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MOLECULAR MECHANISM OF 7TM RECEPTOR ACTIVATION—A GLOBAL TOGGLE SWITCH MODEL

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■ **Abstract** The multitude of chemically highly different agonists for 7TM receptors apparently do not share a common binding mode or active site but nevertheless act through induction of a common molecular activation mechanism. A global toggle switch model is proposed for this activation mechanism to reconcile the accumulated biophysical data supporting an outward rigid-body movement of the intracellular segments, as well as the recent data derived from activating metal ion sites and tethered ligands, which suggests an opposite, inward movement of the extracellular segments of the transmembrane helices. According to this model, a vertical see-saw movement of TM-VI—and to some degree TM-VII—around a pivot corresponding to the highly conserved prolines will occur during receptor activation, which may involve the outer segment of TM-V in an as yet unclear fashion. Small-molecule agonists can stabilize such a proposed active conformation, where the extracellular segments of TM-VI and -VII are bent inward toward TM-III, by acting as molecular glue deep in the main ligand-binding pocket between the helices, whereas larger agonists, peptides, and proteins can stabilize a similar active conformation by acting as Velcro at the extracellular ends of the helices and the connecting loops.

INTRODUCTION

G protein–coupled receptors constitute the largest family of proteins in the human genome (1–4). Based on their multiple signaling pathways, including several that are non-G protein dependent, these receptors should more appropriately be called 7TM receptors, referring to their characteristic common structural element, the seven transmembrane (TM) helical segments (3). These receptors are activated by numerous types of chemical messengers in the body—hormones, neurotransmitters, chemo-attractants, and paracrine agents—and they serve as chemo-sensors

for a multitude of different external chemicals such as odor and taste components and pheromones (2). Owing to their involvement as regulators of key physiological functions throughout the body, 7TM receptors represent one of the main focus areas of the pharmaceutical industry (5). 7TM receptors are the target for a majority of current drugs, which mainly address a rather small fraction of the receptor repertoire, specifically the monoamine receptors and a few peptide and lipid receptors. In general, 7TM receptors are considered to be "drugable" targets, implying that well-established high-throughput screening efforts often result in identification of useful lead compounds for subsequent optimization efforts, i.e., in a classical medicinal chemistry–driven drug discovery process. Nevertheless, it is also well-known in the field that a number of interesting 7TM receptor drug targets have proven to be surprisingly difficult and resistant to this generic, brute-force approach. Moreover, for the majority of 7TM receptors this approach mainly yields antagonist lead compounds, whereas the identification of agonists still is a major problem in most 7TM receptors. In contrast, in a small fraction of receptors, such as the somatostatin, melanocortin, C5a, and ghrelin families of receptors, it is relatively easy to discover agonists—and relatively difficult to discover antagonists/inverse agonists. The reason for this difference is not known. Although many of the agonist-prone receptors often show a high degree of ligand-independent, constitutive activity, this is not in itself the whole explanation. It is expected that detailed knowledge of the molecular mechanism of activation and inactivation of 7TM receptors will be valuable for future drug discovery efforts in this large family of receptors, such as Site Directed Drug Discover®, tethering ligand discovery, and related approaches (6–10).

Although the pharmaceutical industry has major problems in generating agonists for most 7TM receptors, this is apparently not a problem in nature. All kinds of chemical agents, ranging from calcium ions to large glycoproteins, act as endogenous agonists for 7TM receptors. In accordance with the significant differences in size and physiochemical properties of these ligands, they also bind very differently to their respective target receptors (see Agonist Stabilization of the Active Conformation of the 7TM Toggle Switch, below). Nevertheless, it is generally assumed that a common molecular activation mechanism for the 7TM receptor family exists. That is, it is anticipated that agonists acting upon different-yet-homologous receptors will induce or stabilize a common active receptor conformation.[1] Unfortunately, although several X-ray structures of rhodopsin are now available, they all represent the inactive, dark state of the molecule (13–15).

[1]For simplicity, the molecular activation mechanism for 7TM receptors is discussed as if there was only one active conformation, which most certainly is not the case (for review see 11). A given receptor may even be found in the same membrane in different active conformations activating different G proteins in microdomains, which do not readily interchange (12). However, in the present context, it is expected that such different active conformations constitute minor variations over the main toggle switch theme.

The activation-associated conformational changes have been studied using a number of biochemical and biophysical approaches, including site-directed spin labeling and various fluorescent technologies applied mainly to rhodopsin and the β_2-adrenergic receptor model systems (4, 16–22). These studies indicate that the intracellular segments of the TMs, especially TM-VI and -VII, move apart during receptor activation and thereby expose receptor epitopes, such as the TM-III/"DRY" and the TM-VII/helix-VIII motifs, thus allowing recognition by the intracellular signaling molecules, i.e., G proteins, arrestin, etc. (22–24). However, our knowledge of the structural changes, which occur during activation in the extracellular parts of 7TM receptors, i.e., where the agonists bind and act, has until recently been quite limited. The main reason is that the otherwise very informative biophysical methods, because of technical limitations, have not been applied to the extracellular parts of the receptors. However, alternative experimental approaches have recently begun to cast some light on the helical movements in these areas of the receptors. Thus, small, well-defined ligands have been anchored at specific sites in the main ligand-binding pocket through either metal-ion site or disulfide engineering and thereby provided novel, detailed information about the presumed helical movements at this side of the receptor (8, 25–27). These studies indicate that the extracellular segments of the TMs, especially TM-VI and -VII, appear to move in the opposite direction of the intracellular segments, i.e., the TMs move toward each other at the extracellular ends (8, 25–27). Therefore, a toggle-switch activation model for 7TM receptors in which TM-VI and -VII perform "vertical" see-saw movements around the conserved proline bends has been proposed to explain the opposite directed movements of the TMs at the intra- and extracellular ends (27).

The present review focuses on molecular mechanisms of agonist binding and receptor activation in family A, rhodopsin-like 7TM receptors, with an emphasis on the presumed movements of the TM helices.

AGONIST BINDING IN RHODOPSIN-LIKE CLASS-A 7TM RECEPTORS

In the late 1980s, cloning of the first monoamine receptors revealed that they were homologous not only to each other but surprisingly also to the light-sensing 7TM receptor molecule of the eye, rhodopsin (3). It soon became clear that these were only the first members of a very large and highly diverse super-family of receptor proteins, which are activated by a wide variety of different types of agonist molecules. Initially, it was expected that there would be a common active site for agonist binding and action, but subsequent analysis revealed that this was probably not be the case (for reviews see 28, 29). The issue of different agonist binding modes is discussed in more detail in Agonist Stabilization of the Active Conformation of the 7TM Toggle Switch, below, but a couple of basic concepts are introduced here.

A Main Ligand-Binding Pocket Is Located Between the Outer Segments of the Transmembrane Segments

The general organization of the seven-helical bundle was initially identified by cryo-electron microscopy studies with an impressive degree of accuracy (30–34). In 2000, this general topology was confirmed by the first X-ray structure of rhodopsin—an important landmark in the history of 7TM receptors (13)—as well as by subsequent high-resolution X-ray structures of the inactive, dark-state of rhodopsin (14, 15). Importantly, the X-ray structures provided and confirmed a number of important details as well as identified the structure of major parts of the extracellular elements (35–43). The high-resolution X-ray structures of the helical bundle clearly confirmed the localization of the ligand, the chromophore retinal, which is covalently bound in between the extracellular segments of the seven-helical bundle through a protonated Shiff base involving the epsilon amino group of LysVII:10 (Lys256) and with the counter-ion being the carboxylic group of GluIII:04 (Glu113) (13–15). In the dark state, 11-*cis* retinal functions as an inverse agonist that arches around TM-VI and -VII, with the β-ionone ring positioned between the vertical indole side chain of TrpVI:13 and TM-V (Figure 1). Whereas the seven-helical bundle is closely packed at the intracellular side, the extracellular segments deviate away from each other, creating the main ligand-binding pocket, or crevice (2, 28). Removal of 11-*cis* retinal from the inactive structure of rhodopsin reveals that the tilted TM-III opens for a relatively deep pocket between TM-III-IV-V and -VI, and that the proline bend in TM-VII away from TM-II opens for another, smaller pocket between TM-I-II-III and VII (Figure 2). It should be noted that the proline bend in TM-VI is tangential to the center of the receptor, i.e., away from TM-VII toward TM-V (Figure 2). It should also be noted that the main ligand-binding pocket is, in fact, not so deep and empty, as illustrated in Figure 2, except at the position where the β-ionone ring of 11-*cis* retinal has been removed (Figures 1 and 2). In rhodopsin, the main ligand-binding pocket is completely covered by a β-sheet structure that comprises major parts of the extracellular-loop 2 (EC-loop 2), forming the lid of the binding pocket above retinal (Figure 1). It is unclear to what extent EC-loop 2 forms a similar lid over the main ligand-binding pocket in other receptors. However, importantly, the middle of this loop is covalently bound to the top of TM-III through a highly conserved disulfide bridge (see A Global Toggle Switch Model for 7tm Receptor Activation, below, concerning certain constitutively active receptors lacking this disulfide bridge). In several receptors, even monoamine receptors, it has been demonstrated that ligands interact with residues located in EC-loop 2—especially those close to the cysteine in the middle of the loop, which forms the disulfide bridge to CysIII:01 (44, 45). Nevertheless, the main ligand-binding pocket—as defined by the binding site for retinal in rhodopsin—is located rather deep in the 7TM receptor structure, potentially covered by several layers of more-or-less tightly folded protein structure as viewed from the extracellular side.

Through a pioneering mutational mapping exercise performed by Strader & Dixon and their coworkers, this main ligand-binding pocket between the extracellular segments of TM-III, -V, and -VI was identified at an early stage as the binding site for adrenergic agonists (46–55), and subsequently other monoamine agonists (56–60). In this work, a key contribution was the identification of AspIII:08 (Asp113 in the β2-adrenergic receptor) as the binding site for the positively charged amine group of the agonists. This milestone was achieved through an elegant combination of molecular biology and medicinal chemistry (48, 49, 51). Importantly, the anchor point for monoamine agonists is located one helical turn below position III:04, which in the form of GluIII:04 (Glu113) in rhodopsin constitutes the counter-ion for the retinal Schiff base (61–63). Thus, at this point in time it was generally believed that the common binding site for retinal and adrenergic agonists in between TM-III, -V, and -VI was the active site in 7TM receptors, to which all agonists would need to bind to activate these receptors.[2]

There Is No Common Lock for All 7TM Receptor Agonist Keys

7TM receptors are activated by agonists, which range from large glycoprotein hormones over chemokine proteins, peptide hormones and neuro-peptides, lipid messengers, and nucleotides to small monoamines, amino acids, and even metal ions such as calcium. When the binding sites for small peptide agonists, such as substance P (NK1) (67–73) and angiotensin (AT1) (74–78), started to be mapped, the picture changed. It became clear that although small-molecule non-peptide antagonists did bind deep in the main ligand-binding pocket of these receptors—i.e., more or less corresponding to the binding sites of retinal and monoamine agonists and antagonists—the endogenous peptide agonists in fact bound to more extracellular epitopes in the receptors (68, 70, 72–75, 78) (see Agonist Stabilization of the Active Conformation of the 7TM Toggle Switch, below, for more details).

These observations indicated that there may not, in the otherwise common, conserved 7TM structure, be a common lock for all of the chemically highly diverse agonist keys (28, 29). That is, different types of agonists do not need to bind to a common set of specific residues in the receptor, such as the residue located at position III:08, in order to exert their agonist activity. Based on the classical allosteric theory of Monod et al. (79), it was suggested that 7TM receptors, like most other

[2]For a while it was generally believed that Glu113 in rhodopsin and Asp113 in the β2-adrenergic receptor were located at corresponding positions in the two 7TM receptors. This type of misunderstanding was the reason for the development of generic numbering systems for 7TM receptors. In the present review we use the numbering system of Baldwin (64) in a slightly modified form (28, 65), which provides a number for each residue indicating the actual location within each transmembrane helix. Another generic numbering system is based on giving a highly conserved residue in each helix the number 50 (66).

proteins, would interchange between inactive and active conformations and that agonists basically work as agonists by virtue of stabilizing an active conformation of the receptor (28, 29). In such a receptor model, the chemically very diverse agonists can stabilize a similar active conformation in rather significantly different manners depending on their size and chemical nature. However, the over-all molecular activation mechanism would be shared among the 7TM receptors and involve common conformational changes within the seven-helical bundle to convey the activation signal from the extracellular agonist to the intracellular signaling molecules.

TM MOVEMENTS AT THE CYTOPLASMIC FACE OF THE RECEPTOR DURING ACTIVATION

As noted above, the X-ray structures currently available only display the inactive, dark state of rhodopsin (13–15). Nevertheless, Schertler and coworkers have reported the cryo-electronmicroscopy structure of the light-activated photointermediate metarhodopsin I state, in which the isomerization of retinal to the all-trans, active form, has occurred (30). However, this structure does not reveal any noteworthy changes in the overall structure of the seven-helical bundle, in agreement with the notion that during the subsequent transition to the still-elusive metarhodopsin II active signaling state, rather large conformational changes in the rhodopsin protein are expected to occur, as also indicated by a number of different types of biophysical studies (4, 22). The site-directed spin labeling technique, in which the thiol groups of systematically introduced Cys residues are converted into nitroxide-containing side chains and analyzed by electron paramagnetic resonance (EPR) spectroscopy, has generated especially highly informative data concerning the helical movements (80–83). In a series of systematic studies, Hubbell & Khorana and coworkers have in this way performed an exhaustive characterization of side chain accessibility and mobility at essentially all positions throughout the cytoplasmic segments of the transmembrane helices as well as within the intracellular loops and major parts of the C-terminal tail of rhodopsin (17, 19, 22, 84–92). An EPR solution structure of rhodopsin was in this way generated providing important information about functional epitopes, for example, in IC-loop 3, which are too dynamic to be captured well by X-ray analysis. Importantly, changes in the EPR solution structure occurring during activation can be analyzed at the millisecond timescale. For example, the changes in side chain mobility provided a map of the tertiary contact surfaces that rearrange during the transition from the inactive to the active conformation of the receptor (22). The conclusion from these and other studies was that the cytoplasmic parts of the TM helices move apart during 7TM receptor activation to disclose receptor epitopes to intracellular signaling molecules such as G proteins and arrestin (22). Importantly, the movement of certain helices, i.e., especially TM-VI and -VII, are particularly significant.

