Structural Basis of the Diversity of Adrenergic Receptors

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In Brief
Crystal structures of $\alpha_{2A}$ adrenergic receptor ($\alpha_{2A}$AR) reveal the molecular basis for the diversity in adrenergic receptors. Qu et al. define compelling roles for key amino acids in ligand binding, partial agonism, and biased signaling of $\alpha_{2A}$AR.

Highlights
- Partial agonist and antagonist-bound $\alpha_{2A}$AR crystal structures are determined
- F412$^{7.39}$ is essential for $\alpha_{2A}$AR agonist binding, sterically and energetically
- Full agonists but not partial agonists of $\alpha_{2A}$AR form hbonds with Y394$^{6.55}$
- ICL2 plays key role in Gs coupling of $\alpha_{2A}$AR for partial agonists
Structural Basis of the Diversity of Adrenergic Receptors

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SUMMARY

Adrenergic receptors are highly homologous while at the same time display a wide diversity of ligand and G-protein binding, and understanding this diversity is key for designing selective or biased drugs for them. Here, we determine two crystal structures of the α2A adrenergic receptor (α2AAR) in complex with a partial agonist and an antagonist. Key non-conserved residues from the ligand-binding pocket (Phe7.39 and Tyr6.55) to G-protein coupling region (Ile34.51 and Lys34.56) are discovered to play a key role in the interplay between partial agonism and biased signaling of α2AAR, which provides insights into the diversity of ligand binding and G-protein coupling preference of adrenergic receptors and lays the foundation for the discovery of next-generation drugs targeting these receptors.

INTRODUCTION

There are nine human adrenergic receptors (α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3) that mediate the central and peripheral actions of catecholamines (Hein and Kobilka, 1995; Philipp and Hein, 2004a). Numerous compounds targeting adrenergic receptors, such as β-blockers, β2 agonists, and α2 agonists, have proven to be of therapeutic benefit in the treatment of a variety of diseases, including hypertension, angina pectoris, congestive heart failure, asthma, and depression (MacMillan et al., 1996; Philipp and Hein, 2004a; Ruffolo et al., 1993). α2A Adrenergic receptor (α2AAR) agonists have been used for decades in clinic for the treatment of hypertension, attention-deficit/hyperactivity disorder, and anxiety because they have sympatholytic, sedating, and analgesic effects (Ruffolo et al., 1993; Tan et al., 2002).

Although the selectivity of α2AAR is being investigated in an accompanying paper (Chen et al., 2019), we focus on understanding the structural basis of the binding and functional diversity of adrenergic receptors. More specifically, adrenergic receptors couple to different G proteins, with α1, α2, and β types mainly coupling to Gq, Gi/0, and Gs, respectively (Lefkowitz et al., 1988). Exceptionally, α2AAR has a dual pharmacological effect in that it simultaneously couples to Gi and Gs to inhibit or stimulate adenyl cyclase activity (Eason et al., 1992). At low agonist concentrations, α2AAR mainly couples to Gi, whereas at high concentrations, Gs coupling dominates. This unusual dual effect has not been well explained for any G-protein-coupled receptor (GPCR).

Partial agonists of α2AAR, such as clonidine and dexmedetomidine, tend to have better therapeutic benefits than full agonists (Philipp and Hein, 2004a; Tan et al., 2002; Vandergriff et al., 2000). The mechanism of action of these partial agonists of α2AAR remain incompletely understood. Previous research on partial agonism of β1AR and β2AR highlight the diversified roles of three serines (S5.42, S5.43, and S5.46) in transmembrane helix (TM) 5, and an asparagine (N6.55) in TM6 played in differentiating partial agonist from full agonist (superscripts refer to Ballesteros-Weinstein numbering; Ballesteros and Weinstein, 1995; Katritch et al., 2009; Masureel et al., 2018; Warne et al., 2011). Specifically, in β1AR, full agonists form hydrogen bonds with S5.42 and S5.46, but partial agonists form hydrogen bonds only with S5.42 in a series of crystal structures. Whereas in β2AR, different hydrogen bond networks involving S5.42, S5.43, N6.55, and N7.39 were formed upon full agonist and partial agonist binding, in crystal structures and in molecular dynamics simulations. In short, S5.46 in β1AR and N7.39 in β2AR play an essential role in differentiating partial agonist from partial agonist, respectively. The crystal structures of α2AAR were solved, with partial agonist and antagonist bound, further provides the molecular basis for understanding partial agonism of GPCRs and facilitates the structure-based design of novel ligands with desired therapeutic efficacies.

