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Structural Basis for Allosteric Regulation of GPCRs by Sodium Ions^a

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Abstract

Pharmacological responses of G protein-coupled receptors (GPCRs) can be fine-tuned by allosteric modulators. Structural studies of such effects have been limited due to the medium resolution of GPCR structures. We re-engineered the human A_{2A} adenosine receptor by replacing its third intracellular loop with apo-cytochrome b562RIL and solved the structure at 1.8 angstrom resolution. The high-resolution structure identified about 57 ordered waters inside the receptor comprising three major clusters. The central cluster harbors a putative sodium ion bound to the highly conserved Asp^{2.50}. Additionally, two cholesterol molecules stabilize the conformation of helix VI, and one of 23 ordered lipids intercalates inside the ligand-binding pocket. These high-resolution details shed light on the potential role of structured waters, sodium ions and lipids/cholesterol in GPCR stabilization and function.

G protein-coupled receptors (GPCRs) comprise the largest and most diverse family of membrane proteins in eukaryotes, sharing a common architecture of seven transmembrane (7TM) α -helices. GPCRs transduce a variety of signals across the cell membrane that regulate diverse biological and (patho)physiological processes, and, hence, are favored drug targets. Ligand-dependent GPCR activation triggers dramatic conformational changes in the 7TM helical bundle that are coupled to activation of G proteins and other downstream effectors. Over the last four years, breakthroughs in protein engineering and crystallography have yielded structures of fourteen different GPCRs responding to diffusible ligands in different functional states (1–9), providing the basic structural framework for understanding ligand binding and activation mechanisms. However, despite the progress in GPCR stabilization for crystallographic studies, the resolution of GPCR structures has remained in

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the 2.2–3.5 Å range (1). The function of GPCRs relies on a specific lipid environment and often strongly depends on the presence of cholesterol and ions (10–12). Understanding the structural mechanism of such effects has been limited due to the absence of higher resolution GPCR structures.

Here we replaced the third intracellular loop (ICL3) of the human A_{2A} adenosine receptor (A_{2A}AR) with a thermostabilized apocytochrome b₅₆₂RIL (BRIL) (13, 14) and determined the crystal structure of this chimeric protein (referred to as A_{2A}AR-BRIL-ΔC (15)) in complex with a high-affinity, subtype-selective antagonist, ZM241385, at 1.8 Å resolution (table S1). The high resolution enabled us to identify a comprehensive network of 57 internal waters, a highly conserved binding site for a sodium ion, three cholesterol molecules, and 23 lipid acyl chains bound to the receptor.

The ligand binding and functional characteristics of the A_{2A}AR-BRIL-ΔC fusion protein were extensively characterized and compared to the A_{2A}AR-WT (wild type) and the A_{2A}AR-ΔC (C-terminus truncation) constructs. Antagonist [³H]ZM241385 radioligand binding and agonist displacement assays confirmed that the ligand recognition site of the A_{2A}AR-BRIL-ΔC fusion protein is very similar to that of both the A_{2A}AR-WT and the A_{2A}AR-ΔC constructs (fig. S1). The BRIL insertion did not have an impact on receptor expression and trafficking to the cell plasma membrane (fig. S2), but, expectedly, prevented the receptor construct from activating available G_s proteins (fig. S3).

The high-resolution receptor structure is nearly identical to the original 2.6 Å resolution crystal structure of A_{2A}AR-T4L-ΔC/ZM241385 (PDB ID 3EML) (16), with an all-atom RMSD = 0.45 Å over 81% of A_{2A}AR. The conformations of the cytoplasmic ends of helices V and VI near the BRIL junction sites closely resemble the conformations in the A_{2A}AR structure with unmodified ICL3 (17), in contrast to a distorted conformation caused by the T4L fusion in A_{2A}AR-T4L-ΔC/ZM241385 (16). A distal part of the extracellular loop 2 (ECL2), which was missing in all inactive state structures of A_{2A}AR, is fully resolved (fig. S4). Most importantly, the higher resolution revealed atomic details for the structurally conserved regions including intricate networks of water molecules, sodium ion, lipids and cholesterol.

