The G-protein Coupled Receptor Family: Actors with Many Faces

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Abstract: G-protein coupled receptors (GPCRs) comprise the largest family of proteins in our body, which have many important physiological functions and are implicated in the pathophysiology of many serious diseases. GPCRs therefore are significant targets in pharmaceutical research. GPCRs share the common architecture of seven plasma membrane-spanning segments connected to each other with three extracellular and three intracellular loops. In addition, GPCRs contain an extracellular N-terminal region and an intracellular C-terminal tail. GPCRs could stimulate different intracellular G-proteins (internal stimuli) and signaling pathways after their interaction with different ligands (external stimuli). The exceptional functional plasticity of GPCRs could be attributed to their inherent dynamic nature to adopt different active conformations, which are stabilized differentially by different stimuli as well as by several mutations. This review describes the structural changes of GPCRs associated with their activation. Understanding the dynamic nature of GPCRs could potentially contribute in the development of future structure-based approaches to design new receptor-specific, signaling-selective ligands, which will enrich the pharmaceutical armamentarium against various diseases

Keywords: GPCRs, (inverse)agonists, binding site crevice, membrane-spanning segments, structure, conformational changes, G-proteins, signaling, functional plasticity.

INTRODUCTION

G-protein coupled receptors (GPCRs) are one of the most important actors in the "cell" theater. Being on stage, named the plasma membrane, GPCRs interact with many types of audience (internal stimuli), which consists of a large variety of intracellular proteins, including G-proteins. GPCRs convey to audience the information included in various scenarios (external stimuli), which varies from neurotransmitters, peptides, proteases, glycoprotein hormones, purine ligands, chemokines, and many others. GPCRs have been grouped, based on sequence homology, into five main families: rhodopsin, glutamate, adhesion, frizzled/taste2, and secretin, being the rhodopsin family the largest [1]. This review primarily focuses on the rhodopsin family.

THE ACTOR AND THE SCENARIO, "EXTERNAL STIMULI"

Significant advances in crystallization of GPCRs [2, 3] have permitted to elucidate the crystal structures of many receptors (Table **1**). All these structures share the common architecture of seven plasma membrane-spanning (or transmembrane) domains (TMs, which also terms this family of proteins as 7TM receptors) connected to each other with three extracellular (EL) and three intracellular loops (IL), a disulphide bridge between EL 2 and TM 3, and a cytoplasmic C–terminus containing an α-helix (Hx8) parallel to the cell membrane. In addition, GPCRs contain an extracellular N-terminal region (N-region) and an intracellular C-terminal tail (C-tail).

Figure **1A** shows the superimposition of representative crystal structures. Clearly, the structure of the cytoplasmic part is highly conserved (Fig. **1B**), with the exception of CXCR4, which contains very different TM4 and TM7-Hx8 domains. This structural conservation correlates with the fact that most conserved residues are clustered in the central and intracellular regions of the receptor [24]. In contrast, there is a low degree of sequence conservation among different GPCRs at their extracellular domains. Accordingly, the structure of the extracellular part of TM helices is more divergent (Fig. **1C**). We have previously suggested that GPCRs, during their evolution, have evolved to adjust the structural characteristics of their cognate ligands, by customizing a preserved scaffold (7TM receptors) through *conformational plasticity* [25]. We use this term to describe the structural differences among different receptor families within the extracellular side, near the binding site crevices, responsible for recognition and selectivity of diverse ligands.

These crystallographic structures together with previous biophysical and biochemical studies have shown that the TMs of GPCRs fold such as to form a water-filled binding-site crevice, which extends from the extracellular surface of the receptor into the membrane core [23, 26-37]. The binding site crevice is located between the extracellular parts of TMs 3, 5, 6, and 7 and EL 2 for rhodopsin (Fig. **2A**), β_1- (Fig. **2C**) and β_2- (Fig. **2B**) adrenergic receptors, dopamine D3 receptor (Fig. **2F**), and adenosine A_{2A} receptor (Fig. **2D**). In contrast, IT1t binds CXCR4 through TMs 2, 3, and 7 and EL 2 (Fig. **2E**). The key role of this minor binding site between TMs 1-3 and 7 has recently been reviewed [38]. In all these crystal structures, EL 2 defines the binding site crevice as has been previously proposed by a study using site-directed mutagenesis and applying the cysteine-substituted accessibility method (SCAM) [39]. However, EL 2 is highly variable in length, amino acid content, and structure among available crystal structures [40]. EL 2 of rhodopsin, formed by two β-strands, buries the binding site from the extracellular environment (Fig. **2A**), whereas EL 2 of CXCR4, also formed by two β-strands, fully exposes the binding site to the extracellular environment (Fig. **2E**). In contrast, a helical segment forms EL 2 of the β_1- and β_2- adrenergic receptors (Figs **2B** and **2C**). This α-helix is probably not conserved in the other members of the biogenic amine receptor family, as it was not found in the structure of the dopamine D3 receptor (Fig. **2F**). Each receptor subfamily has probably developed a specific EL 2 to adjust the structural characteristics of its cognate ligands. EL 2 plays a key role for the selective affinity of a drug for a given receptor, and, thus, it is highly relevant for structure-based drug design.

THE ACTOR ON STAGE

An actor can take an extremely large number of different positions on stage. Similarly, GPCRs are dynamic rather than static structures, capable to adopt different conformations.

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Table 1.

