Synergism of Constitutive Activity in α_1-Adrenergic Receptor Activation[†]

John Hwa,[‡] Robert Gaivin, James E. Porter, and Dianne M. Perez*

Department of Molecular Cardiology, Cleveland Clinic Research Institute, Cleveland, Ohio 44195

Received August 26, 1996; Revised Manuscript Received November 7, 1996[⊗]

ABSTRACT: Recently a number of mutations have been found *in vitro* which maintain α_1-adrenergic receptors (ARs) in a partially activated form. We have previously identified two amino acid residue positions in the α_{1b}-adrenergic receptor (AR), Cys^{128} and Ala^{204}, one in each of the third and fifth transmembrane segments, that constitutively activate the receptor when substituted for a phenylalanine or valine, respectively [Perez et al. (1996) *Mol. Pharmacol. 49*, 112−122; Hwa et al. (1996) *J. Biol. Chem. 271*, 7956−7964]. Another mutation analyzed previously, Ala^{293}Glu, located in the third intracellular loop, also constitutively activates the receptor [Kjelsborg et al. (1992) *J. Biol. Chem. 267*, 1430−1433]. All three mutations displayed similar manifestations of constitutive activity such as higher binding affinity and potency for agonists as well as higher basal signal transduction as predicted by the revised ternary complex model of receptor activation. We hypothesized that the individual mutations because of their critical location alter the conformation of the transmembrane helices such that mimicry occurs that partially conforms to the activated state, R*. To explore whether these potential conformations are independent, we combined these three mutations in all possible permutations. The combined triple mutation displays 700-fold higher binding affinity for (−)-epinephrine and 20-fold higher basal IP$_3$ release than the wild-type receptor. We also observed that each mutation contributed independently and synergistically to both receptor agonist binding and activation with the combined mutations' basal activity exceeding that of the fully-stimulated wild-type receptor. There was also a direct correlation between epinephrine's binding affinity and the degree of constitutive activity. Because the mutations affect different transmembrane domains, these results are consistent with a mechanism that helical movement acts in a concerted fashion in agonist-induced activation, a synergism predicted if multiple helix movement is involved in receptor activation.

The α_1-adrenergic receptor (α_1-AR) is a G-protein-coupled receptor that mediates many responses of the sympathetic nervous system (Hwa et al., 1996a). α_1-ARs couple via distinct G-proteins to multiple signal transduction pathways, including phospholipase C (PLC) (Perez et al., 1993). Recently a number of mutations have been found *in vitro* which maintain these α_1-ARs in a partially activated form (Perez et al., 1996; Hwa et al., 1996; Kjelsberg et al., 1992). Furthermore, such mutations discovered *in vivo* in other G-protein-coupled receptors have been found to lead to serious disease states (Parma et al., 1993; Shenker et al., 1993). These constitutively active receptors have led to a revision of the widely accepted ternary complex model that relates the active form of a G-protein-coupled receptor to a ternary complex of the hormone, receptor, and the G-protein (Samma et al., 1993). Central to this revised model is that receptors isomerize between a basal state, R, and an active state, R*, and that only the R* state can productively interact with a G-protein to allow effector activation. Based on this model, the wild-type receptors exist predominantly in the R state since the energy barriers to resonate to R* are high. Since agonists bind with higher affinity to the R* state, the agonist either "traps" the receptor in the active conformation or lowers the energy requirements for isomerization. The isomerization of the receptor would require changes in its structure, a mechanism of still unknown nature.

A related G-protein-coupled receptor, rhodopsin, has been used extensively as a model system for the hormone receptors. Because of its purification, biophysical measurements have been employed to study the structure−function relationships in photoactivation. These experiments convincingly show multiple helix movement in a rigid-body motion upon light activation (Farahbakhsh et al., 1995; Altenbach et al., 1996). This is an attractive model for the propagation of structural changes from the site of ligand binding to the intrahelical loops, a mechanism with precedent in the conformational changes of water-soluble proteins (Chothia & Lesk, 1985; McPhalen et al., 1992). Multiple helix movement would predict a concerted motion for activation which may manifest as synergism if helix movement can be isolated, a condition perhaps mimicked by mutagenesis.

We recently reported two mutations, Cys^{128}Phe (Perez et al., 1996) and Ala^{204}Val (Hwa et al., 1996b), located in the third and fifth transmembrane domains (tm) of the α_{1b}-AR, respectively, that lead to its constitutive activation, *i.e.*, activity in the absence of agonists. These mutant α_{1b}-ARs exhibited increases in affinity for agonists but not for antagonists. In accordance with the revised ternary complex model, these mutated receptors also demonstrated increased

---

[†] This work was supported in part by NIH Grant RO1HL52544 (to D.M.P.), an unrestricted research grant from Glaxo Wellcome (to D.M.P.), and a fellowship from the Northeastern Ohio Affiliate of the American Heart Association (to J.P.). This work was done during the tenure of an Established Investigatorship (to D.M.P.) from the American Heart Association.

* To whom correspondence should be addressed at the Department of Molecular Cardiology, FF3, Research Institute, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Telephone: (216) 444-2058. FAX: (216) 444-9263.

