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Uncovering Molecular Mechanisms Involved in Activation of G Protein-Coupled Receptors

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ABSTRACT

G protein-coupled, seven-transmembrane segment receptors (GPCRs or 7TM receptors), with more than 1000 different members, comprise the largest superfamily of proteins in the body. Since the cloning of the first receptors more than a decade ago, extensive experimental work has uncovered multiple aspects of their function and challenged many traditional paradigms. However, it is only recently that we are beginning to gain insight into some of the most fundamental questions in the molecular function of this class of receptors. How can, for example, so many chemically diverse hormones, neurotransmitters, and other signaling molecules activate receptors believed to share a similar overall tertiary structure? What is the nature of the physical changes linking agonist binding to receptor activation and subsequent transduction of the signal to the associated G protein on the cytoplasmic side of the membrane and to other putative signaling pathways? The goal of the present review is to specifically address these questions as well as to depict the current awareness about GPCR structure-function relationships in general. (*Endocrine Reviews* **21:** 90–113, 2000)

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I. Introduction

G PROTEIN-coupled, seven-transmembrane segment receptors (GPCRs or 7TM receptors) comprise the largest superfamily of proteins in the body. More than 1000 different GPCRs have been identified since the first receptors were cloned more than a decade ago (1). The chemical diversity among the endogenous ligands is exceptional. They include biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions, and proteases. Moreover, the sensation of exogenous stimuli, such as light, odors, and taste, is mediated via this class of receptors (1, 2). GPCRs have been named based on their ability to recruit and regulate the activity of intracellular heterotrimeric G proteins. GPCRs act at the heterotrimeric G proteins as guanine-nucleotide exchange factors; thus, the activated receptor induces a conformational change in the associated G protein α-subunit leading to release of GDP followed by binding of GTP (3). Subsequently, the GTP-bound form of the α-subunit dissociates from the receptor as well as from the stable $\beta\gamma$-dimer. Both the GTP-bound α-subunit and the released $\beta\gamma$-dimer can modulate several cellular signaling pathways. These include, among others, stimulation or inhibition of adenylate cyclases and activation of phospholipases, as well as regulation of potassium and calcium channel activity (4). The complexity of GPCR signaling has recently been further underlined by data indicating that GPCRs may not solely act via heterotrimeric G proteins (5–10). Most intriguingly, it has been suggested that agonist-promoted phosphorylation of the receptors by GRKs (G protein-coupled receptor kinases) (11) and subsequent sequestration of the receptors from the cell surface (11) are not only important for turning off signaling, but also play a key role in switching the receptor from G protein-dependent pathways to signaling cascades normally used by growth factor receptors (5–7, 10). Yet another example illustrating the impressive variability of GPCR function is the

observation that human immune deficiency virus (HIV) utilizes G protein-coupled chemokine receptors as cofactors for their cellular entry (12–15).

It is thus clear that extensive experimental work performed over the last decade has uncovered multiple aspects of GPCR function and challenged many traditional paradigms (reviewed in Refs. 7 and 16–22). However, it is only recently that we are beginning to gain insight into some of the most fundamental questions in GPCR function. How can, for example, so many chemically diverse hormones, neurotransmitters, and other signaling molecules activate receptors believed to share a similar overall tertiary structure? What is the nature of the physical changes linking agonist binding to receptor activation and subsequent transduction of the signal to the associated G protein on the cytoplasmic side of the membrane and to other putative signaling pathways? The goal of the present review is to specifically address these questions as well as to depict the current awareness about GPCR structure-function relationships in general.

II. Structural Classification of G Protein-Coupled Receptors

GPCRs do not share any overall sequence homology (1, 23). The only structural feature common to all GPCRs is the presence of seven transmembrane-spanning α-helical segments connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxy terminus on the intracellular side (Fig. 1). Significant sequence homology is found, however, within several subfamilies. The three major subfamilies include the receptors related to the "light receptor" rhodopsin and the β_2-adrenergic receptor (family A), the receptors related to the glucagon receptor (family B), and the receptors related to the metabotropic neurotransmitter receptors (family C) (Fig. 1). Yeast pheromone receptors make up two minor unrelated subfamilies, family D (STE2 receptors) and family E (STE3 receptors). In *Dictyostelium Discoideum* four different cAMP receptors constitute yet another minor, but unique, subfamily of GPCRs (family F) (1).

The subfamily of rhodopsin/β_2 adrenergic receptor-like receptors (family A) is by far the largest and the most studied. Phylogenetically, family A receptors can be subdivided further into six major subgroups as indicated in Fig. 1 (1). The overall homology among all type A receptors is low and restricted to a number of highly conserved key residues (indicated in Fig. 1). The high degree of conservation among these key residues suggests that they have an essential role for either the structural or functional integrity of the receptors. (Fig. 1). The only residue that is conserved among all family A receptors is the arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmic side of transmembrane segment (TM) 3 (Fig. 1) (1, 23).

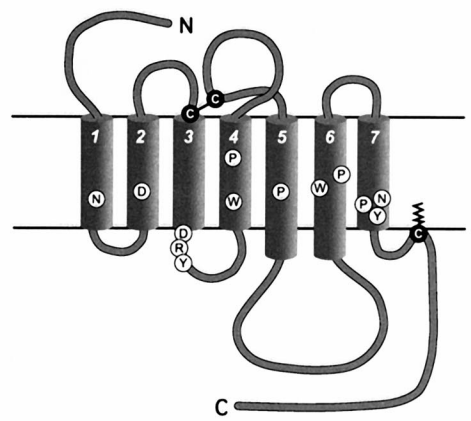
To facilitate comparison of residues between the large number of different receptors belonging to family A there is an obvious need to formulate and use a common numbering scheme. Currently, three different numbering schemes have been suggested but none of them have gained any wide acceptance. The Schwartz and Baldwin numbering schemes are, in principle, identical (24, 25). According to both schemes, the most conserved residue in each helix (*yellow residues* in Fig. 2B and Fig. 3) has been given a generic number describing their predicted relative position in a standard helix of 26 residues (24, 25). A given residue is then described by the helix in which it is located (I–VII) followed by a number indicating its position in the helix. For example, V.16 indicates residue number 16 in TM (transmembrane segment) 5. However, the two numbering schemes are unfortunately incompatible with one another since they do not, except in helix 1, agree on the relative positioning of the conserved residues in the helices (24, 25). This problem is not apparent in the Ballesteros-Weinstein numbering scheme (26). In this scheme, the most conserved residue in each helix has been given the number 50, and each residue is numbered according to its position relative to this conserved residue. For example, 6.55 indicates a residue located in TM 6, five residues carboxy terminal to Pro6.50, the most conserved residue in helix 6 (Fig. 2B and Fig. 3) (26). Since there is no general agreement at this stage in the field on which scheme to use, all residues in this review will be indicated according to the Schwartz scheme followed by the Ballesteros-Weinstein number in superscript.

Family B receptors include approximately 20 different receptors for a variety of peptide hormones and neuropeptides, such as vasoactive intestinal peptide (VIP), calcitonin, PTH, and glucagon (Fig. 1). Except for the disulfide bridge connecting the second (ECL 2) and third extracellular loops (ECL 3), family B receptors do not contain any of the structural features characterizing family A receptors (1) (Fig. 1). Notably, the important DRY motif is absent in family B receptors, and the prolines conserved among the family B receptors are distinct from the ones conserved among the family A receptors (Fig. 1). The most prominent characteristic of family B receptors is a large ($\sim$ 100 residues) extracellular amino terminus containing several cysteines, presumably forming a network of disulfide bridges (27).

Family C receptors are characterized by an exceptionally long amino terminus (500–600 amino acids) (Fig. 1). The receptors include the metabotropic glutamate and γ-aminobutyric acid (GABA) receptors, the calcium receptors, the vomeronasal, mammalian pheromone receptors, and the recently identified putative taste receptors (1, 2). Family C receptors have, like family A and B receptors, two putative disulfide-forming cysteines in ECL 2 and ECL 3, respectively, but otherwise they do not share any conserved residues with family A and B receptors (Fig. 1). The amino terminus of the metabotropic glutamate receptors displays remote sequence homology with bacterial periplasmic binding proteins (PBPs), especially with the leucine/isoleucine/valine binding protein (28). The glutamate binding site has been proposed to be equivalent to the known amino acid binding site of PBPs; therefore, it is believed that the amino terminus of family C receptors contains the ligand-binding site (28, 29).

III. Structural Probing of GPCRs

Due to the inherent difficulties in crystallizing complex membrane proteins, high-resolution structural information



Family A. Rhodopsin/ß2 adrenergic receptor-like

Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)

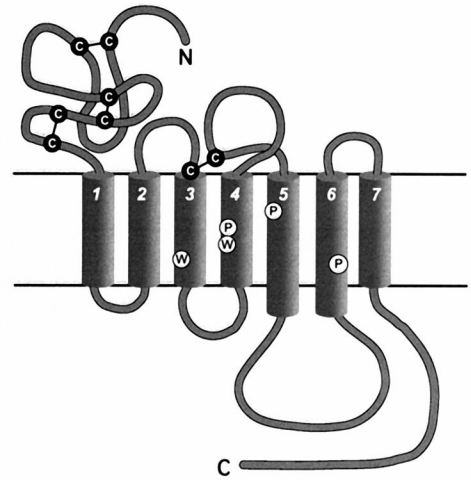
CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin, and growth hormone secretagogues receptors plus vertebrate opsins

Invertebrate opsins and bradykinin receptors

Adenosine, cannabinoid, melanocortin, and olfactory receptors.

Chemokine, fMLP, C5A, GnRH, eicosanoid, leukotriene, FSH, LH, TSH, fMLP, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin, and protease-activated receptors plus others.

Melatonin receptors and other non-classified



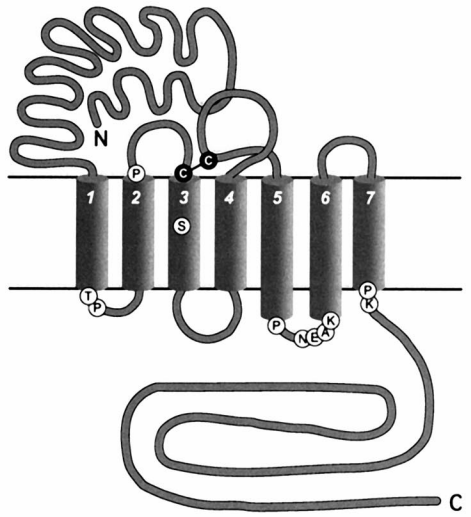
Family B. Glucagon/VIP/Calcitonin receptor-like

Calcitonin, CGRP and CRF receptors

PTH and PTHrP receptors

Glucagon, glucagon-like peptide, GIP, GHRH, PACAP, VIP, and secretin receptors

Latrotoxin



Family C. Metabotropic neurotransmitter/ Calcium receptors

Metabotropic glutamate receptors

Metabotropic GABA receptors

Calcium receptors

Vomeronasal pheromone receptors

Taste receptors

FIG. 1. GPCRs can be divided into three major subfamilies (1). A *snake diagram* for a prototypical member of each subfamily is shown. Family A receptors (*upper panel)* can phylogenetically be subdivided into six subgroups as indicated. Family A receptors are characterized by a series of highly conserved key residues (*black letter in white circles*). In most family A receptors, a disulfide bridge is connecting the second (ECL2) and third extracellular loop (ECL3) (*white letters in black circles*). In addition, a majority of the receptors have a palmitoylated cysteine in the carboxy-terminal tail causing formation of a putative fourth intracellular loop. Family B receptors *(middle panel)* are characterized by a long amino terminus containing several cysteines presumably forming a network of disulfide bridges. The B receptors contain, similar to the A receptors, a disulfide bridge connecting ECL2 and 3. However, the palmitoylation site is missing. Moreover, the conserved prolines are different from the conserved prolines in the A receptors and the DRY motif at the bottom of TM 3 is absent. Family C receptors *(lower panel)* are characterized by a very long amino terminus (~600 amino acids). The amino-terminal domain is thought to contain the ligand-binding site (see *Section IV.F)*. Except for two cysteines forming a putative disulfide bridge, the C receptors do not have any of the key features characterizing A and B receptors. Some highly conserved residues are indicated (*black letter in white circles*). A unique characteristic of the C receptors is a very short and highly conserved third intracellular loop.

