ACTIVATION OF G PROTEIN–COUPLED RECEPTORS

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Abbreviations

β_2AR	β_2-adrenoceptor
GPCR	G protein–coupled receptor
TM	transmembrane segment

Abstract

G protein–coupled receptors (GPCRs) mediate responses to hormones and neurotransmitters, as well as the senses of sight, smell, and taste. These remarkably versatile signaling molecules respond to structurally diverse ligands. Many GPCRs couple to multiple G protein subtypes, and several have been shown to activate G protein–independent signaling pathways. Drugs acting on GPCRs exhibit efficacy profiles that may differ for different signaling cascades. The functional plasticity exhibited by GPCRs can be attributed to structural flexibility and the existence of multiple ligand-specific conformational states. This chapter will review our current

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understanding of the mechanism by which agonists bind and activate GPCRs.

I. Introduction

G protein–coupled receptors (GPCRs) represent the single largest class of membrane proteins in the human genome. Eukaryotic GPCRs have been classified by sequence similarity into five classes (A–F or 1–5) (Attwood and Findlay, 1994; Kolakowski, 1994); however, not all of these classes are represented in humans. A detailed analysis of the human genome reveals at least 800 unique GPCRs, of which ~460 are predicted to be olfactory receptors (Fredriksson *et al.*, 2003). Based on sequence similarity within the seven transmembrane segments (TMs) (Fredriksson *et al.*, 2003), these receptors can be clustered into five families: the rhodopsin family (701 members), the adhesion family (24 members), the frizzled/taste family (24 members), the glutamate family (15 members), and the secretin family (15 members). The physiological function of a large fraction of these 800 GPCRs remains still unknown; these receptors are referred to as orphan GPCRs. However, deorphanization of nonolfactory GPCRs is an ongoing process (Howard *et al.*, 2001), as they are a promising group of targets for the pharmaceutical industry. Therefore, the actual number of orphan GPCRs continues to decline.

GPCRs share a common structural signature of seven hydrophobic segments predicted to be membrane-spanning domains, with an extracellular N-terminus and an intracellular C-terminus (Fig. 1). While the vast majority of GPCRs have been shown to activate one or more cytoplasmic heterotrimeric GTP-binding proteins (G proteins), there is now considerable evidence that some GPCRs can activate signaling pathways that do not involve G proteins (Azzi *et al.*, 2003; Luttrell and Lefkowitz, 2002). For this reason, the terms seven-TM receptor or heptahelical receptors are also been used in place of GPCRs.

This structural and functional similarity stands in contrast to the structural diversity of the natural GPCR ligands (Ji *et al.*, 1998). These range from subatomic particles (a photon) to ions (H^+ and Ca^{2+}), small organic molecules, peptides, or proteins. The location of the ligand-binding domains for many GPCRs has been determined (Ji *et al.*, 1998). While many small organic agonists bind within the TM segments, peptide hormones and proteins often bind to the N-terminus and extracellular sequences joining the TM domains. However, size of the ligand alone cannot be used to predict the location of the binding site: for instance, glycoprotein hormones, glutamate, and Ca^{2+} all activate their respective

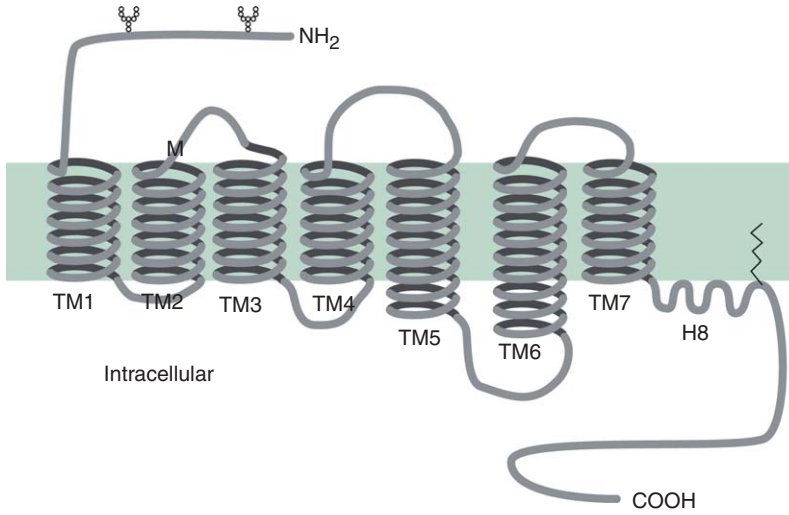

FIG. 1. Secondary structure common to GPCRs.

receptors by binding to relatively large N-terminal domains (Ji *et al.*, 1998; Pin *et al.*, 2003). It is interesting to note that for many GPCRs that bind their native agonists on the extracellular loops or the N-terminus, it has been possible to identify small-molecular-weight allosteric modulators that bind within the TM domains (Knoflach *et al.*, 2001; Ray and Northup, 2002).

In contrast to the diversity in the size of native agonists and the location of the ligand-binding sites, the vast majority of known GPCRs have been shown to activate one or more of the 16 known G protein α subunits (Sprang, 1997). G proteins are structurally homologous, and the mechanism by which GPCRs activate different G proteins is expected to be similar. Therefore, it is likely that the diverse modes of agonist binding to extracellular structures and transmembrane domains result in similar structural changes in cytoplasmic domains that interface with G proteins.

This chapter will review what is known about the mechanism of transmembrane signaling by GPCRs, specifically the process by which agonist binding leads to conformational changes necessary for G protein activation. There is a paucity of experimental data that directly address this process; however, several recent studies are beginning to provide mechanistic insight. These studies suggest that a lock-and-key model of agonist binding does not apply to GPCRs, that is, many of the amino acids that interact with agonists are not optimally positioned for agonist binding in the nonliganded receptor. For agonists to bind, intramolecular interactions

that keep the receptor in an inactive state must be broken. Evidence suggests that agonists bind in stages involving one or more conformational intermediates. If correct, the mechanism will have implications for understanding the physiology of GPCRs and for the development of better drug design strategies.

II. Structural and Mechanistic Homology Among GPCRs

Before focusing on the details about the mechanism of GPCR activation, we will review some of the evidence suggesting that GPCRs are structurally homologous and probably undergo similar structural changes when activating G proteins.

A. Rhodopsin as a Structural Model for GPCRs

There is a wealth of information about the structure and mechanism of activation of rhodopsin. Rhodopsin is a highly specialized GPCR in which the ligand, 11-*cis*-retinal, behaves as a covalently bound inverse agonist that is converted to a full agonist on its photoisomerization to the all-*trans* conformation. This mechanism of agonist activation is highly specific, in contrast to the vast majority of GPCRs that are activated by diffusible agonists. Nevertheless, there is evidence that at least some of the structural changes that occur in rhodopsin are similar to those observed in other GPCRs. Rhodopsin structure and what is known about its light-induced conformational changes have been the subject of several excellent reviews (Hubbell *et al.*, 2003; Ridge *et al.*, 2003; Sakmar, 2002; Sakmar *et al.*, 1991; Schertler, 2005), and some of the main points will be briefly discussed here.