	Crystal Structures of GPCRs	References
1	Bovine (Protein Data Bank accession numbers 1F88, 1HZX, 1GZM, 1L9H, and 1U19) and squid (2Z73) rhodopsin bound to the full inverse agonist *cis*-retinal	[4-7]
2	Ligand-free opsin (3CAP)	[8]
3	Ligand-free opsin in its G-protein-interacting conformation (3DQB)	[9]
4	The metarhodopsin II (3PXO)	[10]
5	β_1-adrenergic receptor bound to the antagonist cyanopindolol (2VT4), partial agonists dobutamine (2Y01) or salbutamol (2Y04), or agonists carmoterol (2Y02), or isoprenaline (2Y03)	[11, 12]
6	β_2-adrenergic receptor bound to the inverse agonist ICI118,551 (3NY8), the partial inverse agonist carazolol (2RH1), the neutral antagonist alprenolol (3NYA), or an irreversible agonist (3PDS)	[13-16]
7	A nanobody-stabilized active state of the β_2-adrenergic receptor (3P0G)	[17]
8	A_{2A} adenosine receptor in complex with the antagonist ZM241385 (3EML), the agonist UK-432097 (3QAK), the endogenous agonist adenosine (2YDO), and the synthetic agonist NECA (2YDV)	[18-20]
9	Dopamine D3 receptor in complex with the antagonist eticlopride (3PBL)	[21]
10	Histamine H_1 receptor in complex with the antagonist doxepin	[22]
11	Chemokine CXCR4 receptor bound to the IT1t antagonist (3ODU) or the CVX15 cyclic peptide (3OE0)	[23]

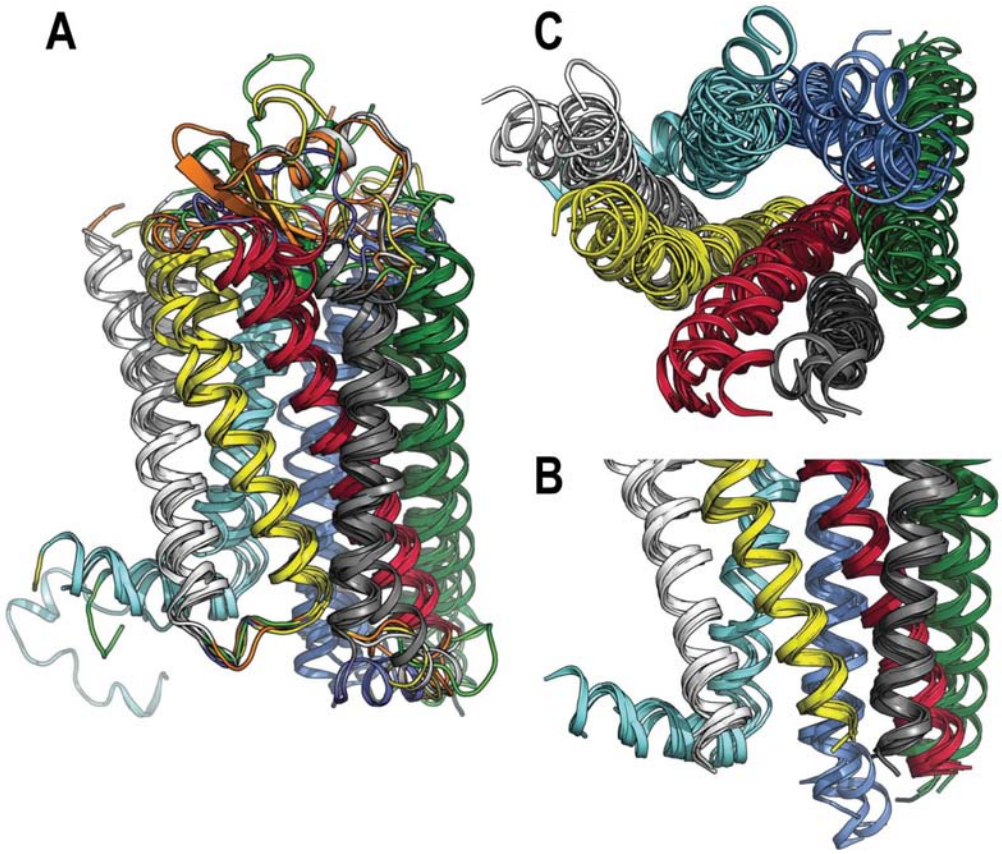

Fig. (1). **A)** Comparison of the currently available crystal structures of GPCRs in the inactive conformation: rhodopsin (PDB accession number 1GZM), the β_1- (2VT4) and β_2- (2RH1) adrenergic, the A_{2A} adenosine (3EML), the dopamine D3 (3PBL), and the chemokine CXCR4 (3ODU) receptors. **B)** The structure of the cytoplasmic part is highly conserved, with the exception of CXCR4, which contains very different TM4 and TM7-Hx8 domains (not shown). **C)**, The structure of the extracellular part of the TM helices is more divergent. The color code of the helices is TMs 1 in white, 2 in yellow, 3 in red, 4 in gray, 5 in green, 6 in dark blue, and 7 in light blue. (The color version of the figure is available in the electronic copy of the article).