The 1.8 Å resolution structure of A_{2A}AR-BRIL-ΔC/ZM241385 contains 177 structured waters, 57 of which occupy the interior of the 7TM bundle. The interior waters form an almost continuous channel extending from the ligand-binding site to the site of G protein interaction (Fig. 1A), which is comprised of three bulky water molecule clusters, as well as several scattered waters. The channel has two narrow “bottlenecks” restricted by Trp246^{6,48} and Tyr288^{7,53} (18) to slightly less than the diameter of one water molecule (2.4 and 2.0 Å, respectively). The rearrangements of the receptor backbone and side chains upon activation disrupt the channel continuity in these sites (Fig. 1B), substantially reducing its volume.

The first of the three water clusters, the EC cluster, located inside the orthosteric ligand-binding pocket, plays a role in ligand binding and selectivity (19) (Fig. 1C and fig. S5). One of the waters in this cluster stabilizes the conformation of a non-proline kink in helix III by forming hydrogen bonds with both the main-chain carboxyl of Ile80^{3,28} and the main-chain nitrogen of Val84^{3,32}. In the active-like state agonist-bound A_{2A}AR structures (20, 21), the kink in helix III straightens, thereby precluding water binding, which points to a role of water rearrangement at this location in A_{2A}AR activation. This ligand-induced and water-mediated conformational change is accompanied by an overall activation-related shift of helix III (20).

The second, central water cluster includes a sodium ion and 10 waters that completely fill a cavity in the middle of the 7TM bundle (Fig. 1D). The central cluster spans a distance of

more than 13 Å between two functionally important and conserved residues, Trp246^{6,48} and Tyr288^{7,53}. Three water molecules from this cluster were previously observed in the A_{2A}AR-T4L-ΔC/ZM241385 complex (16) (fig. S6A), and positions of four of the ten water molecules are similar to those found in bovine rhodopsin (PDB ID 1U19) (22) (fig. S6B), suggesting that such an internal network is common in Class A GPCRs and could be important for their function (23, 24). Finally, the IC cluster containing approximately 20 water molecules is found near the conserved D[E]RY motif (Fig. 1E), which is inferred in the stabilization of different functional receptor states and in interactions with G proteins and other effectors.

Evidence for the presence of a sodium ion in the central water cluster came from a strong spherical electron density with a distorted octahedral coordination and short distances to coordinating atoms in its neighborhood. Such geometry was poorly compatible with the presence of a water molecule. However, Na⁺ modeled in this density is coordinated by five oxygen atoms including OD1 of Asp52^{2,50} (2.45 Å), OG of Ser91^{3,39} (2.54 Å) and three waters (2.21, 2.44 and 2.59 Å) (Fig. 2A, fig. S7), which is in excellent agreement with coordination and distances for Na⁺ in protein structures (25). Moreover, the presence of Na⁺ in the structure is consistent with conditions employed during purification (0.8 M Na⁺) and crystallization (~0.15 M Na⁺). Allosteric effects of Na⁺ on ligand binding, stability and crystallization of A_{2A}AR have been documented in previous studies (11, 16, 26). We confirmed the allosteric effects of physiological concentrations of Na⁺ on agonist and antagonist binding and thermal stability of A_{2A}AR (Fig. 3, table S2).

During the last 40 years allosteric modulation by Na⁺ has been observed for many GPCRs, and was linked to motifs in helix II, including the highly conserved Asp^{2,50} (27). Mutation of this residue has been the subject of many studies on a multitude of receptors; in the GPCRDB database (28), it is mentioned >100 times for dozens of receptors of human origin and from other species. The residue has not been mutated in the A_{2A}AR, but in the closely homologous A₁ and A₃ adenosine receptors, the Na⁺ effect was largely abrogated when the residue was mutated to alanine or asparagine, respectively (29, 30). Despite this indirect evidence, the nature of Na⁺ interactions with GPCRs remained hypothetical (31, 32) and the sodium ion remained undetected in crystal structures of GPCRs solved at medium resolution.