The most detailed information about structural changes associated with activation of a GPCR comes from studies of rhodopsin. This is in part due to its natural abundance and biochemical stability relative to other GPCRs. Electron paramagnetic resonance spectroscopy (EPR) studies provide evidence that photoactivation of rhodopsin involves a rotation and tilting of TM6 relative to TM3 (Farrens *et al.*, 1996). Further support for motion of TM6 during rhodopsin activation was provided by chemical reactivity measurements and fluorescence spectroscopy (Dunham and Farrens, 1999), as well as by ultraviolet absorbance spectroscopy (Lin and Sakmar, 1996) and zinc cross-linking of histidines (Sheikh *et al.*, 1996). Light-induced conformational changes have also been observed in the cytoplasmic domain spanning TM1 and TM2, and the cytoplasmic end of TM7 (Altenbach *et al.*, 1999a,b, 2001).

In addition, rhodopsin is the only GPCR for which high-resolution crystal structures are available (Li *et al.*, 2004; Okada *et al.*, 2000, 2002, 2004;

Palczewski *et al.*, 2000; Teller *et al.*, 2001), with a maximum resolution of 2.2 Å (Okada *et al.*, 2004). While the structures determined by cryoelectron microscopy of 2-D crystals (Krebs *et al.*, 2003; Ruprecht *et al.*, 2004; Schertler *et al.*, 1993) are of lower resolution (up to 5.5 Å), they provide additional information about the orientation of TM segments relative to the lipid bilayer that cannot be obtained from 3-D crystals.

The currently available 3-D structures of rhodopsin correspond to an inactive form of the receptor. While these structures have been a true cornerstone for the study of GPCR structure and function, understanding the activation mechanism demands knowledge of the structure of the active form(s) of the receptor. The publication (Ruprecht *et al.*, 2004) and analysis (Schertler, 2005) of a low-resolution map of metarhodopsin I, an intermediate in the process of rhodopsin activation, reveals that its formation is not accompanied by the large rigid-body movements in TM segments shown to be involved in rhodopsin activation (Farrens *et al.*, 1996). However, a more subtle change, consisting in the rearrangement in the conformation of the Trp residue of the highly conserved CWxxP motif in TM6, has been detected in this intermediate. Thus, it seems that there is no gradual transformation of the inactive protein into the active form, but the activation is initiated through small-scale changes in the conformation of some key residues, which will presumably trigger the larger conformational changes related to the subsequent stages of the activation process. Thus, these key residues can be envisioned as molecular switches that, once turned on, lead to receptor activation.

B. GPCRs Activated by Diffusible Agonists

Rhodopsin is routinely used as a model system for the study of GPCR structure and activation. While high-resolution structures for other GPCRs have not yet been obtained, there is indirect evidence that some rhodopsin family members are structurally very similar to rhodopsin. Ballesteros and Javitch found that structural insights obtained from mutagenesis data and substituted cysteine accessibility studies on monoamine receptors were consistent with the high-resolution structure of rhodopsin, suggesting that rhodopsin serves as a good template for homology modeling (Ballesteros *et al.*, 2001b).

In spite of the remarkable diversity of ligands and ligand-binding domains in the family of GPCRs, there is also considerable evidence for a common mechanism of activation. When comparing sequences, GPCRs are most similar at the cytoplasmic ends of the TMs adjacent to the second and third cytoplasmic domains, the regions known to interact with cytoplasmic G proteins (Mirzadegan *et al.*, 2003). Members of the large family of GPCRs transduce signals by activating one or more members of the

relatively small family of highly homologous heterotrimeric G proteins. For example, the thyroid-stimulating hormone (TSH) receptor is activated by a large glycoprotein hormone that binds to the N-terminus while the β_2-adrenoceptor (β_2AR) is activated by adrenaline (approximately the size of a single amino acid) that binds to the TM segments; yet both of these receptors activate the same G protein (Gs), indicating that the structural changes in the cytoplasmic domains of these two receptors must be very similar. Moreover, many GPCRs exhibit promiscuous coupling to more than one G protein. For example, rhodopsin preferentially couples to transducin while the β_2AR preferentially couples to Gs; however, both are capable of activating Gi (Cerione *et al.*, 1985).

Additional evidence that GPCRs undergo similar conformational changes within TM segments and cytoplasmic domains comes from biophysical and biochemical studies. Fluorescence spectroscopic studies of β_2AR labeled with florescent probes demonstrate movement in both TM3 and TM6 on activation (Gether *et al.*, 1997b). Studies of β_2AR labeled with fluorescent probes at the cytoplasmic end of TM6 provide evidence that agonists induce a rotation or tilting movement of the cytoplasmic end of TM6 similar to that observed in rhodopsin (Ghanouni *et al.*, 2001b; Jensen *et al.*, 2000). Additional support for movement of TM3 and TM6 in the β_2AR comes from zinc cross-linking studies (Sheikh *et al.*, 1999) and chemical reactivity measurements in constitutively active β_2AR mutants (Javitch *et al.*, 1997; Rasmussen *et al.*, 1999). Cysteine cross-linking studies on the M3 muscarinic receptor provide evidence for the movement of the cytoplasmic ends of TM5 and TM6 toward each other on agonist activation (Ward *et al.*, 2002).

Despite the evident similarities between rhodopsin and the rest of Class A GPCRs, it has been proposed that this protein might not be a good template for models of more distantly related rhodopsin family members such as the cholecystokinin CCK1 receptor (Archer *et al.*, 2003). In addition, rhodopsin is unique among GPCRs because of the presence of a covalent linkage between the receptor and its ligand, retinal. Thus, the dynamic processes of agonist association and dissociation common to most GPCRs are not part of the activation mechanism of rhodopsin. Therefore, some caution is needed when extrapolating the information about rhodopsin structure and function to other GPCRs, and a more detailed knowledge of the peculiarities of each system is needed.

