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Dynamics and flexibility of G-protein-coupled receptor conformations and their relevance to drug design

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G-protein coupled receptors (GPCRs) are seven helical transmembrane proteins with functional diversity and form the largest superfamily of drug targets. The functional diversity of these receptors stems from the conformational flexibility of the receptor, the nature of the ligand activating the receptor, and the intracellular protein that the receptor couples to. A molecular level understanding of the influence of each of these factors will greatly aid the design of functional selective drugs. In this review, the current state of our understanding of the conformational flexibility and dynamics of class A GPCRs derived from a confluence of biophysical and computational techniques is elucidated.

Introduction

G-protein-coupled receptors (GPCRs), also known as the seven-transmembrane (TM) receptors, form a superfamily of membrane-bound receptors that mediate cell signaling and are important drug targets. GPCRs share the topology of seven TM helices connected by intracellular and extracellular loops. Despite having a common structural topology, the amino acid sequences of GPCRs are diverse, and so are their functions. This functional diversity could be associated with subtle three-dimensional structural differences such as helical kinks and tilts and the rotation orientation and dynamics of these structural features [1,2]. The GPCR functional diversity is also dependent on the proteins the receptor couples to, such as the G-protein subtypes, the proteins in the G-protein-independent pathways (e.g. the β-arrestin pathway) [3] or other complex regulatory pathways. An understanding of the three-dimensional structural information and its related dynamics for GPCRs is vital to aid drug design.

A seminal breakthrough came with the recent surge of five high-resolution crystal structures of human β2-adrenergic receptor (β2AR), avian β1-adrenergic receptor, adenosine receptor A_{2A}, squid rhodopsin and ligand-free opsin, with and without the G-protein peptide bound [4–10]. This surge facilitates crystallization of other class A GPCRs and opens new doors for understanding the dynamics of GPCR conformations and drug discovery research on class A GPCRs. The remarkable similarity of the structures of β-adrenergic receptors [4,6] and rhodopsin [11,12] with less than 20% sequence similarity reveals high structural similarity in the TM regions in class A GPCRs, in accordance with the results of earlier biophysical studies on biogenic amine receptors. There are, however, subtle but important differences in the structures, especially in the extracellular and intracellular loop regions that are crucial to ligand access to the binding site, ligand binding and G-protein coupling.

Dynamic behavior of GPCR structures

When activated by a ligand, GPCRs in turn activate the G-protein trimer, which leads to secondary messenger production after a cascade of signaling events. It should be noted that certain seven-TM receptors mediate signaling through β-arrestin and not require G-protein activation [13]. Many GPCRs or their constitutive active mutants show a basal activity producing the secondary messenger, even in the absence of an agonist. There is ample experimental evidence showing that GPCR active- and inactive-state conformations are in dynamic equilibrium [14], and the relative concentration of the states is determined by the receptor basal activity. An understanding of the structural features of the various active and inactive conformations of GPCRs and the molecular mechanism of the conformational changes from the inactive to the active states are important in designing functionally specific drugs [15,16]. In fact, this dynamic flexibility is a major bottleneck in obtaining pure protein for biophysical structural studies of GPCRs [17,18].

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2010.08.018

Ligands with different efficacies stabilize varied receptor conformations

In this review, the word 'efficacy' means the measured level of activity of the ligand at a GPCR. Ligands to GPCRs are of different sizes and shapes, ranging from small molecules to large proteins. Because GPCRs are drug targets, there are several known synthetic ligands with different efficacies. An agonist is a ligand, the binding of which leads to full activation of the receptor, resulting in a measurable increase or decrease in the secondary messenger. Partial agonists elicit less than maximal response, even at saturating concentrations of the ligand. Inverse agonists reduce the basal activity of the receptor, and antagonists prevent the agonist binding and activation. Several optical techniques, as well as NMR, have been used to study the dynamics of GPCR conformations in the presence of ligands of varied efficacies, as detailed later in this review.

