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REVIEW

GPCR activation: protonation and membrane potential

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ABSTRACT

GPCR proteins represent the largest family of signaling membrane proteins in eukaryotic cells. Their importance to basic cell biology, human diseases, and pharmaceutical interventions is well established. Many crystal structures of GPCR proteins have been reported in both active and inactive conformations. These data indicate that agonist binding alone is not sufficient to trigger the conformational change of GPCRs necessary for binding of downstream G-proteins, yet other essential factors remain elusive. Based on analysis of available GPCR crystal structures, we identified a potential conformational switch around the conserved Asp2.50, which consistently shows distinct conformations between inactive and active states. Combining the structural information with the current literature, we propose an energy-coupling mechanism, in which the interaction between a charge change of the GPCR protein and the membrane potential of the living cell plays a key role for GPCR activation.

KEYWORDS GPCR, membrane potential, protonation, activation

INTRODUCTION

G-protein coupled receptors (GPCRs) compose the largest family of signaling membrane proteins in eukaryotic cells. By definition, activation of a GPCR protein, upon agonist binding on the extracellular side, results in a state able to interact with cognate guanine nucleotide-binding proteins (G-proteins) on the cytoplasmic side. To date, over 75 three-dimensional (3D) structures of nearly 20 GPCRs have been reported (Rasmussen et al., 2011b; Katritch et al., 2013; Venkatakrishnan et al., 2013), showing a 7-transmembrane helix bundle (TMs 1–7)

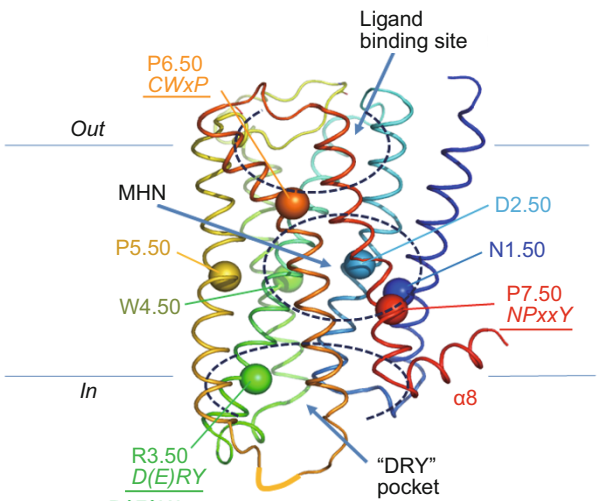


Figure 1. Schematic diagram of a Class-A GPCR protein. Cα-trace of the peptide chain is colored in rainbow, with N-terminus in blue and C-terminus in red. The most conserved position in each TM helix is marked with a sphere and labeled accordingly. Selected regions such as ligand binding site, major hydrogen-bond network (MHN), and G-protein binding site (the "DRY" pocket) are marked. Figures were drawn with the program PyMol.

(Fig. 1). Based on structural comparison of inactive states (also called ground or R states) and active (R*) states of GPCRs, it has been demonstrated that the activation of a GPCR protein involves opening of its cytoplasmic side in order to interact with downstream G proteins and other effectors (Rasmussen et al., 2011b). Activation mechanisms in different GPCRs are believed to share several key features, including movements of TM helices, side-chain micro-switches, and potential rearrangements of a buried solvent cluster (Katritch et al., 2013). In general, GPCRs need at least two sets of conserved intramo-

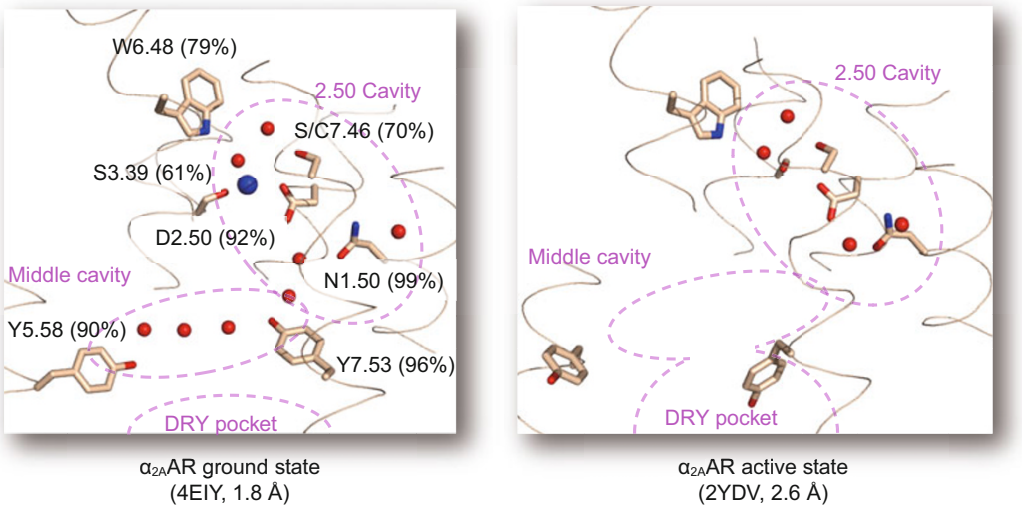
lecular interactions in order to stabilize the ground and active states, respectively. Yet these interactions are unlikely to serve as the driving force for the conformational change because they often cancel each other between the two states. Thus switching between the two states would require a trigger and an additional energy source. Nevertheless, a common mechanism utilized by all GPCRs has not been clearly defined, partially because of the low sequence homology (identity <20%) between GPCR proteins and diversity of their ligands (Katritch et al., 2013). Activation of GPCRs can be induced physiologically by agonist binding, or artificially by introducing constitutively active point mutations. In addition, many factors such as temperature, pH, and lipid molecules of the membrane bilayer have been found to affect the activation of GPCRs (Parkes and Liebman, 1984; Ghanouni et al., 2000; Vogel et al., 2008; Gasperi et al., 2013). Existence of basal activity in many GPCRs suggests that the energy barrier of the transition state can be relatively low, although it should be larger than the thermomotion energy, *RT* (where *R* is the universal gas constant and *T* is absolute temperature). Since a GPCR molecule does not function as a molecular timer similar to some Ras-like small GTPases, the activation process associated with GPCR basal activity is likely to be reversible. In particular, the activation energy is probably less than the energy consumed by a proton-driven transporter in one cycle of substrate transport, which is *ca.* 4 *RT* (≈10 kJ/mol, i.e. the energy gained by a proton moving across a 100 mV membrane potential). An exception is rhodopsin, where the activation energy from a photon can be as high as 80 *RT* (or 200 kJ/mol, at 600 nm wavelength). Furthermore, it has been shown in some GPCRs that membrane potential can modulate activation, and a transient charge movement in the membrane was observed during GPCR activation (Ben-Chaim et al., 2006). However, the energy-coupling mechanism, especially the energy source, of GPCR activation has so far evaded elucidation. In particular, effects of membrane potential, which is a characteristic physical property of cytoplasmic membranes of living cells, have not been widely considered to play part in the activation mechanism of GPCRs.

