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# Mutation of Asn293 to Asp in Transmembrane Helix VI Abolishes Agonist-Induced but Not Constitutive Activity of the $\beta_2$-Adrenergic Receptor

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## ABSTRACT

The $\beta_2$-adrenergic receptor has been shown to display significant constitutive activity (i.e., in the absence of agonist) in addition to agonist-induced activation. Various studies have suggested that a movement in transmembrane helix VI plays a role in activation of various G-protein-coupled receptors. Here we show that a mutation in this domain of the $\beta_2$-adrenergic receptor abolishes agonist activation but not constitutive activity. An Asn293Asp mutant of the human $\beta_2$-adrenergic receptor was expressed either transiently in COS-7 cells or stably in Chinese hamster ovary cells. The mutant receptors were unable to couple to $G_s$, as seen by the lack of high-affinity agonist binding as well as a reduction of the affinities of several agonists correlating with their intrinsic activities. The mutant receptors caused only minimal activation of adenylyl cyclase (2.5% of wild-type activity) and also failed to show agonist-induced phosphorylation by G-protein-coupled receptor kinase 2. In contrast, the mutant receptors were much less affected in their constitutive activity: transient transfection of wild-type and mutant receptors into COS-7 cells caused an increase in intracellular cAMP-levels that was dependent on the level of receptor expression and was maximally 5.4-fold for the mutant and 6.8-fold for the wild-type receptors (67% of wild-type activity). Introduction of the Asn293Asp mutation into a constitutively active mutant receptor did not affect the constitutive activity of this mutant. These results underscore the importance of transmembrane helix VI in controlling agonist-induced activation of the receptor and suggest that constitutive activity is different from agonist-induced activity. Furthermore, they indicate that Asn293 is a key residue in transferring conformational information from the agonist-binding site to the intracellular surface.

$\beta_2$-Adrenergic receptors are often studied as a model system for the large superfamily of G-protein-coupled receptors. These receptors contain seven transmembrane $\alpha$-helices, and their topography has been verified using biochemical and immunological techniques as well as the recently solved X-ray structure of rhodopsin (Dohlman et al., 1987, Wang et al., 1989, Palczewski et al., 2000). The binding of agonists to these receptors is thought to change the receptor into an active conformation, which permits interaction not only with G-proteins (causing the receptor-mediated signal) but also with G-protein-coupled receptor kinases and $\beta$-arrestins (which results in receptor desensitization and internalization). It has recently become clear that in addition to agonist-mediated receptor activation, many G-protein-coupled receptors are active in the absence of agonists; this so-called constitutive activity was first shown for the opioid receptors (Costa and Herz, 1989) but later for many other receptors (Lefkowitz et al., 1993; Scheer et al., 1996; reviewed by Milligan and Bond, 1997). Among the $\beta$-adrenergic receptors, the $\beta_2$-subtype displays far greater constitutive activity than the $\beta_1$-subtype (Zhou et al., 2000; Engelhardt et al., 2001).

The current concept of agonist binding to the human $\beta_2$-adrenergic receptor proposes that the positively charged nitrogen in the ligand interacts with Asp113 in transmembrane helix III (Strader et al., 1987, 1988), and that the two catechol OH-groups form hydrogen bonds with Ser204 and Ser207 in transmembrane helix V (Strader et al., 1989a). Asn293 in transmembrane helix VI seems to bind to the $\beta$-OH group, which defines the chiral center of epinephrine and related agonists (Wieland et al., 1996).

From the latter studies, we have also proposed that the interaction of the $\beta$-OH group with transmembrane helix VI is critical for receptor activation (Wieland et al., 1996). Transmembrane helix VI directly joins the C-terminal end of the third intracellular loop of the receptor, and this region has been shown to be essential for G-protein coupling by a variety of studies (reviewed by Kobilka, 1992; Okada et al.,

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**ABBREVIATIONS:** $\beta_2$AR, (human) $\beta_2$-adrenergic receptor; CYP, cyanopindolol; ICI118,551, 1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(methylethyl)amino]-2-butanol; CHO, Chinese hamster ovary; CAM, constitutively active mutant; CMV, cytomegalovirus; GRK, G-protein-coupled receptor kinase.

2001). Movements of transmembrane helix VI have been shown more directly in the activation of rhodopsin or the $\beta_2$AR, either by creation of immobile helices with artificial zinc-binding sites (Sheik et al., 1996), by electron spin resonance (Farrens et al., 1996), or with site-specific fluorescence labeling of receptors (Gether et al., 1997). The creation of a zinc-binding site in the parathyroid hormone receptor has subsequently been used not only to show the crucial importance of this region in the activation of a class II G-protein-coupled receptor but also that immobilization of this region with zinc can differentiate between active conformations recognized by G-proteins and those recognized by G-protein-coupled receptor kinases and $\beta$-arrestins (Vilardaga et al., 2001).

Many receptors or receptor mutants are "constitutively active" [i.e., active in the absence of agonists (reviewed by Lefkowitz et al., 1993)]. In addition to their spontaneous activity, these receptors are characterized by increased affinity of agonists, decreased affinity of inverse agonists, and phosphorylation by the $\beta$-adrenergic receptor kinase in the absence of agonists (Pei et al., 1994). Constitutive activity can in many instances be produced by mutations in the C-terminal end of the third intracellular loop of receptors (Kjelsberg et al., 1992), the region mentioned above as critical for G-protein-coupling.

Constitutively active receptors have been used to generate models of the receptor activation and signaling processes (Samama et al., 1993; Bond et al., 1995). Furthermore, they have been regarded as good models for the intramolecular mechanisms of receptor activation. In fact, recent molecular modeling studies of such receptors have shown that they closely imitate the agonist-activated state of wild-type receptors (Scheer et al., 1996, 1997; Greasley et al., 2001; Okada et al., 2001). These models support the view that movements in transmembrane helix VI versus III may be critical for the activation process.

From these data, one may conclude that constitutive activity represents a transition (or partial transition) of receptors into the same active conformation that is induced by agonists. In the present study, however, we report a $\beta_2$-adrenergic receptor mutant that essentially retains constitutive activity but is unable to adopt an active conformation in response to agonist binding. We propose that in this mutant, the binding of agonist is uncoupled from the conformational change of the intracellular receptor surface.

