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Advances in Methods to Characterize Ligand-Induced Ionic Lock and Rotamer Toggle Molecular Switch in G Protein-Coupled Receptors

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Abstract

Structural biology of GPCRs has made significant progress upon recently developed technologies for GPCRs expression/purification and elucidation of GPCRs crystal structures. The crystal structures provide a snapshot of the receptor structural disposition of GPCRs itself or with cocrystallized ligands, and the results are congruent with biophysical and computer modeling studies reported about GPCRs conformational and dynamics flexibility, regulated activation, and the various stabilizing interactions, such as “molecular switches.” The molecular switches generally constitute the most conserved domains within a particular GPCR superfamily. Often agonist-induced receptor activation proceeds by the disruption of majority of these interactions, while antagonist and inverse agonist act as blockers and structural stabilizers, respectively. Several elegant studies, particularly for the β 2AR, have demonstrated the relationship between ligand structure, receptor conformational changes, and corresponding pharmacological outcomes. Thus, it is of great importance to understand GPCRs activation related to cell signaling pathways. Herein, we summarize the steps to produce functional GPCRs, generate suitably fluorescent labeled GPCRs and the procedure to use that to understand if ligand-induced activation can proceed by activation of the GPCRs via ionic lock switch and/or rotamer toggle switch mechanisms. Such understanding of ligand structure and mechanism of receptor activation will provide great insight toward uncovering newer pathways of GPCR activation and aid in structure-based drug design.

1. INTRODUCTION

G protein-coupled receptors (GPCRs) are among the largest and most versatile membrane protein receptors in eukaryotes and yeasts (Fredriksson, Lagerstrom, Lundin, & Schioth, 2003). Versatility of GPCRs can be attributed to their large number, diverse physiological roles, and the complex and regulated signal transduction across the plasma membrane (Kobilka & Deupi, 2007). These membrane receptors as key signal transduction players have been implicated in a huge number of pathophysiological conditions, including neurological, immunological, and hormonal disorders (Fredholm, Hokfelt, & Milligan,

2007). Ligands binding to these receptors include hormones, small peptides, neurotransmitters, and endogenous metabolites. Despite the large diversity in physiological roles and associated pathological conditions, these receptors share a high degree of homology in their seven transmembrane structures (Fredholm et al., 2007). Structural differences among these are much more pronounced in their N- and C-terminal domains in terms of both the size and the secondary structures of these domains.

In spite of structural diversity, researchers in the field of GPCR biology have observed a few common mechanisms of GPCR activation. Also many GPCRs (except a few, e.g., Rhodopsin and follicle-stimulating receptors) have a baseline level of constitutive activity (Kudo, Osuga, Kobilka, & Hsueh, 1996). Ligand-induced receptor activation, resulting from agonist or partial agonist binding, results in receptor conformational changes reflecting different stages of receptor activation. Differences in activation affect the nature and extent of pharmacological outcome. Other ligands, for example, antagonist and inverse agonist are blockers or conformational stabilizers, respectively, for the inactive state of the GPCRs. These subtle regulations of receptor activation result in the generation of several activation states and does not fit a simple ON/OFF model (Deupi & Kobilka, 2007; Kobilka & Deupi, 2007). Understanding the underlying mechanisms of this process has enabled us to distinguish the molecular events at the ligand receptor interface that account for the variability in receptor response. Correlation of ligand structures with the receptor activation pathways has revealed ligand pharmacophore features. Several stabilizing interactions within the GPCR transmembrane helical bundles, referred to as “molecular switches,” mediate the regulated activation (Nygaard, Frimurer, Holst, Rosenkilde, & Schwartz, 2009; Schwartz, Frimurer, Holst, Rosenkilde, & Elling, 2006; Trzaskowski et al., 2012).

