Special issue: Allosterism and Collateral Efficacy

Conformational complexity of G-protein-coupled receptors

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G-protein-coupled receptors (GPCRs) are remarkably versatile signaling molecules. Members of this large family of membrane proteins respond to structurally diverse ligands and mediate most transmembrane signal transduction in response to hormones and neurotransmitters, and in response to the senses of sight, smell and taste. Individual GPCRs can signal through several G-protein subtypes and through G-protein-independent pathways, often in a ligand-specific manner. This functional plasticity can be attributed to structural flexibility of GPCRs and the ability of ligands to induce or to stabilize ligand-specific conformations. Here, we review what has been learned about the dynamic nature of the structure and mechanism of GPCR activation, primarily focusing on spectroscopic studies of purified human β_2 adrenergic receptor.

Introduction

G-protein-coupled receptors (GPCRs) for hormones and neurotransmitters are often depicted as bimodal switches with inactive and active states. This depiction might be close to the truth for rhodopsin, where basal signaling is almost non-existent and absorption of a single photon of light is sufficient for maximal activation. Much evidence indicates, however, that GPCR signaling is much more complex than was originally envisaged. GPCRs can activate more than one G protein isoform, and recent evidence suggests that they can also signal through G-protein-independent pathways [1–3]. Moreover, ligands for a given GPCR can show different efficacy profiles for coupling to distinct signaling pathways [4].

Despite advances in the biology and pharmacology of GPCRs, efforts to elucidate the structural basis of this functional plasticity remain limited. So far, only bovine rhodopsin has yielded a high-resolution structure. Nevertheless, both functional studies and low-resolution biophysical studies are providing insights into the structurally dynamic nature of non-rhodopsin GPCRs. Evidence suggests that agonist binding and activation occur through a series of conformational intermediates. Transition to these intermediate states involves the disruption of non-covalent intramolecular interactions that stabilize the basal state of the receptor. Binding of structurally different agonists might

entail the disruption of different combinations of these intramolecular interactions, leading to different receptor conformations and differential effects on downstream signaling proteins.

The dynamic character of GPCRs is likely to be essential for their physiological functions, and a better understanding of this molecular plasticity might facilitate structure-based drug discovery. Such dynamic behavior, however, makes GPCRs challenging experimental subjects and is an obstacle in obtaining diffraction-quality crystals for high-resolution structure determination. Here, we discuss what is known about the dynamic nature of the structure and mechanism of GPCR activation, focusing on spectroscopic studies of the human β_2 adrenergic receptor.

Efficacy and conformational states
Ligand efficacy
With the exception of rhodopsin, most GPCRs do not behave as bimodal switches. Rhodopsin has almost no detectable basal activity in the absence of light, but can be fully activated by a single photon. Many GPCRs show a considerable amount of basal, agonist-independent activity; in other words, the GPCR can activate its G protein in the absence of an agonist. The activity of receptors can be either increased or decreased by different classes of ligands.

The term 'efficacy' is used to describe the effect of a ligand on the functional properties of the receptor (for a more complete discussion of efficacy, see Ref. [5]). 'Agonists' are defined as ligands that fully activate the receptor. 'Partial agonists' induce submaximal activation of the G protein even at saturating concentrations. 'Inverse agonists' inhibit basal activity. Antagonists have no effect on basal activity, but competitively block the access of other ligands. On the basis of functional behavior, therefore, GPCRs behave more like rheostats than simple bimodal switches. Ligands can 'dial in' almost any level of activity from fully active to fully inactive. Although efficacy can be explained by a simple two-state model of receptor activation, evidence from both functional and biophysical studies supports the existence of multiple, ligand-specific conformational states.

Receptor conformations
Proteins are often thought of as rigid structures, as in the lock-and-key model of receptor activation where the agonist

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fits precisely into a complementary pocket in the receptor protein. Proteins, however, are known to be dynamic molecules that show rapid, small-scale structural fluctuations [6].

An intuitive approach for discussing the dynamic nature of protein conformations is an energy landscape (Figure 1a), in which a continuum of conformational states ranges from no activity to maximal activity. For the purpose of this discussion, we ignore the denatured states and consider only functional conformational states of native receptors. The basal conformational state can be defined as a low energy state of the receptor in the absence of a ligand. The width of the energy well reflects the conformational flexibility around a particular conformational state. Within this wide energy well, additional substates can be imagined (Figure 1a, inset). The probability that a protein will undergo a transition to another conformational state is a function of the energy difference between the two states and the height of energy barrier between the two states. For a receptor, the energy of ligand binding can be used either to alter the energy barrier between the two states or to change the relative energy levels between the two states, or both.

Changing the energy barrier would have an effect on the rate of transition between the two states, whereas changing the energy levels would have an effect on the equilibrium distribution of receptors in the two states. Binding of an agonist or partial agonist would lower the energy barrier and/or reduce the energy of the more active conformation relative to the inactive conformation (Figure 1b). Coupling of the receptor to its G protein could further alter the energy landscape. An inverse agonist would increase the energy barrier and/or reduce the energy of the inactive state conformation relative to the active conformation (Figure 1c).

### Basal activity and constitutively active mutants

Some GPCRs such as rhodopsin and the follicle-stimulating hormone receptor [7] have little or no detectable basal activity, whereas others such as the cannabinoid receptors show high basal activity [8,9]. Even for receptors with relatively low basal activity, however, constitutively activating mutations (CAMs) can increase this activity [10].

Basal activity might reflect the inherent flexibility of a GPCR and its tendency to exist in more than one conformational state in the absence of ligands. It could also reflect a highly constrained state with a relatively high affinity for a G protein.

The concept of basal activity and receptor activation can also be considered in terms of an energy landscape (Figure 2). In the absence of agonist, a receptor with low basal activity might be relatively constrained to one inactive conformational state with a deep energy well (Figure 2a). High basal activity might be explained by smaller energy differences between the inactive and active states and a lower energy barrier (Figure 2b), which would increase the probability for spontaneous conformational transitions to the active state. This explanation could also be thought of as a receptor with greater conformational flexibility (fewer conformational constraints). Alternatively, it is possible that a receptor might exist in predominantly one constrained state that has intermediate activity towards its G protein (Figure 2c). Although these two mechanisms might apply to different receptors, there is experimental evidence linking conformational flexibility and structural instability to increased basal activity [11].