C. GPCR Oligomers

There is a growing body of evidence that GPCRs exist as dimers (or oligomers) and that these dimers may be important for G protein activation for at least some GPCR families. This topic has been addressed in

several excellent reviews (Angers *et al.*, 2002; Bulenger *et al.*, 2005; Devi, 2001; Javitch, 2004) and will only be briefly addressed here. Dimerization is clearly an important mechanism of receptor activation for the glutamate family of GPCRs (Pin *et al.*, 2003, 2004), where ligand-induced changes in the dimer interface of the N-terminal ligand-binding domain have been demonstrated by crystallography (Kunishima *et al.*, 2000; Tsuchiya *et al.*, 2002). However, the role of dimerization in the activation of rhodopsin family members is less clear. For instance, cryoelectron microscopy images suggest that rhodopsin may exist as homodimers in rod outer segment membranes (Liang *et al.*, 2003). In addition, neutron scattering studies provide evidence that a pentameric complex forms when purified leukotriene B(4) is reconstituted with purified Gi, suggesting that a receptor homodimer is needed to complex with a heterotrimeric G protein (Baneres and Parello, 2003). Nevertheless, it remains to be seen if a receptor dimer is required for G protein activation. The effect of agonist binding on the formation or disruption of dimers is not consistent among the rhodopsin family members that have been examined (Angers *et al.*, 2002). Moreover, ligands interact with individual receptor monomers, and there is currently no evidence that ligands span the interface between receptor dimers. If changes in dimerization occur, it is likely a secondary consequence of ligand-induced changes in the arrangement of the TM segments. Evidence in support of this comes from biophysical studies on leukotriene B(4) homodimers demonstrating that ligand binding to one protomer leads to conformational changes in its partner (Mesnier and Baneres, 2004). While dimers may be important for G protein activation, it is essential to understand the agonist-induced structural changes that occur in the context of individual GPCR monomers.

III. Conformational States

Proteins are often thought of as rigid structures. The classic model of receptor function is the lock-and-key analogy, where the agonist fits precisely into a complementary pocket in the receptor protein. However, it is known that proteins are dynamic molecules that exhibit rapid, small-scale structural fluctuations. One of the best ways to discuss protein conformations is in terms of an energy diagram (Fig. 2). The basal conformational state is a low-energy state of the protein in a particular environment. The width of the energy well reflects the conformational flexibility. The probability that a protein will undergo transitions to other conformational states is a function of the energy difference between the two states, while the kinetics of the process is function of the height of

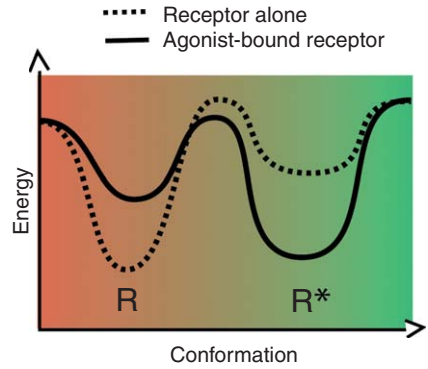

FIG. 2. Energy landscape diagram describing a possible mechanism of GPCR activation by an agonist.

energy barrier between them. In the case of a receptor, the energy provided by ligand binding may change both the depth of an energy well and the height of an energy barrier. The schematic diagram shown in Fig. 2 depicts only two conformational states; however, as discussed below, there is evidence that GPCRs may exist in multiple states.

A. Basal Activity and Ligand Efficacy

Rhodopsin has virtually no detectable basal activity in the absence of light, but can be fully activated by a single photon. But with the exception of rhodopsin, most GPCRs do not behave as simple bimodal (i.e., on–off) switches. In fact, many GPCRs have a considerable amount of basal, agonist-independent activity. The activity of receptors can be either increased or decreased by different classes of ligands (Fig. 3). The term ''efficacy'' is used to describe the effect of a ligand on the functional properties of the receptor [for a more complete discussion of efficacy, refer to Kenakin (2002)]. Agonists are defined as ligands that fully activate the receptor. Partial agonists induce submaximal activation of the G protein even at saturating concentrations. Inverse agonists inhibit basal activity. Antagonists have no effect on basal activity, but competitively block access of other ligands. Therefore, based on functional behavior, GPCRs behave more like rheostats than simple bimodal switches. Different ligands can ''dial in'' virtually any level of activity from fully active to fully inactive.

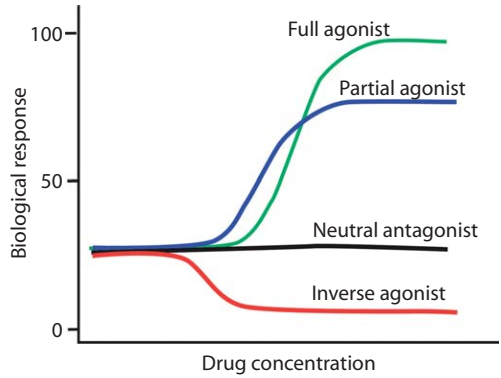

FIG. 3. Ligand efficacy. The effect of different classes of drugs on a GPCR that has some detectable basal activity.

B. Multiple Agonist-Specific States

A number of kinetic models have been developed to explain ligand efficacy, using information derived from indirect measures of receptor conformation such as ligand-binding affinity and the activation of G proteins or effector enzymes (Kenakin, 2001; Leff, 1995; Lefkowitz *et al.*, 1993; Weiss *et al.*, 1996). The simplest of them, the two-state model, proposes that a receptor exists primarily in two states, the inactive state (R) and the active state (R*). In the absence of ligands, the level of basal receptor activity is determined by the equilibrium between R and R*. The efficacy of ligands reflects their ability to alter the equilibrium between these two states. Full agonists bind to and stabilize R*, while inverse agonists bind to and stabilize R. Partial agonists have some affinity for both R and R* and are therefore less effective in shifting the equilibrium toward R*.

The two-state model can describe much of the functional behavior of GPCRs and explain the spectrum of responses to ligands of different efficacy in simple experimental systems consisting of one receptor and one G protein. However, there is a growing body of experimental evidence for the existence of multiple conformational states [summarized in Kenakin (2003)]. Within this framework, each ligand may induce or stabilize a unique conformational state that can be distinguished by the activity of that state toward different signaling molecules (G proteins, kinases, arrestins). In the case of the β_2AR, it has been possible to monitor directly some of these ligand-specific states using fluorescence spectroscopy (Ghanouni *et al.*, 2001a; Swaminath

et al., 2004, 2005). These studies show that β_2ARs labeled at Cys265$^{6.27}$ [1] on the cytoplasmic end of TM6, adjacent to the G protein–coupling domain, is able to report conformational changes in the G protein–coupling domain. These modifications alter the molecular environment around the fluorophore, which is translated to changes in fluorescence intensity and fluorescence lifetime. In these experiments, fluorescence lifetime analysis can detect discrete conformational states in a population of molecules, while fluorescence intensity measurements reflect their weighted average. Our findings show a single broad distribution of fluorescent lifetimes in the absence of ligands, suggesting that this domain oscillates around a single detectable conformation (Fig. 4A). We have also observed that antagonist binding reduces the width of the distribution, but does not change the mean lifetime, suggesting that, while the conformation does not change, the domain flexibility is reduced (Fig. 4A). However, binding of a full agonist to the β_2AR leads to the formation of two lifetime distributions (Fig. 4B and C), suggesting two distinct conformations. Moreover, the conformations induced by a full agonist can be distinguished from those induced by partial agonists (Fig. 4C) (Ghanouni *et al.*, 2001a; Swaminath *et al.*, 2005).