## Materials and Methods

**Materials.** $^{125}$I-cyanopindolol ($^{125}$I-CYP) and [$\alpha$-$^{32}$P]ATP were obtained from PerkinElmer Life Sciences (Dreieich, Germany), and the latter was purified as described by Walseth and Johnson (1979). Stereoisomers of isoproterenol (>99% purity), as well as ICI118,551 and (±)-dobutamine were purchased from Sigma/RBI (Taufkirchen, Germany); stereoisomers of propranolol (>98.5% purity), (−)-epinephrine, (−)-alprenolol, and (±)-terbutaline were obtained from Sigma. Chinese hamster ovary (CHO) 10001 cells were kindly provided by Dr. M. Gottesman (National Institutes of Health, Bethesda, MD). The cDNA for the constitutively active human $\beta_2$-adrenergic receptor (CAM) (Pei et al., 1994) was a kind gift from Susanna Cotecchia (University of Lausanne, Switzerland).

**Mutagenesis of $\beta_2$-Adrenergic Receptor cDNA.** The cDNA for the human $\beta_2$-adrenergic receptor (Kobilka et al., 1987) was cloned into the expression vector pBC-CMV-SK (Lohse 1992) to generate the vector pBC-CMV-$\beta_2$AR. Site-directed mutagenesis of the codon for amino acid 293 was performed essentially as described by Wieland et al. (1996). The vector was linearized with *Hpa*I, directly adjacent to codon 293, the gap was bridged with a 38-mer mutant oligonucleotide containing in its center the codon GAC (Asp) for amino acid 293, and the entire coding region was generated by polymerase chain reactions using the oligonucleotide-annealed linear vector (100 ng) as a template and primers corresponding to nucleotides 1 to 18 (forward) and 1242 to 1225 (reverse) of the receptor cDNA. A 318 base-pair *Bgl*II-*Eco*RV fragment containing the mutated region was excised from the polymerase chain reaction products and inserted into the corresponding sites of pBC-CMV-$\beta_2$AR. The construct was verified by automated sequencing. To construct the Asn293Asp CAM receptor, the same approach was used on a CAM human $\beta_2$-adrenergic receptor (Pei et al., 1994) cloned into pBC-CMV-SK (Lohse, 1992).

**Generation of Transfected Cell Lines.** CHO cell lines stably expressing wild-type and mutant receptors were obtained by transfecting CHO 10001 cells with the respective expression vectors plus pSV2-neo using *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche Applied Science, Mannheim, Germany) as transfection reagent and G418 (Invitrogen, Carlsbad, CA) to select positive clones as described earlier (Lohse, 1992). Several clones were selected for initial experiments, and one wild-type and one mutant clone with comparable densities (about 0.2 pmol/mg of membrane protein) were studied in detail.

Experiments measuring the constitutive activity were done with transiently transfected COS-7 cells. Cells were transfected with various amounts of the respective cDNA by the DEAE dextran method and investigated 48 h after the transfection.

**Radioligand Binding Studies.** Ligand binding to $\beta_2$-adrenergic receptors was analyzed using $^{125}$I-CYP and crude cell membranes prepared as described earlier (Lohse et al., 1990) using an incubation time of 1 h at 30°C. Saturation studies were done with radioligand concentrations from 2 to 200 pM, using 1 $\mu$M (−)-propranolol to define nonspecific binding. Competition studies were done with a radioligand concentration of 30 pM. Unless stated otherwise, all radioligand binding assays contained 100 $\mu$M GTP to uncouple $\beta_2$-adrenergic receptors from $G_s$ and thereby generate monophasic competition curves for agonists as well as antagonists.

**Adenylyl Cyclase Assays.** The function of $\beta_2$-adrenergic receptors was assessed by determining their capacity to stimulate the adenylyl cyclase activity in membranes prepared from the CHO cell lines stably expressing the receptor variants. Membranes were prepared as above, and adenylyl cyclase activity was determined by measuring the generation of [$^{32}$P]cAMP from [$\alpha$-$^{32}$P]ATP as described previously (Pippig et al., 1993). The incubation was done for 30 min at 30°C.

**Receptor Phosphorylation by the $\beta$-Adrenergic Receptor Kinase GRK2.** Receptors were expressed in H5 insect cells grown in suspension culture with the help of recombinant baculoviruses as described earlier (Müller et al., 1997). A virus for the N293D mutant receptor was obtained by cloning the coding region of the cDNA into the vector pVL1393 and cotransfection of Sf9 insect cells (Invitrogen) with this vector and Baculo-Gold (BD Pharmingen, San Diego, CA) virus DNA. Single clones of viruses were obtained by limiting dilution.

Suspension cultures were infected with the respective viruses at a multiplicity of infection of 10, and the cells were harvested 60 to 72 h later. Cell membranes were obtained by lysing the cells in 5 mM Tris-HCl, 2 mM EDTA, pH 7.4, plus protease inhibitors (100 $\mu$M phenylmethylsulfonyl fluoride, 30 $\mu$g/ml benzamidine, 10 $\mu$g/ml soybean trypsin inhibitor, and 5 $\mu$g/ml leupeptin), centrifugation at 40,000*g* for 30 min at 4°C, and subsequent centrifugation of the resuspended pellet at 100,000*g* for 90 min on a discontinuous 40%/20% sucrose gradient in the same buffer. The 20%/40% interface was collected and washed once with buffer containing either 300 mM or 2 M NaCl or 5 M urea and subsequently twice with buffer alone, with

intervening centrifugations at 160,000*g* for 10 min. In GRK2 phosphorylation assays, by far the best signal-to-noise ratio of $\beta_2$AR phosphorylation was obtained when the membranes were washed with urea, so this treatment was used for all subsequent experiments. These membrane preparations contained $\beta_2$AR levels of up to 150 pmol/mg protein. Recombinant bovine GRK2 was expressed in Sf9 insect cells and purified to >95% homogeneity as described earlier (Söhlemann et al., 1993).

The receptors contained in the purified cell membranes were phosphorylated for 60 min essentially as described previously (Müller et al., 1997), using 0.5 to 1 pmol of $\beta_2$-adrenergic receptors, 0.45 $\mu$M GRK2, 0.65 $\mu$M $G_{\beta\gamma}$ purified from bovine brain, and 50 $\mu$M [$\gamma$-$^{32}$P]ATP ($10^6$ cpm per tube) and ligands as indicated in an incubation volume of 40 $\mu$l. The incubation mixture was the centrifuged and the pellet resolved by SDS-polyacrylamide gel electrophoresis. $^{32}$P incorporation into the receptor band was visualized by autoradiography and quantified by PhosphorImaging (Amersham Biosciences, Piscataway, NJ).

