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Review

G protein coupled receptor structure and activation [☆]

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Abstract

G protein coupled receptors (GPCRs) are remarkably versatile signaling molecules. The members of this large family of membrane proteins are activated by a spectrum of structurally diverse ligands, and have been shown to modulate the activity of different signaling pathways in a ligand specific manner. In this manuscript I will review what is known about the structure and mechanism of activation of GPCRs focusing primarily on two model systems, rhodopsin and the β_2 adrenoceptor.

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Keywords: GPCR; 7TM; Structure; Conformational change; Efficacy

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Abbreviations: β_2 AR, beta 2 adrenoceptor; GPCR, G protein coupled receptor; TM, transmembrane

[☆] Amino acid numbering: The position of β_2 AR residues are followed by the Ballesteros general number [1] in superscript, in the form X.YY, where X refers to the TM segment and YY to the position relative to the most highly conserved amino acid in the TM segment, which is assigned an arbitrary position of 50.

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1. Introduction

G protein coupled receptors (GPCRs) represent the largest family of membrane proteins in the human genome and the richest source of targets for the pharmaceutical industry. There has been remarkable progress in the field of GPCR biology during the past two decades. Notable milestones include the cloning of the first GPCR genes, and the sequencing of the human genome revealing the size of the GPCR family and the number of orphan GPCRs. Moreover, there is a growing appreciation that GPCR regulation and signaling is much more complex than originally envisioned, and includes signaling through G protein independent pathways [2–4]. Consequently, it has been proposed that the term GPCR be abandoned in favor of 7 transmembrane or 7TM receptors.

In spite of the remarkable advances in the biology and pharmacology of GPCRs, progress in the area of protein structure has been more limited. To date, the only high-resolution structures of a GPCR have been for bovine rhodopsin. In this manuscript I will briefly review the groundbreaking structural work on rhodopsin and discuss the challenges in obtaining high-resolution structures of other GPCRs. I will also discuss what we know about the structural changes associated with receptor activation.

2. GPCR structure

2.1. Common structural features of GPCRs

G protein coupled receptors (GPCRs) represent the single largest class of membrane proteins in the human genome. A recent and detailed analysis of the human genome reveals over 800 unique GPCRs, of which approximately 460 are predicted to be olfactory receptors [5]. Based on sequence similarity within the 7 TM segments, these receptors can be clustered into 5 families: the rhodopsin family (701 members), the adhesion family (24 members), the frizzled/taste family (24 members), the glutamate family (15 members), and the secretin family (15 members) [5]. The physiologic function of a large fraction of these 800 GPCRs is unknown; these receptors are referred to as orphan GPCRs. However, deorphanization of non-olfactory GPCRs is an ongoing process [6], as they are a promising group of targets for the pharmaceutical industry. Therefore, the actual number of orphan GPCRs continues to decline.

GPCRs share a common structural signature of seven hydrophobic transmembrane (TM) segments, with an extracellular amino terminus and an intracellular carboxyl terminus (Fig. 1). GPCRs share the greatest homology within the TM segments. The most variable structures among the family of GPCRs are the carboxyl terminus, the intracellular loop

spanning TM5 and TM6, and the amino terminus. The greatest diversity is observed in the amino terminus. This sequence is relatively short (10–50 amino acids) for monoamine and peptide receptors, and much larger (350–600 amino acids) for glycoprotein hormone receptors, and the glutamate family receptors. The largest amino terminal domains are observed in the adhesion family receptors.

This structural and functional similarity of GPCRs stands in contrast to the structural diversity of the natural GPCR ligands [7]. These range from subatomic particles (a photon), to ions (H^+ and Ca^{2+}), to small organic molecules, to peptides and proteins. The location of the ligand binding domains for many GPCRs has been determined [7]. While many small organic agonists bind within the TM segments, peptide hormones and proteins often bind to the amino terminus and extracellular sequences joining the TM domains. However, size of the ligand alone cannot be used to predict the location of the binding site: for instance, glycoprotein hormones, glutamate, and Ca^{2+} all activate their respective receptors by binding to relatively large amino terminal domains [7,8]. It is interesting to note that it has been possible to identify small molecular weight allosteric modulators that bind within the TM domains [9–14] for many GPCRs that bind their native agonists on the extracellular loops or the amino terminus.

2.2. Structural insights from rhodopsin

Our understanding of GPCR structure is based largely on the high-resolution structures of the inactive state of rhodopsin. Rhodopsin is better suited for structural studies than most other GPCRs because it is possible to obtain large quantities of highly enriched protein from bovine retina. Rhodopsin is also a remarkably stable GPCR, retaining function under conditions that denature many other GPCRs.

2.2.1. Two-dimensional crystals

The first structures of rhodopsin came from cryoelectron microscopy of two-dimensional crystals of bovine rhodopsin from Gebhard Schertler's group [15–19]. While the resolution of these structures was limited (ranging from 5 to 9 Å), they provided the first picture of the orientation of the TM segments in a lipid environment. These structures, together with the subsequent computational work from Baldwin [20] provided the template from which most molecular models for other GPCRs were generated.

2.2.2. Three-dimensional crystals

More recently, three dimensional crystals structures of rhodopsin have been obtained by several groups [21–26]. Three-dimensional crystals of bovine rhodopsin have been

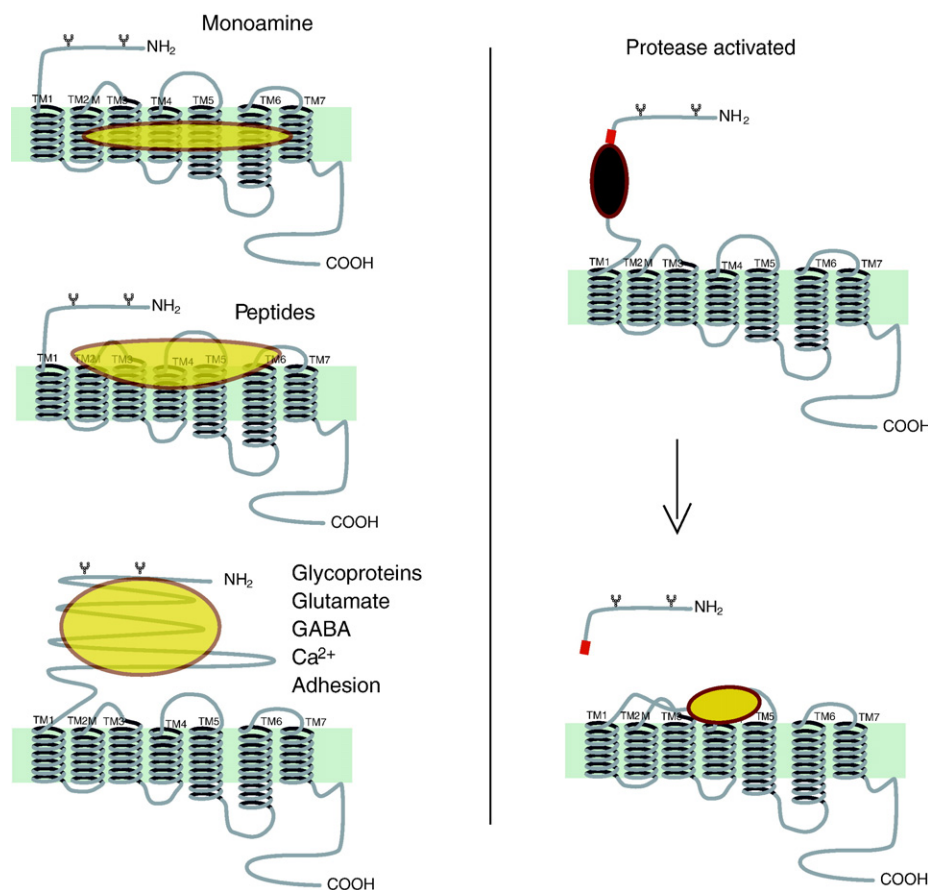


Fig. 1. Cartoons depicting the secondary structure and the location of agonist binding sites for different GPCRs.