RESULTS

Comparison of Two Adrenergic Receptor Structures

Two α2AAR crystal structures were determined and co-crystallized with partial agonist (S)-4-fluoro-2-(1H-imidazo[5,1-b]-1-isoquinolino[2,1-g][1,6]naphthyridine (RS 79948, or in short, RS) (Figures 1, S1, and S2; Table S1). The
Figure 1. Crystal Structures of \( \alpha_{2a} \)AR

(A) Overall structures of RES- (receptor in yellow and ligand in orange) and RS 79948- (receptor in green and ligand in blue) bound \( \alpha_{2a} \)AR. (B and C) The conformational change between the two structures at the extracellular loops (ECLs; B) and intracellular loops (ICLs; C) are shown in the colored cartoon. The disordered region in ECL2 is shown as dotted lines. See also Tables S1 and S2 and Figures S1–S3.

former structure is in an agonist-bound inactive state and the latter is in an inactive state.

To investigate conformational changes upon agonist binding across adrenergic receptors, we compared receptors \( \alpha_{2a} \)AR, \( \beta_1 \)AR (Moukhametzianov et al., 2011; Warne et al., 2011), and \( \beta_2 \)AR (Cherezov et al., 2007; Rasmussen et al., 2011; Rosenbaum et al., 2011) in the inactive, agonist-bound inactive, and active states. Remarkably, the overall structures of antagonist-bound and agonist-bound inactive states are very similar, with \( \alpha \) root-mean-square deviations (RMSDs) of the \( \alpha_{2a} \)AR (PDB: 6KUX/6KUY), \( \beta_1 \)AR (PDB: 2YCW/2Y02), and \( \beta_2 \)AR (PDB: 2RH1/3PDS) pairs at 1.1 Å, 0.5 Å, and 0.6 Å, respectively (Table S2). The slightly greater value of \( \alpha_{2a} \)AR is largely due to variations in the loop regions (Figures 1B and 1C). In contrast, the overall structures of the adrenergic receptors must change significantly when forming complexes with the G protein (i.e., in fully active state), mainly due to the outward movement of the intracellular end of TM6 (Table S2).

Although the overall structures of \( \alpha_{2a} \)AR are similar to those of \( \beta \)ARs, obvious differences are observed in the loop region and especially extracellular ends of TM4 for receptors in the same (inactive, agonist-bound inactive, or active) state (Figure S3). Different from \( \beta \)ARs, the extracellular end of TM4 in \( \alpha_{2a} \)AR is unwound, which is consistent with the fact that the sequence of this region has two consecutive prolines that broke the helix. Connected to the unwound end of TM4, the starting part of ECL2 in \( \alpha_{2a} \)AR has the typical intrinsically disordered sequence “GGGGGPOP,” and indeed the electron density of this part is missing in the structure. The unwound extracellular end of TM4 and the intrinsically disordered region of ECL2 together reflect the highly dynamic nature of ECL2 in \( \alpha_{2a} \)AR. In contrast, in \( \beta \)ARs, the starting part of ECL2 is helical and lacks glycine or proline. Importantly, ELC2 has an extra pair of cysteines that form an intra-ECL2 disulfide bond, which further stabilizes its overall conformation. Other ECLs also have notable differences between \( \alpha_{2a} \)AR and \( \beta \)ARs. The unique composition and conformation of each ECL in \( \alpha_{2a} \)AR may affect the shape and dynamics of the pocket vestibule and, as we found in the case of \( \alpha_{2a} \)AR (Chen et al., 2019), may have an impact on ligand selectivity.