TM-I Through TM-IV Appear to Form a Relatively Rigid Domain

TM-III constitutes a central, highly tilted rod in the 7TM receptor structure as it reaches from the extracellular end of TM-II to the intracellular end of TM-V, with TM-IV being located as a straight helix perpendicular to the membrane on the outside of TM-III (Figure 1). Thus, TM-I through TM-IV appear to form a relatively rigid core structure, which probably includes the intracellular segment of TM-V, as suggested by EPR and cross-linking experiments (93–95). Site-directed spin-labeling studies have demonstrated that during activation the side chain mobility increases for certain residues in TM-II and TM-III, which is interpreted as being a result of a slight outward movement of the intracellular poles of these two helices (22). However, the involved residues in, for example, TM-III are in fact located in the first helical turn after the highly conserved DRY sequence, meaning that these residues could be considered to be partly located in the loop region.

The TMs—Especially TM-VI—Move Away from Each Other on the Inside

In general, the site-directed spin-labeling studies demonstrated a reduced packing of side chains throughout the core of the rhodopsin receptor at the cytoplasmic face during activation. The most significant changes in side chain mobility were measured in and around TM-VI. This was interpreted as a rigid body movement of the intracellular segment of TM-VI away from the center of the receptor toward TM-V (19). To obtain quantitative data for this movement of TM-VI, direct distance measurements were performed through a series of double spin-labeling experiments. Thus, one spin label was introduced in TM-III at position $i + 4$ after the Arg of the DRY motif in combination with a systematic series of spin labels introduced at different positions through a $1^1/_2$ helical turn of TM-VI (17). In the dark state, the interspin distances were consistent with the rhodopsin crystal structure. However, upon light activation, a striking pattern of changes in distances was observed and interpreted to reflect an outward movement of TM-VI away from TM-III of a magnitude of up to approximately 8 Å at the level of the intracellular surface (17) (Figure 3). Similar studies indicated that the intracellular segment of TM-VII also undergoes a rigid body movement outward from the center of the protein, although of a smaller magnitude than seen for TM-VI during activation (22) (Figure 3).

Additional evidence in favor of especially TM-VI but also TM-III movements has been obtained by Kobilka & Gether and coworkers using fluorescence spectroscopy of β_2-adrenergic receptors labeled with fluorescent probes, which are sensitive to changes in the environment (16, 20, 96, 97). From a pharmacological point of view it was particularly interesting that upon treatment with either agonists or inverse agonists, they observed dose-dependent, opposite directed changes in fluorescent signals, suggesting oppositely directed conformational changes (16).

Subsequent studies with fluorescent labels built into specific positions at the intracellular end of TM-VI demonstrated changes in fluorescence, which were interpreted to result from an outward movement of this segment away from the center of the receptor (97–99), i.e., a similar movement to what had been observed in the spin-labeling experiments (17, 22).

A number of other studies have been performed mainly in rhodopsin, but also in the β2-adrenergic receptor and even in the parathyroid hormone, family B receptor, where the cytoplasmic ends of the helices were locked together either by disulfide bridge engineering or by metal ion site engineering (18, 22, 95, 100, 101). Most of these cross-linking experiments—especially those in which residues in TM-VI were linked to other helices—resulted in inhibition of signal transduction and thereby indirectly confirmed that movement of TM-VI is a crucial feature during receptor activation (18, 22, 100, 101). In contrast, disulfide cross-linking of residues in the cytoplasmic segments of TM-III and TM-V was permissive for signal transduction, indicating a lack of relative movement between these helices during activation, as discussed above (22, 95). That TM-VI changes position during receptor activation is also indicated by, for example, studies using the substituted cystein accessibility method of Javitch and coworkers (21) and by tryptophan UV-absorbance spectroscopy studies of Sakmar and coworkers (102).

The relatively large amplitude movements of TM-VI at the cytoplasmic surface are enabled through the flexibility of the IC-loop 3, which connects the intracellular ends of TM-V and -VI (22). IC-loop 3 is very variable in length but usually rather long (2, 103). The EPR solution structure of rhodopsin indicates that the periodic helical structure of both TM-V and -VI continues at the cytoplasmic side and that IC-loop 3 in fact forms a highly flexible helix-loop-helix structure (22). This loop has for many years been known to be involved in interactions with G proteins and arrestin (50); however, this is expected to occur mainly through the membrane-proximal parts (104, 105).

Signal Transduction Through TM-III /DRY and TM-VII /Helix-VIII Recognition

The opening or blossoming of the protein structure at the cytoplasmic side is expected to reveal epitopes such as the TM-III DRY motif and the TM-VII/helix-VIII motif, which change conformation and become accessible for recognition by and activation of G proteins and arrestin (106, 107). This process will not be dealt with in further detail in the present review so the reader is referred to other recent reviews (22, 36, 37, 39, 42, 43, 108).

TM MOVEMENTS IN THE MAIN LIGAND-BINDING POCKET OF THE RECEPTORS

The conformational changes occurring at the cytoplasmic face of the 7TM receptor are responsible for conveying the activation signal to the downstream transducer and signaling molecules. It is the binding of the agonist at the extracellular face of

the receptor that initiates this process, and this is also the site where the vast majority of drugs act. However, for various technical reasons, biophysical studies such as site-directed spin labeling have been directed almost exclusively toward the intracellular domains, and accordingly, very little information is currently available concerning the conformational changes that occur during activation at the pharmacologically important extracellular face of the seven-helical bundle of 7TM receptors.

Most agonists are structurally relatively complicated, and their molecular recognition is accordingly equally ambiguous (see below), thus providing rather vague information about the active conformation of the receptor protein, which they induce or stabilize. This was the basis for the attempt to make the smallest and most well-defined ligand—a metal ion such a Zn(II)—act as an agonist. This approach would provide some well-defined distance constraints in the otherwise elusive, active conformation of the main ligand-binding pocket of a 7TM receptor. The concept was based on the notion that a metal ion can only act as an agonist if it is coordinated in a very well-established geometry by the heavy atoms that constitute the metal ion binding site.

Initially, the technology was established through the exchange of the binding site for a relatively small non-peptide antagonist, CP96345, with a tridentate metal ion site built between the extracellular segments of TM-V and TM-VI of the substance P, NK1 receptor (109). Subsequently, a number of inactivating or inhibitory metal ion sites were built within and between TM-II, TM-III, TM-V, and TM-VI both in the NK1 and the κ-opioid receptor (110–112). These metal ion sites provided useful information about the relative helical orientation and structure of the seven-helical bundle, notably at a time when only low-resolution cryo-electron microscopy structures of rhodopsin were available (110–112). Some of the key distance constraints between the extracellular ends of TM-V and -VI were subsequently confirmed by disulfide engineering at the corresponding positions in rhodopsin (113). However, all of these initial series of metal ion sites were inhibitory.

7TMs Can Be Activated Through Metal Ion Sites Built in the TM-III-VI-VII Pocket

As a starting point for construction of activating metal ion sites, AspIII:08 in the β2-adrenergic receptor, which constitutes the classical anchor point for the positively charged monoamine agonists, was used (51, 114, 115). By either keeping the natural AspIII:08 as one of the metal ion binding residues or substituting it with a His residue, and exchanging AsnVII:06 on the opposing face of TM-VII, which is a known interaction site for partial agonists such as pindolole, with a metal-ion binding Cys residue, an activating metal ion site was constructed (25). In the β2-adrenergic receptor it was difficult to judge how efficacious the metal ions were as agonists, as the receptor no longer could be activated by catecholamines. However, when the III:08-VII:06 activating metal ion site was transferred to the NK1 receptor, the mutated receptor could be activated by metal ions binding to

the metal ion site located relatively deep in the main ligand-binding pocket and by substance P binding one helical turn further up in helices III, VI, and VII, as well as in the loops and the N-terminal extension (26, 116). At the III:08-VII:06 site, Zn(II) was only a 25% partial agonist, which was suggested to be due to the lack of involvement of the important TM-VI in the metal ion site (26).

Through a series of systematic mutational exchanges on the inner face of the extracellular segment of TM-VI, position VI:16 was identified as being ideally situated for construction of high-efficacy and high-potency activating bidentate as well as tridentate metal ion sites in combination with the original III:08-VII:06 site (27). The preferred metal ion binding residue at this position was Cys. Interestingly, when the shorter Asp was used in position III:08, the free metal ions Zn(II) or Cu(II), in a bidentate metal ion site with CysVI:16, could not act as agonists on their own but only in complex with aromatic metal ion chelators such as bipyridine or phenanthroline, which conceivably functioned as coagonists through interaction with hydrophobic, aromatic residues in the binding pocket (27). However, when the longer His was used in position III:08, the free metal ions could function as agonists on their own. Among the different activating metal ion sites constructed between TM-III, -VI, and -VII, the tridentate (HisIII:08, CysVI:16, CysVII:06) metal ion site provided the highest, submicromolar metal ion potency.[3]

TMs—Especially TM-VI—Move Toward Each Other on the Outside During Receptor Activation

Because the metal ion activates the receptor when, and only when, a metal ion binding residue is located in position III:08 together with a metal ion binding residue in position VI:16 and/or one in position VII:06, these studies indicate that the three positions III:08, VI:16, and VII:06 must be in close proximity in the active conformation of the receptor. In fact, based on the coordination geometry for metal ions, the oxygen, nitrogen, and sulfur atoms of the metal ion binding residues introduced at these three positions will have to be within a distance of approximately 3.2 Å from each other to bind a zinc ion well.

No high-resolution X-ray structure was available when the original, bidentate III:08-VII:06 metal ion site was constructed, and accordingly, it was not possible at that time to tell whether the metal ion was pushing or pulling on TM-III and TM-VII to activate the receptor (25). However, it is now clear that the activating metal ion is in fact pulling on TM-III and -VII, and even more so on TM-VI in those activating metal ion sites involving TM-VI (27). The distances were simply much too long between the metal ion binding heavy atoms to effectively coordinate a metal ion in receptor models built over the backbone structure of the inactive conformation of rhodopsin even when all possible rotameric conformations of the

[3]There was no indication of disulfide bridge formation between CysVI:16 and CysVII:06 because the agonistic activity could be stopped by simple washing and activation could be obtained also with the redox inactive Zn(II) (27).

side chains for the metal ion binding residues were probed in positions III:08, VI:16, and VII:06 (27). This was particularly clear for the III:08 to VI:16 distance, where the distribution of possible distances is 6.3–9.8 Å.

Thus, the molecular activation mechanism for 7TM receptors must involve a movement of the extracellular ends of TM-III, TM-VI, and TM-VII toward each other. At the level of positions III:08, VI:16, and VII:06, this movement will have to be at least 1–2 Å (27). Because TM-III appears to be conformationally constrained, or locked, in between the rest of the seven-helical bundle (see above), it is conceivably the extracellular segments of TM-VI and -VII that are moving inward toward TM-III during the activation process (Figures 4 and 5).

A GLOBAL TOGGLE SWITCH MODEL FOR 7TM RECEPTOR ACTIVATION

To accommodate the outward movement of especially TM-VI and -VII at the intracellular ends as well as the opposite, i.e., inward, movement of these helices at the extracellular ends, a global toggle-switch activation model for 7TM receptors has been proposed (27).

TM-VI and -VII Make Vertical See-Saw Movements Around the Proline-Bend Pivots

The basic concept of this model is, as presented above, that activation of a 7TM receptor occurs through an inward movement of the extracellular segments, especially the segments of TM-VI and -VII, which close the main ligand-binding crevice, while the cytoplasmic segments of the helical bundle open up for interaction with signaling molecules. In this model especially, TM-VI, but also to some degree TM-VII, perform a vertical see-saw movement around a fulcrum or pivot located close to the middle of the membrane, which is where the conserved prolines in both of these helices are located (Figure 6). It is tempting to speculate that this see-saw movement is associated with a rotation and partial straightening of the proline bends in the helices, especially TM-VI. In other words, oppositely directed rigid-body movements of the two halves of the proline-kinked transmembrane helices, above and below the highly conserved ProVI:15 and ProVII:17, are coupled and constitute the central component of the overall activation mechanism for 7TM receptors.

Much attention has been directed toward the occurrence and potentially functional importance of the conserved prolines in TM-V, -VI, and -VII of 7TM receptors (2, 22, 117–121) (Figure 6). Proline is a helix breaker that cannot participate in the important periodical hydrogen bond backbone network of an α-helix because it lacks a free α-amino group, and consequently prolines do not occur in α-helices of soluble proteins. Nevertheless, prolines are highly conserved at certain positions

in the middle of TM-V, -VI, and -VII in family A rhodopsin-like 7TM receptors. Consequently, it has been suggested that these prolines serve key roles as conserved, functional switches that allow for dynamic helical kink formations (2, 22, 117–121). In the inactive form of rhodopsin, a strong kink is found at ProVI:15 in which the extracellular segment of TM-VI is maximally bent. This bend is not radial, i.e., pointing away from the center of the receptor, but rather tangential, i.e., the extracellular segment of TM-VI points away from TM-VII and toward TM-V (Figures 2 and 5). Molecular dynamics simulations have shown that the bend in TM-VI is energetically highly unfavorable (119). Thus, in the inactive conformation of rhodopsin, TM-VI could be considered to constitute a kind of spring that is constrained through various molecular interactions (119, 120). In the β2-adrenergic and some related receptors, the constraint appears to encompass an ionic lock between ArgIII:26 of the DRY motif and a glutamic acid in the intracellular extension of TM-VI, which serves to hold the cytoplasmic end of TM-VI in close proximity to TM-III in the inactive form (120). However, Glu is not conserved among 7TM receptors in general at this position, and other constraints on TM-VI movements are therefore expected to be present in different receptors (122).