A detailed account of the similarities of and differences between the crystal structures of rhodopsin, β1- and β2-adrenergic receptors, adenosine receptor and opsin structures has been given in other reviews [19,20]. In brief, comparison of the crystal structures of the four GPCRs solved to date (namely bovine rhodopsin with covalently bound inverse agonist 11-*cis* retinal [12], the human β2AR bound to the inverse agonists carazolol [4] and timolol [21], avian β1AR bound to the antagonist cyanopindolol [6], and the human A_{2A} adenosine receptor bound to the antagonist ZM241385 [7]) shows that the overall structures are similar, with most of the differences stemming from the extracellular surfaces. The root mean square deviation in coordinates for the different receptor is within 3 Å [20]. There are several differences in the location of the inverse agonist or antagonist binding sites in these receptors. Whereas the antagonist ZM241385 binds vertically between TM helices 3 and 6 and extends upwards toward extracellular loop 2 (ECL2) in A_{2A} adenosine receptor, the inverse agonists carazolol and timolol in β2AR and antagonist cyanopindolol in β1-adrenergic receptor bind horizontally in similar locations between TM3, TM5, TM6 and TM7. The 'ionic lock', which is the salt bridge between Arg135$^{3.50}$ and Glu247$^{6.30}$ present in rhodopsin inactive (or dark) state, is not seen in the crystal structures of the adrenergic or the adenosine receptors. This could mean that there are multiple inactive states or simply that the conformations that have been crystallized are not the inactive states of the receptor. Here, we have used the Ballesteros and Weinstein numbering for class A GPCRs.[a]

Conformational flexibility and activation mechanism of class A GPCRs

The substituted-cysteine accessibility method (SCAM) is used to measure the solvent accessibility of residues by mutating them to cysteine residues and measuring the reactivity to sulfhydryl reagents. SCAM was used to map the residues involved in ligand binding, as well as residues inside the TM bundle accessible to water, in the dopamine D_2 receptor. At a time when very little structural information was available on the conformational flexibility of GPCRs, SCAM experiments provided valuable information on the flexibility of the receptor conformations, especially the residues on TM5, in the D_2 dopamine receptor [23]. Ballesteros *et al.* [24,25] proposed that highly conserved motifs in class A GPCRs, characterized as 'functional micro-domains', are involved in the receptor conformational changes and, hence, in the GPCR activation process. They proposed that movements of these functional micro-domains could be conserved in the activation mechanism of class A GPCRs and in constitutively active mutants of GPCRs [24,26–28]. For example, in rhodopsin and adrenergic receptors, movement about the helical kink formed by the highly conserved WXP motif in TM6, the movement in TM7 around the NPXXY motif and the movement in the region around the ionic lock between Arg$^{3.50}$ and Glu$^{6.30}$ are all possibly conserved activation switches triggered in these functional micro-domains.

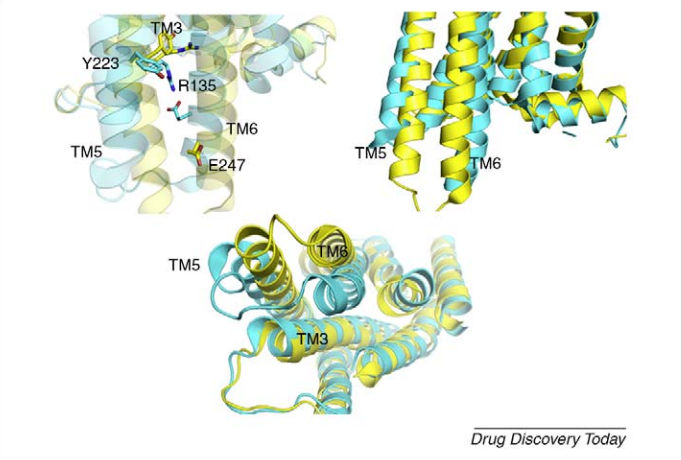

FIGURE 1

Cyan is the rhodopsin (1GZM) crystal structure; yellow is the opsin crystal structure (3DQB). **(a)** Y223$^{5.58}$ in the opsin structure (yellow) breaks the ionic lock between E247$^{6.30}$ and R135$^{3.50}$. **(b)** Elongation of the helical region in the intracellular part of TM5 in the opsin structure. **(c)** TM5 in opsin is more tilted toward TM6, and both TM5 and TM6 are tilted outward from the TM bundle of the receptor.