Further, the transmembrane segments have a greater role than the extracellular and intracellular loops in stabilizing the receptor. For example, in Rhodopsin, the receptor is stabilized by interactions of Glu 134 with Arg 135 in the TM3 and of Glu 247 with Thr 251 in the cytoplasmic end of the TM6 (Stenkamp, Teller, & Palczewski, 2002). Stabilizing interaction of the cytoplasmic end of the TM3 with the TM6 is known as the “ionic lock” (Fig. 7.1). Disruption of the “ionic lock” (stabilizing interaction) leads to receptor activation. Amino acids, which constitute the ionic lock, are highly conserved throughout the Rhodopsin family. Mutation of these key amino acid residues which stabilize the receptor in the inactive state generates constitutively active mutants. The best characterized is the mutation of the highly conserved (D/E)R(Y/W) motif in the members of the Rhodopsin GPCR family. The ionic lock is the ionic interaction of the conserved Asp-Arg-Tyr (DRY) motif of the Rhodopsin family GPCRs with the negatively charged amino acid Asp (D) or Glu (E) (Trzaskowski et al., 2012). These residues are positioned near the cytoplasmic domains of the transmembrane helix 3 and 6, respectively. The interaction of the positively charged guanidium group of the Arg with the negatively charged carboxylic acid group of Aspartic or Glutamic acid is the ionic lock interaction. This has also been demonstrated in the beta 2 adrenergic receptor (β 2AR) (Yao et al., 2006), H2 histamine receptor (Alewijnse et al., 2000), α_{1b} adrenoceptor (Scheer, Fanelli, Costa, De Benedetti, & Cotecchia, 1996, 1997), D3 dopamine receptor (Chien et al., 2010), and the angiotensin (AT1) receptor (Gaborik et al., 2003). This method of receptor activation is referred to as “Ionic Lock disruption” (ILD).

X-ray crystal structure of the β 2AR (Cherezov et al., 2007) and of the turkey β 1AR (Warne et al., 2008) have indicated the presence of the conserved Trp 286 and Trp 303, respectively, which relays ligand interaction in the receptor transmembrane bundle to conformational change in the cytoplasmic domains. This interaction is known as *rotamer toggle switch* (Fig. 7.1). Activation through the rotamer toggle switch was initially reported for Rhodopsin (Bhattacharya, Hall, & Vaidehi, 2008). This is mediated around the conserved Proline

residue in the TM6 of these receptors. For example, the β 2AR mutation of Leu 272 to Alanine in the cytoplasmic end of the TM6 greatly increases receptor basal activity and instability (Gether et al., 1997). Monte Carlo simulation studies have indicated that in the β 2AR, the amino acid residues Cys 285, Trp 286, and Phe 290 (CW $\times$ P motif) constitute the aromatic triad and ligand interaction in this region can couple to and modulate the bend angle kink at the Pro 288 leading to receptor activation (Shi et al., 2002). Rotational toggle of conserved residues also activated agonists in histamine H1 receptors (Jongejan et al., 2005), D2 dopamine receptors (Van Arnam, Lester, & Dougherty, 2011), and trace amine-associated receptors (Tan, Groban, Jacobson, & Scanlan, 2008). Several computer modeling studies with the cannabinoid (CB) receptor were reported. Monte Carlo simulations determined the ligand-induced structural changes around the “aromatic microdomain” of the CB1. These changes refer to the microdomain formed by the aromatic residues from TM3 to 6 (McAllister et al., 2003). Residues Phe 201 and Trp 357 are the toggle pair switches to maintain the receptor in the inactive state (McAllister et al., 2004). Results from this and related studies indicate that the F201/W357 interaction stabilizes the receptor, which can be disrupted by several subclasses of ligands that activate the CB1 receptor. Such detailed molecular dynamics simulation studies were performed with the CB2, strongly argue for the presence of the rotamer toggle switch and other ligand binding domains (Lynch & Reggio, 2006; Xie, Chen, & Billings, 2003). In Rhodopsin family GPCRs, several other molecular switches have been proposed and proven. For example, in Rhodopsin, the isomerization of 11 *cis* Retinal to all *trans* Retinal disrupts the ionic Schiff’s base interaction between the Glu in the TM3 and the lysine in the TM6 (Zhou, Melcher, & Xu, 2012). In several other GPCRs, different pairs of amino acid residues compose the 3–7 lock switch (Fig. 7.1). Recently, many other GPCRs have shown the presence of the 3–7 lock. Examples are the histamine H1 (Shimamura et al., 2011) and dopamine D3 (Chien et al., 2010) receptors as summarized in Table 7.1.

Moreover, it is known that ligand-induced receptor activation proceeds through disruption of one or all interaction stabilizing the receptor in its inactive state (Hurst et al., 2010). Antagonist binding will simply block all ligand binding at that site and prevent binding of the agonist. Inverse agonist ligands will, however, stabilize the receptor in its inactive conformation in a way reinforcing the molecular switches. Receptor activation takes place upon agonist binding by the disruption of some or all stabilizing interactions that hold it in its inactive state. Hence, changes in the particular stabilizing interactions can be monitored within the GPCR by selectively observing conformational changes at the stabilizing points.