In this high-resolution A_{2A}AR structure, we were not only able to determine the precise location of Na⁺, but also resolved the complete network of water molecules around it and conformations of all residues involved in direct or water-mediated coordination. The central water cluster harboring the sodium ion is surrounded by and engaged in hydrogen bonding with several highly conserved residues including Asn24^{1,50}, Asp52^{2,50}, Ser91^{3,39}, Trp246^{6,48}, Asn280^{7,45}, Asn284^{7,49} and Tyr288^{7,53} (Fig. 2A). Structural alignment of these residues from known GPCR structures reveals a well preserved site capable of binding Na⁺ along with several water molecules (Fig. 2C,D). Small deviations were observed in bovine rhodopsin, in which Asn^{7,45} is substituted with a serine, and Asn^{7,49} adopts a different rotamer, and in CXCR4 where Asn^{7,45} is replaced with a histidine. Interestingly, in many olfactory receptors, Ser^{3,39} is substituted with a glutamate, resulting in two acidic side chains pointing inside the central water cluster, which may favor binding of a divalent cation in this site.

The central water cluster with Na⁺ likely plays an important role in receptor activation. Comparison between the inactive and active-like states of A_{2A}AR (PDB ID 3QAK) suggests that the inward movement of helix VII in this region upon activation collapses this pocket from 200 to 70 Å³ and shifts the pocket towards helix VI (Fig. 2B). The resulting pocket in the active-like state can accommodate a maximum of three water molecules and does not

provide sufficient coordination for Na⁺. This comparison suggests that high-affinity agonist binding and the presence of Na⁺ are indeed mutually exclusive, which is consistent with the observed negative allosteric effects of Na⁺ on binding of agonists (Fig 3 and table S2) (16, 20).

The high resolution structure also allows us to investigate off-target interactions of the diuretic and sodium channel blocker amiloride with GPCRs that were first documented by Howard et al. (33) and Nunnari et al. (34). They demonstrated that amiloride and derivatives non-competitively displaced a radiolabeled antagonist from the α_2 -adrenergic receptor by enhancing the radioligand's dissociation rate. Moreover, Howard et al. (33) provided evidence that NaCl competed with amiloride, suggesting that Na⁺ and amiloride share an allosteric binding site. We docked amiloride into the central cluster cavity (fig. S8), with only minor changes in the conformations of the surrounding side chains, i.e. Trp246^{6,48}. Multiple docking runs consistently led to an amiloride orientation, in which its charged guanidinium group interacts with Asp52^{2,50}, while other polar groups form a network of hydrogen bonds with residues in the pocket, mimicking polar interactions in the Na⁺/water cluster. Successful docking of bulkier amiloride derivatives, such as 5-(N,N-hexamethylene)amiloride (HMA), required more substantial rearrangements of several side chains and possibly the main-chain (not shown), which would potentially perturb the conformation of the deepest part of the orthosteric ligand-binding pocket. This result is consistent with the higher potencies of such derivatives in accelerating orthosteric antagonists' off-rates (11). This docking study also corroborates the results of our experiments (Fig. 3), in which amiloride reduced [³H]ZM241385 binding (probably due to the conformational changes in Trp246^{6,48}), inhibited binding of the agonist [³H]NECA, and competed with Na⁺ ions for the same binding site.