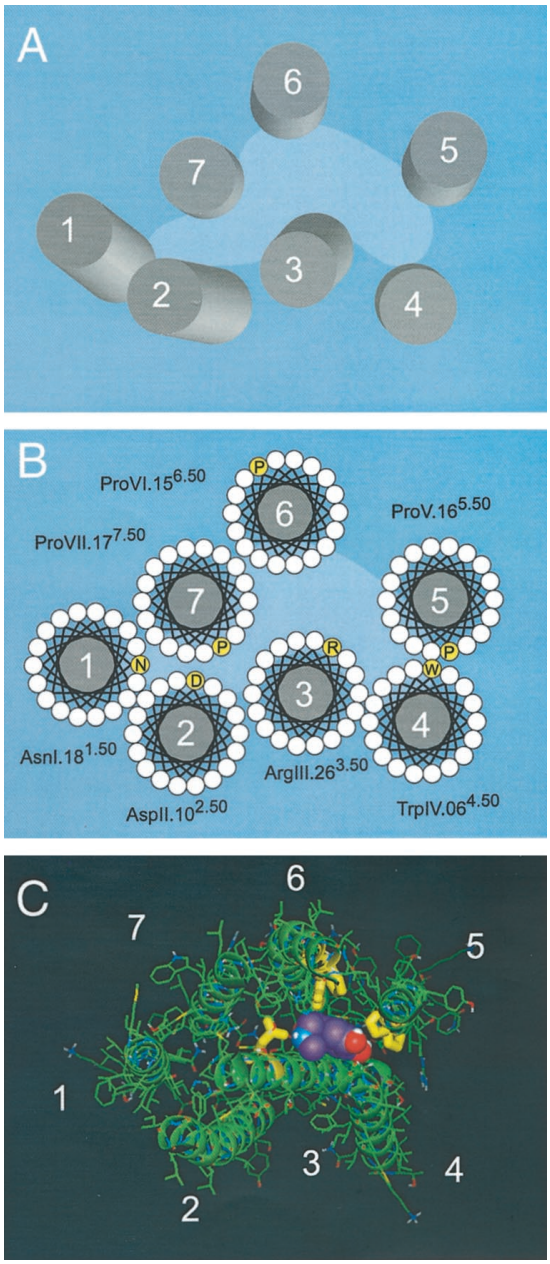


FIG. 2. The predicted structure of rhodopsin-like GPCRs. A, Diagram of a rhodopsin-like GPCR as seen from the extracellular side with each helix represented by a cylinder. The helices are positioned according to the projection maps of frog rhodopsin (37, 38). The helices are organized sequentially in a counterclockwise fashion with helix 3 being almost in the center of the molecule (37, 38). B, "Helical wheel" diagram of a rhodopsin-like GPCR as seen from the extracellular side. The helices are positioned according to the projection maps of frog rhodopsin (37, 38). The conserved fingerprint residues are shown in *yellow*. These residues have been given a general number to facilitate comparison of residues between the receptors. According to the Schwartz numbering scheme, the number is given according to its predicted relative position in the helix (24). For example, ProV.16 indicates residue number 16 in TM 5. In the Ballesteros-Weinstein numbering scheme the most conserved residue in each helix has been given the number 50 (26). The residues are indicated according to the Schwartz scheme followed by the Ballesteros-Weinstein number in superscript. C, Molecular model of the β_2-adrenergic receptor, as seen from the extracellular side, based on the projection map of rhodopsin (26) and structural analysis of multiple GPCR sequences (26). The full agonist epinephrine is shown in the binding crevice with key interactions *highlighted* (see *Section IV.A.2* for further details). Dr. Juan Ballesteros is thanked for preparing the figure. Susan L. Glick and Julie Bryant from Molecular Simulations, Inc., are thanked for technical assistance.

is not yet available for GPCRs. A high-resolution structure of the light-driven proton pump from *Halobacterium halobium*, bacteriorhodopsin, has been available for several years (30). Since bacteriorhodopsin, similar to the GPCRs, possesses seven-transmembrane α-helices and uses retinal as its chromophore, it has been considered a bacterial homolog of vertebrate rhodopsin. The bacteriorhodopsin structure has accordingly been widely used as a template for tertiary structure models of GPCRs (31–35). However, bacteriorhodopsin is a proton pump, is not linked to a G protein, and does not even display remote sequence homology with any GPCR. Moreover, the structural information that recently has become available for rhodopsin indicated clear differences between bacteriorhodopsin and rhodopsin (30, 36–39). Overall, the use of bacteriorhodopsin as a template for molecular models should now be considered obsolete.

Using electron cryomicroscopy of two-dimensional crystals, Schertler and co-workers (36–39) have succeeded in obtaining low-resolution structures of both bovine and frog rhodopsin. In addition, a low-resolution structure of squid rhodopsin has become available (40). The first projection map of bovine rhodopsin at 9 Å resolution provided the first direct insight into how the predicted seven helices are organized relative to one another in the tertiary structure of the receptor (36). Importantly, a very similar arrangement of the transmembrane helices was found in the projection maps of frog and squid rhodopsin at 7 Å and 8 Å resolutions, respectively (38, 40). The projection maps are characterized by an arc-shaped feature, which has been interpreted as reflecting the presence of three tilted helices (36, 38, 40). Four additional peaks were interpreted as the remaining four transmembrane helices (36, 38, 40). The structural information achieved from aligning multiple receptor sequences permitted assignment of the individual peaks in the projection maps to the individual helices in the receptor (25, 41). As shown in Fig. 2, it is believed that the helices are organized sequentially in a counterclockwise fashion as seen from the extracellular side, with helix 3 being almost in the center of the molecule. Further insight into the packing of the seven-helix bundle and calculation of the tilting angles of the helices have been achieved by detailed analysis of tilted two-dimensional crystals of bovine and frog rhodopsin, allowing generation of the first three-dimensional maps (37, 38). The resolution of the map based on the frog rhodopsin crystals was 7.5 Å in the plane of the membrane and 16.5 Å perpendicular to it (38). According to the map, helices 1, 2, and 3 are tilted 27–30 degrees, helix 5 is tilted 23 degrees, whereas helices 4 and 7 are almost perpendicular to the plane of the membrane (38). Helix 6 appears almost perpendicular to the plane of the membrane in the cytoplasmic half but is bent toward helix 5 on the extracellular side (38). The structure also shows that the helices are tightly packed on the intracellular side with helices 2 and 3 packed between helix 4, 6,

and 7 (38). On the extracellular side the helical arrangement opens up and forms a cavity that serves as a binding pocket for retinal. The cavity is lined by helices 3, 4, 5, 6, and 7 and is closed toward the intracellular side by the tilted helix 3 (38). A recent projection map of bovine rhodopsin with an improved resolution (5 Å) suggests that the two-dimensional crystallography technique may lead to even more detailed understanding of the tertiary structure of GPCRs (39).

Guided by the rhodopsin projection maps and the structural information that has been acquired from extensive analysis of multiple GPCR sequences, several tertiary structure models of receptors belonging to family A have been developed over the last few years (25, 26, 41, 42) (Fig. 2C). The models are, of course, still somewhat uncertain but they do provide a believable general picture of the receptor structure and thus a reliable framework within which the structure and molecular function of GPCRs can be further debated and experimentally explored. Importantly, a large number of experimental studies, aimed at probing tertiary structure relations in GPCRs, have been highly critical for refining and validating the molecular models. First of all, this includes identification of several distance constraints in the receptor structure. The close proximity between TM 1 and 7 has, for example, been established based on rescue of nonfunctional adrenergic α_2/β_2 receptor chimeras and muscarinic M_2/M_5 chimeras (43–46). An important series of helix-helix interactions have also been identified by engineering of histidine zinc(II) binding sites in the neurokinin 1 (NK-1) (substance P) receptor and the κ-opioid receptor (47–49). In the NK-1 receptor bis-zinc(II) binding sites were constructed by introducing pairs of histidines in positions predicted to be in close proximity, and in this way it was possible to define the proximity and orientation of TM 3 relative to TM 2 and 5 (47). The distance constraints inferred from the engineered zinc(II) binding sites, as well as from the rescue of nonfunctional chimeras, strongly supported a counterclockwise organization of the seven helices as seen from the extracellular side (45–47). Additional distance constraints in the tertiary structure of the receptors have been identified by formation of intramolecular disulfide bridges between engineered pairs of cysteines in rhodopsin (50, 51) and lately in the M_3 muscarinic receptor (52). Notably, the use of biophysical techniques has also allowed insight into tertiary structure relationships. Turcatti *et al.* established a system, based on suppression of UAG nonsense codons and the use of modified tRNAs, allowing biosynthetic introduction of a fluorescent, unnatural amino acid at known sites in the tachykinin NK-2 receptor during heterologous expression in *Xenopus* oocytes. In this way, they were able to define a set of distances in the tertiary structure by measurement of fluorescence resonance energy transfer between a fluorescent peptide antagonist and different sites containing the fluorescent amino acid (53).

In the GnRH receptor, the proximity between TM 2 and 7 was suggested based on identification of an evolutionary reciprocal mutation (54). In nearly all family A receptors there is a conserved aspartic acid in TM 2, AspII.10$^{2.50}$ (II.10 according to the Schwartz numbering scheme, 2.50 according to the Ballesteros-Weinstein scheme), and a conserved asparagine in TM 7 (VII.16$^{7.49}$) (Fig. 3), but in the GnRH receptor an asparagine is found in the corresponding position in TM 2 and an aspartic acid in TM 7. Since replacement of the asparagine in TM 2 with aspartic acid eliminated detectable ligand binding, but high-affinity agonist binding was restored by additional mutation of the aspartic acid in TM 2 to asparagine, it was proposed that the two residues are in close spatial proximity (54). The observation is not readily compatible with the receptor model proposed by Baldwin *et al.* (25). In this model the distance between the α-carbons of the two residues is 10.4 Å, which is too large for their side chains to form a direct hydrogen-bonding interaction (25). However, if the proposed kink at ProVII.17$^{7.50}$ also causes a twisting of the helix, the two residues can be in sufficiently close proximity to form a direct interaction (26, 55). Remarkably, the presence of both a kink and twist in helix 7 is experimentally supported by the observed cysteine accessibility pattern in TM 7 (55).