A series of well-designed studies have systematically determined ligand-induced effects in several stabilizing interactions, particularly in the β 2AR (Swaminath et al., 2004; Yao et al., 2006). In this chapter, we describe a general guideline of experimental procedures to determine receptor activation induced by ligands. We summarize methods to generate mutated, purified, and functional GPCR receptors to determine if receptor activation can take place by ILD or rotamer toggle switch activation.

2. EXPERIMENTAL PRINCIPLES

We briefly summarize here the entire experimental procedure to study effects of ligands on the GPCR Ionic Lock and rotamer toggle switch. The procedure is divided into three parts. First is the generation of suitable receptors for the biophysical studies. Second is to determine effects induced by ligands on the receptor ionic lock. Third is to determine activation of receptors by rotamer toggle switch.

2.1. Generation of chimeric GPCR to determine the mechanism of receptor activation

To determine the effect of agonist, partial agonist, antagonist, and inverse agonist binding on the ionic lock switch and the rotamer toggle switch of the GPCR, it is necessary to determine whether ligand dose-dependently changes the receptor conformation. A purified and functional receptor, reconstituted in lipid bilayer, therefore needs to be generated. Flag and histidine-tagged GPCR, expressed in Sf9 cells, purified by detergent extraction and chromatographic separation, provide the recombinant receptor for the biophysical studies. Pharmacological properties and subcellular localization of the recombinant GPCR should be verified prior to purification and after purification and reconstitution in the lipid bilayers. The verification determines if the receptor maintains all properties of the wild-type (WT) GPCR (receptor expressed on transfected mammalian cells) and no structural and functional differences that arise from its expression in Sf9 membrane and from its purification and reconstitution in lipid bilayers. Selective labeling of specific reactive cysteine residues in the juxtamembrane positions of the GPCR can be done by mutating and removing other reactive cysteines within the receptor accessible regions. For example, in the β 2AR, the mutations A271C and I135W were carried out to determine ligand-induced effects on ionic lock. Trp(135) served as a fluorescence quencher of monobromobimane to determine the activation of ionic lock (Yao et al., 2006). In another study, Cys 265 was labeled with fluorescein maleimide or tetramethyl maleimide for the investigation of receptor activation by rotamer toggle switch (Swaminath et al., 2004). These mutants were expressed and purified from the Sf9 cells. All labeling was performed on the mutated receptor reconstituted in lipid bilayers. To determine if labeling can change the receptor structure and function, ligand displacement assay and GTP γ -binding assay are needed to ensure integrity of the receptor function. If all pharmacological properties are preserved, these receptors can then be used to determine the effects of the ligand on the receptor Ionic Lock and rotamer toggle switch.

2.2. Determination of ligand-induced effects on the receptor ionic lock

The Ionic Lock is formed and is intact in inactive GPCRs by the ionic interaction of the Arg (R) residues from the conserved DRY motif with the Asp/Glu in the TM6. Monobromobimane (MBB)- labeled GPCR can be suitably used to detect the disruption of the Ionic Lock (Fig. 7.2). For example, in the absence of any activating ligand, the ionic lock stays intact and maintains a distance between the TM3 and the TM6, separating the MBB from the Trp. The MBB has strong emission signal at 400 nm. When the ionic lock is activated and disrupted by the ligand, the MBB and Trp move closer to each other, thereby decreasing the intensity of the MBB emission signal by fluorescence quenching. A dose-dependent decrease of MBB signal intensity should be observed which will saturate at a given ligand concentration.

2.3. Determination of ligand-induced effects on the receptor rotamer toggle switch

Probing into the receptor activation by the rotamer toggle mechanism was accomplished by placing fluorophore tetramethylrhodamine maleimide (TMRM) at Cys 265 the cytoplasmic end of the TM6 in the β 2AR (Fig. 7.2). Activation of the receptor by the rotational toggle will lead to conformational change in the cytoplasmic domain of the TM6 and subsequently in the intracellular loop 3 (IL3) leading to receptor activation. It was observed that the aromatic ring of catechol and catecholamine derivative ligands is the necessary and sufficient parameter for RTS activation in the β 2AR. However, ligands without the catechol ring and otherwise capable of activating the receptor show no effect in the change of TMRM fluorescence (Kobilka & Deupi, 2007; Swaminath et al., 2005, 2004). Mutated receptor bearing TMRM at Cys 265 do not show any difference in radioligand binding compared to the WT receptor.

To conduct the studies described above, the corresponding experiments are suggested as the following protocols.

