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Review

Structural features of adenosine receptors: From crystal to function ☆

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ABSTRACT

The important role that extracellular adenosine plays in many physiological processes is mediated by the adenosine class of G protein-coupled receptors, a class of receptors that also responds to the antagonist caffeine, the most widely used pharmacological agent in the world. The crystallographic model of the human adenosine A_{2A} receptor was recently solved to 2.6 Å in complex with the antagonist ZM241385, which is also referred to as "*super-caffeine*" because of its strong antagonistic effect on adenosine receptors. The crystallographic model revealed some unexpected and unusual features of the adenosine A_{2A} receptor structure that have led to new studies on the receptor and the re-examination of pre-existing data. Compared to other known GPCR structures, the adenosine A_{2A} receptor has a unique ligand binding pocket that is nearly perpendicular to the membrane plane. The ligand binding site highlights the integral role of the helical core together with the extracellular loops and the four disulfide bridges in the extracellular domain, in ligand recognition by the adenosine class of GPCRs. This article is part of a Special Issue entitled: "Adenosine Receptors".

Contents

1. Introduction

There are four subtypes of adenosine receptor – adenosine A_1, A_{2A}, A_{2B} and A_3 – all of which are members of the heterotrimeric guanine nucleotide-binding protein (G protein) coupled receptor (GPCR) family A (Table 1). Each receptor subtype has a unique ligand binding profile, activation profile, subcellular localization and G protein binding preference (reviewed extensively elsewhere in this special issue on adenosine receptors).

The adenosine A_1 and A_{2A} receptor subtypes modulate neuronal activity by regulating neurotransmitter release and depolarization both presynaptically (adenosine A_1 receptor) and postsynaptically (adenosine A_{2A} receptor) in specific cell types throughout the central and peripheral nervous system. They are implicated in a number of neurological diseases including Alzheimer's disease, Parkinson's

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Table 1
Primary sequence and key properties of human adenosine receptors.

Subtype	Pubmed symbol	PID ID # (PDB ID #)	Chromosomal location	G protein coupling	Predicted MW (by amino acid sequence) [Da]	Length [Residues]	# of potential glycosylation sites	Predicted pI	Predicted phosphorylation	
									Server 1[a]	Server 2[b]
A_1	ADORA1	P30542.1 (−)	1q32.1	i/o	36,512	326	1	8.9	-	Ser176/261/218/219; Thr78/120; Tyr106
A_{2A}	ADORA2A	P29274.2 (3EML)	22q11.2	s, olf	44,707	412	1	8.34	Thr298	Ser156/223/374/387; Thr224/298/319
A_{2B}	ADORA2B	P29275.1 (−)	17p12-p11.2	s, q	36,333	332	2	8.62	Tyr113	Ser115/149/283; Thr40/161/225; Tyr10/229
A_3	ADORA3	P33765.2 (−)	1p13.2	i/o	36,185	318	3	9.11	Ser317; Pro305, Asn316	Ser215/275/317; Thr122/123/228/312; Tyr15/109/293

[a] www.phosphosite.org.
[b] http://www.cbs.dtu.dk/services/NetPhos/.

disease, Lesch–Nyhan syndrome, Creutzfeldt–Jakob disease, Huntington's disease and multiple sclerosis, as well as the brain damage associated with stroke, and have been pursued as therapeutic targets to treat these diseases as well as insomnia, pain and drug addiction [1]. In non-neuronal tissues the adenosine A_{2A} receptor is also involved in inflammation and regulation of myocardial oxygen consumption and coronary blood flow, and the United States Food and Drug Administration recently approved the adenosine A_{2A} receptor agonist Regadenoson (CTV-3146, Lexiscan™) as a coronary vasodilator for use in myocardial perfusion imaging [2].

Activity of the adenosine A_1, $A_{2A,}$ and A_{2B} receptor subtypes is inhibited by naturally occurring methylxanthines such as caffeine or theophylline [3]. Recent epidemiological studies suggest that coffee and tea consumers have a lower risk of Parkinson's disease [4], an effect that has been linked to the blockade of the adenosine A_{2A} receptor subtype by caffeine as the adenosine A_{2A} receptor controls locomotor behaviour and neurotransmitter release in basal ganglia [5,6]. Furthermore, it has recently been observed that the non-xanthine synthetic adenosine A_{2A} receptor antagonist ZM241385, a triazolotriazine, enhanced L-DOPA derived dopamine release [7,8]. Not surprisingly, many drug discovery teams have developed antagonists for the adenosine A_{2A} receptor as potential therapeutic agents to control the impairment of motor skills that occurs in Parkinson's disease.

In this review we summarize the main structural features of the human adenosine A_{2A} receptor:ZM241385 complex, and compare the main similarities and differences of this structure to other solved GPCR structures.

2. Primary sequence and covalent modifications of adenosine receptors

The overall sequence similarity between human adenosine receptors is relatively high (Table 2). The adenosine A_{2A} receptor has higher sequence identity to the adenosine A_{2B} receptor (46%) than to either the adenosine A_1 (37%) or A_3 (31%) receptors. All subtypes have potential N-linked glycosylation sites, and the adenosine A_1, A_{2B} and A_3 receptor subtypes have potential palmitoylation sites at the end of helix 8 that are lacking in the adenosine A_{2A} receptor (Table 1 and Fig. 1; Arg309(8.60)). Glycosylation(s) state does not alter ligand binding properties but may be important in targeting receptors to the plasma membrane. Removal of palmitoylation sites by mutagenesis had no effect on binding to G protein or receptor down-regulation but did affect receptor degradation after synthesis [9,10]. In contrast to the adenosine A_1 receptor, depalmitoylation of the adenosine A_3 receptor increases phosphorylation of the receptor by GPCR kinases (GRKs) causing rapid desensitization of the receptor [9–11]. There are several predicted phosphorylation sites in both the cytoplasmic and carboxy terminal domains of all four adenosine receptor subtypes Table 1.