C. *Defining the "Active State"*

As we learn more about the complexity of GPCR signaling, it is becoming more difficult to define exactly what is meant by activation and ''active state.'' Works from several laboratories have shown that GPCRs can activate signaling pathways by G protein–independent mechanisms such as through arrestin and possibly other signaling molecules (Azzi *et al.*, 2003; Baker *et al.*, 2003; Lefkowitz and Shenoy, 2005), and that the ''active state'' for receptor activation of arrestin or other G protein–independent pathways may differ from that for receptor activation of a G protein (Azzi *et al.*, 2003). Thus, a drug classified as an inverse agonist when monitoring receptor activation of a G protein–dependent signaling pathway may behave as a partial agonist for a G protein–independent signaling pathway (Azzi *et al.*, 2003; Baker *et al.*, 2003). For example, a modified angiotensin

[1] Note: The residues of β_2AR are numbered according to their position in the sequence followed by the Ballesteros general number (Ballesteros *et al.*, Methods Neurosci. 1995; 25: 366–428) in superscript. In this numbering scheme, each residue is identified by two numbers: the first (1–7) corresponds to the helix where it is located; the second indicates its position relative to the most conserved residue of the helix, arbitrarily assigned to 50. For instance, Trp286$^{6.48}$ is the tryptophan in TM6 located two residues before the highly conserved proline Pro288$^{6.50}$. This general method can be applied to all rhodopsin-like GPCRs and allows easy comparison among residues in the 7-TM segments of different receptor families.

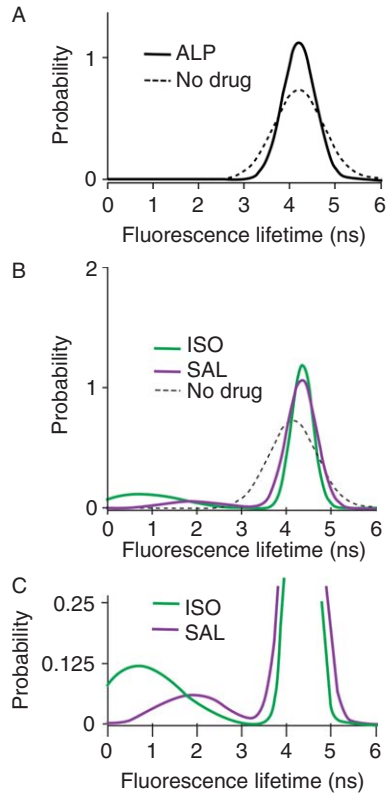

FIG. 4. Fluorescence lifetime distributions of β_2AR labeled at Cys265$^{6.27}$ with fluorescein maleimide (Ghanouni *et al.*, 2001a). (A) Single lifetime distributions are observed for unliganded receptor and receptor bound to the neutral antagonist alprenolol (ALP). (B and C) Two lifetime distributions are observed for β_2AR bound to the full agonist isoproterenol (ISO) and the partial agonist salbutamol (SAL). The short lifetime distribution for ISO is different from that for SAL, consistent with a different active conformation.

peptide has been shown to promote arrestin-dependent activation of ERK1/2, but not activation of Gq (Wei *et al.*, 2003). Moreover, the fully active state may differ for different G proteins (Kenakin, 2003). For a GPCR capable of activating more than one G protein, a drug may act as a full agonist toward one G protein and as a partial agonist toward another. To simplify the following discussion, the activity of a particular conformational state of a GPCR will be defined here by the effect that conformational state has on the activity of the receptor's cognate (or preferred)

G protein. Thus, a full agonist maximally activates the cognate G protein while an inverse agonists maximally inhibits any basal activation of the G protein by the receptor.

IV. Activation by Agonists

A. *Insights from Constitutively Active Mutants*

To understand the process of receptor activation, we must first understand the properties of the basal or nonliganded state of the receptor. As outlined above, some GPCRs, such as rhodopsin and the FSH receptor (Kudo *et al.*, 1996), have little or no detectable basal activity. Conversely, other GPCRs, such as cannabinoid receptors, exhibit a high degree of basal activity in the absence of ligands (Nie and Lewis, 2001; Sharma and Sharma, 1997). This basal activity could reflect an inherent flexibility of the receptor and, thus, a tendency to exist in more than one conformational state in the absence of ligands. It could also reflect a highly constrained state that has a relatively high affinity for a G protein. The concept of basal activity and receptor activation can be considered in terms of an energy landscape (Fig. 5). In the case of a receptor with low basal activity, in the absence of agonist, the receptor may be relatively constrained into one inactive conformational state having a deep energy well (Fig. 5A). High basal activity might be explained by a smaller energy difference between the inactive and active states, with a lower energy barrier (Fig. 5B). This might also be thought of as a receptor with greater conformational flexibility (i.e., fewer conformational constraints). Alternatively, it is possible that a receptor may exist in predominantly one constrained state that has intermediate activity toward its G protein (Fig. 5C). While both of these mechanisms may apply to different receptors, there is experimental evidence linking conformational flexibility and structural instability to elevated basal activity (Gether *et al.*, 1997a).

How are these concepts translated into receptor structure? TM domains are held in the basal state by intervening loops and noncovalent interactions between side chains. However, proteolysis and split receptor studies suggest that the noncovalent interactions appear to play a greater role in determining the specific basal arrangement of the TM segments relative to each than do some of the intervening loop structures. For example, the cotransfection of a plasmid encoding the N-terminus through TM5 with a plasmid encoding TM6 through the C-terminus [i.e., excluding the third intracellular loop (IC3)] generates a functional β_2AR (Kobilka *et al.*, 1988), where the fragments assemble and are held together by noncovalent interactions. Similar

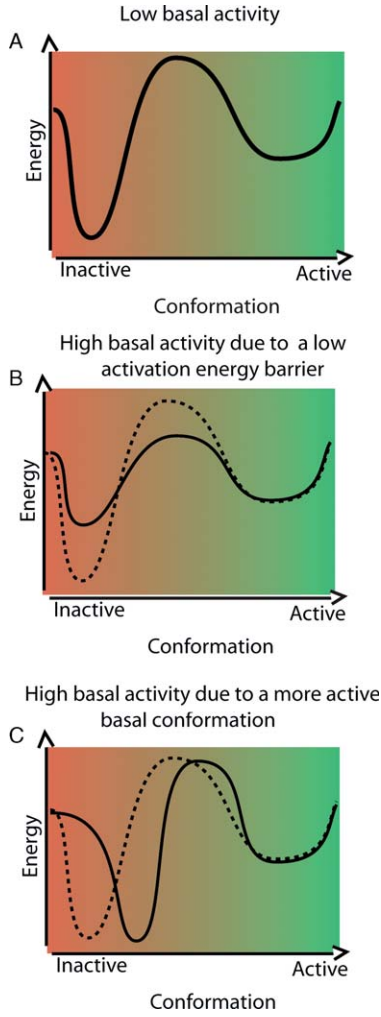

FIG. 5. Energy landscapes used to explain possible mechanisms of elevated basal activity. (A) Receptor with low basal activity. (B and C) Receptors with high basal activity, relative to panel A (shown as a dotted line). (B) The basal activity is elevated because of a reduced energy barrier separating the basal and active conformational states. (C) The basal activity is higher because the basal conformational state has a higher activity toward the G protein.

observations have been made for the muscarinic receptor. Schoneberg and Wess were able to generate functional M3 muscarinic receptors with discontinuity within the loop connecting TM3 and TM4, the loop connecting TM4 and TM5, and the loop connecting TM5 and TM6 (Schoneberg *et al.*, 1995).

