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Similar Structures and Shared Switch Mechanisms of the β_2-Adrenoceptor and the Parathyroid Hormone Receptor

Zn(II) BRIDGES BETWEEN HELICES III AND VI BLOCK ACTIVATION*

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The seven transmembrane helices of serpentine receptors comprise a conserved switch that relays signals from extracellular stimuli to heterotrimeric G proteins on the cytoplasmic face of the membrane. By substituting histidines for residues at the cytoplasmic ends of helices III and VI in retinal rhodopsin, we engineered a metal-binding site whose occupancy by Zn(II) prevented the receptor from activating a retinal G protein, G_t (Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) *Nature* 383, 347–350). Now we report engineering of metal-binding sites bridging the cytoplasmic ends of these two helices in two other serpentine receptors, the β_2-adrenoreceptor and the parathyroid hormone receptor; occupancy of the metal-binding site by Zn(II) markedly impairs the ability of each receptor to mediate ligand-dependent activation of G_s, the stimulatory regulator of adenylyl cyclase. We infer that these two receptors share with rhodopsin a common three-dimensional architecture and an activation switch that requires movement, relative to one another, of helices III and VI; these inferences are surprising in the case of the parathyroid hormone receptor, a receptor that contains seven stretches of hydrophobic sequence but whose amino acid sequence otherwise shows no apparent similarity to those of receptors in the rhodopsin family. These findings highlight the evolutionary conservation of the switch mechanism of serpentine receptors and help to constrain models of how the switch works.

Serpentine receptors are key signaling molecules that relay extracellular signals from hormones and sensory stimuli to heterotrimeric G proteins located on the cytoplasmic face of the plasma membrane. Ligand-activated receptors activate G proteins by promoting exchange of GTP for GDP bound to the α subunit ($G\alpha$) of the heterotrimer, causing liberation of both α-GTP and free $\beta\gamma$ complexes, which in turn activate effector enzymes and ion channels (1, 2). Patterns of conserved amino acid sequence distinguish three separate families of serpentine receptors in mammals; these include the rhodopsin-like receptors, with more than 1000 members, and two smaller families, related to the secretin receptor or to metabotropic glutamate receptors, respectively (3–6). Although the three families share no similarities of primary structure, all members of each family activate heterotrimeric G proteins, and all contain seven stretches of hydrophobic amino acids, which are thought to be folded into a bundle of transmembrane α-helices.

Baldwin *et al*. (7) have proposed a three-dimensional model of the transmembrane helices of receptors in the rhodopsin family. Based on analysis of the amino acid sequences of ~500 rhodopsin-like receptors and guided by a projection density map of frog rhodopsin (8), the model places each individual helix in the density map and specifies its position relative to the lipid bilayer, tilt in the plane of the membrane, and position and orientation relative to other helices. Thus experiments that define distance constraints between specific positions in individual helices can test the model and may enhance its precision. In addition, the model provides a starting point for designing experiments to determine the molecular mechanism by which the helix bundle transmits signals across the membrane from ligand to G protein. In such a mechanism, it seems likely that occupancy of the ligand-binding pocket induces a switch-like movement in the relative positions of two or more helices.

At present we have only a fragmentary notion of how one part of such a receptor switch may work. Site-directed spin labeling experiments with retinal rhodopsin suggest that activation causes the cytoplasmic end of helix VI to move, as a rigid body, away from helix III (9). In accord with this idea, activation of rhodopsin is blocked by either of two kinds of biochemical constraints that prevent movement of helices III and VI relative to one another. These constraints include disulfide bonds (9) or a metal ion bridge (10), engineered by substituting cysteines or histidines, respectively, at appropriate positions in the two helices.

Does ligand-induced separation of helices III and VI play a key role in activation of other serpentine receptors? To answer this question, and to test the generality of the Baldwin-Schertler model, we have constructed Zn(II) bridges connecting the cognate helices of two additional serpentine receptors, the β_2 adrenoreceptor (β_2AR)[1] and the parathyroid hormone recep-

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[1] The abbreviations used are: β_2AR, β_2-adrenoreceptor; PTH, parathyroid hormone; PTHR, parathyroid hormone receptor; G_s, trimeric G protein that stimulates adenylyl cyclase; G_t, trimeric G protein that mediates vision in rod cells (also called transducin); CYP, cyanopindolol; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; TM, transmembrane; CHO, Chinese hamster ovary.

tor (PTHR). The β_2AR, one of the best studied serpentine receptors, belongs to the rhodopsin family but is stimulated by different ligands (norepinephrine or epinephrine, rather than light-activated retinal) and activates a different G protein (G_s rather than G_t). A member of the secretin-like receptor family, the PTHR regulates calcium homeostasis, is stimulated by a polypeptide hormone, and activates both G_s and G_q; its primary structure shows no resemblance to that of either rhodopsin or the β_2AR (11), whereas amino acid sequences of the latter two receptors are 16% identical. Thus the PTHR furnishes an opportunity to probe a distinct family of serpentine receptors, whose evolutionary relation to the rhodopsin family is unknown.

The results of our experiments indicate that both the β_2AR and the PTHR share with rhodopsin a conserved structure and activation mechanism. Thus it is likely that the secretin-like and rhodopsin families evolved from a common serpentine precursor.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium H21, minimal essential medium, and fetal bovine calf serum were obtained from the UCSF Tissue Culture Facility. GTPγS, GDP, leupeptin, and phenylmethylsulfonyl fluoride were purchased from Roche Molecular Biochemicals, isoproterenol, cholic acid, and Lubrol from Sigma, and synthetic bovine PTH-(1–34) from Bachem (Irvine, CA). Nitrocellulose filters and the vacuum manifold used for GTPγS binding assays were from Millipore. The β_2AR antagonist ^{125}I-CYP was obtained from Amersham Pharmacia Biotech and [^{35}S]GTPγS from NEN Life Science Products. HitrapChelate and HitrapQ columns were obtained from Amersham Pharmacia Biotech. $ZnCl_2$ and other materials were of reagent grade and purchased from Sigma or Fisher.

Construction of Receptor Mutants—Point mutations were generated either by Kunkel mutagenesis, as described (12), with a mutagenesis kit (CLONTECH), or by polymerase chain reaction in two steps, using *Pfu* polymerase and wild type receptor cDNA as probes. In the first polymerase chain reaction, we generated overlapping fragments containing the desired mutation and a diagnostic silent restriction site, using wild type cDNA as a template. In the second polymerase chain reaction the generated fragments were annealed by virtue of their overlapping sequence, amplified in the absence of wild type cDNA, and subcloned into the pcDNA1 expression vector containing wild type receptor cDNA. Each mutation was verified by DNA sequencing.

Cell Culture and Transfection—COS-7 and CHO cells were maintained in Dulbecco's modified Eagle's medium H21 or modified Eagle's (minimal essential medium) α medium, containing 10% fetal calf serum, Fungizone, and 10 μg/ml gentamycin. Transient transfections with wild type and mutant receptors were performed using a DEAE-dextran/adenovirus method as described (12).