The Ligand-Binding Diversity of Adrenergic Receptors

Electron densities for RES and RS 79948 in the two structures of \( \alpha_{2a} \)AR enable us to convincingly make the placement of the two ligands (Figure S1). The two ligands show strong interactions with residues in the pocket of \( \alpha_{2a} \)AR, both including a salt bridge between the ligand’s positively charged nitrogen atom and D113(3.32)—the conserved residue involved in ligand binding in all amineergic receptors and opioid receptors (Figures 2A–2D). The binding pocket of \( \alpha_{2a} \)AR is composed of 12 residues (Figure S4). Although the backbone conformations of the pocket-forming residues are rather similar in the two \( \alpha_{2a} \)AR structures, the shapes of the two binding pockets are strikingly different due to the repacking of four sidechains (F412(7.39), Y416(7.43), Y394(6.55), and D113(3.32)) upon binding with different ligands (Figures 2C, 2E, and S4A–S4D). These dramatic sidechain rearrangements reflect the highly plastic nature of the receptor to accommodate ligands with very different chemical scaffolds, such as RES and RS 79948 (Figures 2A and 2B).

Interestingly, when we tried to dock agonists and antagonists of \( \alpha_{2a} \)AR into the two structures, we found that agonists (especially when containing an imidazole ring head like RES) fit better in the RES-bound structure (i.e., agonist-bound inactive state), whereas antagonists fit better in the RS-bound structure (i.e., inactive state) (Table S3). This finding implies that the shapes of the pockets of the two structures are specific to the agonists and antagonists of this receptor. Conversely, in \( \beta \)AR, generally agonists can be docked into the pocket of antagonist-bound structures, and vice versa, which reflects the relative rigidity of the binding pocket of \( \beta \)AR (Beuming and Sherman, 2012).

F412(7.39) Is Essential for \( \alpha_{2a} \)AR Agonist Binding as a Switching Lid of an Aromatic Cage

Among these conformationally rearranged residues, the most intriguing is F412(7.39), which functions as a switching lid for the pocket. Large ligands, like RS 79948 with a saturated ring system, push F412(7.39) aside, making the pocket large and open. Conversely, small ligands, like RES, can induce the closure of the lid (i.e., F412(7.39) to form an aromatic cage together with residues W387(6.48), F390(6.51), and Y416(7.43) (Figure S4E). By forming the aromatic cage around the cation in the ligand not only is the steric of the site tightly defined but also the energetics. Notably, the residue at 7.39 is one of the three non-conserved residues (the other two are 6.55 and 5.43) (Figure S5) in the binding pocket of adrenergic receptors (Figures 2C and 2D; Table S4). Thus, the “switching lid” phenomenon seems unique to \( \alpha \)ARs. In fact, even among amineergic and opioid receptors, only \( \alpha \)ARs, muscarinic receptors, and two histamine receptors (H3 and H4) have aromatic residues at the four positions 6.48, 6.51, 7.39, and 7.43 (Table S4). Unlike \( \alpha_{2a} \)AR, in muscarinic receptors, there is no dramatic repacking of Y7.39 upon agonist binding; thus, Y7.39 forms a rather static cage restricting the
muscarinic ligands while isolating the allosteric modulator from the ligand-binding pocket (Figures S4E and S4F). Such a rigid or flexible aromatic cage plays a substantial role in cation recognition in these receptors. Thus, it has particular implications for ligand discovery.