grown using at least two different approaches. The first published crystals of rhodopsin were obtained from rhodopsin selectively solubilized from rod outer segments using a combination of alkyl(thio)glucoside detergents and divalent cations [21,27]. The procedure used no additional purification steps. The mixture likely contained more rod outer segment lipids than would be expected from protein purified by column chromatography, and it was speculated that the presence of these lipids may influence crystal formation [21]. Crystals grown from this preparation of rhodopsin initially diffracted at 2.8 Å [22] and subsequent improvements have led to diffraction at 2.2 Å [25]. All of these crystals have a $P4_1$ space group, and crystal contacts form between hydrophilic domains of the receptor.

Three-dimensional crystals have also been obtained from bovine rhodopsin solubilized from rod outer segments using the detergent lauryldimethylamine-oxide (LDAO) and subjected to lectin chromatography followed by detergent exchange into n-octyltetraoxyethylene (C8E4) followed by anion exchange chromatography [26,28]. This more extensive purification procedure, which would be expected to remove all but tightly bound lipid, resulted in crystals diffracting at 2.6 Å [26]. These crystals have a $P3_1$ space group and crystal contacts form primarily within the transmembrane domains and the intracellular and extracellular loop structures point into solvent filled cavities. As a result, the loop structures may assume a more

native structure in the $P3_1$ crystal form compared to the $P4_1$ crystals [29].

2.2.3. Comparison of $P4_1$ and $P3_1$ rhodopsin structures

The structures obtained from $P4_1$ and $P3_1$ crystals are very similar overall, particularly in the transmembrane, and extracellular domains (Fig. 2, for a detailed comparison of these structures see Schertler [29]). However, there are significant differences in the cytoplasmic loop linking TM5 and TM6, which is known to be involved in G protein coupling. These differences are highlighted in red in Fig. 2. The structure from the $P3_1$ crystals is in agreement with structures obtained from electron diffraction of two-dimensional crystals and with electron paramagnetic resonance spectroscopy studies [29]. As discussed above, this loop may assume a more native structure in the $P3_1$ crystals because none of the cytoplasmic domains are involved in crystal lattice contacts.

2.3. Structure of other GPCRs

Recently the amino terminal ligand binding domain of the FSH receptor was crystallized in complex with its ligand [30]. This provides important structural insights into glycoprotein hormone binding; however, the structure does not include the transmembrane domains. With this exception, there are no other high-resolution crystal structures of GPCRs. Structure analysis

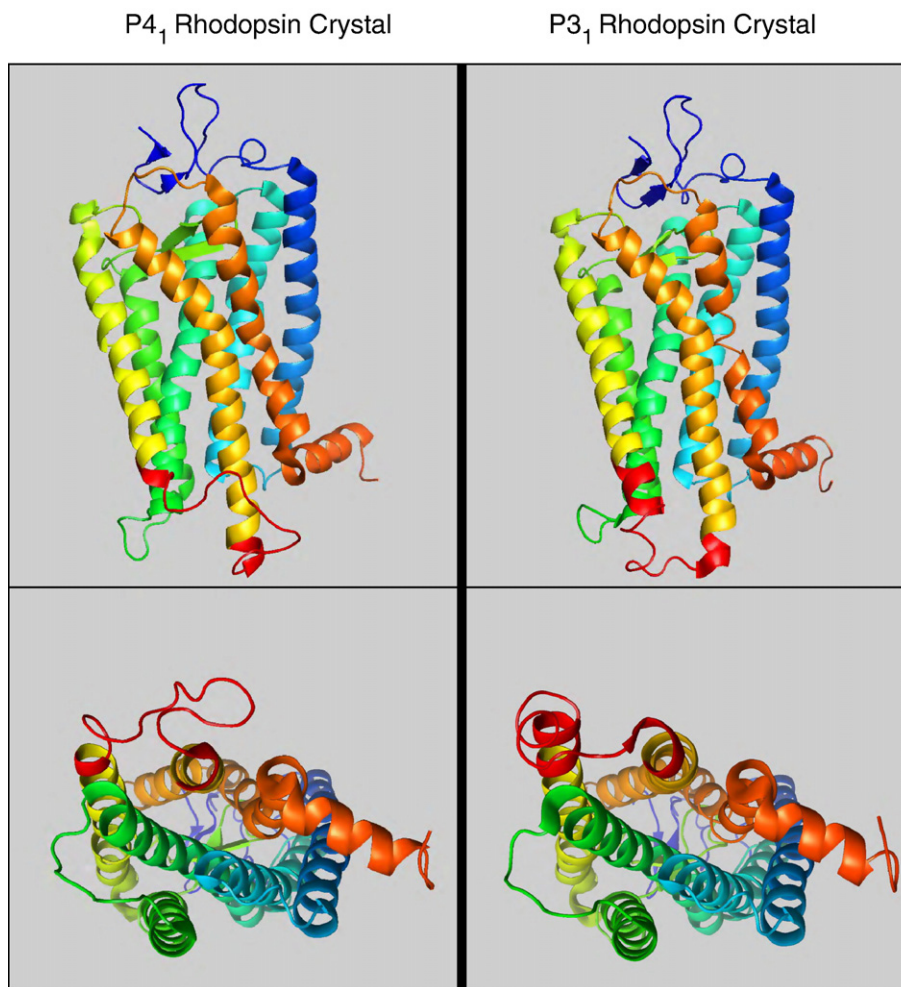


Fig. 2. A comparison of the rhodopsin structures determined from the P4₁ [25] and P3₁ [26] crystal forms. The loop connecting TM5 and TM6 (shown in red) is the most divergent sequence.

of other GPCRs has largely been limited to the use of site-directed mutagenesis and cysteine scanning mutagenesis [31] to detect receptor–ligand interactions, and the use of engineered metal ion binding sites to probe intramolecular interactions [32]. While these approaches provide low-resolution structural information, this information can be used to verify and improve the accuracy of homology models based on rhodopsin. Ballesteros and Javitch found that structural insights obtained from mutagenesis data and substituted cysteine accessibility studies on monoamine receptors were consistent with the high-resolution structure of rhodopsin, suggesting that rhodopsin serves as a good template for homology modeling [33]. However, rhodopsin might not be a good template for models of more distantly related family rhodopsin family members such as the cholecystokinin CCK1 receptor [34].

