and 2), still displayed an imidazoline phenotype for both receptor binding and activation.

We next considered whether this differential binding of ligands was unique to the $Cys^{128}Phe$ mutation or also occurred with other constitutively active receptors. We therefore evaluated an Ala^{293} α_{1B}AR mutation ($Ala^{293}Glu$) that had previously been shown to render the α_{1B}AR constitutively active. It had also been shown that this mutant binds phenethylamine agonists with higher affinity and that this effect was not due to enhanced formation of the R*G complex because of an effect of the binding of G protein on agonist affinity (16). However, it had not been investigated whether this mutant has differences in imidazoline versus phenethylamine binding. As shown in Table 1, the $Ala^{293}Glu$ mutant displayed a similar ligand binding phenotype to that observed with the $Cys^{128}Phe$ mutation, i.e., an increase in affinity for phenethylamine agonists but no changes in affinity for imidazoline agonists or for antagonists.

In keeping with the effects of both $Cys^{128}Phe$ and $Ala^{293}Glu$ mutants on phenethylamine binding, both mutants were constitutively active for inositol phosphate signaling. This is evident both by an increase in basal inositol phosphate generation and by an increase in the potency of phenethylamines for activating this pathway. Furthermore, the affinity for phenethylamine agonists was increased with the mutants to an extent related to their degree of intrinsic activity at the wild-type receptor (Table 3). Thus, the increased affinity of the mutants for phenethylamines is not due merely to changes in the ligand binding pocket but rather indicates that the mutations induce conformations that partially mimic the R* state.

These changes in agonist activation are predicted from the ternary complex model that has been revised both to account for the agonist-independent isomerization of receptors between R and R* states (3), with the R* state forming the high affinity ternary complex HR*G that is productive for effector activation, and to explain why the increase in agonist affinity observed here and with other constitutively active receptors is directly proportional to the intrinsic activity of these agonists at wild-type receptors. However, in keeping with the lack of effect of these mutants on the binding of imidazoline agonists, neither the potency nor the intrinsic activity of either the phenethylamine SKF 89748, which likely behaves as an imidazoline because of a constrained amine, or the imidazoline agonists was altered with the mutants. Because

the mutants are constitutively active, i.e., formation of the R* state and, thus, the R*G complex is enhanced, this lack of effect on imidazoline binding, potency, and intrinsic activity call into question the generality of the revised ternary complex model.

Although the mutants displayed similar ligand binding and inositol phosphate activation properties, they differed markedly in their effects on arachidonic acid release. For this effector pathway, only the $Ala^{293}Glu$ mutation displayed constitutive activity as demonstrated by increased basal activity in the absence of agonist as well as an increased potency and intrinsic activity for phenethylamine agonist but not for SKF 89748 or imidazolines. Moreover, the increased basal arachidonic acid release observed with the $Ala^{293}Glu$ mutant could be blocked with PTX, indicating this effect is due to selective precoupling to a PTX-sensitive G protein and not to promiscuous coupling to additional PTX-insensitive G proteins. This argument also holds for the increased basal Ins(1,4,5)P_3 production observed with both mutants that was completely insensitive to PTX.