Membrane Preparation—Membranes from COS-7 or CHO cells, transfected with cDNAs encoding PTHRs or β_2ARs, respectively, were prepared by a modification of a previously described method (12). Briefly, cells were harvested, lysed in a buffer containing 20 mM NaHepes, pH 7.4, with protease inhibitors (phenylmethylsulfonyl fluoride, bacitracin, pepstatin, and leupeptin), and homogenized by passing 12 times through a 27-gauge needle. Membrane fractions were obtained by centrifugation at 4 °C, first at 900 × *g* for 10 min and then at 100,000 × *g* for 30 min. The membranes were stripped of GTP-binding proteins essentially as described (13), by incubation in 6 M urea buffered by 25 mM NaHepes, pH 7.4, for 30 min on ice, followed by sedimentation at 100,000 × *g* for 30 min at 4 °C. After a second urea wash and centrifugation, the membranes were reconstituted in 250 mM sucrose, 5 mM Tris/HCl, pH 7.4, frozen in liquid nitrogen, and stored at −70 °C.

G Protein Purification—α_s was purified from cytosol of Sf9 cells infected with baculovirus encoding the wild type protein, exactly as described (14). In some experiments we used His_6-tagged α_s, purified without detergents in two steps at 4 °C. Sf9 cell cytosol, prepared by nitrogen cavitation, was passed over a nickel-charged HiTrapChelate column (5-ml bed volume), and α_s was eluted with 0.5 M imidazole, followed by chromatography on a HitrapQ column (5 ml bed volume) with a NaCl gradient. Gβγ was purified from Sf9 cells using His_6-tagged α_{i2}, as described (15).

G_s Activation—Exchange of GTPγS for GDP bound to G_s was measured by a modification of a previously described procedure (14, 16). Briefly, membranes containing receptors (~5 nM) were preincubated with purified α_s (50 nM) and βγ (100 nM) for 15 min on ice in a buffer containing 20 mM NaHepes, pH 7.6, 1 mM Tris/HCl, pH 7.6, 100 mM NaCl, 0.1 mM ascorbic acid, 2 mM $MgCl_2$, 1 μM GDP, and 1 mM β-mercaptoethanol. Assays were initiated by addition of agonist and 1 μM [^{35}S]GTPγS (10^5 cpm per tube), in a total volume of 20 μl. After incubation for the indicated times at 30 °C, reactions were terminated by adding 400 μl of ice-cold stop solution containing 20 mM Tris/HCl, pH 8, 100 mM NaCl, and 10 mM $MgCl_2$, and filtered over nitrocellulose membranes on a vacuum manifold; filters were then washed 5 times with 250 μl of stop solution. Radioactivity was quantitated by liquid scintillation in a β-counter. Nonspecific binding (binding to the filter in the absence of membranes) was less than 10% of total binding. Specific binding was defined as the difference between total binding and nonspecific binding.

Ligand Binding—Binding of ^{125}I-cyanopindolol (CYP) was determined as described (17). Binding was initiated by suspending membranes (5 μg of protein in a final volume of 500 μl) in a mixture of ^{125}I-CYP (75 pM), increasing concentrations of isoproterenol, a buffer consisting of 25 mM NaHepes, pH 7.6, 0.05% (w/v) bovine serum albumin, and 0.1 mM ascorbic acid. Zn(II) (10 μM) and GTPγS (30 μM) were present or absent, as indicated. Nonspecific binding was assessed in the presence of 10 μM isoprotenerol. Reactions were conducted for 45 min at 30 °C, stopped by adding 2 ml of ice-cold binding buffer, and filtered over Whatman GF/C filters. Membranes used for binding assays were not subjected to washes with urea.

Alignments Using the Evolutionary Trace Method—In the absence of recognizable sequence identity, the evolutionary trace method can be used to align positions so as to match their functional importance during evolution.[2] Here, 58 animal visual opsins, 56 adrenergic receptors, and 33 members of the secretin-like family were gathered from Swiss-Prot version 34.0. These sequences are shown in Table I. The seven transmembrane regions, recognizable by their hydrophobicity, were excised from the rhodopsin and adrenergic receptors. PILEUP (from the GCG8.0 Wisconsin Sequence Analysis Package) then produced an alignment and a sequence identity dendrogram of each helix. The evolutionary trace computed for each position of each helix its evolutionary rank, which is the minimum number of branches that span the dendrogram so that this position is invariant in each branch. This ranking measures the functional importance of this position during evolution relative to the other positions in the multiple sequence alignment (18). The same procedure was followed with sequences from the secretin-like receptor family, except that, in the absence of a clearly defined alignment to the other receptors, nine possible alignments were considered for each transmembrane helix, each shifted one residue further toward the C terminus. Table II shows the alignments for PTHR helices III and VI that showed, in comparison to the other eight alignments, the highest non-parametric (Pearson) correlation of ranks between the secretin family transmembrane helices and those from the opsin and adrenergic receptors.

RESULTS

Receptor Activation Assay—To assess activation of G_s by wild type and mutant receptors, we measured ligand-dependent binding of radioactive GTPγS in a mixture containing pure α_s, pure βγ, and urea-washed membranes from cells expressing the recombinant receptor. Washing the membranes with urea, a procedure modified from previous assays (13, 19, 20), removes contaminating GTP-binding proteins from the membrane preparation without inactivating the recombinant serpentine receptors. We expressed wild type and mutant versions of the PTHR and the β_2AR in COS-7 cells and CHO K1 cells (which lack the endogenous β-adrenoreceptors present in COS-7 membranes), respectively.

In the β_2AR assay, GTPγS binding required the presence of receptor, α_s, βγ, and the agonist, isoproterenol (Fig. 1*A*). The effect of isoproterenol was rapid (complete within 3 min; Fig. 1*B*), saturable by increasing concentrations of α_s (Fig. 1*C*) or βγ (not shown), and dependent on concentration (Fig. 1*D*); the EC_{50} for isoproterenol, 68 nM, is comparable to values reported previously (*e.g.* Ref. 17). Isoproterenol increased GTPγS binding 5–20-fold in different experiments; at maximal stimulation,

[2] O. Lichtarge, A. Philippi, R. L. Dunbrack, S. R. Coughlin, H. R. Bourne, and F. E. Cohen, manuscript in preparation.