**Determination of Constitutive Receptor Activity.** Experiments measuring the constitutive activity of wild-type and mutant receptors were done in transiently transfected COS-7 cells, as described recently (Engelhardt et al., 2001). In brief, COS-7 cells were transfected with various amounts of plasmids containing the cDNA coding for the wild-type or the mutant receptors in the pcDNA3 plasmid. Expression levels of the receptors were determined 48 h later by radioligand binding, and cAMP-accumulation was determined in the absence of agonists to measure constitutive activity. To this end, cells were washed twice with HEPES buffer (137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES, pH 7.3) and resuspended in the same buffer with 0.5 mM 3-isobutyl-1-methylxanthine. The cells were incubated for 20 min at 37°C, the reaction was stopped by addition of boiling water, and the cellular cAMP was determined by radioimmunoassay (Immunotech, Marseilles, France).

**Data Analysis.** Radioligand binding data were analyzed by nonlinear curve-fitting using the program SCTFIT, which allows analysis for multiple binding sites as described previously (Lohse et al., 1984). Concentration-response curves for adenylyl cyclase stimulation were analyzed by nonlinear curve-fitting to the Hill equation as described earlier. Intrinsic activities of agonists and inverse agonists were determined in concentration-response adenylyl cyclase experiments, and the maximal extent of stimulation (agonists) or inhibition (inverse agonists) of the calculated curve was taken as the intrinsic activity, which was expressed as percentage of the activity of (−)-isoproterenol.

Signaling-efficiencies of different receptor mutants in adenylyl cyclase experiments were determined by simultaneous curve-fitting and calculation of the "transducer ratio" $\tau$ (Black et al., 1985) using the algorithm $E = E_o + E_{max} \times (\tau \times A)/[(K_A + A) + \tau \times A]$, where $E_{max}$ denotes the maximum possible effect of the system (which was the same for the wild-type and mutant curves), and $K_A$ the agonist dissociation constant (which was determined independently in radioligand binding experiments). $\tau$ describes the signal transduction efficacy of the respective receptor and was estimated individually for each curve as described previously (Lohse, 1990).

## Results

Wild-type and N293D mutant $\beta_2$-adrenergic receptors were transfected into CHO cells, and stably expressing clones were selected. To avoid clonal artifacts, in initial studies, several clones were studied for both receptor types, but subsequently only two clones were characterized in more detail. These clones had similar expression levels, as determined in saturation experiments with the antagonist radioligand $^{125}$I-CYP (Table 1).

The ability of the wild-type and the N293D mutant receptors to generate a signal was investigated by measuring the isoproterenol-stimulated adenylyl cyclase activity in membranes prepared from the CHO cells (Fig. 1). Wild-type receptors were capable of generating a signal that was slightly greater than that caused by direct stimulation of adenylyl cyclase with 10 $\mu$M forskolin. In contrast, the stimulation via N293D mutant receptors was only minimal, with a maximum of less than 10% of that by wild-type receptors plus a ≈10-fold rightward shift of the concentration-response curve. These changes were observed with several clones expressing the mutant receptors (data not shown). The signal transduction efficacy ($\tau$) was estimated by simultaneous curve fitting according to Black et al. (1985) as described under *Materials and Methods*. This analysis gave an average signal transduction efficacy of 2.83 ± 0.39 for the wild-type receptors, and a value of 0.072 ± 0.03 for the N293D mutant receptors. Thus, the mutant receptors have only a very limited ability (2.5% of the wild-type) to generate a signal in response to (−)-isoproterenol. Similarly, the full or partial agonists (−)-epinephrine, terbutaline, clenbuterol, and dobutamine failed to activate the N293D mutant receptors (data not shown).

To investigate whether the reduced signaling was caused by an inability of the mutant receptors to couple to their G-protein, $G_s$, we measured the competition of $^{125}$I-CYP binding by the agonist (−)-isoproterenol in the absence and presence of GTP (Fig. 2). In such experiments, receptors can couple to $G_s$ in the absence of GTP, resulting in a biphasic curve consisting of a high-affinity $G_s$-coupled and a low-affinity uncoupled component (Kent et al., 1980). In membranes containing wild-type $\beta_2$-adrenergic receptors, the two components could easily be detected in the absence of GTP, whereas in the presence of GTP, only the uncoupled, low-affinity form was present. In contrast, in the case of the N293D mutant receptors, the curves in the absence and presence of GTP were virtually indistinguishable and no high-affinity $G_s$-coupled component was discovered. Thus, within the detection limit of this assay, the N293D mutant receptors were completely unable to form a high-affinity state. In addition, the low-affinity component of the mutant receptors was of ≈20-fold lower affinity than that of the wild-type receptor (see below). The lack of a high-affinity component is compatible with the interpretation that the mutant receptors fail to couple to $G_s$ and that this explains their inability to generate a signal.

Alterations of agonist affinities have been described for constitutively active mutants of various receptors. In these cases, increases in agonist affinities were observed that correlated with their respective intrinsic activities. We therefore sought to investigate whether a similar but opposite effect was seen in the N293D $\beta_2$AR. Competition experiments similar to those shown in Fig. 2 were therefore done with several compounds with different intrinsic activities. The assays were done in the presence of GTP to measure only binding to

TABLE 1
Parameters for $^{125}$I-CYP binding to membranes from CHO cells stably expressing wild-type or N293D mutant $\beta_2$-adrenergic receptors

Saturation studies were done with 2 to 200 pM $^{125}$I-CYP, and the data were analyzed by nonlinear curve fitting to obtain estimates for the affinity ($K_D$) and receptor number ($B_{max}$). Data are means ± S.E.M., $n$ = 3.

| Receptor | $K_D$ | $B_{max}$ |
|---|---|---|
| | *pM* | *fmol/mg membrane protein* |
| Wild-type | 52 ± 8 | 285 ± 20 |
| N293D | 57 ± 20 | 200 ± 80 |

the receptors themselves. These experiments revealed that the N293D mutant receptors had a reduced affinity not only for (−)-isoproterenol, but also for many other agonists (Table 2). In contrast, the affinities of the mutant receptors for the isomers of propranolol and many other antagonists or inverse agonists were only modestly affected and, in some cases, even increased (Table 2).