Applying the substituted cysteine accessibility method to the dopamine D_2 receptor has provided further highly useful structural information about GPCRs (55–59). Javitch and coworkers (55–58) have systematically substituted residues in TM 2, 3, 5, 6, and 7 with cysteine and determined their accessibility in the predicted binding crevice by reacting with charged sulfhydryl-specific methanethiosulfonate (MTS) derivatives. Their data have allowed mapping of residues facing the binding crevice and estimation of the relative orientation of individual helices (55–58). The accessibility patterns were consistent with TM 2, 3, 6, and 7 forming regular α-helices in agreement with the predictions from the rhodopsin projection maps (55, 56, 58). In TM 6 and 7, the data also supported the presence of kinks corresponding to the conserved prolines, ProVI.15$^{6.50}$ and ProVII.17$^{7.50}$, respectively (Figs. 2 and 3) (55, 58). The accessibility pattern in TM 5 differed from that observed in the other helices (57). A stretch of 10 consecutive residues in the outer portion of TM 5 were found exposed in the binding crevice, which is inconsistent with the prediction that TM 5, like the other helices, should form a regular helix with one side exposed and one side hidden form the crevice (57). There is no obvious explanation for this observation. One explanation could be that the exposed stretch of residues is nonhelical and loop out into the lumen of the binding crevice, making all the residues accessible to the MTS reagents. Alternatively, the outer portion of TM 5 may be structurally flexible and rapidly shift between different conformations, exposing different sets of residues to the binding crevice (57). In both cases, it is of notable interest that the exposed region contains residues believed to form key contacts with the small-molecule agonists (60).

In rhodopsin, the application of EPR (electron paramagnetic resonance) spectroscopy has provided information about structural features, particularly in the cytoplasmic loop regions. Consecutive residues in the cytoplasmic loops and the carboxy-terminal tail have been substituted with cysteine and each of the cysteine mutants was labeled with sulfhydryl-specific nitroxide spin labels (61–65). By determining the accessibility of the attached nitroxide labels to collisions with paramagnetic probes in solution, information about aqueous/hydrophobic boundary zones and secondary structure relations was obtained. The accessibility pattern in the third intracellular loop connecting TM 5 and 6 provided

important evidence that these two α-helices extend two to three turns beyond the cytoplasmic surface of the membrane (62). In the second intracellular loop connecting TM 3 and 4, the analysis indicated that the TM 3 α-helix extends at least 1.5 turns past the important D/ERY motif (Figs. 1 and 3) and that much of the helix surface at the cytoplasmic side forms contacts with protein rather than with the lipids (61). Analysis of the "fourth intracellular loop" between the cytoplasmic end of TM 7 and the palmitoylation site indicated that helix 7 extends around 1.5 turns beyond the membrane surface and that the remaining part of the loop forms very strong tertiary contacts with the protein (64). It was therefore suggested that the loop beyond the helix may be folded over the body of rhodopsin, allowing interactions with residues in the first loop between TM 1 and 2 (64).

IV. Ligand-Binding Domains

Numerous studies have been carried out to identify domains involved in ligand binding to various subclasses of GPCRs. The binding sites for endogenous "small-molecule" ligands in family A receptors, such as the binding site for the retinal photochromophore in rhodopsin and the binding site for catecholamines in the adrenergic receptors, are perhaps the most well characterized. They have been described in detail several times (16, 17, 19, 21) and will therefore be reviewed here only briefly. It is, however, only recently that we have gained insight into binding domains for other classes of ligands. In particular, the ligand-binding domains in peptide receptors are of interest due to the discovery of many small-molecule nonpeptide ligands that can act with high potency at peptide receptors as antagonists and agonists. Intriguingly, it has appeared that the small-molecule agonists and antagonists of peptide receptors may not necessarily share an overlapping binding site with the endogenous peptide agonist. Since these findings have wide implications for receptor activation models, the current knowledge about ligand-binding domains in peptide receptors will be described in more detail. Specifically, the focus will be on the tachykinin system, which has been extensively investigated and served as an important model system for peptide GPCRs.

A. Rhodopsin and the biogenic amines

1. Rhodopsin. The photochromophore of rhodopsin and the opsins, 11-*cis*-retinal, is unique among the endogenous ligands for GPCRs in that it is *covalently* attached to the receptor within a binding crevice formed by the transmembrane helices (reviewed in Ref. 66). Through formation of a Schiff base, 11-*cis*-retinal is coupled to a lysine in TM 7 (Lys296, VII.10$^{7.43}$). The protonated Schiff base is paired with a glutamic acid (Glu113, III.04$^{3.28}$) in the outer portion of TM 3 (67). Additional interactions are found in TM 3 between the C9 group of retinal and Gly121 (III.12$^{3.36}$) (68), and between retinal and aromatic residues in the outer portion of TM 6 (69). Upon exposure to light, 11-*cis*-retinal undergoes an isomerization to all-*trans*-retinal, which leads to formation of the metarhodopsin II state and thus receptor activation (66). While all-*trans*-retinal behaves like the rhodopsin agonist, 11-*cis*-retinal behaves as an inverse agonist (*i.e.*, an antagonist with negative intrinsic activity), keeping the receptor quiescent in the absence of light (70).

2. Classical small-molecule transmitter family A receptors. The binding sites for the classical "small-molecule" transmitters (epinephrine, norepinephrine, dopamine, serotonin, histamine, and acetylcholine) are contained in a binding crevice formed by the transmembrane helices. The residues involved in binding of agonists and antagonists to the β_2- adrenergic receptor are found in TM 3, 5, 6, and 7 (Figs. 2C and 3). The binding crevice is deeply buried in the receptor molecule as evidenced by spectroscopic analysis of the fluorescent antagonist carazolol bound to the β_2 -adrenergic receptor (71). The energetically most important interaction is most likely a salt bridge between the charged amine of adrenergic ligands and the carboxylated side chain of Asp113 (AspIII.08$^{3.32}$) in TM 3 (72) (Figs. 2C and 3). This aspartic acid is conserved among the biogenic amine receptors and is thought to interact also with the positively charged head group of dopamine (73), serotonin (74, 75), histamine (76), and acetylcholine (77). Additional key interactions of the agonists in the β_2-adrenergic receptor include hydrogen bonding between the hydroxyls of the catechol ring in epinephrine and two serines one α-helical turn apart in TM 5, Ser204 (V.09$^{5.43}$) and Ser207 (V.12$^{5.46}$) (60) (Figs. 2C and 3). In TM 6, Phe290 (VI.17$^{6.52}$) may stabilize the catechol ring (78) while recent evidence suggests that Asn293 (VI.20$^{6.55}$) forms a hydrogen bind with the β-hydroxyl of epinephrine (79) (Figs 2C and 3). In the case of the β_2-adrenergic antagonists, which are structurally related to the endogenous agonists, evidence suggests that they share the Asp113 (III.08$^{3.32}$) ionic interaction with the agonists, but that other key interactions differ. For aryloxyalkylamine antagonists, such as alprenolol and propranolol, an asparagine in TM 7 (Asn312, VII.06$^{7.39}$) has been identified as a critical interaction point (80) (Fig. 3). Even though the majority of ligands for small-molecule transmitter receptors seems to bind deep within the binding crevice, there are indications that some antagonists, which show no structural relationship with their corresponding agonist, may partly interact with residues closer to the surface of the membrane. For example, α_{1B}-antagonists, such as phentolamine and WB4101, may interact with three residues in ECL 2 immediately adjacent to the top of TM 5 (81).

B. The binding domains for peptide ligands in peptide receptors belonging to family A

More than 50 different neuropeptides and peptide hormones have been identified. With only a few exceptions, these peptide messengers all act through receptors belonging to the GPCR superfamily and, at present, more than 100 different peptide GPCRs, including subtypes, have been identified (1). In contrast to the general picture obtained for the small-molecule ligands, mutational mapping of ligand-binding sites in many of the peptide receptors has demonstrated the critical involvement of the extracellular domains for binding of the larger peptide ligands.

1. The tachykinin system. The mammalian tachykinins include substance P, neurokinin A, and neurokinin B, which act at the NK-1 receptor, the neurokinin-2 (NK-2) receptor, and the

neurokinin-3 (NK-3) receptor, respectively (82). In addition, a variant of the NK-3 receptor, NK-3B, has recently been identified (83). These receptors are homologous but display significant differences in their pharmacological profile (82, 84, 85). The initial analyses of chimeric NK-1/NK-2 receptors and NK-1/NK-3 receptors suggested that multiple epitopes scattered throughout the receptor structures contribute to the subtype selectivity of the tachykinin peptides and that different receptor domains contribute in varying degrees to the receptor specificity (84, 85). This suggests that the binding sites for the tachykinin peptides are not fully identical (85). Exchange of extracellular loop segments between the NK-1 and NK-3 receptors revealed the involvement of the extracellular domains in binding of the tachykinins (86, 87). Subsequent point-mutational analysis of the NK-1 receptor identified three residues in the amino terminus (Asn23, Glu24, and Phe25), a residue at the top of TM 3 (His108), and a residue at the top of TM 7 (Tyr287) as putative points of interaction for substance P (Fig. 3) (86–88). The importance of the loop regions in substance P binding has been directly supported by affinity cross-linking of a photolabile and radioactively labeled substance P analog to Met181 in the third extracellular loop (89, 90) (Fig. 3). At present, there is no clear evidence that substance P, like the small-molecule ligands, enters deeply into a binding crevice formed by the transmembrane helices. Despite extensive mutational analysis of residues facing the putative binding crevice, no residues have convincingly been identified as potential sites of interactions for substance P (24, 91).

Mutational analysis of neurokinin A binding to the NK-2 receptor also demonstrated evidence for interactions with residues in the extracellular domains (92, 93). However, the residues affecting neurokinin A binding in the NK-2 receptor differed partly from the residues affecting substance P binding in the NK-1 receptor (92, 93). Moreover, mutation of residues in the transmembrane regions, *e.g.*, Leu202 ($V.09^{5.43}$) in the middle of TM 5, was found to affect neurokinin A binding (92, 93). Thus, neurokinin A may partially enter the transmembrane binding crevice. In agreement with the initial chimeric studies (85), these findings indicate that there may be clear differences in the binding modes even among homologous peptides acting at homologous receptors.

Mutation of four residues situated on the same face of helix 2 in the NK-1 receptor has been reported to substantially impair the ability of substance P to compete for binding of radiolabeled nonpeptide antagonists (88). It was therefore initially concluded that these residues are involved in substance P binding. However, it has later been shown that radiolabeled substance P itself could bind with essentially unaffected affinity to the mutated receptors (94). The most likely explanation is that these mutations, rather than affecting the peptide-binding site, affect the ability of the receptor to freely interchange between distinct receptor conformations, which bind the nonpeptide antagonist and peptide agonist with high affinity, respectively (94). Notably, similar observations have been done in the κ-opioid receptor (95), and recently mutation of yet another residue in the NK-1 receptor (Gly166, $IV.21^{4.65}$) has been shown to affect interconversion between different receptor states that display distinct selectivity for the tachykinin peptides (96). These observations underline the importance of direct determination of binding affinity or testing second messenger coupling ability for an agonist before it is reasonable to consider whether the effect of a mutation reflects a real interaction between the ligand and the receptor or is due to an indirect effect.

2. Other family A peptide receptors. For the majority of receptors studied, there is evidence for major interactions in the amino terminus and predicted extracellular loop regions. This includes the receptors for angiotensin (97–99), neuropeptide Y (100), chemokines (interleukin-8) (101), vasopressin/oxytocin (102), GnRH (103), TRH (104–106), complement factor C5A (107, 108), formyl-Leu-Met-Phe (109), somatostatin (110), opioids (111–115), bradykinins (116), cholecystokinin/gastrin (117–121), and neurotensin (122). Importantly, the significance of the extracellular domains for binding of peptide ligands has been directly documented using affinity cross-linking techniques in the GnRH receptor (103), the bradykinin B2 receptor (116), and the cholecystokinin CCK-A receptor (118, 121).