[‡] Current address: Department of Biology and Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139.

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1997.

S0006-2960(96)02141-1 CCC: $14.00

intrinsic activity for partial agonists, and increased potency (decreased EC_{50}) in signal transduction. The $Cys^{128}Phe$ mutant is located one-helical turn below the important Asp^{125} residue that forms a counterion with the protonated amine of catecholamines (Porter et al., 1996; Strader et al., 1987). The $Ala^{204}Val$ mutant is located one-helical turn above the critical Ser^{207} residue that binds the *m*-hydroxyl of catecholamines (Hwa & Perez, 1996) and is required for receptor activation. These results suggested that each individual mutation because of its critical location in the transmembrane domains alters the conformation of the receptor such that mimicry occurs that partially conforms to the activated state, R*.

To explore whether these potential conformations are different and whether there are limitations in constitutive activity, we combined these activating mutations with the $Ala^{293}Glu$ mutant, previously characterized by Kjelsberg et al., in all possible permutations. We observed that each mutation contributed independently and synergistically in receptor activation and the combined mutations' basal activity exceeded that of the fully-stimulated wild-type receptor. These results are consistent with a mechanism of multiple helix movement acting in a concerted fashion in receptor activation.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The construct used was the hamster α_{1b}-AR (Cotecchia et al., 1988). Site-directed mutagenesis was performed as previously described utilizing the oligonucleotide-mediated double primer method (Sambrook et al., 1989). Positive plaques were purified and sequenced to verify the mutation. The mutagenic construct was subcloned into the expression vector, pMT2′, and resequenced to verify the mutation.

Cell Culture and Transfection. COS-1 cells (ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transient expression in COS-1 cells was accomplished by the DEAE-dextran method (Sambrook et al., 1989). Transfection efficiency ranged from 10 to 20% of total cells used. Cells were harvested or assayed 60 h post-transfection.

Radioligand Binding. COS-1 membranes were prepared as previously described (Perez et al., 1991). Competition reactions 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. Nonspecific binding was determined in the presence of 10^{-3} M oxymetazoline. Reactions were stopped after 1 h by the addition of cold HEPES buffer and were filtered with a Brandel cell harvester. Binding data were analyzed by the iterative curve-fitting program LIGAND. Hill coefficients were determined by the slope of the log-logit curve. For saturation binding studies, [^{125}I]HEAT concentrations ranging from 25 to 2000 pM were used.

Energetics. $\Delta\Delta G^{\ddagger}_{\text{binding}}$ was calculated using the equation $\Delta\Delta G^{\ddagger}_{\text{binding}} = -RT \ln [K_i(\text{wt})/K_i(\text{mutant})]$, where R is the gas constant and T is the absolute temperature (Wells, 1990). For single mutants, the one-site K_i was used. For multiple mutants, the high-affinity site K_H was used. $\Delta\Delta G^{\ddagger}_{\text{binding}}$ represents the difference in free energy of binding between a wild-type receptor versus its mutant. ΔG_I, a term used to identify the free energy change due to interactions between components, was calculated by subtracting $\Delta\Delta G^{\ddagger}_{\text{binding}}$(observed) from the summation of the $\Delta\Delta G^{\ddagger}_{\text{binding}}$ of the components (Carter, 1984).

IP_3 Production. For basal measurements, IP_3 production was determined on 60 mm dishes (3×10^6 transfected cells/dish) using a [^{3}H]IP_3 radioreceptor assay kit (Dupont) according to the manufacturer's specifications. IP_3 release was normalized to the receptor density by dividing the picomoles of IP_3 produced by the $B_{\max}$ (picomoles per milligram of membrane protein) as determined by Scatchard analysis of membranes prepared from parallel plates.

Statistics. Statistical testing was performed using an ANOVA and Student−Newman−Keuls multiple comparison test to determine significant differences ($p < 0.05$) for both ligand binding and functional assays. Correlations were generated using iterative regression analysis (Multulsky & Ransnas, 1987) (GraphPad Prism).

Materials. Drugs were obtained from the following manufacturers: (−)-epinephrine, oxymetazoline, methoxamine, phenylephrine, and prazosin, Sigma; WB4101, Research Biochemicals; [^{125}I]HEAT and the [^{3}H]IP_3 radioreceptor kit, Dupont-New England Nuclear (Boston, MA).