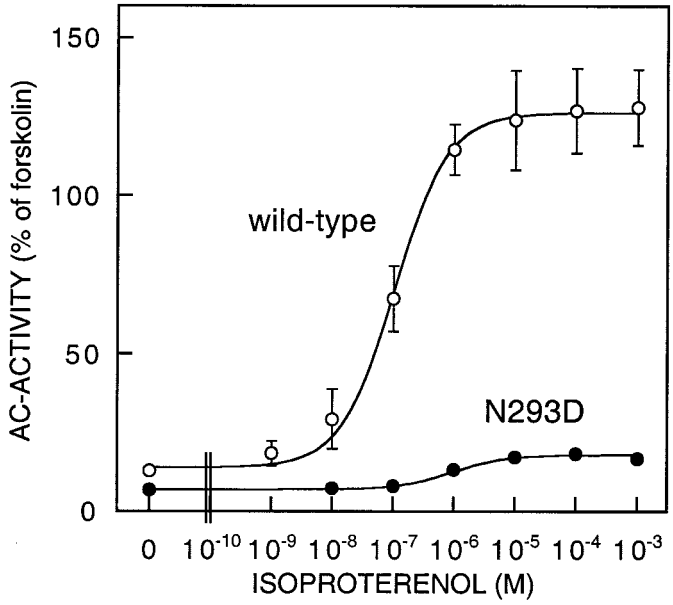


**Fig. 1.** Stimulation of adenylyl cyclase activity in membranes prepared from CHO cells expressing wild-type $\beta_2$AR (○) or the N293D mutant (●) by (−)-isoproterenol. Reference values (100%) were the stimulation by 10 $\mu$M forskolin in the same membrane preparations; these were 202 ± 7 and 229 ± 15 pmol cAMP/mg protein/min for wild-type and N293D, resp. The curves were obtained by nonlinear curve-fitting as described under *Materials and Methods*. These gave a signal transduction efficacy $\tau$ of 2.83 ± 0.39 for wild-type and 0.072 ± 0.003 for N293D mutant receptors. Data are means ± S.E.M. of four independent experiments with duplicate samples. Error bars are not shown when smaller than symbol size.

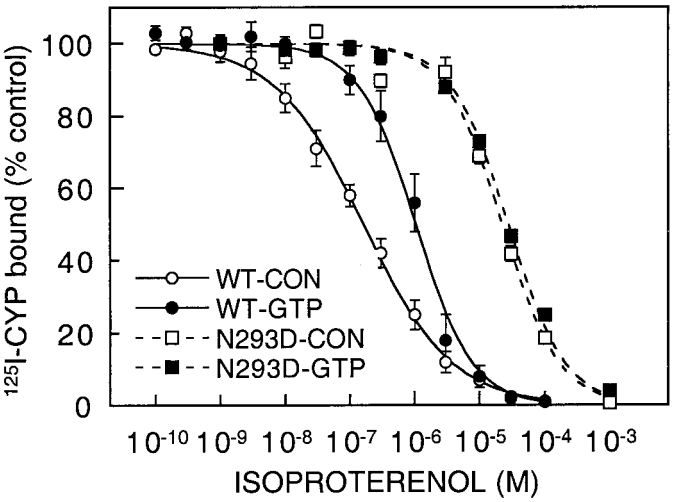


**Fig. 2.** Inhibition of $^{125}$I-CYP binding to wild-type (WT) and N293D mutant $\beta_2$-adrenergic receptors by (−)-isoproterenol. $^{125}$I-CYP binding was measured with membranes prepared from CHO cells expressing wild-type and N293D mutant $\beta_2$-adrenergic receptors. Nonlinear curve-fitting showed the presence of two affinity states (with $K_H$ and $K_L$) in the absence of GTP for wild-type receptors, whereas all other curves were monophasic (only one $K_i$). Estimated $K_i$-values were: wild-type receptors: no GTP, $K_H$ = 15 nM, $K_L$ = 560 nM; 100 $\mu$M GTP, $K_i$ = 810 nM. $\beta_2$AR-N293D: no GTP, $K_i$ = 19 $\mu$M; 100 $\mu$M GTP, $K_i$ = 22 $\mu$M. Data are means ± S.E.M. of four independent experiments with duplicate samples.

To investigate whether the alterations in the affinity for the N293D mutant receptors were indeed related to the intrinsic activity of the various compounds, their intrinsic activities were determined in adenylyl cyclase experiments (using wild-type receptors). These assays revealed a greater loss of affinity for full than for partial agonists, and little change (or even increase) in affinity for most inverse agonists. Figure 3 shows a correlation between the intrinsic activities and the alterations in affinity induced by the N293D mutation. This correlation was indeed highly significant as indicated by a correlation coefficient $r^2$ of 0.98. These data are compatible with the notion that the N293D mutant receptors were unable to adopt an active conformation.

The data shown so far suggest that the N293D mutant receptors cannot assume an active conformation when probed with the G-protein $G_s$. A second strategy to probe an active receptor conformation is their phosphorylation by G-protein-coupled receptor kinases (GRKs). This phosphorylation is strictly agonist-dependent and it is assumed that only the active conformation of the receptors is a substrate for the kinase (Benovic et al., 1986, 1988). For these experiments, the receptors were expressed in H5 insect cells with the help of recombinant baculoviruses and cell membranes were prepared on sucrose density gradients. Before the phosphorylation by purified GRK2, the membranes were washed with either different concentrations of NaCl or 5 M urea to strip the membranes of peripheral proteins and to inactivate endogenous protein kinases. By far the best signal/noise ratio for the receptor band was obtained when the membranes had been treated with urea (data not shown), indicating that the receptors can tolerate this treatment, whereas most membrane-associated kinases do not. Under these conditions, clear phosphorylation of the wild-type $\beta_2$AR was observed in the presence of the agonist (−)-isoproterenol, although none was seen in the presence of the inverse agonist (−)-propranolol (Fig. 4A). In five such experiments, the agonist-induced phosphorylation of the wild-type $\beta_2$AR was on average 8-fold higher than the signal detected in the presence of (−)-propranolol, and the latter was not statistically significantly different from 0 (Fig. 4B). In contrast to the data seen with the wild-type $\beta_2$AR, there was absolutely no phosphorylation of the N293D mutant by GRK2, in the presence of neither

TABLE 2

Affinities of agonists and antagonists for wild-type and N293D mutant $\beta_2$-adrenergic receptors