3. DETAILED EXPERIMENTAL PROCEDURE

3.1. Generation of chimeric GPCR for the determination of the mechanism of receptor activation

3.1.1 Expression and purification of GPCR using Baculovirus-infected insect cell lines—Baculovirus-infected insect cell lines (particularly Sf9) are suggested here as they have many advantages for GPCR overexpression. Although *Escherichia coli* has obvious advantages of fast and low-cost production of GPCR and/or GPCR fragments, receptor may not be translocated to the membrane and hence render nonfunctional. Substantial success has been achieved for the production of the GPCR fragments in the *E. coli* (Chowdhury et al., 2012; Xie, Zheng, & Zhao, 2004; Zhang & Xie, 2008; Zheng et al., 2005). Expression and posttranslational modification of recombinant GPCR is similar to that of mammalian cells, and GPCR produced are almost identical in insect and mammalian cells except for some minor differences. Compared to mammalian cells, these cells can be cultivated much easily and scaled up at much cheaper costs. Steps to produce recombinant biologically active GPCR from Sf9 cells are listed as follows.

1. *Generation of expression vector for GPCR production in Sf9 cells.* We recommend the Invitrogen Bac to Bac system for GPCR expression, however any other related products or in-house procedures may be equally competent or better. The procedures listed here are similar to those in the *Invitrogen Bac to Bac Baculovirus Expression System*. cDNA encoding the GPCR gene should be cloned into the pFastBac vector. Two different fusion tags with the GPCR will allow two separate chromatographic purifications on the same protein. Generated pFastBac vector (sequence confirmed) should be transformed to the *E. coli* DH10 Bac using the standard heat and cold shock procedure. The DH10 Bac allows the recombination of the GPCR from the pFastBac (donor plasmid) to generate a high molecular weight Bacmid DNA. Healthy and log phase Sf9 cells are the best for the transfection procedure. For transfection of the Sf9 with the Bacmid, the cell number should be approximately 2×10^6 cells/ml. Following, stain the cells with Trypan Blue (cell viability should be ~95%). Follow manufacturer's instructions to transfect the Sf9 cells and generate P1, P2, and P3 viral stocks.
2. *Preparation of membrane fractions.* Harvested Sf9 cells should be subjected to membrane lysis by a nitrogen cavitation pump in a hypotonic buffer (10 mM HEPES (pH 7.5), 20 mM KCl, and 10 mM MgCl₂) (Xu et al., 2011, supplementary materials). Wash isolated membranes in high osmotic buffer (1.0 M NaCl, 10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl), supplemented with protease inhibitor cocktail (1:100 dilution). Washed membrane fractions should be resuspended in the same buffer by Dounce Homogenizer. The soluble proteins and the membrane-bound proteins will now be separated in the soluble fraction and pellet, respectively. Resuspend the pellet in buffer containing the concentrated membrane fraction in 10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, and 40% glycerol (base buffer) and flash-freeze with liquid nitrogen and store at -80°C until further use. Verification of receptor expression and characterization will be done as described later before carrying out purification of the recombinant GPCR.
3. *Purification of recombinant protein.* Receptor isolated by this procedure will be subjected to methods that have been described in following sections to determine structural and functional intactness of the receptor produced. As reported (Krepkiy, Gawrisch, & Yeliseev, 2007), it is recommended to use the detergent cocktail

mixture such as 0.5% w/v *n*-dodecyl- β -D-maltopyranoside (DDM), 0.01% w/v cholesteryl hemisuccinate (CHS) supplemented in the base buffer (solubilization buffer), and incubate the membrane fractions for extractions at 4 °C for the solubilization of the membrane proteins. Solubilized membrane proteins will first be purified by immobilized metal affinity chromatography (IMAC) using a Ni²⁺/Co²⁺ column and the specific protein will be analyzed by SDS PAGE and Western Blotting using anti-His/GPCR/Flag Ab. Following IMAC, the proteins will be loaded into antibody column for the purification of the Flag-tagged protein. Such purifications can be performed with M2-Flag column, and analysis of protein purity and characterization should be done after purification. Successive rounds of affinity purification will allow increasing the protein homogeneity and the two tags at the two terminals of the recombinant protein will allow ensuring that only intact recombinant proteins are isolated. Purified and concentrated recombinant GPCR will be incubated with the TEV protease according to the manufacturer's protocol. The eluted protein will be subjected to concentration by using the 10 kDa molecular weight cut-off concentrator (Amicon) which will first concentrate the protein of interest and second remove contaminants and salts of lower molecular weights and will then be further purified by SEC (Sephadex column). The concentration of the purified receptor is then estimated by the BCA assay and by receptor saturation binding assay.