TABLE I
Receptors used in the evolutionary trace alignments

Opsins	Adrenergic	Secretin-like	No.
OPS1_CALVI	A1AA_HUMAN	CALR_HUMAN	1
OPS1_DROME	A1AA_RAT	CALR_PIG	
OPS1_DROPS	A1AB_HUMAN	CALR_RAT	
OPS1_LIMPO	A1AB_MESAU	CRF2_RAT	
OPS2_DROME	A1AB_RAT	CRFR_HUMAN	
OPS2_DROPS	A1AC_BOVIN	CRFR_MOUSE	
OPS2_LIMPO	A1AC_HUMAN	CRFR_RAT	
OPSB_ANOCA	A1AC_RAT	GIPR_HUMAN	
OPSB_ASTFA	A2AA_HUMAN	GIPR_MESAU	
OPSB_BOVIN	A2AA_MOUSE	GIPR_RAT	10
OPSB_CARAU	A2AA_PIG	GLPR_HUMAN	
OPSB_CHICK	A2AA_RAT	GLPR_RAT	
OPSB_GECGE	A2AB_HUMAN	GLR_HUMAN	
OPSB_HUMAN	A2AB_MOUSE	GLR_RAT	
OPSB_MOUSE	A2AB_RAT	GRFR_HUMAN	
OPSD_ALLMI	A2AC_DIDMA	GRFR_MOUSE	
OPSD_ANOCA	A2AC_HUMAN	GRFR_PIG	
OPSD_ASTFA	A2AC_MOUSE	GRFR_RAT	
OPSD_BOVIN	A2AC_RAT	PACR_HUMAN	
OPSD_CANFA	A2AD_HUMAN	PACR_RAT	20
OPSD_CARAU	A2AR_CARAU	PTR2_HUMAN	
OPSD_CHICK	AA2A_CANFA	PTRR_DIDMA	
OPSD_CRIGR	B1AR_HUMAN	PTRR_HUMAN	
OPSD_CYPCA	B1AR_MACMU	PTRR_MOUSE	
OPSD_HUMAN	B1AR_MELGA	PTRR_RAT	
OPSD_LAMJA	B1AR_MOUSE	PTTR_PIG	
OPSD_LOLFO	B1AR_RAT	SCRC_HUMAN	
OPSD_MOUSE	B2AR_CANFA	SCRC_RAT	
OPSD_OCTDO	B2AR_HUMAN	VIPR_HUMAN	
OPSD_POMMI	B2AR_MESAU	VIPR_RAT	30
OPSD_PROCL	B2AR_MOUSE	VIPS_HUMAN	
OPSD_RABIT	B2AR_RAT	VIPS_MOUSE	
OPSD_RANCA	B3AR_BOVIN	VIPS_RAT	
OPSD_RANPI	B3AR_HUMAN		
OPSD_RAT	B3AR_MOUSE		
OPSD_SHEEP	B3AR_RAT		
OPSD_SPHSP	B4AR_MELGA		
OPSD_TODPA	D1DR_CARAU		
OPSD_XENLA	D3DR_CERAE		
OPSG_ASTFA	D3DR_HUMAN		40
OPSG_CARAU	D3DR_MOUSE		
OPSG_CHICK	D3DR_RAT		
OPSG_GECGE	D4DR_RAT		
OPSG_HUMAN	DADR_DIDMA		
OPSH_ASTFA	DADR_HUMAN		
OPSH_CARAU	DADR_PIG		
OPSI_ASTFA	DADR_RAT		
OPSL_CALJA	DADR_XENLA		
OPSP_CHICK	DBDR_RAT		
OPSP_COLLI	DBDR_XENLA		50
OPSR_ANOCA	DOPR_DROME		
OPSR_ASTFA	HH1R_CAVPO		
OPSR_CARAU	HH1R_RAT		
OPSR_CHICK	HH2R_CANFA		
OPSR_HUMAN	HH2R_CAVPO		
OPSU_BRARE	HH2R_RAT		
OPSV_CHICK			
OPSV_XENLA			58

radioactive GTPγS bound to 10–30% of the total α_s present in the assay. Concentrations of α_s and $\beta\gamma$ required in the PTHR assay (not shown) were similar to those in the β_2AR assay, but a longer time (10 min) was required for maximal PTH-dependent binding of GTPγS.

Choosing Sites for Histidine Substitutions—In the β_2AR and PTHR we sought to reproduce metal-binding sites cognate to the site we had constructed in rhodopsin (10). To do so, we used the alignments of helix III and VI amino acid sequences shown in Table II. Similar primary structures made it straightforward to align these segments of the β_2AR with those of rhodopsin. In TM III of the β_2AR we substituted a histidine for Ala-134, which is cognate in sequence to Val-138 of rhodopsin, a position that participated in the helix III–helix VI metal ion-binding site we created in that receptor (10). In TM VI, we substituted histidine, in separate mutant receptors, for each of six consecutive amino acids. These six residues (Table II) cover more than a full turn of the putative α helix; they include Leu-272, which is cognate to the position in helix VI of rhodopsin (residue 251) at which a substituted histidine participated in the engineered metal ion-binding site (10).

In the absence of obvious similarities of amino acid sequence, aligning the PTHR and rhodopsin sequences (Table II) was more difficult. We based this alignment on an evolutionary trace analysis (18, 21), as described under "Experimental Procedures." This method has already successfully identified Gα surfaces that interact with $\beta\gamma$ and with serpentine receptors (21, 22). Briefly, the analysis assumes that shared structures

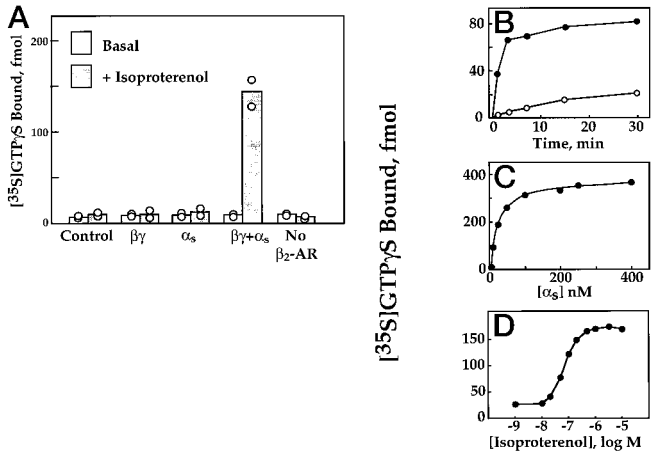

FIG. 1. **β_2AR-dependent activation of G_s in a reconstituted system.** Urea-washed membranes from CHO cells expressing the recombinant β_2AR were treated with or without isoproterenol in the presence of G_s subunits, and binding of [^{35}S]GTPγS to α_s was measured, as described under "Experimental Procedures." *A,* binding of [^{35}S]GTPγS after incubating membranes for 4 min at 30 °C in the presence or absence (as indicated) of isoproterenol (1 μM), α_s (50 nM), and βγ (100 nM); the concentration of β_2AR (assessed by binding radioactive antagonist) was 5 nM, except for the two columns on the *right,* which represent incubations of membranes from cells not expressing the β_2AR. The total volume of each incubation was 20 μl. The *bars* represent means of duplicate determinations, shown in *circles*. This experiment is representative of four separate experiments using two different membrane preparations. *B–D,* binding of [^{35}S]GTPγS under conditions identical to those described for *A*, except that incubations were conducted for different times (*B*), at different concentrations of α_s (*C*), or at different concentrations of isoproterenol (*D*). Values in *B* represent determinations in the presence or absence of 1 μM isoproterenol (*filled* and *open symbols,* respectively). *C,* the concentrations of isoproterenol (1 μM), β_2 adrenoreceptors (5 nM), and Gβγ (250 nM) were fixed.