The affinities were determined in competition experiments with 30 nM $^{125}$I-CYP in the presence of 100 $\mu$M GTP and were calculated by nonlinear regression. Data are means ± SEM, $n$ = 3.

| | Affinity ($K_i$) | |
|---|---|---|
| | Wild-type | N293D |
| | *nM* | |
| (−)-Isoproterenol | 280 ± 76 | 5760 ± 460 |
| (+)-Isoproterenol | 280 ± 76 | 5760 ± 460 |
| (+)-Isoproterenol | 10,600 ± 2,390 | 87,500 ± 11,000 |
| (−)-Epinephrine | 1,450 ± 50 | 38,600 ± 10,000 |
| (±)-Terbutaline | 10,900 ± 5,400 | 51,900 ± 12,900 |
| (±)-Dobutamine | 66,000 ± 21,000 | 80,000 ± 39,000 |
| (−)-Alprenolol | 2.1 ± 0.4 | 2.4 ± 0.4 |
| (−)-Propranolol | 1.04 ± 0.28 | 0.86 ± 0.02 |
| (+)-Propranolol | 108 ± 30 | 66 ± 14 |
| ICI118,551 | 0.32 ± 0.04 | 0.82 ± 0.13 |

(−)-isoproterenol nor (−)-propranolol (Fig. 4B). Variations of the experimental conditions (protein content, concentrations of GRK2, G-protein βγ-subunits, ATP, incubation time) never resulted in the detection of phosphorylation of the mutant receptors (data not shown), even though the wild-type receptors were phosphorylated under all these conditions. This suggests that the N293D receptor mutant failed to adopt an active conformation also toward GRK2.

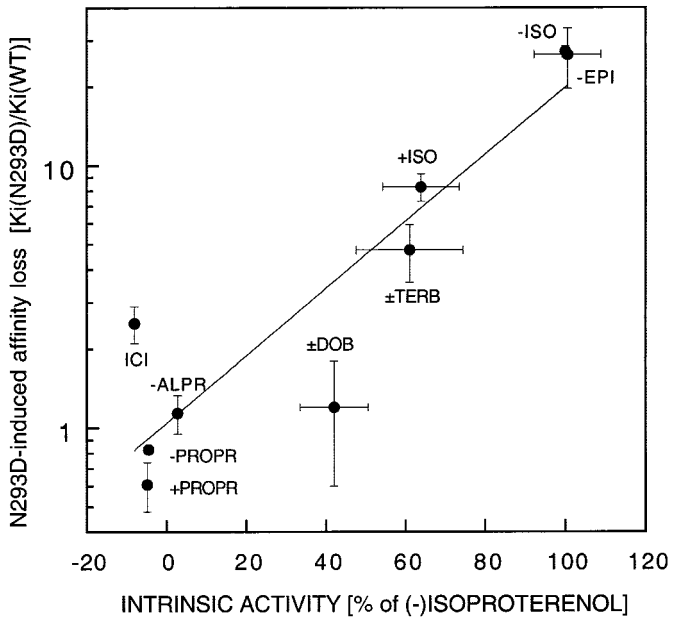


**Fig. 3.** Correlation between the alterations in affinity induced by the N293D mutation in the human $\beta_2$-adrenergic receptor and the intrinsic activity of the respective compounds. The mutation-induced alteration in affinity was determined from inhibition of $^{125}$I-CYP binding to wild-type and N293D mutant receptors and is expressed as log($K_i$ mutant/$K_i$ wild-type). Intrinsic activities were determined as the maximal stimulation of adenylyl cyclase activity as in Fig. 1. They were normalized to the maximal activity of (−)-isoproterenol (for agonists) tested in the same experiments. The correlation coefficient $r^2$ of the fit was 0.98. ALP, alprenolol; EPI, epinephrine; ISO, isoproterenol; TERB, terbutaline; PROP, propranolol; ICI, ICI118,551. Data are means ± S.E.M. of three to six independent experiments.

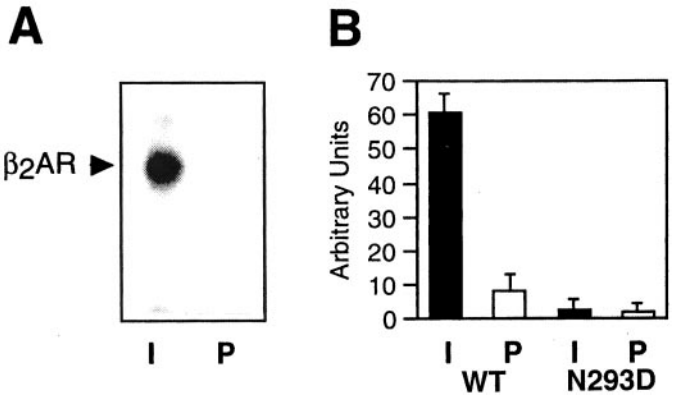


**Fig. 4.** Phosphorylation of $\beta_2$-adrenergic receptors by GRK2. $\beta_2$-Adrenergic receptors in purified H5 insect cell membranes were phosphorylated with 0.45 μM purified GRK2. $^{32}$P-Incorporation into the receptors was visualized and quantified by SDS-polyacrylamide gel electrophoresis of the membranes and autoradiography/PhosphorImaging. A, autoradiogram of the phosphorylated receptors. The membranes were phosphorylated in the presence of 100 μM (−)-isoproterenol (I) or 10 μM (−)-propranolol (P). The receptor band is marked ($\beta_2$AR). B, phosphorylation of wild-type (WT) and N293D mutant $\beta_2$-adrenergic receptors. The experiments were done as in A. GRK2-catalyzed $^{32}$P-incorporation into the receptor band was quantified by PhosphorImaging and is expressed in arbitrary units. The results are means ± S.E.M. of five (wild-type) or three (N293D) independent experiments.