3. Overall three-dimensional structure and comparison to other GPCRs

High-resolution crystallographic entries in the protein data bank are available for four unique GPCRs: human adenosine A_{2A} receptor (one entry; with ZM241385 bound), turkey β_1-adrenoceptor (one entry; with cyanopindolol), human β_2-adrenoceptor (five entries; with carazolol and timolol bound) and bovine/squid rhodopsin/opsin (14 entries; with and without retinal) (www.pdb.org/). All of these receptors have seven transmembrane α-helices (7TM, helices 1–7) followed by one short membrane-associated helix (helix 8), an extracellular amino-terminus (N-terminus), a cytosolic carboxy terminus (C-terminus), three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3, Fig. 2). Although the primary sequence variation within GPCR family A members is quite large and only a few residues in the transmembrane domains are highly conserved (Fig. 2, Ballesteros–Weinstein residues and so-called microdomains, see section 4.1), the overall structure of the 7TM domain is very similar among all the solved GPCR structures, and differs considerably from the bacterial counterparts such as bacteriorhodopsin ground-state and the photocycle intermediate structures halorhodopsin and sensory rhodopsin [12]. The helical domain of the adenosine A_{2A} receptor can be superimposed over the same domain in the other solved GPCR structures with an r.m.s.d. < 2.6 Å (c-alpha) (Table 3). The r.m.s.d. decreases to ~1.2 Å if only the most conserved residues within these structures are selected (97 residues), indicating that all these GPCRs possess very similar arrangements of their 7TM domains [13]. In contrast, the structures of bacterial rhodopsin and the solved GPCRs do not align very well (Table 3). Despite the high structural

Table 2
The percentage of residues that match exactly (identical residues) between the two sequences. The amino acid sequences were aligned using the TCoffee (www.tcoffee.org) and GeneDoc (http://www.nrbsc.org/gfx/genedoc) programs.

Adenosine receptor subtype (PID ID #)	A_1 (P30542.1)	A_{2A} (P29274.2)	A_{2B} (P29275.1)	A_3 (P33765.2)
A_1 (P30542.1)		37%	42%	46%
A_{2A} (P29274.2)			46%	31%
A_{2B} (P29275.1)				35%
A_3 (P33765.2)				

similarity among GPCR transmembrane domains, the extramembrane domains show significant structural diversity.

4. Heptahelical microdomains in GPCRs

The most conserved residues and sequence motifs among GPCR family A members can be classified as microdomains. These microdomains include: i) the most conserved residues in the transmembrane helices, ii) the DR(3.50)Y-motif, iii) the rotamer toggle residues, iv) the NP(7.50)XXY(X)5,6F-motif and v) Cys-bridge between helix 3 and ECL2.

4.1. The most conserved residues among family A GPCRs

The most conserved helical residues among family A GPCRs are usually referred to as Ballesteros–Weinstein residues and indexed by the numbering system proposed by Ballesteros and Weinstein [14]. Transmembrane helices V, VI and VII have conserved proline residues in the helix. These residues are indexed as X.50, where X is the transmembrane helix number (5–7). Other residues in the same transmembrane helix are indexed relative to the.50 reference number.

Interestingly, the conserved proline residue is typically located in the middle of the transmembrane helix, and the conformation of the proline residue causes bending, irregularities (*phi*-helical and 3(10) conformations) and broken hydrogen bonds in the alpha helix. Because of the high conservation of this residue, it is possible that the strong effects that this mid-helix proline has on helix structure are relevant and important for the conformational changes that the family A GPCRs undergo during activation.

4.2. "Ionic lock" and DR(3.50)Y-motif

The "*ionic lock*" and DR(3.50)Y-motif (and its extension D[E]RY[W]) are highly conserved (>65%) among family A GPCRs, and likely play a role in controlling the activity of the receptor. Rhodopsin in its inactive state exhibits very low basal activity in the dark, which is essential for proper vision. In the structure of bovine rhodopsin in its inactive state, Arg(3.50) of helix 3 makes salt bridges with the equally conserved Glu(6.30) of helix 6 and Glu(3.49) of helix 3. This arrangement is referred to as the "*ionic lock*," and is proposed to play an important role in mainting rhodopsin in the fully inactive conformation. Electron paramagnetic resonance (EPR) and other biophysical methods show that when bound to a short, G protein-mimicking peptide, helices 3 and 6 in the bovine opsin structure are farther apart than in the unbound structure, resulting in disruption of the salt bridge between Arg(3.50), Glu(6.30) and Glu(3.49) [15–19].

In the human adenosine A_{2A} receptor and the β_1-adrenoceptor structures, the conserved residues that correspond to the "*ionic lock*" are arranged differently than in the other GPCR family members solved to date. In the high affinity antagonist-occupied structures of adenosine A_{2A} receptor (ZM241385) and turkey β_1-adrenoceptor (cyanopindolol), Asp(3.49) from the DR(3.50)Y motif interacts with the tyrosine (Tyr3.60) residue from ICL2 instead of Glu(6.30) from helix 6, and constrains the helical conformation of ICL2 (Fig. 3B). Although the human β_2-adrenoceptor has the same sequence motif and a tyrosine at the 3.60 position of ICL2, in both the timolol and carazolol occupied structures, Asp130(3.49) interacts with Ser143 (3.62), and the short helical motif found in ICL2 in the adenosine A_{2A} receptor structure is lacking from both solved β_2-adrenoceptor structures (carazolol and timolol). Despite this lack of a stabilizing salt bridge in the two β_2-adrenoceptor structures, previous observations have suggested that an "*ionic lock*" stabilizes other family A GPCR