Evidence indicates that some of the peptides have additional points of interactions in the transmembrane domains and therefore, to different degrees, may enter the transmembrane binding crevice. These include both the small tripeptides TRH (123, 124) and fMLP (125) and larger peptides such as angiotensin (126, 127), endothelin (128–130), somatostatin (131–133), opioids (134), and bradykinin (135). The residues identified are found in the outer portions of TM 2, 3, 5, 6, and 7. They differ considerably among the receptors and are, except in a very few cases (128, 131), different from the key positions believed to interact with the biogenic amines. However, it is remarkable to note that almost all of the residues identified appear to be on the surface of the predicted binding crevice as assessed by the cysteine accessibility method (55–59). This supports a high degree of structural similarity between the receptors, even though they bind chemically very different ligands.

C. The binding domains for nonpeptide ligands in peptide receptors belonging to family A

The large group of peptide receptors represents an impressive pool of potential drug targets; however, until recently this has been an almost unexplored area due to the low bioavailability and metabolic instability of the peptide ligands. It has been a long sought goal to develop small-molecule nonpeptide compounds that are orally active and can act at peptide receptors with high potency. The first and most significant discovery, indicating that this would be feasible, was the identification in the 1970s of a family of peptides, the enkephalins and endorphins, as the endogenous ligands of the opioid receptors (136). Until then, the only known ligands for the opioid receptors were nonpeptide exogenous compounds, such as morphine and naloxone. The finding directly showed that small nonpeptide compounds can act with high affinity at peptide receptors both as agonists and antagonists. It is only within recent years, however, that high-affinity nonpeptide compounds have been discovered for an increasing number of peptide recep-

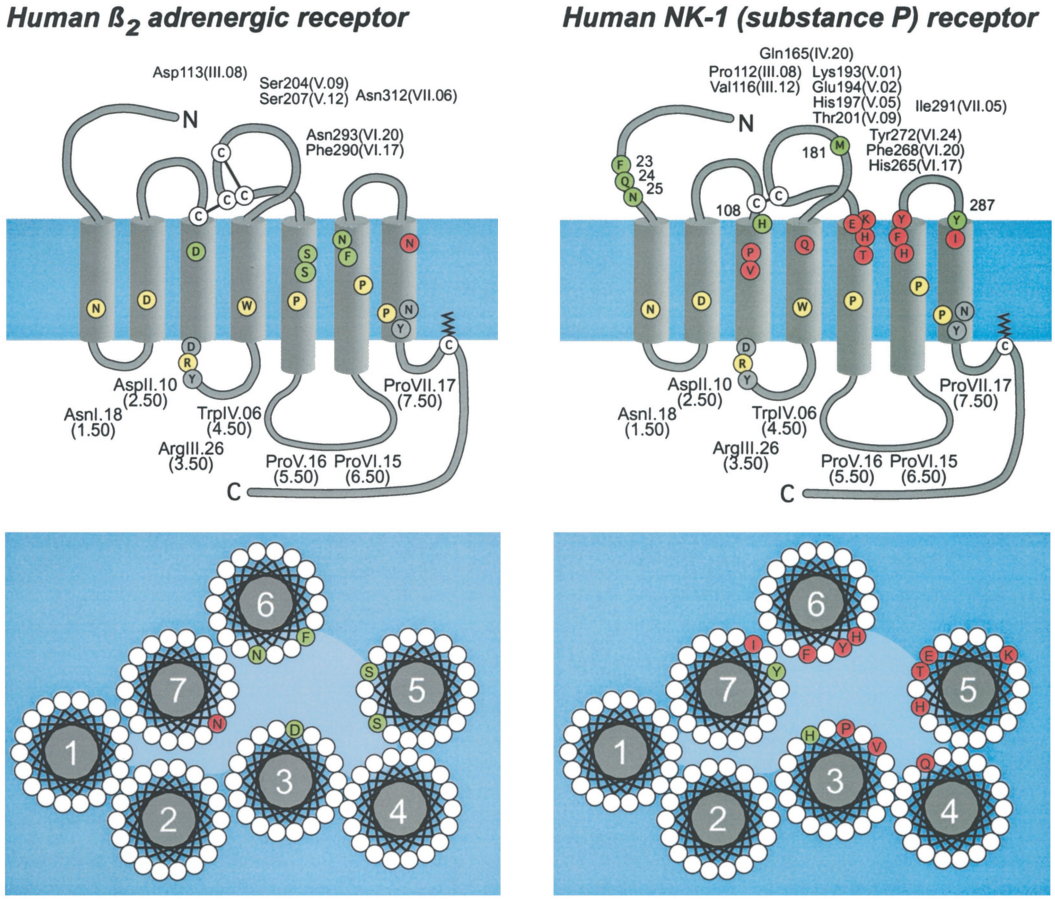


FIG. 3. Comparison of ligand-binding domains in a prototype small-molecule family A receptor (the β_2-adrenergic receptor, β2AR) with a prototype family A peptide receptor (the NK-1 receptor). *Upper panels, Snake diagrams* of the human β2AR and the human NK-1 receptor. *Lower panels,* Helical wheel diagrams of the receptors as seen from the extracellular side. The helices are positioned in a counterclockwise fashion according to the projection map of rhodopsin (25, 36, 38, 41). In the *upper panels* the most highly conserved residue in each helix is indicated in *yellow*. These so-called "finger print" residues have been given a general number to facilitate comparison of residues between the receptors. According to the Schwartz numbering scheme, the number is given according to its predicted relative position in the helix (24). For example, ProV.16 indicates residue number 16 in TM 5. In the Ballesteros-Weinstein numbering scheme, the most conserved residue in each helix has been given the number 50 (26). The numbers for each key residue, according to both numbering schemes, are indicated on this figure below the receptors. Otherwise, the residues shown in the figure are indicated by their "real" number in the receptor followed by the number according to the Schwartz numbering scheme. The amino acids predicted to form the contact points for the agonists are shown in *green* while residues involved in small-molecule antagonist binding are shown in *red* (see text for details). The residues in the β2AR (*left panels*) that form the agonist binding site for the epinephrine are found in a binding crevice between TM 3, 5, and 6 (72, 78, 79). In contrast, the presumed major contact points for the peptide agonist, substance P, in the NK-1 receptor (*right panels*) are found in the extracellular domains or at the top of the helices (86–88). In the β2AR, an asparagine in TM 7 (AsnVII.06) has been shown to interact specifically with aryloxyalkylamine antagonists (80). Notably, the aspartic acid in TM 3 (AspIII.08) (shown in *green*) is a common interaction point for both adrenergic agonists and antagonists (72). The residues shown in *red* in the NK-1 receptor are positions of point mutations shown to affect binding of the prototype nonpeptide antagonist CP 96345 (91, 142–146). Mutation of these residues, clustering in a crevice formed by TM 3, 4, 5, and 6, does not affect peptide agonist binding (91, 142–146).

tors and changed the peptide receptor field into a rapidly expanding area for drug development (24). The majority of the nonpeptide compounds [mostly antagonists but recently, in some cases, also agonists (137)] are developed into high-affinity compounds from "leads" identified by screening of large chemical files (24). In almost all cases, the resulting compounds exhibit no obvious structural similarity to the endogenous peptide ligands, despite an apparent classical competitive mode of action and despite the ability of both the peptide agonist and nonpeptide antagonists to bind with often subnanomolar affinity to the same receptor (24). Interestingly, these nonpeptide compounds have turned out to be valuable for understanding the molecular function of GPCRs.

1. Tachykinin nonpeptide antagonists. An initial series of chimeric NK-1/NK-3 receptors provided the first evidence that the binding mode for the prototype nonpeptide NK-1 receptor antagonist, CP 96,345, was distinct from the binding mode of the endogenous agonist substance P (138). Several chimeric exchanges that dramatically affected CP 96,345 affinity did not affect binding of substance P (138). Overall, the chimeric analyses indicated that CP 96,345 and several other structurally distinct nonpeptide NK-1 receptor antagonists,

but not substance P itself, interact in different ways with a domain located around TM 5 and 6 (138, 139). Moreover, data from a series of NK-1/NK-2 receptor chimeras indicated that SR 48,968, an NK-2 receptor-selective nonpeptide antagonist, has critical interactions in the same region of the NK-2 receptor (140). The different binding modes of the nonpeptide antagonists and the peptide agonists have also been supported by comparing fluorescent analogs of substance P and CP 96,345 bound to the NK-1 receptor. Most significantly, it was found that while the environment surrounding the nonpeptide antagonist was highly hydrophobic and inaccessible to hydrophilic quenchers, the peptide was directly exposed to the solvent (141).

Comprehensive point-mutational analysis has further defined the nonpeptide antagonist binding site in the NK-1 receptor (Fig. 3). The residues predicted to be involved in nonpeptide antagonist binding are located in a transmembrane crevice lined by TM 3, 5, and 6 (91, 142–146), although interactions for some compounds also may occur in TM 4 (147) and 7 (88). The most well documented putative direct interactions of the prototype compound CP 96,345 are Gln165 (IV.20$^{4.64}$) (147), His197 (V.05$^{5.39}$) (142), His265 (VI.17$^{6.52}$) (143, 144), Phe268 (VI.20$^{6.55}$) (91, 146), and Tyr272 (VI.24$^{6.59}$) (145). It should be emphasized here that it is highly difficult with mutational analysis techniques to distinguish direct interactions between the ligand and the receptor from indirect structural effects caused by the mutation. For example, mutation or deletion of Lys193 (V.01$^{5.35}$) and Glu194 (V.02$^{5.36}$) substantially affect CP 96,345 binding affinity (145) (Fig. 3). It is nevertheless unlikely that they participate in a direct interaction since they can be interchanged without affecting CP 96,345 affinity (145). Conceivably, these two residues form a salt bridge that stabilizes the CP 96,345 binding pocket (145). Two other residues, Val116 (III.12$^{3.36}$) and Ile290 (VII.05$^{7.38}$), which are nonconserved between the human and rat receptor, have been shown to be responsible for the species selectivity of CP 96,345 and three other structurally distinct nonpeptide antagonists (148–150) (Fig. 3). It was concluded that these two residues indirectly affected the geometry of a common binding crevice for nonpeptide ligands (148–150). However, Val116 would be predicted to face the binding pocket and could, in fact, be involved in a direct interaction with CP 96,345 (Fig. 3).

Summarized, the studies on the tachykinin receptors suggest the presence of a small-molecule binding pocket, similar to the binding pocket found in the biogenic amine receptors, where structurally distinct nonpeptide compounds can be accommodated through distinct sets of interactions. Surprisingly, this binding pocket is most likely not occupied by substance P, and thus an actual overlap in the binding sites is not required for a competitive mode of action of the nonpeptide antagonists.

2. Nonpeptide ligands for other family A peptide receptors. Considerable differences in binding modes between nonpeptide antagonists and endogenous peptide agonists have been demonstrated in other peptide receptor systems as well. These include the angiotensin (97, 151, 152), opioid (153, 154), CCK/gastrin (155–157), neurotensin (122, 158), and endothelin systems (159). The general conclusions emerging are similar to the ones from the studies of the tachykinin system; hence, the small-molecule nonpeptide compounds interact with residues in the transmembrane binding crevice and, in most cases, there is no evidence that these residues are overlapping with peptide agonist binding. In the neuropeptide Y system, however, there is evidence for several overlapping contact points in the binding site for the peptide agonist and the first available nonpeptide antagonist of the Y1 receptor, BIBP 3226 (160). Similarly, nonpeptide antagonists of the endothelin ET-A and ET-B receptors may share interactions with the endothelin peptides in TM 2 and TM 3 (128, 130). On the other hand, thorough mutagenesis of 18 amino acids in the predicted transmembrane binding crevice of the ET-A receptor revealed no indication of other overlapping contact points between the nonpeptide antagonist bosentan and endothelin-1 (159).