The 1.8 Å structure includes 23 ordered lipid chains and 3 cholesterol molecules per receptor. Together, they form an almost complete lipid bilayer around each protein molecule, mediating crystal contacts (Fig. 4A,B and fig. S9). Lipids on the extracellular side have stronger electron densities and appear to be more ordered. All three cholesterol molecules in this structure are bound to the extracellular half of the receptor and have low B-factors (25 to 27 Å²) in comparison to other lipids (41 to 61 Å²). Two of these cholesterol molecules (CLR1 and CLR3) are bound to symmetry-related receptors and mediate crystal lattice packing by forming face-to-face interactions. The third cholesterol molecule (CLR2) does not participate in crystal contacts. Interestingly, CLR2 and CLR3 occupy hydrophobic grooves along helix VI and form extensive contacts with the aromatic ring of Phe255^{6,57}, which is sandwiched between them (Fig. 4C, fig. S10). In the adenosine family of receptors, position 6.57 is conserved as Ile, Val, or Phe: hydrophobic residues that could all support the type of stacking interaction observed in this structure. In addition, CLR2 forms a hydrogen bond (2.7 Å) with the main-chain carboxyl of Ser263, and the hydroxyl of CLR3 has a polar interaction with sulfur of Cys259 (3.8 Å) in ECL3, the loop that is stabilized by the Cys259-Cys262 disulfide bond. Specific binding and conformational stabilization of this region of helix VI by cholesterol molecules may play a functional role in A_{2A}AR by fixing the position of Asn253^{6,55} in the ligand binding pocket of the receptor (Fig. 4C). This key residue exists in all adenosine receptors and anchors the exocyclic amine of the ligand's central core in both agonist and antagonist complexes (20). Such direct cholesterol binding to A_{2A}AR is also consistent with observations that addition of cholesterol hemisuccinate (CHS) increases the thermostability of the receptor purified in detergent micelles (Fig. 3).

While most lipid chains form non-specific hydrophobic contacts filling grooves on the protein surface, several interactions of lipids with A_{2A}AR may be specific: for example, interactions between the lipids' polar groups and the main chain and side chain polar groups of ECL1 and ECL3. In addition, one lipid (OLA11) intercalates inside the TM bundle

between helices I and VII, thereby protruding into the ligand-binding pocket (Fig. 4D). This lipid molecule apparently stabilizes the conformation of the first eight N-terminal residues of helix I, which do not make any direct contacts with the rest of the helical bundle.

It is intriguing to speculate that GPCRs are allosteric machines (35–38), controlled in part by ions like sodium, membrane components such as lipids and cholesterol, as well as water molecules. Although this concept of allostery is different, with more and more high resolution eukaryotic membrane protein structures and complementary biophysical studies, it seems likely we will start to observe the control of membrane proteins by more than just the pharmacological ligands or the traditional definition of allostery being a singly defined binding site that directly perturbs the orthosteric binding site. The small molecules mentioned can dramatically affect the stability of a protein as well as the function, and this effect can have a pronounced effect on the physiological signaling of GPCRs in very important ways.

Supplementary Material

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Acknowledgments

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Fig. 1. Distribution of ordered waters in A_{2A}AR

In all panels, the antagonist-bound high-resolution structure is shown in cyan and the agonist-bound, active-like state structure (PDB ID 3QAK) is shown in yellow, waters are represented as red spheres, and salt bridges and hydrogen bonds as small green spheres. (A) Interior waters in A_{2A}AR-BRIL-ΔC/ZM241385 structure form an almost continuous water channel (grey; calculated using the program Hollow (37)) containing three major water clusters. (B) The channel is disrupted in the active-like state structure (PDB ID 3QAK). (C) Close-up of the extracellular (EC) water cluster deep in the ligand binding pocket. The water molecule W15 shown as a large red semitransparent sphere stabilizes the kink in helix III. (D) Close-up of the central cluster, which includes waters and a sodium ion (blue transparent sphere). Water molecules W34 and W33 stabilizing the Pro-induced kinks in helices VI and VII are shown as large red semitransparent spheres. (E) Close-up of the intracellular (IC) cluster around the D[E]RY motif in helix III. Despite of close proximity Arg102^{3.50} and Glu228^{6.30} do not form an ionic interaction, instead both amino acids form hydrogen bonds with neighboring waters. An alternative rotamer of Glu228^{6.30}, making a potential 3.0 Å contact with Arg102^{3.50} side chain, is shown in a grey stick representation.