A number of models for 7TM receptor activation have focused on various types of rigid body movement of the intracellular segment of TM-VI, often involving straightening of the proline kink (17, 22, 97, 119, 121). In these models little, if any, attention has been directed toward the extracellular segment of TM-VI. It has generally been assumed that this part of the receptor would not participate in the dynamic conformational interchanges during activation. For example, the fact that disulfide bridge formation between certain positions in the extracellular segments of TM-V and TM-VI were permissive for receptor signaling in rhodopsin (113) has been taken as evidence indicating that this part of TM-VI does not move during activation (22). However, TM-V may be relatively flexible or may even participate in the inward movement (see below).

For the moment, schemes accounting for the detailed trajectory of the helical movements, especially those related to the extracellular parts, will merely be speculative. However, because the bend in TM-VI has a tangential orientation toward TM-V, a simple straightening of the segment above the ProVI:15 kink would in fact not bring the metal ion binding CysVI:16 closer to position III:08, as the metal ion site engineering data has indicated (Figure 5). Likewise, straightening of this kink by an outward movement of the intracellular segment would bring this segment closer to TM-VII. However, the spin-labeling studies indicate that this segment should move outward—but in the direction of TM-V (22). On the other hand, position VI:16 can be brought closer to III:08 by rotating or swinging the extracellular segment of TM-VI inward toward TM-III in the direction indicated in Figure 5. If the rotation of the extracellular segment is followed by/coupled to a straightening of the energetically unfavorable proline bend then the intracellular segment of TM-VI will move outward in the direction toward TM-V, as suggested by spin-labeling experiments (Figure 5). A similar combined rotation of the

extracellular segment of TM-VII and a straightening of the bend in this helix would again be in agreement with both the metal ion site distance constraints at the extracellular pole and the spin-labeling experiments performed at the intracellular pole of this TM (Figure 5). NMR or X-ray crystallographic analysis of the active conformation(s) will eventually show whether these speculations do indeed reflect the real activation process of 7TM receptors.

TrpVI:13 of the CWLP Motif as a Rotamer-Switch Allowing TM-VI Inward Movement and Proline Bend Straightening

The residues located in the mini-loop between IleVI:11, with the free carbonyl group that cannot make a backbone hydrogen bond, and ProVI:15 constitute the conserved so-called CWLP finger-print motif for TM-VI. Besides ProVI:15 [close to 100% conserved (122)], it is CysVI:12 (74% Cys and 9% Ser) and TrpVI:13 (71% Trp and 16% Phe) that are the highly conserved residues of this motif. In the inactive structure of rhodopsin, the side chain of TrpVI:13 (Trp265) is situated vertically at the interface between TM-III and -VI (Figure 2), conceivably stabilized by a hydrogen bond to the preceding Cys residue or to a water molecule toward TM-VII (119). This location of the TrpVI:13 side chain appears to prevent the suggested inward movement of the outer segment of TM-VI (Figure 2). NMR studies of rhodopsin have demonstrated that in the dark state, especially the Trp side chains are restricted to a single specific conformation (30, 93). However, it has been suggested by Javitch & Ballesteros that TrpVI:13, together with CysVI:12, could function as a rotamer switch in the activation of 7TM receptors. The idea was that these residues in a concerted manner would change rotameric state, which was suggested to be involved in the modulation/straightening of the proline-kink of TM-VI (29). Importantly, both cryo-electron microscopy and NMR studies indicate that TrpVI:13 changes position as well as interaction partners during receptor activation (29). We propose that the rotation of the side chain of TrpVI:13 away from the interface between TM-III and TM-VI into the empty pocket between TM-III and -V, where the β-ionone ring or 11-*cis* retinal is located in the inactive state of rhodopsin, releases a steric constraint on TM-VI by allowing the inward movement of the outer segment of TM-VI and the straightening of the helix (Figures 5 and 6). So, what keeps TrpVI:13 in the active rotamer configuration?

The Conserved PheV:13 Could Function as a Lock for the Trp-Switch

In 81% of all rhodopsin-like 7TM receptors, either a Phe or Tyr residue is found in position V:13 (122), strongly indicating that the large aromatic side chain is of functional importance at this particular position located at the interface between TM-V and TM-VI. In accordance with this notion, substitution of PheV:13 with an Ala totally eliminates the very high constitutive activity of the ghrelin receptor and shifts the agonist dose-response curve to the right. This is also the case in

GPR39—another highly constitutively active receptor—as well as the β2-adrenergic receptor (123). Because PheV:13 is clearly critical not only for the ligand dependent but, importantly, especially for ligand-independent signaling, and because this residue is located in the middle of TM-V next to the presumed important weak point in the helix (see below), it was suggested that the conserved PheV:13 constitutes a structural element in the basic activation switch (123). As shown in Figure 7*B*, when TrpVI:13 is rotated into the presumed active rotamer conformation, the indole side chain makes an edge-to-face aromatic-aromatic interaction with the benzyl side chain of PheV:13 (123). Thus, PheV:13 could function as a lock for the side-chain of TrpVI:13 in the active conformation of the rotamer switch. PheV:13 is located in the mini-loop or weak point in TM-V generated by ProV:16, and it could be speculated that an interaction between the side chain of PheV:13 with TrpVI:13 could affect the backbone configuration around PheV:13 and thereby the bend in TM-V.

TM-V Movements as Part of the Conformational Activation Mechanism

ProV:16 is the third highly conserved proline residue among rhodopsin-like 7TM receptors, and accordingly— analogous with ProVI:15 and ProVII:17—ProV:16 would be expected also to play an important functional role. However, if TM-V participates in the activation mechanism, it is probably mainly the extracellular segment that is involved because, as discussed above, the biophysical characterization would tend to indicate that the cytoplasmic end of TM-V does not participate much in the activation-associated conformational changes (22).

In the inactive form of rhodopsin there is only a rather minor bend around ProV:16, with the extracellular segment deviating slightly outward, away from the center of the receptor. In a proline bend in a transmembrane helix, the backbone hydrogen bond network breaks at the *i-4* position, where the free carbonyl group cannot hydrogen bond to the proline, and consequently, the following part of the transmembrane segment, including the proline, will be able to bend in a direction away from the free carbonyl (see Figure 7). Although there is no major bend in TM-V in the X-ray structure of the inactive state of rhodopsin, the backbone of the helical turn preceding ProV:16 in the middle of the helix does constitute a non-helical bulge, or mini-loop, in which the free carbonyl group of HisV:12—located in the *i-4* position relative to ProV:16—is highly twisted and makes a hydrogen bond almost perpendicular, away from the helical rod toward TM-III to the side chain of GluIII:13, which is bending backward into the main ligand binding pocket to make this contact (Figure 7). Thus, one possibility could be that receptor activation is associated with an increased bend formation around ProV:16, or rather around the weak point at the highly distorted, free carbonyl in position V:12.

It is interesting to note that the helical stability of the extracellular segment of TM-V appears to vary among receptors, as indicated by the different results obtained with cysteine accessibility studies in different receptors. In, for example,

the dopamine D2 receptor, a clear picture of only the expected inner face of the extracellular end of TM-V is obtained by use of a polar, ligand-like thiol-reactive probe (124). In contrast, in the β2-adrenergic receptor, basically all positions are labeled in the corresponding part of TM-V (124, 125), suggesting that in this receptor, which is more constitutively active, the helical structure of the extracellular end of TM-V is lacking, or rather that it is more flexible or dynamic.

The stability of an α-helix can be increased through metal ion binding between two histidine residues located in an *i* and *i* + *4* configuration, which is ideal for a bidentate metal ion site (126, 127). Interestingly, when such a bis-His site is built between positions V:01 and V:05—both facing the main ligand-binding pocket—in the highly constitutively active ORF-74 receptor, Zn(II) acts as potent, full inverse agonist on the receptor (128). This effect cannot be related to a direct or indirect influence on ligand binding because it is the ligand-independent, constitutive activity of this virally encoded 7TM oncogene that is inhibited (128). Thus, in the ORF-74 receptor, stabilization of the helical structure of the extracellular segment of TM-V by metal ion binding in an intrahelical metal ion site effectively silences all signaling of the receptor, strongly indicating that TM-V, at least in this receptor, is involved in the 7TM activation mechanism. This notion is supported by observations in the NK1 and the κ-opioid receptors, where metal ion binding in the corresponding V:01 to V:05 bis-His sites blocked ligand binding and receptor activation (109, 111). Interestingly, in the NK3 tachykinin receptor, His residues are naturally found at positions V:01 and V:05, and binding of Zn(II) at this natural metal ion site enhances agonist binding without having any effect on its own (129). Thus, in this case it appears that a regular, stable helical structure in the extracellular segment of TM-V is compatible with the binding of the agonist peptide, which also is known to interact with other residues in TM-V and the adjacent loop (130, 131). Together, all of these observations indicate that the extracellular segment of TM-V is somehow involved in the activation process for 7TM receptors.

However, despite numerous and systematic attempts, it has not been possible to construct an activating metal ion site between TM-V and any other helix. A relatively high affinity bis-His metal ion site could be built between position 05 in TM-V and position 05 in TM-III in the NK1 receptor—even though the relatively long distance between these two positions in the rhodopsin structure suggests that this would require an inward tilt of the extracellular segment of TM-V (112). However, in contrast to the inter-helical metal ion sites built between TM-VI and TM-III, this site was inhibitory. Similarly, all the sites that have been built between the extracellular segments of TM-V and -VI also blocked receptor activation (109, 111).

Whereas the proline in TM-V is very well conserved among rhodopsin-like receptors in general, there are two receptor families in which this is not the case—and interestingly, members of both of these families are agonist prone and easy to activate. Thus, ProV:16 is not found in the family of glycoprotein hormone receptors, including the TSH, LH, and FSH receptors. These receptors can, for example, be activated just by removal of their large N-terminal domain (132).

Moreover, a multitude of naturally occurring, activating mutants, some of which even are located in the extracellular loops, have been described in these receptors as the cause of various diseases (133). The other major family of receptors lacking ProV:16 is the relatively large melanocortin (MC) receptor family, which also comprises the cannabinoid receptors; the sphingolipid receptors; and the GPR-3, -6, and -12 lipid receptors (134, 135). In fact, members of this family are also lacking the otherwise highly conserved disulfide bridge extending from the top of TM-III to the middle of EC-loop 2 (134). In the MC receptors, TM-V is instead kept in close proximity to TM-IV by an extremely short EC-loop 2, which basically consists of only a couple of polar residues. In the cannabinoid and the other lipid receptors EC-loop 2 is longer, but it is characterized by the presence of two additional Cys residues, which very likely form an intra-loop disulfide bridge that constrains the movements of TM-V relative to TM-IV, analogous to the ultra-short loop in MC receptors (134). Interestingly, members of this family of receptors that lack ProV:16 are also easy to activate and are characterized by a relatively high degree of constitutive signaling activity (134–136). It could be speculated that in this family, the lack of EC-loop 2, which normally is covalently attached to the extracellular end of TM-III, provides an open main ligand-binding pocket, which allows for relatively free movement of TM-VI and -VII inward to the active conformation.

It is at present difficult to predict a general role for TM-V in the activation mechanism of 7TM receptors. Nevertheless, it is suggested that it does play an important, but probably more secondary, role, which may differ depending on the receptor (sub)family. The extracellular end of TM-V could, for example, take part in constraining the receptor in the inactive conformation by preventing the extracellular end of TM-VI from moving inward. Because of the weak point created by ProV:16, TM-V could change conformation upon ligand binding—or straighten out or even perhaps bend further away from the center of the receptor—and in this way perhaps allow the extracellular end of TM-VI to move inward.

AGONIST STABILIZATION OF THE ACTIVE CONFORMATION OF THE 7TM TOGGLE SWITCH

The constitutive activity observed in many 7TM receptors indicates that the receptors are able to adapt the active signaling conformations on their own, and, therefore, the energy barrier between inactive and active conformations is rather small. Thus, the role of an agonist is basically to stabilize the receptor in the active signaling conformation by binding selectively to this receptor conformation. Importantly, the global toggle switch activation mechanism allows ligands to act as agonists at different sites in the receptor depending on their size and chemical nature. However, certain small agonists that bind relatively deep in the main ligand-binding pocket present particularly important insight into their molecular mechanism of action, as discussed in the following sections.

The β-Ionone Ring of Retinal Leaves the Inner Face of TM-VI During Rhodopsin Activation

Retinal functions both as an inverse agonist and as an agonist for the opsin 7TM receptor molecule—depending on the conformation around the double bond in position 11. In the dark, inactive state of rhodopsin, 11-*cis* retinal is an efficient antagonist, or rather inverse agonist, which keeps rhodopsin signaling completely silent. The positioning of the β-ionone ring deep in the pocket between TM-V and TrpVI:13 (Trp265) appears to be highly appropriate for a blocker of receptor activation, as it efficiently prevents the rotameric interchange of TrpVI:13 into the proposed active conformation and sterically blocks the inward movement of the extracellular segment of TM-VI (Figure 1). Moreover, the outer segments of TM-VI and -VII are tied closely together by the covalent molecular string, which retinal constitutes as it extends from the β-ionone ring located between TM-V and -VI to the helical backbone on the opposite side of TM-VII at position VI:10 (LysVII:10) (Figure 1). Light turns the inverse agonist 11-*cis* retinal into the agonist, all-*trans* retinal. Although there is some debate concerning the position of the β-ionone ring immediately following the light-induced isomerization (137–140), it appears that retinal during the formation of the active metarhodopsin-II form changes position rather significantly. For example, NMR analysis indicates that the β-ionone ring during activation makes a translational movement toward TM-V (93, 94). Moreover, a photo-active analog of 11-*cis* retinal, which in the dark state exclusively labels TrpVI:13, does not at all label TM-VI in the active state, but instead labels a residue in TM-IV (137). Thus, during receptor activation, the β-ionone ring of retinal at least appears to leave the pocket between TM-VI and -III next to TrpVI:13. From a toggle switch point of view, the interpretation will be that the strong steric constraint or lock on TM-VI is released, as free space is provided for TrpVI:13 to rotate and thereby allows for an inward movement of the extracellular segment of TM-VI (Figure 7) (27).