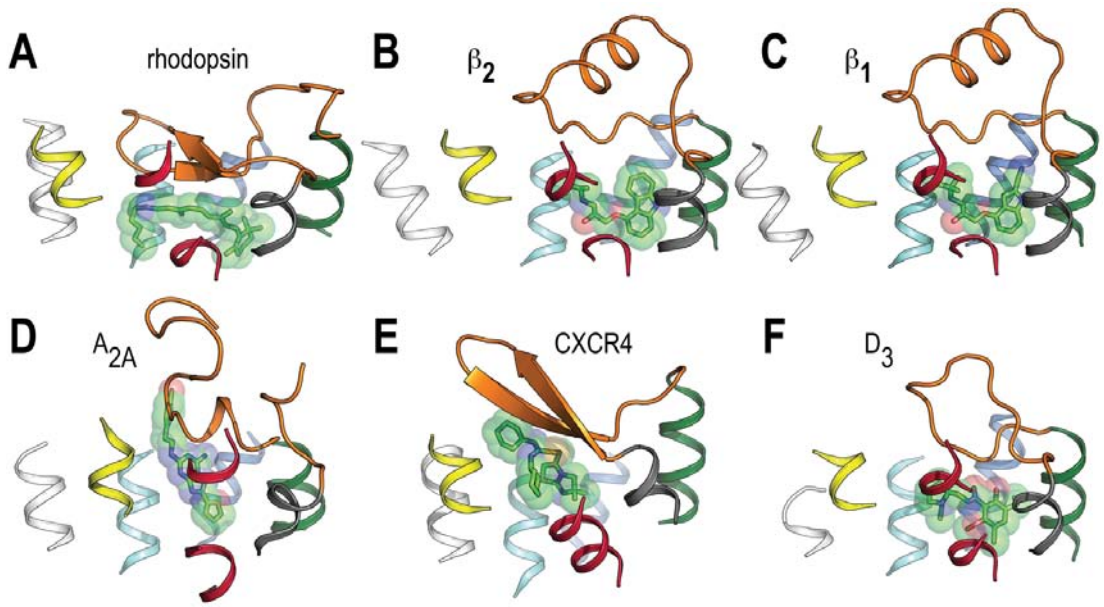

Fig. (2). Detailed view of the binding site crevice for rhodopsin (**A**), β_2- (**B**) and β_1- (**C**) adrenergic, adenosine A_{2A} (**D**), chemokine CXCR4 (**E**), and dopamine D3 (**F**) receptors. Extracellular loops are not shown with the exception of EL 2 in orange. Ligands are shown as spheres. The color code of TMs is as in Fig. **1**. (The color version of the figure is available in the electronic copy of the article).

The Intrinsic Property of GPCRs to Adopt an Active State

A plethora of studies have experimentally demonstrated that several mutations constitutively activated different GPCRs [41-60]. This has led to propose that GPCRs exist as ensembles of different conformations in equilibrium. In the simplest model, GPCRs exist in an equilibrium between two conformations (two-state model), the active, R*, and the inactive, R [61]. The agonist "external stimuli" binding at the native (or orthosteric) site stabilizes active state(s), R*. Conversely, the inverse agonist "external stimuli" decreases the basal, agonist-independent level of signaling by stabilizing inactive state(s), R. These findings also suggest that the ability of GPCRs to adopt their active states is most likely an intrinsic property of receptors. This was further supported by the fact that the high affinity for agonists of constitutively active mutants of the alpha1 adrenergic receptor did not require interaction with G proteins; the high affinity binding of these constructs was not significantly affected by the absence of magnesium or the presence of GTP or Gpp(NH) [44]. Similarly, the constitutive activity of a D2 construct, as measured by its increased affinity for agonists, was not affected by the presence of GTP [45]. Furthermore, the affinity of isoproterenol was greater at a "G-protein-free" purified constitutive active mutant of beta2-adrenergic receptor as compared with its wild-type counterpart, thus further supporting that the ability of GPCRs to adopt an active conformation is an inherent property of these receptors, which does not reflect an altered interaction with G-proteins [62]. Accordingly mutation-associated constitutive activation of beta2-adrenergic receptor has been observed in a receptor overexpressing system, such as the membranes of HEK 293 or COS-7 cells in which only a negligible fraction of receptors was coupled to G-proteins [54, 63].

GPCRs Adopt Many Conformations

In agreement with the concept that GPCRs have the inherent property to adopt active conformations by themselves, Kenakin [64, 65] and Kobilka [66, 67] have proposed that GPCRs, similar to other proteins [68], are dynamic molecules. This permits rapid small-scale structural fluctuations and pass through an energy landscape to adopt a number of conformations. In this energy landscape a large number of conformational states, ranging from no activity to maximal activity, are represented as energy wells. The width of each well reflects the conformational flexibility around a particular state. The transition probability from one state to another state depends on the energy difference between both states and the energy barrier between them. Based on this concept a GPCR could adopt more than two conformations.

The ability of a receptor to adopt different active states has also been proposed by Leff in his three-state model, which included an inactive and two different active states of a receptor [69]. Moreover Liapakis [63] and Feng [70] have suggested that the β_2-adrenergic and the angiotensin receptor, respectively exist in more than two states [63, 70]. In agreement with this concept, biophysical and computational studies have revealed the presence of different conformations of the beta2 adrenergic, which were stabilized differentially by agonists with different efficacies and had different functional properties [71-73]. Similarly, the NK1 receptor can occur in at least two distinct active conformations, which do not interchange readily [74].

Structural Changes Associated with Activation of GPCRs

GPCRs are maintained within the ensemble of inactive conformations through non-covalent interactions between side chains, mostly located in the TM segments. Mutation of these side chains, disrupting these stabilizing interactions, and leading to gain-of-function mutant receptors, has been a useful tool to study structure-function relationships [75]. However, a more clear understanding of the mechanisms that shift the equilibrium of the ensemble to the active conformations has been possible thanks to the recent crystal structure of the ligand-free opsin, which contains several distinctive features of the presumed active state. Comparison of this "active" opsin structure [8], with the structure of "inactive" rhodopsin [6] leads to the conclusion that during the process of GPCR activation the intracellular part of TM 6 tilts outwards by 6-7 Å, TM 5 nears TM 6, and Arg135 or $R^{3.50}$ within the (D/E)RY motif in TM 3 adopts an extended conformation pointing towards the protein core, to interact with the highly conserved Tyr223 or $Y^{5.58}$ in TM 5 and Tyr306 or $Y^{7.53}$ of the NPxxY motif in TM 7 (Fig. **3**). The superscripts refer to a generalized numbering system that indexes TM residues relative to the most conserved residue in the TM in which it is located [76]. This allows easy comparison among residues in the 7TM segments of different receptors. As shown in the original