and molecular mechanisms dictate similarly located interfaces between helices and therefore similar patterns of functionally important residues in each helix; this should be true even if the sequences themselves show no identical amino acids. The analysis (to be described in detail elsewhere)[2] identified apparent functionally important positions (as indicated by patterns of sequence conservation) in serpentine receptors related to rhodopsin and compared distributions of these positions to those of similarly important positions in receptors related to the PTHR. The evolutionary trace approach revealed putative structural and functional similarities between the rhodopsin and secretin-like receptor families. Based on the analysis, we substituted histidines for Leu-303 in helix III and at each of six consecutive positions in helix VI of the PTHR (Table II). In addition, we tested the Zn(II) sensitivity of mutants containing the histidine at position 301 in helix III of the PTHR (Table II).

Zn(II) Sensitivity of β_2AR Receptors—To our surprise, a relatively low concentration of Zn(II) (IC_{50} ~10 μM) inhibited the ability of the wild type β_2AR to activate G_s (Fig. 2). This suggested that the wild type receptor contains a cryptic endogenous site where Zn(II) can bind and block activation. One partner in such a site may be a naturally occurring histidine (His-269) in helix VI (see Table II). Several observations suggest that this residue participates in a metal ion-binding site. Replacement of His-269 by an alanine reduced the sensitivity of the receptor to inhibition by Zn(II); moreover, substitution of a histidine for Ala-134 in helix III increased the Zn(II) sensitivity of the receptor containing His-269 ~10-fold (Fig. 2 and Table III). A 30-fold higher Zn(II) concentration was required for half-maximal inhibition of the control receptor, containing the histidine substituted in helix III but lacking histidine at position 269 in helix VI (Table III). Taken together, these observations indicate that histidines in helices III and VI can form a Zn(II) bridge that inhibits activation by the β_2AR and that the metal-binding site is in fact tridentate, involving an unidentified third amino acid (see below) somewhere nearby.

We next tested the abilities of histidines placed at successive positions around helix VI to cooperate with the histidine substituted at position 134 in helix III (Fig. 2 and Table III). Zn(II) blocked receptor activation (IC_{50} ~1 μM) when the histidine in helix III (*red* in Fig. 3*A*) was paired with a histidine at position 268, 269, or 272 (*yellow* in the same figure). Relative to a receptor lacking His-269 and the substituted histidine in helix III, a histidine at position 268 or 272 in helix VI produced a receptor with intermediate sensitivity to inhibition by Zn(II) (IC_{50} ~10 μM; Table III); this suggests that residues at all three positions (268, 269, and 272) may cooperate with the same cryptic residue to produce a bidentate metal-binding site with intermediate Zn(II) binding affinity. In contrast, a histidine at each of the other positions (270, 271, or 273; *green* in Fig. 3*A*) produced a receptor that was quite insensitive to Zn(II) inhibition (IC_{50} ~30 μM; Table III), suggesting that these positions cannot cooperate either with the cryptic residue or with the histidine at position 134 of helix III to form a metal-binding site. According to the Baldwin-Schertler model, positions 268, 269, and 272 are clustered on one side of helix VI (Fig. 3*A*). Moreover, histidines at positions 268 and 272 would occupy locations one turn apart in an α-helix, in keeping with evidence (23) that the cytoplasmic end of helix VI in rhodopsin projects as an α-helix beyond the sequence that is buried in the lipid core of the membrane.

We have not identified the cryptic third member of this putative tridentate binding site. A likely possibility is Asp-130, which forms part of a highly conserved DRY/ERY motif in helix III; Asp-130 is one turn (4 residues) away from the site of our histidine substitution (position 134).

Zn(II) Sensitivity of PTH Receptors—We first probed the PTHR with histidines substituted at positions that correspond (according to the evolutionary trace analysis) to those we mutated in the β_2AR, that is Leu-303 in helix III and six positions in helix VI. Each of the helix III–helix VI double histidine mutants activated G_s poorly, however, even in the absence of Zn(II) (results not shown). To our surprise, each of the helix VI substitutions by itself (in the absence of a substitution at position 303 in helix III) produced a functioning receptor that was inhibited by Zn(II). The susceptibility to Zn(II) turned out to depend on a histidine residue naturally present in helix III of the PTHR, at position 301. Replacement of the histidine at position 301 by alanine produced receptors whose sensitivity to Zn(II) was unaffected by histidines substituted at any of the six positions in helix VI (Table III). The Zn(II) sensitivity of this H301A mutant (lacking histidines substituted into helix VI) was identical to that of the wild type PTHR, that is, to that of a receptor with a histidine at position 301 but no histidine at any of the six positions in helix VI (Table III).

From these results we infer that a histidine at position 301 in helix III can partner with histidines at six different positions in helix VI to form metal-binding sites that inhibit the ability of the receptor to activate G_s. Table III shows that the Zn(II) sensitivity of two of these putative metal-binding sites is much greater than those of others: combinations of His-301 in helix III with histidines at positions 401 or 402 in helix VI created receptors that were 20- or 80-fold more sensitive to Zn(II) than the wild type receptor (Table III and Fig. 2).

Fig. 3*B* depicts the Baldwin-Schertler receptor model, highlighting the predicted positions of histidines that create metal-binding sites in the PTHR. In the model the key histidine (*red*) in helix III of the PTHR, His-301, is located on the opposite face

TABLE II

Alignments of bovine rhodopsin, the human β_2AR, and the opossum PTHR

The evolutionary trace method (18) was used to align the sequences of amino acid residues in helices III and VI, as described under "Experimental Procedures." Underlined residues are those we replaced by histidines; boldface type indicates residues that are identical in two or more of the sequences.