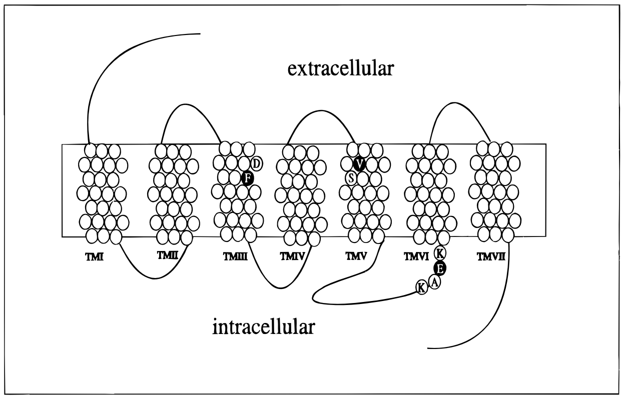

FIGURE 1: Schematic diagram of the α_{1b}-AR highlighting the three amino acid residues that render constitutive activation and their location. TMI, -II, -III, -IV, -V, -VI, and -VII represent the seven transmembrane domains. The mutations (in black) were as follows: TMIII, $Cys^{128}Phe$ (F); TMV, $Ala^{204}Val$ (V); third intracellular loop, $Ala^{293}Glu$ (E). Important amino acids involved in receptor activation are indicated in white: TMIII, Asp^{125} (D) that forms a counterion with the protonated amine of catecholamines; TMV Ser^{207} (S) that hydrogen bonds with the *m*-hydroxyl of the catechol; third intracellular loop, Lys^{291} (K), Ala^{292} (A), Lys^{294} (K), a region known to interact with G-proteins.

RESULTS AND DISCUSSION

Figure 1 shows a representation of the putative seven transmembrane-spanning topography of the α_{1b}-AR and indicates the three amino acids which were replaced in the mutated receptors and previously shown to constitutively activate. The $Cys^{128}Phe$ mutant is located one-helical turn below the important Asp^{125} residue that forms a counterion with the protonated amine of catecholamines (Porter et al., 1996). The $Ala^{204}Val$ mutant is located one-helical turn above the critical Ser^{207} residue that binds the *m*-hydroxyl of catecholamines and is required for receptor activation (Hwa & Perez, 1996). The previously reported $Ala^{293}Glu$ α_{1b}-AR mutant (Kjelsberg et al., 1992) was also analyzed since this mutation is located in the third intracellular loop in the proposed amphipathic helix shown to interact with G-protein. To explore whether these potential conformations are independent, we combined these mutations in all possible permutations.

When transiently expressed in COS-1 cells either individually or in the various combinations, receptor expression levels ranged from 3 to 0.10 pmol/mg of membrane protein (Table 1). The Ala^{293}Glu mutation, whether individually or in combination, consistently had lower expression levels that could not be rescued by the other higher expressing mutations. Competition binding experiments (Table 1, Figure 2A) indicated that as the mutations were combined, there was an increased complexity of the inhibition curves. The wild-type and individual point-mutated receptors displayed binding isotherms with Hill coefficients near unity. When the mutations were combined, however, the Hill coefficients gradually decreased to 0.2−0.6. There was a linear correlation ($r^2 = 0.9$) of the lower Hill coefficients with the higher binding affinity (pIC$_{50}$) of (−)-epinephrine (Figure 2B). The possibility that differences in the apparent affinity of agonists and the lower Hill coefficients might be due to enhanced 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 combined mutants (data not shown). The lower Hill coefficients were also noted in the antagonist binding isotherms. Thus, it appears that the shallow binding is an intrinsic property of the receptor. As seen in Figure 2A, the binding curves for epinephrine shift to the left but also become more complex when combining mutations. One interpretation is that the multiple mutations caused a reduction in the energy of isomerization. Thus, these receptors may freely resonate between high- and low-affinity states which reflect as multiple binding sites.

Indeed, the epinephrine competition curves for the double and triple mutants significantly fitted better to a two-site model (Table 2). The affinity of the full agonist, (−)-epinephrine, already significantly higher in the individual mutations, had a synergistic effect in the various combinations (Figure 2C). Similar trends were also seen for various partial agonists (Table 1). The triple mutation's high-affinity site showed a 700-fold increased binding affinity for (−)-epinephrine as compared to the wild-type receptor with intermediate higher affinities in the double mutations (Table 2). The increased binding affinity for agonists is a documented manifestation of constitutive activity in hormone/neurotransmitter receptors (Perez et al., 1996; Hwa et al., 1996b; Kjelsberg et al., 1992; Samma et al., 1993). In contrast, the affinity for α_1-antagonists displayed an opposite trend in the various combinations with the triple mutation having greater than 100-fold decreased binding affinity (Table 1). However, the iodinated analog of HEAT used as the radioligand displayed no changes in affinity. The decreased antagonist binding is predicted by the revised ternary complex model. Antagonists are classified as neutral antagonists if they bind with similar affinity to both R and R*, as suggested by [^{125}I]Heat. In this situation, effector activation is unaltered, but further activation by the addition of agonist is blocked. In contradistinction, antagonists are referred to as inverse agonists if they bind with higher affinity to R than to R* and trap the basal R conformation. These compounds actively inhibit effector activation by R*. Therefore, based upon this model, one would predict that WB4101 and prazosin should be inverse agonists while HEAT would

Table 1: Binding Profiles of Wild-Type α_{1b}-ARs and Constitutively Active Mutants[a]