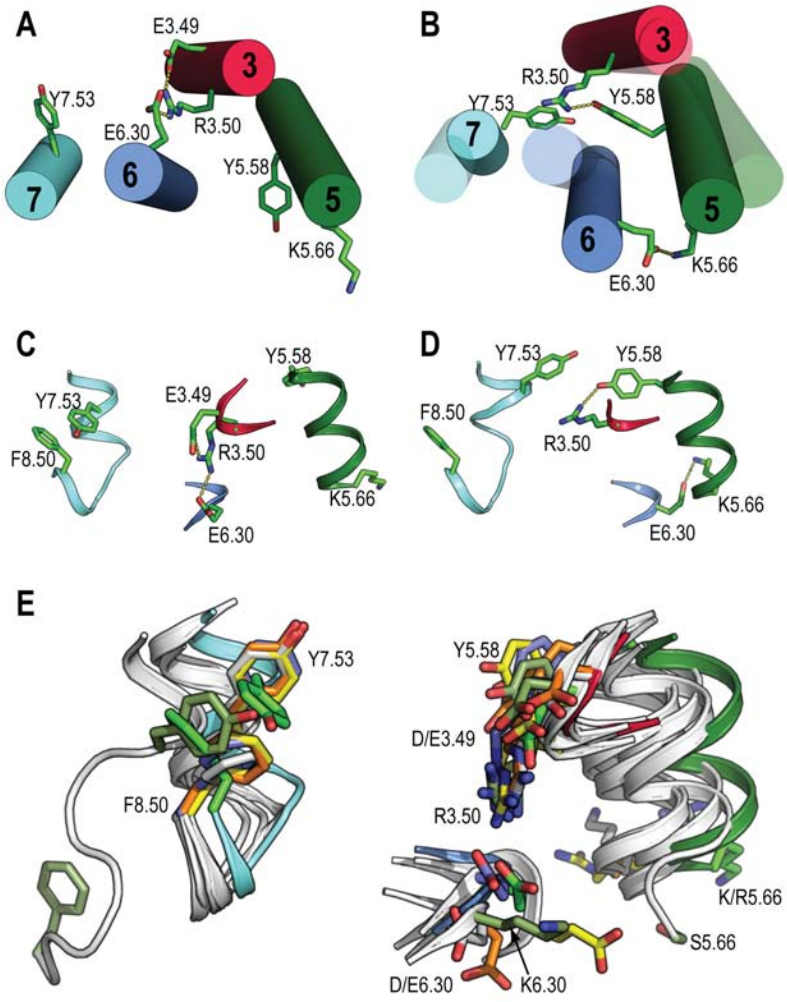

Fig. (3). Comparison of (**A, C**) the crystal structure of inactive rhodopsin (1GZM) with (**B, D**) the crystal structure of the ligand-free opsin (3CAP), which contains several distinctive features of the presumed active state, in views parallel (**C, D**) and perpendicular (**A, B**) to the membrane. Panel B shows the positions of TMs 3, 5-7 in rhodopsin (transparent cylinders) and opsin (opaque cylinders) for comparison purposes. **E**, Comparison of intracellular domains of TMs 3 and 5-7, and key D/E$^{3.49}$, R$^{3.50}$, Y$^{5.58}$, K/R$^{5.66}$, D/E/K$^{6.30}$, Y$^{7.53}$, and F$^{8.50}$ side chains in the currently available crystal structures of GPCRs in the inactive state. The helices of rhodopsin are shown in color while the helices of the other receptors are shown in white. Side chains of rhodopsin are shown in green, of β_2- in orange, of β_1- in white, of A_{2A} in yellow, of D3 in purple, and of CXCR4 in olive. (The color version of the figure is available in the electronic copy of the article).

publication of the opsin structure, these conformational changes disrupt the ionic interaction between R$^{3.50}$ with negatively charged side chains at positions 3.49 in TM3 and 6.30 in TM6 (Figs **3A** and **3C**) and facilitates the interaction between K$^{5.66}$ in TM 5 and E$^{6.30}$ in TM 6 (Fig. **3B** and **3D**).

Figure **3F** shows the comparison of these conserved regions in the currently available crystal structures of GPCRs in the inactive state. The following findings can be summarized from the observed conformational variability of these side chains. The ionic interaction between D/E$^{3.49}$ and R$^{3.50}$, restraining the Arg side chain towards the cytoplasm, is present in all crystal structures. Accordingly, charge-neutralizing mutations of D/E$^{3.49}$ resulted in constitutive activation [47, 54, 77]. This extended conformation of R$^{3.50}$, in the active state, was suggested earlier using site-directed mutagenesis on D$^{3.49}$ [78] and on the hydrophobic side chain at position 6.40 in the vicinity of R$^{3.50}$ [60]. The "ionic lock" interaction between R$^{3.50}$ and D/E$^{6.30}$ in TM 6 is only present in rhodopsin and dopamine D3 receptor. However, mutations weakening this interhelical lock between TMs 3 and 6 lead to constitutive activation, in a variety of GPCRs [77, 79] including the β_2-adrenergic receptor [55]. Dror *et al*., using microsecond-timescale molecular dynamics simulations, have suggested that the inactive β_2-adrenergic receptor alternates between a major conformation, with an ionic lock formed between R$^{3.50}$ and E$^{6.30}$, which brings TMs 3 and 6 close together, and a minor conformation, with the "ionic lock" broken and both helices separated as found in the crystal structure [80]. Importantly, the acidic residue at position 6.30 is only present in 32% of GPCRs [24]. Chemokine receptors, as well as about 34% of class A GPCRs, contain a basic residue at position 6.30. The structure of CXCR4 has shown that although the interaction between R$^{3.50}$ in TM3 and K$^{6.30}$ in TM 6 is not feasible the relative intracellular positions of TMs 3 and 6 in CXCR4 is not very different from those determined in the other crystal structures. Nevertheless, this electrostatic repulsion between both helices seems important for receptor activation since mutation of the positively charged residue in CCR5 to a negatively charged amino acid (R$^{6.30}$D/E), restoring the ionic lock between position 6.30 and R$^{3.50}$, results in an almost silent receptor devoid of constitutive activity [81]. Furthermore, the conformation of Y$^{5.58}$ and Y$^{7.53}$ in TMs 5 and 7, respectively, in all crystal structures is different from rhodopsin. Y$^{5.58}$ points toward the lipid environment in rhodopsin, whereas in the other structures it points toward TM 3. Similarly, Y$^{7.53}$ in rhodopsin hydrogen