CONSERVED BURIED HYDROGEN-BOND NETWORK

A movement of electric charges within a membrane-embedded protein would require a path, a source, and a regulation mechanism. The path can be composed of either an ion channel, a network of polar groups, or functional groups that can change their charge statuses (e.g. through protonation) and/or conformations under influence of the electrostatic field of the membrane potential (DeCoursey, 2003).

GPCRs are signaling proteins and thus in general do not possess properties of typical ion channels such as a high transfer rate reaching diffusion limit. Instead, analysis of 3D structures of Class-A GPCRs has revealed a conserved, hydrogen-bond network that is buried in the middle of the transmembrane (TM) region (Palczewski et al., 2000; Cherezov et al., 2007; Roth et al., 2008; Nygaard et al., 2009; Wu et al., 2010; Zhang et al., 2012; Katritch et al., 2013), and existence of a similar hydrogen-bond network has been postulated for Class-C GPCRs as well (Yanagawa et al., 2013). Such an extensive, buried, hydrogen-bond network is unusual for membrane proteins (Illergard et al., 2011) and is likely to be influenced by the membrane potential. Thus, it has been postulated that this conserved network, referred to here as a 'Major Hydrogen-bond Network' (MHN) of GPCRs, could possibly play a role in charge transfer during GPCR activation (Angel et al., 2009). In the following, we use the B-W numbering scheme (Ballesteros and Weinstein, 1995), X.YY, to refer positions of amino acid residues, in which X stands for the TM helix number (i.e. 1–7) and YY is the position relative to the most conserved one (defined as position 50) in the corresponding TM helix. For proteins within the Class-A GPCR group, the MHN is composed of Asn1.50, Asp2.50, Ser3.39, Tyr5.58, Trp6.48 (in the conserved CWxP motif (Healy, 2002)), Asn7.45, Ser7.46, Asn7.49, and Tyr7.53 (in the conserved NPxxY motif) as well as a number of highly conserved, structured, water molecules (Angel et al., 2009; Nygaard et al., 2009; Liu et al., 2012) (Figs. 1 and 2). The existence of water molecules may strongly influence dynamic distribution of the dielectric constant in the interior of the protein thus the micro-environment of the buried polar residues (Nie et al., 2005). In addition, the MHN, which is located in the middle of the TM region, links the ligand binding pocket on the extracellular side to the G-protein binding site (including the conserved D(E)RY motif) on the cytoplasmic side (Venkatakrishnan et al., 2013). The latter is referred to as DRY pocket hereafter. Therefore, the MHN connects nearly all of the most conserved structural elements of GPCRs (Fig. 1). Although it is almost certain that the MHN contributes to the overall thermo-stability of GPCR, maintaining stability is clearly not the only, and possibly not even the major function of such a network. It is reasonable to argue that if it was to fulfill solely such function, a hydrophobic core might be more effective. The major difference between hydrogen-bond interaction and hydrophobic interaction is that the former shows bonding directionality and donor/acceptor saturation while the latter does not. As a consequence, a hydrogen-bond network can alternate between two (or more) constellations that may have similar free-energy levels but distinct geometries, as illustrated in the MHN during GPCR activation (Fig. 2). In addition, some amino acid residues within the MHN are able to change their charge status. Thus, the hydrogen-bond network may potentially provide a path of charge movement (e.g. a Grotthuss proton wire (DeCoursey, 2003)) within the TM core of the GPCR protein.

The MHN can be divided into two cavities. One is around the conserved Asp2.50, termed as 2.50 cavity hereafter; and the other is located between the 2.50 cavity and DRY pocket, thus referred to as middle cavity (Fig. 2). In the inactive state (e.g. in α_{2A} adenosine receptor (α_{2A}AR), PDB ID: 4EIY), the middle cavity has an elongated shape and holds three structured water molecules. It was referred to as TM6-clamp previously (Hulme, 2013). The cavity walls are formed by conserved



α_{2A}AR ground state (4EIY, 1.8 Å)

α_{2A}AR active state (2YDV, 2.6 Å)

Figure 2. Conserved proton wire in the major hydrogen-bond network of α_{2A}AR. Key residues are shown in stick models, and water molecules and Na^+ are shown in red and blue spheres, respectively. For clarity, only components along the major path are shown. Percentage conservativeness of the involved residues is included in parentheses. In the active state (the right panel), the proton wire breaks in the middle, and the side chain hydroxyl group of Ser3.39 replaces a solvent molecule of the inactive state (i.e. the blue sphere in the left panel).

hydrophobic residues L/I/V-2.43 (88%, i.e. 48% + 29% + 11%) (where percentage conservativeness of a given position in all Class-A GPCRs is estimated according to the online database *7TM Alignment Explorer* (Van Durme et al., 2006)), Leu2.46 (95%), L/I-3.43 (89%, i.e. 72% + 17%), and I/L/M/V-3.46 (97%, i.e. 51% + 26% + 17% + 3%) on one side and by L/V/I/A-6.37 (83%, i.e. 31% + 25% + 20% + 7%) and I/V/M/L-6.40 (86%, i.e. 33% + 29% + 13% + 11%) on the opposite side. Conserved Tyr5.58 (90%) and Tyr7.53 (96%) form the two ends of the elongated cavity. This semi-hydrophobic cavity appears energetically unfavorable for water binding, yet it associates with TMs 2, 3, 5, 6, and 7. In addition, it is connected to the more hydrophilic 2.50 cavity through Tyr7.53. In the active state (e.g. in β_2 adrenergic receptor (β_2AR), PDB ID: 3SN6), on the other hand, the middle cavity is deformed because of movements of TM6 and Tyr5.58, two characteristic features of the active conformation; and it becomes connected with the DRY pocket but disconnected from the 2.50 cavity. Releasing of water molecules from the middle cavity to the DRY pocket may correlate with activation of the DRY motif. In a large scale point-mutation screening for ligand-binding variants of neurotensin receptor-1 (NTR-1), of the previously identified most conserved residues in Class-A GPCRs the majorities are shown individually to be non-sensitive to mutations (Schlinkmann et al., 2012). Thus, these conserved residues are not essential for both the folding of GPCRs and formation of the ligand-binding site but are more likely to be important for activation which was not part of the selection pressure in this particular experiment. Exceptions include conserved Pro6.50 (92%), Pro7.50 (98%), short side chain G/T/S/A-1.46 (91%, i.e. 50% + 17% + 13% + 11%), Leu2.46, and I/L/M/V-3.46. These observations suggest that, together with helix packing between TMs 1 and 7, and kinks in TMs 6 and 7, the middle cavity formed by TMs 2 and 3 is essential for GPCR folding and/or stability. Therefore, the middle cavity is likely to contribute to the stability of ground conformation as well as to the activation process.