Helix III					
Rhodopsin	108	T**GC**NLEGFFA	TLGGEIALWS	L**V**V**L**A**I**E**RY**V	V̲VCK**P**
β_2AR	104	FW**C**EFWTSID	VLCV**T**ASIET	L**C**V**I**A**V**D**RY**F	A̲**I**TS**P**
PTHR	273	V**GC**RVAVTVF	LYFL**T**TNYYW	ILV̲EGLYL̲HS	L̲**I**FMA
Helix VI					
Rhodopsin	246	A**E**KEVT̲RMVI	**I**MVIA**F**LI**CW**	**LP**YAG**V**AFYI	F
β_2AR	267	K**E**H̲K**A**L̲K̲TLG	**I**IMGT**F**TLC**W**	**LPFFIV**NIVH	**V**
PTHR	396	Q̲YR̲K̲L̲L̲K̲STL	VLMPL**F**GVHY	IV**F**MATPYTE	**V**

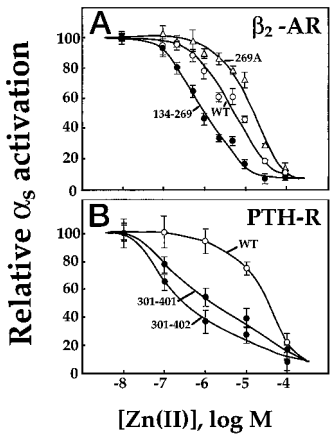

FIG. 2. **Effect of different concentrations of Zn(II) on G_s activation (assessed by binding of [^{35}S]GTPγS) stimulated by wild type and mutant β_2AR (*top*) and PTHR (*bottom*).** Concentration of [^{35}S]GTPγS, receptors, α_s, and $\beta\gamma$ are described under "Experimental Procedures." The concentrations of both isoproterenol and PTH were 1 μM. *Circles* and *bars* represent means ± S.E. of 3–5 separate experiments.

of this helix from the position that creates a Zn(II)-binding pocket in the β_2AR (position 134 in Fig. 3*A*); as noted above, substitution at a different site was necessary because histidine at position 303 inactivated the PTHR. Nonetheless, the ability of His-301 to form Zn(II)-binding sites in cooperation with histidines in helix VI agrees both with the Baldwin-Schertler model and with the alignment of PTHR sequence with those of receptors in the rhodopsin family. This is because the α-carbon of each histidine substituted in helix VI would be almost equidistant in the model from α-carbons at either position 301 or 303 of helix III.

Fig. 3 highlights an apparent difference between the β_2AR and the PTHR. All six histidines in helix VI of the PTHR enhanced its sensitivity to inhibition by Zn(II) (Fig. 3*B*), whereas in the β_2AR only three of the six histidine residues tested did so (Fig. 3*A*). This may indicate that the cytoplasmic end of helix VI in the PTHR is more mobile than its counterpart in the β_2AR. It should be pointed out, however, that substitutions at two of the helix VI sites in the PTHR (indicated by *yellow balls* in Fig. 3*B*) produce receptors that are much more sensitive to inhibition by Zn(II). One of these, at position 401, is precisely cognate to the position in the β_2AR where histidine substitution (L272H) induced the greatest sensitivity to Zn(II); in rhodopsin a histidine substituted (T251H) at the corresponding position also produced a Zn(II)-sensitive receptor (10).

Agonist Binding Affinity of Wild Type and Mutant β_2ARs—Interaction of G_s with the β_2AR is known to enhance the receptor's affinity for agonists (for review, see Ref. 24), presumably via a conformational change transmitted to the ligand-binding pocket from the G protein-binding cytoplasmic surface of the receptor. The effect of G_s on agonist binding affinity can be reversed by adding a GTP analog, such as GTPγS, as shown for the β_2AR(H269A) mutant in Fig. 4; this reversal is thought to reflect GTP-induced dissociation of α_s from $\beta\gamma$ and of both α_s-GTP and $\beta\gamma$ from the receptor. If occupancy of the Zn(II) metal-binding site in appropriate β_2ARs prevents them from interacting with G_s, Zn(II) should partially or completely mimic the effect of GTPγS. This prediction was not fulfilled (Fig. 4). In the absence of GTPγS, addition of Zn(II) (10 μM) caused small increases (~2-fold) in the apparent agonist binding affinities of both the β_2AR(H269A) mutant and the β_2AR(H134/H269) double mutant; addition of GTPγS caused equivalent decreases in agonist binding affinities of both mutant receptors, measured either in the absence or presence of 10 μM Zn(II), despite the ~30-fold difference in sensitivity of the same receptors to inhibition by Zn(II) (Table III). From these results we infer that occupancy by Zn(II) of the metal-binding site does not prevent the receptor from associating with G_s, although it does block agonist-dependent activation of G_s by the receptor.

It is worth noting that failure of Zn(II) to affect agonist binding affinity of the β_2AR(H134/H269) double mutant rules out the possibility that the cation inhibits stimulation of G_s by denaturing the receptor.

DISCUSSION

In these experiments we engineered potential metal-binding sites into two serpentine receptors as probes for elucidating the structure and molecular mechanism of the receptor switch. This approach, which has been applied to serpentine receptors (25–27) and many other proteins (28), depends upon the ability of Zn(II) (or certain other metals) to be chelated by side chains of two or more amino acids in a protein. Because the imidazole group of histidine chelates metals rather well, potential Zn(II) bridges are often constructed by substituting histidines at appropriate positions in a mutant protein. A Zn(II) bridge between appropriately oriented histidines in separate structural elements of the protein will link the two elements together. If Zn(II) inhibits a function of such a mutant protein, we infer that normal function requires movement of one or both of the two structural elements, relative to the other. For this inference to be valid, Zn(II) must inhibit function of the mutant protein at a considerably lower concentration than that required to inhibit function of the wild type protein; moreover, neither histidine substitution should mediate the Zn(II) effect on its own, and the histidine substitutions should not alter function of the mutant protein in the absence of Zn(II). Our results meet these criteria.

From the effects of Zn(II) bridges in the β_2AR and the PTHR, we infer that helices III and VI of each receptor lie close to one another and also that the two helices must move, relative to one another, for the receptor to activate the G protein. These inferences extend our understanding of the serpentine receptor switch in four ways. First, experiments with the β_2AR confirm

TABLE III

Effects of Zn(II) on activation of G_s by wild type and mutant receptors

Ligand-stimulated activation of G_s was assessed by measuring the specific binding of [^{35}S]GTPγS to recombinant α_s, as described under "Experimental Procedures." Results indicate the maximal ligand-induced binding of [^{35}S]GTPγS (fmol per tube) and the Zn(II) concentration that inhibited activation by 50% (IC_{50}, μM). Data for each receptor construct represent mean values from 3 to 12 separate experiments, conducted on 2–6 different membrane preparations. Distances between the α-carbons of mutated residues are those specified in the Baldwin-Schertler model (7).