bonds the partly conserved $N^{2.40}$ in TM 2 and forms aromatic-aromatic interactions with $F^{8.50}$ in Hx8, whereas $Y^{7.53}$ points towards the protein bundle in the other structures (Fig. **3E**). Remarkably, the conformation of $Y^{5.58}$ and $Y^{7.53}$ in all crystal structures resembles the conformation of active opsin more than inactive rhodopsin. The importance of these aromatic side chains was predicted from mutagenesis studies. Random mutagenesis on the M3 muscarinic receptor showed a conformational link between the highly conserved $D^{2.50}$, $R^{3.50}$, and $Y^{5.58}$ residues [82]. Moreover, in the $5HT_{2C}$ receptor, mutation of $Y^{7.53}$ to all naturally occurring amino acids revealed the presence of three distinct constitutively active receptor phenotypes, a moderate, a high, and a "locked-on" ($Y^{7.53}N$) constitutive activity [83].

Mechanism of Ligand-induced Receptor Activation

The mechanism by which binding of the extracellular ligand triggers these conformational rearrangements near the G-protein binding domain is not fully understood. Fluorescence spectroscopy in the β_2-adrenergic receptor has shown that agonists disrupt the "ionic lock" between TMs 3 and 6 [84]. The disruption of this important molecular switch is necessary, although not sufficient, for full activation of the beta2-AR suggesting that several pathways of activation must exist [84]. In the following paragraphs we detail key findings of this process of ligand-induced receptor activation.

The crystal structures of the β_1-adrenergic receptor bound to antagonists, partial agonists, and full agonists [11, 12] have revealed small structural differences in binding (Fig. **4A**). The hydrogen bond interactions between the protonated secondary amine and $D^{3.32}$ and the β-OH and $N^{7.39}$ are present in all types of ligands. In addition, both antagonists and agonists form a hydrogen bond interaction with $S^{5.42}$. The major difference between the binding of full agonists compared to partial agonists or antagonists is that only full agonists make a hydrogen bond to the side chain of $S^{5.46}$. This results in a contraction of the catecholamine-binding pocket by 1 Å that is associated with receptor activation. Importantly, the role of many of these side chains in ligand binding and receptor activation was previously proposed by extensive mutagenesis [85, 86].

Similarly, the crystal structure of a nanobody-stabilized active state of the β_2-adrenergic receptor bound to BI-167107 [17] shows hydrogen bonding interactions with $S^{5.42}$ and $S^{5.46}$ (Fig. **4B**). These interactions stabilize a receptor conformation that includes a 2.1Å inward movement of TM5 at position 5.46 and 1.4Å inward movement of the conserved $P^{5.50}$ relative to the inactive, carazolol-bound structure. $P^{5.50}$ in TM5 (conserved in 77% of the rhodopsin-like sequences) induces a local opening of the helix (*proline-unwinding*, in contrast to *proline-kink* [87, 88]). This key distortion is stabilized in the known crystal structures by a bulky hydrophobic side chain at position 3.40 (Fig. **4B**), highly conserved in the whole Class A GPCR family. Mutation of $I^{3.40}$ to either Ala or Gly, i.e. removing the bulky side chain at this position, abolishes the constitutive activity of the histamine H_1 receptor, the effect of constitutive-activity increasing mutations, as well as the histamine-induced receptor activation [88]. Therefore, as revealed in the original publication of the nanobody-stabilized active state of the β_2-adrenergic receptor [17], this inward movement of TM5 upon agonist binding destabilizes the packing of $I^{3.40}$ and $P^{5.50}$, contributing to a rotation and outward movement of TM6 and an inward movement of TM7 for receptor activation.

Schwartz *et al*., using engineered GPCRs with metal ion binding sites have proposed the global toggle switch, which is characterized by an inward movement of the extracellular end of TM 6 towards TM 3, simultaneously with an outward motion of the intracellular end of TM 6 towards TM 5 [89]. In this mechanism, the interaction between agonists and TM 6 (mainly a hydrogen bond interaction with the polar side chain at position 6.55) would trigger the active conformation of TM 6.

Active states are also accomplished by the rearrangement of side chains forming different networks of interactions between helices, often named microswitches [90]. The structure of metarhodopsin I by electron crystallography [91], spectroscopic studies of rhodopsin [92], solid-state NMR measurements of metarhodopsin II [93, 94], and computer simulations associated with mutagenesis studies of β_2-adrenergic [56], cannabinoid CB_1 [95], histamine H_1 receptors [59], and serotonin 5-HT_4 receptor [96] have shown that agonist binding might also trigger the toggle switch of $W^{6.48}$ (conserved in 71% of class A GPCRs) of the CWxP(F/Y) motif in TM 6. Importantly, the non-conserved side chain at position 3.36 has also been suggested to act as a toggle switch simultaneously with $W^{6.48}$ [59, 95, 96]. In this mechanism, the side chain at position 3.36 moves away from TM 6 and reorients towards TM 7 while $W^{6.48}$ breaks its water-mediated hydrogen bond interactions with TM 7 and reorients towards TM 5 (Fig. **4C**). Importantly, the structure of opsin has shown that TM 3 rotates anticlockwise (viewed from the extracellular side), facilitating the movement of the 3.36 side chain towards TM 7, and $W^{6.48}$ moves toward TM 5 (Fig. **4C**). Binding of agonists trigger these rotations/movements required for receptor activation through the formation of aromatic-aromatic interactions or specific hydrogen bonds interactions with the side chains at position 3.36 and 6.48 in the active conformation [59, 96, 97]. Moreover, it was proposed that the highly conserved $F^{5.47}$ (70% of rhodopsin-like GPCRs) in TM 5 serves as an aromatic lock for this proposed active conformation of $W^{6.48}$ (Fig. **4C**) [98]. Mutation of either $F^{5.47}$ or $W^{6.48}$ in several GPCRs eliminated their constitutive activation and also impaired agonist-induced signaling.