For residues within the MHN, Asp2.50 is the only conserved acidic residue in the GPCR superfamily, *ca.* 92% being Asp with additional 1.2% substituted by Glu. In fact, Asp2.50 is one of the two most conserved acidic residues in Class-A GPCRs, and the other one is Asp/Glu3.49 in the conserved D(E)RY motif (see below). Being the only conserved titratable residue in the MHN, Asp2.50 is deeply buried inside the TM region, and its side chain rotamor conformation is stabilized by another highly conserved residue, Asn1.50 (99%), through a water bridged hydrogen bond. Asp2.50 is located close to the mass center of the TM core, is beneath the ligand-binding site, but is not directly involved in ligand binding (Rasmussen et al., 2011b). Thus this residue is more likely to be involved in a common mechanism in GPCR activation. Numerous point mutations in a variety of GPCRs have shown a critical role of Asp2.50 in GPCR activation (Strader et al., 1988; Ceresa and Limbird, 1994; Martin et al., 1999; Proulx et al., 2008). For example, a mutation of D2.50N abolishes agonist activation in both A_{T2} receptor (Bihoreau et al., 1993) and PAF receptor (Parent et al., 1996). In some GPCRs, even a substitution as conservative as Glu at this position reduces agonist binding (Ringholm et al., 2004), indicating that compromising the precise geometry of the MHN may critically impair its function. Moreover, both D2.50N and D2.50S point mutations are observed in a type of so-called decoy GPCRs and some virally encoded GPCRs, both of which show defective signaling

functions (Daiyasu et al., 2012). In contrast to the conservative character of the Asp2.50 residue, many other membrane-embedded acidic residues outside of the MHN appear to be tolerant to point mutations, at least in relation to GPCR activation (Ghanouni et al., 2000). Interestingly though, the functional loss of the D2.50N mutation in the α_{2A}AR can be partially rescued with a mutation of N7.49D (Wilson et al., 2001), the side chain of which forms a hydrogen-bond with Asp2.50 in the native protein. The same Asn2.50-Asp7.49 arrangement also occurs naturally (0.6%) in gonadotropin releasing hormone (GnRH) receptors (Zhou et al., 1994), and so does an Asp2.50-Asp7.49 acidic residue pair in *ca.* 10% GPCRs (Stitham et al., 2007). These observations suggest that an acidic residue in the MHN is essential for the activation of this group of GPCRs. In addition, many point mutations of other residues within the MHN interrupt GPCR activation. For example, mutations of S3.39A and Y7.53F in A_{T2}R reduce the activation and/or inhibit G-protein coupling (Marie et al., 1994; Monnot et al., 1996); and a mutation N7.49A of the N-formyl peptide receptor inhibits its signaling (Gripentrog et al., 2000). Moreover, the conserved Asp2.50 and Ser3.39 were observed to bind with Na^+ in the crystal structures of the ground states of the protease-activated receptor-1 (PAR1, PDB ID: 3VW7) (Zhang et al., 2012) and α_{2A}AR/4EIY (Liu et al., 2012), supporting the notion that similar cation binding may affect their activation in the cell membrane (Wilson et al., 2001). Taken together, although the molecular mechanisms behind the involvement of the MHN in GPCR activation are ill-defined at present, MHNs certainly provide the necessary structural elements to explain putative charge binding and movement, allowing for charge-dependent information to transfer across the membrane upon agonist-binding.

PROTONATION IN GPCR ACTIVATION

Protonation has been shown to be important for activation in some GPCRs (Parkes and Liebman, 1984; Ghanouni et al., 2000; Ratnala et al., 2007; Mahalingam et al., 2008; Rodriguez et al., 2011). In general, buried acidic residues (e.g. Asp2.50) are of higher pK_a and become protonated more easily than solvent exposed ones. Besides the potential Asp2.50, protonation of Asp/Glu3.49 in the conserved D(E)RY motif has been reported in rhodopsin activation (Mahalingam et al., 2008) (see below), and ligand protonation is also widely observed (e.g. in Ref. (Ratnala et al., 2007)). A classic example of ligand protonation is the activation of rhodopsin, where light-induced isomerization of the ligand, 11-*cis*-retinal, has been shown to be associated with protonation. An earlier model of rhodopsin activation suggested that a light induced *cis-trans* isomerization of the retinal chromophore triggers the conformational transition of rhodopsin; and later it was shown that protonation of the chromophore precedes to and is required for its isomerization (Eyring and Mathies, 1979). In addition, the retinal isomerization promotes protonation of the protein, e.g. at the Glu3.37-His5.46 pair by reducing their side chain distance (Choe et al., 2011). Furthermore, studies on pH-sensing GPCRs, which exhibit higher activities in lower pH (Ghanouni et al., 2000; Radu et al., 2005), suggest a general mechanism of protonation-based activation (Im, 2005).

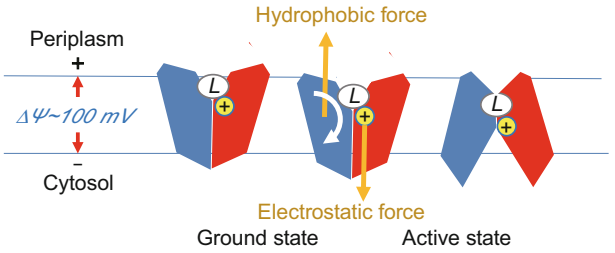


Figure 3. Schematic diagram of GPCR activation taking into account the presence of a negative-inside membrane potential.