2.3.1. Obstacles for obtaining GPCR structures

The major obstacles to obtaining structures of other GPCRs include protein production and purification, and protein stability and homogeneity. In terms of production, it is now possible to generate sufficient quantities (tens of milligrams) of several GPCRs for crystal screening using bacterial, yeast, insect cell,

and mammalian cell expression systems [35–40]. The availability of robotic systems for preparing setups of 100 nl volumes (or smaller) has enabled large parameter screens with relatively small amounts of protein. As such, protein production is no longer the major limitation for crystallography efforts.

Perhaps a greater problem is the stability of purified GPCRs in detergents compatible with crystallography. For example, the β_2 adrenoceptor (β_2 AR) and many other GPCRs are not stable in the detergents used to obtain rhodopsin crystals; and thus far rhodopsin crystals have not been obtained in dodecylmaltoside, a detergent in which the β_2 AR is relatively stable. GPCRs tend to be more stable in non-ionic detergents with relatively long alkyl chains. These detergents may form larger micelles that prevent the formation of crystal contacts [41]. Another problem is the potential for both structural and conformational heterogeneity in GPCRs. By structural heterogeneity I mean heterogeneity in posttranslational modifications such as glycosylation, phosphorylation and palmitoylation. These sources of heterogeneity can often be eliminated by site directed mutagenesis of the protein, or enzymatic removal of sugars and phosphates. This source of heterogeneity is minimized if the GPCR can be expressed in bacteria.

The greatest obstacle to the formation of crystals may be conformational heterogeneity due to the inherent flexibility of GPCRs. This flexibility may be functionally important, enabling structural changes associated with agonist binding and activation of a membrane bound receptor; however, this property may lead to the existence of different protein conformations and to denaturation, particularly in detergent solutions. There is evidence that the conformational heterogeneity due to relative movements of the TM segments can be reduced by specific ligands [42]. Other sources of conformational heterogeneity are the amino and carboxyl termini, and the loops connecting the TM segments. These sequences are often longer than the homologous segments in rhodopsin and may be unstructured. In some cases the problem of conformational heterogeneity can be mitigated by structural modifications; however, identifying these poorly structured domains can be challenging. Moreover, removing hydrophilic sequences may eliminate potential crystal lattice sites.

While the challenges facing GPCR structural biologists are formidable, I believe structures will begin to appear within the next several years. The problems outlined above are solvable. This may require a highly focused effort on a specific target, where structural modifications and/or associated proteins (antibodies, arrestins, G proteins) are used to facilitate crystal formation. Another approach is to screen a large number of GPCRs to identify those with the best characteristics for structural studies. This approach has been used by a European consortium known as the Membrane Protein Network (MEP-NET, <http://www.mepnet.org/>) consisting of academic and industrial groups. This program has made significant progress in identifying the best GPCR candidates for crystallography trials.

2.4. GPCR oligomers

There is a growing body of evidence that GPCRs exist as dimers (or oligomers) and that these dimers may be important for G protein activation for at least some GPCR families. This topic has been addressed in several excellent reviews [43–46] and is the subject of another review in this series. Therefore, GPCR dimers will be only briefly discussed here. Dimerization is clearly an important mechanism of receptor activation for the glutamate family of GPCRs [8,47], where ligand-induced changes in the dimer interface of the amino terminal ligand binding domain has been demonstrated by crystallography [48,49]. However, the role of dimerization in the activation of rhodopsin family members is less clear. For instance, recent cryoelectron microscopy images suggest that rhodopsin may exist as homodimers in rod outer segment membranes [50]. In addition, neutron scattering studies provide evidence that a pentameric complex forms when purified leukotriene B(4) is reconstituted with purified G_i , suggesting that a receptor homodimer is needed to complex with a heterotrimeric G protein [51]. Nevertheless, it remains to be seen if a receptor dimer is required for G protein activation. The effect of agonist binding on the formation or disruption of dimers is not consistent among the rhodopsin family members that have

been examined [43]. Moreover, ligands interact with individual receptor monomers, and there is currently no evidence that ligands span the interface between receptor dimers. If changes in dimerization occur, it is likely a secondary consequence of ligand-induced changes in the arrangement of the TM segments. Evidence in support of this comes from biophysical studies on leukotriene B(4) homodimers demonstrating that ligand binding to one protomer leads to conformational changes in its partner [52]. While dimers may be important for G protein activation, it is essential to understand the agonist-induced structural changes that occur in the context of individual GPCR monomers.

3. GPCR activation

3.1. Activation of rhodopsin

The currently available three-dimensional, high-resolution structures of rhodopsin correspond to an inactive form of the receptor. The most detailed information about structural changes associated with activation of a GPCR comes from lower-resolution biophysical studies on rhodopsin. Rhodopsin structure and what is known about its light-induced conformational changes have been the subject of several excellent reviews [29,53–56], and some of the main points will be briefly discussed here. Electron paramagnetic resonance spectroscopy (EPR) studies provide evidence that photoactivation of rhodopsin involves a rotation and tilting of TM6 relative to TM3 [57]. Further support for motion of TM6 during rhodopsin activation was provided by chemical reactivity measurements and fluorescence spectroscopy [58], as well as by ultraviolet absorbance spectroscopy [59] and by zinc crosslinking of histidines [60]. Light-induced conformational changes have also been observed in the cytoplasmic domain spanning TM1 and TM2, and the cytoplasmic end of TM7 [61–63].

Obtaining a high-resolution structure of metarhodopsin II, the active form of rhodopsin has been hindered by the instability of rhodopsin crystals following light activation. However, there has been some progress towards this goal. Shertler's group succeeded in obtaining two dimensional crystals and a low resolution map of metarhodopsin I [64,29]. Metarhodopsin I is an intermediate in the process of rhodopsin activation that occurs after photoisomerization of 11-Cisretinal, but before structural changes required for transducin activation (metarhodopsin II). Electron diffraction studies reveal that the formation of metarhodopsin I is not accompanied by the large rigid-body movements in TM segments shown to be involved in rhodopsin activation [57]. However, a more subtle change, consisting in the rearrangement in the conformation of the Trp residue of the highly conserved CWxxP motif in TM6, has been detected in this intermediate. Thus, it seems that there is no gradual transformation of the inactive protein into the active form, but the activation is initiated through small scale changes in the conformation of some key residues, which will presumably trigger the larger conformational changes related to the subsequent stages of the activation process [29]. Recently, conditions for growing more light stable three-dimensional crystals of rhodopsin have been identified [65], suggesting that

a high-resolution structure of metarhodopsin II may be available in the near future.