3.1.2 GPCR Reconstitution in liposomes and validation of receptor structure–function Integrity

1. *Preparation of proteoliposomes.* Reconstitution of the purified receptor within lipidic micelles generates a membrane mimetic environment. The receptor contained within the lipid should be in its native state and should show ligand binding and G protein coupling. The procedure to reconstitute the GPCR to functional form within the liposomes has been reported in detail (Krepkiy et al., 2007; Oates & Watts, 2011; Yeliseev, Wong, Soubias, & Gawrisch, 2005; Yoshiura et al., 2010). Use lipid mixture 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) (Berger et al., 2010). Recombinant protein from the size exclusion chromatography should be subjected to extensive washings through the detergent removing resin which will remove detergent (or detergent micelles if used above CMC), however, will not be able to completely remove the lipidic cholesterol component. Proteoliposomes will be prepared by mixing purified GPCR (containing CHS) with the lipid bolus (POPC/POPS). The proteoliposomes obtained from this step can now be subjected to receptor saturation binding assay by incubating the proteoliposomes with successive dilution of [³H]-Ligand. Modification of the ratio of the protein/lipid complex and other parameters will be done based on the results of the receptor saturation binding assay. Proteoliposomes once prepared can then be subjected to analysis of receptor pharmacological properties to ensure its native structure and function as described in the next section.
2. *Determination of GPCR subcellular localization.* Expression and localization of the recombinant receptor in the Sf9 cells can be verified by various methods. We recommend the use of Western blot and immunofluorescence (IF) techniques to determine the expression and localization of the recombinant receptor in the Sf9 cells. Membrane fractions are prepared from the Sf9 cells by a standard procedure that has been reported very well (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). Western blotting of the membrane fraction lysates with anti-His, anti-GPCR, and anti-Flag antibodies (considering the construct has Flag

and His tags) confirms expression of the intact recombinant protein. For best confidence, we recommend stripping and reprobing the same membrane with each antibody one by one. Next, to also confirm the membrane-targeted expression of the protein of interest, carry out IF assay. Fixed Sf9 cells can be probed with anti-Flag Ab and fluorescent dye-conjugated secondary antibody to determine and confirm the subcellular location of the recombinant protein. Cells may also be stained with DAPI to visualize and locate the nucleus and further confirm the membrane-targeted expression of the recombinant receptor. Consult any general IF protocol for more information on optimization. Pharmacological properties of the recombinant receptor in membrane fractions can be verified by the following methods.

3.1.3 Determination of recombinant and reconstituted receptor pharmacological properties

1. *Assay for competitive ligand displacement.* The assay procedure has been described in Zhang et al. (2011). Briefly, estimate the protein content in proteoliposomes and incubate small amounts of liposomes (protein amounts ~1–2 µg) with ~4 nM [³H]-ligand with increasing concentration of unlabeled ligand in a 96-well plate to a total reaction volume of 200 µl. Incubate reaction at 30 °C for 1 h (with mild shaking) and then harvest to Perkin Elmer 96-well GF/C filter plates using Perkin Elmer Filter Mate Harvester (or any other suitable 96-well plate harvester). GF/C plates should be dried overnight, soaked in liquid microscint liquid, and read in a Perkin Elmer top count reader. Binding data should be normalized to define 0–100% and then analyzed in Graph Pad Prism 5.0 software using one point receptor competitive displacement curve fitting. Depending on the molecular weight of the receptor or the harvester machine GF/B, plates may be alternatively used for the harvesting purpose.
2. *GTP gamma assay.* A general method is first to incubate proteoliposomes (~10–15 µg) with receptor agonist and [³⁵S] GTPγS. Then, add purified G_{ai} to the reaction mixture to allow G protein binding. The reaction mixture should be harvested and radioactivity of GF/C (or GF/B) plates will be read in the same procedure as previously described for the ligand binding assay. It is necessary to normalize the plate for [³⁵S] and set up the plate to the [³⁵S] counting mode.

The optimal condition for generating physiologically relevant reconstituted GPCR is to begin with approximately 95% receptor localization in the Sf9 cell membrane. This increases the probability of obtaining functional receptor in the subsequent steps. Receptor localized in the membrane should have pharmacological properties similar to the WT receptor from transfected mammalian cells. These pharmacological characteristics should also be preserved in the purified and reconstituted receptor. Also, of note, ligands of different pharmacological profiles, for example, agonist, antagonist, and inverse agonists, should be used to test for receptor activity.