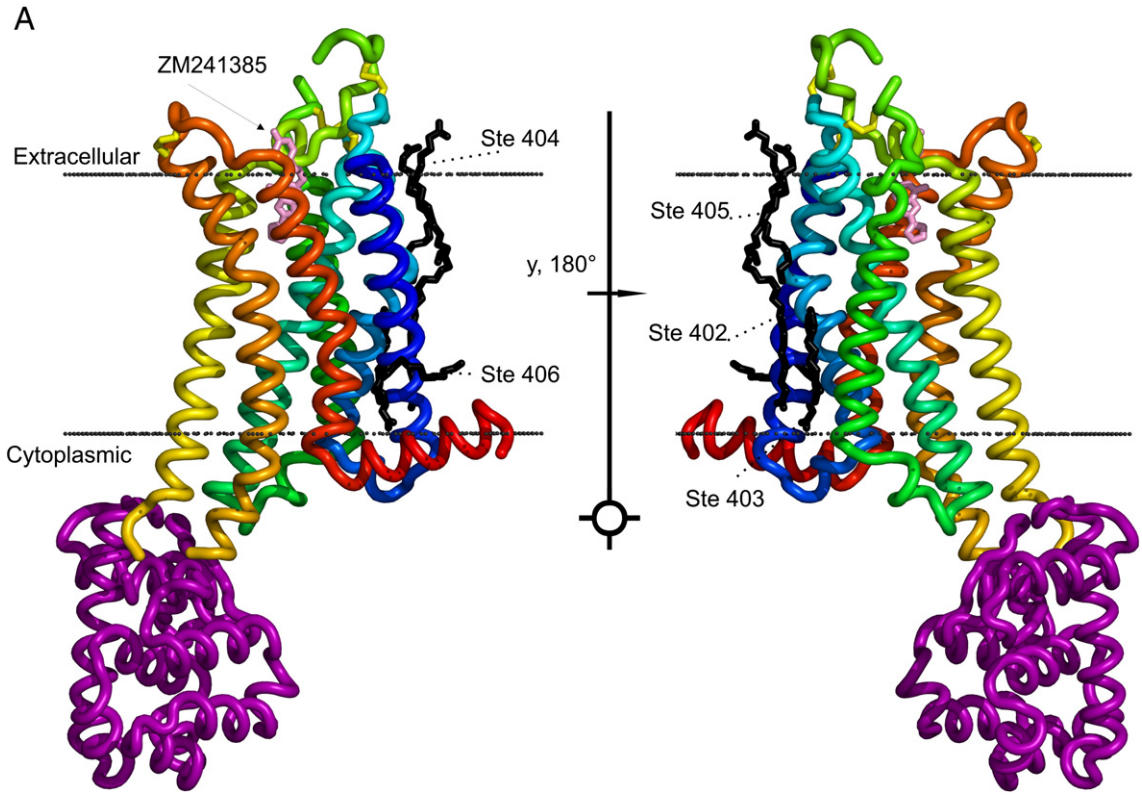

Fig. 1. Three-dimensional structure of human adenosine A_{2A} receptor:antagonist ZM241385 complex (PDB ID # 3EML). A. The overall structure is represented as a rainbow coloured cartoon diagram (N-terminus, blue; C-terminus, red). The T4 lysozyme fusion protein domain is shown in purple. The membrane boundary planes are obtained from http://opm.phar.umich.edu/ and marked as grey coloured "dummy" atoms. Bound ZM241385 (pink) and lipids (black, Ste #402 – Ste #406) are shown as stick models. B. Schematic ligand-plot representation of the hydrogen bonding and aromatic interactions between the receptor and ZM241385 in the antagonist binding cavity.