These processes show how the ligand-encoded extracellular signal is propagated from the binding site into intracellular microdomains known to be important in receptor activation. Specifically, agonist-induced receptor activation *via* $W^{6.48}$ disrupts a conserved hydrogen bond network linking $W^{6.48}$ and $D^{2.50}$ [6, 99-101], triggering the conformational transition of $N^{7.49}$ of the NPxxY motif towards $D^{2.50}$ [58, 102], the conformational transition of $Y^{7.53}$ [8], and ultimately leading to the disruption of the "ionic lock" of $R^{3.50}$ with the adjacent $D/E^{3.49}$ and $D/E^{6.30}$ (see above) (Fig. **4D**).

THE ACTOR AND THE AUDIENCE, "INTERNAL STIMULI"

Like an actor who could perform in front of many different types of audience, even a single GPCR could interact with many different G-proteins and thus stimulating different signaling pathways.

Receptor / G-protein Interaction

The structure of the ligand-free opsin bound to a synthetic peptide derived from the C-terminus of the α-subunit of transducin has recently been obtained [9]. This structure has shown that the α5 helix of $G_{\alpha t}$ binds to a site in opsin that is opened by the movement of the cytoplasmic end of TM 6 away from TM 3 and towards TM 5 (see above). The C-terminal domain of the G protein interacts with the extended conformation of $R^{3.50}$, the short loop connecting TM 7 and Hx8, and the inner side of the cytoplasmic TMs 5 and 6 (Fig. **5**). Notably, both the G protein family (positions i-2 and i-7 relative to the final amino acid) and TMs 5 (positions 5.61 and 5.65) and 6 (position 6.33) of class A GPCRs contain highly conserved hydrophobic amino acids that form key hydrophobic contacts between the receptor and the G protein. Thus it seems reasonable to assume that the mode of recognition of the G protein by the other members of the GPCR family resembles this structure found for opsin.

Receptor / G-protein Interaction is Associated with Conformational Changes that Activate the GPCR

It has been shown that GPCRs change their conformation after interaction with the G-protein, in the absence of either agonists or