Because the N293D mutant receptors could not be activated by agonists, it would seem reasonable to assume that they also had no constitutive activity. However, Fig. 5 shows that this was clearly not the case: constitutive activity was assessed by the well established model of increases in cAMP in COS-7 cells transiently transfected with various amounts of the cDNA for the $\beta_2$AR. In this model, increasing amounts of cDNA led to increasing expression of receptors, and this caused increases in basal cAMP even in the absence of agonists. These cAMP increases follow the law of mass action and therefore are a hyperbolic function of the receptor levels (Fig. 5). Both the wild-type and the N293D mutant receptors were capable of eliciting such cAMP increases. The potency of the N293D mutant receptors (apparent $K_{act}$ 3.4 ± 0.7 versus 2.4 ± 0.7 pmol/mg membrane protein) as well as their efficacy (maximal stimulation, 5.4 ± 0.7-fold versus 6.8 ± 0.8-fold) were only modestly (and statistically not significantly) reduced compared with the wild-type values. Calculation of the transducer ratio indicated that—relative to the wild-type receptors—the constitutive activity of the mutant receptors was 67%, compared with an agonist-induced activity of less than 3% (Fig. 1). These data show that the constitutive activity of the N293D $\beta_2$AR was largely maintained, whereas the agonist-induced activation was almost completely abolished.

To find out whether the intracellular part of the receptor was still able to adopt an active conformation, we combined the N293D mutation with a CAM generated by mutations at the C-terminal end of the third intracellular loop [i.e., the

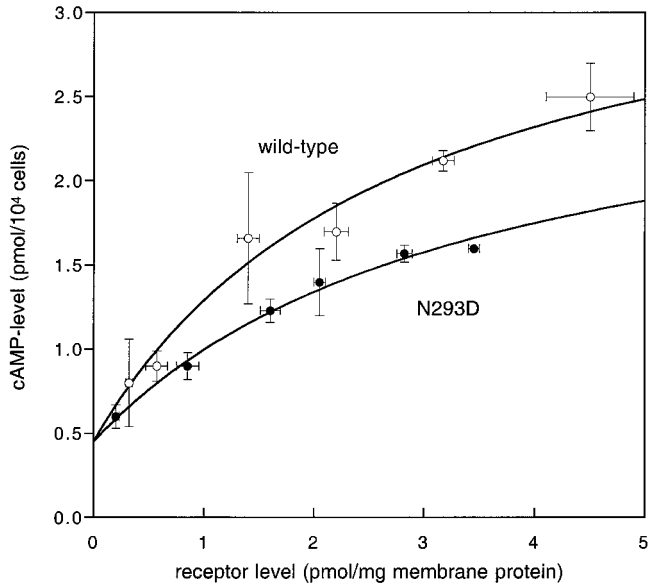


**Fig. 5.** Constitutive activity of wild-type and N293D mutant $\beta_2$-adrenergic receptors. Constitutive activity was assessed by transfecting various amounts of the respective cDNA into COS-7 cells and measuring receptor expression (by $^{125}$I-CYP binding) and cAMP-levels in the absence of agonists (by radioimmunoassay) 48 h later. Shown is the relationship between receptor expression and cAMP levels (means ± S.E.M. of three independent experiments). The fitted hyperbolic curve gave cAMP levels in the absence of receptors of 0.45 pmol/10$^4$ cells, and apparent $K_{act}$-values of 2.4 ± 0.7 (wild-type) and 3.4 ± 0.7 (N293D) pmol/mg membrane protein, and $E_{max}$ values of 3.1 ± 0.4 (wild-type) and 2.4 ± 0.3 (N293D) pmol/10$^4$ cells.

region adjacent to transmembrane helix VI (Pei et al., 1994)]. Constitutive activity of the N293D/CAM receptor was compared with N293D, wild-type, and CAM receptors in transiently transfected COS-7 cells as described above. Because we could not obtain high expression levels of the N293D/CAM receptor, all receptors were studied at an expression level of ≈200 fmol/mg of membrane protein. At this level, the amount of cAMP produced by the N293D/CAM receptor in the absence of agonists was similar to that produced by the CAM receptor and significantly higher than the constitutive activity of either the wild-type or the N293D receptor (Fig. 6). Thus, the N293D mutation prevents receptor activation by agonists but has no effect on either basal or mutation-induced constitutive activity.

## Discussion

A large set of observations indicates that motions between helix III and VI of G-protein-coupled receptors play an essential role in the activation of G-protein-coupled receptors. This seems to be true also for the $\beta_2$-adrenergic receptor, because it has been shown that 1) fluorescence-labeling of Cys125 in helix III and/or Cys285 in helix VI results in agonist-dependent changes in fluorescence compatible with a movement of helix VI during activation (Gether et al., 1997), 2) creation of a Zn(II)-binding pocket between helices III and VI blocks activation (Sheikh et al., 1999), 3) as in many other receptors, mutations in the transition between the third intracellular loop and helix VI result in constitutive receptor activation (Pei et al., 1994), and 4) an interaction between the $\beta$-OH group of agonists and Asn293 in helix VI seems to play a role in the activation process of the receptors by catecholamines (Wieland et al., 1996).

The present study supports this proposal by showing that replacement of Asn in position 293 by Asp results in a receptor that can no longer be activated to a significant extent by agonists. This lack of ability to assume an active conformation is evident from four independent sets of data: 1) an almost complete loss of adenylyl cyclase activation, 2) an inability to form a high-affinity state for agonists in the absence of GTP, 3) a loss in affinity for ligands that correlates with the intrinsic activity of these ligands, and 4) a lack of agonist-induced phosphorylation by GRK2. These assays assess different activation-dependent properties [i.e., coupling to $G_s$ (1 and 2), agonist binding (2 and 3), and coupling to GRKs (4). Taken together, the data clearly indicate that the N293D $\beta_2$AR is unable to assume an agonist-induced active conformation, presumably because of an inability to move helix VI in a manner required for agonist-dependent activation.

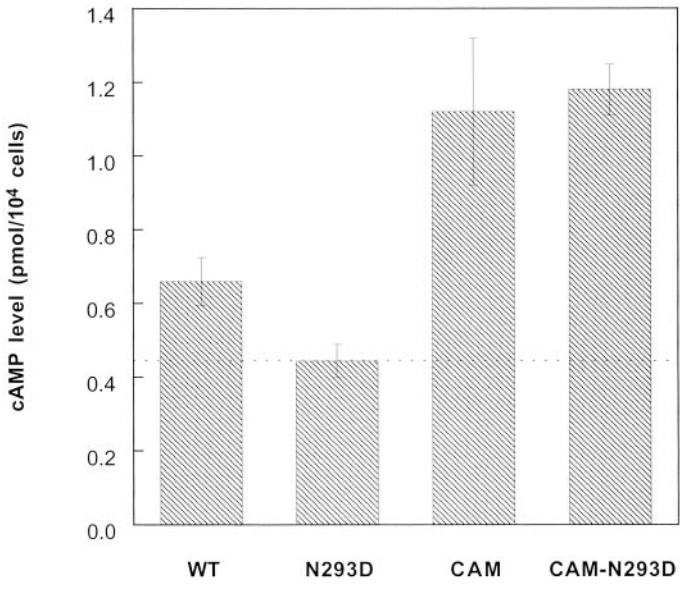


**Fig. 6.** Constitutive activity of CAM and CAM-N293D $\beta_2$-adrenergic receptors. COS-7 cells were transfected with the respective cDNAs that were titrated to reach a receptor expression levels of approximately 200 fmol/mg of membrane protein (determined by radioligand binding 48 h after transfection). Constitutive activity was assessed by measuring cAMP-accumulation in the absence of agonist but in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine 48 h after transfection. Shown are means ± S.E.M. of three independent experiments. WT, wild-type.