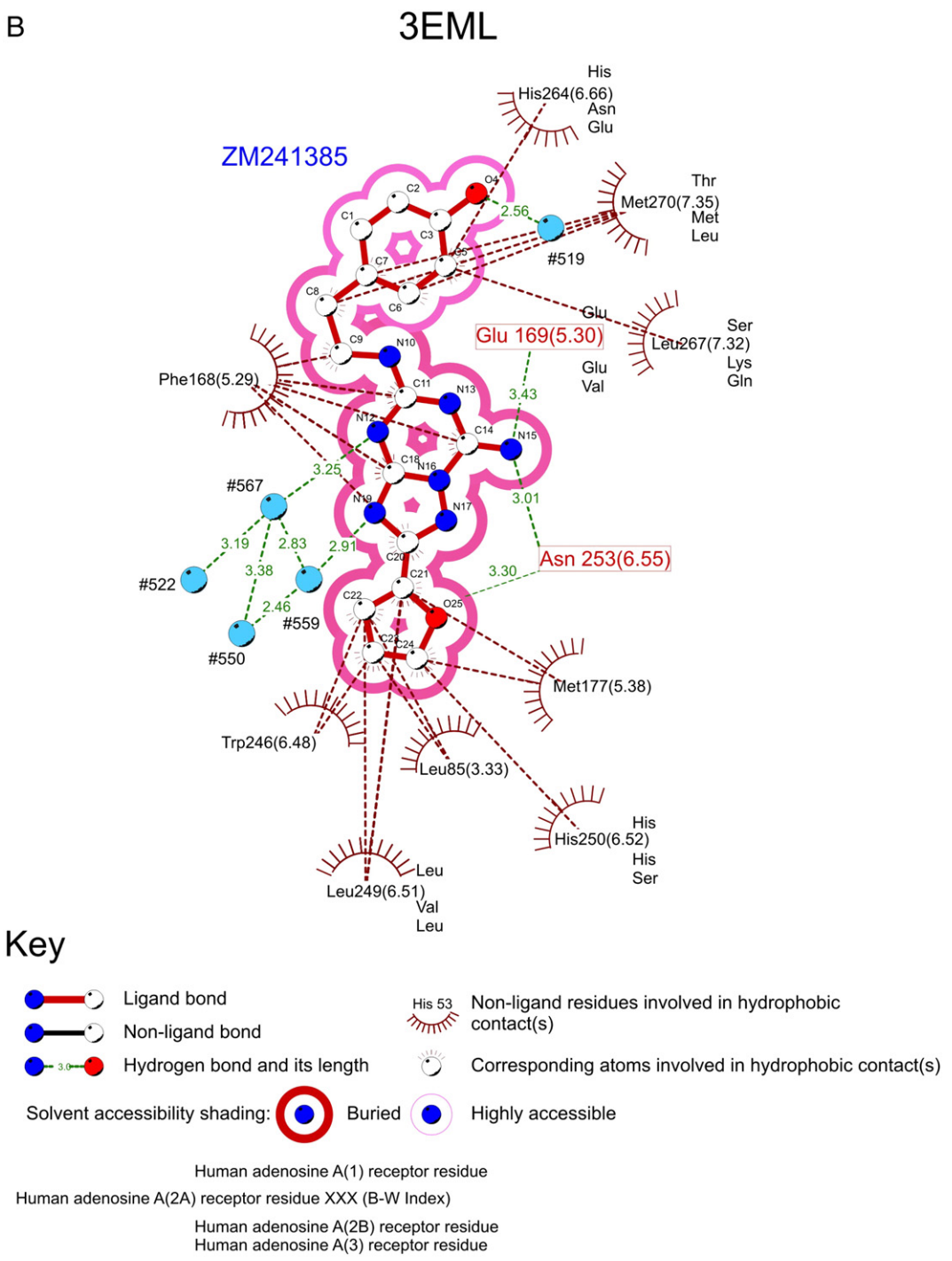

Fig. 1 (*continued*).

members, including adrenoceptors [17,18]. Molecular dynamic simulations with β_2-adrenoceptor indicated the helices 3 and 6 formed an ionic lock similar to that of inactive rhodopsin. This suggests that the β_2-adrenoceptor exists in equilibrium where ionic lock is formed or broken. Other secondary elements are also formed during simulations including the formation of helix in ICL2 [20,21]. Three possible reasons why ionic lock is not observed in any of the non-visual GPCRs is that the trapped crystal structures might not have

Fig. 2. A two dimensional schematic representation of human adenosine receptor subtypes. **Upper panel:** Transmembrane domains. Residues conserved in all four human adenosine subtypes are shown in the green boxes. Residues which bind to the antagonist ZM241385 are in bold framed boxes with their corresponding Ballesteros–Weinstein indexes [14]. **Lower panel**: Extramembrane domains. Conserved residues among human adenosine receptors are indicated as green boxes. The residue variation between human subtypes is indicated as follows; adenosine A_{2A} receptor residues are denoted using white square boxes, adenosine A_1 receptor residues using light grey circles, adenosine A_{2B} receptor residues using grey circles and adenosine A_3 receptor residues using dark grey circles. All human adenosine A_{2A} receptor residues have been numbered in the corresponding boxes. The residues not defined in electron density are indicated in pink boxes. Cysteine residues are shown in yellow colour and bonds between them are in bold green dotted line. The carboxy terminal domain is excluded (Δ317–412). The lipid-bilayer boundaries are indicated as dashed lines. The model is based on the crystal structure of human adenosine A_{2A} receptor subtype (PDB ID # 3EML).