This aromatic cage was not observed in βARs (Figures 2Fa and 2G) where the residue at 7.39 is a much smaller asparagine and shows no obvious conformational changes during various ligand binding because N7.39 is involved in a stable hydrogen bond with the conserved positively charged ligand nitrogen (Figure 2D). Interestingly, the F4127.39N mutation in α2AR (i.e., mutation to the residue at 7.39 in βARs) abolished the function of all four partial and full agonists we tested (clonidine, guanabenz, UK14,304, and epinephrine), reflecting the essential p-π and cation-π interactions provided by F4127.39 for the activity of α2AR agonists (Figure S6A). Similarly, the F3127.39N mutation in α1AAR also abolishes the binding of ligands containing an imidazoline ring (Waugh et al., 2001), which is indicative of the critical function of the phenylalanine at 7.39 for βARs. Interestingly, the F4127.39N mutation in α2AAR has no response to two β2AR selective agonists, namely salmeterol and salbutamol (Figure S6A), implying the other two non-conserved residues (Y3946.55 and C2015.43) in the binding pocket also play important roles in ligand activity. Nevertheless, F4127.39N and Y3946.55N/F4127.39N abolish the activation activity of α2AAR agonists and partial agonists, possibly due to the key role F4127.39 plays in agonist binding; yet, they are still unable to trigger the activity of β2AR ligands (Figures S6). This finding implies that other regions, such as ECLs, may also be important for the binding and function of β2AR ligands.

Full but Not Partial Agonists Form Hydrogen Bonds with Y3946.55 and S2005.42/S2045.46

In both α2AR structures, Y3946.55 - another non-conserved position in the ligand-binding pocket of adrenergic receptors, has no direct interaction with ligands, whereas in βAR, N6.55 forms a strong and conserved hydrogen bond with full agonist but not partial agonist (Figure 2D) (Masureel et al., 2018). In fact, hydrogen bond formation between N6.55 and S5.43 is believed to be a hallmark of the activation of βARs (Masureel et al., 2018). As a result, the role of Y3946.55 in α2AR is particularly intriguing. From the RES-bound structure, we knew that
the partial agonist RES has no interaction with Y3946.55. We further checked the docking poses of representative full agonists (epinephrine, norepinephrine, and UK14,304) and partial agonists (clonidine, guanfacine, guanabenz, and dexmedetomidine) to see if Y3946.55 contributes to partial agonism in a2AR as N6.55 does in b2AR. The docking results show that all three full agonists of a2AR formed hydrogen bonds with Y3946.55 and S2005.42/S2045.46, but all partial agonists do not (Figure S7). The reason is that the tails of partial agonists generally are more hydrophobic, whereas tails of full agonists are more hydrophilic and, thus, are prone to hydrogen bond forming (Figures S7 and S8). Because the movements of the extracellular end (which participates in ligand binding) and the intracellular end of TM6 (which interacts with the G protein) are more or less coupled, the strong interaction between full agonists and the extracellular end of TM6 could, in turn, push TM6 to be more kinked, which leads to more outward movement of the extracellular end of TM6 (i.e., more activated). In contrast, partial agonists can only form weaker interactions with TM6, which will lead to less outward movement of intracellular end of TM6 (i.e., less activated).

Notably, the roles of residues at positions 6.55, 5.42, and 5.46 in differentiating full agonist from partial agonist are diverse in adrenergic receptors. In a2AR and b2AR, full agonists form a hydrogen bond with 6.55 but partial agonists do not, although position 6.55 is not reversed in the two receptors (Table S5). In b2AR, full agonists form a hydrogen bond with S5.46 but partial agonists do not (Warne et al., 2011), while in a2AR, full agonists cannot reach S5.46 (Table S5).

Role of Non-conserved Residues in Dual Coupling of a2AR to Two G Proteins

The movement of the intracellular end of TM6 is the hallmark of GPCR activation. Recent electron microscopy (EM) structures of GPCR-G complexes revealed that TM6 are less kinked compared to those in GPCR-Gs complexes (Draper-Joyce et al., 2018; García-Nafria et al., 2018; Kang et al., 2018; Koehl et al., 2018). Thus, we hypothesized that TM6 of a2AR is in two different states when the receptor couples to different G proteins. Using functional studies of a2AR mutants, we investigated the role of the two non-conserved residues Y3946.55 and C2015.43 in dual coupling of a2AR to Gs and Gs by mutating them to residues in b2AR.