	α_{1b}-AR	C128F	A204V	A293E	C128F/A204V	C128F/A293E	A204V/A293E	C128F/A204V/A293E
agonists								
epinephrine	13800 ± 210(0.8)	1725 ± 62(0.8)*	3200 ± 48(0.8)*	546 ± 14(0.7)$^{\#}$	336 ± 5(0.5)$^{\#}$	243 ± 3(0.55)$^{\#}$	93 ± 2(0.37)$^{\#}$	75 ± 3(0.26)$^{\#}$
phenylephrine	88500 ± 2400	5990 ± 210*	8400 ± 75*	2800 ± 58*	5300 ± 100*	1680 ± 56$^{\#}$	1767 ± 22$^{\#}$	3770 ± 180*
methoxamine	645000 ± 12000	143500 ± 4000*	328900 ± 4900*	198200 ± 11000*	70300 ± 600*	44300 ± 800*	55800 ± 1400*	377600 ± 9600*
antagonists								
prazosin	1 ± 0.01(0.8)	0.97 ± 0.03(0.8)	0.80 ± 0.001(0.8)	0.97 ± 0.001(0.8)	3 ± 0.1(0.4)*	6 ± 0.03(0.5)*	5 ± 0.06(0.2)*	181 ± 10(0.2)$^{\#}$
WB4101	8 ± 0.1(0.8)	34 ± 0.6(0.8)	20 ± 0.3(0.8)	8 ± 0.05(0.8)	48 ± 0.2(0.5)*	38 ± 0.4(0.5)*	378 ± 11(0.4)*	1190 ± 50(0.3)$^{\#}$
[^{125}I]HEAT	0.3 ± 0.002	0.47 ± 0.01	0.24 ± 0.003	0.44 ± 0.01	0.95 ± 0.02	0.24 ± 0.003	0.3 ± 0.004	0.84 ± 0.02
B_{max} (pmol/mg of protein)	2.87	1.37	2.60	0.24	2.76	0.13	0.43	0.10

[a] Competition binding studies were used to determine IC$_{50}$ values (nM, ±SEM), as described under Materials and Methods, using [^{125}I]HEAT as the radioligand and membranes prepared from COS-1 cells expressing the various constructs. Receptor densities (B_{max}) were determined on the same membranes from equilibrium binding studies. An ANOVA followed by a Student−Newman−Keuls multiple comparison test was performed to assess significance from the wild-type receptor ($^{*}p < 0.05$, $^{\#}p < 0.001$). Values in parentheses represent Hill coefficients.

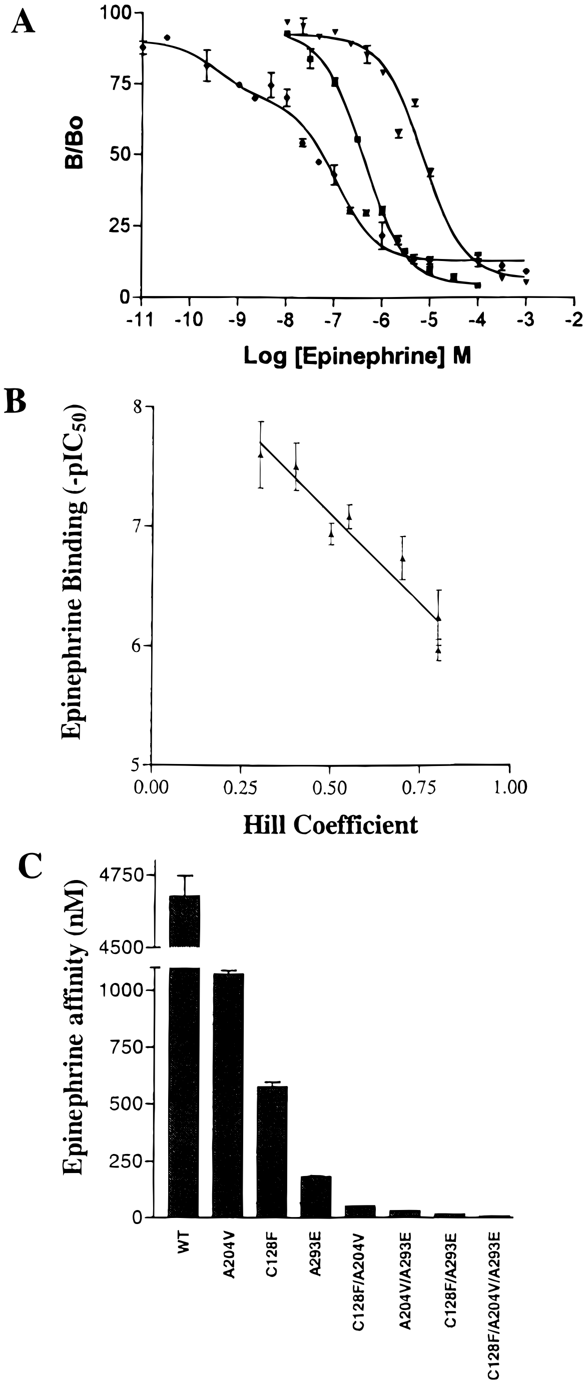

FIGURE 2: Synergism of high-affinity epinephrine binding. Panel A: Epinephrine inhibition binding curves for WT (▼) and single (A293E, ■) and double mutation (A204V/A293E, ●). All single mutants displayed similar curves to A293E while combined mutants displayed similar curves to A204V/A293E. Panel B: Correlation of epinephrine pIC_{50} in constitutively active α_{1b}-ARs with their Hill coefficients. The epinephrine pIC_{50} for each mutant receptor construct (taken from Table 1) was plotted versus its Hill coefficient. A correlation was best fit to a linear regression ($r^2 = 0.9$). Panel C: Synergistic relationship of epinephrine binding affinity in combining constitutively active mutations. Affinity values converted to nanomolar were taken from Table 2. For point mutations, the single-site value was used. For combinatorial mutants, the high-affinity site, K_H, was used.

be a neutral antagonist. We (Perez et al., 1996) and others (Kjelsberg et al., 1992) have previously shown that prazosin is indeed an inverse agonist at constitutively active α_1-ARs induced by these point mutations. However, we are currently assessing the issue of inverse agonism on these combinatorial constructs.