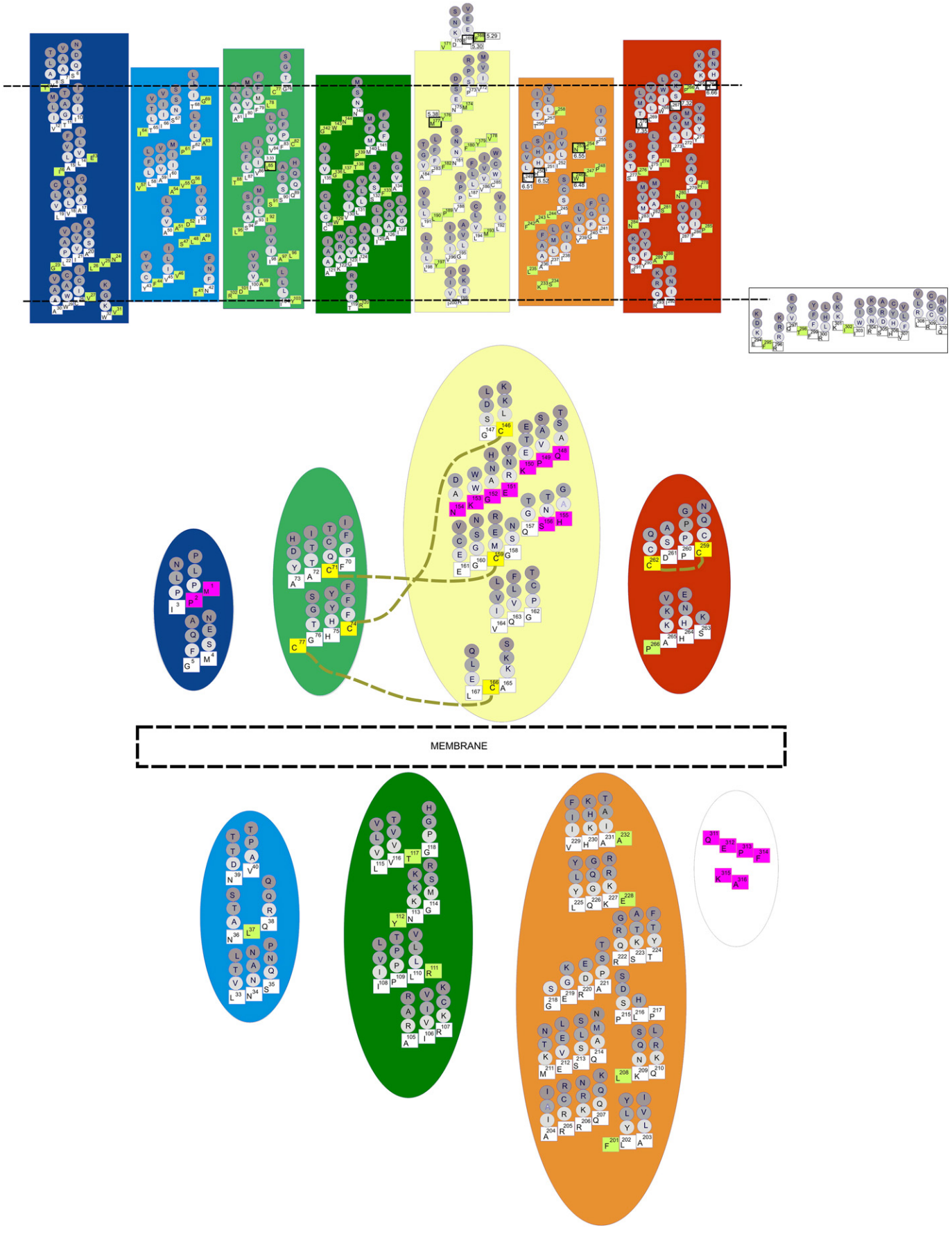
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Table 3
Overall structural similarity between the human adenosine A_{2A} receptor (3EML) and the presentable members of heptahelical receptors.

Receptor	PDB ID # (Chain)	Nominal resolution [Å]	Receptor r.m.s.d. Cα [Å]	# of Cα aligned
H. salinarum Bacteriorhodopsin (*proton pump*)	1M0L	1.47	3.9	183
Human beta2-adrenoceptor	2RH1	2.4	1.2	166
Turkey beta1-adrenoceptor	2VT4 (A)	2.7	1.3	183
	2VT4 (B)		1.4	192
	2VT4 (C)		1.5	180
	2VT4 (D)		1.2	182
Bovine opsin (unligated)	3CAP (A)	2.9	2.5	184
	3CAP (B)		2.5	184
Bovine opsin in complex with a C-terminal peptide of G protein (*active or partially active*)	3DQB (A)	3.2	2.6	186
Bovine rhodopsin	1U19 (A)	2.2	2.0	174
	1U19 (B)		2.0	175

any ionic locks or the modifications required for crystallization such as T4 lysozyme fusion, deletion of ICL3 and addition of antibody fragment might have disrupted the ionic lock or alternatively crystal packing forces could have overcome this interaction.

4.3. The NP(7.50)XXY(X)5,6F motif

In family A GPCRs, the conserved NP(7.50)XXY(X)5,6F sequence motif makes several hydrogen bonds to internal water molecules (Fig. 3A). Some of these waters are conserved among the rhodopsin, β_2-adrenoceptor and adenosine A_{2A} receptor structures. It has been speculated that these water molecules play an important role in the activation process [22,23]. In the adenosine A_{2A} receptor structure, ordered waters form a network connecting the extracellular binding cavity to the intracellular space (Fig. 3A).

5. Adenosine A_{2A} receptor structure

5.1. Engineering, purification and crystallization

The existence of multiple conformations and intrinsically disordered regions in GPCRs makes them difficult to crystallize. Majority of the receptor lies in the lipid phase and this prevents the formation of crystal contacts. Thus native receptor is unsuitable for crystallization. The published crystal structure of the human adenosine A_{2A} receptor is not of the native protein but rather is of an engineered receptor, and the successful crystallization is likely due to the introduction of these critical changes. The engineered receptor construct is modified at the amino-terminus by addition of a signal sequence (hemagglutinin) and a detection tag (FLAG-M2) [24]. The hemagglutinin signal sequence and FLAG-M2 tag as well as the first two residues of the receptor are not visible in the electron density maps. The carboxy terminal domain of the engineered receptor was truncated after position Ala316 (Δ316–412), and a histidine purification epitope (6X-His tag) was added. C-terminal truncation removed the predicted disordered regions and improved the likelihood of crystallogenesis. The residues between Gln311(8.62) and Ala316(8.67) as well as the purification tag are not visible in the electron density map. A glycan [Asn154 (4.75)] was enzymatically removed during purification. The helix 5–third intracellular loop–helix 6 interface is stabilized by inserting T4 lysozyme (T4L) sequences between residues Leu209(5.70) and Ala221(6.23). T4L fusion removed the flexible ICL3, increased the thermostability of the receptor and provided intermolecular contacts required for crystallization.

This engineered receptor construct was expressed in Sf9 insect cells using a baculovirus vector. Raw membranes were separated from soluble protein by extensive high salt washing, the membranes were solubilized in dodecyl maltoside, and the engineered receptor purified *via* the 6-His tag using TALON/Co^{2+}/NTA-matrix affinity chromatography. The receptor was stabilized throughout the purification process by maintaining high-concentrations of sodium chloride, and including cholesterol hemisuccinate and the non-specific, low affinity antagonist theophylline. Theophylline was exchanged to ZM241385 in the last purification step and the purified receptor:ZM241385 complex was reconstituted into a mono-olein:cholesterol mixture. Crystallization occurred at relatively low pH (<6) in the presence of Li_2SO_4 and ~30% PEG400 as precipitant. Final diffraction data from 13 crystals was merged to obtain a complete data set at 2.6 Å resolution, and a number of crystals diffracted beyond 1.8 Å resolution. Excellent electron density was observed for the 7-transmembrane domain and T4 lysozyme. The residues Met1-Pro2, Gln148-Ser156 and Glu311-Ala316 were not modeled.