Consistent with previous reports, wild-type (WT) a2AR simultaneously coupled to both Gs and Gi proteins ( termed “dual effect”: Eason et al., 1992) when stimulated by full agonist adrenaline, UK14,304, or partial agonists clonidine and guanabenz (Figure 3A). Conversely, full or partial agonists of b2AR could only activate the Gs signal (Figure 3B). We hypothesized that the non-conserved residues in the pocket may cause this dual effect. Therefore, we designed mutations to minimize the dual effect of a2AR. Surprisingly, with the single mutation Y3946.55N, the dual effect disappeared (Figures 3C and 3D), and the same result was achieved when adding another mutation: C2015.43S (Figure 3E). Both mutations caused an approximately 10-fold half maximal effective concentration (EC50) loss (Figure 3F). These results confirm the key role Y3946.55 plays in G-protein selectivity of a2AR.

G Protein Binding Site Diversity in Adrenergic Receptors

The activation of G protein is affected not only by the ligand-binding pocket but also by the G protein binding site. One region that may impact the G protein binding preference is ICL2, which often has more contacts with Gs than Gi (Figure 4A). Based on structural analysis of the b2AR-Gs complex (PDB: 3SN621), we predicted that I34.51 would form intensive hydrophobic interactions with H41 and F376 in Gs (Figure 4B). Similarly, residue K14434.56 would form electrostatic interactions with D139 in Gs. As a result, two mutations, namely, I13934.51A and K14434.56A, were designed to see if they would hamper the coupling of Gs when the receptor binds with full or partial agonists. To evaluate the Gs signaling without the interference of Gi signaling, pertussis toxin (PTX), which can selectively block the Gi signaling pathway, was introduced. With PTX, we can see that WT a2AR has strong Gs signaling for full agonists and rather weak Gs signaling for partial agonists (Figure 4C). The I13934.51A mutant can abolish Gs signaling for both partial agonists and full agonists (Figure 4D). Unlike I13934.51A, K14434.56A could selectively diminish the Gs potency of partial agonists clonidine and guanabenz, leaving the Gs potency of full agonists
UK14,304 and epinephrine less affected (Figure 4E). Notably, the Gi potencies of I13934.51A and K14434.56A mutants were more or less similar to that of WT a2AAR for both partial and full agonists (Figures 4D, 4E, and 3F).

The distinct pharmacological consequence of the two mutants (I13934.51A and K14434.56A) for partial and full agonists sheds light on the interplay between partial agonism and biased signaling of a2AAR. Both mutants completely abolished the weak Gs signaling for partial agonists, and they convert partial agonists clonidine and guanabenz to Gi-biased agonists from the perspective of pharmacology. Unlike full agonists, partial agonists of a2AAR lack of hydrophilic tails (Figure S8) that can form hydrogen bonds with polar residues (Y6.55, S5.42 or S5.46) in the extracellular end of TM5 and TM6 (Figure S7); thus, it is difficult for them to promote the outward movement of the intracellular end of TM5 and TM6 as large as that required for Gs coupling. The two mutations on ICL2 push the energy barrier for Gs coupling induced by partial agonists even higher, and they become the last straw. On the other hand, full agonists can form strong interactions with the extracellular end of TM5 and TM6 (Figure S7); thus, they may push the receptor to have a higher population in the Gs-coupling state. The Gs signaling pathway of a2AAR induced by full agonists was largely unaffected by K14434.56A but was nearly abolished by I13934.51A. The difference highlights the crucial role of I13934.51 on Gs coupling, and it infers that the I13934.51A mutant may lose the Gs coupling ability. Taken together, these results demonstrate the essential role of ICL2 in Gs coupling for partial agonists and reflects the allosteric communication between extracellular ligand binding and intracellular G protein coupling.