Because α_1ARs expressed in COS-1 cells activate both PLA_2 and PLC by coupling to two distinct G proteins, one PTX sensitive and one insensitive (8), respectively, these findings indicate that the $Cys^{128}Phe$ mutation results in only a partially activated conformational coupling to the PTX-insensitive G protein and allows preferential activation of PLC by phenethylamine agonists but not imidazoline agonist. The $Ala^{293}Glu$ mutant, on the other hand, partially mimics an activated conformation that can promiscuously couple to both effector pathways. There are several explanations for these findings; one is that the α_{1B}AR merely couples more efficiently to the PTX-insensitive/PLC pathway than to the PTX-sensitive/PLA_2 pathway. Therefore, a mutation that only partially mimics the activated state, as observed with the $Cys^{128}Phe$ mutation, unmasks this perferential receptor/G protein/effector coupling and results in selective activation of only the PLC pathway. With the $Ala^{293}Glu$ mutant, which is more constitutively active, as judged by its greater effect on phenethylamine binding affinity and intrinsic activity, both effector pathways are constitutively active. However, for several reasons, this explanation appears to be unlikely. Rather, we suggest that the R state isomerizes not to a single active conformation but rather to at least two active conformations, R^*_1 and R^*_2. First, the EC_{50} values, which can be influenced by the efficiency of G protein/effector coupling and the presence of spare receptors, were almost identical for both the PLC and PLA_2 pathway with various full and partial agonists. Second, to eliminate possible spare receptors as a confounding variable, we further evaluated the ability of the $Ala^{293}Glu$ mutation to constitutively activate the PLA_2 pathway when expressed at lower levels (Fig. 5). These studies demonstrate that the ability of the $Ala^{293}Glu$ mutation to constitutively activate the PLA_2 pathway and its potency of activation are independent of receptor density and, thus, represent an intrinsic property of the mutant. Third, not only phenethylamine full agonists but also partial agonists showed selective activation of the PLC pathway as opposed to the various imidazoline agonists. Thus, selective phenethylamine activation is not related merely to the intrinsic activity of the agonists but likely involves structural determinants. In this regard, the use of the phenethylamine agonist SKF 89748, which has a constrained basic amine in a manner similar to imidazolines, essentially acts like an imidazoline. Previous X-ray diffraction studies on SKF 89748 indicate that the ethylamine side chain is not perpendicular to the aromatic ring, as is the case for phenethylamines, but is only slightly out of the plane of the ring (17). Our results with SKF 89748 are in agreement with the imidazoline phenotype observed for this compound by others (17). Thus, it appears that the structure and, specifically, the orientation of the basic amine in the phenethylamines are responsible for the selective phenotype. Confirming this hypothesis, another constitutively active α_{1B}AR mutant, $Ala^{204}Val$, which is located in transmembrane five and near the proposed region of hydrogen bonding of the catecholhydroxyls of agonists with the transmembrane five serine residues, displays a high affinity state for both phenethylamine and imidazoline agonists and activates both the PLA_2 and PLC pathways, although it is less constitutively active than the $Cys^{128}Phe$ mutant.[1] Thus, $Ala^{204}Val$, which directly effects a common pharmacophore between the two classes of agonists and at the opposite end of the ligand from the protonated amine, displays a common high affinity state recognized by both classes of agonists, providing evidence that the disparity between the two classes of agonists and the selective activation of effectors in $Cys^{128}Phe$ is not due merely to the intrinsic activity of these agonists or to the degree of activated receptor but to structural determinants. Finally, the concept that a receptor can isomerize to more than one active state conformation is supported by the findings of studies with other ARs (15, 18, 19), even though these studies were unable to provide evaluation of agonist-independent receptor isomerization.

Because the alanine residue involved in the $Ala^{293}Glu$ mutation is located in the highly conserved carboxyl end of the third intracellular loop and is contained in all ARs, this could be an explanation for its precoupling to both pathways. This region may not be able to adopt a fixed conformation; in fact, mutation of the Ala^{293} residue in this region with any other amino acid results in constitutive activity (16). This suggests that the native receptor is constrained and that any mutation at that site releases this constraint. In contrast, preliminary results from site-saturation studies of the Cys^{128} residue indicate that only selected uncharged residues are able to confer constitutive activity (data not shown). Recent data have also suggested that the third intracellular loop contains separate and distinct molecular determinants that divergently stimulate separate signaling pathways. For the βAR, activation of Na^+-H^+ exchange is discrete from that for adenylate cyclase activation and involves coupling via distinct regions of the third intracellular loop to G_{i3} and G_s, respectively (20, 21). The third intracellular loop has also been implicated in G protein promiscuity (22, 23), and the $Ala^{293}Glu$ mutation could perturb this region more readily because it is located directly in this loop. The second intracellular loop, on the other hand, has been implicated in determining signaling specificity (22, 23), and the $Cys^{128}Phe$ mutation could perturb this region via its location on the third transmembrane helix. Therefore, it is plausible that specific conformations of the receptor are needed for each

[1] J. Hwa, R. M. Graham, and D. M. Perez. Chimeras of α_1-adrenergic receptor subtypes identify critical residues that modulate active state isomerization. Submitted for publication.

distinct signaling pathway that use the second intracellular loop in conjunction with different regions of the third intracellular loop.