5.2. Extracellular domains

The extracellular loops of the adenosine A_{2A} receptor form a funnel-like domain that allows open access to the ligand binding cavity (Fig. 1B). The electrostatic surface distribution is more even when compared to β-adrenoceptors, where the negatively charged binding cleft is very prominent. The binding cleft is primarily formed by ECL2 and assumes a random coil structure. The electron density is missing for the amino acids [Gln148(4.69)-Ser156(4.77)] that forms the tip of the extracellular region. In the adenosine A_{2A} receptor model, the end of ECL2 contains a very short alpha helical segment that forms a critical aromatic π-stacking interaction between Phe168 (5.29) and bound ligand (Fig. 1B). The coil structure is held together by four disulfide bridges [71(2.69):159(5.20), 74(3.22):146(4.67) 77 (3.25):166(5.27) and 259(6.61):262(6.64)] (Fig. 2B), the first three of which are unique to the adenosine A_{2A} receptor structure. The human adenosine A_1 and A_{2B} receptors have the corresponding primary sequence for all four disulfide bridges and may also form a coil stabilized by these disulfides, but the adenosine A_3 receptor only has the pair that are conserved among the family A GPCRs (Fig. 2).

5.3. Antagonist binding pocket

The prototypical adenosine receptor antagonist ZM241385 binds to the engineered adenosine A_{2A} receptor in an extended conformation perpendicular to the plane of the membrane bilayer (Fig. 1). This orientation is very different from the retinal:rhodopsin and the beta-blockers:β-adrenoceptor structures in which ligands bind parallel to the plasma membrane. The bicyclic trizolotriazine (BCT) core of ZM241385 is located in the middle of the binding cavity, the furan ring is in the lower part of the binding cavity, and the 4-hydroxyphenylethyl (4-HFE) substituent is in the upper part of the binding cavity, closer to the extracellular space (Fig. 1B).

The BCT core unit forms a number of stabilizing interactions with residues in the binding cavity. The exocyclic nitrogen of BCT core unit makes two polar interactions with the conserved Asn253(6.55) and Glu169(5.30) residues. These polar interactions are important for designing adenosine A_{2A} receptor antagonists because in adenosine A_3 receptor, a valine residue takes the place of Glu169(5.30), eliminating this stabilizing interaction and potentially contributing to the low binding affinity of ZM241385 for the adenosine A_3 receptor. The BCT core unit makes a strong hydrophobic π-stacking interaction with conserved Phe168(5.29), and a hydrophobic interaction with Ile274 (7.39). The BCT core unit makes also several hydrogen bond interactions (directly or indirectly) with crystallographic water molecules (#559, #567, #550 and #522) in the binding cavity (Fig. 1B).