DISCUSSION

Our comparative study of two a2AAR crystal structures in binding poses with very different ligands has identified key non-conserved residues from the ligand binding pocket (F4127.39 and Y3946.55) to the G protein coupling region (I13934.51, K14434.56) that control adrenergic diversity through ligand
binding, partial agonism, and G protein preference. Remarkably, we found that G protein signaling can be dramatically altered or even abolished by several single mutations, revealing how evolution has been able to achieve the diversity of adrenergic receptor function with minimal modifications. Together with accompanying insights on adrenergic selectivity (Chen et al., 2019), our results will lay the foundation for next-generation drug discovery efforts targeting adrenergic receptors.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.10.086.

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## STAR METHODS

### KEY RESOURCES TABLE

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<td>z2aAR-RS 79948 complex structure</td>
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<td>Experimental Models: Cell Lines</td>
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(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Suwen Zhao (zhaosw@shanghaitech.edu.cn). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
S. frugiperda (Sf9) cells were used for α2AAR expression and crystallization. CHO-K1 cells (ATCC) were used for Split luciferase biosensor cAMP assay.
METHOD DETAILS

Protein Engineering for Structure Determination

The sequence of the human \( \alpha_{2A} \)AR gene was synthesized by GenScript. The modified thermostabilized apocytochrome \( b_{562} \)RIL (BRIL) was inserted into the receptor’s third intracellular loop (IL3) at Thr227 and Arg365 of the human \( \alpha_{2A} \)AR gene as a fusion partner, by overlapping PCR. The construct was further optimized by truncation of N-terminal residues 1-19 and C-terminal residues 446-450. The \( \Delta N-\alpha_{2A} \)AR-RS-BRIL-\( \Delta C \) DNA sequence was subcloned into a modified mammalian expression pTTS5 vector, which contained a hemorrhagglutinin (HA) signal sequence, a FLAG tag and 10 × His tag, followed by a tobacco etch virus (TEV) protease cleavage site, before the N terminus of the truncated \( \alpha_{2A} \)AR-RS gene. The \( \Delta N-\alpha_{2A} \)AR-RES-BRIL-\( \Delta C \) DNA sequence was subcloned into a modified pFastBac1 vector for expression in Spodoptera frugiperda (Sf9) cells. The chimera sequence has a hemorrhagglutinin (HA) signal sequence followed by a FLAG tag at the N terminus, a PreScission protease site and a 10×His tag at the C terminus. Two rationally designed mutations, I1213.40A and V1223.41W, and three mutations, I1213.40A, V1223.41W, and I1554.47L, were further introduced into the \( \alpha_{2A} \)AR-RES and \( \alpha_{2A} \)AR-RS genes, respectively, by standard QuickChange PCR.

Protein Expression

\( \alpha_{2A} \)AR-RS construct was transfected and expressed in HEK293F cells (Invitrogen) (Passage number is 12-20) using the FreeStyle TM293 Expression system (Invitrogen). HEK293F cells (Invitrogen) were cultured in suspension starting from densities of 0.2-0.3 × 10^6 cells/ml in a humidified incubator with 5% CO\(_2\) at 37°C with a shaking speed of 130 rpm. Passage cells when cell density reached 1.6-1.8 × 10^6 cells/ml. Briefly, HEK293F cells were seeded on day 0 at 6 × 10^5 cells/ml in freeStyle 293 expression medium (Invitrogen). On day 2 the transduction was performed at a cell density of 1.0 to 1.2 × 10^6 cells/ml and cell viability over 95% using PEI-DNA complexes. Approximately 48 h post-transfection, cells were harvested by centrifugation at 400 g for 20 min at 4°C. Cells were stored at −80°C for future use.