Nonpeptide *agonists* have recently been discovered for the angiotensin receptors. The nonpeptide agonists of the AT-1 angiotensin receptor were found among a series of biphenylimidazole antagonists, of which some turned out to possess agonistic properties (161). Surprisingly, it appeared that the binding mode of the biphenylimidazole agonist differed both from the binding mode of the *peptide* agonist angiotensin, as well as that of the structurally related biphenylimidazole antagonists (161). Mutations in TM 3 and 7, known to severely affect binding of biphenylimidazole antagonists, did not affect binding of the biphenylimidazole agonist. Moreover, binding of the biphenylimidazole agonist was also unaffected by mutation of residues in the extracellular domains known to affect binding of the peptide agonist angiotensin (161).

D. Ligand-binding sites in other family A receptors

While the binding sites for eicosanoids (leukotrienes and prostanoids) and purines mainly are contained within the transmembrane binding crevice (reviewed in Ref. 21), high-affinity binding of glycoprotein hormones such as LH/CG, FSH, and TSH to their receptors occurs in the large extracellular amino terminus that characterizes this receptor subgroup (21, 162–167). It is believed that after the initial binding to the extracellular domain, the amino-terminal part of the hormone undergoes a conformational change leading to secondary contacts with the extracellular loop regions of the membrane-associated part of the receptor and to subsequent receptor activation (21).

The protease-activated thrombin receptors also belong to family A (168, 169). The unique activation mechanism of the thrombin receptor involves cleavage of the amino-terminal segment by thrombin (168). The resulting 33-amino acid amino terminus subsequently acts as tethered peptide ligand, which, through interactions with the extracellular loop regions of the receptor, is able to activate the receptor (170, 171).

E. Ligand-binding domains in family B receptors

Similar to peptide receptors belonging to family A, the binding sites for peptide ligands in family B receptors involve the extracellular domains. The large amino terminus

that characterizes family B receptors seems to play a key role for most ligands, including secretin, VIP, pituitary adenylate cyclase-activating polypeptide (PACAP), glucagon, glucagon-like peptide-1, PTH, and CRF (172–182). The amino terminus is not sufficient for binding of these ligands, and additional interactions are found in the extracellular loops (173, 175, 178, 180, 183–187). However, there is at present no evidence that any of the peptides have interactions deep in a transmembrane binding pocket. Generally, nonpeptide antagonists are still not available for type B receptors. One exception is the CRF receptor for which a few nonpeptide compounds have been recently developed (185, 188). Clear evidence has already been obtained that these may bind very distinctly from the peptide and penetrate into a transmembrane-binding crevice (185).

F. Ligand-binding domains in family C receptors

In the metabotropic glutamate and GABA receptors, the ligand-binding sites are contained within the large extracellular domain characterizing family C receptors, thereby clearly distinguishing this subclass from the biogenic amine family A receptors (28, 189, 190). The calcium-binding site in the calcium-sensing receptors is also found in the large amino terminus (reviewed in Ref. 191). The extracellular amino terminus of the metabotropic glutamate receptors shares remote structural similarity with bacterial periplasmic amino acid-binding proteins (28, 29). A high-resolution x-ray structure of the extracellular glutamate-binding domain of an ionotropic glutamate receptor has recently been published (192). This structure represents the first x-ray structure of a neurotransmitter receptor-binding domain. Based on the x-ray structure, a mechanism was proposed for the propagation of the activation signal in the ionotropic receptors after agonist binding (192). Whether a similar mechanism also accounts for how the signal in metabotropic receptors is transmitted from the extracellular domain to the receptor core region remains elusive.

V. Molecular Mechanisms Involved in Activation of GPCRs

As described in detail in the previous section, the binding modes for agonists acting at GPCRs are almost as diverse as the chemical nature of the ligands. Even agonists acting at the same receptor may not necessarily share an overlapping binding site. Therefore, it seems clear that there are multiple ways of propagating activation of GPCRs or, in other words, there is no common "lock" for all agonists' "keys" (193). Further support for this has been obtained by the identification of receptor-activating antibodies, directed against the extracellular loop regions of the α_1- and β_1-adrenergic receptors, in serum from patients with malignant hypertension and idiopathic dilated cardiomyopathy, respectively (194, 195). The apparent ability of these antibodies to induce receptor activation represents an intriguing example that even in the small-molecule biogenic amine receptors, docking of an activating ligand in the transmembrane-binding crevice is not a prerequisite for ligand-induced receptor activation. Additional examples of activating antibodies, such as monoclonal antibodies against the muscarinic receptors (196) and the bradykinin B2 receptor (197), as well as autoantibodies directed against the extracellular domains of the TSH receptor in Grave's disease (198), also provide strong evidence that there are multiple ways of activating GPCRs. It is still most likely, nevertheless, that the underlying fundamental mechanisms of activation for GPCRs have been conserved during evolution given the ability of the receptors to activate the same intracellular signaling pathways through the same classes of G proteins. In the following section, our current insight into these mechanisms will be discussed.

A. GPCRs are kept silent by constraining intramolecular interactions

An important discovery has been the observation that many GPCRs have a certain basal activity and thus can activate the G protein in the absence of agonists (199–201). Interestingly, it has also been encountered that discrete mutations are able to dramatically increase this constitutive agonist-independent receptor activity (42, 202–205). The majority of the constitutively activating mutations were initially identified after mutational substitutions in the C-terminal part of the third intracellular loop of adrenergic receptors (202–205), but currently activating mutations have been identified in almost any receptor domain in an increasing number of receptors (representative examples in Refs. 42 and 206–218). In a few cases, activating mutations have been found even in the exterior part of the receptors, such as the second extracellular loop of the TSH receptor (214) and the third extracellular loop of the thrombin receptor (213). In the β_2-adrenergic receptor constitutive activation has been observed in a chimeric construct where ECL2 was substituted with the corresponding loop of the α_{1B}-receptor (219). Of interest, some constitutively active mutations have arisen naturally and have been linked to genetic diseases. This includes mutations in the TSH receptor associated with hereditary thyroid adenomas (208, 211, 214); mutations in the LH receptor leading to male precocious puberty (209); and mutations in rhodopsin associated with development of retinitis pigmentosa (210).

A crucial clue about the molecular mechanisms underlying constitutive receptor activation came from a study carried out by Lefkowitz and co-workers in which the naturally occurring Ala293 (VI.0$^{6.34}$) residue in the C-terminal part of third intracellular loop of the α_{1b}-adrenergic receptor was substituted with all other possible residues. They found that substitution of the alanine with any other residue resulted in higher agonist-independent receptor activity (203). This led to the suggestion that constraining intramolecular interactions have been conserved during evolution to maintain the receptor preferentially in an inactive conformation in the absence of agonist. Conceivably, these inactivating constraints could be released as a part of the receptor activation mechanism, either after agonist binding or due to specific mutations, causing key sequences to be exposed to G protein. The hypothesis has been indirectly supported by the recent observation that constitutively activated β_2-adrenergic receptor mutants are characterized by a marked structural instability and enhanced conformational flexibility of the

purified receptor proteins (218, 220). The data imply that the mutational changes have disrupted important stabilizing intramolecular interactions in the tertiary structure, allowing the receptor to undergo conversion more readily between its inactive and active state (218, 220).

Experiments performed in other receptors have also indicated that constraining intramolecular interactions have been conserved during evolution to keep the receptors preferentially silent in the absence of agonists. Hsueh and colleagues (221) obtained evidence using a series of chimeric LH/FSH receptors that stabilizing interactions between TM 5 and 6 are critical for the resistance of the FSH receptor to constitutively activating mutations. A stabilizing role of TM 6 has also been suggested from a random mutagenesis study in the muscarinic M5 receptor where substitutions on one face of the helix conveyed constitutive activity to the receptor (222). Similarly, mutation of polar residues in TM 6 of the α-factor pheromone receptor (STE2p) conveyed constitutive activation to this receptor (223). Molecular modeling and analysis of naturally occurring activating mutations in the LH receptor also strongly point to the importance of the helical packing of TM 6 for maintaining the receptor in an inactive configuration (224). In rhodopsin there is evidence suggesting that opsin, the apoprotein form of rhodopsin, is maintained in an inactive configuration by interactions between a methionine in TM 6 (Met257, VI.05$^{6.40}$) and the conserved NPXXY motif in TM 7 (225), as well as by a salt bridge between Lys296 (VII.10$^{7.43}$) (the retinal attachment site in TM 7) and Glu113 (GluIII.04$^{3.28}$) (the Schiff base counterion in TM 3) (206). Stabilizing interactions between TM 3 and 7 have also been suggested in the angiotensin AT-1 receptor between Asn111 (III.11$^{3.35}$) and Tyr292 (VII.10$^{7.43}$) (226), and in the α_{1b}-adrenergic receptor between the conserved aspartic acid (Asp125, III.08$^{3.32}$) and Lys331 (VII.03$^{7.36}$) (212).

B. Protonation is a key element in GPCR activation

If receptor activation involves disruption of stabilizing intramolecular interaction, an obvious question is how this may be initiated after agonist binding. At present, this question cannot be fully answered; however, substantial evidence suggests that at least one of the key events in the activation process among family A GPCRs involves protonation of the aspartic acid in the highly conserved D/E RY (Glu/Asp-Arg-Tyr) motif at the cytoplasmic side of TM 3 (Fig. 3). The most direct evidence has been obtained by Sakmar and co-workers (227) who compared wild-type rhodopsin and rhodopsin mutated in position Glu134 (III.25$^{3.49}$) by flash photolysis, allowing simultaneous measurement of photoproduct formation and rates of pH changes. Their data strongly suggested that proton uptake of Glu134 (III.25$^{3.49}$) accompanies formation of the metarhodopsin II state (227). The "protonation hypothesis" has been further supported by the observation that charge-neutralizing mutations, which mimics the unprotonated state of the aspartic acid/glutamic acid, cause dramatic constitutive activation of both the adrenergic α_{1b}-receptor and the β_2-adrenergic receptor (42, 218, 228). Similarly, improved coupling has been observed by mutation of the aspartic acid in the GnRH receptor (229). Mutation of the aspartic residue in the M_1 muscarinic receptor resulted in phosphoinositide turnover responses of the mutant that were quantitatively similar to the wild-type despite markedly lowered levels of expression (230). In parallel, constitutive activation was observed in rhodopsin after mutation of the glutamic acid found in the corresponding position of this receptor (231). Finally, it was found that charge-neutralizing mutations of the aspartic acid (Asp130; III.25$^{3.49}$) in the β_2-adrenergic receptor are linked to the overall conformation of the receptor (218). Thus, mutation of Asp130 to asparagine did not only activate the receptor but also caused a cysteine in TM 6 (Cys285, VI.12$^{6.47}$), which is not accessible in the wild-type receptor, to become accessible to methanethiosulfonate ethylammonium (MTSEA), a charged, sulfhydryl-reactive reagent (218). This observation is consistent with a counterclockwise rotation (as seen from the extracellular side) or tilting of TM 6 in the mutant receptor. Importantly, this conformational rearrangement is identical to the movement of TM 6, which biophysical studies have indicated to be essential for agonist-induced receptor activation (see next section).