MEMBRANE POTENTIAL

In extension to those earlier finding, we ask as to whether it is energetically possible for a protonation in the ligand binding site and/or middle of the TM region to induce a conformational change of the GPCR protein on the cytoplasmic side, thereby achieving activation. A living cell usually has a strong membrane potential across its cytoplasmic membrane (*ca.* 100 mV across 30 Å), with its cytoplasmic side being negatively charged (Fig. 3). Any electric charge-carrying membrane proteins must experience electrostatic forces from this membrane potential. The ground state of a GPCR protein is in fact a balance between the forces of this electric field and other forces such as hydrophobic interactions with the lipid environment. Upon protonation, the GPCR protein experiences an extra electrostatic force; thus, the existing equilibrium is interrupted, and a new equilibrium is to be established. The strength of this electrostatic force for one proton is $F\Delta\Psi/d$ (where F is the Faraday constant, DY is the membrane potential, and d is the thickness of the membrane) which can be as strong as 3×10^{12} N/mol (i.e. 5 pN/proton). (Note that it could be even stronger if a focused electric field is assumed (Tombola et al., 2005)). On the other hand, in addition to non-specific hydrophobic interactions between the GPCR protein and the surrounding lipid bilayer, the forces that hold a GPCR within the membrane usually include interactions of a number of Trp residues with lipid molecules in the water-membrane interface region; a specific interaction of the conserved Trp4.50 (94%, accompanied by conserved N/S/H-2.45 (94%, i.e. 60% + 28% + 6%)) with cholesterol molecules in the middle of the lipid bilayer (Hanson et al., 2008); and interactions of both the C-terminal amphipathic helix α8 and a palmitoylation site with the inner leaflet of the membrane (Shimamura et al., 2008; Standfuss et al., 2011). These (semi-) specific interactions are mainly located on one side of the TM core of the GPCR protein that is composed of TM helices 1, 2, 3, 4, and 7, whereas TMs 5 and 6 are left for less specific protein-membrane interactions. Such a conserved architecture of GPCRs allows TMs 5 and 6 to move relatively independently

from the rest of the protein, particularly when the interaction between the two parts is weakened during activation (see below). The above-mentioned electrostatic force, if applied on the side chain of the protonated Asp2.50, would pass the interface of both TMs 5 and 6 with the rest of the GPCR structure and would point toward the cytoplasmic side of the membrane, resulting in a putative 'inward' shift of the protein. Such a shift would in turn induce a hydrophobic mismatch of the protein relative to the membrane (Mouritsen and Bloom, 1984) and result in unbalanced forces on the protein, producing mechanical torques that could rotate the two parts of the TM core relatively to each other. Therefore, a protonated GPCR protein must seek a new equilibrium, e.g. by moving TM6 as a rigid body (Standfuss et al., 2011) and opening the interface between TM6 and the main body of the GPCR protein, in order to alleviate the hydrophobic mismatch. Taken together, we hypothesize that the conformational change of a GPCR upon activation requires, in general, the negative-inside membrane potential and a change of electric charge.

Since membrane potential is an evolutionarily highly conserved property of the cellular membrane, it is reasonable to argue that signaling proteins such as GPCRs are likely to take advantages of the membrane potential in their functions, as do membrane proteins involved in energy metabolism and transporting (Jiang et al., 2013). In fact, membrane potential-driven conformational changes have been observed in voltage-gating ion channels, and some TM helices of the voltage-sensing domain (e.g. the helix S4 in Kv channels) are estimated to move across half of the lipid bilayer upon voltage inverse (Tombola et al., 2005). Additionally, although some *in vitro* GPCR functional assays have been reported, the activation kinetics is usually significantly slower in isolated membranes or reconstituted systems than in intact cells (Lohse et al., 2007, 2008), suggesting that membrane components in addition to the lipid bilayer may play important roles in GPCR activation. Moreover, in molecular dynamic (MD) simulation of GPCR activation, agonist-bound active conformations almost always revert back to an inactive one (Taddese et al., 2013). Based on the principle of micro-reversibility, it was believed that such a result infers the nature of the activation process. However, since membrane potential is missing in most current MD simulations, such a result may also suggest that an extra energy source from an electrostatic interaction between the GPCR protein and membrane potential is required for the activation to proceed in the forward direction. Future MD analyses that take electrostatic membrane potential into account may be useful to address these questions.

Besides triggering a conformational change upon a charge variation, membrane potential also influences orientation of TM helices during folding (von Heijne, 1989) and perhaps adjusts the equilibrium position of a charge-carrying membrane protein. Charge distribution can be altered by either ligand binding or point mutations; and the membrane potential itself may naturally vary, depending on the type and the status of the cell. For instance, from patients of the hypo-gonadotropic hypogonadism disease, 14 of the 21 reported mutants in the related GnRH receptor involve change of charge of single amino acid residues (Conn et al., 2007). Moreover, the charge-variation mutant N7.49D in α_{2A}AR results in profound conformational instability and more rapid internalization of the receptor from the surface of cells, whereas receptor activation and allosteric modulation properties of this mutant remain similar to the wild-type one (Wilson et al., 2001). Interestingly, the loss-function mutant D2.50N in M3 muscarinic acetylcholine receptor can be rescued by either R3.50M, R3.50W, or Y5.58D (Li et al., 2005). Individually, none of these secondary mutations alone are active. Arg3.50 and Tyr5.58 are close to each other in active conformations of known GPCR 3D structures (e.g. with a distance of 4.7 Å in β_2AR/3SN6) but 12-Å away from Asp2.50. One possible explanation for a secondary mutation to rescue D2.50N from such a long distance is the following. Each of these secondary mutations presumably introduces a net change of −1 electric charge. Thus, they would shift the equilibrium position of the M3 receptor towards the extracellular direction under the influence of a negative-inside membrane potential and thus would possibly compensate the effect of the D2.50N mutation which contains a net change of +1 electric charge comparing with the deprotonation status of the native Asp2.50. In general, by regulating the equilibrium position of a GPCR protein, membrane potential can affect the status of the protein.

In addition, it should also be noted that effects of membrane potential on GPCR-G protein coupling are likely to be complicated. For example, depolarization of the membrane potential reduces both binding of a positively charged acetylcholine and G-protein coupling to M2 receptors (consistent with our prediction) but enhances the G-protein coupling for M1 receptors, albeit the ligand binding sites are conserved between the two receptors. This discrepancy was attributed to their distinct G-protein binding modes, which might be affected by the membrane potential (Mahaut-Smith et al., 2008). It might also be explained by differences in the shift of their equilibrium positions upon voltage changes.