Comparison of the rhodopsin and opsin crystal structures also shows considerable conformational changes that occur upon activation. The ionic lock between Arg135$^{3.50}$ and Glu247$^{6.30}$ present in rhodopsin inactive state is broken in the opsin structure. The residue Tyr227$^{5.58}$ is pointing toward the lipid in the inactive rhodopsin structure, whereas this residue points toward the core of the TM region, between TM3 and TM6, in the opsin structure. This, in turn, leads to the disruption of the ionic lock in the partial active opsin structure (Fig. 1a). This is a possible conformational switch for the activation of class A GPCRs. Another activation conformational switch, known as the 'rotamer toggle switch', was originally observed in rhodopsin [29,30]. Upon activation, the side-chain rotamer of the residue Trp265$^{6.48}$ flips, breaking the water-mediated hydrogen bond with Asn302$^{7.49}$. Turning on the rotamer toggle switch upon activation leads to more flexibility in the active state than in the inactive state of rhodopsin, showing again that GPCRs have conformational flexibility. The differences between the partially active state opsin crystal structure and the crystal structure of rhodopsin in the inactive state are substantial and comparable to the differences between different GPCRs with less than 30% sequence

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[a] The first number in the superscript represents the TM helix in which the residue is located, and the second number refers to the residue number with respect to the most conserved residue in each helix among class A GPCRs [22].

identity. There are substantial rearrangements in all three intracellular loops in the opsin structure, and the intracellular end of TM5 shows elongation of the helical regions (Fig. 1b). As shown in Fig. 1c, TM5 is also more tilted toward TM6, and both TM5 and TM6 are tilted outward from the TM bundle of the receptor [10]. Thus, it is evident that activation is associated with considerable conformational changes in the receptor.

Spin labeling and EPR studies on rhodopsin before the publication of the opsin structure have shown that the ionic lock breaks upon activation and the rotamer toggle switch is also used for the activation of rhodopsin [29,31]. Solid-state NMR studies showed that the interhelical contact between the side chain of Glu122$^{3.37}$ and the backbone oxygen of His211$^{5.46}$ breaks upon activation of rhodopsin [32,33]. More recent NMR experiments combined with NMR-derived distance-restrained molecular dynamics (MD) simulations elucidated a chain of molecular events leading to activation in rhodopsin [34]. The light-activated isomerization of 11-*cis*-retinal to all-*trans* retinal leads to changes in the conformation of ECL2, which triggers the movement of TM5, TM6 and TM7 [35]. Movement of these helices leads to breaking of the ionic lock between Arg135$^{3.50}$ on TM3 and Glu247$^{6.30}$ on TM6. Side-chain conformations of Trp265$^{6.48}$, Tyr223$^{5.58}$ and Tyr306$^{7.53}$ change to assist the breakage of the ionic lock [36]. Vogel and coworkers studied the effect of membrane on the activation of rhodopsin and concluded that the membrane environment is enthalpically less favorable for the receptor activation steps such as the coupling of the breaking of the ionic lock and the uptake of protons by the cytoplasmic Glu134$^{3.49}$ [37].

Experiments engineering metal ion binding sites and tethered ligands show that the extracellular regions of TM6 and TM7 move inward toward TM3 upon activation as the intracellular regions of TM6 and TM7 move outward from the protein core [38].

Biophysical experiments on purified fluorescently labeled β2AR to study the time evolution of the receptor conformational change upon exposure to various ligands of varied efficacies have shown that partial and full agonists stabilize conformationally distinct active states of the receptor [39]. Full agonists show a biphasic behavior in fluorescence intensity change with time, with a fast step preceding the slow step, whereas a strong partial agonist showed only a slow step and a weak partial agonist showed only a fast step. Yao *et al.* [40] showed that the conformational switches in the functional micro-domains used for activation by agonists of varied efficacies could also be different. Several other fluorescence experiments, such as fluorescence resonance energy transfer and bioluminescence resonance energy transfer in intact cells, have shown that peptide or protein agonist binding shows biphasic behavior. Kinetic fluorescence resonance energy transfer studies have shown that the efficacy of an agonist is also dependent on the rate of conformational change in the GPCRs [41]. More recently, Kobilka and coworkers have used NMR experiments to show that the extracellular loop conformations are distinct and vary when an agonist is bound as compared to an inverse agonist [42]. Thus, there is ample evidence that ligands of different efficacies could stabilize or select distinct receptor conformational states that have varied affinities to other downstream proteins such as the G proteins. The conformational flexibility, therefore, poses considerable complexity to drug design because it requires the knowledge of various conformational states to design a drug to shut down a particular active state of the receptor [43].