3.2. Common structural changes among GPCRs

In spite of the remarkable diversity of ligands and ligand binding domains in the family of GPCRs, there is also considerable evidence for a common mechanism of activation. When comparing sequences, GPCRs are most similar at the cytoplasmic ends of the transmembrane segments adjacent to the second and third cytoplasmic domains, the regions known to interact with cytoplasmic G proteins [66]. Members of the large family of GPCRs transduce signals by activating one or more members of the relatively small family of highly homologous heterotrimeric G proteins. For example, the follicle stimulating hormone (FSH) receptor is activated by a large glycoprotein hormone that binds to the amino terminus while the β_2 AR is activated by adrenaline (approximately the size of a single amino acid) that binds to the TM segments; yet both of these receptors activate the same G protein (Gs), indicating that the structural changes in the cytoplasmic domains of these two receptors must be very similar. Moreover, many GPCRs exhibit promiscuous coupling to more than one G protein. For example, rhodopsin preferentially couples to transducin while the β_2 AR preferentially couples to Gs; however, both are capable of activating Gi [67].

Additional evidence that GPCRs undergo similar conformational changes within TM segments and cytoplasmic domains comes from biophysical and biochemical studies. Fluorescence spectroscopic studies of β_2 AR labeled with fluorescent probes demonstrate movement in both TM3 and TM6 upon activation [68]. More recent studies of β_2 AR labeled with fluorescent probes at the cytoplasmic end of TM6 provide evidence that agonists induce a rotation or tilting movement of the cytoplasmic end of TM6 similar to that observed in rhodopsin [69,70]. A key structural change involving the disruption of an ionic interaction between the highly conserved D(E)RY sequence at the cytoplasmic end of TM3 and an acidic residue at the cytoplasmic end of TM6 is observed upon activation of both rhodopsin and the β_2 AR [57,71]. Additional support for movement of TM3 and TM6 in the β_2 AR comes from zinc crosslinking studies [72,73], and chemical reactivity measurements in constitutively active β_2 AR mutants [74,75]. Cysteine crosslinking studies on the M3 muscarinic receptor provide evidence for the movement of the cytoplasmic ends of TM5 and TM6 toward each other upon agonist activation [76–78].

3.3. Activation by diffusible agonists

While the structural changes associated with activation may be similar for rhodopsin and other GPCRs, the mechanism by which these changes are brought about is quite different. In inactive rhodopsin, there is virtually no activity towards the G protein transducin. Absorption of a photon of light converts covalently bound 11-Cisretinal (an inverse agonist) to all trans retinal (a full agonist) within femtoseconds. Rhodopsin then rapidly undergoes a series of conformational changes that have

been characterized spectroscopically (rhodopsin > bathorhodopsin > lumirhodopsin > metarhodopsin I > metarhodopsin II). The structural changes associated with the formation of metarhodopsin II, the active form of rhodopsin, are observed within microseconds of photoactivation [79]. Photoisomerization of bound retinal is extremely efficient in using the energy of the captured photon to induce protein structure changes. It is interesting that free trans-retinal is not a very effective agonist for opsin (the unliganded form of rhodopsin) [80,81], producing only about 14% of the response observed for light-activated rhodopsin [80].

For the vast majority of other GPCRs, activation occurs when an agonist diffuses into an unliganded receptor. In many cases the unliganded receptor has some basal (constitutive) activity towards a G protein. The term “efficacy” is used to describe the effect of a ligand on the functional properties of the receptor [For a more complete discussion of efficacy refer to [82]]. Agonists are defined as ligands that fully activate the receptor. Partial agonists induce submaximal activation of the G protein even at saturating concentrations. Inverse agonists inhibit basal activity. Antagonists have no effect on basal activity, but competitively block access of other ligands. The efficacy of a given drug may vary depending on the signaling pathway being examined [83].

A number of kinetic models have been developed to explain GPCR activation using information derived from indirect measures of receptor conformation, such as ligand binding affinity and the activation of G proteins or effector enzymes [84–87]. The simplest of them, the two-state model proposes that a receptor exists primarily in two states, the inactive state (R) and the active state (R*). In the absence of ligands, the level of basal receptor activity is determined by the equilibrium between R and R*. The efficacy of ligands reflects their ability to alter the equilibrium between these two states. Full agonists bind to and stabilize R*, while inverse agonists bind to and stabilize R. Partial agonists have some affinity for both R and R* and are therefore less effective in shifting the equilibrium towards R*.

The two-state model can describe much of the functional behavior of GPCRs and explain the spectrum of responses to ligands of different efficacy in simple experimental systems consisting of one receptor and one G protein. However, there is a growing body of experimental evidence for the existence of multiple conformational states [summarized in [83]]. Within this framework, each ligand may induce or stabilize a unique conformational state that can be distinguished by the activity of that state towards different signaling molecules (G proteins, kinases, arrestins).

3.4. β_2 AR as a model system for studying ligand-induced conformational changes

With the exception of rhodopsin, the β_2 AR is perhaps the most extensively characterized member of the GPCR family. The β_2 AR is a good model system for studying agonist binding because much is known about the sites of interactions between catecholamine ligands and the receptor (Fig. 3). Moreover,