Non-covalent intramolecular interactions define the activity and stability of the basal state

Transmembrane (TM) domains are held in the basal state by intervening loops and non-covalent interactions between side chains. The non-covalent interactions seem to have a greater role in determining the specific basal arrangement of the TM segments relative to each other than do some of the intervening loop structures, as assessed by proteolysis and split receptor studies. For example, co-transfecting a plasmid encoding the amino terminus to TM5 of the β_2 adrenoceptor (β_2AR), and a plasmid encoding TM6 to the carboxyl terminus of β_2AR generates a functional 'split' receptor [12], comprising two non-covalently bound receptor fragments. In addition, Schoneberg *et al.* [13] have generated functional M3 muscarinic split receptors with discontinuity in the loop connecting TM3 and TM4, the loop connecting TM4 and TM5, and the loop connecting TM5 and TM6. Similarly, the
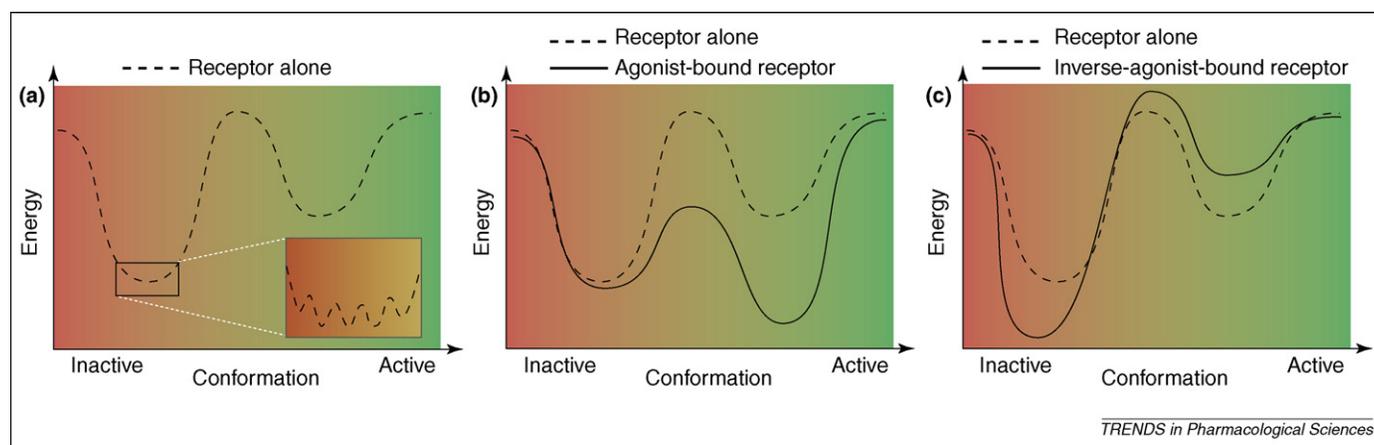
Figure 1. Theoretical energy landscape of a GPCR. **(a)** Conformational states of an unbound GPCR. **(b)** Binding of an agonist or partial agonist lowers the energy barrier and/or reduces the energy of the more active conformation relative to the inactive conformation. **(c)** Binding of an inverse agonist increases the energy barrier and/or reduces the energy of the inactive state conformation relative to the active conformation.
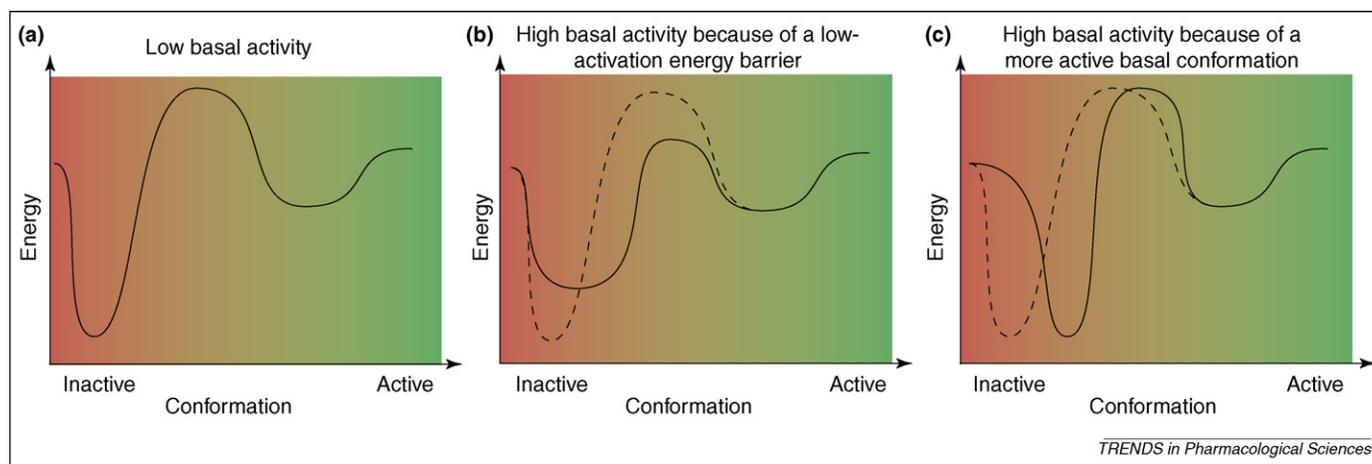
Figure 2. Theoretical energy landscape of a GPCR with low or high basal activity. **(a)** Conformational states of a GPCR with low basal activity. **(b)** Conformational states of a GPCR with high basal activity owing to a low activation energy barrier. **(c)** Conformational states of a GPCR with high basal activity owing to a more active basal conformation.

α_{2A} adrenoceptor can still bind ligands after proteolytic cleavage of its loop structures [14].

It might be predicted that disrupting one of the stabilizing intramolecular interactions would favor either a more active conformation or denaturation of the receptor. This prediction is consistent with the observation that the extent of basal activity for GPCRs can be markedly enhanced by single-point mutations in various structural domains [10]. Mutations that disrupt intramolecular interactions would increase the 'flexibility' of the protein (movement of TM domains relative to each other) and thus the probability that the receptor can assume an active conformation.