Similarly, both α_2-adrenergic receptor (Wilson *et al.*, 1990) and the β_2AR (Swaminath and Kobilka, unpublished data) are capable of binding ligands after proteolytic cleavage of loop structures.

It has also been observed that the degree of basal activity can be dramatically enhanced by single point mutations in a variety of structural domains (Parnot *et al.*, 2002). These constitutive active mutations (CAMs) provide insight into the structural basis of basal activity and of receptor activation. For instance, the fact that CAMs can be generated in virtually any structural domain (Parnot *et al.*, 2002) suggests that in the inactive state the receptor structure is constrained by multiple intramolecular interactions that link TM segments or link TM segments with inter-TM segment loops. Thus, mutations that disrupt these interactions would increase the ''flexibility'' of the protein (movement of TM domains relative to each other) and the probability that the receptor can assume a more active conformation.

One might predict that mutations that lead to enhanced basal activity by disrupting intramolecular interactions could also lead to decreased structural stability. Mutation of Leu272$^{6.34}$ at the cytoplasmic end of TM6 in the β_2AR to alanine results in elevated basal activity (Samama *et al.*, 1993) as well as biochemical instability (Gether *et al.*, 1997a). Purified L272A β_2AR denatures two to three times faster than wild-type receptor (Gether *et al.*, 1997a). The increased basal activity observed in the native β_2AR at reduced pH is also associated with an increased rate of denaturation (Ghanouni *et al.*, 2000). These denaturation processes can be attenuated by both agonists and antagonists (Gether *et al.*, 1997a). Instability has also been reported in constitutive active mutants of the β_1-adrenoceptor (McLean *et al.*, 2002) and the H_2 histamine receptor (Alewijnse *et al.*, 2000). It is worth mentioning that ligands, both agonists and antagonists, can stabilize the receptor against denaturation and act as biochemical chaperones (McLean *et al.*, 2002; Petaja-Repo *et al.*, 2002), suggesting that they form stabilizing bridges between TM segments.

B. Molecular Switches

So what do CAMs tell us about GPCR activation? We have seen how active states can be achieved by destabilizing the normal arrangement of TM domains by mutations at several different sites. As discussed above, TM domains are held in the basal state primarily by a network of noncovalent interactions between side chains. Thus, any compound that disrupts one of the many intramolecular interactions that stabilize the basal state could have, in principle, agonist activity. The process of disrupting a stabilizing

intramolecular interaction can be thought of as activating a *molecular switch*. Importantly, these molecular switches can be activated by mutations or ligands. It is expected that for any given GPCR, there will be numerous molecular switches. Evidence for this is apparent in constitutively active mutants that can be further activated by agonists, suggesting that the mutation activated only one of several switches and that other switches still must be triggered by agonist binding. While some of these molecular switches will be specific for a given GPCR, common molecular switches have been proposed for members of the rhodopsin family of GPCRs. Two of these switches, a rotamer toggle switch in TM6 and an ionic lock between TM3 and TM6, are briefly described below in Section IV.B.1 and 2.

1. *Rotamer Toggle Switch*

Using site-directed mutagenesis studies and computer simulations, it has been suggested that rotameric positions of Cys285$^{6.47}$, Trp286$^{6.48}$, and Phe290$^{6.52}$ of the β_2AR are coupled, and modulate the bend angle of TM6 around the highly conserved proline kink at Pro288$^{6.50}$, leading to the movement of the cytoplasmic end of TM6 (Shi *et al.*, 2002) (Fig. 6). Authors

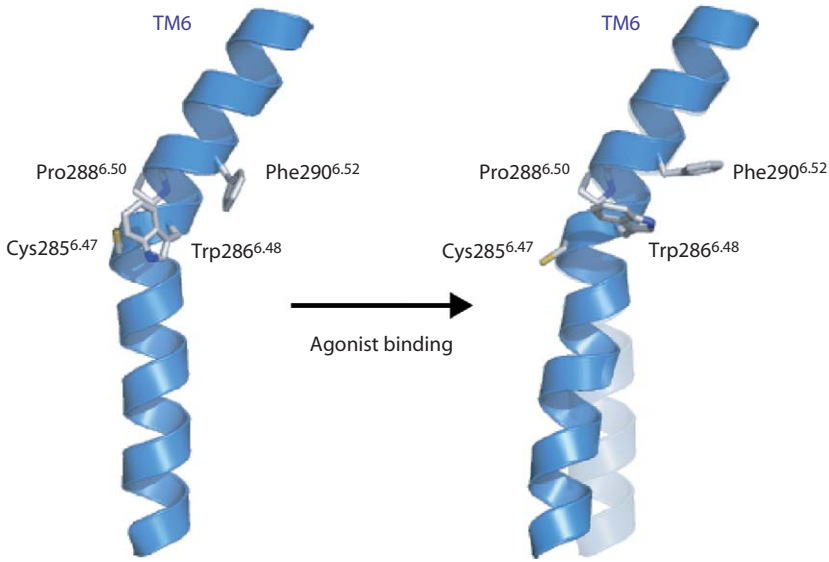

FIG. 6. The rotamer toggle switch. Agonist binding leads to changes in the rotameric states of aromatic amino acids in TM6 resulting in a change in the angle of the helical kink formed by the highly conserved Pro288$^{6.50}$.

also proposed that interactions between the aromatic ring of catecholamine agonists and Phe290$^{6.52}$ in TM6 play a role in the stabilization of the active form of this switch. While this mechanism was initially defined for catecholamine receptors, this sequence motif is highly conserved in amine and opsin receptors, so it is expected that this step in the activation mechanism will be conserved within these families.