The experimental data have been supported by molecular modeling and computational simulations. Two distinct hypotheses have been proposed to define the specific role of Asp/GluIII.25$^{3.49}$ protonation in receptor function, the "polar pocket" hypothesis proposed by Scheer *et al.* (42) and the "arginine cage" hypothesis proposed by Ballesteros *et al.* (229). According to the polar pocket hypothesis, the invariably conserved ArgIII.26$^{3.50}$ is in the inactive state of the receptor constrained in a pocket formed by conserved polar residues in TM 1, 2, and 7, including AsnI.18$^{1.50}$, AspII.10$^{2.50}$, AsnVII.16$^{7.49}$, and TyrVII.19$^{7.52}$ (Fig. 3). Upon protonation (or mutation to alanine) of the adjacent AspIII.25$^{3.49}$, the simulation indicated that the arginine shifts out of the polar pocket leading to long-range conformational changes in the receptor molecule (228). In their model, they highlighted that the ionic counterpart of the arginine in the inactive receptor state was the conserved aspartic acid in TM 2 (AspII.10$^{2.50}$, Fig. 3), and that this interaction is broken after receptor activation (228). Alternatively, based on computational simulations in the GnRH receptor, the arginine-cage hypothesis suggests that the ionic counterpart of ArgIII.26$^{3.50}$ in the inactive state of the receptor could be the adjacent AspIII.25$^{3.49}$ and not AspII.10$^{2.50}$ (229). It was hypothesized that during receptor activation, AspIII.25$^{3.49}$ becomes protonated and that AspII.10$^{2.50}$ substitutes for AspIII.25$^{3.49}$ in forming an ionic interaction with ArgIII.26$^{3.50}$ (229). Thus, an ionic interaction between ArgIII.26$^{3.50}$ and AspII.10$^{2.50}$ was associated with the active receptor state instead of with the inactive state as proposed by Scheer *et al.* (42). An indirect support for this alternative hypothesis is the observation in several GPCRs that mutations, which eliminate the charged character of AspII.10$^{2.50}$ and in this way conceivably destabilize the Asp-Arg interaction, also disturb functional coupling of the receptor (232–236). Spectroscopic experiments in rhodopsin have also indicated that AspII.10$^{2.50}$ is more strongly hydrogen bonded upon activation, consistent with its potential interaction with another residue in the active state of the receptor (237).

C. Conformational changes involved in receptor activation

An ultimate understanding of the receptor activation mechanism requires development of techniques that can provide insight into the character of the physical changes accompanying transition of the receptor from the inactive to the active state. Sheikh *et al.* (238) have undertaken an approach where bis-histidine metal ion-binding sites were generated between the cytoplasmic extensions of TM 3 and 6 in rhodopsin. In this way, they were able to show that cross-linking pairs of histidines with Zn^{2+} prevented transducin activation, providing indirect evidence that movements of these two domains are important for activation. Recently, they have obtained similar results in the β_2-adrenergic receptor and in the PTH receptor of which the latter belongs to family B (Fig. 1) (239). This suggests that the activation mechanism may be conserved among both family A and family B receptors (239). Javitch and co-workers (240) have applied the substituted cysteine accessibility method, in which specific advantage was taken of a constitutively activated β_2 adrenergic receptor, CAM. Their main observation in CAM was that a cysteine in TM 6 became accessible in the binding crevice to a charged, sulfhydryl-reactive reagent (240). This indicated a conformational rearrangement of TM 6 with CAM consistent with a counterclockwise rotation or tilting of the helix (240). Assuming that the conformation of CAM mimics the agonist-activated state of the receptor, the data thus indicated that movements of TM 6 are a critical element in the receptor activation mechanism.

Recently, biophysical techniques have also been implemented, allowing direct time-resolved analysis of conformational changes in the receptor molecule. It is not surprising that a majority of the studies initially have been carried out in rhodopsin. There are abundant natural sources of rhodopsin, and the inherent stability of the rhodopsin molecule makes it possible to produce and purify relatively large quantities of recombinant protein. Accordingly, several spectroscopic techniques have been applied to rhodopsin, including Fourier transform infrared resonance spectroscopy (FTIR) (241, 242), surface plasmon resonance (SPR) spectroscopy (243), tryptophan UV-absorbance spectroscopy (244), and EPR spectroscopy (61, 62, 64, 65). All approaches have consistently provided evidence for a significant conformational rearrangement accompanying transition of rhodopsin to metarhodopsin II. Using tryptophan UV-absorbance spectroscopy, Lin and Sakmar (244) were able to obtain the first direct evidence that photoactivation may involve relative movements of TM 3 and 6 (244). Thus, mutation of tryptophans in TM 3 and 6 eliminated the spectral differences in the UV absorbance spectra that distinguished rhodopsin from metarhodopsin II (244).

In a series of very elegant studies, carried out by Khorana, Hubbell, and co-workers (50, 61–65, 245), the use of EPR spectroscopy in combination with multiple cysteine substitutions has led to further insight into the character of conformational changes accompanying photoactivation of rhodopsin. Site-directed labeling of single cysteines inserted at the cytoplasmic side of the transmembrane helices with sulfhydryl-specific nitroxide spin labels provided evidence for movements particularly of the cytoplasmic termination of TM 6 upon light-induced activation of rhodopsin (50, 61–65). The spectroscopic analyses also showed evidence for smaller movements in the loop connecting TM 1 and 2 as well as at the cytoplasmic ends of TM 3 and TM 7 (61, 64, 246). Only minor or no structural changes appeared to occur at the cytoplasmic end of TM 4 and 5 (61, 62). To investigate the character of the conformational changes, Khorana, Hubbell, and co-workers have taken advantage of the magnetic dipole interaction between two nitroxide spin labels causing spectral line broadening if the two probes are less than 25 Å apart (50). Pairs of sulfhydryl-reactive spin labels were incorporated into a series of double-cysteine mutants enabling measurement of changes in relative distance between TM 3 and TM 6 (50). While the movement of TM 3 was interpreted as relatively small, the data pointed to a significant rigid-body movement of TM 6 in a counterclockwise direction (as viewed from the extracellular side) and a movement of the cytoplasmic end of TM 6 away from TM 3 (Fig. 4) (50). Importantly, movements of TM 6 in rhodopsin upon photoactivation have recently been additionally documented by site-selective fluorescent labeling of cysteines inserted at the cytoplasmic termination of the helix (247).

The first direct structural analysis of conformational changes in a GPCR activated by a diffusable ligand was recently carried out in the β_2-adrenergic receptor using fluorescence spectroscopic techniques (220, 248, 249). The spectroscopic technique that initially was applied used the sensitivity of many fluorescent molecules to the polarity of their local molecular environment (248). The sulfhydryl reactive fluorophore IANBD (*N,N′*-dimethyl-*N* (iodoacetyl)-*N′*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) ethylene-diamine) was used to label free cysteine residues in purified detergent-solubilized β_2-adrenergic receptor (248). Both the quantum yield of the emission spectrum and the decreased accessibility to hydrophilic quenchers strongly suggested that one or more of the naturally transmembrane cysteines were labeled. Exposure of IANBD-labeled receptor to agonist led to a reversible and dose-dependent decrease in emission consistent with movements of the fluorophore to a more hydrophilic environment after binding of the full agonist isoproterenol (248). Interestingly, exposure of the IANBD-labeled receptor to inverse agonists (*i.e.,* antagonists with negative intrinsic activity) led to an apparent increase in fluorescence, suggesting that not only agonists but also inverse agonists can promote structural changes in a GPCR (248).

To identify the cysteines labeled with IANBD that gave rise to the spectral changes, a series of mutant β_2 receptors with one, two, or three of the natural cysteines available for fluorescent labeling was generated (249). The fluorescence spectroscopy analysis of the purified and site-selectively labeled mutants showed that IANBD bound to Cys125 (III.20$^{3.44}$) in TM 3 and Cys285 (VI.12$^{6.47}$) in TM 6 were responsible for the observed changes in fluorescence (249). This suggests that movements of TM 3 and 6 may occur during receptor activation (249) (Fig. 4). The possible spatial orientation of IANBD bound to Cys125 (III.20$^{3.44}$) and Cys285 (VI.12$^{6.47}$) in TM 6 was explored in a series of computational simulations to define the character of the putative movements of TM 3 and 6. In a rhodopsin-based model of the

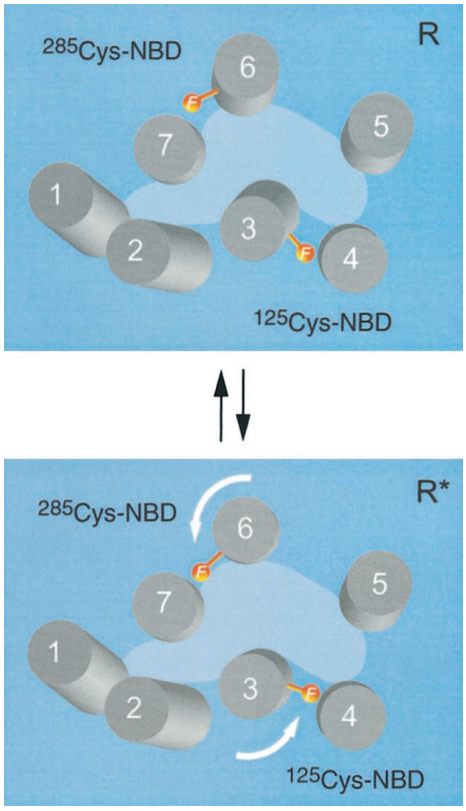


FIG. 4. Predicted conformational changes accompanying activation of family A GPCRs. The figure shows a simplified model of the β_2-adrenergic receptor based on the projection map of frog rhodopsin as seen from the extracellular side (25, 36, 38, 41). The *upper panel* illustrates the inactive receptors' state (R) while the *lower panel* indicates the anticipated conformation of the activated state (R*). The NBD fluorophore (shown in *orange*) bound to Cys125 (III.20$^{3.44}$) in TM 3 is in the inactive state of the receptor predicted to lie at the helix 3–4 interface, oriented predominantly toward the lipid (249). NBD bound to Cys285 (VI.12$^{6.47}$) is predicted to be at the helix 6–7 interface in a boundary zone between the lipid bilayer and the more polar interior of the protein (249). An agonist-induced rigid-body movement of TM 6 involving a counterclockwise rotation and a movement of the cytoplasmic end of the helix away from TM 3, as indicated by the *arrow*, would cause Cys285-NBD to be exposed to a more polar environment in the interior of the protein. Similarly, a counterclockwise rotation and/or tilting of TM 3 would cause Cys125-NBD to be exposed to a more polar face of TM 4 and/or the more polar interior of the receptor as indicated by the *arrow*. These movements explain the observed changes in fluorescence (249) and is consistent with the spin-labeling studies in rhodopsin (50).

β_2–receptor, the preferred conformation of IANBD attached to Cys125 (III.20$^{3.44}$), as defined by the computational simulations, is bounded by the lipid bilayer and the interface of TM 3 and TM 4, while the IANBD attached to Cys285 (VI.12$^{6.47}$) is predicted to be at the helix 6-7 interface in a boundary zone between the lipid bilayer and the more polar interior of the protein (249) (Fig. 4). In the framework of this model, the change in fluorescence of IANBD-labeled β_2-adrenergic receptor can best be explained by a counterclockwise rotation of both TM 3 and TM 6, which would move the IANBD molecules from the nonpolar lipid environment to the more polar interior of the protein (249) (Fig. 4). Of interest, Cys285 (VI.12$^{6.47}$) is situated one α-helical turn below Pro288 (VI.15$^{6.50}$), which is highly conserved among GPCRs and provides a flexible hinge in TM 6. It has been speculated, therefore, that the movement of Cys-NBD to a more polar environment in the protein interior is directly facilitated by this flexible hinge connecting residues involved in agonist binding in the outer part of TM 6 with the putative G protein-coupling domain in the cytoplasmic extension of the helix (249). Notably, site-selective incorporation of the NBD fluorophore in a new series of single-cysteine mutants of the β_2-adrenergic receptor has recently documented significant agonist-promoted conformational changes corresponding to this cytoplasmic extension of TM 6 (A.D. Jensen and U. Gether, to be published).