A PUTATIVE CONFORMATIONAL SWITCH AT ASP2.50

To identify a possible mechanism of ligand-induced protonation of Asp2.50, we analyzed available crystal structures of GPCRs. When comparing the β_2AR active (PDB ID: 3SN6) and inactive (2RH1) structures, we found that, roughly speaking, TMs 3 and 4 maintain one rigid body and that TMs 1, 2, and 7 (N-terminal 2/3) maintain another during the conformational change of activation. In addition, the MHN resides near the interface between the two rigid bodies, facilitating conformational changes between them. Moreover, both TMs 3 and 7 are involved in ligand binding in almost all reported GPCR structures (Venkatakrishnan et al., 2013), thus the two corresponding 'rigid bodies' may sense ligand binding and respond to it through a relative movement. Indeed, TM3 moves towards TM2 upon agonist binding (Lebon et al., 2012; Hulme, 2013). In particular,

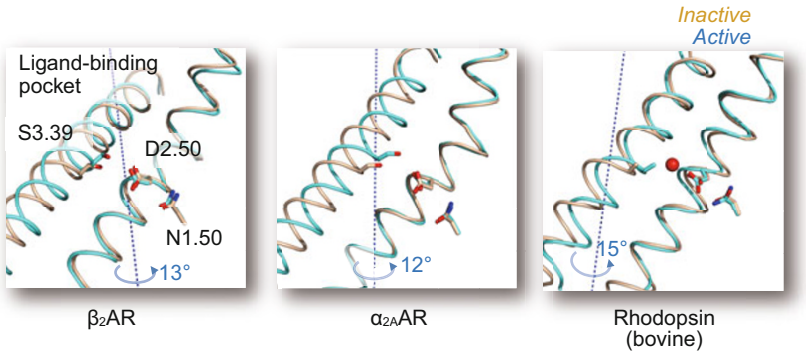


Figure 4. A conformational switch at Asp2.50. Structures of antagonist-bound (colored in wheat) and agonist-bound (in cyan) conformations of β_2AR (2RH1:3SN6), α_{2A}AR (4EIY:3QAK), and bovine rhodopsin (1F88:3PQR) are compared. All three structure pairs show a consistent shift of Ser/Ala3.39 in TM3 relative to Asp2.50 in TM2. The corresponding rotation axes are shown as dotted blue lines.

from the point of view of TM2, the rigid body of TMs 3 and 4 rotates *ca.*13° about an axis roughly parallel to the normal of the membrane plane (Fig. 4). More importantly, this conformational change allows Ser3.39 to be positioned closer to the side chain of Asp2.50 in the agonist-binding structure than in the antagonist-binding structure. In the inactive state, Ser3.39 forms a water-mediated hydrogen bond with Asp2.50; yet in the active state, the hydroxyl group of Ser3.39 side chain replaces the solvent molecule and forms a direct hydrogen bond with the Asp2.50 side chain. Similar relative movements between TMs 2 and 3 have been observed in other GPCR structures as well (Fig. 4). Table 1 lists the distances between Asp2.50 and Ser3.39 side chains calculated from 28 crystal structures of 17 non-rhodopsin GPCRs, including all agonist-bound structures as well as all unique GPCR structures in the current literature. All 8 structures that were assigned by the original researchers as either active or partially active possess a direct hydrogen bond between Asp2.50 and Ser3.39 (bond length ranging from 2.3–3.2 Å, average 2.9 Å). In contrast, in all inactive structures, the distance ranges between 3.4–4.8 Å (average 4.2 Å). Although the residue at the position 3.39 is alanine in bovine rhodopsin, a similar movement between Asp2.50 and TM3 upon activation is also observed. The distance from the side-chain tip of Asp2.50 to the Cβ atom of Ala3.39 decreases from 6.3 to *ca.* 4.2 Å upon activation (Fig. 4 and Table 1). In active bovine rhodopsin structures, a water molecule is observed at the position of the hydroxyl group of the Ser3.39 side chain of other active GPCR structures. Interestingly, squid rhodopsin possesses a Ser residue at the position 3.39. Although its crystal structure (PDB ID: 2Z73) was assigned to represent the inactive state (Murakami and Kouyama, 2008), the Asp2.50-Ser3.39 pair clearly forms a direct hydrogen bond, with a 2.6-Å distance. It suggests that the crystal structure of this detergent-solubilized rhodopsin may present an intermediate state. In fact, the ligand retinal molecule in this crystal structure assumes a less distorted configuration than in bovine rhodopsin structures, although no other sign of activation was observed (Murakami and Kouyama, 2008). Therefore, it seems that the information of agonist binding is transferred from the ligand binding pocket to the conserved Asp2.50 through a relative movement between TMs 2 and 3. As a consequence of the movement, some solvent molecules must be squeezed out of the central solvent cavity (particularly the 2.50 cavity) upon activation (Liu et al., 2012), and the micro-environment of Asp2.50 changes (Fig. 4). Taken together, the relative movement between TMs 2 and 3 that is coupled with agonist binding appears to be a necessary step for GPCR activation, and the distance between Asp2.50 and Ser3.39 may provide a useful structural criteria to distinguish an active state from an inactive state of a GPCR protein.

Asp2.50 is the most probable candidate for a change of protonation status during GPCR activation. As discussed earlier, Asp2.50 is the only conserved acidic residue, thus titratable, in the MHN. In general, buried acidic residues in the TM region are likely to be neutralized in order to minimize the energy cost for protein folding (which may be as high as ~15 kJ/mol (Wimley et al., 1996)). Exceptions may include those residues in the middle of a proton wire (or a solvent channel), where protonation may be dynamic (Nie et al., 2005). Since Asp2.50 is located right beneath the ligand-binding pocket, extracellular solution may serve as the proton source to titrate Asp2.50. Hypothetically, it is possible that Asp2.50 maintains deprotonation during the activation (i.e. requiring a negatively charged residue). If this was true, however, re-shuffling of polar residues among the central cluster of polar residues should be observed more often during evolution, provided that a net negative charge was maintained. Another possibility might be that Asp2.50 maintained its protonation status, which was suggested by an early FTIR-difference spectra experiment on rhodopsin (Fahmy et al., 1993). However, if a protonated Asp2.50 was the only status required for this residue during the entire activation process, a D2.50N substitution might fulfill such a function. Note that rhodopsin is special in that, unlike most GPCRs, the D2.50N mutation does not abolish the ability of rhodopsin to activate its downstream G-protein (i.e. transducin) (Fahmy et al., 1993). Therefore, we attempt to argue that a change of the protonation status of Asp2.50 is essential for the