3.1.4 Generation of mutated receptor suitable for fluorescence labeling and biophysical studies—To determine if ligand activation can proceed through the ILD and the rotamer toggle switch mechanism, it will be necessary to generate fluorescently labeled receptor wherein the change of fluorescence intensity will act as reporter of receptor conformational change. In the previous section, we have discussed the steps to produce recombinant receptor and reconstitute in lipid bilayer. In this section, we will discuss the steps involved in the design and generation of mutant GPCR which would be suitable for fluorescence labeling. The exact selection of the amino acid residues mentioned here are dependent on the particular GPCR.

1. *Site-directed mutagenesis of the GPCR.* Site-directed mutagenesis of the GPCR can be performed directly on the pFastBac vector backbone. Mutagenesis can be conveniently done with any kit-based protocol (e.g., Stratagene Quick Change). Optimization of the process should be done by following steps from the manufacturer's protocol. To ensure higher success for generating mutant GPCR, we recommend the use of high-fidelity DNA polymerase enzymes (Pfu/Pfx). cDNA encoding mutant receptors once generated should be confirmed by automated DNA sequencing and then will be used to express and purify mutated receptor in the Sf 9 cells as mentioned in the sections before.
2. *Selection of diverse ligands to carry out receptor activation.* To determine the role of the ionic lock switch and the rotamer toggle switch in receptor activation, it will be necessary to monitor ligand-induced effects on these interactions. However, to confirm the stabilizing role of these interactions, it will be necessary to observe the differential effects of full agonist, partial agonist, antagonist, and inverse agonist on these stabilizing interactions. As described in Yao et al. (2006), a partial agonist, for example, catechol was used for the β 2AR as it may preferably interact with the ionic lock or rotamer toggle switch; on the other hand, a full agonist will disrupt all stabilizing interactions.

3.2. Determination of ligand-induced effects on the disruption of GPCR ionic lock

To verify whether a ligand can activate the GPCR by disruption of the ionic lock, reactive cysteine (indicated by *Cys) residue on the GPCR backbone with all other reactive cysteines removed will be labeled with monobromobimane (MBB) at *Cys (Fig. 7.2). Procedures for generating suitably mutated and fluorescence-labeled receptors to investigate disruption of ligand-induced ionic lock have been described previously in this chapter. Following receptor modification, radioligand binding assay ensures structural and functional integrity of the receptor. The following nine steps determine effects of the ligand on the ionic lock. These methods have been reported for the β 2AR (Yao et al., 2006).

1. It has already been reported that MBB shows maximum absorption/emission peak at 370/460 nm, respectively (Yao et al., 2006). However, it will be necessary to carry out a wavelength scan from $\sim\pm 100$ nm to account for any changes the receptor microenvironment may have on the bimane fluorescence.
2. Prepare ligand concentration gradients to include $10^{\pm 5}$ Log [ligand concentration] around their respective EC_{50} values. An adequate window thereby is provided for ligand-induced activation and saturation in the assay. The ligand can be diluted in the assay buffer (50 mM Tris-HCl, 2.5 mM EGTA, 5 mM $MgCl_2$ supplemented with 3 mg/ml BSA) to prepare respective dilutions.
3. It will be necessary to carry out a wavelength scan from $\sim\pm 100$ nm to account for any changes the receptor microenvironment may have on the bimane fluorescence. This should be performed in two steps. First, determine the optimum amount of MBB-receptor liposomes that should be used for the assay. Fluorescence scanning of GPCR-MBB in different amounts (~ 5 –50 fmol) should be used in different wells to determine the optimum receptor concentration for the highest signal to noise ratio. In this step, monitoring the fluorescence emission maxima should be done in the spectrum scanning mode. Set up the start and end points at 400 and 500 nm, respectively. Fluorescence intensity at the emission maxima (E_{max}) will be measured in the absence of any ligand, normalized with the receptor concentration, and will be designated as 100%. Normalization will allow compensating for the reduction of signal intensity due to dilution upon addition of ligand.