The \( \alpha_{2A} \)AR-RES construct was transfected and expressed in Sf9 cells. The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to generate high-titer recombinant baculovirus (> 10^9 viral particles/ml). Recombinant baculovirus was produced by transfecting recombinant bacmids (2.5-5 mg) into Sf9 cells (2.5 mL, density of 106 cells/ml) using 5 mL of X-tremeGENE HP DNA Transfection Reagent (Roche) and Transfection Medium (Expression Systems). After 4 d of shaking at 27°C, P0 viral stock (~10^9 virus particles/ml) was harvested as the supernatant of the cell suspension to produce high-titer viral stock. Viral titers were analyzed by flow cytometry on cells stained with gp64-PE antibody (Expression Systems). \( \alpha_{2A} \)AR-RES was expressed by infecting Sf9 cells at a cell density of 2-3 × 10^6 cells/ml with P1 virus at MOI (multiplicity of infection) of 5. Cells were harvested by centrifugation for 48 h post infection and stored at −80°C for future use.

Protein Purification

Frozen cell pellets were thawed and lysed by repeated washing and centrifugation in the hypotonic buffer of 10 mM HEPES (pH 7.5), 10 mM MgCl2, 20 mM KCl, and the high osmotic buffer of 10mM HEPES (pH 7.5), 1.0 M NaCl, 10mM MgCl2, and 20 mM KCl, with EDTA free complete protease inhibitor cocktail tablets (Roche). The washed membranes were suspended in hypotonic buffer with 30% glycerol and flash-frozen with liquid nitrogen and stored at −80°C until further use. Purified membranes were thawed at room temperature and incubated with 50 µM RES or RS 79948 and inhibitor cocktail at 4°C for 3 h. The membranes were further incubated with 1.0 mg/ml iodoacetamide (Sigma) for 1 h. The membranes were solubilized in the buffer containing 50 mM HEPES (pH 7.5), 200 mM NaCl, 1% (v/v) n-dodecyl-beta-D-maltopyranoside (DDM, Anatrace), and 0.2% (w/v) cholesterol hemisuccinate (CHS, Sigma-Aldrich) at 4°C for 2.5-3 h. The supernatants containing the solubilized \( \alpha_{2A} \)AR proteins were isolated by high-speed centrifugation, and then incubated with TALON IMAC resin (Clontech) and 20 mM imidazole, at 4°C overnight. The resin was washed with 15 column volumes of washing buffer I containing 25 mM HEPES (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 30 mM imidazole, and 50 µM RES or RS, and then 5 column volumes of washing buffer II containing 25 mM HEPES (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 50 mM imidazole, and 50 µM RES or RS 79948. The protein was eluted using 2.5 column volumes of elution buffer containing 25 mM HEPES (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 0.01% (w/v) DDM, 0.002% (w/v) CHS, 250 mM imidazole, and 50 µM RES or RS 79948. A PD MiniTrap G-25 column (GE Healthcare) was used to remove imidazole. The protein was treated overnight with TEV protease to cleave the N-terminal FLAG/His tags from the proteins.

Lipidic Cubic Phase Crystallization

The purified \( \alpha_{2A} \)AR protein in complex with RES or RS 79948 was screened for crystallization in lipidic cubic phase (LCP) with mixed molten lipid (90% (w/v) monolein and 10% (w/v) cholesterol) at a protein/lipid ratio of 2.3 (w/v) using a mechanical syringe mixer (crystallizing membrane proteins using lipidic mesophases). LCP crystallization trials were set up using an NTB-LCP crystallization robot (Formulatrix). 96-well glass sandwich plates were incubated at 20°C in an automatic incubator/imager (RockImager 1000, Formulatrix) and imaged. Crystals were obtained in condition of 0.1M HEPES sodium pH 7.4, 310mM Ammonium tartrate dibasic, 36.5% PEG400 (RES) and condition of 0.1M Sodium citrate tribasic dihydrate pH 5.0, 290mM Ammonium chloride, 30% PEG400, 7% glycerol (RS 79948) and grew to full size in around two weeks. The crystals were harvested using micromounts (MITEGen) and flash-frozen in liquid nitrogen.
Data Collection and Structure Determination