Table 1. Distance variation between Asp2.50 and Ser3.39

Names	PDB IDs	Originally assigned states	Ligands	Resolution (Å)	D2.50-S3.39 (Å)	D2.50-Cb @3.39 (Å)	References
α_{2A}AR	3EML	R	Antagonist	2.6	4.6		Jaakola et al., 2008
	4EIY	R	Antagonist	1.8	4.4		Liu et al., 2012
	2YDO	R* intermediate	Agonist	3.0	3.0		Lebon et al., 2011
	2YDV	R* intermediate	Agonist	2.6	3.0		Lebon et al., 2011
	3QAK	R* intermediate	Agonist	2.7	3.1		Xu et al., 2011
β_1AR	2VT4 (turkey)	R	Antagonist	2.7	4.4		Warne et al., 2008
β_2AR	2Y03	R	Agonist	2.9	4.2		Warne et al., 2011
	2Y02	R	Agonist	2.6	4.4		Warne et al., 2011
	2Y00	R	Partial agonist	2.5	4.5		Warne et al., 2011
	2Y04	R	Partial agonist	3.1	4.4		Warne et al., 2011
	2RH1	R	Partial inverse agonist	2.4	4.1		Cherezov et al., 2007
	3PDS	R	Covalent agonist	3.5	4.0		Rosenbaum et al., 2011
	3P0G	R*	Agonist	3.5	3.2		Rosenbaum et al., 2011
	3SN6	R*	Agonist	3.2	3.0		Rasmussen et al., 2011b
NTR1	4GRV	R* intermediate	Agonist	2.8	3.0		White et al., 2012
κOR	4DJH	R	Antagonist	2.9	4.0		Wu et al., 2012
μOR	4DKL	R	Antagonist	2.8	3.9		Manglik et al., 2012
δOR	4EJ4	R	Antagonist	3.4	3.4		Granier et al., 2012
NOP	4EA3	R	Antagonist	3.0	4.7		Thompson et al., 2012
D3R	3PBL	R	Antagonist	2.9	3.7		Chien et al., 2010
CXCR4	3ODU	R	Antagonist	2.5	3.8		Wu et al., 2010
H1R	3RZE	R	Antagonist	3.1	3.4		Shimamura et al., 2011
S1P1R	3V2Y	R	Antagonist	2.8	3.5		Hanson et al., 2012
M2R	3UON	R	Antagonist	3.0	4.5		Haga et al., 2012
M3R	4DAJ	R	Antagonist	3.4	4.7		Kruse et al., 2012
PAR1	3VW7	R	Antagonist	2.2	4.0		Zhang et al., 2012
$5HT_{1B}$R	4IAR	R* intermediate	Agonist	2.7	2.7		Wang et al., 2013
$5HT_{2B}$R	4IB4	R* intermediate	Agonist	2.7	2.3		Wacker et al., 2013
Opsin	1F88	R	Covalent antagonist	2.8		6.3	Palczewski et al., 2000
	2X72	R*	Agonist	3.0		4.1	Standfuss et al., 2011
	3PQR	R*	Covalent agonist	2.9		4.3	Choe et al., 2011
	3PXO	R*	Covalent agonist	3.0		4.2	Choe et al., 2011
	3DQB	R*	None	3.2		4.2	Standfuss et al., 2011
	3CAP	R*	None	2.9		4.2	Park et al., 2008
	2Z73 (squid)	R(*)	Covalent antagonist	2.5	2.6	3.8	Murakami and Kouyama, 2008

activation of non-rhodopsin GPCRs, and a tightly regulated micro-environment is critical for the change.

Ser3.39 is the only residue that is both located in the vicinity of Asp2.50 and observed consistently to change its distance from Asp2.50 during GPCR activation at least in all non-rhodopsin GPCR crystal structures currently available. In addition, the side chain of Ser7.46 (51% with additional 19% Cys) maintains a direct hydrogen bond with Asp2.50 from the extracellular direction in both the ground and active states. Together, such an Asp-Ser/Cys network centered at Asp2.50 may facilitate changes of the protonation status of the latter. In particular, protonation of the Asp residue by extracting a proton from a neighboring Ser/Cys residue (e.g. 7.46) would in turn make the nearby Ser/Cys residue to become nucleophilic (i.e. a proton acceptor) to attract to a proton from the environment. Similar Asp-Ser or Asp-Cys pairs have been found at the active sites in some enzymes (Dessen et al., 1999; Mixcoha et al., 2012), indicating proton transfer ability of such pairs. Structural

and/or functional roles of such pairs have also been implicated in membrane proteins. For example, in the ground state of bacteriorhodopsin, which is a 7-TM helix, light-driven, proton pump, there are only three protonated acidic residues, Asp96, Asp115, and Glu194, being both suggested by theoretical calculations and demonstrated experimentally (Spassov et al., 2001). In the crystal structure of bacteriorhodopsin (e.g. PDB ID: 2NTU at 1.53-Å resolution), Asp96 and Asp115 are buried in the TM region, while Glu194 forms an acidic residue pair with Asp204 near the extracellular surface. In particular, Asp96 and Asp115 are accompanied by Thr46 and Thr90, respectively. Being accompanied by a Thr or Ser residue is not uncommon for acidic residues buried in the TM region of a membrane protein. One role of such accompanying Thr/Ser residues may be to facilitate the acidic residue to become protonated, thus neutralizing the energetically unfavorable buried charge (Wimley et al., 1996; Madathil and Fahmy, 2009) in addition to forming a hydrogen bond with the protonated acidic residue. Theoretical calculation on bacteriorhodopsin has shown that pK_a of a buried acidic residue can be dramatically changed (up to 5.5 pH units) by varying its distance from a nearby Ser/Thr residue (Onufriev et al., 2003). In the case of GPCRs, by replacing more freely orientated water molecules with the polar side chain of Ser3.39, the apparent dielectric constant of the micro-environment of Asp2.50 decreases, thus favoring protonation (Nie et al., 2005). It is noted that unlike the conservative nature of Asp2.50, more than one third of GPCR proteins do not have a Ser/Thr residue at the position 3.39. Instead, substitutions at the position 3.39 mainly include Gly, Ala, or acidic residues (e.g. Glu) (Montaner et al., 2013). Among these substitutions, the first two are short side chain residues, which allow a water molecule to replace the side chain hydroxyl group of the more conserved Ser residue thus maintaining the Ser-like function on Asp2.50 to some extent, as observed in the crystal structure of active bovine rhodopsin. In addition, according to the website *7TM Alignment Explorer* (Van Durme et al., 2006), 53 out of 293 Gly3.39-containing variants and 105 out of 122 Ala3.39-containing variants are actually a variety of rhodopsin proteins, which may not require Asp2.50 for their functions (Fahmy et al., 1993). In case of an acidic residue at the position 3.39, on the other hand, a direct hydrogen bond with Asp2.50 would favor protonation of the acidic residue pair. In addition, hydrophobic substitutions to Leu or Val at the position 3.39, accompanied by mutations at Asp2.50, are found in virally encoded, constitutively active GPCRs (Montaner et al., 2013). Taken together, Ser3.39 is likely to play an important role in GPCR activation by changing the micro-environment of Asp2.50.