The energetics of virtually all binding functions in proteins are the culmination of the individual molecular interactions. A review of the data base for free energy changes shows that the sum of the free energy changes derived from a single mutation is nearly equal to the free energy change measured in a multiple mutant (Wells, 1990). Simple additivity reflects the modularity (independence) of the amino acids in function. Deviation from simple additivity can be measured through the calculation of the term ΔG_I, called the coupling energy, which reflects the extent of the energy change that is due to an interactive component (Carter et al., 1984). It is possible for ΔG_I to be positive or negative depending upon whether the interactions reduce or enhance (synergism) the functional property measured. Using the K_i or K_H measured in Table 2, the energetics of binding can be calculated and correlated to what was observed between the individual versus the multiple mutants. As shown in Table 3, the multiple mutants are not simple additives of the individual components but display negative ΔG_I values, suggesting that these mutations are not merely independent but are synergistic in behavior. This component of synergism ranged from 11 to 37% with the C128F/A204V combination displaying the greatest degree of synergism. This could be due to their direct influence on the binding of epinephrine since both mutations are located adjacent to critical ligand contact points and are located on opposing ends of the agonist binding pocket where the enhanced docking of one end of the epinephrine may enhance the docking of the opposite end.

To see if the higher agonist binding affinity was merely a binding pocket change or that the conformational changes could also be associated with signal transduction, the binding affinity was correlated to its degree of constitutive activation by analyzing the constructs for their basal ability to activate PLC. Since receptor densities were different between receptor constructs, the IP_3 release was normalized to the receptor number determined by Scatchard analysis from membranes prepared from parallel plates. As seen in Figure 3A, there was also a synergistic relationship in IP_3 release for three of the four receptor constructs. The $Cys^{128}Phe/Ala^{293}Glu$ double mutant and the triple mutant display at least 20-fold more basal activity than the wild-type receptor. Figure 3B represents the correlation of binding affinity for (−)-epinephrine for all of the receptor constructs to the amount of basal IP_3 release. The relationship was best fit to a power function ($r^2 = 0.96$) indicating a direct relationship between the higher agonist binding affinity and the degree of constitutive activity. The correlation of affinity change to functional activity suggests that these mutations are not "abnormalities" *per se* but are mimicking part of the natural mechanism of receptor signal transduction.

Since basal signal transduction is highly dependent on the receptor density and Figure 3A normalization assumes a linear relationship between receptor density and constitutive activity, we analyzed the $Cys^{128}Phe/Ala^{293}Glu$ and the triple mutant, which had the two most poorly expressed receptors, for basal IP_3 production at different receptor number by varying the amount of DNA used in the transfection protocol (Figure 4). From these results, a linear regression was indeed found to best represent the data, and a slope was calculated which reflects the IP_3 production per expressed receptor. These numbers approximated the normalized values in Figure

Table 2: Analysis of Epinephrine Binding Profiles[a]

	one-site	two-site		
construct	$-pK_i$	$-pK_H$	$-pK_L$	R_L
WTα_{1b}	5.33 ± 0.8			
A204V	5.97 ± 0.09			
C128F	6.24 ± 0.23			
A293E	6.74 ± 0.18			
C128F/A204V		7.3 ± 0.2	5.67 ± 0.25	69 ($p < 0.005$)
C128F/A293E		7.8 ± 0.3	6.10 ± 0.12	59 ($p < 0.04$)
A204V/A293E		7.53 ± 0.15	5.55 ± 0.22	29 ($p < 0.006$)
C128F/A204V/A293E		8.18 ± 0.3	4.0 ± 0.2	40 ($p < 0.001$)

[a] Composite inhibition curves were subjected to two-site analysis using the program LIGAND. The equilibrium binding constants for high- (K_H) and low-affinity (K_L) sites were determined if a two-site model was significantly better ($p < 0.05$) fit than a one-site model. Values in parentheses represent the significance. R_L is the percentage of low-affinity sites.