Some of the best-characterized examples of CAMs are those that disrupt the highly conserved (D/E)R(Y/W) amino acid sequence, present in 72% of GPCRs belonging to the rhodopsin family (http://lmc.uab.cat/gmos/). In rhodopsin, there is a network of interactions between $Glu134^{3.49}$ and $Arg135^{3.50}$ at the cytoplasmic end of TM3, and between $Glu247^{6.30}$ and $Thr251^{6.34}$ at the cytoplasmic end of TM6 [15] (note that the position of residues are followed by the Ballesteros general number [16] in the form superscript X.YY, where X refers to the TM segment and YY to the position relative to the most highly conserved amino acid in the TM segment, which is assigned an arbitrary position of 50.) This network, known as the 'ionic lock' is one of the non-covalent interactions that stabilize the receptor in the basal state. Disruption of this network by mutating $Glu134^{3.49}$ to glutamine leads to constitutive activity in opsin [17]. Experimental evidence indicates that $Glu134^{3.49}$ is protonated during activation of rhodopsin, demonstrating that disruption of this network is part of the normal activation process [18].

Amino acids constituting this ionic lock are conserved in other GPCRs, and mutations of the acidic amino acid have been reported to increase basal activity in various GPCRs, including β_2AR [19], the H_2 histamine receptor [20], the α_{1b} adrenoceptor [21,22] and the angiotensin (AT_1) receptor [23]. My co-workers and I have recently used fluorescence spectroscopy to monitor movement of the cytoplasmic end of TM3 relative to TM6 in β_2AR [24]. We observed an agonist-induced conformational change similar to that seen on light

activation of rhodopsin [25]. This conformational change was observed on binding of almost all agonists and partial agonists [24]. We also observed that β_2AR shows higher basal activity when the pH is reduced from 7.5 to 6.5, presumably owing to the disruption of the ionic lock and/or other intramolecular interactions as a result of protonation of an acidic amino acid [26].

It might be predicted that mutations that cause enhanced basal activity by disrupting intramolecular interactions could also lead to structural instability. Mutation of $Leu272^{6.34}$ to alanine at the cytoplasmic end of TM6 in β_2AR results in increased basal activity [27] and biochemical instability [11]. Purified $Leu272 \rightarrow Ala$ β_2AR denatures 2–3 times faster than wild-type receptor [11]. The increase in basal activity observed in native β_2AR at reduced pH is also associated with a higher rate of denaturation [26]. Instability has also been reported in constitutively active mutants of the β_1 adrenoceptor [28] and the H_2 histamine receptor [20]. Of note, ligands (both agonists and antagonists) can stabilize the receptor against denaturation and act as biochemical chaperones [11,28,29], suggesting that they form stabilizing bridges between TM segments.

Agonist binding and activation
Agonists disrupt stabilizing intramolecular interactions
The energy of agonist binding is used to change the energy landscape by altering the network of stabilizing intramolecular interactions to favor an active conformation. Figure 3 shows two possible ways in which ligands might disrupt intramolecular interactions and thereby influence the arrangement of TM domains.

First, agonists might effect a conformational change by simply disrupting existing intramolecular interactions (Figure 3a), thereby favoring a new set of interactions that stabilize a new conformational state. This effect is analogous to a mutation that produces high basal activity. The angiotensin AT_1 receptor provides an example of an agonist binding to and displacing stabilizing interactions. Experimental evidence suggests that $Asn111^{3.35}$ interacts with $Asn295^{7.46}$ in TM7 of the angiotensin AT_1 receptor to
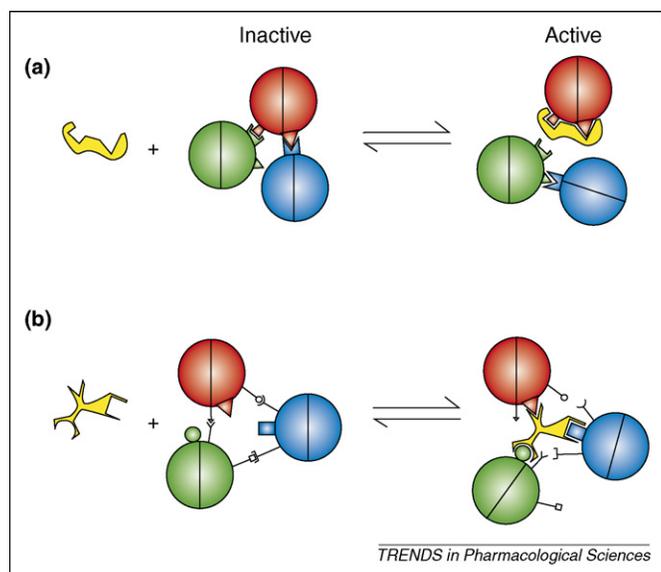
Figure 3. Possible mechanisms by which agonist binding disrupts intramolecular interactions that stabilize the inactive state. **(a)** The agonist binds directly to amino acids involved in stabilizing the inactive state. **(b)** Agonist binding stabilizes a new set of intramolecular interactions.

stabilize the inactive state [30]. Consistent with this interpretation, mutation of Asn111$^{3.35}$ to alanine leads to constitutive activity [31]. Asn111$^{3.35}$ also seems to interact with Tyr4 of the agonist angiotensin [32]; therefore, angiotensin binding might disrupt the interaction between Asn111$^{3.35}$ and Asn295$^{7.46}$.

Second, agonists might serve as bridges that create new interactions between TM domains that stabilize a more active state (Figure 3b). For example, catecholamines can disrupt the ionic lock of β_2AR without directly interacting with amino acids involved in forming the ionic lock [24].

It is likely that a combination of the mechanisms shown in Figure 3 is operable for any given ligand, particularly for larger ligands such as peptide agonists. Notably, in both models shown in Figure 3, there is no pre-existing agonist-binding site in the basal state of the receptor. Spontaneous conformational transitions are needed to expose the amino acids that form the binding site.