Relevance of GPCR conformational flexibility to drug design

The GPCRs constitute the largest single group of targets for approved drugs, but their importance for future drug design has increased with the awareness that new approaches, such as functional selectivity [44], can permit novel approaches to drug design, even for 'old' GPCR targets. The receptor conformations selected or stabilized by ligands of varied efficacies could be different. There is also a growing body of literature that GPCRs signal through β-arrestin [45], a G-protein-independent pathway. The receptor conformations adapted for β-arrestin binding could be different from that conformation when coupled to the G protein [46]; hence, knowledge of the dynamics of various receptor conformations would immensely aid the design of biased ligands or functionally selective ligands.

Computational methods in studying GPCR conformational flexibility

Obtaining a static image of any of the GPCR conformations through X-ray crystallography is by itself a tedious task, largely because of the conformational flexibility of these receptors. It is considerably more difficult to fully describe the dynamics of the receptor conformational states, using any single experimental method. Computational methods have a vital role in providing an atomistic-level model of the GPCR conformational dynamics and thereby aid design of conformation-specific drugs [47–55]. Predicted GPCR structural models can also be used to generate hypotheses as to which residues to label for activation studies. Here, I detail the state of the computational methods and demonstrate how they could be used to gain insights into conformational dynamics of GPCRs.

Molecular dynamics simulations for understanding early events in the activation of class A GPCRs

Starting from crystal structures of the inactive states, MD simulations provide an atomistic-level mapping of the conformational changes representing the early events leading to activation of GPCRs. There have been several MD simulation studies, ranging from several hundreds of nanoseconds to a few microseconds, to study the early events in the activation of rhodopsin [56–60]. Crozier *et al.* [57] found substantial conformational changes in the TM helices TM5 and TM6, apart from shifting of counterions of the Schiff base in retinal from Glu113$^{3.28}$ to Glu181 on the ECL2. But the counterion switch mechanism was not supported by the microsecond simulations by Grossfield *et al.* [59], stating that in the metarhodopsin-I state, it is more probable that the Schiff base forms a complex counterion with both Glu113$^{3.28}$ and Glu181. The activation mechanism of rhodopsin from MD trajectories has also been analyzed in light of structural changes that occur in functional micro-domains. Saam *et al.* [56] observed that the ionic lock between Arg135$^{3.50}$ on TM3 and Glu247$^{6.30}$ on TM6 is weakened upon activation during the MD simulations. Although it is known from experiments that receptor lipid interactions are crucial to the function of GPCRs [4,61,62], atomic-level insights on the role of lipid–receptor interactions were provided by long-time-scale MD simulations. Khelashvili *et al.* [60] have shown recently that cholesterol interaction with rhodopsin modulates the functional micro-domain formed by TM1–TM2–TM7–helix8. Although many of these conformational changes are known to occur from experiments, MD simulations provide detailed atomic-level understanding of the experimental data on the mechanism of activation.

Unlike in the rhodopsin crystal structure, the ionic lock is not present in any of the other GPCR crystal structures solved so far. Dror *et al.* [63] performed microsecond-level MD simulations on the β2AR crystal structure with and without T4L lysozyme bound. They showed that both the inverse agonist carazolol-bound β2AR and the apo protein β2AR show a statistically significant population of the ionic lock formation in the inactive state of β2AR, although it was not found in the crystal structure. The formation of the ionic lock, however, was less likely to occur during the MD simulations of β2AR with carazolol and T4L lysozyme attached, as observed in the crystal structures. Thus, studying the dynamics complements the information obtained from the crystal structures.