Although the above hypothesis that Asp2.50 becomes protonated upon GPCR activation appears attractive to us, another possibility exists in which the relative movement between TMs 2 and 3 facilitates deprotonation of a pre-protonated Asp2.50 upon activation. Under the influence of a negative-inside membrane potential, the released proton would be transferred through the MHN to the cytoplasmic side, e.g. to the acidic residue at the position 3.49 (94%) of the conserved D(E)RY motif or the partially conserved 'ionic-lock' between Arg3.50 and Glu6.30 (37%) (Ballesteros et al., 2001) which stabilizes the inactive state (see below). Consistent with such a possibility, a molecular dynamics study suggested that deprotonation of Asp2.50 is correlated with dissociation of the 'ionic-lock' (Vanni et al., 2010). This possibility is also consistent with observations that either the D2.50N mutation (non-titratable) or Na^+ binding to Asp2.50 (i.e. a positive charge non-movable along a Grotthuss proton wire) favors the inactive/ground state. Nevertheless, with this possibility, the usage of the membrane electric potential would not be via a direct force on the protonated GPCR structure; instead, some of the electrostatic energy gained from proton movement along the electric field needs to be converted through a dynamic form to mechanical energy to be used for the conformational change of activation. As the proton released from deprotonation of Asp2.50 on the high energy side of the focused electric field pushes its way to the negative side of the electric field, conformational changes may occur that open the cytoplasmic side of the GPCR molecule. Further, by filling the ligand binding pocket with an agonist and opening the cytoplasmic side of the GPCR, the focused electric field shifts towards the extracellular direction. In this process, the energy gained by the proton across the field would roughly be 4 *RT* (≈10 kJ/mol). Remaining questions about detailed mechanisms warrant further studies to elucidate the precise protonation status of Asp2.50 during the GPCR activation process, e.g. using FTIR spectroscopy techniques (Nie et al., 2005).

PROTONATION SWITCH OF THE DRY MOTIF

Activation of rhodopsin from an intermediate (the Meta I) state to the active Meta II state is pH dependent. Particularly rhodopsin becomes inactive above pH 7.0 (Matthews et al., 1963; Vogel et al., 2008). Two protonation switches have been identified in rhodopsin and are found to be partially uncoupled (Mahalingam et al., 2008). One is located in the Schiff-base of the chromophore ligand buried in the middle of the TM core, which is unique for rhodopsin. This protonation switch probably plays a role similar to that of Asp2.50 in non-rhodopsin GPCRs. The second protonation switch is the D(E)RY motif on the cytoplasmic side of the TM core, which is conserved during evolution of Class-A GPCRs. Similarly, the DRY motif and another putative protonation site have been postulated to be essential for the pH-dependent activation of β_2AR (Ghanouni et al., 2000).

In the ERY motif of rhodopsin, Glu3.49 is directly responsible for the proton uptake during activation (Mahalingam et al., 2008). In the ground state, deprotonated Glu3.49 forms an intra-helix salt-bridge bond with Arg3.50. Once Glu3.49 is protonated, the salt-bridge is weakened, and the local active conformation including the deformation of the middle cavity becomes energetically favored (Mahalingam et al., 2008; Vogel et al., 2008). Neutralizing mutations at Asp/Glu3.49, e.g. E3.49Q in rhodopsin, D3.49N in β_2AR, and D3.49A in α_{1B}AR, shift the corresponding GPCRs to the active conformation (Scheer et al., 1996; Ballesteros et al., 2001; Vogel et al., 2008). Thus, the

effects of protonation of Asp/Glu3.49 on GPCR activation are largely energetic rather than structural. Similarly, a mutation of R3.50L shifts rhodopsin to the active conformation and makes the activation to become pH-independent (Vogel et al., 2008). These observations suggest that the strength of the salt-bridge bond but not the net charge change at this site plays a key role in opening the GPCR cytoplasmic part. This is consistent with the fact that the D(E)RY motif is located on the cytoplasmic side thus being less affected by the membrane potential. A mechanism of coupling the breaking of the Asp/Glu3.49-Arg3.50 salt-bridge bond with GPCR activation is likely to be shared by most GPCRs, yet remains to be unveiled. One possibility is that the salt-bridge bond constrains the two side chains in a spring-like, high energy state; and by eliminating the salt-bridge bond, the local conformational energy is released to contribute partially to the formation of the G-protein binding site. In fact, in nearly all GPCR crystal structures of inactive conformations, the Arg3.50 assumes a side chain conformation of high energy χ_2 torsion angle, while in the fully activated β_2AR/3SN6 crystal structure Arg3.50 assumes an extended side chain conformation. Thus protonation of Asp/Glu3.49 may serve as a trigger to convert the D(E)RY switch.

Another general question about functions of the D(E)RY motif concerns how agonist binding on the extracellular side of the TM core triggers the protonation of Asp/Glu3.49 on the cytoplasmic side. Beside the above-mentioned, putative mechanism of proton transfer from Asp2.50, the following probability exists as well. The conserved D(E)RY motif is "strategically" located in the membrane-cytosol interface (Periole et al., 2004). In general, acidic residues are the most potent residues in sensing the termination positions of TM helices (Krishnakumar and London, 2007). In the 1.8-Å resolution crystal structure of inactive α_{2A}AR (PDB ID:4EIY), Asp3.49 is located in vicinity of the carboxyl head group of a fatty acid molecule, suggesting existence of a similar interaction in the lipid-cytosol interface when the protein is embedded in the cell membrane. Consistently, lipid molecules in the membrane bilayer are found to have a strong effect on protonation of Asp/Glu3.49 (Vogel et al., 2008; Madathil and Fahmy, 2009). In particular, the pK_a of the side-chain carboxyl group of Glu3.49 may critically depend on the lipid-protein interface (Periole et al., 2004). In addition, in *ca.* 38% GPCRs the position 2.38 is occupied by a Thr residue which is located in the vicinity of Asp/Glu3.49 in the ground state (e.g. in α_{2A}AR/4EIY). Therefore, it is probable that, upon agonist binding on the extracellular side, the TM3 movement relative to the membrane as well as to TM2 changes the microenvironment of Asp/Glu3.49 on the cytoplasmic side as it does to Asp2.50. Such a movement might couple agonist binding to protonation of the D(E)RY motif, and the latter might in turn cause structural re-equilibration at the protein-lipid interface in favoring the activation.

LIGAND BINDING

Ligand binding induces conformational change in the GPCR protein, including the above-mentioned movement of TM3 relative to both TM2 and the membrane. However, this structural change does not necessarily result in transition from the ground state to a fully active state. For instance, to obtain the crystal structure of a GPCR in its fully activated conformation, the presence of both an agonist on the extracellular side and a G-protein (alternatively, either its key structural elements or antibodies directed specifically against the activated conformation) on the cytoplasmic side is required (Scheerer et al., 2008; Rasmussen et al., 2011a, 2011b). Several available crystal structures of agonist-bound GPCRs show either partially activated, or essentially ground-state conformations (Warne et al., 2011; Xu et al., 2011; White et al., 2012). One probable explanation for such bias in conformation is the lack of an intact membrane in the crystallization experiment. It is widely believed that crystal structures are snapshots of the corresponding proteins in their functional states. While this might hold true for soluble proteins, some bias towards particular, even partially artificial conformation might be present in structures of detergent-solubilized membrane proteins. In fact, while they are essential for our understanding of ligand functions, crystal structures of ligand-bound GPCRs only reflect the low energy states of the complexes acquired under the specific crystallization conditions. They do not, however, necessarily mirror the low energy state of the protein when embedded in a membrane that bears an electrostatic potential.