Binding of Adrenergic Agonists Is Compatible with an Inward Movement of Helices in the Main Ligand-Binding Pocket

As discussed above, the binding sites for the classical monoamine agonists are found in the pocket between the extracellular segments of TM-III, -V, and -VI (46–55). It was quickly realized that this in many ways closely corresponds to the binding site for retinal in rhodopsin, although with more emphasis on the importance of residues in TM-V (48, 49). Thus, the main interaction points for the prototype monoamine agonist epinephrine are believed to be (*a*) AspIII:08 (Asp113 in the β2-adrenergic receptor) for the positively charged amine; (*b*) a cluster of serines on TM-V consisting of SerV:08 (Ser203), SerV:09 (Ser204), and SerV:12 (Ser207) for the two hydroxyl groups on the catechol-ring; (*c*) PheVI:17 (Phe290) for the catechol-ring itself; and (*d*) AsnVI:20 (Asn293) for the β-hydroxyl group (Figure 8). It should be noted that there are possibilities for

alternative or additional interactions in the binding pocket, for example, one of the catechol-ring hydroxyls could very well interact with ThrIV:16, as shown in Figure 8.

A number of recent studies all support the notion that the binding site for epinephrine is not a solid mold in which the ligand fits like a key in a lock (141–144). Rather, it appears that the different functional groups of the ligand bind sequentially to the residues indicated above, and that, for example, the binding of the β-hydroxyl group is a late but crucial event in the binding process (143). Importantly, energy calculations based on systematic variations of the functional groups of the ligand and analysis of their binding affinity to several receptor mutants has revealed a substantial degree of cooperativity or synergy between the individual interactions rather than plain additive effects (141, 144). This would indicate that the agonist binds in an induced fit manner, involving a sequential series of conformational changes. Based on a joined analysis of epinephrine analogs and receptor mutants, Costa and coworkers concluded that the free energy couplings had to reflect global conformational changes that altered the mutual distances among several receptor domains and the entire three-dimensional configuration of the ligand-binding subsites (141). It was suggested that the best explanation is that during the agonist binding process the ligand-binding subsites would move closer to each other (141, 142). This type of binding mode is obviously in complete agreement with the present toggle switch model for 7TM activation, where the vertical see-saw movements of especially TM-VI and -VII result in a closing of the extracellular segments of the TMs in the main ligand-binding pocket around the stabilizing agonist—for example, the metal ion in the metal ion site-engineered receptors or a small catecholamine in the wild-type adrenergic receptor. The binding event for epinephrine could therefore, from the point of view of the toggle switch model, be envisioned to occur in a series of sequential steps as follows:

1. The initial event is assumed to be the binding of the positively charged amine group of the ligand to the negatively charged carboxylic acid side chain of AspIII:08. Charge-charge interactions are relatively long-ranged and not very dependent on the direction, making this a likely early event in the formation of the ligand-receptor complex.
2. This interaction enables the hydroxyl groups of the catechol ring to orient the ligand through interactions with the cluster of hydrogen bond donor-acceptor side chains located around the extracellular segments of TM-V and TM-IV. The ligand can probably, at least initially, interchange between different binding modes, for example, with the para-hydroxyl group interacting with SerV:08 and the ortho-hydroxyl interchanging between interactions with SerV:09, SerV:12, and ThrIV:16 through a more-or-less simple rotation of the catechol ring (Figure 8). The free back-bone carbonyls of SerV:12 and ThrIV:16, which are located in an *i-4* position relative to ProV:16 and ProIV:20, respectively, could be alternative hydrogen partners. Importantly, however, the observed cooperative effects indicate that

conformational changes will occur between the binding site for the ethylamine in TM-III and the binding site for the catechol hydroxyl groups in TM-V (and -IV?) and even between SerV:09 (Ser204) and SerV:12 (Ser207) within TM-V (141). It is at present unclear what these conformational changes in the receptor around TM-V represent; however, one possibility could be that the interaction of the two catechol hydroxyl groups could stabilize the otherwise rather unstable helical structure of the outer segment of TM-V through binding to the serines located in *i, i + 3*, and *i + 4* positions, and/or the agonist could alter the proline bend of this helix and/or alter the interaction between TM-V and TM-IV through binding in between these helices.

3. To reach from position III:08 to TM-V, the backbone of epinephrine is proposed to establish van der Waal interactions with the side chain of ValIII:09 (Figure 8). At this frozen point in the agonist binding process, the extracellular segment of TM-III could be regarded as being decorated with an extra aromatic group (the catechol ring) and an extra hydroxyl group (the β-hydroxy group of the agonist), both pointing toward TM-VI.
4. As reflected in the constitutive activity of the receptor, TM-VI will occasionally bend into its active conformation toward TM-III; but, in the absence of the agonist, TM-VI will not bind stably to TM-III, and consequently rapidly move back into the inactive conformation. However, with the agonist attached to TM-III, PheVI:17 will, in an inwardly bent conformation of TM-VI, be able to make an aromatic-aromatic interaction with the catechol ring. In fact, binding of just the catechol-ring fragment of the agonist, presumably between TM-V and TM-VI, will lead to partial activation of the receptor and to conformational changes in the cytoplasmic segment of TM-VI, as determined by fluorescent probes (143, 145).
5. AsnVI:20, located one helical turn above PheVI:17, will, in the presumed fully active conformation of TM-VI—in contrast to the inactive conformation—be able to make a hydrogen bond to the β-hydroxyl group on epinephrine. These interactions in which the agonist functions as a glue between TM-VI and TM-III will lock TM-VI in the active, inward conformation and lead to = full agonist-mediated receptor signaling, i.e., through the coupled outward movement of the cytoplasmic segment of TM-VI, as described above.
6. TM-VII is normally not envisioned to participate in the binding of epinephrine. However, it is here presumed that TM-VII also performs a see-saw movement, as described above, during activation of the β2-adrenergic receptor. Conceivably, the movements of TM-VI and -VII are more or less coupled. In this context, it should be noted that in high-affinity agonists such as isoproterenol, the N-methyl group of epinephrine is substituted with N-isopropyl, which is believed to bind in between TM-VI and TM-VII, around position VII:06 (146).

The schematic, step-wise binding process for epinephrine described above obviously represents an oversimplification. In reality, the binding event is a highly dynamic process where the individual steps involving the different subsites will engage and disengage, and not necessarily in the consecutive order suggested here. The most important issue in the present context is that there is a considerable degree of cooperativity between these events that involves conformational changes within the binding pocket, and a major component appears to be the inward movement of the agonist-binding residues on TM-VI toward TM-III (141–144)—in agreement with the global toggle switch model for receptor activation (27).

Agonism Through Metal Ion Site Anchoring of Aromatic Chelators in the TM-III-IV-V Pocket of a Chemokine Receptor

An important point in the schematic, dynamic binding process for epinephrine presented above is the frozen picture in which the catechol ring of the agonist and the neighboring β-hydroxyl group are tethered to TM-III in the TM-III-IV-V pocket as interaction partners for residues in TM-VI to bind to and thereby holding TM-VI in its active conformation(s). Although it is still unclear precisely where the β-ionone ring is located in the active signaling conformation of rhodopsin, it is likely that it is similarly positioned in this TM-III-IV-V pocket, as it apparently leaves its former position close to the inner face of TM-VI and translates toward TM-V (94). To test the hypothesis that this pocket between the extracellular segments of TM-III, -IV, and -V is a favorable binding site for small-molecule agonists to act as interaction partners for residues in the extracellular segment of TM-VI, an artificial attachment point for small aromatic metal ion chelators was constructed between TM-III and -IV in the chemokine CXCR3 receptor (147). In this receptor, the combination of the natural Asp in position IV:20 and a His residue introduced at position III:05 created a high-affinity bidentate metal ion site (147). That is, the metal ion site was not readily apparent, as free metal ions had no effect on receptor activation, in agreement with the notion that the interface between TM-III and TM-IV does not participate significantly in the movements during receptor activation (see above). Similarly, the small aromatic chelators, phenanthroline and bipyridine, did not affect receptor activation on their own. However, in complex with Zn(II) or Cu(II), bipyridine or phenanthroline functioned as high-potency and highly efficacious agonists for the metal-site engineered CXCR3 receptor—even with higher efficacy than the natural ligand, the chemokine protein I-TAC (147). It is envisioned that the metal ion basically functions as a bridge or attachment vehicle for the aromatic chelator, which hereby becomes tethered in the TM-III-IV-V pocket at a position corresponding to the location of the catechol ring of epinephrine (Figure 9). Mutational mapping identified TyrVI:16 as the second-site, or interaction partner, for the agonistic metal ion chelators (147). In molecular models in which the extracellular segment of TM-VI had been rotated closer to TM-III, i.e., into a presumed active conformation, the phenol side chain of TyrVI:16 is able to make a $\pi - \pi$ interaction with the aromatic

chelator that is tethered to the metal ion bound between HisIII:05 and AspIV:20 (Figure 9) (147).

The second site interaction point for the chelators in the metal ion site-engineered CXCR3 receptor—the natural TyrVI:16—corresponds to CysVI:16, which was engineered into the β2-adrenergic receptor to establish the bi- and tridentate activating metal ion sites with residues at position III:08 and VII:06 (see above) (27). According to the molecular models, an inward movement of the extracellular segment of TM-VI is required in both cases to make a good connection, in accordance with the toggle switch model for 7TM receptor activation.

Agonism Through Disulfide Anchoring of Small Organic Compounds in the TM-III-VI-VII Pocket of the C5a Receptor

The most convincing insight into the molecular mechanism of action of a ligand is obtained when the compound is covalently coupled to the receptor at a specific position because the location of the ligand in that case is truly well defined. In an elegant study, Buck & Wells tethered ligands to specific positions within the main ligand-binding pocket of the C5a receptor obtained through the introduction of reactive Cys residues (8, 9). Especially interesting results were obtained when the thiol-reactive small organic molecules were tethered to TM-VI through a Cys residue introduced at position VI:20, i.e., corresponding to the interaction point discussed above for the β-hydroxyl group of the catecholamine agonists in the β-adrenergic receptor (52). Mutational analysis revealed that in this case position III:08 on the opposing face of TM-III functioned as an important second-site interaction point for the disulfide tethered ligands (8). Interestingly, it was observed that removal of the large aliphatic side chain of the natural IleIII:08 (Ile116) through mutation to an Ala residue improved the affinity for certain compounds and, importantly, this substitution changed the compounds from functioning as antagonists to instead being agonists (8) (Figure 10). Conversely, introduction of the larger, more conformationally constrained Trp residue at position III:08 decreased the affinity as well as the agonism of the small organic compounds, which were covalently tethered at position VI:20 (8). The III:08 position, at which the affinity and efficacy of the TM-VI-attached compounds was altered depending on the size of the residue, is identical to the anchor point for the positively charged monoamines (Figure 8) and for the activating metal ions in the metal ion site-engineered β2-adrenergic receptor (Figure 4). Moreover, this position is located one helical turn below the attachment site for the agonistic metal ion chelators in the CXCR3 receptor, HisIII:05 (Figure 9) (147).

Thus from a toggle switch point of view, small organic compounds tethered to position VI:20 function as agonists for the C5a receptor by mediating TM-VI to bind to the opposing face of TM-III and consequently be held in an active, inward-bend conformation. This notion is supported by the fact that the agonistic effect is increased through substitution of Ile with the smaller Ala residue at position III:08 at the interface between TM-III and -VI, presumably because this small

residue provides sufficient space for the attached agonistic compounds. In contrast, introduction of the larger Trp residue at this presumed docking site for the ligand-decorated inner face of TM-VI results in decreased affinity and decreased agonism, for the same compounds, presumably through a steric hindrance mechanism (8).

Constitutive Toggle Switch Activation by Large Aromatic Side Chains Tethered in the TM-III-VI-VII Pocket of the Ghrelin Receptor Family

An important aspect of the classical Monod-Wyman-Changeux model for protein activation is that the receptor by itself is able to interchange between active and inactive conformations, which is associated with constitutive, ligand-independent activity (148). Most 7TM receptors show a low degree of constitutive signaling activity (149), but a few receptors, such as the receptor for the appetite-inducing hormone ghrelin, are in the absence of agonist signaling with 50% or even more of the maximal signaling capacity (135, 150).

In the ghrelin receptor and in other constitutively active members of this receptor family, the ligand-independent signaling activity appears to be controlled by a cluster of aromatic residues located at the interface between TM-III, -VI, and -VII, where many small molecule agonists bind and where the activating bi- and tridentate metal ion sites were built (150). Thus, the constitutive activity of the ghrelin receptor, as well as the closely related GPR39 receptor, can systematically be tuned up and down depending on the size and hydrophobicity/aromaticity of the side chain presented in position VI:16 in the presence of a large hydrophobic residue in position VII:06 and especially position VII:09 (150). It is presumed that aromatic-aromatic interactions of PheVI:16, PheVII:06, and PheVII:09, the latter of which in the majority of 7TMs is a residue with a very small side chain (122), are sufficiently strong to stabilize the active, inward-bend conformation of TM-VI, even in complete absence of ligand, resulting in the observed high degree of constitutive activity (150) (Figure 11). It could be envisioned that the ghrelin family of receptors uses the large aromatic amino acid side chains at the core of the main ligand-binding pocket as tethered agonists to obtain constitutive activation of the toggle switch, analogous to the metal ions and metal ion chelator complexes (25, 27, 147) and the disulfide tethered ligands of Buck & Wells (8).

There Are Different Locks for the Different Keys in a Common Toggle Switch Activation Mechanism for 7TM Receptors

In the sections above, the similarities in interaction points were emphasized for the various small-molecule agonists, whether these ligands were binding in a classical sense, tethered to the receptor at specific sites, or were natural, large side chains functioning as tethered ligands. According to this view, small-molecule agonists utilize similar interaction modes to obtain agonism as a consequence of their binding to residues at these key positions in the main ligand-binding pocket

(Figure 12). A novel, recently characterized example of this is the small dicarboxylic acid, succinate, which was identified to act as an agonist for the orphan receptor GPR91 through binding to three arginine residues located at positions well-known from the activating metal ion sites and tethered agonist studies: III:05 (Arg99), VI:20 (Arg252), and VII:06 (Arg281) (151).