Constitutive activity of receptors is usually explained in a framework assuming two states of a receptor (Lefkowitz et al., 1993): R (inactive) and R* (active). In the R* state, receptors couple to $G_s$ and to GRKs and have high affinity for agonists. Agonists increase the probability that the receptors are in the R* state and thus cause receptor activation. Constitutive activity of a receptor then means that even in the absence of agonists, a receptor assumes the R* state with a certain probability. Inverse agonists, finally, reduce this probability. Constitutive activity and inverse agonism are well-documented properties of the $\beta_2$-adrenergic receptor (Chidiac et al., 1994; Bond et al., 1995; Zhou et al., 2000; Engelhardt et al., 2001).

If R and R* were the only two states of the receptor, the N293D mutant should display no constitutive activity. However, the constitutive activity of the N293D mutant was only slightly lower than that of the wild-type $\beta_2$AR. This suggests that constitutive activity is not dependent on the agonist-induced R* state. This was confirmed by combining the N293D mutation with a constitutively active mutant (CAM). There was no difference in the constitutive activity of the N293D/CAM receptor and the CAM alone. Thus, in neither the wild-type nor in the CAM $\beta_2$AR did the N293D mutation cause a significant reduction in constitutive activity. These data suggest that there is more than one conformation of the receptor that can couple to $G_s$.

Our data show that the N293D mutant displays constitutive activity (i.e., that it can adopt a conformation capable of activating G-proteins at the cytosolic interface). However, agonists fail to promote this state, suggesting that the conformational changes in the agonist binding pocket are uncoupled from the conformational changes at the intracellular receptor surface. This is confirmed by the combination of the N293D mutation with a constitutively active $\beta_2$AR (CAM). This mutant showed an increased constitutive activity, but still no agonist-mediated stimulation of G-protein.

The mechanism for the uncoupling remains currently unknown. One might speculate that the N293D mutant cannot be switched by agonists into the R* conformation because it lacks the Asn side chain in position 293 required for the interaction with the $\beta$-OH group of (−)-isoproterenol. However, this is surprising, because the N293D mutant was also not activated by agonists of a different chemical structure and because a 293L mutant receptor, which also lacks the interaction with the $\beta$-OH group, can fully activate $G_s$ (Wieland et al., 1996).

In an earlier study of mutant receptors for parathyroid hormone, we described receptor mutants that could adopt an active conformation toward GRKs and β-arrestins but not toward G-proteins (Vilardaga et al., 2001). In the current study, we report that constitutive activity of a $\beta_2$-adrenergic receptor mutant seems to be mediated by a conformation that is partially active toward $G_s$ but is not recognized by GRK-2. This supports the idea that receptors can adopt multiple conformations with various degrees of activity versus different effectors. This contention is supported by the finding—contrary to earlier data with $\beta_2$-adrenergic receptor agonists (Benovic et al., 1988)—that in the case of the μ-opioid receptor, some agonists can induce coupling to G-proteins without inducing desensitization (Whistler et al., 1999). It seems, therefore, that agonist-induced activation of receptors is far more complex than a simple R − R* transition and involves multiple receptor conformations, with some structural changes occurring in the transmembrane regions comprising the ligand binding pocket and others involving the cytosolic receptor parts that contact the G-protein.

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**References**

Benovic JL, Staniszewski C, Mayor F Jr, Caron MG, and Lefkowitz RJ (1988) β-Adrenergic receptor kinase. Activity of partial agonists for stimulation of adenylate cyclase correlates with ability to promote receptor phosphorylation. *J Biol Chem* **263:**3893–3897.

Benovic JL, Strasser RH, Caron MG, and Lefkowitz RJ (1986) β-Adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc Natl Acad Sci USA* **83:**2797–2801.

Black JW, Leff P, and Shankley NP (1985) An operational model of pharmacological agonism: the effect of E/[A] curves shape on agonist dissociation constants. *Br J Pharmacol* **84:**561–571.

Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, McMinn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF, et al. (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the $\beta_2$-adrenoceptor. *Nature (Lond)* **374:**272–276.

Chidiac P, Hebert TE, Valiquette M, Dennis M, and Bouvier M (1994) Inverse agonist activity of beta-adrenergic antagonists. *Mol Pharmacol* **45:**490–499.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity for opioid receptors coupled to G proteins. *Proc Natl Acad Sci USA* **86:**7321–7325.

Dohlman HG, Bouvier M, Benovic JL, Caron MG, and Lefkowitz RJ (1987) The multiple membrane spanning topography of the $\beta_2$-adrenergic receptor. Localization of the sites of binding, glycosylation and regulatory phosphorylation by limited proteolysis. *J Biol Chem* **262:**14282–14288.

Engelhardt S, Grimmer Y, Fan G-H, and Lohse MJ (2001) Constitutive activity of the human $\beta_1$-adrenergic receptor in $\beta_1$-adrenergic receptor transgenic mice. *Mol Pharmacol* **60:**1–6.

Farrens DL, Altenbach C, Yang K, Hubbell WL, and Khorana HG (1996) Requirement of rigid body motion of transmembrane helices for light activation of rhodopsin. *Science (Wash DC)* **274:**768–770.

Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H, and Kobilka BK (1997) Agonists induce conformational changes in transmembrane domains III and VI of the $\beta_2$-adrenoceptor. *EMBO (Eur Mol Biol Organ) J* **16:**6737–6747.

Greasley PJ, Fanelli F, Scheer A, Abuin L, Nenniger-Tosato M, De Benedetti PG, and Cotecchia S (2001) Mutational and computational analysis of the $\alpha_{1b}$-adrenergic receptor. Involvement of basic and hydrophobic residues in receptor activation and G protein coupling. *J Biol Chem* **276:**46485–46494.