TM-III			TM-VI						
	Maximum	IC_{50}	Mutation	α-Carbon distance	134A		134H		IC_{50} ratio
					Maximum	IC_{50}	Maximum	IC_{50}	
	fmol	*μM*		*Å*	*fmol*	*μM*	*fmol*	*μM*	
β_2-Adrenoceptor									
134H	154	23.1	H269A		169	32	163	29	1.1
			E268H	8.69	126	9.1	178	0.9	10
			H269	10.3	212	7.5	144	0.7	11
			K270H	12.6	81	27	157	25	1.1
			A271H	10.3	93	31	143	28	1.1
			L272H	8.76	154	8.2	101	0.7	12
			K273H	12.48	137	30	149	32	0.9

TM-III			TM-VI						
	Maximum	IC_{50}	Mutation	α-Carbon distance	301A		301H		IC_{50} ratio
	fmol	*μM*		*Å*	*fmol*	*μM*	*fmol*	*μM*	
PTH receptor									
301H	104	32	Y397H	10.7	84	39	101	11	3.5
			R398H	10.7	73	27	114	9.6	2.8
			K399H	12.5	52	33	94	8.8	3.8
			L400H	10.4	89	38	132	7.2	5.3
			L401H	7.33	65	26	143	1.3	20
			K402H	10.3	47	25	76	0.3	83

that the Baldwin-Schertler structural model, based on density maps made from rhodopsin, applies to other receptors in the rhodopsin family of serpentine receptors. Second, experiments with the PTHR show that a member of the secretin-like family of serpentine receptors shares a common three-dimensional structure and activation mechanism with receptors in the rhodopsin family. Third, our results confirm the notion (10, 29) that ligand-induced movement of helix VI relative to helix III is necessary for the receptor to catalyze efficient replacement by GTP of GDP bound to the G protein. Finally, however, the surprising failure of Zn(II) bridges to alter the agonist binding affinity of β_2AR mutants (Fig. 4) indicates that relative movement of helix VI *versus* helix III is not required for the G protein to associate with the receptor and to regulate the affinity of its ligand-binding site.

A Test of the Baldwin-Schertler Model—In known three-dimensional structures, α-carbons of histidines that form Zn(II) bridges are found no more than 13 Å apart (30, 31). The Baldwin-Schertler model (7) predicts that the α-carbon of the amino acid (Ala-134) replaced by histidine in helix III of the β_2AR (Fig. 3) lies within 8–10.3 Å of the α-carbons of helix VI positions at which a second histidine substitution makes β_2AR(A134H) sensitive to inhibition by Zn(II). Although details of the coordination of the metal ion remain to be elucidated, these results confirm a key prediction of the model, that the cytoplasmic ends of helices III and VI are located relatively close to one another. Abundant evidence has already indicated proximity of the cytoplasmic ends of helices III and VI in retinal rhodopsin; this evidence includes the Zn(II) bridge we engineered into rhodopsin (10), distances between the helices as assessed with site-directed spin labels (9), and formation of disulfide bonds between cysteines substituted into the same regions of these helices (9).

These geometrical constraints constitute useful tests of the Baldwin-Schertler model. This is because projection density maps obtained from electron cryomicroscopic studies of rhodopsin (8) indicate probable locations and tilts of α-helices, but the low resolution of these maps does not make it possible to identify a specific density with a specific helix or to know whether the density map is being viewed from the cytoplasmic or the extracellular side. The Baldwin-Schertler model, however, does identify the helices and predicts that they are arranged in a clockwise fashion, as viewed from the cytoplasm (7, 32). Effects of Zn(II) sites on the β_2AR mutants, along with earlier biochemical studies of rhodopsin (9, 10), support the predicted proximity of helices III and VI as well as the predicted clockwise arrangement. Baldwin identified helix III with the centrally located density of the rhodopsin map (32), based on its amino acid sequence; among all the helices, the hydrophilic and conserved residues of helix III show the least tendency to be distributed on one face; this is the pattern expected of a helix in the center of the helix bundle, where it must interact with other helices on all sides. If the central density corresponds to helix III, the Zn(II) bridges indicate a clockwise arrangement of the helices; this is because in the opposite (counterclockwise) arrangement, helix VI would be too far (25 Å) from helix III for formation of a Zn(II) bridge (not shown).

Other data can similarly be explained only if the helices are ordered in a clockwise fashion. In the tachykinin NK-1 receptor, agonist binding was blocked by either of two bidentate Zn(II) sites on the extracellular side of the helix bundle (between helix III and helix II or helix V) (26); formation of these Zn(II)-binding sites would have been much less likely in a counterclockwise helix bundle. In addition, functional folding of chimeric muscarinic receptors required alteration of threonine residues in helices I and VII, suggesting that these residues meet at an interface between these two helices (33).

The Zn(II) bridges we engineered into the β_2AR involve positions in helices III and VI cognate to positions that participated in function-inhibiting Zn(II) bridges (10) engineered into retinal rhodopsin. Thus it is likely that these two receptors share both a similar three-dimensional architecture and a highly conserved activation switch, even though they stimulate different G proteins, G_s and G_t, respectively. By extension, this architecture and switch mechanism are probably common to all receptors in the rhodopsin family. The same overall inference

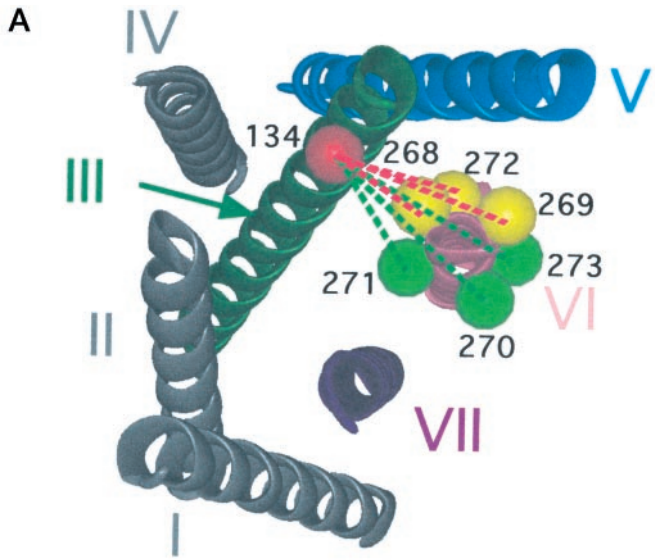

FIG. 3. **Mutated positions and Zn(II) bridges.** *Ribbons* represent the seven helices of serpentine receptors in the rhodopsin family, according to the Baldwin-Schertler model (7). *Balls* represent β-carbons of the positions where histidine substitutions were tested for their ability to participate in Zn(II) bridges. *A,* β_2AR, in which a histidine at position 134 (*red*) in helix III formed a Zn(II) bridge with histidines at each of three positions (*yellow*) in helix VI, but did not form bridges with three other substituted histidines (*green*) in helix VI. *B,* PTHR, in which a histidine at position 301 (*red*) in helix III formed a Zn(II) bridge with histidines at each of six positions in helix VI; two helix VI positions (*yellow*) formed bridges at especially low Zn(II) concentrations, whereas four (*green*) formed bridges at intermediate Zn(II) concentrations.