Structurally different agonists disrupt distinct combinations of stabilizing intramolecular interactions

Evidence from both cell-based and biophysical studies suggests that structurally different agonists (and partial agonists) of a given GPCR can induce distinct conformational states, rather than simply altering the equilibrium between two states (inactive and active) [3,4]. Moreover, studies in HEK293 cells show that, for β_2AR, an inverse agonist that inhibits basal signaling through G_s and adenylyl cyclase is a partial agonist of arrestin-mediated activation of the ERK signaling pathway [4].

Over the past several years, my co-workers and I have developed biophysical approaches that detect agonist-induced conformational changes in purified β_2AR. We've compared the ability of a set of structurally related agonists (Figure 4) to induce conformational changes in different assays. These experiments provide evidence that different agonists disrupt distinct networks of stabilizing intramolecular interactions.

Molecular switches: the ionic lock and the rotamer toggle switch

For the purpose of discussion, we define 'molecular switches' as non-covalent intramolecular interactions that exist in the basal state of a GPCR and that must be disrupted to achieve an active state. For a given GPCR, there are likely to be several molecular switches. Two that have been proposed to exist in β_2AR are the ionic lock and the rotamer toggle switch. As discussed earlier, the ionic lock consists of the (D/E)R(W/Y) sequence at the cytoplasmic end of TM3 and an acidic amino acid at the cytoplasmic end of TM6 (Figure 5a). The ionic lock is highly conserved among the rhodopsin family of GPCRs. These amino acids form a stabilizing network of non-covalent intramolecular interactions that retain the cytoplasmic ends of TM3 and TM6 in an inactive conformation.

Another molecular switch, known as a 'rotamer toggle switch', has been proposed to be involved in activation of the amine and opsin receptor families [33]. This switch involves a change in the bend of TM6 at the highly conserved residue Pro288$^{6.50}$. In β_2AR, the aromatic catechol ring of catecholamines would interact directly with the aromatic residues of the rotamer toggle switch, Trp286$^{6.48}$ and Phe290$^{6.52}$. Monte Carlo simulations suggest that rotamer configurations of Cys285$^{6.47}$, Trp286$^{6.48}$ and Phe290$^{6.52}$ – the residues that comprise the rotamer toggle switch – are coupled and modulate the bend angle of TM6 around the highly conserved proline kink at Pro288$^{6.50}$, leading to movement of the cytoplasmic end of TM6 on receptor activation [33].

Recent biophysical experiments on purified β_2AR suggest that these two switches can be activated independently of each other, and that agonists differ in their ability to activate these two switches. Moreover, they provide evidence that additional switches must be activated to achieve the fully active state of the receptor.

Catechol does not disrupt the ionic lock

It is possible to monitor disruption of the ionic lock in β_2AR by placing a fluorophore (monobromobimane) at amino acid position 271$^{6.33}$ at the cytoplasmic end of TM6, and a quenching amino acid (tryptophan) at position 135$^{3.45}$ at the cytoplasmic end of TM3 (Figure 5b). In the unbound receptor, the ionic lock separates bimane and tryptophan, and prevents quenching of bimane fluorescence. On agonist binding, the ionic lock is disrupted and tryptophan quenches bimane fluorescence by ~50% (Figure 5c). Maximal quenching has been observed for almost all agonists and partial agonists, including salbutamol and dopamine; however, catechol (a weak partial agonist) has no effect (Figure 5d). These results show that disruption of the ionic lock is necessary, but not sufficient, for full activation of β_2AR.

Salbutamol does not activate the rotamer toggle switch

The conformational change associated with activation of the rotamer toggle switch can be detected by a rapid increase in fluorescence of purified β_2AR labeled at Cys265$^{6.27}$ (at the cytoplasmic end of TM6) with tetramethylrhodamine [34,35]. All agonists that have a catechol ring induce this rapid conformational response, including catechol itself [34] (Figure 6a), whereas non-catechol
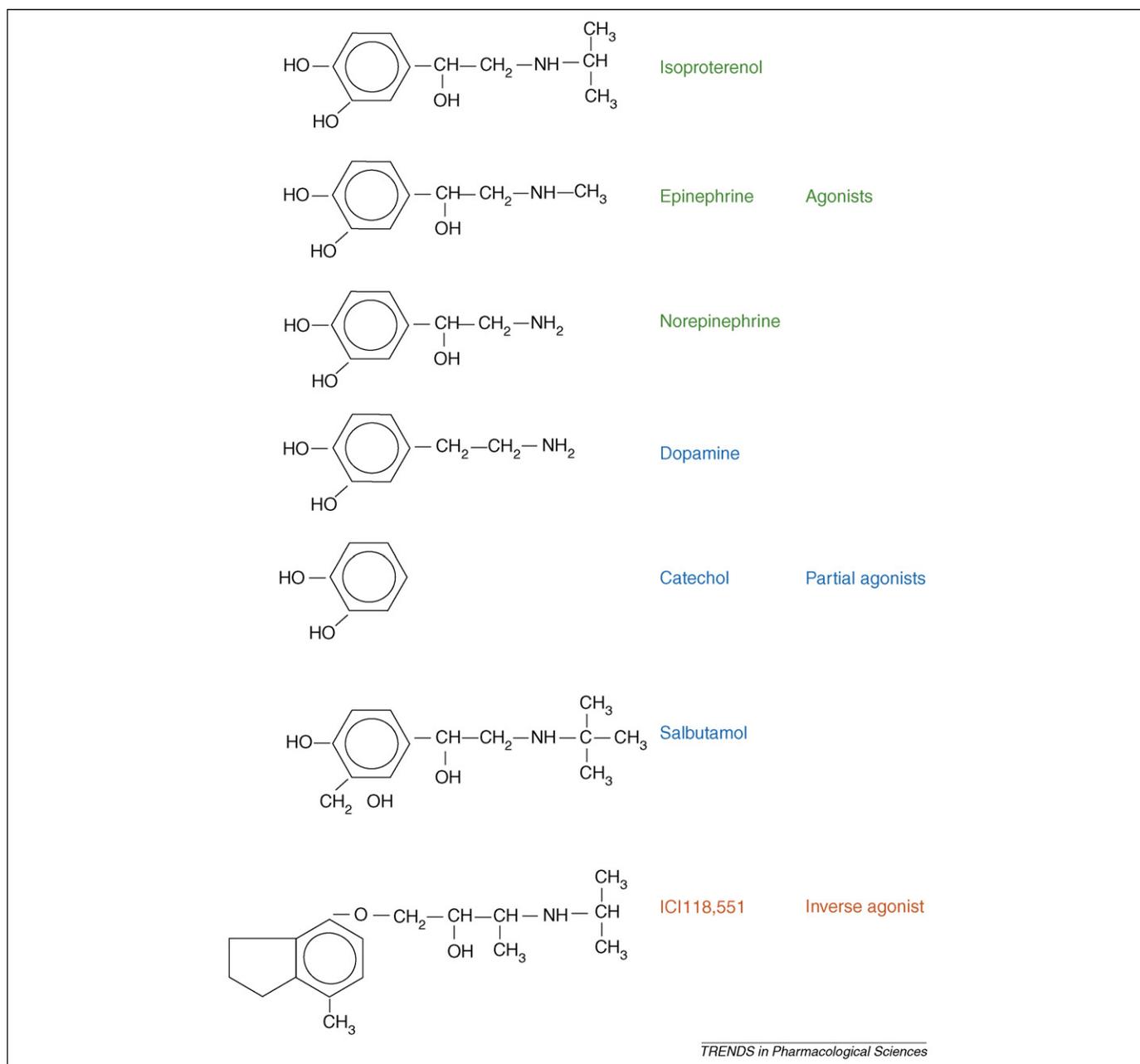
Figure 4. Ligands of the β_2 adrenoceptor. Agonists, partial agonists and inverse agonists are shown.