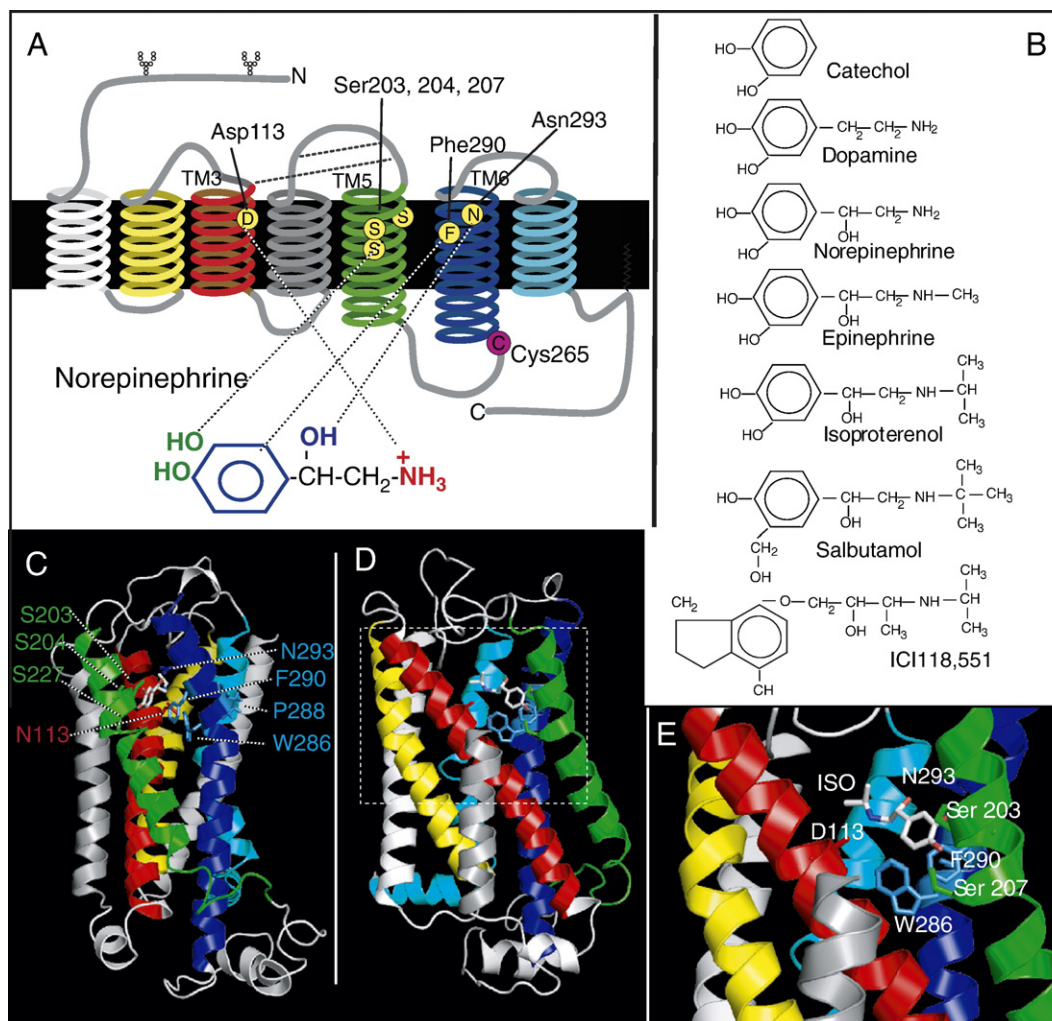


Fig. 3. Agonist binding site in the β_2 AR. (A) Amino acids involved in forming the agonist binding site for the β_2 AR. The sites of interaction between catecholamines and the β_2 AR have been extensively characterized [100–102]. The amine nitrogen interacts with Asp 113 in TM3 [103], the catechol hydroxyls interact with serines in TM5 [100–102]. Interactions with the aromatic ring and the chiral β -hydroxyl have both been mapped to TM6 [101]. (B) A panel of β_2 AR ligands discussed in this review. Catechol is a very weak partial agonist. Dopamine and salbutamol are partial agonists. Norepinephrine, epinephrine and isoproterenol are full agonists. ICI118,551 is an inverse agonist. (C–E) Isoproterenol docked into a three-dimensional model of the β_2 AR. Illustrations were made with MacPyMOL software.

there is a rich source of structurally similar ligands having a spectrum of efficacies ranging from inverse agonists to full agonists (Fig. 3B).

In the case of the β_2 AR it has been possible to monitor directly some of these ligand-specific states using fluorescence spectroscopy [42,70,71,88,89]. These studies reveal that for the β_2 AR ligand binding and activation is a kinetically and conformationally complex process. Based on these studies we have proposed a working model (Fig. 4) where agonist binding and conformational changes occur through a sequence of conformational intermediates. Biophysical support for this model will be briefly summarized below.

To study ligand induced conformational changes, purified β_2 AR is labeled with relatively small fluorescent probes (less than 500 Da) at specific sites on the cytoplasmic ends of transmembrane domains involved in ligand binding and G protein coupling [42,70,71,88,89]. Selective labeling is accomplished using a modified β_2 AR in which the most reactive

cysteines have been mutated to alanine, valine or serine [71]. The resulting minimal cysteine receptor exhibits normal ligand binding and G protein coupling behavior. A new reactive cysteine is introduced into a specific structural domain by site-directed mutagenesis. The modified β_2 AR is expressed in insect cells, solubilized from insect cell membranes with detergent (dodecylmaltoside), purified by affinity chromatography, and labeled with a cysteine reactive fluorescent probe. The fluorescent probes are sensitive to their local molecular environment such that changes in the β_2 AR structure lead to changes in the fluorescent properties of the fluorophores (intensity, lifetime, mobility). Therefore it is possible to monitor ligand-induced conformational changes in different receptor domains, and to correlate ligand structure with specific structural changes in the receptor.

Fig. 3 shows the amino acid residues that form the ligand binding site for the β_2 AR deduced from mutagenesis studies, and illustrates the location of Cys 265, one of the labeling sites

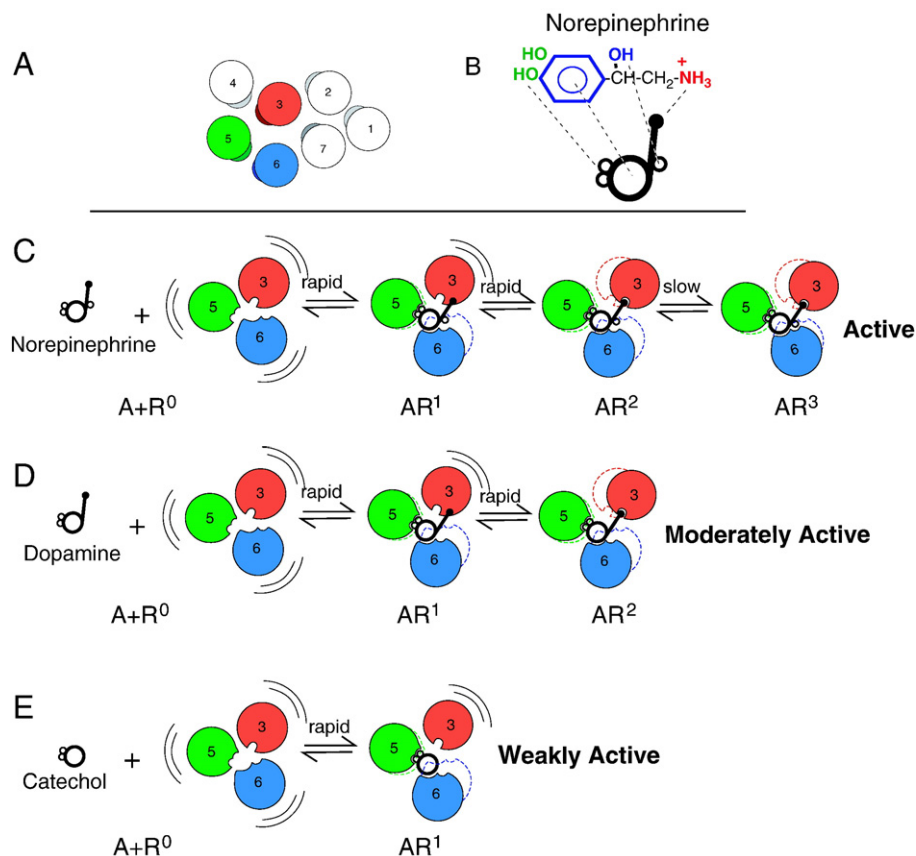


Fig. 4. Sequential Binding Model. (A) Arrangement of the TM domains of the β_2 AR as viewed from the extracellular surface. The agonist binding domains are shown in red (TM3), green (TM5) and blue (TM6). (B) Cartoon representing structural components of norepinephrine. (C–E) In the absence of ligand, the receptor (R) is conformationally flexible. Conformational state R^1 is stabilized by interactions between TMs 5 and 6 and the catechol ring. The transition to state R^2 occurs when Asp 113 in TM3 binds the amine nitrogen. The transitions from R to R^2 are rapid. The slow transition from R^2 to R^3 involves interactions between the chiral β -hydroxyl and Asn293 on TM6. Adapted from Swaminath et al. [88].

we have used for our spectroscopy studies. An environmentally sensitive fluorophore covalently bound to Cys265 is well positioned to detect agonist-induced conformational changes relevant to G protein activation. Based on homology with rhodopsin, Cys265 is located in the third intracellular loop (IC3) at the cytoplasmic end of the TM6. Mutagenesis studies have shown this region of IC3 to be important for G protein coupling [90,91]. Moreover, TM6, along with TM3 and TM5 contain amino acids that form the agonist binding site.