Fig. 2. Structural details of the Na^+ allosteric site in the inactive and active-like state A_2AAR
(A) Sodium ion (blue sphere) in the middle of the 7TM bundle coordinated by highly conserved Asp52^{2.50}, Ser91^{3.39} and 3 water molecules. Receptor is shown as a ribbon, with residues lining the Na^+ cavity shown as sticks and transparent spheres with carbon atoms colored cyan and oxygen atoms red. Water molecules in the cluster are shown as small red spheres, while the salt bridge between Na^+ and Asp52^{2.50} and hydrogen bonds are shown as green dotted lines. **(B)** The pocket collapses in the active-like state $\text{A}_2\text{AAR-T4L-}\Delta\text{C/UK432,097}$ structure, precluding Na^+ binding at this site (hatched sphere designates the position of Na^+ in the inactive structure). **(C)** Structural conservation of the allosteric pocket among solved GPCR structures. (A_2AAR - cyan, CXCR4 - green, rhodopsin - magenta, all other - grey). **(D)** Sequence conservation of the pocket residues among all class A GPCRs (shown as a residue profile in the top row), and among the solved GPCR structures.

Figure 3. Modulation of A_{2A}AR by sodium ions, amiloride and cholesterol
 (A) [³H]ZM241385 or (B) [³H]NECA equilibrium binding to A_{2A}AR-WT and A_{2A}AR-BRIL-ΔC constructs transiently expressed on HEK293 cell membranes in the presence of buffer (control) or buffer supplemented with 150 mM NaCl, 100 μM amiloride, combinations of 100 μM amiloride and 150 mM NaCl, and 150 mM choline chloride. The figures represent data combined from three separate experiments performed in duplicate. Differences in specific binding were analyzed by a Student's t-test. Significant differences were observed for the effect of modulators on control binding and are noted as follows: ** p < 0.01, *** p < 0.001. Significant differences were observed for the effect of NaCl on amiloride modulation and noted as follows: # p < 0.05, ## p < 0.01. There was no significant

effect of choline chloride on [^3H]ZM241385 or [^3H]NECA binding, further proof that Na^+ rather than Cl^- ions caused the effect of NaCl. (C) Shifts in thermostability of $\text{A}_{2\text{A}}\text{AR}$ -BRIL- ΔC construct purified in detergent micelles upon addition of 150 mM NaCl, 100 μM amiloride, combinations of 100 μM amiloride and 150 mM NaCl, 1 μM ZM241385, 1 μM ZM241385 and 150 mM NaCl, and 0.01% CHS. Experiments with ZM241385 have been repeated 6 times, with a standard deviation of less than 1 $^{\circ}\text{C}$. The composition of control buffer was 25 mM Hepes pH 7.5, 0.05% DDM, 0.01% CHS for all samples except for the study of the effect of CHS, in which the control buffer was 25 mM Hepes pH 7.5, 0.05% DDM, 800 mM NaCl.

Fig. 4. Lipid-receptor interactions

(A) Top view of A_{2A}AR (cyan ribbon) including crystallographic neighbors (green: translational symmetry, magenta: rotational, yellow: antiparallel arrangement). Cholesterol molecules are shown as balls with yellow carbons, lipid molecules are shown as sticks with grey carbons. (B) Side view of A_{2A}AR. (C) Potential stabilizing effect of two cholesterols, CLR2 and CLR3, on the conformation of helix VI. Side chains Phe255^{6.57} and Asn253^{6.55} of the A_{2A}AR-BRIL-ΔC/ZM241385 complex are shown as sticks with cyan carbons. The superimposed active-like state A_{2A}AR-T4L-ΔC/UK432,097 is shown as orange ribbon (helix VI only) and sticks (Asn253^{6.55} side chain and NECA scaffold of the UK432,097 agonist only). (D) Lipid molecule (OLA11, grey balls) is inserted in between helices I and VII inside the ligand-binding pocket.