partial agonists do not induce this conformational change [35]. Moreover, saturating concentrations of salbutamol do not block the conformational change ind-uced by catechol (Figure 6b). By contrast, no response to catechol is observed in labeled receptor bound to saturating concentrations of norepinephrine (Figure 6c). These results indicate that the aromatic ring of salbutamol occupies a different space in β_2AR than does the aromatic ring of catecholamines; consequently, salbutamol does not acti-vate the rotamer toggle switch.

Taken together, these experiments show that disruption of the ionic lock and activation of the rotamer toggle switch are not tightly coupled conformational changes. They show that ligands induce or stabilize differ-ent conformational changes by disrupting distinct intra-molecular interactions. Lastly, they provide evidence for

the existence of other molecular switches, because dopa-mine activates both switches but is only a partial agonist.

An inverse agonist does not inhibit β_2AR activation by catechol It might be expected that inverse agonists not only compete with binding of agonists, but also stabilize an inactive conformation. As expected, the inverse agonist ICI118551 does not produce a significant change in fluorescence in β_2AR labeled with tetramethylrhod-amine on Cys265[6.27] [35] (Figure 6d), although it does inhibit the response to agonists and partial agonists with one exception: catechol can bind and induce a conf-ormational response in tetramethylrhodamine-labeled β_2AR bound to saturating concentrations of ICI118551 [35] (Figure 6d). The fluorescence response is associ-ated with a functional response in a G protein acti-