2. *Ionic Lock*

Another molecular switch, the ionic lock, involves the interaction between Glu$^{6.30}$, highly conserved in amine and opsin receptors (>93%), and the Asp$^{3.49}$/Arg$^{3.50}$ pair, in the highly conserved (D/E)RY motif found in virtually all Class A GPCRs (Ballesteros *et al.*, 2001a) (Fig. 7). This ionic interaction is proposed to hold together the cytoplasmic ends of TM3 and TM6 in the resting state of different amine receptors (Ballesteros *et al.*, 2001a; Greasley *et al.*, 2002; Shapiro *et al.*, 2002). This interaction is also observed in the crystal structures of inactive rhodopsin (Li *et al.*, 2004; Okada, 2004; Okada *et al.*, 2002; Palczewski *et al.*, 2000; Teller *et al.*, 2001), and disruption of this interaction during activation is suggested by various biophysical (Farrens *et al.*, 1996; Gether *et al.*, 1997b), biochemical (Arnis *et al.*, 1994; Ghanouni *et al.*, 2000; Sheikh *et al.*, 1996, 1999), and mutagenesis (Alewijnse *et al.*, 2000;

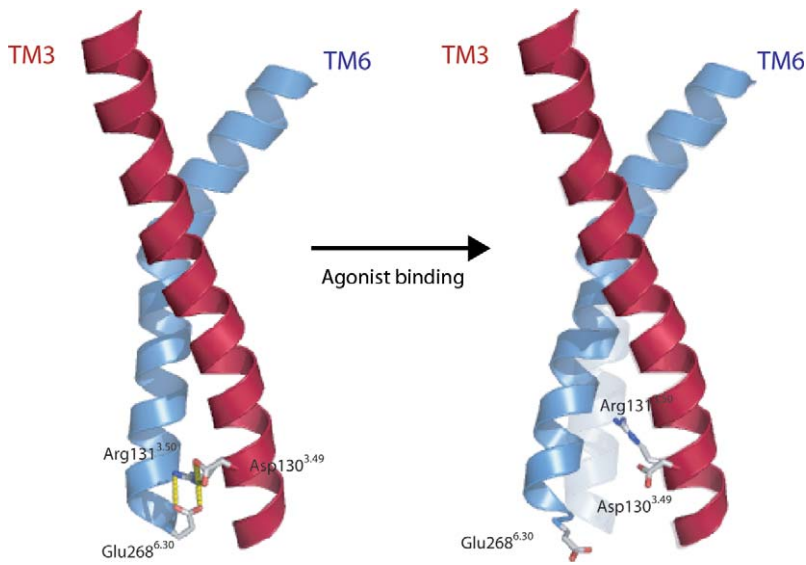

FIG. 7. The ionic lock stabilizes interactions between the cytoplasmic ends of TM3 and TM6 in the inactive state. Agonist binding disrupts these interactions.

Gaborik *et al.*, 2003; Kim *et al.*, 1997; Rasmussen *et al.*, 1999; Scheer *et al.*, 1996) studies. This ionic lock is key in the structure of the receptor, probably in combination with other inter- and/or intrahelical interactions, as it keeps TM6 in a relatively distorted conformation with a marked decrease of the helical twist at the level of the proline kink at Pro288$^{6.50}$. As a result, the cytoplasmic end of TM6 is much closer to TM3 than would be expected from the distortion induced solely by the proline.

C. Activation of Molecular Switches by Ligands

Figure 8 shows two possible ways that ligands may influence the arrangement of TM domains. Ligands may serve as bridges that stabilize new interactions between TM domains (Fig. 8A). In doing so, ligands may move specific TM domains closer to each other, push them further apart, or rotate one relative to the other. At the other end of the spectrum, ligands may act by simply disrupting existing intramolecular interactions (Fig. 8B). An example of an agonist binding to and displacing stabilizing interactions can be found in the AT1 receptor. Experimental evidence suggests that Asn111$^{3.35}$ interacts with Asn295$^{7.46}$ in TM7 to stabilize the inactive state of the receptor (Balmforth *et al.*, 1997). Other evidences suggest that Asn111$^{3.35}$ interacts with Tyr4 of angiotensin (Noda *et al.*, 1996). Thus, during activation, angiotensin would replace Asn111$^{3.35}$ as the interacting partner with Asn295$^{7.46}$. It has also been shown that

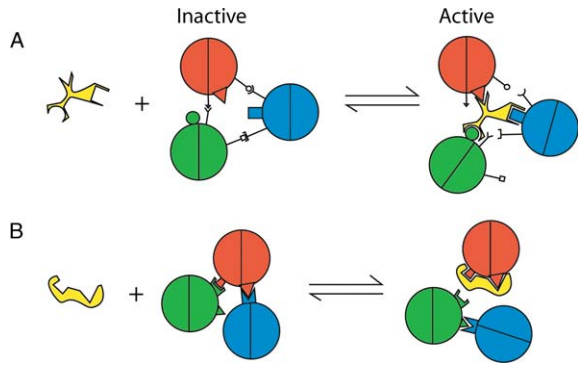

FIG. 8. Mechanisms by which agonist binding may change the relative arrangement of TM segments. (A) The agonist binding requires disruption of intramolecular interactions and the formation of new interactions with the ligand. (B) The agonist binds directly to amino acids involved in forming stabilizing intramolecular interactions.

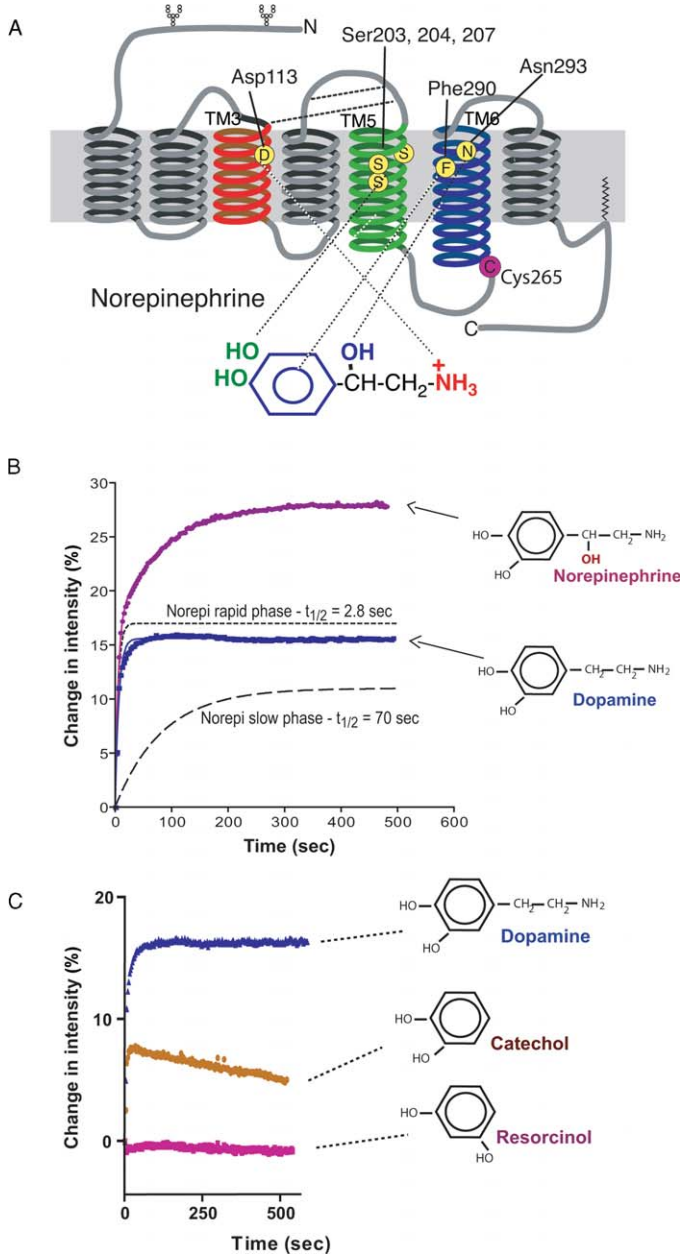

FIG. 9. Agonist-induced conformational changes in the β_2AR. (A) Sites of interaction between norepinephrine and the β_2AR identified by site-directed mutagenesis. The catecholamine nitrogen interacts with Asp113$^{3.32}$ in TM3 (Strader *et al.*, 1989a).

histamine binding to the H_1 receptor induces $Ser^{3.36}$ to interact with $Asn^{7.45}$ on activation (Jongejan *et al.*, 2005). It is likely that most ligands use a combination of mechanisms shown in Fig. 8.