X-ray diffraction data of α2AR-RES and α2AR-RS crystals were collected at beam line 41XU at SPring-8, Japan, using a Pilatus3 6M detector. The data collection strategy was designed based on rastering results as previously described (Cherezov et al., 2009). Diffraction images were indexed, integrated and scaled using XDS (Kabsch, 2010) and merged using SCALA (Collaborative Computational Project, Number 4, 1994). Initial phases were obtained by the molecular replacement (MR) method with Phaser® using the receptor and BRIL portions of turkey β1AR (PDB entry: 2Y02) as independent search models. Refinement was carried out with Phenix (Adams et al., 2010) and Buster (Smart et al., 2012) alternately followed by manual examination and adjustments of the refined structures in the program COOT (Emsley et al., 2010) with both 2|Fo|-|Fc| and |Fo|-|Fc| maps. In the final refined 2|Fo|-|Fc| maps, most of the 7TM structure and BRIL were ordered in both structures. The relatively high average B-factor of both structures (α2AR-RS complex: 124.9; α2AR-RES complex: 160.4) may have been due to the particular molecule packing in crystals (Figure S2) with weak contact among transmembrane domain and strong contact among BRILs.

Cell culture and transfection for cAMP assay

To determine GPCR mediated cAMP production, we use Promega’s split luciferase based GloSensor cAMP biosensor technology. CHO-K1 cells (ATCC), were maintained in F-12 supplemented with 10% FBS 1% penicillin/streptavidin. When cells reached 70% confluency in a 10 cm dish, 1 μg of target receptor DNA and 1 μg of GloSensor cAMP DNA were co-transfected by TransIT 2020(Mirus bio). On the following day, cells were seeded into 384-well white clear bottom cell culture plates (Greiner) at a density of 10-15,000 cells in 40 μL growth medium per well. To selectively ablate receptor- Gi coupling, 125 ng/mL pertussis toxin (PTX, Tocris) was added 18 hours before assay. The plates could be used for assays the next day.

Split luciferase biosensor cAMP assay

After removing supernatant from 384 well plates, wells were loaded for 60 min at 37°C with 20 μL of 2 mg/ml Luciferin prepared in HBSS with 0.1% BSA, pH 7.4. All the following steps were carried out at room temperature. To measure agonist activity on α2AR, 10 μL 4 x test drug solution was added for 15 min before the addition of 10 μL of forskolin (Sigma Aldrich) at a final concentration of 20 μM, followed by counting of the plate for chemiluminescence after 15 minutes. To measure antagonist activity, cells were preincubated with test drug for 15 min before 4 x 350 nM of UK14,304 and forskolin at a final concentration of 20 μM was added. Counting was undertaken after 15 min. Chemiluminescence signal was measured on an EnVision plate reader (Perkin Elmer).

Ligand docking

The whole process was done in Schrodinger Suite 2018-3. RES-bond and RS 79948-bond structures were prepared by Protein Preparation Wizard. Fusion partners were deleted, proteins were capped, and missing side chains were added. Protonation states were assigned by PROPKA. The directions of polar hydrogens were optimized and the whole structures were minimized. Grids were generated via Receptor Grid Generation in Glide (Friesner et al., 2004, 2006), hydroxyols in residues at positions 6.55, 5.42 and 5.46 were allowed to rotate during docking; RES or RS 79948 were select to define the center of the grid box. Ligands docked were downloaded from IUPHAR. LigPrep was used to prepare ligands so they will be ready to be docked. Epik was used to determine ligand’s ionization states at pH 7.0 ± 2.0. Glide XP was used to dock ligands into two structures. After getting the result, those poses do not have a salt bridge between ligand and the residue at 3.32 were dropped.

QUANTIFICATION AND STATISTICAL ANALYSIS

The data obtained for individual experimental pEC50 between WT and mutated α2AR, WT β2AR presented as the mean ± SEM (N ≥ 3) in Prism 7 (GraphPad, La Jolla, CA).

DATA AND CODE AVAILABILITY

The accession numbers for the structures of α2AR-RS 79948 complex and α2AR-RES complex reported in this paper are PDB: 6KUX and 6KUY, respectively. All other data are available from the corresponding authors upon request.