4. All assays should be conducted with either the proteoliposomes or detergent micelles added to assay buffer. To ensure receptor structural and functional intactness, use only proteoliposomes from the batches which have been verified for receptor functional properties. Dose-dependent activity of representative ligands will be assessed by monitoring changes in MBB emission intensity at $E_{\max}$.
5. Perform a blank scan to determine the basal level of GPCR activation (due to the ground state flexibility of the ionic lock).
6. Add diluted ligands (in ~20–50 μ l volume) to proteoliposomes in the 96-well plates and incubate the plate at 30 °C in dark with gentle shaking.
7. Assay in 96-well plates in triplicates. Read plates on the plate reader instrument, which allows adjustment of parameters like band pass, temperature, and time-resolved fluorescence which will be used for fine tuning the assay and improve the signal/noise ratio. The signal intensity may also differ slightly from the usage of flat-/round-bottom 96-well plates.
8. Take out the plate from dark after 30 min and 1 h, respectively, and measure the fluorescence intensity at $E_{\max}$. An alternative procedure is to measure and determine the differences in receptor activation kinetics by adding ligands to proteoliposomes and by determining changes in MBB fluorescence at $E_{\max}$ using time-resolved fluorescence. The time interval for this process should be optimized for a given GPCR ligand pair.
9. Increased fluorescence quenching will indicate disruption of ionic lock in response to ligand binding. This can be observed by the dose-dependent reduction of MBB emission intensity. We recommend use of full agonist, partial agonist, antagonist, and inverse agonist sequence. To make sure the properties of such ligands, coinubate the agonist with antagonist and determine any rescue effect of antagonist and inverse agonist on MBB fluorescence intensity. The key to understanding of preferential receptor activation is to use partial or weak partial agonist. For example, weaker ligands (e.g., GW 40533 for the CB2 or Catechol for the β 2AR) were used for this purpose.
10. All data will be plotted in the Prism One point receptor activation curve. Error values should be included as mean $\pm$ S.E.M. (Fig. 7.3)

3.3. Determination of GPCR activation by the rotamer toggle switch

To verify whether the ligand can activate the receptor by the rotamer toggle switch mechanism, the receptor will be labeled at the *Cys with TMRM to detect relative movement in the IL3 due to the rotational toggle at the tryptophan (TM6). Generation of the mutated and labeled GPCR with TMRM is described in previous sections. The positions of the *Cys are important for effective determination of the respective molecular switches (Fig. 7.4). Cysteine residue selected for the investigation of rotamer toggle activation should be located at the IL3 or TM5-IL3 interface to effectively transmit the rotational movement at the TM regions and also be able to amplify the movement due to its flexibility. Also, this will allow the fluorescent dye attached to the Cysteine to move between relatively hydrophobic and polar environment which can then be monitored by the use of any environmentally sensitive fluorophore. Following receptor modification, radioligand binding and GTP γ assay will be done to ensure receptor structural and functional integrity. TMRM excitation and emission wavelengths are reported to be at 541/571 nm; however, we will still perform fluorescence absorption emission scan from $\pm$ 100 nm to account for any influence on fluorophore properties due to the receptor microenvironment concerned. Methods

described here have already been reported for the β 2AR labeled at Cys 265 (Swaminath et al., 2004) and briefly summarized below.

1. Determine the optimum amounts of GPCR–TMRM in the proteoliposomes. This can be done by varying the amount of protein micelles (10–100 pmol) in separate wells to obtain the highest signal to noise ratio. Any other high signal observed in the assay different than TMRM can be considered as noise.
2. TMRM shows emission maxima at 571 nm (Neumann, Wohland, Whelan, Zare, & Kobilka, 2002). Set up the instrument in the spectrum scanning mode (540–640 nm) to determine the $E_{\max}$ of the GPCR–TMRM in the proteoliposomes. For all successive steps, monitor changes in fluorescence at the wavelength of maximum emission.
3. It is desirable to determine whether participating ligands have fluorescence Abs/Emm at the $E_{\max}$ of the GPCR–TMRM complex. If yes, such ligands should be preferably excluded from the assay. Alternatively, the TMRM fluorescence should be measured at a slightly different wavelength to avoid ligand interference.
4. Fluorescence emission will be normalized by dividing the fluorescence intensity by the amount of receptor (as described in the previous section).
5. A blank scan can be done to determine the basal level of GPCR activation (due to the ground state flexibility of the rotamer toggle switch).
6. Ligand dilutions should be prepared to include $10^{\pm 5}$ Log (ligand concentration) around their EC_{50} values to provide adequate window of ligand-induced activation and saturation in the assay.
7. It is also important to determine any time-dependent effects on the TMRM fluorescence, by measuring the changes in TMRM fluorescence as a function of time. The fluorometer must be in time-resolved measurement mode. The TMRM fluorescence should be recorded at 10, 50, and 100 s after addition of the ligand.
8. All assays are with either the proteoliposomes or detergent micelles. Dose-dependent activity of representative ligands are assessed by monitoring changes in TMRM fluorescence. Ligand concentration gradients need to include $10^{\pm 5}$ Log (ligand concentration) around their EC_{50} values for the assay to provide an adequate window of ligand-induced activation and saturation.
9. All studies should be performed in 96-well plates in triplicates. Plates will be read on the plate reader instrument, which allows fine tuning of band pass, temperature, and other conditions.
10. To determine the effect of TMRM fluorescence on GPCR activation, the effect of full agonist on TMRM fluorescence should be monitored against ligand concentration. The TMRM fluorescence signal should increase upon addition of the agonist and also upon movement of the IL3, which exposes the TMRM to a more water-accessible environment.
11. Similar to our recommendation for the ionic lock, we recommend sequential use of full agonist, partial agonist, antagonist, and inverse agonist. This sequence will help to distinguish the properties of respective ligands. Coincubation the agonist with antagonist and/or inverse agonist with the receptor will help to determine if the ligand pair can activate and stabilize the receptor through the same molecular switch. The key to understanding preferential receptor activation by a single molecular switch is to use a partial or weak partial agonist. For this purpose,