However, although there are clear similarities between the binding modes for many small-molecule agonists deep within the main ligand-binding pocket, 7TM receptors, including monoamine receptors, can be activated by other mechanisms involving, for example, antibodies directed against extracellular loops and by mutations in these loops, especially EC-loop 2 (152–155). This in fact led to introduction of the concept that there is no common lock for all agonist keys in 7TM receptors (29). Importantly, the global toggle switch activation model provides a truly general mechanism that encompasses activation by all of the different locks for the chemically diverse agonist keys. That is, irrespective of the binding site of the agonist, a common or rather a similar active conformation is stabilized in which the outer segments of TM-VI and -VII are docked in an inward, activating conformation toward TM-III.

A small metal ion can be an agonist for a 7TM receptor, for example, through coordination of residues located at positions III:08, VI:16, and VII:06 deep in the main ligand-binding pocket (Figure 4) (25–27). The larger, but still relatively small, organic compounds act as agonists through binding to one or more of these residues and/or residues located further up on the inner face of the same helices. Or they may bind within the TM-III-IV-V-VI pocket and still be agonists, provided that the compound is able to stabilize an active, inward-bend conformation of TM-VI (Figures 8 and 9). Peptide hormones and neuropeptides stabilize similar receptor conformations through binding mainly to residues located toward the extracellular ends of the helices. Such peptides often have additional interaction points, for example, in the loop regions. Similarly, the large chemokines obtain most of their binding affinity through interactions with the N-terminal domain and extracellular loops (156, 157).

A persistent and recurring question in the field is whether, for example, peptide hormones and neuro-peptides somehow reach down and touch proposed trigger residues at key positions, such as III:08 deep in the main ligand-binding pocket, or even further down toward position II:10 (the conserved Asp). Some of the early mutational studies indicated that this was indeed the case, for example, for a classical neuropeptide agonist such as substance P (see for example 158). However, when these mutants were probed not only in heterologous but also in homologous binding assays, it surprisingly turned out that the actual affinity of the peptide agonists was not affected (159). Rather, these mutations had impaired the ability of the receptor to interchange between different conformations, each binding preferentially the different ligands, and in this way impaired the apparent affinity of the peptide agonist binding to one conformation, whereas the radioactive antagonist tracer bound to the other conformation. Importantly, the actual or real affinity of the peptide agonist as measured in homologous binding assays had not

been affected (159). The fact that some peptides can be efficacious agonists on a 7TM receptor without reaching deep down in the main ligand-binding pocket was probably most clearly demonstrated by steric hindrance mutagenesis performed at key positions lining the main ligand-binding pocket of the NK1 receptor (116). Bulky substitutions at, for example, positions III:08 or VII:09 impaired the binding of a number of small, nonpeptide antagonists 100–1000-fold, yet did not affect the binding and the signaling function of the agonist peptides, including substance P (116).

Thus, although substance P functions well as an agonist without interacting with receptor sites located deep in the main ligand-binding pocket, other peptides may still have such interaction sites in addition to their usually multiple interaction points in the receptor loops and N-terminal domain. The important issue is that in the global toggle switch activation model, there is no requirement for an agonist to touch residues at particular positions to activate the receptor. Instead and irrespective of the exact interaction points, the agonist only needs to bind to and stabilize an active state of the receptor in which a key feature is suggested to be an inward-bend conformation of TM-VI and -VII. This can be achieved in multiple ways depending on the size and physiochemical properties of the agonist: At one end of the spectrum are the metal ions and small-molecule agonists, which function as molecular glue deep between TM-III, -VI, and VII, and at the other end of the spectrum are peptides and proteins, which often function as Velcro at the extracellular ends of the helices or their connecting loops.

KOSHLAND INDUCED FIT FOR LIGAND BINDING—BUT MONOD-WYMAN-CHANGEUX CONFORMATIONAL SWITCH FOR THE ACTIVATION MECHANISM

Ligand binding generally occurs at the extracellular face of the 7TM receptor, and activation of intracellular signaling molecules obviously occurs at the cytoplasmic face; thus, an allosteric mechanism within the receptor molecule connects these two events. Two main theories for allosteric mechanisms have been suggested. According to the conformational-switch model of Monod, Wyman & Changeux (148), as applied to a receptor, the protein by itself is in equilibrium between active and inactive states, to which agonists and antagonists, respectively, preferentially bind. In contrast, according to the sequential, induced-fit model of Koshland (160), again as applied to a membrane receptor, the actual binding of the agonist to its binding site will induce conformational changes within the molecule, which eventually will lead to the conformational change at the cytoplasmic site, which is sensed as being active.

For 7TM receptors the induced-fit model was particularly attractive at the time when it was generally believed that there was a particular and unique trigger area or active site in the receptor, e.g. position III:08, which agonists needed to bind to in order to initiate the subsequent intramolecular activation process

(161–163). It is clear today that this is not the case. Accordingly, the conformational switch model, where the receptor molecule by itself can adopt the global active conformation, which agonists can stabilize through binding at multiple different sites, appears more attractive for the activation process as such. This can explain the constitutive activity observed among most 7TM receptors. Moreover, it can explain why agonists for 7TM receptors can be so highly different in size and chemistry.

Nevertheless, as discussed above in some detail for the adrenergic agonists, the binding process as such is probably in many cases characterized by a high degree of sequential induced fit. That is, the binding site is not a solid mold that the agonist fits into.

Often the conformational switch model for allostery is taken too literally, especially concerning the issue that the ligands function through binding to a preformed binding site presented by either the active or the inactive conformation of the receptor. It has, for example, been argued that the conformational switch model cannot account for the function of the extracellular fly-trap domain of the Family C 7TM receptors because the X-ray structures show that the ligand cannot get access to the binding pocket in the closed, active state (164–166). However, the fly-trap domain by itself interchanges between the open and closed states and the agonist probably binds with low affinity to the open, inactive, state and is subsequently trapped by the closing of the two halves of the fly-trap, and stabilizes the closed, active state by gluing the two halves together (166, 167). This might suggest an induced-fit mechanism. However, it is also very clear that the actual activation mechanism is an open-closed, "Klick-Klack," Monod-Wyman-Changeux-type of allosteric mechanism, as the activation occurs independent of the agonist binding. With an agonist bound, the receptor just stays in the active conformation for a longer time.

It is proposed that the global toggle switch mechanism of the seven-helical bundle functions in a similar manner and that the vertical see-saw movements of especially TM-VI and -VII occur independent of agonist binding. However, in most receptors an active conformation is only adopted by a small fraction of the receptors at a given time. In other words, a given receptor molecule only rather infrequently adopts an active conformation. This is reflected in the relatively low degree of constitutive activity observed for most receptors. Small-molecule agonists will bind in the deep pocket more or less as described for epinephrine in Agonist Stabilization of the Active Conformation of the 7TM Toggle Switch, above, and will in this way be trapped by the inward movement of the helices and, through interactions with the inner faces of the different helices, glue the helical bundle in the active conformation for an extended period of time. The larger ligands, peptides, etc. will bind to a number of sites located in the extracellular domains and at the ends of the helices, and they will, like Velcro, hold these extracellular domains together when the receptor is in its active toggle switch state, and hold it there for a longer time than would have been the case in the absence of the agonist.

It has been argued that the evolutionary success of 7TM receptors could be related to the allosteric activation mechanism and the possibility for ligands to stabilize an active conformation in multiple ways between the helices and loops (29). It could thus be envisioned that during evolution, the constitutively active receptors have adjusted to or picked up all kinds of available molecules as agonists because the only requirement for being an agonist is being able to bind somewhere between the helices and loops to stabilize the active receptor conformation.

TOGGLE SWITCH ACTIVATION IN DIMERIC 7TM RECEPTORS – DYNAMIC VERSUS RIGID INTERFACES?

According to the prevailing concept, 7TM receptors form stable dimers—mostly homodimers, but also heterodimers—during their biosynthesis in the ER (166, 168, 169). Subsequent to the dimer formation, the receptors are apparently sorted to the plasma membrane, where they function as constitutive dimers. Although this view is still a matter of debate (170–172), it is generally believed that the 7TM receptor dimers are stable and that the ligands induce conformational changes within and between the protomers, which often is registered by biophysical probes introduced at various positions (168, 169). These signals are interpreted, however, in some studies as either formation or breakage of dimers. In 7TMs of family C, the evidence that the receptors function as preformed, stable dimers is very strong. For example, the X-ray structures of various active and inactive forms of the homodimeric, ligand-binding, extracellular Venus fly-trap domains from metabotrobic glutamate receptors have been characterized in great detail (164, 165, 173). Moreover, the GABAb receptor has very convincingly been demonstrated not to be a single 7TM receptor, but instead a heterodimer, where the endogenous agonist binds in one protomer and where the other protomer is responsible for the correct cell surface sorting and for signaling (166). The prevailing concept has become that family A receptors also function as preformed stable dimers.

Although the actual molar ratio between receptor and the relevant G protein in a cell often is in the order of 1:100 (173a), both molecular modeling and biophysical data suggest that the functional stoichiometry of receptor to G protein in fact is probably 2:1 (174–176). That is, two 7TM receptors appear to function in complex with one heterotrimeric G protein in a pentameric complex. In such a complex, it could be envisioned that one receptor protomer, perhaps the one responsible for the signaling specificity, etc., interacts with the α-subunit, whereas the other receptor protomer is attached to the $\beta\gamma$-subunit. Similarly, the two beta-sheet domains of a single molecule of arrestin could very well each bind a 7TM receptor protomer of a dimer, i.e., also forming a 2:1 complex (176).

It has been surprisingly difficult, however, to reach a consensus concerning the actual dimerization interface. Thus, the alpha-factor receptor of the yeast *Saccharomyces cerevisiae* has very convincingly been demonstrated to dimerize through a TM-I / TM-I glycophorin-A like interface (177). In contrast, mutational analysis suggests that the dopamine D2 receptor dimerizes through a TM-IV/TM-IV interface with the highly conserved TrpIV:10, which importantly is facing outward, as a key structural element (178). For the β2-adrenergic receptor, it was at an early stage suggested that dimerization occurred through a TM-VI/TM-VI interface (179). Bioinformatic analysis combined with molecular modeling indicates that there is no consensus dimeric interface, and that many different dimerization interfaces may exist among 7TM receptors (180). Still, if the receptors do function in a 2:1 complex with the G protein, the interaction face toward the asymmetric intracellular, heterotrimeric transducer protein will very likely require a particular organization of the receptor dimer.

In light of the global toggle switch activation mechanism, the elusive dimeric interface between the two 7TM receptors is important, as the activation-associated movement of the exo-faces of the helices varies significantly among the seven transmembrane helices. According to the site-directed spin-labeling studies, rather small changes are believed to occur in, for example, TM-I and TM-IV (22). Thus, if these TMs constitute the dimeric interface, as suggested by certain studies, rather limited allosteric interactions would be expected between the two protomers within the dimer (Figure 13). On the other hand, if a TM-VI/TM-VI interface is involved in the dimerization process, it would be expected that the considerable conformational changes with an up to 6–8 Å movement outward of the intracellular segment of this helix that occurs during activation would either break the dimer or prevent activation and/or even agonist binding in the other protomer (Figure 13). Interestingly, there is increasing evidence that, at least in certain receptors, agonist binding can only occur in one protomer of a dimeric receptor at a time (181–184). Moreover, Pin and coworkers have presented evidence that within family C receptors only one of the two seven-helical bundle domains of the receptor dimer actually reaches the active state during agonist binding and activation (185); although, in the case of the metabotrobic glutamate receptor, the agonist may bind in both of the extracellular ligand-binding domains to obtain full agonism (167). The negative cooperative effect of agonist binding in one protomer upon binding in the other protomer, observed especially with the relatively large agonists such as chemokines and glycoprotein hormones (181, 182), could obviously occur through other types of steric-hindrance mechanisms not involving the seven-helical bundle domains.

Nevertheless, the structural basis for the allosteric effects occurring within and between receptor protomers during agonist binding and especially receptor activation will be highly interesting to identify, especially in the light of the dynamic versus rigid interfaces that may exist between the protomers of dimeric 7TM receptors.

CONCLUDING REMARKS

The number of 7TM receptors and their involvement in basically all parts of physiology and patho-physiology as well as their huge potential as drug targets contrasts sharply to the very limited structural information, which currently is available in relation to receptor activation mechanisms and ligand binding. We are still waiting for the first X-ray structure of a "normal" 7TM receptor, i.e., apart from rhodopsin. and we are still waiting for an X-ray structure of an active form of a receptor, including rhodopsin. Not to mention, we totally lack structural information concerning Family-B receptors and information concerning the seven-helical bundle of Family-C receptors as well as the other minor families of 7TM receptors.

Thus, the toggle switch activation mechanism presented above represents a relatively bold attempt to interpret mainly biochemical and biophysical data related to the position and movements of the transmembrane segments in light of the X-ray structure of the inactive form of rhodopsin. However, it should be appreciated that actually a large amount of relatively solid biophysical data has accumulated concerning the movements of the intracellular segments of the helices, especially from EPR analysis combined with site-directed spin labeling technology. Hopefully, this and similar biophysical types of analysis will soon be extended to also include the extracellular parts of rhodopsin and other 7TM receptors. Importantly, although EPR and NMR spectroscopic analysis may not at present provide high-resolution total structures of these elusive membrane proteins, these techniques are nevertheless very informative in respect to answering specific questions concerning, for example, conformational changes in specific side chains and conformational changes of helices, etc., at the millisecond timescale. Moreover, it is clear that the EPR structure of rhodopsin—obtained through a massive effort over several years—in fact gives a much more relevant and true picture of the structure and dynamics of, for example, the extended intracellular helical segments of TM-V and -VI (22) than the original X-ray structure indicated (13), as confirmed by a later X-ray structure of rhodopsin (14). Thus, the high-resolution X-ray structures, which, hopefully, in the nearer future will appear in the literature with increasing frequency and applied to a broader spectrum of 7TM receptors in complex with various agonist and antagonist ligands, will serve as the basis for biophysical and biochemical studies to elucidate the real-life, dynamic function of these receptors during ligand binding and receptor activation.