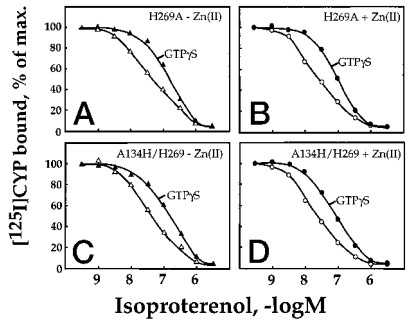

FIG. 4. **Competition between isoproterenol and ^{125}I-cyanopindolol (*CYP*) for binding to the β_2AR(H269A) (*A* and *B*) or the β_2AR(A134H/269H) mutant (*C* and *D*).** Membranes of CHO cells expressing the wild type or mutant receptor were incubated, as described (14), with ^{125}I-CYP (75 pM) and the indicated concentrations of isoproterenol for 45 min, in the presence (*circles, B* and *D*) or absence (*triangles*, *A* and *C*) of 10 μM Zn(II) and in the presence or absence of 30 μM GTPγS (*filled* and *open symbols*, respectively).

can be drawn from the effects of engineered metal-binding sites on the extracellular sides of two other receptors in this family (25–27); the engineered metal ion-binding site between helices V and VI on the extracellular side of the tachykinin NK-1 receptor (25, 26) was duplicated by mutations at cognate positions in the same helices of the κ-opioid receptor (27).

Although the geometric constraints imposed by histidine-histidine Zn(II) bridges (30, 31) confirm the low resolution model (7) proposed by Baldwin and colleagues, they do not enhance its precision. The helix VI substitutions that did enhance sensitivity appear to be situated on one face of the helix (Fig. 3), as the model would predict. The low "resolution" of these experiments, however, is evident from comparing the effects of Zn(II) on mutant β_2ARs *versus* the predicted α-carbon distances between the positions substituted in helix III and helix VI. The model shows short α-carbon distances (~8.7 Å) for two of the mutants that were strongly inhibited by Zn(II) and considerably longer distances (~12.5 Å) for two of the mutants without enhanced sensitivity to inhibition by Zn(II); the model predicted an intermediate distance (10.3 Å) for the other two mutants, only one of which showed enhanced sensitivity to Zn(II).

The PTHR Versus Rhodopsin and the β_2AR—In mammals, the receptors coupled to G proteins can be grouped into three families, which resemble, respectively, rhodopsin, the secretin receptor, and "metabotropic" receptors for glutamate (4–6, 32). Within each of these families, deduced amino acid sequences of individual receptors show clear-cut patterns of conserved and identical amino acids. No such sequence conservation or identity has been found in comparisons of receptors in any one of the three families *versus* receptors in another. Receptors in all three families do, however, exhibit seven stretches of hydrophobic amino acids, each of which is thought to constitute an α-helix that crosses the plasma membrane (4–6, 32).

The shared seven-helix topology suggests the hypothesis that all three receptor families evolved from a common precursor and share a common three-dimensional architecture and mechanism for transducing signals from the agonist-binding site to the G protein. Based on this hypothesis we used the evolutionary trace approach (18) to align sequences in the secretin-like receptor family with apparently cognate positions in helices III and VI of the rhodopsin family (for details, see "Experimental Procedures" and "Results"). As an initial test of the hypothesis, we compared effects of metal-binding sites engineered into a member of each family, the PTHR and the β_2AR, respectively. The results suggest that these two receptors and, by extension, the two receptor families do share a common origin in evolution, as well as similar three-dimensional architectures and switching mechanisms.

The effects of Zn(II) on the PTHR mutants indicate that the cytoplasmic ends of helices III and VI are close to one another in this receptor, much like the corresponding helices of the β_2AR and other receptors in the rhodopsin family. Indeed, the "strongest" Zn(II) bridges (those at which the lowest Zn(II) concentrations inhibit G protein activation) link positions that would be quite close to one another in a PTHR that conforms to the Baldwin-Schertler model and the alignment based on the evolutionary trace approach, that is, α-carbon distances between position 301 in helix III and positions 401 or 402 in helix VI are 7.3 or 10.3 Å, respectively (Table III); these are within the range of distances (30, 31) that allow histidine residues to

form metal-binding sites. In comparison to the wild type PTHR, histidine substitutions at these two positions in helix VI markedly enhanced Zn(II) sensitivity (20- and 83-fold, respectively). Substitutions at the other four positions tested in helix VI also increased Zn(II) sensitivity, although to a lower degree (3.5–5.3-fold; Table III). We do not know how histidines at six positions around the entire circumference of helix VI can form Zn(II) bridges with a single histidine in helix III. Indeed, residues 397–402 in the PTHR may not belong to an α-helix at all; alternatively, the putative helix may be unusually flexible. The latter possibility is consistent with a rhodopsin experiment (9), in which a cysteine at each of five positions in helix VI could form a disulfide bond with a cysteine in helix III.

Overall, Zn(II) sensitivities of the PTHR mutants generally agree with the Baldwin-Schertler model, which would predict distances between the appropriate positions ranging from 7.3 to 12.5 Å (see Table III). Accordingly, we propose that receptors in the secretin receptor-like family share the overall three-dimensional architecture of receptors in the rhodopsin family. Extension of the model to this second family of receptors was not anticipated (7) but is in keeping with the basic topology shared by the two families and with their apparently similar signaling functions.

Donnelly (4) has proposed a molecular model for the transmembrane helices of another member of the secretin-like receptor family, the glucagon-like peptide 1 receptor. In this model, as compared with the Baldwin-Schertler model, helix III is less buried within the helix bundle; helix VII, lying closer to the receptor core, is located between helices III and VI. Donnelly's tentative model, which includes loops and tilts of helices, predicts much longer α-carbon distances between positions 301 (helix III) and positions 401 or 402 (helix VI) of the PTHR, 20.73 and 20.71 Å, respectively. These distances substantially exceed those that allow histidines to form effective metal ion-binding sites (28). Consequently, our results are much more congruent with the Baldwin-Schertler model than with that of Donnelly.

Functional Role of Helices III and VI in Activating the G Protein—How does Zn(II) prevent receptors with the appropriate histidine substitutions from activating the G protein? A straightforward interpretation, previously applied to the inhibitory effect of a cognate Zn(II) bridge in rhodopsin (10), is that the Zn(II) ion prevents movement of helix VI relative to helix III; the relative motion of the two helices results from activation of the serpentine receptor switch and is necessary for effective catalysis of GDP-GTP exchange on the G protein. This notion is strongly supported by results of site-directed spin labeling experiments (9), which were interpreted as showing that photo-excitation of rhodopsin causes the cytoplasmic end of helix VI to move 10–15 Å away from helix III and to rotate on its own axis, in a clockwise direction.[4]

Our speculative extension of this scenario (29) includes two additional inferences: the stimulus-induced separation of helix VI from helix III opens a cleft or pocket in the cytoplasmic surface of the receptor, and occupancy of the cleft by the C-terminal tail of the G protein's α subunit is required for the receptor to catalyze GDP-GTP exchange. The first of these additional inferences implies that the Baldwin-Schertler model represents the inactive conformation of serpentine receptors, and is in keeping with both the spin labeling results and our experiments with metal-binding sites. The second inference, that helix VI interacts specifically with the C terminus of Gα, was suggested by results of an experiment (34) that tested the ability of chimeric muscarinic receptors to interact with chimeric Gα subunits; a 4-residue epitope in helix VI functionally complemented a similarly short sequence in the Gα C terminus. One of the complementing receptor residues in that study (34) is cognate to residue 272 of the β_2AR, a position at which substitution of a second histidine made the β_2AR(A134H) mutant susceptible to inhibition by Zn(II) (Table III); this raises the possibility that a Zn(II) bridge may inhibit G protein activation not only by immobilizing helix VI relative to helix III but also by steric hindrance.