3.4.1. Ligand-specific conformational states detected by fluorescence lifetime studies

We examined ligand-dependent changes in fluorescence lifetime of purified β_2 AR labeled at Cys265 with fluorescein maleimide in an effort to identify the existence of agonist-specific conformational states [42]. Fluorescence lifetime analysis can detect discrete conformational states in a population of molecules, while fluorescence intensity measurements reflect the weighted average of one or more discrete states. Fluorescence lifetime, τ , refers to the average time that a fluorophore that has absorbed a photon remains in the excited state before returning to the ground state. The lifetime of fluorescein (nanoseconds) is much faster than the predicted off-rate of the agonists we examined (μ s–ms), and much shorter

than the half-life of conformational states of bacteriorhodopsin (μ s)[92], rhodopsin (ms) [79,93] or of ion channels (μ s–ms) [94]. Therefore, lifetime analysis of fluorescein bound to Cys265 is well suited to capture even short-lived, agonist-induced conformational states. We observed that the full agonist isoproterenol induced a conformation that was distinct from the conformations induced by the partial agonists salbutamol and dobutamine [42]. These studies also revealed the existence of an intermediate state in equilibrium with the active state for both full agonists and partial agonists. In contrast, the neutral antagonist appeared to stabilize a state that was indistinguishable from the unliganded receptor.

3.4.2. Intermediate conformational states detected by kinetic studies

Agonist-induced conformational changes in purified β_2 AR lead to an increase in the fluorescence intensity of tetramethylrhodamine bound to Cys265 [88,89]. The increase in fluorescence intensity as a function of time following activation by the agonist norepinephrine is best fit with a two component exponential function [88] (Fig. 5A). In contrast, the response to dopamine (a partial agonist) is adequately fit by a one component exponential function. Of interest, the rapid component of norepinephrine is very similar to the response to dopamine (Fig. 5A), which

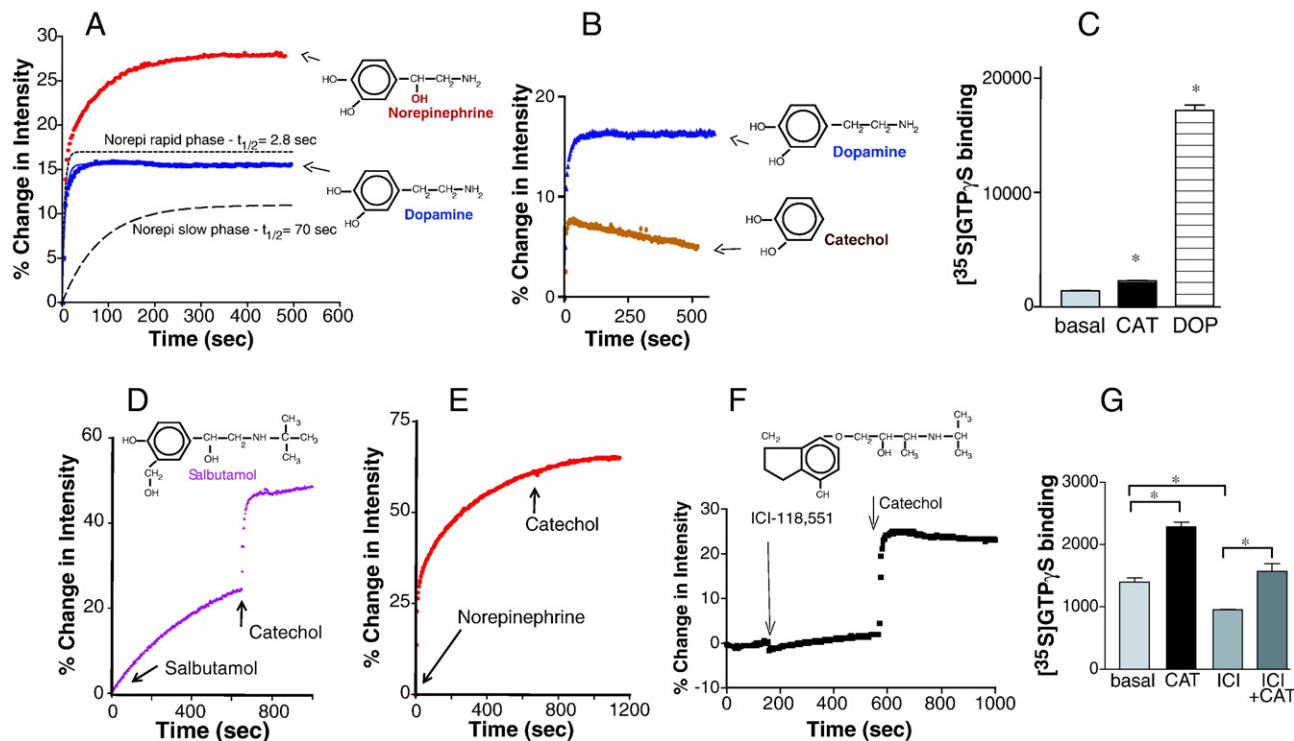


Fig. 5. Agonist-induced conformational changes detected by fluorescence spectroscopy in β_2 AR labeled at Cys265 with tetramethylrhodamine maleimide (TMR- β_2 AR). (A) Change in the intensity of TMR- β_2 AR in response to norepinephrine and dopamine. The response to norepinephrine is best fit by a two-site exponential function. The rapid and slow components of the response are illustrated by the dotted lines. (B) Change in the intensity of TMR- β_2 AR in response to dopamine and catechol. (C) Catechol and dopamine stimulated [35 S]GTP γ S binding to purified β_2 AR reconstituted with purified Gs. (D) The change in the intensity of TMR- β_2 AR in response to the non-catechol partial agonist salbutamol followed by the addition of catechol. Catechol can induce a conformational change in TMR- β_2 AR bound to a saturating concentration of salbutamol, indicating that salbutamol and catechol occupy non-overlapping binding sites. (E) The change in the intensity of TMR- β_2 AR in response to norepinephrine followed by the addition of catechol. No catechol response is observed in TMR- β_2 AR bound to a saturating concentration of norepinephrine indicating that these ligands share a common binding site. (F) There is no significant change in the intensity of TMR- β_2 AR in response to the inverse agonist ICI118,551. Catechol can induce a conformational change in β_2 AR bound to a saturating concentration of ICI118,551, indicating that these ligands do not occupy the same binding space. (G) [35 S]GTP γ S binding to purified β_2 AR reconstituted with purified Gs. Catechol weakly stimulates [35 S]GTP γ S binding and ICI118,551 inhibits basal [35 S]GTP γ S binding. Of interest, catechol can stimulate [35 S]GTP γ S binding in β_2 AR occupied by a saturating concentration of ICI118,551. The data presented here are adapted from Swaminath et al. [88,89].

suggested that the dopamine-induced conformation might represent an intermediate in the conformational response to norepinephrine (Fig. 4D). A rapid component of fluorescence change is observed with all ligands containing a catechol ring and can be observed with catechol alone (Fig. 5B). In fact, catechol is a weak partial agonist (Fig. 5C). Based on this observation we proposed that the catechol-induced conformation might represent an intermediate in the conformational response to dopamine and norepinephrine (Fig. 4E).