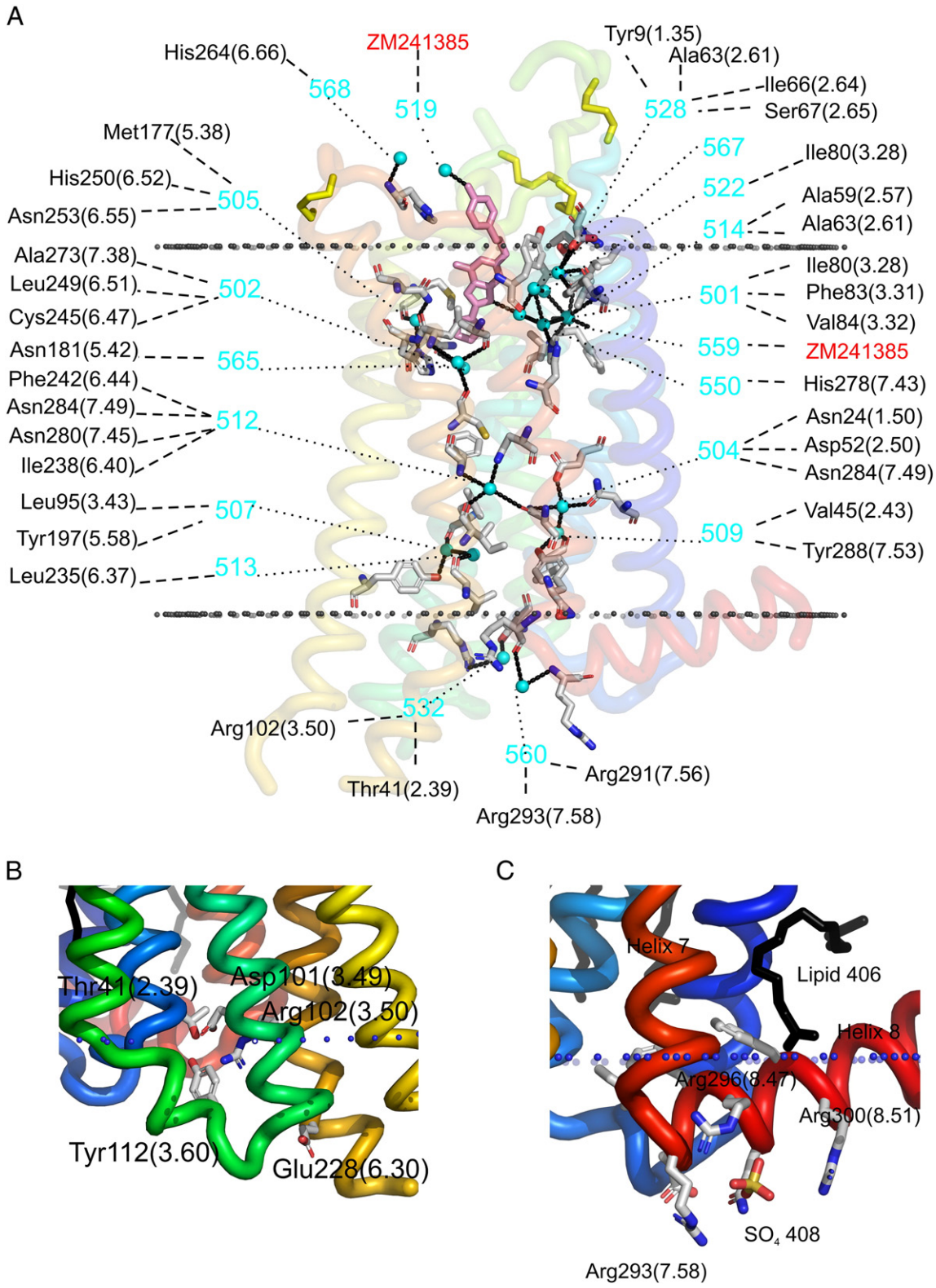

Fig. 3. Structural details of key regions of the human adenosine A_{2A} receptor bound to the antagonist ZM241385. A. Polar interactions between internal water molecules and either receptor residues or ZM241385. Water molecules are shown as blue dots, interacting side chains as white sticks and bound ZM241385 as a pink stick model. Water molecules #519, #559 and #567 directly interacts with antagonist ZM241385. B. Interactions between the DRY motif located in helix 3 and the second intracellular loop. In the adenosine A_{2A} receptor structure the DRY motif does not form the classical "ionic lock" between helixes 3 and 6, but instead Arg102(3.50) interacts with Asp101(3.49) stabilizing its deprotonated state that in turn interacts with Thr41(2.39) of helix two and Tyr112(3.60) of the second intracellular loop. The membrane boundary is indicated as blue dotted line. C. Interactions between three Arg residues [Arg293(7.58), Arg296(8.47)] and Arg300(8.51)] of helix 8 and sulfate #408 ion. The Arg to Ala mutations disrupt the receptor interactions with Calmodulin [31]. The membrane boundary is indicated as blue dotted line.

The furan ring is buried deeply in the binding cavity and forms several hydrophobic interactions [Leu85(3.33), Met177(5.38), Leu249 (6.51), Trp246(6.48) and His250(6.52)] and one polar [Asn253(6.55)] interaction. The furan ring interactions may directly and indirectly restrict the movement of the "*toggle switch*" Trp246(6.48). The rotamer switch of this residue and the nearby aromatic residues [in the CW(6.48)XP(6.50)aa sequence] are predicted to be coupled during activation [25,26]. ZM241385 has a 14 $Å^2$ contact area with Trp246(6.48) compared to 32 $Å^2$ in the rhodopsin:retinal (inactive) structure and 0 $Å^2$ in the β_2-adrenoceptor:carazolol (reduced basal activity; apo-β_2-adrenoceptor have high basal activity *ca*. 50%) structure.

The 4-HFE moiety faces the solvent and makes hydrophobic interactions with His264(6.66), Leu267(7.32) and Met270(7.35), as well as a hydrogen bond with crystallographic water molecule #519 (Figs. 2 and 3). The 4-HFE moiety has relatively high crystallographic temperature factors indicating a larger structural flexibility in this part of the ZM241385 molecule. Correspondingly, larger substitutions are tolerated in this part of the ZM241385 pharmacophore.

Several key interactions involved in ZM241385 and other antagonist binding to the adenosine A_{2A} receptor were recognized by mutagenesis and binding studies prior to the solution of the crystal structure (Table 4), including Glu169(5.30), His250(6.52), Asn253 (6.55) and Ile(7.39). ZM241385 makes several interaction that had not been characterized before, including critical BTC core interactions with Phe168(5.29) and Leu249(6.51). We have started systematic mutagenesis of the binding cavity residues to further validate their roles in ligand binding. Thus far we have validated the critical roles of Phe168(5.29), Leu249(6.51) and Met177(5.38) for ZM241385 and agonists such as NECA/CGS15943. Our data suggest that the BCT core, and the heterocyclic scaffolds generally found in both adenosine receptor agonists and antagonists, interact with the same core group of residues in the receptor [27]. We speculate that the selectivity of ZM241385 for adenosine A_{2A} receptor over adenosine A_{2B} is probably due to very small sequence variations in the residues of the binding cavity (positions 6.51 and 7.32, for example). The selectivity of ZM241385 for the adenosine A_{2A} receptor over the adenosine A_3 receptor subtype is much clearer and most likely caused by differences at positions 6.30, 6.52 and 7.32. Mutagenesis studies provide evidence that there are several residues that influence ligand binding but are not in direct contact (within 5 Å) or close vicinity to ZM241385 in the crystal structure. We speculate based on *in silico* molecular modeling that some of these residues, such as Thr88(3.36), Phe180(5.41), Asn181(5.48, Phe182(5.34), Ser277(7.42), His278 (7.43) and Ser281(7.46), might interact with small molecule agonists such as NECA and CGS15943. Most interestingly, the adenosine A_{2A} receptors have an ordered water cluster (waters #501, #514, #522, #528 and #567) in the binding cavity (Fig. 3). This water cluster makes interactions with some of the residues that were identified as a "*binding residues*" prior to the antagonist-bound x-ray structure but are not in direct contact with or in the vicinity of (5 Å) ZM241385 (Table 4 and Fig. 3). The presence of these waters raises several important questions: What is the role of these waters for antagonist selectivity and agonist binding? Do these waters define the allosteric modulation site? Are these ordered waters specific for ZM241385 binding? How to handle these waters during receptor modeling, *in silico* screening and drug design?