weaker ligands (e.g., GW 40533 for the CB2 or Catechol for the β 2AR) may be considered.

12. All data can be plotted and analyzed in the Prism one-point receptor activation curve. Error values should be included as mean $\pm$ S.E.M.

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Figure 7.1.

Graphic representations of ionic lock switch and rotamer toggle switch. (A) Model illustrating generalized GPCR structure, (B) Ionic lock between the positively charged Arg in TM3 and the negatively charged Asp/Glu residue in TM6, (C) Rotamer toggle switch in rhodopsin family GPCR composed of the aromatic microdomain within residues from TM3 to 5 and 7, and (D) The 3–7 lock is functional in the rhodopsin/rhodopsin family GPCRs by interaction between the Lys in TM3 and Glu in TM7.

Figure 7.2.

Figure 7.2. Schematic diagram of the β_2 adrenergic receptor. Mutations were done in pairs to remove reactive cysteines and insert cysteine residues at points of selective fluorescent labeling. Solid arrows indicate residue pair used to determine ligand effects on the receptor ionic lock. The dashed arrow indicates residue used for rotamer toggle switch. Cys 271-MBB and W135 were used as donor-acceptor pair to determine the ligand-induced disruption of the ionic lock. Cys 265-TMRM was used to determine Trp-rotamer toggle and resulting changes in IL3 disposition after receptor activation (Swaminath et al., 2004). *Adapted from* Yao et al. (2006).

Figure 7.3.

Monitoring ligand-induced receptor activation of the ionic lock. (A) TM3 and TM6 are separated in an inactivated receptor, but reorganization of the helix 3 and 6 occurs in the activated receptor. (B) In the inactive receptor, the cytoplasmic juxta-membrane domains of TM3 and TM6 are separated by ionic interaction between the Arg in the conserved DRY motif in the TM3 and the negatively charged Asp/Glu in the TM6. Receptor activation leading to receptor conformational change leads to the breakage of this noncovalent interaction and brings MBB and Trp closer, leading to fluorescence quenching. Fluorescence of the MBB can be monitored in the (C) absence of ligand, (D) presence of activating ligands, and (E) dose-dependent effects on the ionic lock can be observed. *Adapted from Yao et al. (2006).*

Figure 7.4. GPCR activation by rotamer toggle switch can be observed by (A) placing an environment-sensitive fluorophore (TMRM) in the TM3 and (B) monitoring the ligand-induced conformational changes by measuring the fluorescence of the TMRM. Adapted from Swaminath et al. (2004).

Table 7.1

List of representative rhodopsin family GPCRs and correspondent Ionic lock and rotamer toggle switch molecular switches were characterized

Receptor	Stabilizing interaction	PDB ID	Reference
β 2 Adrenergic receptor	Ionic lock (Arg 131–Glu 268)	2RH1	Ballesteros et al. (2001)
	Rotamer toggle switch (Phe 290–Trp 286)	2RH1	Swaminath et al. (2005)
Human histamine H4 receptor	Ionic lock (Arg 112–Glu 298)	NA	Schneider, Schnell, Strasser, Dove, and Seifert et al. (2010)
D3 dopamine receptor	Ionic lock (Arg 128–Glu 324)	3PBL	Chien et al. (2010)
	Rotamer toggle switch	3PBL	Chien et al. (2010)
A2a adenosine receptor	Ionic lock (Arg 102–Glu228)	3EML	Jaakola et al. (2008)
Rhodopsin	Ionic lock (Glu134–Arg135–Glu247)	1U19	Goncalves et al. (2010)

In most crystal structures except the dopamine D3 receptor, intact ionic lock was not observed. Residues reported for the A2a adenosine receptor represent a potential ionic lock, which may have been disrupted in the crystal due to the presence of the T4L fusion in the ICL3 region.