Kent RS, De Lean A, and Lefkowitz RJ (1980) A quantitative analysis of β-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Mol Pharmacol* **17:**14–23.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG, and Lefkowitz RJ (1992) Constitutive activation of the $\alpha_{1B}$-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J Biol Chem* **267:**1430–1433.

Kobilka BK, Dixon RAF, Frielle T, Dohlman HG, Bolanowski MA, Sigal IS, Yang-Feng TL, Francke U, Caron MG, and Lefkowitz RJ (1987) cDNA for the human $\beta_2$-adrenergic receptor: A protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc Natl Acad Sci USA* **84:**46–50.

Kobilka B (1992) Adrenergic receptors as models for G protein-coupled receptors. *Annu Rev Neurosci* **15:**87–114.

Lefkowitz RJ, Cotecchia S, Samama P, and Costa T (1993) Constitutive activity of receptors coupled to guanine regulatory proteins. *Trends Pharmacol Sci* **14:**303–307.

Lohse MJ (1990) Quantitation of receptor desensitization by an operational model of agonism. *J Biol Chem* **265:**3210–3211.

Lohse MJ (1992) Stable overexpression of $\beta_2$-adrenergic receptors in mammalian cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **345:**444–451.

Lohse MJ, Benovic JL, Caron MG, and Lefkowitz RJ (1990) Multiple pathways of rapid $\beta_2$-adrenergic receptor desensitization: Delineation with specific inhibitors. *J Biol Chem* **265:**3202–3209.

Lohse MJ, Lenschow V, and Schwabe U (1984) Two affinity states of $R_i$ adenosine receptors in brain membranes: analysis of guanine nucleotide and temperature effects on radioligand binding. *Mol Pharmacol* **26:**1–9.

Milligan G and Bond RA (1997) Inverse agonism and the regulation of receptor number. *Trends Pharmacol Sci* **18:**468–474.

Müller S, Straub A, and Lohse MJ (1997) Selectivity of β-adrenergic receptor kinase 2 for G protein βγ subunits. *FEBS Lett* **401:**25–29.

Okada T, Ernst OP, Palczewski K, and Hofmann KP (2001) Activation of rhodopsin: new insights from structural and biochemical studies. *Trends Biochem Sci* **26:**318–324.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.

Pei G, Samama P, Lohse M, Wang M, Codina J, and Lefkowitz RJ (1994) A constitutively active mutant $\beta_2$-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc Natl Acad Sci USA* **91:**2699–2702.

Pippig S, Andexinger S, Daniel K, Puzicha M, Caron MG, Lefkowitz RJ, and Lohse MJ (1993) Overexpression of β-adrenergic receptor kinase and β-arrestin augment homologous desensitization of $\beta_2$-adrenergic receptors. *J Biol Chem* **268:**3201–3208.

Samama P, Cotecchia S, Costa T, and Lefkowitz RJ (1993) A mutation-induced activated state of the $\beta_2$-adrenergic receptor: extending the ternary complex model. *J Biol Chem* **268:**4625–4636.

Scheer A, Fanelli F, Costa T, De Benedetti PG, and Cotecchia S (1996) Constitutively active mutants of the $\alpha_{1B}$-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *EMBO (Eur Mol Biol Organ) J* **15:**3566–3578.

Scheer A, Fanelli F, Costa T, De Benedetti PG, and Cotecchia S (1997) The activation process of the $\alpha_{1B}$-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate. *Proc Natl Acad Sci USA* **94:**808–813.

Sheikh SP, Vilardarga JP, Baranski TJ, Lichtarge O, Iiri T, Meng EC, Nissenson RA, and Bourne HR (1999) Similar structures and shared switch mechanisms of the $\beta_2$-adrenoceptor and the parathyroid hormone receptor. Zn(II) bridges between helices III and VI block activation. *J Biol Chem* **274:**17033–17041.

Sheikh SP, Zvyaga TA, Lichtarge O, Sakmar TO, and Bourne HR (1996) Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. *Nature (Lond)* **383:**347–350.

Söhlemann P, Hekman M, Elce JS, Buchen C, and Lohse MJ (1993) Purification and functional characterization of the β-adrenergic receptor kinase expressed in insect cells. *FEBS Lett* **324:**59–62.

Strader CD, Candelore MR, Hill WS, Sigal IS, and Dixon RAF (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J Biol Chem* **264:**13572–13578.

Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, and Dixon RAF (1988) Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263:**10267–10271.

Strader CD, Sigal IS, Register R, Candelore MR, Rands E, and Dixon RAF (1987) Identification of residues required for ligand binding to the β-adrenergic receptor. *Proc Natl Acad Sci USA* **94:**4384–4388.

Vilardaga J-P, Frank M, Krasel C, Dees C, Nissenson RA, Lohse MJ (2001) Differential conformational requirements for activation of G proteins and regulatory proteins, arrestin and GRK in the G protein-coupled receptor for parathyroid hormone (PTH)/PTH related protein. *J Biol Chem* **276:**33435–33443.

Walseth TF and Johnson RA (1979) The enzymatic preparation of [α-$^{32}$P]nucleoside triphosphates, cyclic [$^{32}$P]AMP and cyclic [$^{32}$P]GMP. *Biochim Biophys Acta* **526:**11–31.

Wang H, Lipfert L, Malbon CC, and Bahouth S (1989) Site-directed anti-peptide antibodies define topography of the β-adrenergic receptor. *J Biol Chem* **264:**14424–14431.

Wieland K, Zuurmond HM, Andexinger S, IJzerman AP, Lohse MJ (1996) Stereospecificity of agonist binding to $\beta_2$-adrenergic receptors involves Asn-293. *Proc Natl Acad Sci USA* **93:**9276–9281.

Whistler JL, Chuang HH, Chu P, Jan LY, and von Zastrow M (1999) Functional dissociation of μ-opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23:**737–746.

Zhou YY, Yang D, Zhu WZ, Zhang SJ, Wang DJ, Rohrer DK, Devic E, Kobilka BK, Lakatta EG, Cheng H, et al. (2000) Spontaneous activation of $\beta_2$- but not $\beta_1$-adrenoceptors expressed in cardiac myocytes from $\beta_1\beta_2$ double knockout mice. *Mol Pharmacol* **58:**887–894.

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