The slow component of the response to norepinephrine (Fig. 5A) is also observed with the full agonists epinephrine and isoproterenol [88]. The only structural difference between dopamine and norepinephrine is the β -hydroxyl (Fig. 3B). Thus conformational changes associated with interactions between the β -hydroxyl and the β_2 AR are much slower than those involving interactions with catechol ring in our experimental system. Functionally, these slower conformational changes correlate with efficient agonist-induced β_2 AR internalization, most likely due to interactions between the β_2 AR and G protein coupled receptor kinases and/or arrestins. While dopamine is a relatively good partial agonist for G protein activation (approximately 60% of isoproterenol), it is much

less efficient at inducing internalization (approximately 20% of isoproterenol) [88].

3.4.3. Relevance of slow conformational changes

The slow component of the agonist-induced change observed by fluorescence spectroscopy in purified β_2 AR (Fig. 5A) is considerably slower than expected from some physiologic responses to GPCR activation, and slower than the conformational changes observed in a modified alpha 2 adrenergic receptor containing a fluorescein arsenical hairpin binder (FIAsH) site in the third intracellular loop and cyan fluorescent protein (CFP) fused to the carboxyl terminus [95,96]. This modified receptor was expressed in HEK293 cells and agonist-induced conformational changes were detected as changes in fluorescence resonance energy transfer (FRET) between FIAsH and CFP. There are several possible explanations for these observed differences in kinetics. The use of a large fluorescence reporter such as CFP does not allow one to determine the nature of the structural change responsible for the change in FRET; therefore, these FRET experiments may be detecting a different conformational switch, possibly similar to the rapid response observed with dopamine and catechol. However, the different

rates observed could also be due to the fact that the β_2 AR experiments use purified receptor in the absence of G protein, while the FRET experiments were performed on receptor expressed in cell membranes that contain G proteins. It is known that receptors form complexes with G proteins in the plasma membrane (precoupling), and that these complexes have a higher affinity for agonists than do receptors alone. Other factors that might contribute to differences between experiments using purified receptor and those on receptors in cells could be the influence of the pH and salt gradients across the plasma membrane in living cells, as well as the asymmetry of plasma membrane lipids. Nevertheless, while the slow component of the conformational response to agonists may be attributable to the use of purified receptor protein, we believe the structural changes are physiologically relevant because they can be linked to specific interactions between the ligand and the receptor (e.g., the β -hydroxyl of catecholamine agonists interacting with Asn293 in TM6, Fig. 3), and they correlate with specific functional behaviors stimulated by the agonists in β_2 AR expressed in cells (e.g., agonist-induced internalization [88]).

3.4.4. Catechol activates of the rotamer toggle switch

Based on what is known about the binding site for the catechol ring of catecholamines (Fig. 3), we proposed that the rapid component of the conformational response associated with catecholamine and catechol binding involves changes in the rotameric position of aromatic amino acids surrounding the highly conserved proline Pro288^{6,50} in TM6 (Fig. 3C–E). This conformational change, known as a rotamer toggle switch, has been proposed to be involved in the activation of amine and opsin receptor families [97]. Upon binding, the aromatic catechol ring of catecholamines would interact directly with the aromatic residues of the rotamer toggle switch, Trp286^{6,48} and Phe290^{6,52}. Molecular dynamics simulations suggest that rotamer configurations of Cys285^{6,47}, Trp286^{6,48} and Phe290^{6,52}, the residues that comprise the rotamer toggle switch, are coupled and modulate the bend angle of TM6 around the highly conserved proline kink at Pro288^{6,50}, leading to the movement of the cytoplasmic end of TM6 upon activation [97]. This movement could be detected by tetramethylrhodamine bound to Cys265 at the cytoplasmic end of TM6 (Fig. 5B).

3.4.5. Non-catecholamine partial agonists do not activate the rotamer toggle switch

In the same experimental system, activation of tetramethylrhodamine-labeled β_2 AR by salbutamol, a noncatechol partial agonist, produced only a slow monophasic increase in fluorescence intensity [89] (Fig. 5C). Catechol could induce a further increase in fluorescence in β_2 AR bound to saturating concentrations of salbutamol, but not in β_2 AR bound to norepinephrine (Fig. 5C and D). This suggests that the aromatic ring of salbutamol does not occupy the same binding space as catechol and does not activate the rotamer toggle switch [89]. Thus, the active state induced by salbutamol would be different from that induced by catecholamine agonists [89]. This is in agreement with fluorescent lifetime experiments discussed above [42].

3.4.6. The inverse agonist ICI118,551 does not inhibit activation of the rotamer toggle switch

The neutral antagonist alprenolol and the inverse agonists ICI118,551 and timolol do not produce a detectable change in fluorescence in β_2 AR labeled with tetramethylrhodamine on Cys 265 [89], although they do inhibit the response to agonists and partial agonists with one exception. In tetramethylrhodamine-labeled β_2 AR bound to saturating concentrations of these antagonists there is no effect on the response to catechol. This is shown in Fig. 5F for ICI118,551. The fluorescence response to catechol in β_2 AR bound to ICI118,551 is associated with a functional response in a G protein activation assay (Fig. 5G). This suggests that ICI118,551 does not occupy the catechol binding pocket and does not prevent activation of the rotamer toggle switch by catechol.

3.4.7. Activation of the ionic lock

We investigated another proposed molecular switch, the ionic lock between the Asp130^{3,49}/Arg131^{3,50} pair at the cytoplasmic end of TM3 and Glu268^{6,30} at the cytoplasmic end of TM6 (Fig. 6A) [71]. We used a modified β_2 AR to introduce a single cysteine labeling site for the cysteine-reactive fluorophore monobromobimane at the cytoplasmic end of TM6 (A271C) adjacent to a Trp introduced at the cytoplasmic end of TM3 (I135W) (Fig. 6B). We took advantage of the ability of tryptophan to quench the fluorescence of bimane, with measurable effects at distances smaller than 15 Å [98], and used bimane fluorescence to monitor structural changes associated with disruption of the ionic lock between TM3 and TM6 in purified β_2 AR. In the three-dimensional model of the β_2 AR based on rhodopsin, bimane bound to Cys271 would be separated from Trp135 by the ionic lock. Disruption of the ionic lock would allow Trp to contact and quench bimane fluorescence. This is what we observed upon activation of the modified receptor by isoproterenol, which reduced the intensity of bimane by approximately 50% (Fig. 6C). Our results demonstrated that the disruption of the ionic lock is an obligatory step for maximal receptor activation and is triggered by nearly all agonists, independent of efficacy (Fig. 6D). However, we found that disruption of the ionic lock is not directly coupled to the rotamer toggle switch in TM6 since catechol, which is capable of activating the rotamer toggle switch, was not able to activate the ionic lock [71]. Moreover, salbutamol which does not activate the rotamer toggle switch [89] is able to fully activate the ionic lock [71] (Fig. 6D).