Rather than providing the activation energy *per se*, ligand binding is likely to play multiple roles in the process that leads to GPCR activation. First, agonist binding may promote a contraction of the ligand binding pocket in some GPCRs (Nygaard et al., 2009; Lebon et al., 2011), thus favoring the relative movement between TMs 2 and 3 in activation. It is to note, however, that the binding affinities of GPCR agonists vary by at least 6 magnitudes (with pK_i ranging between 3 and 9), and many GPCR agonists have low binding affinities (Niedernberg et al., 2003; Smith, 2012), indicating that a high binding energy may not be a universal trigger for GPCR activation. In fact, in rhodopsin, the antagonist 11-*cis*-retinal is more rigid than the agonist all-*trans*-retinal, and maintaining the active conformation does not necessarily require the binding of an agonist (Choe et al., 2011). It appears that the activation process of rhodopsin is achieved simply by removing constrains imposed by the antagonist. In addition, structural differences between an agonist and an inverse agonist for a given GPCR protein can be subtle in geometry (Miura et al., 2012), thus it may not be straight forward to explain their functional differences simply by comparing their geometries. According to our hypothesis of the Asp2.50 switch, inducing a proper relative movement between TMs 2 and 3 is what an agonist must do in order to activate a non-rhodopsin GPCR protein.

Secondly, some ligands themselves carry electric charges, thus creating a structure point to apply an electrostatic force. For example, most agonists for dopaminergic GPCRs are found to contain one or more basic nitrogen groups that are positively charged under physiological conditions (Selent et al.,

2010). Interestingly, an analysis of pK_a distribution of pharmaceutical drugs showed that (positively charged) amine groups are mostly present in ligands that target GPCRs (Manallack, 2008). Moreover, a study on structural differences of a group of ligands for $A_{T2}R$ showed a trend that negatively charged ligands exert more inhibitory effects than neutral but otherwise structurally similar ones. This charge effect seems independent from binding affinity (Miura et al., 2012). This observation may be explained by altered equilibrium positions of the ligand-GPCR complexes in the membrane. Nevertheless, some GPCRs do have negatively charged agonists. In principle, a negatively charged ligand can be accompanied by more neutralizing cations to achieve net positive charge binding during activation. In addition, for some agonists of negative charges (e.g. glutamate), ligand-binding and GPCR activation can be separated in different domains (e.g. in Class-C of GPCRs), and the activation signal from the ligand binding in an extracellular domain indirectly triggers the conformational change of the TM domain (Jones et al., 1998).

Thirdly, ligand binding may alter the so-called focused electric field by expelling solvent molecules from the deep, ligand-binding pocket, therefore decreasing the dielectric constant. Consequently, ligand binding may stimulate GPCR protonation by manipulating the micro-environment of residues to be protonated. Taken together, agonist binding more likely functions as a latch opener instead of a major energy source for the GPCR activation, particularly for the large conformational change on the cytoplasmic side.

Similar to ligand binding, protonation of the GPCR protein alone may not be sufficient for activation. The above-mentioned D2.50N mutations would be equivalent to a protonated Asp residue. Yet, in some of these GPCR variants, the agonist-induced activation appears to be inhibited but the basal activity increases slightly (Shenker, 1995; Wilson et al., 2001). This could be explained by an altered equilibrium position of the ground state in the mutant GPCR protein, unfavorable for agonist binding. Moreover, Asp2.50 assumes another important role in Na^+ inhibition. In an *in situ* (in membrane) agonist binding experiment, α_{2A}AR is shown to be inhibited by Na^+ at 100 mmol/L level, while D2.50N-containing variants become insensitive to Na^+ inhibition (Wilson et al., 2001). This observation indicates that Asp2.50 is deprotonated in the ground state, and this status is essential for Na^+ inhibition. It is also consistent with the observation that lowering pH facilitates for β_2AR activation (Ghanouni et al., 2000). There appears a positive correlation between the basal activity and resistance to Na^+ inhibition (Wilson et al., 2001). Residues that are involved in this Na^+ binding are conserved in GPCRs (Liu et al., 2012), and physiological Na^+ concentration on the extracellular side is at the level of *ca.* 100 mmol/L or higher in general (Page and Di Cera, 2006). Therefore, Na^+ inhibition may be a common phenomenon for GPCRs in order to improve signal to noise ratio. As most D2.50N mutations show lower agonist affinity and become less active in response to agonist (Wilson et al., 2001), eliminating the Na^+ inhibition by protonation of Asp2.50 appears necessary but not sufficient for GPCR activation.

CONCLUSION MARKS

The energy source for large conformational changes associated with activation is a fundamental question in the GPCR field. Membrane potential is a conserved physical property of cell membrane, thus likely being used by signal transduction membrane proteins including GPCRs.

Membrane potential affects the equilibrium position of a charged membrane protein, and such an equilibrium position in turn determines the status of that protein, including its ligand-binding specificity and affinity, transition state energy barrier, and interactions with downstream effectors. Both a charge variation of the membrane protein and a change in the membrane potential can alter the equilibrium position, namely by re-balancing hydrophobic (mismatch) forces with electrostatic forces.

Protonation is one of the common mechanisms to change electric status of a membrane protein. GPCRs contain a conserved MHN, and Asp2.50 in MHN is one of the two most conserved titratable residues in GPCR. Asp2.50 is most likely to change its protonation status during the activation process. In particular, agonist binding could change the micro-environment of Asp2.50, thus changing its protonation status. Similarly, the other conserved titratable residue, Asp/Glu3.49 of the D(E)RY motif is also shown to become protonated during activation. Either protonation or deprotonation could be used to promote large conformational changes required by GPCR activation.

Therefore, we propose that GPCRs contain two protonation switches in general: First, a change of a net positive charge at Asp2.50 provides a structural point, on which the negative-inside membrane potential applies an electric force; and secondly, protonation of Asp/Glu3.49 releases side-chain constraining energy. Consequently, they synergistically trigger the conformational change of the agonist-bound GPCR protein during the process of GPCR activation. We hope that our hypothesis will add a new dimension to the research of the GPCR structure-function relationship.

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This article does not contain any studies with human or animal subjects performed by the any of the authors.

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