Table 3: Comparison of Sums of $\Delta\Delta G^{\ddagger}_{\text{binding}}$ from Component Mutants vs the Multiple Mutant

mutant	$\Delta\Delta G^{\ddagger}_{\text{binding}}$[a] (kJ·mol^{-1})	$\Sigma\Delta\Delta G^{\ddagger}_{\text{binding}}$(calculated)[b] (kJ·mol^{-1})	$\Delta\Delta G^{\ddagger}_{\text{binding}}$(observed)[a] (kJ·mol^{-1})	$\Delta G_I^{\ddagger}$(coupling energy)[c] (kJ·mol^{-1})
WT	0			
A204V	−3.62			
C128F	−5.16			
A293E	−8.01			
C128F/A204V		−8.78	−13.93	−5.15 (37)
C128F/A293E		−13.17	−16.74	−3.57 (21)
A204V/A293E		−11.63	−15.20	−3.57 (23)
C128F/A204V/A293E		−16.79	−18.82	−2.03 (11)

[a] $\Delta\Delta G^{\ddagger}_{\text{binding}} = -RT \ln [K_i(\text{wt})/K_i(\text{mutant})]$; in multiple mutants, the K_H value was used. [b] $\Sigma\Delta\Delta G^{\ddagger}_{\text{binding}}$ is calculated by the summation of the $\Delta\Delta G^{\ddagger}_{\text{binding}}$ of the individual mutants. [c] $\Delta G_I^{\ddagger}$ term (also called the coupling energy) reflects the extent to which the change in the energy of interaction between mutant 1 and mutant 2 affects the functional property measured by, i.e., $\Delta\Delta G^{\ddagger}_{\text{binding}}(\text{C128F/A204V}) = \Delta\Delta G^{\ddagger}_{\text{binding}}(\text{C128F}) + \Delta\Delta G^{\ddagger}_{\text{binding}}(\text{A204V}) + \Delta G_I$. Value in parentheses indicates percent of energy change due to an interactive (synergistic) component.

3A. The slopes of the wild-type, Ala^{204}Val, Cys^{128}Phe/Ala^{293}-Glu, and the triple mutation graphs were 36 ± 10, 118 ± 36, 825 ± 306, and 4910 ± 886, respectively, reflecting changes of 3, 23, and 136 times the wild-type receptor value. To test whether the receptor conformations of the double and triple constitutively active mutations could be further stimulated by (−)-epinephrine, IP$_3$ release was measured after a 30 min incubation with a saturating level of (−)-epinephrine (10 μM final). Because of the high basal activity, the mutant receptors could not accurately generate a dose−response curve. As shown in Figure 5, (−)-epinephrine produced significant but small increases in IP$_3$ release for both Cys^{128}Phe/Ala^{293}Glu (20% over basal, $p < 0.05$) and the triple mutant (17% over basal, $p < 0.05$). The other combined mutations did not shown any increases in IP$_3$ production with (−)-epinephrine stimulation. One interpretation is that the conformations adopted by these super active mutations already contain most of that induced in the wild-type receptor by the binding of (−)-epinephrine, with a small component due to an atypical conformation. When stimulated with (−)-epinephrine, the wild-type receptor displays 3−4-fold increased IP$_3$ production (Figure 5). Three of the combined mutations has basal activity exceeding that of the stimulated wild-type receptor, suggesting that these combined mutations indeed achieved a "super-activated" state.

Together, the agonist binding data and basal second messenger levels suggest that each mutation created a conformation that is different, independent, but cooperative for the binding of agonists and its activation of effector proteins. Within the experimental scope of these studies, it does not appear that the G-protein and/or effector becomes rate-limiting as evidenced by the linear relationship between receptor density and constitutive activity. Since the mutations cause intrinsic changes in the receptor proteins and are located in helices III and V or, in the case of Ala^{293}Glu, proximal via the third intracellular loop, the results are consistent with a mechanism that changes/movement in these helices occur(s) during receptor activation which correlate(s) to critical contact points known to be involved in receptor binding to the catecholamine. Recent evidence in the rhodopsin receptor in which site-directed spin-labels were employed to measure physical movements of the receptor domains during light activation revealed that both helix C and helix F indeed move in a rigid-body fashion (Farahbakhsh et al., 1995; Altenbach et al., 1996). Similar results also occur in bacteriorhodopsin (Brown et al., 1995). It was suggested in rhodopsin activation that the concerted motion of these helices would present new surfaces for interaction with transducin. In the α_1-AR system, the synergism presented in this paper would also support this mechanism since synergism could be derived from concerted helical movement. This notion is based upon the synergism observed in transcriptional activation of steroid receptors in which multiple domains of the receptor synergistically are required to achieve full activation (Kraus et al., 1995). In addition, the high transforming potential of the v-erbB oncogene is a consequence of synergism among multiple constitutively active mutations located in different domains of the protein (Shu et al., 1991).