There are several caveats in the simulation methods that should be noted when interpreting the results. MD simulations on GPCRs use an explicit description of the lipid bilayer and water. Only one type of lipid is used in these simulations, however, and this in no way reflects the variety of lipids and their composition in the cell membrane. The time scale of simulations is a serious bottleneck for MD simulations. Very few research groups can afford microsecond-level simulations in explicit membrane and water. The long-time-scale MD simulations (low microseconds) starting from an inactive state of the receptor have not shown any features of the active-state receptor conformation. If the energy barriers involved in conformational changes are high enough for MD simulations at room temperature, the sampling could be limited to only those conformations that are accessible at room temperature. This limitation in MD simulations can be overcome using steered MD simulation techniques. Steered MD simulations enable transition from one conformational state to another using constraints. Such simulations, however, require prior knowledge of the active-state conformation or the activation process. Simulated annealing MD techniques combined with the available experimental data on the active-state conformation as constraints to optimize the active-state model provide atomic level insight but are limited by the constraints imposed on the system [64]. The methods described in the section below do not have the limitation of crossing energy barriers.

Other computational methods to understand the activation of GPCRs

Elastic network models (ENMs) have been used to study the possible alternate conformations sampled by a protein in its native state, around the global minimum. This method involves computing the principal components of molecular motion by inverting the Hessian matrix calculated from applying a uniform harmonic potential applied to inter-residue contacts from the native state or from experimental data. Application of the ENM to study rhodopsin active state has shown good agreement in predicting the residues in the binding site of all-*trans* retinal and the motion opening the TM bundle in the intracellular regions [58].

Starting with the intermediate structure of lumirhodopsin, Costanzi and coworkers simulated the activation pathway of going from lumi to meta-II rhodopsin by combining MD simulations and ENM coarse-grain modeling, in two steps [65]. The first step was to map the local changes in the binding site of all-trans-retinal using restraints obtained from experimental measurements combined with MD simulations. Subsequently, the global changes in conformations were mapped by deriving an ENM model from the MD simulation results of the first step. The simulation of the pathway to meta-II structure showed a strong correlation between the forward and backward transition rates and the phenotype of several rhodopsin mutants. Some of the constitutively active mutants showed faster transitions from the inactive to the meta-II state compared to the mutations that reduce the activity of the receptor [65].

LITiCon method and its applications to study activation mechanisms

GPCRs have a seven-TM helical topology that enables the receptor conformations to be sampled on a coarse-grain level, by performing systematic rigid body motion of the seven helices allowing a certain level of flexibility in the helices. Such methods could overcome energy barriers and enable sampling of various conformational states that are not accessible by MD simulations. The ligand-induced transmembrane conformational changes (LITiCon) computational method is one such method [66–68]. It optimizes the ligand-selective receptor conformation for a given ligand, by simultaneous optimization of the rotational orientations of the seven TM helices (including the side-chain rearrangements), while allowing for the ligand to reorient and redock into the reshaped binding site. The change in the ligand-binding energy for each sampled receptor conformation is calculated. This procedure generates a multidimensional binding energy surface from which all the local minima are identified. The local minima are then sorted, and the best minimum energy conformation – ranked by the binding energy and receptor stability – is chosen as the ligand-stabilized GPCR conformation. The LITiCon procedure enables the backbone and the side chains of the receptor to optimize to the ligand conformation and hence can be used as a receptor-flexible ligand-docking program for GPCRs. Because it is more difficult to obtain structural information for the active state of the receptor, the LITiCon method can be used to map the gross conformational changes in the receptor upon ligand binding.