In summary, the spectroscopic studies in rhodopsin and in the β_2-adrenergic receptor clearly support a critical role of TM 3 and 6 for transition of GPCRs to their activated state (Fig. 3). Importantly, the agreement between the data obtained in rhodopsin and in the β_2-adrenergic receptor also strongly indicates that the activation mechanism in many aspects is similar at least among type A GPCRs. It should, however, be emphasized that the established importance of TM 3 and 6 does not exclude that movements of other domains may contribute to receptor activation. For example, there is evidence based on EPR spectroscopy in rhodopsin that movements of TM 7 may also occur in response to photoactivation (64). The possible importance of TM 7 in receptor activation is also indirectly supported by the very recent observation that an activating metal ion-binding site can be generated between TM 3 and 7 in the β_2-adrenergic receptor (250).

D. How is the activation signal transmitted to the G protein?

A myriad of studies involving chimeric substitutions, various other mutational approaches, and the use of synthetic peptides have in many receptors provided considerable insight into the structural elements important for the interaction with the G protein. The literature describing these studies have been reviewed several times (16, 17, 20, 251–253) and will therefore be discussed only briefly here. Summarized, the studies have established the pivotal roles of the second (ICL2) and the third intracellular (ICL3) loops plus, at least in some receptors, the proximal part of the carboxy terminus in G protein coupling (16, 17, 20, 251–253). Chimeric approaches, applied in the adrenergic and muscarinic systems, clearly defined that ICL3 is the key determinant of coupling specificity among the different G protein α-subunits (16, 17, 20, 251–253). Subsequent point mutational analyses in many receptors have identified residues crucial for selective G protein coupling clustering in the amino-terminal part of ICL3 adjacent to TM 5 (252, 254–257) and in the carboxy-terminal part of ICL3 adjacent to TM 6 (258–260). In contrast to ICL3, ICL2 is less important for determining G protein specificity but is important for the efficiency of G protein activation (16, 17, 20, 251–253). The role of ICL2 has recently been convincingly substantiated by Brann and co-workers, who developed a random mutagenesis approach for their study of muscarinic receptor coupling (215). In ICL2 of the M5 muscarinic receptor, they found that substitution of residues clustering on one side of a presumed ICL2 α-helix extending

from TM 3 caused constitutive activation, while substitutions of residues clustering on the opposite side of the helix compromised G protein coupling. Taken together, the data suggest that the residues on the constitutively activating side were critical for maintaining the receptor in an inactive state, whereas the residues on the opposing side were important for G protein activation (215). It was therefore inferred that ICL 2 could act as a switch that enables G protein coupling (215). Notably, this hypothesis is consistent both with role in receptor activation of the adjacent DRY motif (42, 218, 229) and the predicted movements of TM 3 relative to TM 6 from spectroscopic analyses (50, 249). Interestingly, the aspartic acid of the DRY motif (AspIII.25$^{3.49}$), which is believed to undergo protonation during receptor activation (see *Section V.B.*), is located on the same side of the helix as the residues found to cause constitutive activation (Fig. 2).

Despite the abundance of information acquired over the last decade, the mechanisms by which the signal is transmitted from the activated receptor to the G protein heterotrimer remains, nevertheless, surprisingly elusive. Recently, x-ray crystallography has provided substantial insight into the tertiary structure of the heterotrimeric G proteins (261, 262), but still little is known about the actual points of interactions between the receptor and the G protein and, thus, how the two proteins are oriented relative to one another. So far, only the interaction between the carboxy terminus of the G protein α-subunit and the carboxy-terminal part of IC3 seems reasonably well substantiated from mutagenesis studies (259). Based on the currently available data, an orientation of the G protein relative to the plasma membrane has been proposed placing the nucleotide-binding domain of the α-subunit approximately 30 Å away from the membrane (261–263). According to this, the receptor must induce GDP release from the α-subunit without directly interacting with the nucleotide-binding domain. It has been speculated that the suggested movements of TM 3 and 6 apart from each other during receptor activation (Fig. 4) could allow insertion of the α-subunit carboxy terminus into a cavity in the seven-helix bundle (263). Conceivably, this could trigger structural changes in the adjacent α5-helix and β6-strand that are transmitted to the nucleotide-binding domain via the α5/β6 loop, which is in the immediate vicinity of the guanine nucleotide (263).

E. Receptor dimerization—an artifact or a functional necessity?

It is well known that receptor dimerization is required for signal transduction in other classes of receptors, *e.g.*, receptor tyrosine kinases. An increasing number of studies have shown that many GPCRs also form dimers. For example, formation of receptor *homodimers* has been reported for the β_2-adrenergic receptor (264), the δ-opioid receptor (265), the dopamine D_1, D_2, and D_3 receptors (266–268), the chemokine receptors CCR2b, CCR4, and CCR5 (269, 270), the extracellular calcium-sensing receptor (271, 272), and the metabotropic glutamate receptor 5 (273). It has been demonstrated moreover that functional receptor dimers can be formed by coexpressing two reciprocal nonfunctional chimeras constructed between the α_{2C}-adrenergic receptor and the M_3 muscarinic receptor (274). However, the molecular mechanisms of dimer formation seem to differ considerably among the receptors. In the β_2-adrenergic receptor, dimerization most likely involves interactions between transmembrane segments since a peptide derived from transmembrane segment 6 has been shown to inhibit dimer formation (264). Similarly, peptides derived from the transmembrane domains of the dopamine D_2 receptor dissociated dimers to monomers (266), but a peptide derived from TM 6 of the dopamine D_1 receptor did not affect dimerization of this receptor (268). For the δ-opioid receptor, dimerization was eliminated by deletion of 15 amino acids in the carboxy terminus, indicating the involvement of this part of the receptor in dimerization (265). In contrast, dimerization of the metabotropic glutamate receptors and the extracellular calcium-sensing receptor was found to be dependent on intermolecular disulfide bonds between cysteines in their large amino-terminal domains (271–273).

An intriguing observation has been that agonist can stabilize the dimeric form of several receptors including the β_2-adrenergic receptor (264) and the chemokine receptors CCR2b, CCR4, and CCR5 (269, 270). This suggests that homodimerization could have a role either directly in the receptor activation mechanism or, alternatively, in the subsequent agonist-dependent densitization and internalization process. For the CCR2b receptor, evidence suggests that dimerization does have a direct role in agonist-mediated receptor activation (270). First, it was found that the CCR2b receptor can only be activated by the bivalent form of an agonistic monoclonal antibody directed against the CCR2b receptor and not by the corresponding monovalent Fab fragment (270). Second, it was demonstrated that coexpression of wild-type CCR2b with a coupling-deficient mutant (CCR2/Y139F) eliminated any functional coupling in response to the endogenous agonist of the CCR2b receptor, monocyte chemoattractant protein 1 (MCP-1) (270). Hence, the mutant acted as a dominant negative mutant, indicating that dimerization is a prerequisite for ligand-induced CCR2b signaling (270). For the calcium-sensing receptor there is also experimental support for a role of dimerization in receptor activation (275). In a recent study it was shown that elimination of dimerization, by mutating the two cysteines believed to form intermolecular disulfide bridges between the extracellular domains, resulted in a receptor with lowered calcium affinity and much slower kinetics of the responses to calcium. However, as yet there is no evidence supporting a universal role of dimerization for GPCR activation. In the case of the δ-opioid receptor, it has been observed, for example, that agonists *decrease* the level of dimer formation (265).

Recently, substantial evidence has accumulated demonstrating the possible importance of *heterodimerization* between closely related receptor subtypes (276–279). The $GABA_B$ R1 receptor subtype is mostly retained inside the cell as an immature glycoprotein when expressed in mammalian cells and displays low affinity for agonists (276–279). However, if it is coexpressed with the newly discovered $GABA_B$ R2 receptor, a fully functional and terminally glycosylated receptor can be detected at the cell surface (276–279). The data indicate that heterodimerization can be critical for targeting functional receptors to the cells surface and, thus, that

the *in vivo* functional $GABA_B$ receptor could be a heterodimer of $GABA_B$ R1 and $GABA_B$ R2 (276–279). An additional intriguing example, which indicates a functional relevance of heterodimerization between receptor subtypes, is the observation that formation of heterodimers between two fully functional opioid receptors, δ and κ, results in a new receptor that displays binding and functional properties distinct from those of either of the receptors (280). It is of interest moreover to note that heterodimerization between the wild-type CCR5 receptor and the naturally occurring nonfunctional mutant of the CCR5 receptor, ccr5δ32, has been shown to inhibit targeting of the wild-type receptor to the cell surface after coexpression in HeLa cells (281). Since CCR5 acts as a coreceptor for HIV infection, the inhibition of wild-type receptor surface expression by the mutant was proposed as a molecular explanation for the delayed onset of AIDS in heterozygotic (CCR5/ccr5δ32) individuals (281). Finally, it should be mentioned in this context that a family of accessory single-transmembrane proteins, RAMPs (receptor-activity-modifying proteins), has been identified and found to complex with the calcitonin-receptor-like receptor (CRLR). The association of CRLR with RAMPs was found not only to play a role in targeting the receptor to the cell surface, but also to modify the pharmacological properties of the receptor. While RAMP1 converted CRLR into a calcitonin-gene-related-peptide (CGRP) receptor, RAMP2-associated receptors display the properties of an adrenomedullin receptor (282). To what degree such mechanisms also may account for the function of other GPCRs remains obscure and needs to be clarified in the future.

VI. Models of Receptor Activation

A. The two-state model of receptor activation vs. multistate models of receptor activation

The currently most widely accepted model for GPCR activation is the extended ternary complex model (often referred to simply as the two-state model) (205, 283, 284). This model was proposed in light of the discovery that receptors in the absence of agonist spontaneously can adopt an active conformation and couple to the G protein (205, 283, 284). Importantly, the model both accommodates the phenomenon of agonist-independent receptor activity and the complex behavior of various classes of ligands (agonists, partial agonists, neutral antagonists, and inverse agonists). According to the model, the receptor exists in an equilibrium between an inactive conformation (R) and an active conformation (R*) (205). In the absence of agonist, the inactive R state is prevailing; however, the energy barrier between the R and R* state is sufficiently low, allowing a certain fraction of the receptors spontaneously to assume the R* state. Agonists are predicted to bind with highest affinity to the R* conformation and in this way shift the equilibrium and increase the proportion of receptor in R*. Conversely, inverse agonists (also called negative antagonists), *i.e.*, compounds possessing the ability to inhibit agonist-independent receptor activity, are predicted to stabilize the inactive R state, shifting the equilibrium away from R*. Neutral antagonists, according to the model, are defined as compounds that bind with the same affinity to both R and R* and thus cause no change in the equilibrium (205).