The analogy of these constitutively active mutations in the α_1-AR to rhodopsin structure−function is apparent. Helix C in rhodopsin contains half of the salt-bridge (Glu^{113}) previously shown to constrain the receptor in the inactive state and which is broken during light activation (Robinson et al., 1992), a mechanism consistent with the movement of this helix. Helix F is thought to move to accommodate the strain introduced during the *cis*- and *trans*-conversion of

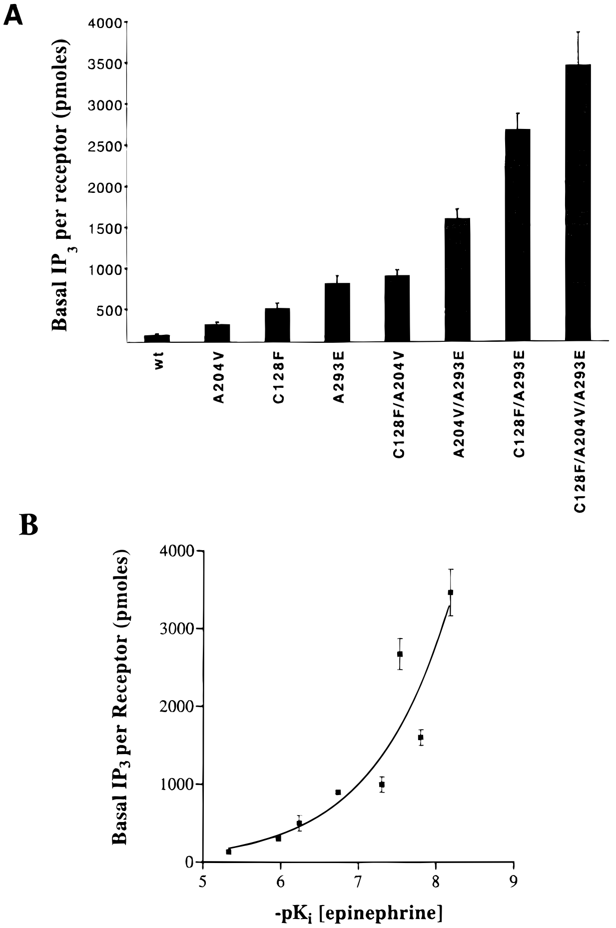

FIGURE 3: Synergism in basal IP_3 production. Panel A: Relationship of basal IP_3 activity in combining constitutively active mutations. Basal IP_3 was determined as described under Materials and Methods and normalized to the receptor density by dividing the picomoles of IP_3 produced by the B_{max} as determined by Scatchard analysis of membranes prepared from parallel plates. Values shown are the mean ± SE for 12 determinations from 3 separate transfections. Panel B: Relationship between epinephrine affinity versus basal IP_3 production. Epinephrine affinity values were taken from Table 2 (pK_H) and basal IP_3 values from above. Correlation (solid line) was best fit to a power function ($r^2 = 0.96$).

retinal and the resulting rotation of the ionone ring (Altenbach et al., 1996). Helices C and F correspond to helices III and VI in the α_1-AR. The $Cys^{128}Phe$ mutation in helix III is thought to impart its constitutive activity through enhancement of the salt-bridge disruption (between Asp^{125} in helix III and Lys^{331} in helix VII) in the activation of the α_1-AR, in an equivalent mechanism to rhodopsin (Porter et al., 1996). Likewise, the $Ala^{204}Val$ constitutive activity involves a disturbance in the helical packing between helices V and VI because of the strain introduced by a bulkier valine (Hwa et al., 1996b). Hence, these mutations in the α_1-AR correlate to motions and mechanisms established in rhodopsin, and the synergism is consistent with a concerted motion of these helices. If multiple helix movement is involved in agonist-induced activation, this paradigm also provides a molecular explanation of partial agonism.

The changes in agonist and antagonist affinity as well as the increased basal signal transduction are predicted from the ternary complex model that has been revised to account for the isomerization of receptors between R and R* states (Samma et al., 1993), with the R* state forming the high-affinity ternary complex, HR*G. Thus, the results indicate that the increased affinity of the mutants for agonists is not due merely to changes in the ligand binding pocket, *per se*, but indicates that the mutations induce conformations that mimic the R* state. Since the mutations, in combination, produced basal activity greater than the fully-stimulated wild-type receptor, this reveals a greater activational potential in G-protein-coupled receptors than nature has regulated. If this potential can eventually be induced by ligands, we speculate on the possibility of designing "super agonists" which have intrinsic activities greater than the natural agonist, an example being isoproterenol which has a higher intrinsic activity at the β-AR than the natural hormones. This possibility is also suggested from the unprecedented high-affinity values for (−)-epinephrine seen in the combined

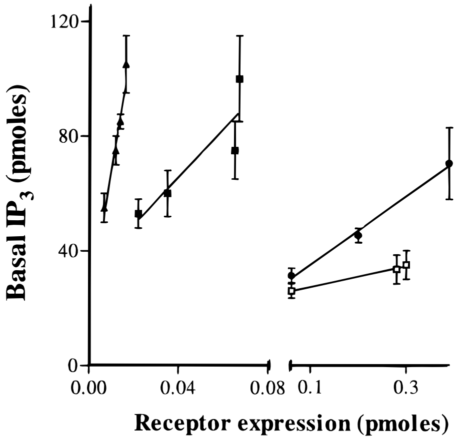

FIGURE 4: Effect of receptor density on basal IP_3 production. Wild-type (□), A204V (●), C128F/A293E (■), or C128F/A204V/A293E (▲) constructs were expressed in COS-1 cells at different receptor densities by varying the amount of cDNA used in each transfection. Receptor expression levels of the various constructs were determined from equilibrium binding studies. The slope of the wild-type receptor was 36 ± 10 pmol of IP_3/pmol of receptor while that of A204V, C128F/A293E, or C128F/A204V/A293E was 118 ± 36, 825 ± 306, and 4910 ± 886, respectively.