Besides providing information of basic scientific interest, it is expected that insight into the activation and inactivation mechanisms for 7TM receptors to a major degree will be exploited in structure-based drug discovery efforts. However, with these difficult membrane proteins, it is far from likely, even in a positive scenario, that it will be possible in the foreseeable future to produce X-ray structures of receptor-ligand complexes in a rapid, repetitive, iterative manner, as required according to classical structure-driven medicinal chemistry programs. Nevertheless, especially in the 7TM receptor field, the pharmaceutical and biotech industry

has had much success in efficiently utilizing the rather sparse available structural information to support their knowledge-based drug discovery process through the development of approaches such as Site Directed Drug Discover®, tethering ligand discovery, and related procedures (6–10). We expect that the present toggle switch model for 7TM receptor activation will provide novel input for the generation of molecular models of the elusive active receptor conformations to enable structure-based discovery programs aiming at the discovery of not only antagonists but also of small-molecule, drug-like agonists for this large class of drug targets.

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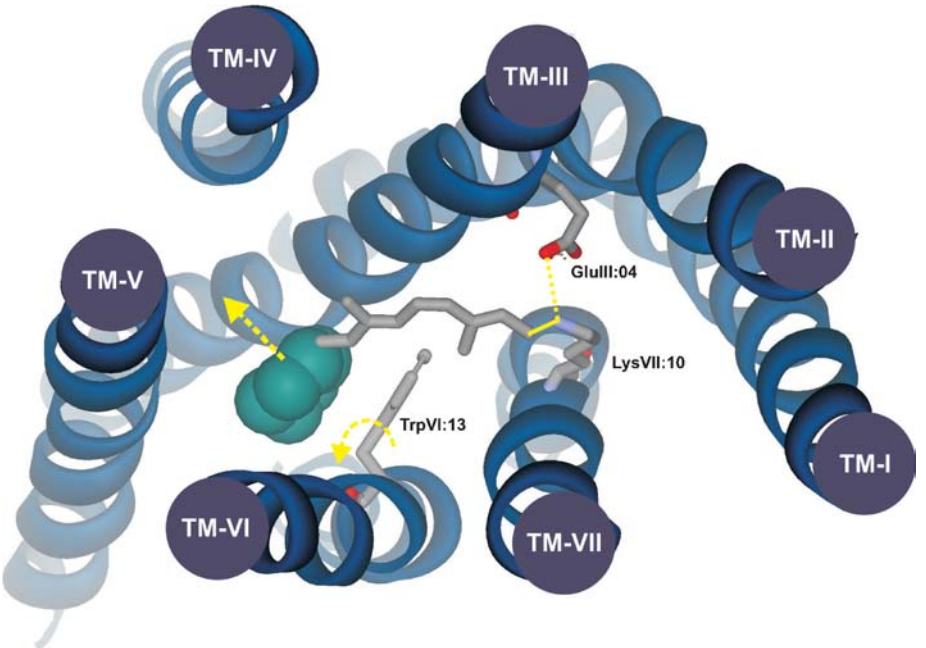

Figure 1 Rhodopsin as viewed from the extracellular side with the inverse agonist 11-*cis* retinal located in what in 7TM receptors generally corresponds to the main ligand-binding pocket. The molecular model represents the X-ray structure of rhodopsin, but only the seven-helical bundle is shown (TM-I to TM-VII) and only three side-chains: LysVII:10, to which retinal is attached through a Schiff base, for which GluIII:04 forms the counter-ion, and TrpVI:13, which is believed to be part of an important rotameric switch in the activation mechanism for 7TM receptors (see text). The β-ionone ring is shown in a space-filling model in green and the rest of the retinal molecule is shown in a gray stick model. The straight yellow arrow indicates that retinal, upon light activation and during conversion to the metarhodopsin-II state, is believed to translate toward TM-V, as indicated by NMR experiments. This movement is here interpreted to allow for the rotameric interchange in the conformation of TrpVI:13 and the inward movement of TM-VI (see text).

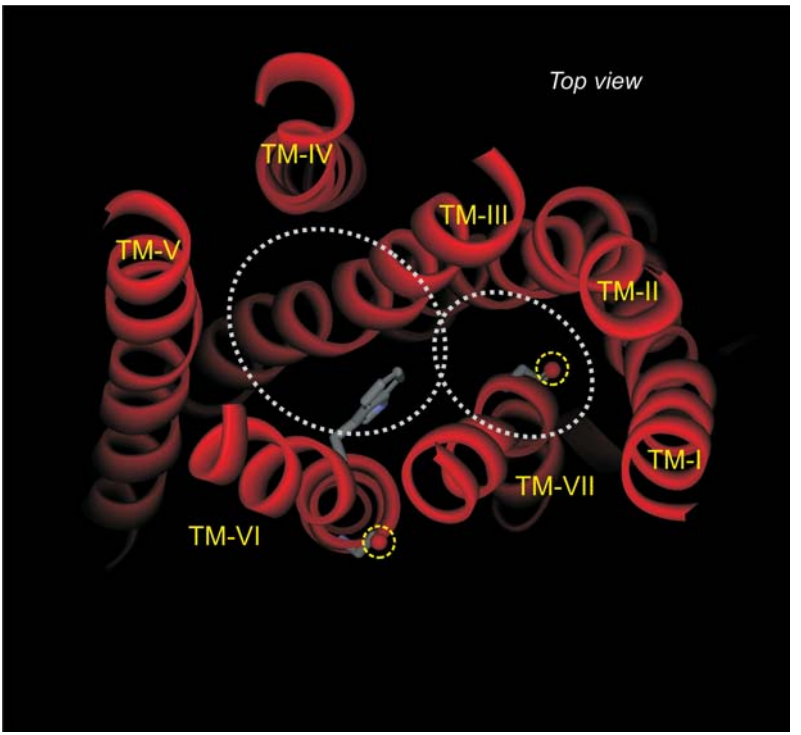

Figure 2 The main ligand-binding pockets of a generic 7TM receptor as viewed from the extracellular side. The two parts of the main ligand-binding pocket are indicated with gray dotted circles: a rather deep pocket in between the extracellular segments of TM-III, -IV, -V, -VI, and -VII and a more shallow pocket between TM-II, -III, and -VII. The kink or bend in TM-VII generates the shallow part of the main ligand-binding pocket and the free backbone carbonyls at the *i-4* position relative to the conserved Pro residues in TM-VI and -VII are indicated by yellow dotted rings.

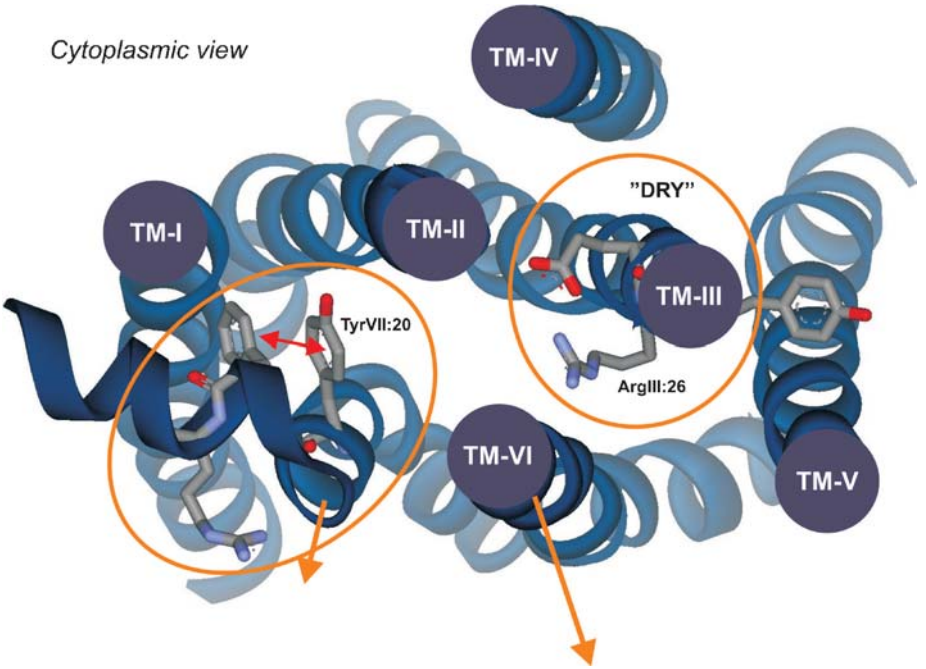

Figure 3 Rhodopsin as viewed from the intracellular side where the two main motifs believed to be involved in the signal transduction process to downstream signaling molecules are highlighted: the DRY-TM-III and the Helix-8/TM-VII motifs. The molecular model represents the X-ray structure of rhodopsin, but only the seven helical bundle is shown (TM-I to TM-VII). In TM-III, the residues constituting the DRY motif corresponding to AspIII:25, ArgIII:26, and TyrIII:27 are indicated in stick models and Helix-8, indicated by the highly conserved Arg facing the cytoplasm and the conserved Phe, which interacts with TM-VII, is highlighted. TyrVII:20, which interacts with Helix-8, is similarly highlighted. Orange arrows indicate the direction of the supposed outward movement of the intracellular segments of TM-VI and TM-VII as indicated by site-directed spin-labeling experiments (22).

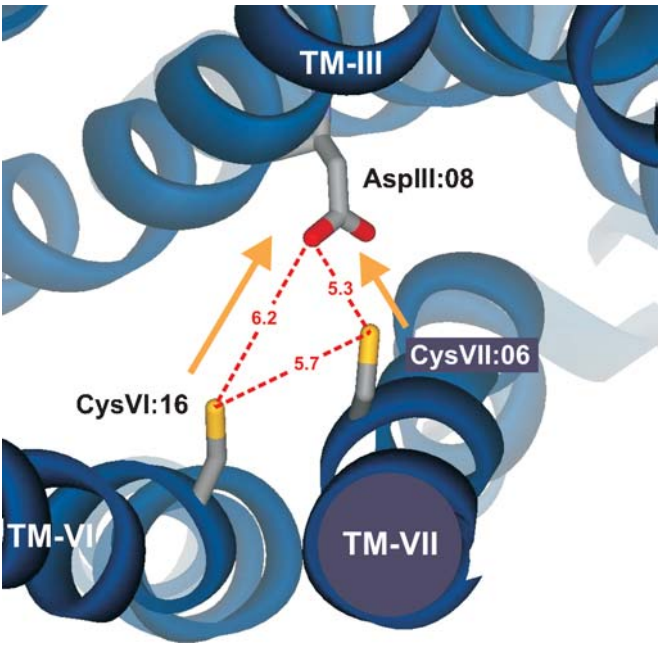

Figure 4 Molecular model of a highly efficacious tridentate activating metal ion site: AspIII:08-CysVI:16-CysVII:06 shown in a molecular model of the β2-adrenergic receptor built over the X-ray structure of the inactive state of rhodopsin. The three residues constituting the activating metal ion site are shown in a likely rotameric state and the distances between the metal ion–binding heavy atoms are indicated in Angstroms (see Reference 27 for details). Because Zn(II) or Cu(II) act as efficacious agonists through binding to these three residues and because the distances between the heavy atoms when binding these metal ions will have to be in the order of 2–3 Å (depending on the coordination geometry), the three helices will have to move toward each other during receptor activation. The two orange arrows indicate that especially TM-VI and TM-VII move toward TM-III as this transmembrane segment appears to be rather immobile in the middle of the seven-helical bundle.

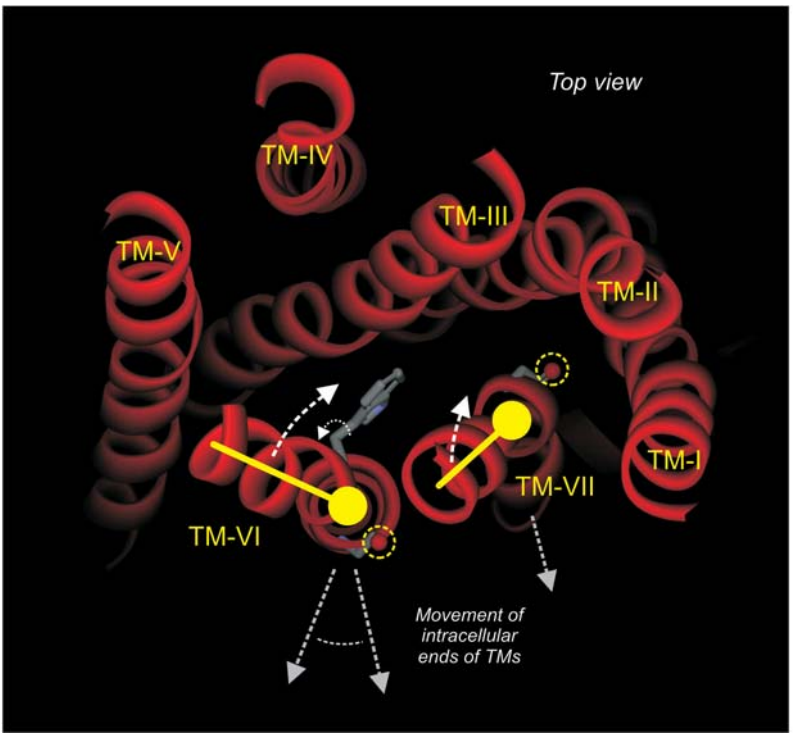

Figure 5 Proposed activation-associated movements in TM-VI and TM-VII around the weak points generated by the two highly conserved prolines—as viewed from the extracellular side. The extracellular segments of TM-VI and TM-VII are highlighted by two yellow rods with a yellow ball placed in the proposed pivot or fulcrum around which the movement is proposed to take place. Indicated by yellow dotted rings are the two free backbone carbonyls at the *i-4* position relative to the conserved Pro residues, i.e., in the backbone of position VI:11 (*pointing upward*) and in position VII:12 (*pointing downward to the right*). The two white arrows indicate the proposed activation-associated rotational inward movements of the extracellular segments of TM-VI (more rotational) and TM-VII (more vertical helical straightening). Gray arrows indicate the previously proposed outward movements of the intracellular segments of these two helices (for review, see Reference 22). The rotameric interchange of the conserved TrpVI:13, which is located in the middle of the weak point, and the bulge in TM-VI are also indicated. The figure is redrawn from Reference 27.