Agonist Binding Affinities of Mutant β_2ARs—Surprisingly, Zn(II) failed to reduce the agonist binding affinity of the histidine-substituted β_2AR mutants whose ability to activate G_s was sensitive to inhibition by Zn(II) (Fig. 4). We had expected the contrary result, based on the scenario for G protein activation described above, in combination with a number of observations in many laboratories. Thus in the absence of added guanine nucleotide the β_2AR (like many other receptors) exhibits an enhanced affinity for binding agonists (reviewed in Ref. 24). This high affinity is thought to result from association of the receptor with the appropriate G protein, because addition of GTP analogs reduces agonist binding affinity and at the same time causes G protein α and $\beta\gamma$ subunits to dissociate from each other and from the receptor. The affinity of the β_2AR for agonists is similarly low in cells genetically lacking the α subunit of G_s (35, 36). In contrast, neither GTP analogs nor genetic absence of the G protein affect the receptor's affinities for binding pharmacological antagonists. The ability of the G protein to enhance binding of agonists, but not antagonists, suggests a reciprocal interaction between the receptor-G protein interface and the agonist-binding pocket. A similar reciprocity is thought to account for the ability of retinal transducin (G_t) to stabilize a spectral form of photorhodopsin called metarhodopsin II (see references in Ref. 10); that is, hormone and light induce a conformational change that enhances affinity of their respective receptors for binding G proteins, and G proteins reciprocate by enhancing stability of this agonist-bound "activated" conformation of the receptors. For this reason, we had expected Zn(II) bridges to prevent G_s from increasing the affinity of the β_2AR for binding agonist, just as the same bridges inhibited agonist-induced activation of G_s.

Instead, Zn(II) had little or no effect on agonist binding affinity (Fig. 4); wild type and mutant receptors showed nearly identical affinities for binding isoproterenol, whether or not Zn(II) was present. Moreover, a GTP analog reduced isoproterenol binding affinity to the same degree in both wild type and mutant receptors, again in a fashion that was unaffected by Zn(II). Thus transmission of conformational change from the G protein to the ligand-binding site is unaffected by the same Zn(II) bridges that inhibit transmission of conformational change in the other direction, from the ligand-binding site to the G protein. Moreover, the Zn(II) bridges prevent the G protein from activating the receptor but *not* from interacting with the receptor.

Can these asymmetric effects of Zn(II) tell us something useful about how the receptor switch works? If Zn(II) bridges prevent movement of helix VI relative to helix III, as described above, then this movement does not mediate the effect of G protein on agonist binding affinity. For example, Zn(II)-induced immobilization of helices III and VI may prevent the receptor from promoting release of GDP from the G protein trimer, whereas the GDP-bound form of the trimer interacts with a separate site on the cytoplasmic face of the receptor to initiate the conformational change that enhances agonist binding affinity. In this regard, several rhodopsin mutants furnish an instructive precedent (37, 38); these mutations prevent the

[4] The spin labels were attached to a cysteine substituted at position 139 in helix III and to individual cysteines engineered into positions cognate to those where we substituted histidines in helix VI of the β_2AR and the PTHR.

receptor from promoting GDP release from the trimer (38), but their association with the trimer nonetheless stabilizes the metarhodopsin II spectral form of rhodopsin. The locations of these mutations suggest that, like the engineered Zn(II) bridges, they affect a function mediated by the cytoplasmic ends of helices III and VI. One of the mutations substituted a different sequence (which included the cytoplasmic end of helix III) for a part of the second intracellular loop of rhodopsin; the other deleted most of the third intracellular loop, including the cytoplasmic end of helix VI.

Peptides representing the 11 C-terminal amino acids of two different Gα subunits are reported (39, 40) to reproduce the effects of G proteins on ligand-binding sites of the corresponding receptors. Such a peptide from α_t stabilized the metarhodopsin II state of rhodopsin (39), and the cognate peptide from α_s enhanced affinity of the β_2AR for binding isoproterenol (40). These results conflict with the proposal (29), described above, that the C termini of Gα subunits associate with a cleft between helices III and VI on the cytoplasmic face of the receptor: if Zn(II) bridges prevent formation of such a cleft, how can a G protein use the C terminus of Gα to regulate the ligand-binding site? One possibility is that our interpretation (29) of the receptor-Gα complementation experiments (34) is wrong, *i.e.* the Gα C terminus does not in fact associate with this region of the receptor but regulates the ligand-binding site by contacting the receptor at a different site. Regardless of whether the Gα C terminus interacts with the putative cleft between helices III and VI, it is likely that G proteins can exert their effects on ligand-binding sites by contacting either of two (or more) separate sites on the receptor. This is suggested by the observation (39) that the metarhodopsin II state of rhodopsin is stabilized by a peptide representing residues 311–328 of α_t. Because these residues contribute to a surface of the G protein near but quite distinct from the C-terminal tail (41), it is likely that the receptor makes multiple contacts with the G protein trimer; some of these may suffice to transmit conformational change to the ligand-binding site of the receptor, whereas a different (but overlapping) subset of these contacts mediates receptor-induced release of GDP.

Finally, the failure of Zn(II) to alter high agonist affinity of the β_2AR raises two questions. The first question relates to the ability of GTPγS to shift the agonist binding curve to the right, even in the presence of Zn(II). Does this suggest that the receptor is promoting GDP/GTP exchange, contrary to our demonstration that Zn(II) prevents the receptor from activating G_s? We think not, because it is well established that G_s releases GDP spontaneously and binds GTPγS even in the absence of receptor stimulation. Although this basal exchange process is slower (minutes rather than seconds) than that catalyzed by receptors, it could certainly reach completion during the 45-min ligand binding assay. Thus the rightward shift does not necessarily reflect receptor activation of G_s.

A second, more difficult question relates to the fact that we assessed β_2AR binding affinity and G_s activation under quite different conditions. In the first case we added ligands to G_s-containing particulate fractions, while we assessed activation by adding pure G proteins to urea-washed membranes that lack endogenous G_s. Thus it is possible that endogenous G_s, "pre-coupled" to β_2ARs in the particulate extracts, could prevent entry of Zn(II) into the metal-binding site and thereby prevent Zn(II) from altering any effect of the G protein on agonist binding affinity. We cannot rule out this possibility at present, although the extent of pre-coupling in these (or any) membranes is unknown. Resolution of this question will require that the binding and activation assays be performed under similar conditions. This has proved difficult because (*a*) the high background of guanine nucleotide-binding proteins in intact membranes can obscure receptor-catalyzed binding of GTPγS, and (*b*) we have found it extremely difficult to reconstitute high affinity receptor binding by adding pure G protein subunits to receptors *in vitro*.

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