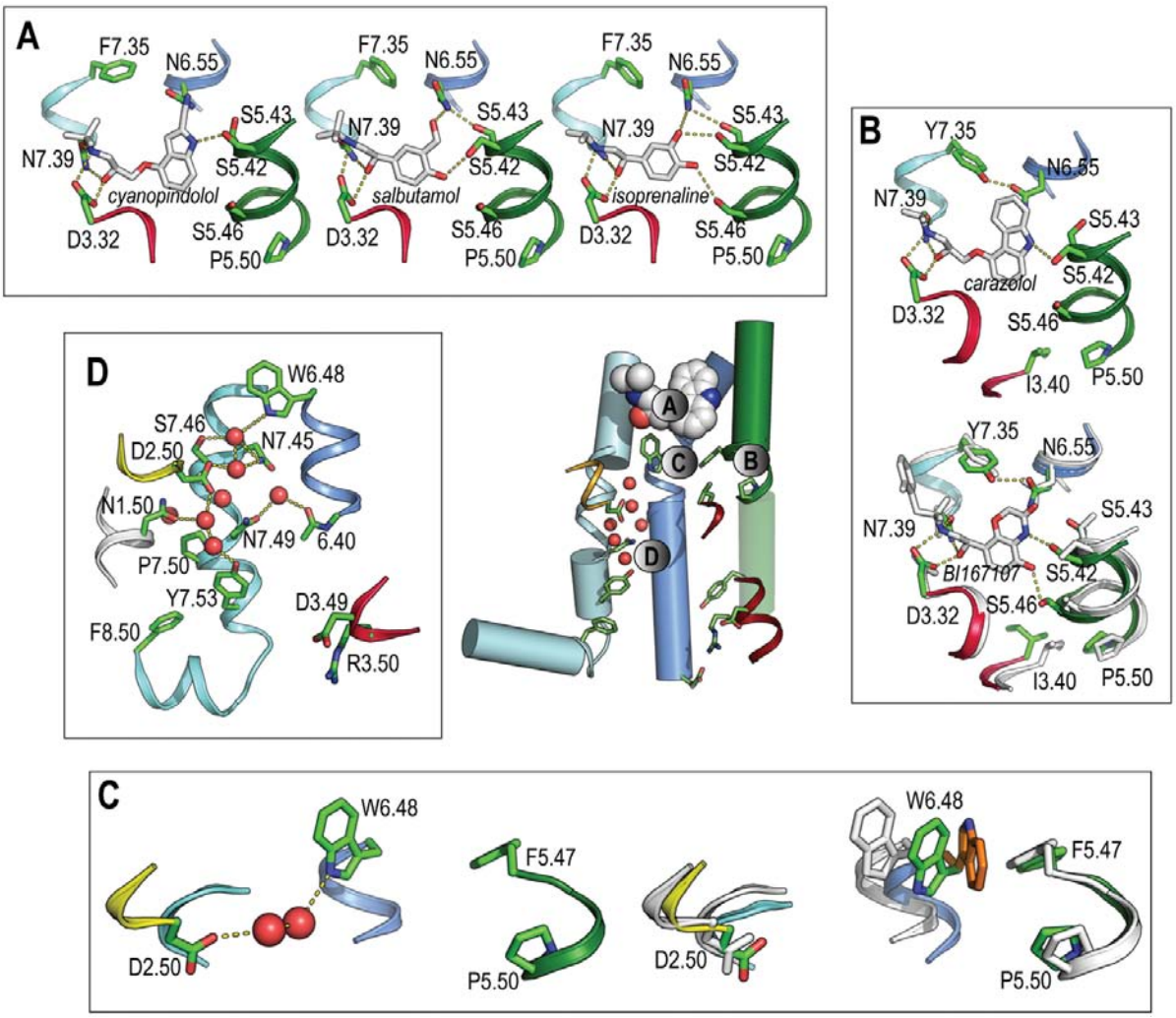

Fig. (4). Pathways of activation in Class A GPCRs. (**A**) Detailed view of the β_1-adrenergic receptor bound to the antagonist cyanopindolol (left panel, PDB id 2VT4), the partial agonist salbutamol (middle panel, 2Y04) and the full agonist isoprenaline (right panel, 2Y03). In contrast to partial agonists or antagonists, full agonists make a hydrogen bond to the side chain of $S^{5.46}$. **B**) Detailed view of the β_2-adrenergic receptor bound to the partial inverse agonist carazolol (top panel, 2RH1) and the full agonist BI-167107 (bottom panel, 3P0G). The hydrogen bond interaction between full agonists and $S^{5.46}$ stabilizes a receptor conformation that includes an inward movement of TM5 relative to the inactive (shown in white in the bottom panel for comparison purposes), carazolol-bound structure. **C**) Detailed view of the rhodopsin crystal structure (left panel, 1GZM) showing the conserved hydrogen bond network linking $D^{2.50}$ and $W^{6.48}$ between TMs 2 and 6; and comparison of the structure of rhodopsin (white, 1GZM), opsin (in green, 3CAP), and a computational model of $W^{6.48}$ (orange) (right panel). Importantly, the structure of opsin has shown that the $W^{6.48}$ side chain moves (green), relative to rhodopsin (white), toward TM 5. $W^{6.48}$ can adopt either the *gauche+* conformation (green) as observed in opsin, or the *trans* conformation (orange) as it has been proposed from site-directed mutagenesis experiments [96]. **D**) Location of highly conserved motifs in class A GPCRs and structural water molecules in their vicinity, involved in receptor activation. (The color version of the figure is available in the electronic copy of the article).

mutations inducing constitutive activity. For instance, fusion of Gs alphaL to the β_2-adrenergic receptor constitutively activate the receptor, as shown by the increased potency and intrinsic activity of partial agonists, increased efficacy of inverse agonists, and increased basal GTPase activity [103]. In addition, fusion of G-alphaS to the β_2-adrenergic receptor provided to it properties representing a unique conformation being in the transition from high- to low-affinity forms rather than properties ascribed to a constitutively activated state [104]. In a different GPCR, the human 5-hydroxytryptamine (5-HT1A) receptor, the findings of a study which fused this receptor with wild-type and pertussis toxin-resistant forms of Gi1 alpha proteins, led to the suggestion that alteration of a single amino acid in the G-protein regulated agonist-independent constitutive activity of this receptor [105]. Similarly, fusion of glucagon receptor with Gs-alpha proteins constitutively activated the receptor, causing elevated basal levels of cAMP even in the absence of glucagon [106]. Moreover, overexpression of G-proteins in cells expressing GPCRs was able to constitutively activate the latter. Specifically, Burstein [107] have demonstrated that overexpression of Gq-alpha proteins induced constitutive activity of the m1, m3, and m5 subtypes of muscarinic receptor.

Direct evidence for the ability of G-proteins to couple and change the conformation of a GPCR in the absence of ligands has been provided by the group of Kobilka [108]. In this elegant study, purified beta2-adrenergic receptor, reconstituted into recombinant HDL particles, was labeled with an environmentally sensitive fluorophore at position $Cys265^{6.27}$, adjacent to the G protein-coupling region of TM6. It was shown that receptor conformational changes (changes in fluorescence) induced by Gs, in the absence of the agonist isoproterenol, were similar to those observed for an isoproterenol-activated receptor in the absence of Gs [108]. These

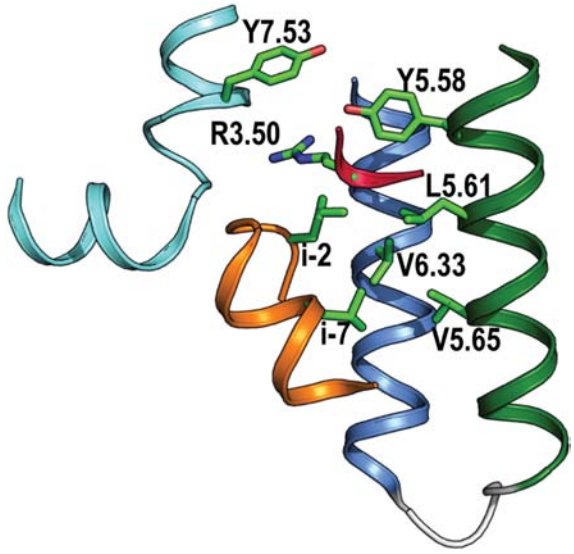

Fig. (5). Crystal structure of the ligand-free opsin in its G-protein-interacting conformation (3DQB). The C-terminal domain of the G protein (in orange) interacts with $R^{3.50}$, the short loop connecting TM7 and Hx8, and the inner side of the cytoplasmic TMs 5 and 6. The color code of TMs is as in Fig. **1**. (The color version of the figure is available in the electronic copy of the article).

results are compatible with the concept that G-proteins form complexes with the receptor, and induce changes in their structure even in the absence of agonists. This process also occurs in living cells as demonstrated by Audet [109]. This is remarkable because the presence of guanine nucleotides in *in-vitro* experiments destabilizes GPCR-G-protein complexes.

GPCRs Adopt Different Conformations After their Interaction with Different G-Proteins

Evidence for G protein-induced changes in GPCR conformation was further provided by a previous study, in which overexpressed different Galpha-subunits (Galpha16 or Galphai2) with the kappa-opioid receptor, displayed different conformational changes of TM6, TM7 and EL2, as revealed by the cysteine-substituted accessibility method [110]. Thus as an actor on stage, who could change positions depending on the type of audience, similarly a single GPCR is able to adopt different conformations when it interacts with different intracellular G-proteins. The ability of different G-proteins to stabilize different conformations of GPCRs was further supported by a study, which used fusion proteins constructed between either Galpha(s) or Galpha(q) and a construct of NK1 neurokinin receptor with a truncated tail [74]. The findings of this study suggested that the heterogeneous pharmacological phenotype displayed by wild type NK1 receptor was a reflection of the occurrence of two active conformations or molecular phenotypes representing complexes with the Galpha(s) and Galpha(q) proteins. In addition, Wenzel-Seifert *et al*. (2000) constructed fusion proteins of the beta2 adrenergic receptor with the long and short splice variants of Galpha(s) proteins, as well as with Galpha(i2), Galpha(i3), Galpha(q) and Galpha(16) and tested their pharmacological properties [111]. The experimental findings of this study revealed substantial differences in the interaction of this receptor with different G-proteins, supporting the existence of receptor-specific G-protein conformational states.