We have demonstrated the use of the LITiCon method for providing a molecular level insight integrating all experimental data available on the activation of rhodopsin. For example, although it was known from experiments that the TM6 in rhodopsin undergoes a dynamic change in the kink angle, that the rotamer of Trp265$^{6.48}$ toggles upon activation [29] and that the ionic lock between TM3 and TM6 breaks upon activation, the mechanistic link behind these processes and their energetics was not known. Using the LITiCon method, Bhattacharya *et al.* [66] showed that the isomerization of 11-*cis*-retinal to all-*trans*-retinal frees up the side chain of Trp265$^{6.48}$, leading to a change in its side-chain conformation, which in turn modulates the bend angle of TM6 around the conserved Pro267$^{6.50}$. They showed a direct correlation between the change in the helical kink angle and the rotamer side-chain angle of Trp265$^{6.48}$; the energy required for the rotamer toggle comes from water-mediated hydrogen bonds. As a result, the intracellular ends of TM5 and TM6 move outward from the protein core, causing large conformational changes at the cytoplasmic interface. The predicted outward movements of TM5 and TM6 together as a unit were found to be in good agreement with the crystal structure of opsin that was published subsequent to this computational study [66] (Fig. 1c). The predicted active-state conformation of rhodopsin also leads to the formation of new stabilizing interhelical hydrogen-bond contacts, such as those between Trp265$^{6.48}$ and His211$^{5.46}$ and Glu122$^{3.37}$ and Cys167$^{4.56}$. These hydrogen-bond contacts serve as potential conformational switches, offering new opportunities for future experimental investigations.

Monte Carlo method for calculating the activation pathways

The LITiCon method has been used to predict the active-state model of β2AR with ligands of varied efficacies. Using a biased Monte Carlo method on the multidimensional coarse-grain binding energy surface generated by LITiCon, Bhattacharya and Vaidehi calculated the possible activation pathways taken by the receptor in the presence of ligand of varied efficacies [68]. Analysis of the ligand-binding energy landscapes showed that the inverse agonist carazolol stabilizes the receptor in a deep energy well with high barriers to access the agonist-stabilized states (shown in Fig. 2a). Agonist-bound binding energy landscape, however, such as that of norepinephrine and epinephrine, are highly flexible with an ensemble of degenerate states, showing a favorable energy channel connecting the carazolol-bound conformational state to the norepinephrine-bound state (Fig. 2b). This implies that the receptor is flexible and able to sample the inverse agonist states while bound to the agonist, norepinephrine. On the contrary, the inverse agonist carazolol trapped the receptor in the inactive conformation, making the agonist-bound states inaccessible, thus reducing the basal activity of the receptor. In the salbutamol, a partial agonist stabilized binding energy surface (Fig. 2c), the carazolol and the salbutamol states were parts of two distinct energy wells separated by a barrier and the salbutamol-bound receptor potential well was deeper and broader than the carazolol-bound well. Binding of partial agonists such as salbutamol stabilizes different receptor conformations. Subsequent virtual ligand screening calculations on the epinephrine-bound predicted model showed enrichment of agonists, and the crystal structure showed poor enrichment for agonists and substantial enrichment for the antagonists. This is a clear demonstration of the usefulness of these predicted receptor state models for drug design. Mapping of