In separate experiments (Fig. 4), we also measured Ins(1,4,5)P_3 production using a highly sensitive radioreceptor assay in which most of the Ins(1,4,5)P_3 released is measured rather than just a proportion of a labeled phospholipid pool. This showed increases in Ins(1,4,5)P_3 production of ~130% and ~300% for the $Cys^{128}Phe$ and $Ala^{293}Glu$ mutant receptors, respectively, and this basal activity could be blocked by the extended exposure (16–24 hr) to the α_1-specific antagonist prazosin or phentolamine. Blockade of this constitutive response with prazosin and phentolamine indicates that these compounds are inverse agonists that can shift the equilibrium from R* by stabilizing the "inactive" (R) or ground state conformation of the receptor. This is in agreement with previous studies on the $Ala^{293}Glu$ mutation in which blockade of the constitutive inositol phosphate signal was achieved with 5 μM prazosin added with the [^{3}H]inositol 18–24 hr before assay (16). It can be predicted from computer simulations (24) that the binding affinity of an inverse agonist in this system is lower. This could be due to a slower on-rate because negative agonists bind to the inactive (ground) state (R) of the receptor, and with constitutively active receptors the resident time in the R state compared with the R* state can be predicted to be shorter than with wild-type receptor. Thus, it is not surprising that we found that prolonged incubation with antagonist is required to achieve blockade of constitutively active receptors. However, no significant changes in affinity for any antagonists for either mutant were noted by competition or Scatchard analysis. In this regard, we also perform saturation binding analysis of the mutants using [^{3}H]prazosin and found no evidence of a two-site binding curve or changes in equilibrium. Consistent with this observation are the one-site fit and similar K_D values generated for the mutants with saturation binding analysis using [^{125}I]HEAT (data not shown). This phenomenon can be explained in that these mutations are not intrinsically fully activated; therefore, the proportion of receptors in the R* state is not sufficiently large to allow quantification of a change in antagonist affinity. In support of this notion, we combined the $Cys^{128}Phe$ and $Ala^{293}Glu$ mutations. In this double mutant, a cooperative effect was observed for constitutive activity and agonist binding affinity (data not shown). Moreover, with this double mutant the affinity for some antagonists was 8-fold lower than that of the wild-type receptor.

This is the first reported documentation that a G protein-coupled receptor can actually be induced into a conformation that constitutively signals through only a particular pathway. Thus, the $Cys^{128}Phe$ mutation has induced a conformational change in the receptor structure that partially mimics the activated state for the PLC pathway but not for the PLA_2 pathway. This conformational change is associated with an increase in the affinity of the receptor for phenethylamine agonists. In contrast, the affinity of this receptor mutant for imidazolines is unaltered. This indicates that at least some of the residues binding imidazolines are distinct from those binding phenethylamines. In keeping with this latter conclusion, the binding affinity of imidazolines is also unaltered in the $Ala^{293}Glu$ mutation but altered in the $Ala^{204}Val$ mutation, even though both mutants are constitutively activated for both PLC and PLA_2 pathways but located spacially in different parts of the receptor.

A possible mechanism of constitutive activation for the $Cys^{128}Phe$ mutation could be due to the bulky Phe residue pushing the third transmembrane domain away from the second, providing translational movement of the helix and allowing a closer and tighter contact of the Asp^{125} counterion to the protonated amine on the phenethylamine ligand (Fig. 1A). This transmembrane movement could partially mimic the conformational change observed with full agonist-induced activation and could potentially represent the PLC-specific conformation. Imidazolines, on the other hand, might be binding farther back in the binding pocket and their protonated amine might be located farther from the counterion (25), thus accounting for why many imidazolines are partial agonists and for the differential binding affinities for phenethylamines and imidazolines seen in these mutations. This is also supported by the SKF 89748 compound, whose protonated amine structure is different from the phenethylamines.

Based on these findings with the $Cys^{128}Phe$ mutation, it can be predicted that it should also be possible to develop a mutant that induces the receptor into a conformation that mimics the activated state for PLA_2 and its coupled PTX-sensitive G protein rather than for PLC and the PTX-insensitive G protein.[2] Together with the $Cys^{128}Phe$ mutation, such mutants should be uniquely useful for ultimately designing signaling-specific drugs (agonists or negative agonists) that can induce the receptor to adopt a single activated conformation, thus achieving pharmacological diversity through a single receptor subtype. Such compounds not only would allow the contribution of each distinct receptor-activated pathway to be evaluated but also may form an important new class of therapeutic agents.

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[2] This prediction assumes an independent model of receptor activation in which R can isomerize to either R^*_1 or R^*_2. Although unlikely, the possibility that isomerization to R^*_2 (PLA_2 pathway) is dependent on initial isomerization to R^*_1 (PLC pathway) cannot be excluded. In this case, it would not be possible to develop a mutant that mimics the activated state for PLA_2 signaling.

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