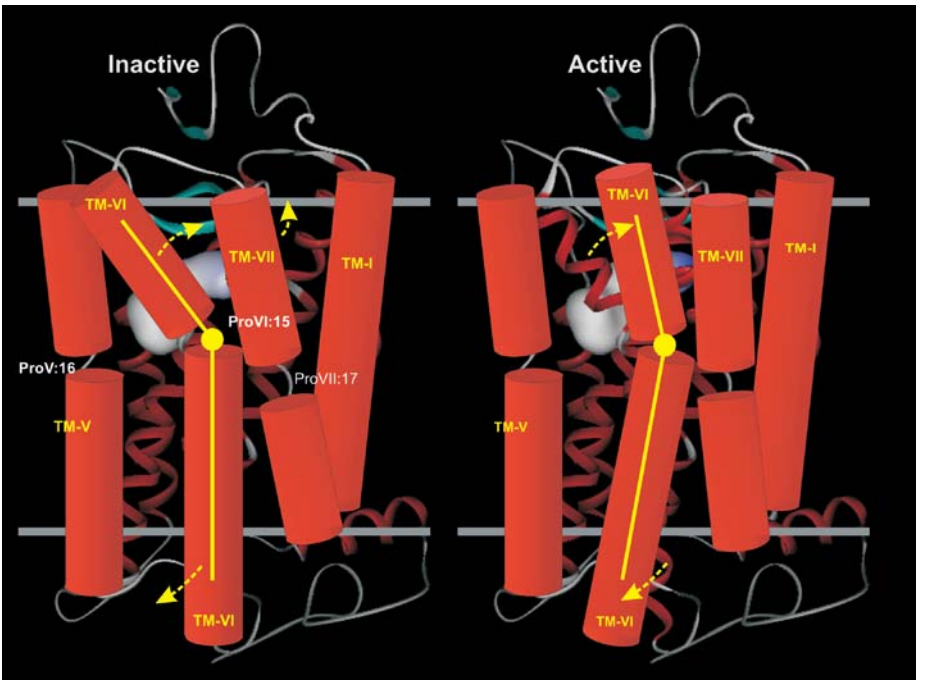

Figure 6 Proposed activation-associated movements in TM-VI and TM-VII around the weak points generated by the two highly conserved prolines, as viewed from the membrane (*side view*). Left (inactive): The helices of TM-I, -V, -VI, and -VII are highlighted by simplified red cylinders upon the X-ray structure of the inactive form of rhodopsin, with retinal shown as a surface model in the middle. Right (active): The proposed activation-associated see-saw movements in TM-VI and TM-VII occurring around the pivot or fulcrum corresponding to the proline-induced weak point in these helices are shown in the helical cylinders upon the still inactive state of rhodopsin. The location and helical break induced by the highly conserved ProV:16, ProVI:15, and ProVII:17 are indicated. The extracellular and the intracellular segments of TM-VI are highlighted by two yellow sticks or levers that meet in a yellow ball placed in the proposed pivot around which the see-saw like movements are proposed to take place. Redrawn from Reference 27.

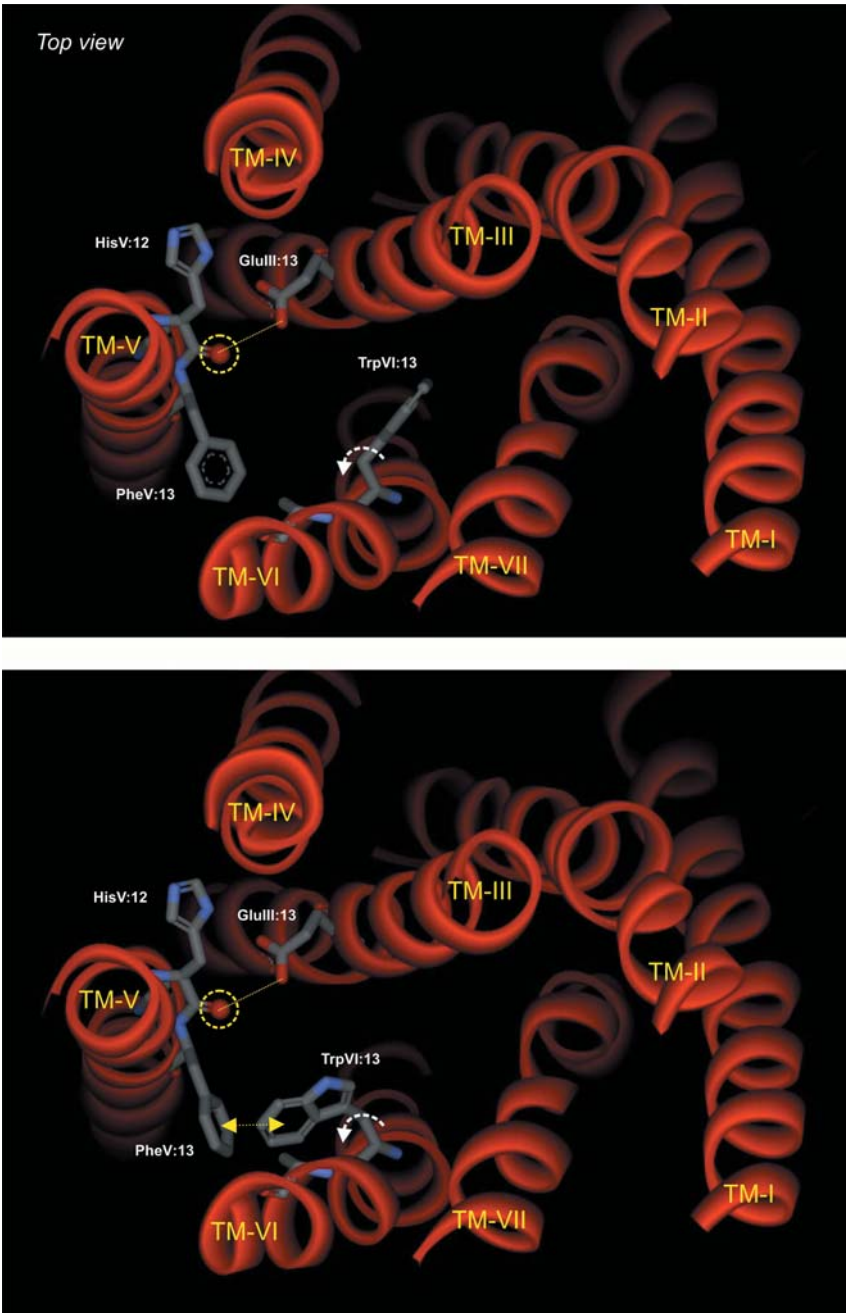

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Figure 7 The rotameric TrpVI:13 activation switch and its proposed interaction with the PheV:13 lock located in the ProV:16 induced bulge or weak point in TM-V in the active state. Upper panel: The inactive state of rhodopsin with TrpVI:13 shown in TM-VI in its vertical position between TM-III and TM-VI. Highlighted in TM-V are the highly conserved residue PheV:13 as well as HisV:12 located in the i-4 position relative to ProV:16 (*not shown*) with its free backbone carbonyl group twisted outward in an almost 90 degree angle relative to the helical axis (*indicated with a yellow dotted circle*). Shown in TM-III is GluIII:13, which bends back and appears to make a stabilizing hydrogen bond to the twisted, free carbonyl group from the backbone of position V:12. Lower panel: The same structure as shown above but with TrpVI:13 in its proposed active rotameric configuration making an edge-to-face aromatic-aromatic interaction with the conserved PheV:13, which in this way is proposed to form a lock for the active conformation of the TrpVI:13 switch. It is proposed that the TrpVI:13 to PheV:13 side chain interaction could influence the backbone conformation in the ProV:16-induced bulge or weak point in TM-V, and thereby it could affect the whole conformation of TM-V. Note that the helix-based generic numbering (64, 65) of the highlighted residues reflects that they all are located at the same level around the middle of their respective helices. Redrawn from Reference 123.

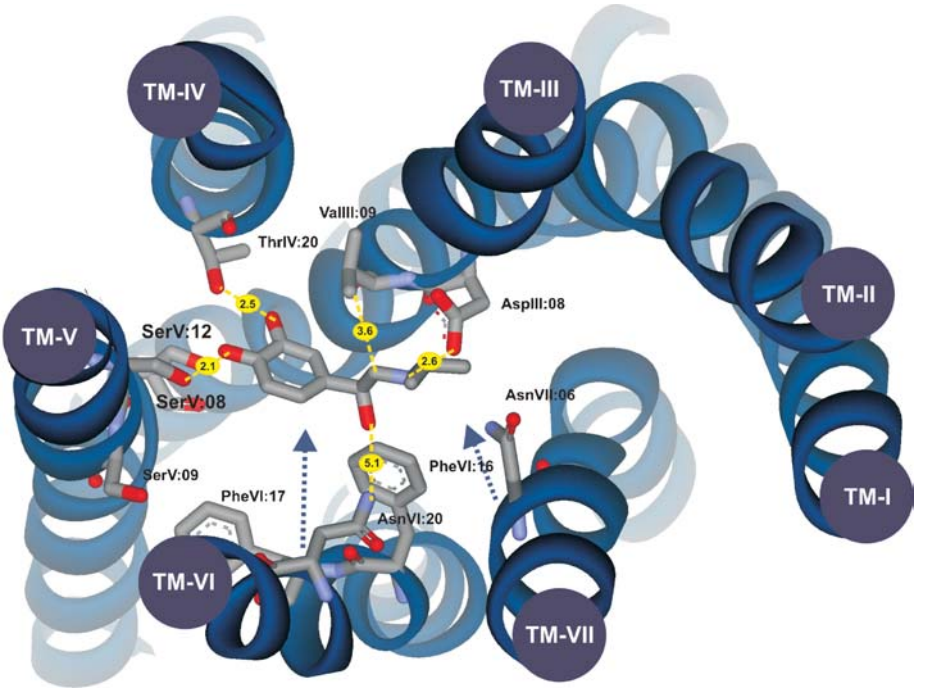

Figure 8 Agonist binding in the β2-adrenergic receptor in a molecular model built over the inactive state of rhodopsin as viewed from the extracellular side. Isoprenalin (isoproterenol) has been docked manually into the model in a configuration where it makes optimal interactions with residues in TM-III, -IV, and -V. As discussed in the text, it is proposed that the charge-charge interaction between the positively charged amine group of the ligand and the side chain of AspIII:08 is an initial important event followed by orientation of the ligand through interchanging hydrogen bond interactions with donor acceptor groups located in the backbone and side chains, indicated in TM-IV and especially TM-V (ThrIV:20, SerV:08, SerV:09, and SerV:12). In such a frozen picture of the dynamic binding event, the distance from, for example, the β-hydroxyl group in the ligand to its hydrogen bond interaction point in the receptor, AsnVI:20, is too long (5 Å), which also is the case for the catechol ring and its aromatic-aromatic interaction partner in the receptor, the side chain of PheVI:17. The proposed toggle switch inward movement of TM-VI (*indicated by the blue arrow*) would bring these interaction partners in close and optimal position for forming the assumed ligand-receptor complex. AsnVII:06, which is presumed to interact with certain agonists (as well as antagonists) is also highlighted, with a blue arrow indicating the proposed toggle switch inward movement of TM-VII. A similar type of induced fit or cooperative interactions during agonist binding in this receptor has recently been proposed by several groups (141–144).

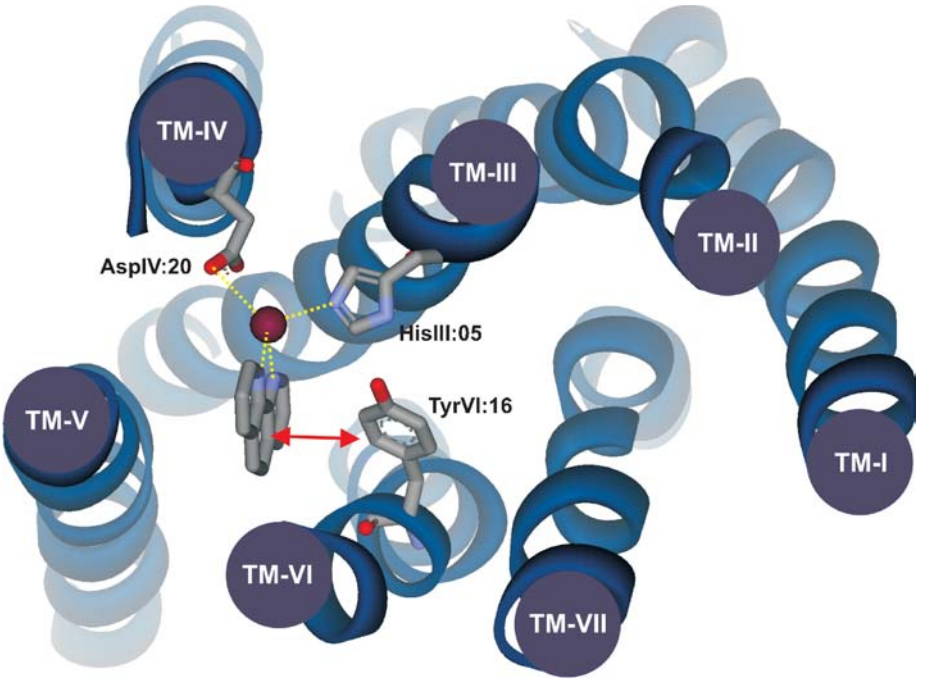

Figure 9 Illustration of the metal ion site anchoring of a small aromatic chelator in the pocket between TM-III, -IV, and -V to act as a high-efficacy agonist in the chemokine CXCR3 receptor through second-site interactions with TyrVI:16. Phenanthroline has been docked manually into a molecular model of the [HisIII:05] metal ion site-engineered mutant form of the CXCR3 receptor built over the inactive X-ray structure of rhodopsin in which TM-VI has been moved inward toward TM-III. A metal ion tetrahedrally coordinated by the side chains of HisIII:05 and AspIV:20 and by the two nitrogens of the phananthroline ligand is indicated in purple. The phenanthroline ring system has been docked in a vertical orientation to a position close to where the cathechol ring is assumed to be located, for example, the isoproterenol complex with the β2-adrenergic receptor (see Figure 8). The red double arrow indicates the presumed aromatic-aromatic second-site interaction between the phenanthroline ring system and the side chain of TyrVI:16 as indicated by the mutational analysis of the metal ion site-engineered CXCR3 receptor. Redrawn from Reference 147.