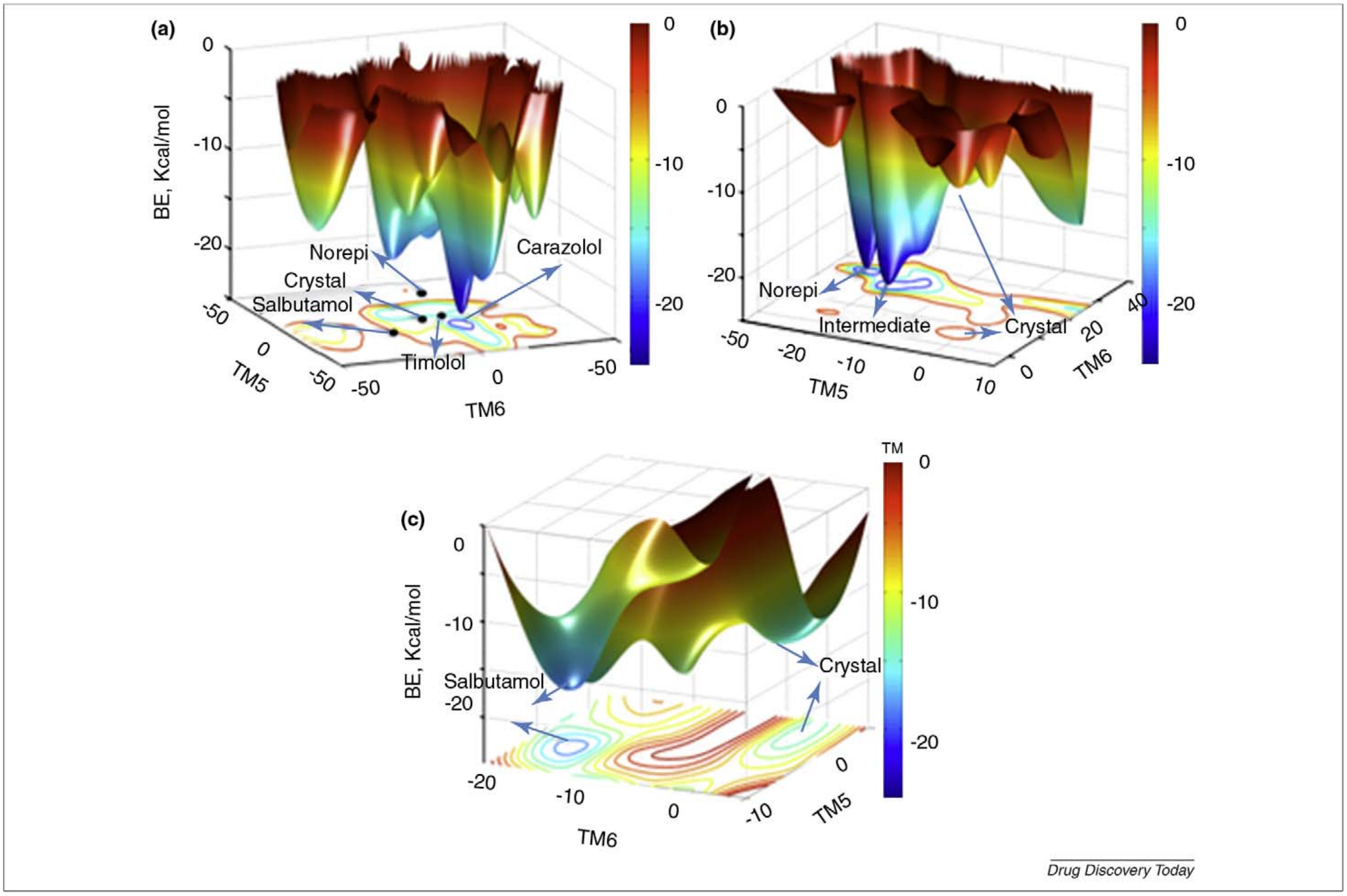

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FIGURE 2

Binding energy surfaces for β2AR with **(a)** inverse agonist carazolol; **(b)** full agonist norepinephrine; **(c)** partial agonist salbutamol. The various calculated ligand-stabilized states are marked. Norepinephrine is abbreviated as norepi. **(d)** Structures of β2AR ligands. The inverse-agonist-bound surface shows distinct separations between conformational states, whereas the agonist-bound surface shows more degeneracy among the conformation states. The above figure has been taken from reference [68] with permission.

the flexibility of the energy surface is also important in designing mutants that stabilize various receptor conformations [18,69].

Concluding remarks

GPCRs exhibit dynamic conformational flexibility, leading to a dynamic equilibrium between several inactive- and active-state conformations. On the basis of our calculation of the potential energy surfaces of GPCR crystal structures, I postulate that there is a continuum of GPCR conformational states that could be stabilized by ligands of different efficacies for a given signaling pathway and the proteins that couple to the receptor. For example, the G-protein-coupled signaling pathways could select a receptor conformation that is different from the states selected by β-arresting coupled pathways. An understanding of the continuum of states and the energetics of the dynamics between them is important in designing ligands with functional specificity to a signaling pathway, the so-called 'biased ligand' design. I have enumerated various computational methods available in literature to model the dynamics of the GPCR conformations. Of course, there are several approximations involved in all these calculations that I have enumerated in each section, and these approximations should be improved upon in the future. However, there is a strong need for computational methods: first, to generate an ensemble of structural models that can be used for drug design and structure–activity studies, and second, to identify residues to be labeled for activation mechanism studies. Computational methods to calculate the potential energy surface of these receptors will also aid in understanding the various discrepancies observed in the crystal structures that stem from the artifacts of the protein construct used for the crystallization of GPCRs.

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