It is becoming increasingly clear that the two-state model cannot sufficiently explain the complex behavior of GPCRs. Several lines of evidence have provided strong support that GPCRs may exist in possibly multiple conformational states (42, 146, 201, 285–288). For example, the nonoverlapping binding sites between peptide agonists and nonpeptide antagonists, proposed for some receptors (see *Section IV.C.*), cannot be reconciled with a simple two-state model (24). Similarly, a two-state model cannot explain how mutation of certain serines in TM 5 of the dopamine D_2 receptor can lead to loss of functional coupling in response to some agonists, but not others, with only modest effect on their affinity (287). Furthermore, different synthetic agonists of the *Drosophila* D_1-like dopamine receptor have been shown to induce selective coupling to distinct second messenger pathways (286). It is also difficult to explain within a simple two-state model how β_2 receptor ligands can act as partial agonists or inverse agonists depending on whether the functional assay is performed in membranes or intact cells (201). An additional interesting finding, strongly supporting the existence of more than one active receptor state, has been the observation that different constitutively active mutants of the α_{1B}-receptor are differentially phosphorylated and internalized although they convey a similar agonist-independent activity to the receptor (288). Finally, more direct structural evidence has been obtained by fluorescence spectroscopy analysis of the purified β_2-adrenergic receptor, which indicated that most ligands promote alterations in receptor structure consistent with the existence of multiple ligand-specific conformational states (146).

Evidently, receptor activation models that incorporate the existence of several or multiple conformational states have recently been suggested (24, 42, 285, 289). In the multistate model proposed by Schwartz *et al.* (24) the receptor is proposed to alternate spontaneously between multiple active and inactive conformations. The key element in this model is that the biological response to a given ligand is determined by the conformation to which the ligand binds with highest affinity. If the preferred conformation is recognized by the G protein as active, the compound would behave like an agonist, and if the preferred conformation is inactive, the ligand would behave like an inverse agonist. The important impact of the model is, obviously, that there is no requirement for a common binding mode for agonist to trigger receptor activation. Even two agonists acting at the same receptor do not have to share (although they probably often would) an overlapping binding site; they both must stabilize an active conformation (24). For example, a peptide agonist may be able to stabilize an active state by interacting with the extracellular loop regions while a small molecule agonist of the same receptor could stabilize the same or another active configuration by penetrating into the transmembrane-binding crevice. Similarly, the model does not require any overlap in binding site between the agonist and a competitive antagonist. The agonist and antagonist can be envisioned simply to stabilize distinct receptor conformations to which the agonist and antagonist bind in a mutually exclusive fashion (24). Kinetically this would be indistinguishable from a classical

competitive situation with overlapping binding sites between the agonist and antagonists (24).

B. Implications from biophysical studies on receptor activation models

The recent biophysical analyses of conformational changes in rhodopsin and in the β_2-adrenergic receptor have provided novel insight into the critical conformational changes accompanying receptor activation. However, the data also raise new interesting questions about molecular modes of agonist-induced receptor activation. As discussed in *Section V.C*, spectroscopic studies of conformational changes in both rhodopsin and the β_2-adrenergic receptor suggest that similar movements are important for activation of both receptors. Otherwise, there are substantial differences underlying activation of rhodopsin compared with the β_2-adrenergic receptor. Rhodopsin is unique in that its ligand, *cis*-retinal, is covalently bound to the receptor as an inverse agonist and upon absorption of a photon isomerizes to an agonist (*trans*-retinal) within the binding pocket (reviewed in Ref. 66). In other words, ligand binding is not part of the activation process. This specialized mechanism of activation may be necessary to facilitate the very rapid response of rhodopsin to light. Thus, formation of the activated metarhodopsin II state occurs essentially within microseconds even in detergent solution in the absence of transducin (290). Interestingly, metarhodopsin II subsequently undergoes a slow ($t_{1/2} \sim 6$ min) transition to the inactive metarhodopsin III (290). During this inactivating transition *trans*-retinal undergoes hydrolysis and release from the binding pocket (291). Remarkably, free *trans*-retinal is not a very effective agonist for opsin, producing only approximately 14% of the response observed for light-activated rhodopsin (292). This shows that efficient activation of rhodopsin by *trans*-retinal requires that *cis*-retinal is prebound and that *cis*-retinal can be rapidly converted to *trans*-retinal by photoisomerization. The less efficient activation of opsin by free *trans*-retinal may more closely reflect the process of activation of other GPCRs.

In contrast to the rapid activation and the slow inactivation kinetics observed for rhodopsin, spectroscopic analyses of the purified β_2-adrenergic receptor labeled with a conformationally sensitive fluorophore revealed slow agonist-induced conformational changes ($t_{1/2} \sim 2$–3 min), significantly slower than the predicted association rate of the agonist (220, 248, 249). However, the reversal of the agonist-induced conformational change was relatively fast ($t_{1/2} \sim 30$ sec) (220, 248, 249). It should be emphasized that the slow activation kinetics now have been observed in several different readouts. Thus, the agonist-induced spectral changes observed after labeling of cysteines introduced at the cytoplasmic side of TM 6 occur with similar kinetics as that observed after labeling of the endogenous cysteines (Cys125 and Cys285) (A.D. Jensen and U. Gether, to be published). It is possible that the differences between rhodopsin and the β_2-adrenergic receptor are caused by differences in the methodological approach. However, since the measurements were performed under similar conditions (in detergent solution in the absence of G protein) it is more likely that they reflect inherent differences between rhodopsin and a receptor activated by a diffusable ligand.

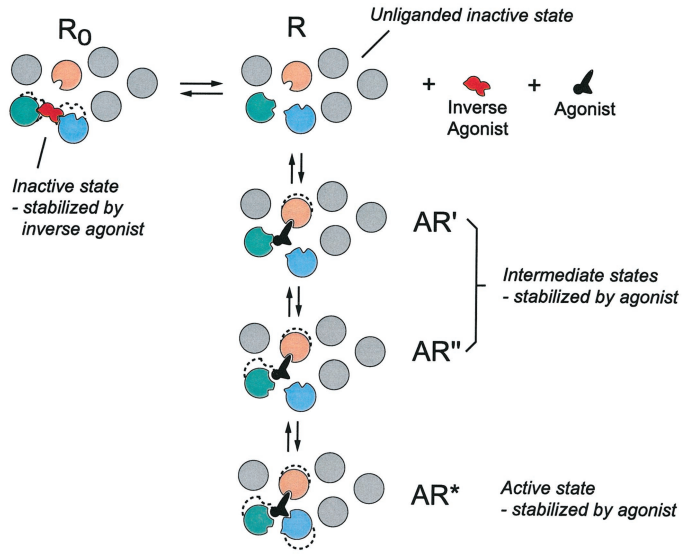


FIG. 5. Sequential binding and conformational stabilization model for the molecular mechanisms of ligand action in GPCRs. The hypothetical receptor is illustrated by seven apparent helices seen from above. The model predicts that the unliganded receptor exists in a unique state R that can undergo transitions to at least two other states R_0 and R*. R_0 is stabilized by inverse agonists and R* is stabilized by agonists. R may undergo spontaneous transitions to the R* state, explaining the high basal activity observed for some GPCRs, and it may undergo spontaneous transition to the R_0. As discussed in the text, binding of the agonist is suggested to occur sequentially, resulting in a series of conformational states that are intermediates (R′ and R″) between R and R*. The agonists are known to have several functionally important sites of interaction with the receptor. Binding may involve an initial interaction between receptor and one structural group of the agonist. After the initial binding of one structural group, binding of the remaining groups occurs in a sequential manner as a result of random and spontaneous movements of TM domains to positions that permit interaction with the functional groups. Each interaction between the receptor and the agonist stabilize one or more TM domains until the receptor has been stabilized in the active R* state. A similar mode of binding can be envisioned for inverse agonists resulting in stabilization of the R_0 state. The model would be consistent both with a rapid association rate for agonists (formation of AR′) and the relatively slow rate of conformational change observed spectroscopically (formation of AR*). Importantly, the G protein may substantially affect the kinetics of the transition from AR′ over AR″ to AR*. Similar to the multistate model described in Ref. 24, the model also readily accommodates the concept of "allosteric competitive antagonism" (24) *i.e.,* that a competitive antagonist does not have to share an overlapping binding site with the agonist. Hence, an "allosteric competitive" antagonist, according to the model, would simply act by stabilizing the receptor in R_0, which would not be expected to bind the agonist. Conversely, the agonist could stabilize the receptor in R*, which would not be expected to bind the antagonist. In this way, by stabilizing different receptor conformations, the agonist and the antagonist can mutually exclude the binding of each other to the receptor.

The observed slow activation kinetics cannot be readily accommodated into a simple "two-state model". According to this model the affinity of a full agonist for the R state is negligible; thus, agonist binding occurs selectively to the activated state R*, thereby pulling the equilibrium toward R*. This would predict that the association rate for agonist binding is limited by the rate of transition from R to R*. This is

not readily compatible with the observation in the β_2-adrenergic receptor that the conformational change, and not the binding event, is the rate-limiting step. We have therefore suggested the "sequential binding and conformational selection" model shown in Fig. 5 (22). This model predicts, similar to the two-state model (205) and the multistate model suggested by Schwartz *et al.* (24), that the receptor spontaneously alternates between different receptor conformations (active and inactive). However, a major difference is that binding of agonist does not occur directly to R* but is suggested to occur sequentially, resulting in a series of conformational states that are intermediates (R′ and R″) between R and R* (Fig. 5). Agonists are known to have several functionally important sites of interaction with the receptor (See *Section IV.A*). As illustrated in Fig. 5, binding may involve an initial interaction between receptor and one structural group of the agonist. After the initial binding of one structural group, binding of the remaining groups occurs in a sequential manner as a result of random and spontaneous movements of TM domains to positions that permit interaction with the functional groups. Each interaction between the receptor and the agonist stabilize one or more transmembrane domains until the agonist finally stabilizes the receptor in the active R* state. Such a model would be consistent both with a rapid association rate for agonists (formation of AR′) and the relatively slow rate of conformational change observed spectroscopically (formation of AR*). Importantly, the G protein may substantially affect the kinetics of the transition from AR′ over AR″ to AR*. The slow kinetics of the agonist-induced conformational change in the absence of G protein strongly suggests the existence of a high activation energy barrier for the transition from AR′ through AR″ to AR*. The R* state can from a thermodynamic point of view be considered a high-energy intermediate that can be stabilized energetically by the G protein and/or the agonist (220). It is conceivable that the G protein stabilizes the AR* state and, in addition, substantially lowers the activation energy barrier, causing the transition from AR′ through AR″ to AR* to occur much faster. The hypothesis awaits experimental evaluation in a reconstituted system with purified receptor and G protein. Nevertheless, it provides an intriguing explanation for the apparent discrepancy between the slow kinetics of agonist-induced conformational changes observed for the purified β_2-adrenergic receptor with the rapid responses to agonist stimulation of GPCRs in cells, such as, for example, activation of ion channels.

VII. Concluding Remarks

The wealth of information gained over the last decade has substantially improved our understanding of GPCR function and changed the way we look at receptors. Importantly, it has been conceptualized that GPCRs are not simple "on/off" switches but highly dynamic structures that exist in equilibriums between active and inactive conformations. In this framework, an agonist is recognized as a molecule that can stabilize an active conformation while an inverse agonist (*i.e.*, an antagonist with negative intrinsic activity) is a molecule that can stabilize an inactive conformation. Thus, it has become clear that not only agonist but also antagonists are capable of actively modulating receptor function. Moreover, it has become evident that neither agonists nor antagonists necessarily have to share an overlapping binding site, even if they act at the same receptor. An important implication of this in clinical endocrinology is the prospect of developing small-molecule antagonists and agonists for, in principle, any GPCR. Recent biophysical studies allowing direct structural analyses of conformational change in the receptor molecule represent an important first step toward a more profound understanding of GPCR function at a molecular level. However, our present knowledge about the physical changes in the receptor structure, distinguishing inactive from active states, is still very limited. Furthermore, our insight into the molecular basis for transmission of the signal to the G protein remains rather poor. The further clarification of these mechanisms represents a daunting task together with efforts aimed at obtaining high-resolution x-ray crystals.

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