3.4.8. Binding energy and conformational change

It is interesting to note that catechol and dopamine have nearly the same binding affinity for purified β_2 AR [71]. The K_i of dopamine, as determined by competition binding, is 350 μ M, while the K_d for catechol, determined by a conformational assay, is 160 μ M [71]. This is surprising considering that the interaction between the primary amine of dopamine and Asp113 makes the strongest contribution to the binding energy. Since dopamine and catechol bind with the same affinity, but only dopamine disrupts the ionic lock, part of the binding energy associated with the interaction between dopamine and Asp113

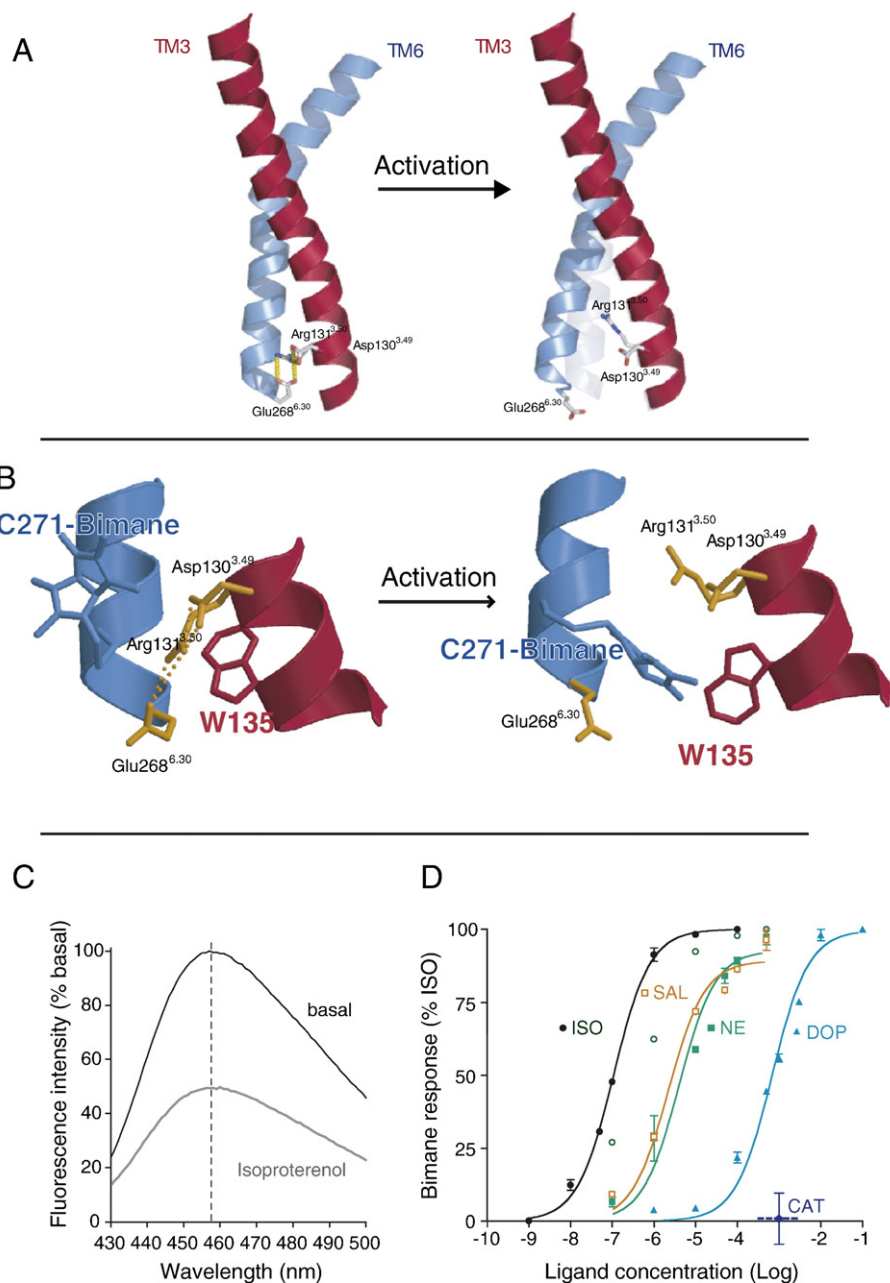


Fig. 6. Fluorescence spectroscopy to monitor disruption of the ionic lock in the β_2 AR. (A) Model of TM3 (red) and TM6 (blue) from the β_2 AR depicting the amino acids that comprise the ionic lock at the cytoplasmic end of these TM segments. (B) Close up view of the ionic lock and the modifications made to monitor conformational changes in this region. Alanine 271 was mutated to cysteine (C271) and isoleucine 135 was mutated to tryptophan (W135). C271 was labeled with monobromobimane in purified β_2 AR. Upon activation, W135 moves closer to bimane on C271 and quenches fluorescence. (C) Emission spectrum of bimane on C271 before and after activation by the agonist isoproterenol. (D) Effect of different ligands on disruption of the ionic lock as determined by bimane fluorescence. The partial agonists dopamine and salbutamol are as effective at disrupting the ionic lock as the full agonists norepinephrine and isoproterenol. Only catechol has no effect on the ionic lock. These data are adapted from Yao et al. [71].

may be used to offset by the energetic cost of breaking the ionic lock.

4. Conclusions

Based on these fluorescence studies we proposed a model where agonists stabilize partially or fully active states by using different chemical groups to activate different combinations of

molecular switches, which are not necessarily interdependent. In the unliganded inactive state of a GPCR, the arrangement of TM segments is stabilized by non-covalent interactions between side chains. Structurally distinct ligands are able to break different combinations of the basal state stabilizing interactions either directly by binding to amino acids that are involved in these intramolecular interactions, or indirectly by stabilizing new intramolecular interactions. These ligand-

specific conformational changes may be responsible for differential activation of the signaling cascades of the receptor. The affinity of a particular ligand will then be dependent on the energy costs and gains associated with each disrupted and created interaction, while its efficacy will be dependent on the ability to trigger the switches associated with activation. These molecular switches are normally activated by agonist binding, but will also be revealed in constitutively active mutants, where single point mutations in virtually any structural domain can lead to elevated basal activity[99]. A better understanding of the process by which ligands bind and modify GPCR structure may ultimately help in the design of more selective drugs with the appropriate efficacy for the desired physiologic function.

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