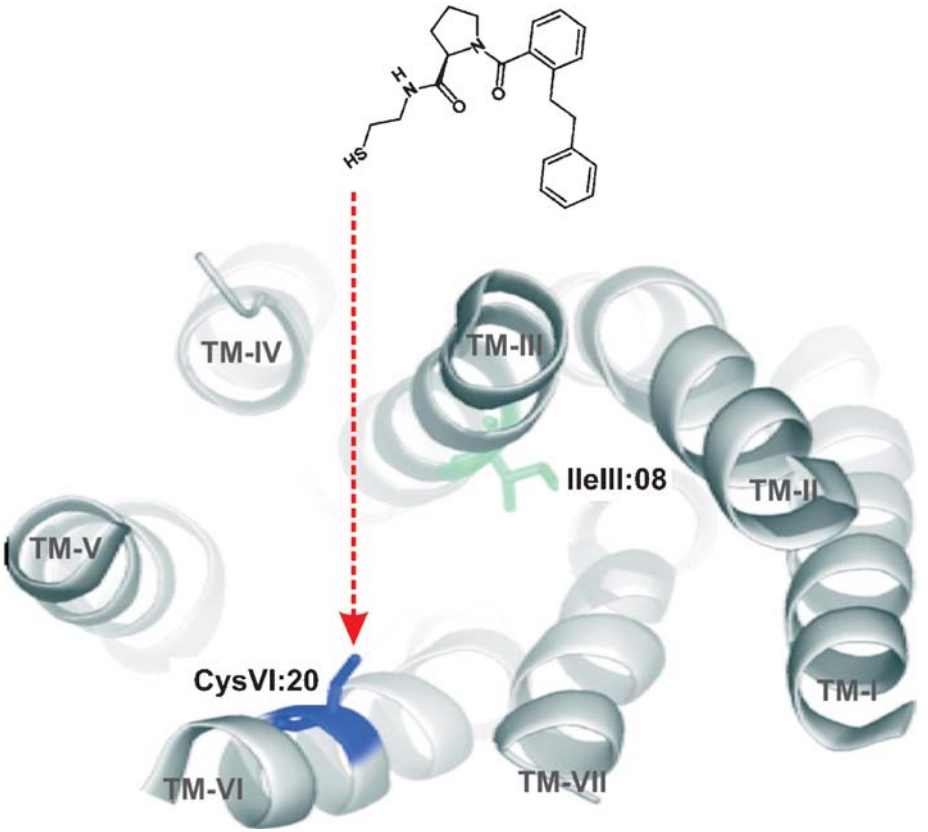

Figure 10 Illustration of covalent disulfide anchoring of small organic compounds to CysVI:20, which through second-site interactions with the inner face of TM-III acts as an efficacious agonist for the complement C5a receptor. Thiol-containing compounds, as the one indicated at the top, were covalently attached to a Cys residue introduced at position VI:20 to act as agonists or antagonists for the C5a receptor. Through exchange of the relatively large IleIII:08 with a small Ala residue at the proposed second-site interaction point on the opposing inner face of TM-III, the affinity and agonistic property of the compounds could be increased. In contrast, introduction of a large rigid Trp residue at this position III:08 decreased the affinity of the compounds and turned them into antagonists. These observations are compatible with the toggle-switch mechanism for 7TM receptor activation, as the space-giving Ala substitution could be envisioned to allow for a better inward movement of TM-VI toward TM-III, corresponding to agonism and receptor activation (provided that the compounds otherwise fit appropriately), whereas the space-filling Trp would counteract such a movement and lead to antagonism. Redrawn from Reference 8.

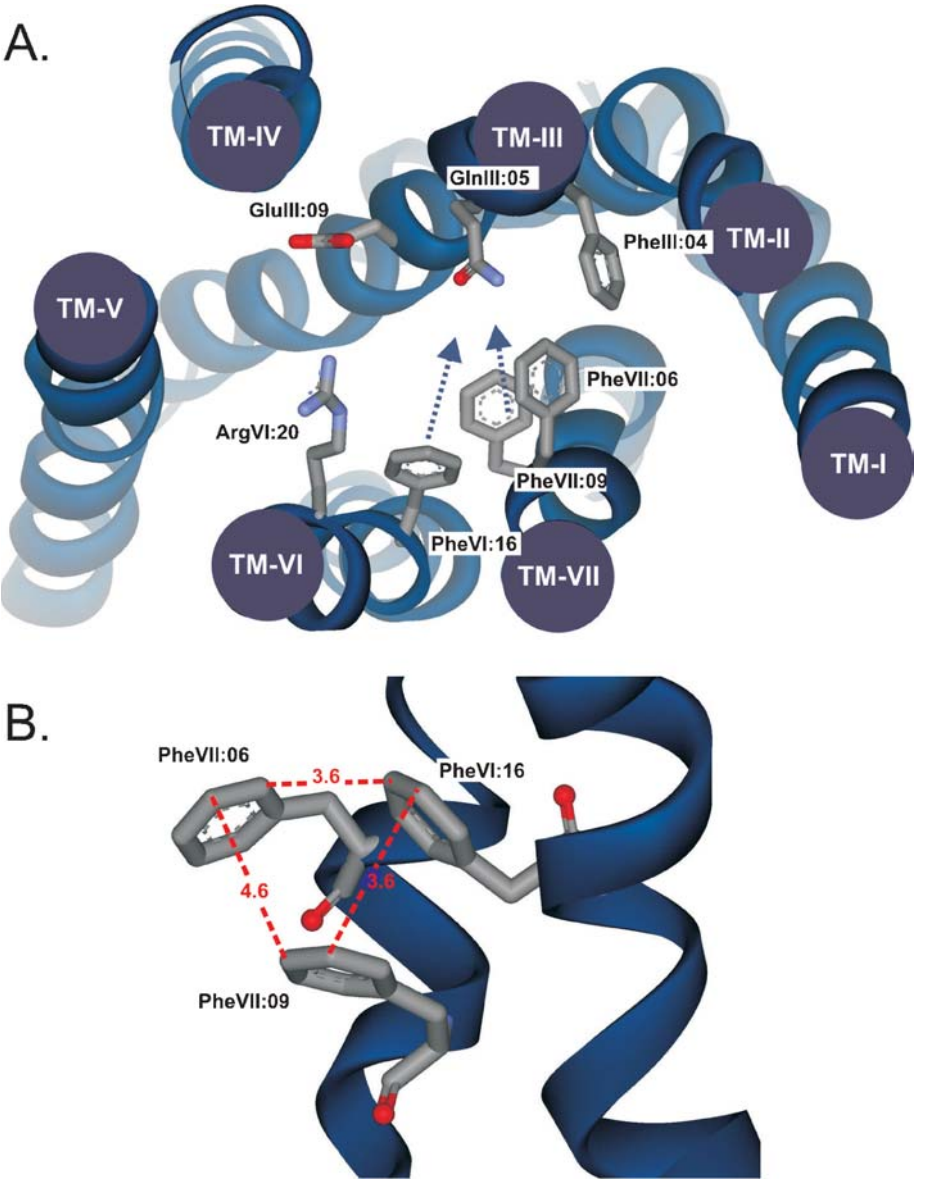

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Figure 11 The cluster of aromatic residues at the interface between the extracellular segments of TM-VI and TM-VII, the side chains of which are proposed to function as tethered ligands responsible for the very high degree of constitutive activity of the ghrelin family of receptors. Upper panel: A molecular model of the ghrelin receptor built over X-ray structure of the inactive state of rhodopsin as viewed from the extracellular side. A number of key residues in TM-III, -VI, and -VII are highlighted. Mutational analysis has demonstrated that the constitutive activity of the ghrelin receptor systematically could be tuned up and down depending on the size and aromaticity of PheVI:16 in the context of large aromatic residues in positions VII:06 and VII:09 (150). It should be noted that although a Phe or Tyr frequently is found in position VI:16, the residue in position VII:09 is a residue with a small side chain in the vast majority of 7TM receptors (122). The blue arrows indicate the inward direction of activation-associated movements of the extracellular segments of TM-VI and -VII. Lower panel: A close-up view of the aromatic cluster at the interface between TM-VI and TM-VII in the ghrelin receptor with the close relationship between Phe-VI:16, PheVII:06, and PheVII:09. A few selected distances are indicated in Angstroms.

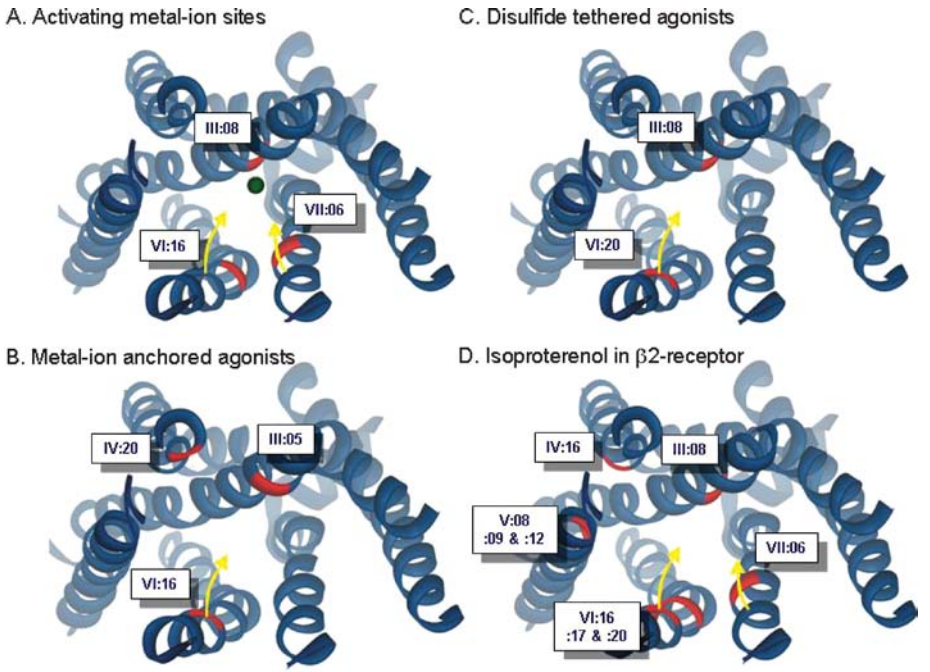

Figure 12 Comparison of a collection of small-molecule agonist interaction sites in the main ligand-binding pocket of 7TM receptors. All panels show a simple filled ribbon model of the seven-helical bundle in blue, and the position of the agonist interaction sites are highlighted in red. Panel A: The tridentate metal ion site built between positions III:08, VI:16, and VII:06 in the β2 adrenergic receptor as shown in Figure 4, where Zn(II) or Cu(II) act as agonists requiring inward movement of TM-VI and –VII, as indicated by the yellow arrows (27). Panel B: The metal ion site built between positions III:05 and IV:20 in the CXCR3 receptor through which aromatic chelators, such as bipyridine and phenanthroline, can be anchored, as shown in Figure 9, to act as agonists involving second-site interactions with residue PheVI:16 requiring inward movement of the extracellular segment of TM-VI (147). Panel C: Covalent disulfide tethering of small organic compounds at position VI:20 that act as agonists through second-site interactions with position III:08, where space-giving substitutions provide agonism and space-filling substitutions provide antagonism (8) in agreement with an activation associated inward movement of TM-VI (Figure 10). Panel D: The assumed interaction sites for isoproterenol in the β2 adrenergic receptor, with the charge-charge anchor site for the amine group being AspIII:08 and second-site interactions located in the TM-IV-V region and in TM-VI, the latter of which appears to require an induced-fit interaction in agreement with an inward movement of TM-VI, as shown in Figure 8.

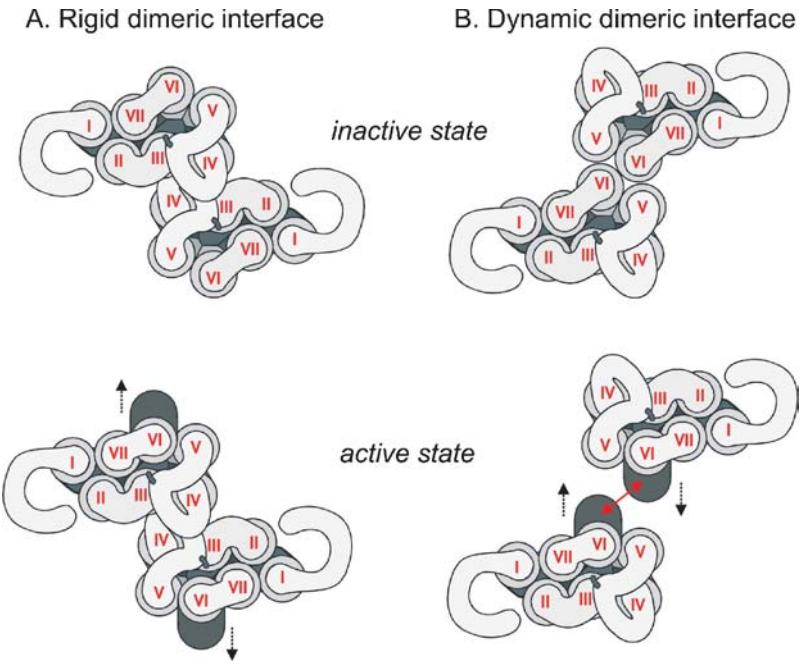

Figure 13 Schematic illustration of the implications of different types of dimerization interfaces and the proposed toggle-switch TM movements for cooperative effects between protomers. (*Left*) A dimeric 7TM receptor with a protomeric interface involving a TM-IV/TM-IV interaction as suggested for the dopamine D2 receptor (178)—at the top shown in the inactive state and at the bottom in the active state with the intracellular ends of the two TM-VIs moved outward. Owing to the limited movements of TM-III and -IV, such a dimerization form would probably not involve much cooperative effect between the protomers. (*Right*) A dimeric 7TM receptor with a protomeric interface involving a TM-VI/TM-VI interaction as suggested for the β2-adrenergic receptor (179)—at the top in the inactive state and at the bottom in the active state where the outward movement of the intracellular segment of TM-VI will occur at the dimeric interface and consequently very likely will result in strong cooperative effects between the protomers.

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CONTENTS