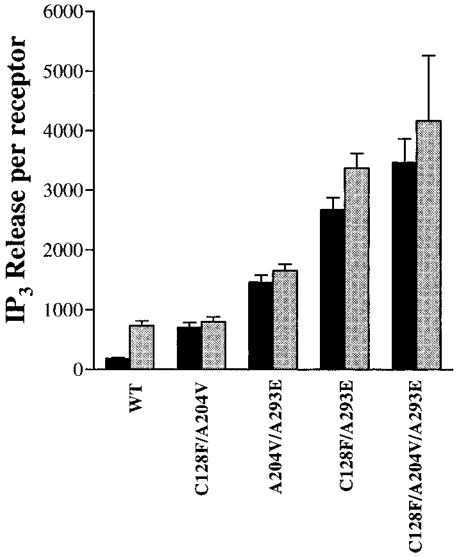

FIGURE 5: Effect of (−)-epinephrine stimulation on combinatorial constitutively active mutations. IP_3 was determined with (gray bars) or without epinephrine (10 μM) (black bars) as described under Materials and Methods and normalized to the receptor density by dividing the picomoles of IP_3 produced by the B_{max} as determined by Scatchard analysis of membranes prepared from parallel plates. Values shown are the mean ± SE for 2−5 determinations from 2 separate transfections.

mutations and the direct correlation to the degree of constitutive activity. The design of the "super agonist" may simply enhance the known interactions of the agonist−receptor complex (i.e., Asp^{125} and the protonated amine of catecholamines or Ser^{207} and the *m*-hydroxyl of the catechol) or take advantage of yet undiscovered interactions between receptor residues and the agonist. The design of super agonists may have therapeutic potential in diseases such as heart failure where the need for increased inotropic response is apparent.

REFERENCES

Altenbach, C., Yang, K., Farrens, D. L., Khorana, H. G., & Hubbell, W. L. (1996) *Biochemistry 35*, 12470−12478.

Brown, L. S., Varo, G., Needleman, R., & Lanyi, J. K. (1995) *Biophys. J. 69*, 2103−2111.

Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835−840.

Chothia, C., & Lesk, A. M. (1985) *J. Mol. Biol. 115*, 116−118.

Cotecchia, S., Schwinn, D. A., Randall, R. R., Lefkowitz, R. J., Caron, M. G., & Kobilka, B. K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7159−7163.

Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G., & Hubbell, W. L. (1995) *Biochemistry 34*, 8812−8819.

Hwa, J., & Perez, D. M. (1996) *J. Biol. Chem. 271*, 6322−6327.

Hwa, J., DeYoung, M. B., Perez, D. M., & Graham, R. M. (1996a) in *The Autonomic Nervous System: The Nervous Control of the Heart* (Shepherd, J., & Vatner, S. F., Eds.) Vol. VIII, Harwood Academic Press, Amsterdam, The Netherlands.

Hwa, J., Graham, R. M., & Perez, D. M. (1996b) *J. Biol. Chem. 271*, 7956−7964.

Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G., & Lefkowitz, R. J. (1992) *J. Biol. Chem. 267*, 1430−1433.

Kraus, W. L., McInerney, E. M., & Katzenellenbogen, B. S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12314−12318.

McPhalen, C. A., Vincent, M. G., Picot, D., Jansonius, J. N., Lesk, A. M., & Chothia, C. (1992) *J. Mol. Biol. 227*, 197−213.

Multulsky, H. J., & Ransnas, L. A. (1987) *FASEB J. 1*, 365−374.

Parma, J., Duprez, L., Van Sande, J., Cochaux, P., Gervy, C., Mockel, J., Dumont, J., & Vassart, G. (1993) *Nature 365*, 649−651.

Perez, D. M., Piascik, M. T., & Graham, R. M. (1991) *Mol. Pharmacol. 40*, 876−883.

Perez, D. M., DeYoung, M. B., & Graham, R. M. (1993) *Mol. Pharmacol. 44*, 784−795.

Perez, D. M., Hwa, J., Gaivin, R., Mathur, M., Brown, F., & Graham, R. M. (1996) *Mol. Pharmacol. 49*, 112−122.

Porter, J. E., Hwa, J., & Perez, D. M. (1996) *J. Biol. Chem. 271*, 28318−28323.

Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., & Oprian, D. D. (1992) *Neuron 9*, 719−725.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Samma, P., Cotecchia, S., Costa, T., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 4625−4636.

Shenker, A., Laue, L., Kosugi, S., Merendino, J. J., Minegishi, T., & Cutler, G. B. (1993) *Nature 365*, 652−654.

Shu, H. K., Pelley, R. J., & Kung, H. J. (1991) *J. Virol. 65*, 6173−6180.

Strader, C. D., Sigal, I. S., Register, R. B., Candelore, M. R., Rands, E., & Dixon, R. A. F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4384−4388.

Wells, J. A. (1990) *Biochemistry 29*, 8509−8517.

BI962141C