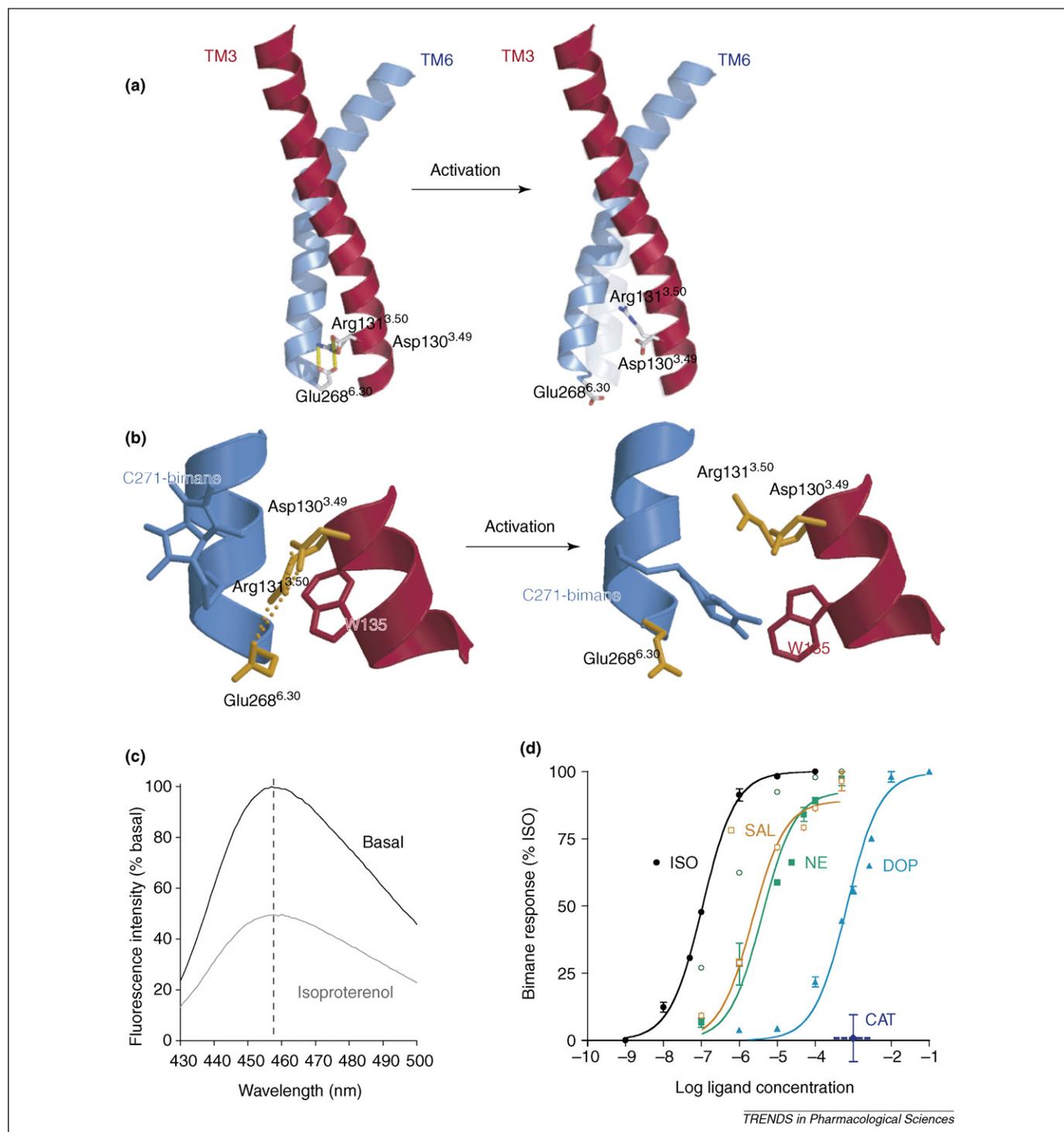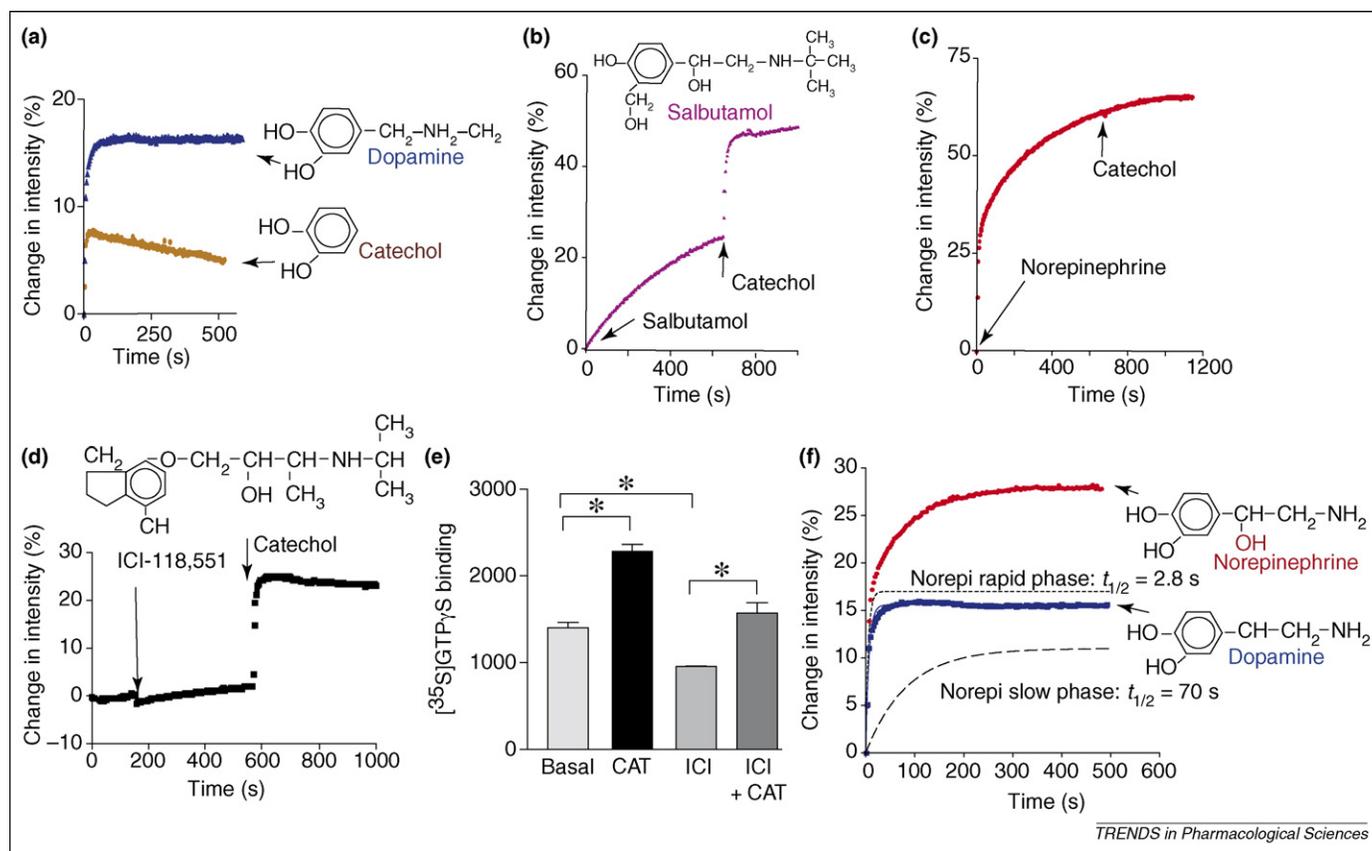
Figure 5. Fluorescence spectroscopy of disruption of the ionic lock in β_2AR. **(a)** Model of TM3 (red) and TM6 (blue) from β_2AR, highlighting the amino acids that comprise the ionic lock at the cytoplasmic end of these TM segments. **(b)** Close-up view of the ionic lock and the modifications made to monitor conformational changes in this region. Ala271 was mutated to cysteine (C271) and Ile135 was mutated to tryptophan (W135). C271 was labeled with monobromobimane in purified β_2AR. On activation, W135 moves closer to bimane on C271 and quenches fluorescence. **(c)** Emission spectrum of bimane on C271 before and after activation by the agonist isoproterenol. **(d)** Effect of different ligands on disruption of the ionic lock, as determined by bimane fluorescence. The partial agonists dopamine (DOP) and salbutamol (SAL) are as effective at disrupting the ionic lock as the full agonists norepinephrine (NE) and isoproterenol (ISO). Only catechol (CAT) has no effect on the ionic lock. Data adapted from Ref. [24].

vation assay (Figure 6e). This finding suggests that ICI118551 does not occupy the catechol-binding pocket and does not prevent activation of the rotamer toggle switch by catechol.