D. Agonist Binding and Activation Is a Multistep Process

In both of the models shown in Fig. 8, the ability of the ligand to bind the receptor depends on the dynamic nature of the noncovalent interactions between TM segments. That is, agonist activation cannot be explained by a simple lock-and-key model, and there is no preformed binding site for the agonist (Del Carmine *et al.*, 2004; Liapakis *et al.*, 2004). In the basal state, sites of contact for the ligand either are not optimally aligned to bind all structural components of the agonist (Fig. 8A) or are involved in intramolecular interactions (Fig. 8B). Thus, interactions must break and reform on a timescale compatible with rapid binding and activation of receptors. In the case of the model shown in Fig. 8A, ligands may first bind to one interacting site and be poised to bind to a subsequent site on disruption of intramolecular interactions between TM segments. This would involve the formation of one or more intermediate conformational states.

E. The β_2AR as a Model System for Ligand Binding and Activation: Biophysical Analysis of Agonist-Induced Conformational Changes

The β_2AR is a good model system for studying agonist binding because the sites of interaction between catecholamine ligands and the β_2AR have been extensively characterized (Liapakis *et al.*, 2000; Strader *et al.*, 1989c; Wieland *et al.*, 1996) (Fig. 9A). In summary, the amine nitrogen interacts with

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Hydroxyls on the catechol ring interact with serines $203^{5.42}$ (Liapakis *et al.*, 2000), $204^{5.43}$, and $207^{5.46}$ (Strader *et al.*, 1989b) in TM6. The chiral β-hydroxyl interacts with $Asn293^{6.55}$ in TM6 (Wieland *et al.*, 1996) and the aromatic ring interacts with $Phe290^{6.52}$ in TM6 (Strader *et al.*, 1989c). Also shown is the relative position of $Cys265^{6.27}$, the labeling site for tetramethylrhodamine. (B) Agonist-induced conformational changes in purified β_2AR labeled with tetramethylrhodamine at $Cys265^{6.27}$. Conformational response to norepinephrine and dopamine was examined by monitoring changes in fluorescence intensity as a function of time. The response to norepinephrine was best fit with a two-site exponential association function, while there was no significant difference between a one-site and a two-site fit for the response to dopamine. The rapid and slow components of the biphasic response to norepinephrine are shown as dotted lines.

Asp113$^{3.32}$ in TM3 (Strader *et al.*, 1989a), the catechol hydroxyls interact with serines in TM5 (Liapakis *et al.*, 2000; Strader *et al.*, 1989b; Wieland *et al.*, 1996). Interactions with the aromatic ring and the chiral β-hydroxyl both have been mapped to TM6 (Wieland *et al.*, 1996).

Several studies investigating the process of catecholamine binding to the β_2AR provide evidence supporting a multistep process for agonist binding (Ghanouni *et al.*, 2001a; Swaminath *et al.*, 2004, 2005). For instance, fluorescence lifetime studies reveal the existence of at least one intermediate conformational state in the presence of the full agonist isoproterenol or partial agonists dobutamine and salbutamol (Ghanouni *et al.*, 2001a) (Fig. 4C). The existence of an intermediate conformational state can also be demonstrated kinetically in the β_2AR. In these studies, β_2AR was labeled at Cys265$^{6.27}$ at the cytoplasmic end of TM6 with tetramethylrhodamine (TMR-β_2AR). Based on homology with rhodopsin, Cys265$^{6.27}$ is located in the IC3 at the cytoplasmic end of the TM6. Mutagenesis studies have shown this region of IC3 to be important for G protein coupling (Liggett *et al.*, 1991; O' Dowd *et al.*, 1988). Moreover, TM6, along with TM3 and TM5, contains amino acids that form the agonist-binding site. Thus, an environmentally sensitive fluorophore covalently bound to Cys265$^{6.27}$ is well positioned to detect agonist-induced conformational changes relevant to G protein activation. Using this experimental system, agonist-induced conformational changes can be observed by monitoring fluorescence intensity of TMR-β_2AR over time. Our results show how binding of the catecholamine norepinephrine results in curve that is best fit by a two-site exponential association function (Fig. 9B) (Swaminath *et al.*, 2004). This suggests that on catecholamine binding, β_2ARs undergo transitions to two kinetically distinguishable conformational states through a combination of a fast and a slow conformational change. Using a panel of chemically related catechol derivatives, we identified the specific chemical groups on the agonist responsible for the rapid and slow conformational changes in the receptor. In the presence of catechol and dopamine, only rapid conformational changes were observed (Fig. 9B and C). In contrast, both rapid and slow conformational changes were observed on binding to norepinephrine, epinephrine, and isoproterenol. These results suggest that formation of interactions between the catechol ring and the amine group of the ligand with the Ser residues in TM5 and Asp113$^{3.32}$ occurs rapidly, while interactions between the β-hydroxyl of the ligand and Asn293$^{6.55}$ occur more slowly, possibly due to the need to overcome a strong stabilizing intramolecular interaction.

Interestingly, the conformational changes observed in these biophysical assays were correlated with biological responses in functional assays. Dopamine, which induces only a rapid conformational change, is efficient at activating Gs but not receptor internalization. In contrast, norepinephrine

and epinephrine, which induce both rapid and slow conformational changes, are efficient at activating Gs and receptor internalization.

The process of activation by dopamine can be further dissected using catechol alone as a ligand. As discussed above, the catechol ring of catecholamines is predicted to interact with serines in TM5 and the aromatic residues of the rotamer toggle switch in TM6 (Fig. 9A). We found that catechol alone was able to induce a rapid conformational change in TMR-β_2AR similar to the response observed with dopamine (Fig. 9C). Moreover, we found that catechol is a weak partial agonist (Swaminath *et al.*, 2005). Both ligand binding and conformational studies demonstrated that catechol occupied the same binding space as the catechol component of catecholamines (Swaminath *et al.*, 2005). Based on these observations, we speculate that catechol binding is sufficient to activate the rotamer toggle switch, inducing the fast change in fluorescence, but not other molecular switches required for full activation. Catechol has a remarkably high affinity ($K_D = 160$ μM, based on a conformational assay) considering its size (formula weight 110), which is consistent with an agonist fragment where a high proportion of the catechol atoms are involved in binding interactions with the receptor. Moreover, the relatively high binding affinity suggests that energetic cost of the conformational changes required for optimal interactions between the β_2AR and catechol is small.