SCENARIO, AUDIENCE AND ACTOR'S POSITIONING: THE PERFORMANCE

Similar to the interrelationship between the nature of the scenario, the type of audience and the positioning of the actor; the nature of ligand, the conformation of receptor and the type of G-protein are tightly associated to one another. In agreement with this concept there is a plethora of different studies suggesting that GPCRs could exist in different active states which are differentially stabilized by different agonists and trigger different signaling pathways [96, 112-123]. Berg *et al*. [124] have proposed that several agonists of the serotonin 5-HT_{2C} receptor activate differentially two signal transduction pathways: the PLC-IP3 and the PLA2-arachidonic acid. Interestingly, these signaling-selective ligands were partial agonists, stimulating the different signaling pathways with different efficacies. For example, lysergic acid diethylamide (d-LSD) barely stimulated (displaying very low efficacy) the IP3 accumulation pathway, whereas it stimulated release of arachidonic acid with considerable efficacy but still lower than the full agonist, serotonin [124]. Similarly the (±)-1-(2,5-dimethoxy-4- iodophenyl)-2-aminopropane (DOI) was a partial agonist in stimulating the IP3 accumulation pathway compared to serotonin. In marked contrast DOI was a full agonist in stimulating the arachidonic release, displacing similar efficacy (maximal effect) with that of serotonin [124]. Conversely, quipazine preferentially activated the IP3 pathway, being almost a full agonist in stimulating this pathway and a partial one in stimulating arachidonic release [124]. Likewise, Eason *et al*. have demonstrated that the alpha2-adrenergic receptors (alpha 2AR) were coupled to Gi and Gs proteins and that different agonists displayed different efficacies for these signaling pathways [125]. Although each compound among those tested in this study was found to be a full agonist for alpha 2AR-Gi coupling, the efficacy of these agonists to elicit alpha 2AR-Gs coupling was markedly different. Thus, oxymetazoline displayed no stimulation of Gs protein-mediated adenylyl cyclase for the alpha 2C4 adrenergic receptor, whereas for this subtype UK-14304 significantly stimulated this pathway.

The extent of stimulation of a GPCR could, therefore, dictate the type of G proteins and the signaling pathways that are activated by this receptor. This is most likely due to the ability of different agonists with different efficacies for a particular GPCR to stabilize different active conformations of the receptor. Triggering specific pathways along the activation process might be an explanation for the observed collateral efficacy of GPCR agonists [126]. In agreement with this concept, a fluorescence lifetime spectroscopy study has suggested that the region around $Cys265^{6.27}$ of the agonist-bound beta2-adrenergic receptor exists in two distinct conformations, and that the conformations induced by a full agonist can be distinguished from those induced by partial agonists [72]. Also, biophysical, pharmacological and molecular modeling approaches

have proposed that the full agonist isoproterenol and the partial agonist salbutamol induce different active states [127]. Similarly, different conformational arrangements occur during stabilization of the active state of the 5-HT_4 receptor [96]. Benzamides have been proposed to stabilize an active state by interacting with $T^{3.36}$, and BIMU8 by interacting with $W^{6.48}$. In contrast, the active state stabilized by the natural agonist serotonin is attained through other routes of activation than those of benzamides and BIMU8.

The ability of different ligands to stabilize different conformations of a receptor has also been observed in other GPCRs. The different binding modes of GnRH I and GnRH II to the GnRH receptor differentially simulate different signaling pathways [128]. Similarly, different active conformations of the angiotensin II type 1 receptor (AT1) are stabilized by different peptide-receptor interactions, which preferentially affect particular receptor-mediated signaling pathways [129]. In addition, a plasmon-waveguide resonance (PWR) spectroscopy study has also proposed that binding of the structurally different cannabinoid agonists CP 55,940 and WIN 55,212-2 leads to different hCB1 receptor conformations [113]. These agonists shifted the PWR spectra in opposite directions and differ in their ability to activate Galpha(i1) proteins.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

GPCRs are disordered allosteric proteins that exhibit modulator behavior with a number of guests in both the extracellular (ligand) and intracellular (G protein) spaces [130]. As an actor, this is only achievable by adopting different positions (conformations) and faces (extracellular and intracellular). This considers GPCRs as monomeric transmembrane receptors that form a ternary complex: a ligand, the GPCR and its associated G protein [131, 132]. Nevertheless, it is now well accepted that many GPCRs have been observed to oligomerize in cells [133-139]. Most likely, an actor alone would perform in a different manner than being on stage with other actors. Similarly, as experimentally demonstrated, the structural and functional properties of GPCRs can be largely affected by its interaction with the same or different GPCRs. It has recently been shown that receptor activation is modulated by allosteric communication between protomers of dopamine class A GPCR dimers [137]. The minimal signaling unit, two receptors and a single G protein, is maximally activated by agonist binding to a single protomer. Inverse agonist binding to the second protomer enhances signaling, whereas agonist binding to the second protomer blunts signaling. Thus, GPCR dimer function can be modulated by the activity state of the second protomer, which for a heterodimer may be altered in pathological states. Therefore, drug development may consider GPCRs, in addition to proteins being able to adopt different active conformations and trigger different signaling pathways, as homo- and hetero-oligomers with different functional properties, opening new opportunities for novel drug discovery [140].

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