Agonist binding and activation is a multistep process
Agonist binding involves the formation of interactions between chemical substituents on the agonist and specific amino acids in the GPCR, in addition to the disruption of intramolecular interactions stabilizing the basal state and the formation of a new set of intramolecular interactions between TM segments. This process is complex, and evidence from both functional [36] and biophysical [24,34,35,37,38] studies on β_2AR suggests that it occurs through kinetically distinct steps involving conformational intermediates. β_2AR is a good model system in which to

Figure 6. Agonist-induced conformational changes in β_2AR. Conformational changes were detected by fluorescence spectroscopy in β_2AR labeled at Cys265 with tetramethylrhodamine maleimide (TMR–β_2AR). **(a)** Change in intensity of TMR–β_2AR in response to dopamine and catechol. **(b)** Change in intensity of TMR–β_2AR in response to the non-catechol partial agonist salbutamol, followed by the addition of catechol. Catechol induces a conformational change in TMR–β_2AR bound to a saturating concentration of salbutamol, indicating that salbutamol and catechol occupy non-overlapping binding sites. **(c)** Change in intensity of TMR–β_2AR in response to norepinephrine, followed by the addition of catechol. No catechol response is observed in TMR–β_2AR bound to a saturating concentration of norepinephrine, indicating that these ligands share a common binding site. **(d)** There is no significant change in the intensity of TMR–β_2AR in response to the inverse agonist ICI118551. Catechol can induce a conformational change in β_2AR bound to a saturating concentration of ICI118551, indicating that these ligands do not occupy the same binding space. **(e)** [^{35}S]GTPγS binding to purified β_2AR reconstituted with purified G_s. Catechol weakly stimulates [^{35}S]GTPγS binding and ICI118551 inhibits basal [^{35}S]GTPγS binding. Notably, catechol can stimulate [^{35}S]GTPγS binding in β_2AR occupied by a saturating concentration of ICI118551. **(f)** Change in intensity of TMR–β_2AR in response to norepinephrine and dopamine. The response to norepinephrine is best fitted by a two-site exponential function. The rapid and slow components of the response are indicated. Data adapted from Refs [34,35].

study agonist binding because much is known about the sites of interaction between catecholamine ligands and the receptor. Moreover, it has a rich source of structurally related ligands with a spectrum of efficacies ranging from inverse agonists to full agonists.

Evidence from biophysical studies As discussed earlier, agonist-induced conformational changes in purified β_2AR lead to an increase in the fluorescence intensity of tetramethylrhodamine bound to Cys265$^{6.27}$ [34,35]. Cys265$^{6.27}$ is located at the cytoplasmic end of TM6 in a pocket formed by TM3, TM5 and TM6. A fluorophore bound to Cys265$^{6.27}$ is ideally positioned to detect changes in β_2AR conformation relevant to G-protein activation. The increase in fluorescence intensity as a function of time after activation by the agonist norepinephrine is best fitted by a two-component exponential function [34] (Figure 6f). By contrast, the response to dopamine (a partial agonist) is adequately fitted by a one-component exponential function.

Of interest, the rapid component of norepinephrine-induced fluorescence change is very similar to the response to dopamine (Figure 6f), which suggests that

the dopamine-induced conformation might represent an intermediate in the conformational response to norepinephrine. Functionally, these slower conformational changes correlate with a higher efficacy towards activation of G_s and efficient agonist-induced β_2AR internalization, most probably owing to interactions between β_2AR and GPCR kinases and/or arrestins. Although dopamine is a relatively good partial agonist of G-protein activation (achieving ~60% of activation by the agonist isoproterenol), it is much less efficient at inducing β_2AR internalization (~20% of isoproterenol activation) [34].

The results of these studies suggest that binding of agonists such as norepinephrine occurs through at least one conformational intermediate, and that this intermediate state is similar to the conformational state induced by dopamine. Further evidence for the existence of an intermediate conformational state comes from fluorescence lifetime experiments on the β_2AR [38]. The results from these kinetic and lifetime studies can be depicted using an energy landscape (Figure 7). The simplest model involves one intermediate state representing the rapid component of the norepinephrine response. This state can also be stabilized by dopamine and, to a lesser extent, by catechol.
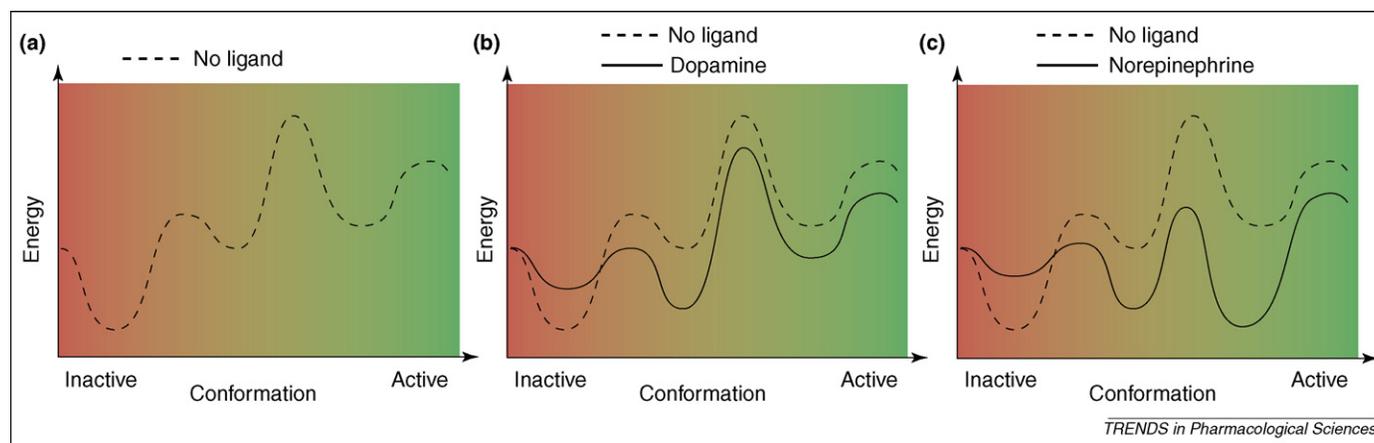
Figure 7. Theoretical energy landscapes of β_2AR. **(a)** Conformational states of unbound β_2AR. **(b)** Conformational states of β_2AR bound to dopamine. **(c)** Conformational states of β_2AR bound to norepinephrine. The partial agonist dopamine stabilizes a conformational intermediate observed in β_2AR bound to the agonist norepinephrine.