The binding of the catechol ring of dopamine results in the same structural change that occurs on binding of catechol alone, but the interaction between the amine group and Asp113$^{3.32}$ also stabilizes a specific arrangement of TM3 relative to TM5 and TM6. This additional conformational change imparts a much greater activity toward Gs. Note that binding affinity for dopamine ($K_i = 350$ μM) is similar to that for catechol. This is surprising considering that the interaction between the primary amine and Asp113$^{3.32}$ makes the strongest contribution to the binding energy. Part of the binding energy associated with the interaction between dopamine and Asp113$^{3.32}$ might be offset by the energetic cost of the conformational change needed for the binding interaction to occur. Thus, in the inactive state, TM5 and TM6 are positioned such that little energy is needed to accommodate the binding of the catechol ring. In contrast, the movement of TM3 relative to TM5 and TM6 required for binding of dopamine may involve breaking of intramolecular interactions, thereby consuming part of the energy provided by the ionic interaction between the ligand and the receptor. Evidence from unpublished studies suggests that this added energy is required to disrupt the ionic lock.

Using the information obtained through these biophysical studies on the β_2AR (Ghanouni *et al.*, 2001a; Swaminath *et al.*, 2004, 2005), we proposed a model whereby agonist binding and activation occur through a series of conformational intermediates (Fig. 10). Within this model, catechol and

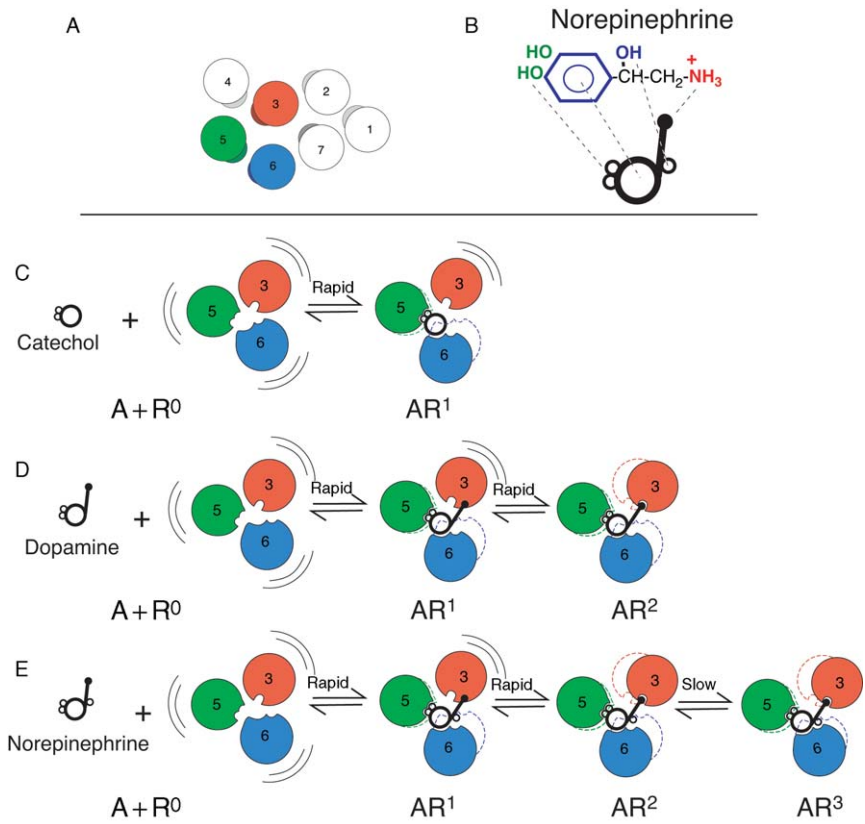

FIG. 10. Sequential binding model. (A) Arrangement of the TM domains of the β_2AR as viewed from the extracellular surface. The agonist-binding domains are shown in red (TM3), green (TM5), and blue (TM6). (B) Diagram representing structural components of norepinephrine. (C–E) In the absence of ligand, the receptor (R) is conformationally flexible. Conformational state R^1 is stabilized by interactions between TM5 and TM6 and the catechol ring. The transition to state R^2 occurs when Asp113$^{3.32}$ in TM3 binds the amine nitrogen. The transitions from R to R^2 are rapid. The slow transition from R^2 to R^3 involves interactions between the chiral β-hydroxyl and Asn293$^{6.55}$ on TM6.

dopamine can be considered as fragments of full catecholamine agonists (for instance, norepinephrine), which are capable of stabilizing intermediate states that have only partial activity.

Evidences for intermediate conformational states have also been observed in other receptors. Time-resolved peptide-binding studies on the neurokinin receptor revealed that an agonist peptide binds with biphasic

kinetics. The rapid binding component was associated with a cellular calcium response while the slow component was required for cAMP signaling (Palanche *et al.*, 2001). These results support a mechanistic model for GPCR activation where contacts between the receptor and structural determinants of the agonist stabilize a succession of conformational states with distinct cellular functions.

V. Concluding Remarks

This chapter has addressed activation of GPCRs using data from only a small subset of rhodopsin family members in which the agonist-binding site is formed by the TM segments. At the other end of the spectrum are receptors for glycoprotein hormones and the glutamate family of receptors in which the ligand-binding site is found within a large N-terminal domain. Nevertheless, glycoprotein hormone receptors can be activated by mutations within TM segments, and glutamate receptor activity can also be modulated by small organic compounds that bind within the TM segments (Pin *et al.*, 2003), suggesting that agonist binding ultimately leads to the disruption of interactions that stabilize the arrangement of the TM segments. In the case of the glycoprotein hormones, evidence suggests that part of the N-terminus interacts with sequence between TM4 and TM5 to stabilize the inactive state and that this interaction is disrupted by agonist binding (Nishi *et al.*, 2002; Yi *et al.*, 2002). In the glutamate family, receptors are homodimers held together by the N-terminal Venus flytrap domain. Agonist binding leads to large structural changes in the Venus flytrap motifs and would be predicted to alter the relative arrangement of the 7-TM segments from each monomer (Pin *et al.*, 2003; Tateyama *et al.*, 2004). This may in turn alter the orientation of the TM segments within each monomer. Therefore, the mechanism linking ligand binding to receptor activation for these receptors is likely to be more complex (Pin *et al.*, 2003; Yi *et al.*, 2002).

While we have learned a great deal about GPCR structure and the cellular signaling pathways activated by GPCRs over the past 20 years, much remains to be learned about the mechanism of activation of this fascinating family of membrane proteins. A better understanding of the complex process of agonist binding and activation may facilitate the design of more effective and selective pharmaceuticals.

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