Neither dopamine nor catechol, however, stabilizes the more active state.

Insight from binding studies The biophysical studies on β_2AR are supported by an elegant series of experiments examining the binding properties and efficacy of a set of ligands representing components of the catecholamine epinephrine [36]. By comparing the affinity of these different compounds, it is possible to determine the contribution of each chemical substituent (catechol hydroxyls, β-hydroxyl and N-methyl) of the agonist to binding affinity and efficacy. A simple lock-and-key model (Figure 8a) predicts that the contribution of each substituent to binding affinity would not be dependent on the presence of the other substituents. This

is not the case, however. The binding energy associated with each of the substituents has been found to depend on the presence of other substituents. The data suggest that there is no pre-formed binding site for the agonist epinephrine in unbound β_2AR.

These results might be explained by a model in which the agonist binds through a series of conformational intermediates (Figure 8b). In the basal state, a minimal, low-affinity binding site forms interactions between the receptor and a few structural features on the agonist (e.g. the aromatic ring and the amine). Binding to this site increases the probability of a conformational transition that is stabilized by an interaction between the receptor and the catechol hydroxyls. The binding energy gained by inter-
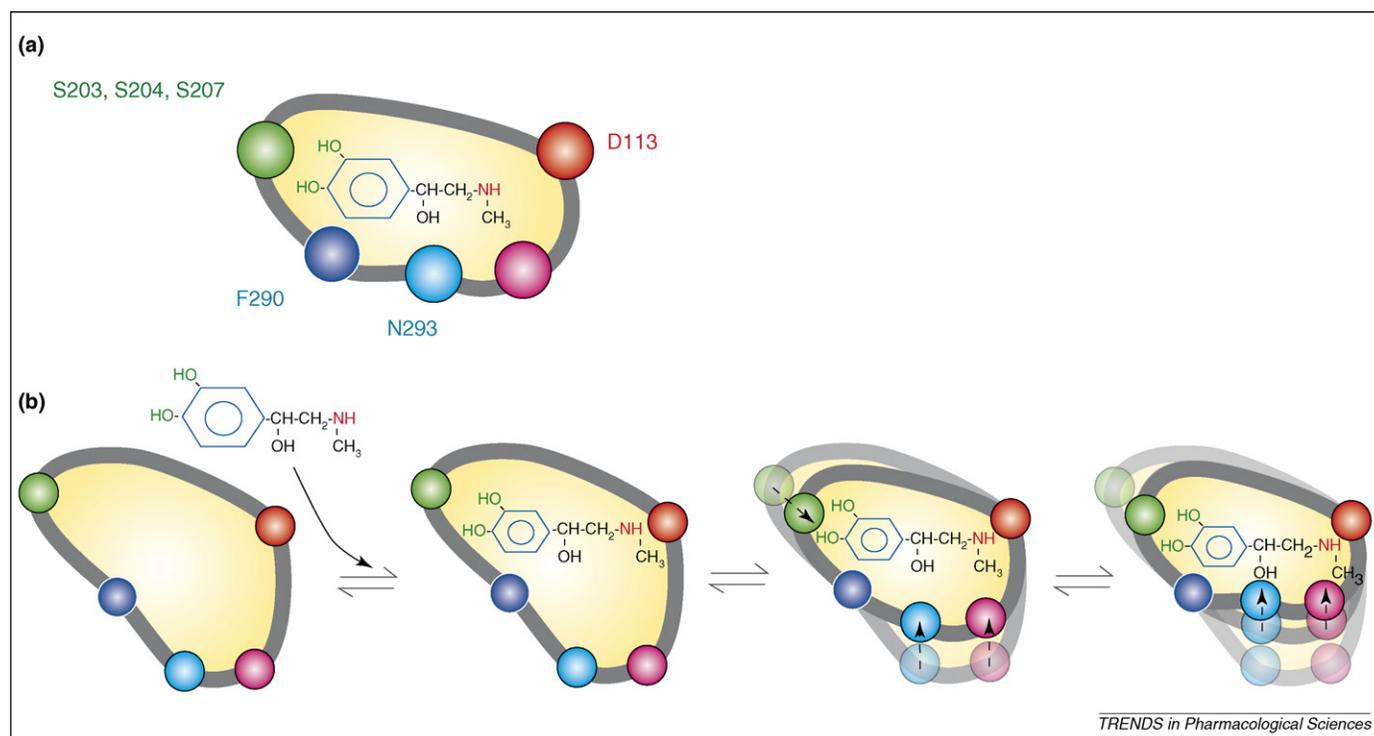
Figure 8. Models of agonist binding and activation. **(a)** Lock-and-key model of agonist binding to β_2AR. Receptor sites that interact with specific substituents of the ligand are shown as colored circles. **(b)** Sequential model of agonist binding to β_2AR. The models are based on a study by Liapakis *et al.* [36] and are reproduced, with permission, from Ref. [40].

actions between the receptor and the catechol hydroxyls pays for the energetic costs of the conformational change. This conformational transition increases the probability of another conformational change stabilized by interactions between receptor and the β-hydroxyl and/or the *N*-methyl group. Thus, the receptor becomes activated through a series of conformational intermediates, and the energetic costs of receptor activation are paid in installments.

Evidence for conformational intermediates has also been obtained for binding of agonist peptides to the neurokinin A receptor [39]. In these studies, the rapid component of binding was associated with an increase in cytosolic Ca^{2+} and the slow component was associated with an increase in cAMP.

Concluding remarks

GPCRs are remarkably versatile signaling molecules. This versatility can be attributed to a flexible and dynamic three-dimensional structure. Better understanding of this dynamic character might prove valuable for structure-based drug discovery efforts; unfortunately, however, such dynamic behavior is particularly challenging for high-resolution structure analysis. Growing diffraction-quality crystals requires stable, conformationally homogenous protein. As such, diffraction-quality crystals of a native, unbound GPCR will be difficult to obtain and, even when this goal is achieved, the crystal structure will represent only one of the many native conformations. Although it is technically not feasible to obtain a high-resolution structure of a GPCR with current NMR technology, this approach might hold the greatest promise for characterizing the dynamic nature of these fascinating proteins.

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