The adenosine A_{2A} receptor:ZM241385 complex structure was also used for conducting *in silico* drug screening with validation of "hits" through *in vivo* ligand binding and G protein activation [28]. Over 4,000,000 commercially available lead compounds were computationally docked to the adenosine A_{2A} receptor structure. The main interaction residues and waters near ZM241385 were used to define and optimize the binding cavity prior docking. Based on the docking results, 56 chemically diverse top lead compounds were tested for activity using radioligand binding and G protein functionality. Out of the 56 compounds tested, 23 showed activity in the micromolar to nanomolar range. All compounds were verified as antagonists and a limited number displayed subtype selectivity. These compound are relatively small (<500 Da) and are derivitazable, making them excellent starting points for drug discovery and development. Very similar results were obtained by Kobilka and co-workers using β_2-adrenoceptor as a template structure [29]. The identification of only antagonists and not agonists by the screen might imply that either the chemical collections are biased towards inactivating compounds or that the agonist-bound receptor structure truly differs from the inactivated structures.

5.4. Intracellular domains, carboxy terminal domain and co-factors

The intracellular domain that mediates G protein binding and signaling is perturbed in the adenosine A_{2A} receptor structure by insertion of T4 lysozyme and truncation of the carboxy terminal domain, both of which were necessary for highly ordered crystal formation. The same is true for β_2-adrenoceptors (with T4 lysozyme and antibody complex) and β_1-adrenoceptor (truncations and receptor mutations). Nevertheless, there are still features of interest to note. The role of the ICL2 and the DRY motifs has already been discussed above (section 4.2) (Fig. 3B). Another interesting feature is the extension of helix 6. Both the adenosine A_{2A} receptor and squid rhodopsin structures have an extended helix 6 compared to the other GPCR structures. In the case of the adenosine A_{2A} receptor, this might be an artifact caused by T4 lysozyme. The lysozyme tilting is different between adenosine A_{2A}:ZM241385, β_2-adrenoceptor:carazolol and β_2-adrenoceptor:timolol receptor structures, although the insertion sequences are overall quite similar, and identical in the case of β_2-adrenoceptors. In the case of squid rhodopsin, the extension might have a role in Gαq protein coupling specificity vs. bovine rhodopsin G protein-transducin specificity.

Removal of the carboxy terminus of the adenosine A_{2A} receptor improved receptor behaviour (such as aggregation, stability and concentration dependent solubility) in detergent micelles. The truncated portion potentially contains long amino acid sequences (about 96 aa) that may lack three-dimensional structure in the absence of accessory/regulatory proteins. Fascinatingly, it has been shown that the carboxy terminus of the adenosine A_{2A} receptor is responsible for G protein-independent signal transduction by PDZ-like and PDZ-independent scaffold proteins. (This is extensively reviewed by Dr. M. Freissmuth and co-workers in the later part of this issue.) It has been also demonstrated in many studies that the carboxy terminus of the adenosine A_{2A} receptor interacts with alpha-actinin (type 2), dopamine receptors (types 2 and 3), glutamate mGlu5 receptors and many other regulatory proteins such as NECAB2 (N-terminal EF-hand calcium binding protein 2), ARNO (ARF-nucleotide binding site opener), TRAX (translin-associated factor X) and calmodulin [30]. Interestingly, arginine residues in helix 8 are believed to contribute to these protein-protein interactions, and arginine residues in helix 8, Arg293(7.58), Arg296(8.47) and Arg300 (8.51), coordinate a SO_4 ion (#408) in the adenosine A_{2A} receptor: ZM241385 complex structure, raising the possibility that the antagonist's inactivating/allosteric effects in *cytosolic* site may be mediated in part through binding of these arginines. These same arginines are also shown to interact with the Ca^{2+}-controlled protein calmodulin [31].

Five lipid molecules were bound to the solved adenosine A_{2A} receptor structure (Fig. 4) and modeled as stearic acid [24]. The electron density maps did not support modeling of cholesterol molecules at this receptor whereas in high resolution β_2-adrenoceptor structures cholesterol moieties were clearly evident [32–34]. Based on the β_2-adrenoceptor structures and sequence similarity to rhodopsin-like GPCRs, a cholesterol consensus motif (CCM) [R/K(4.39–4.40), W/Y(4.50), I/V/L(4.46), F/Y(2.41)] was proposed. The CCM motif is

Table 4
Analysis of artificial mutations for the adenosine A_{2A} receptor residues with respect to ligand binding properties. Table was modified from [38–41].

Residue	Mutation analysis for adenosine A_{2A} receptor ligands			Ligand	Reference
	Mutation	Antagonist/Agonist	Effect on affinity		
Glu13 (1.39)	Gln	Antagonist	No effect	SCH58261, CGS15943, Theo, XAC	[42,43]
		Agonist	Decrease	DPMA, CGS21680, NECA	
Val84 (3.32)	Leu	Antagonist	Decreases	CGS15943, XAC, DPCPX	[44]
		Agonist	No effect	DPMA, NECA, IB-MECA	[44]
Thr88 (3.36)	Asp, Glu	Antagonist	No effect	CGS15943	[39]
		Agonist	No binding	NECA	[39]
	Ala, Ser, Arg	Antagonist	Decreases	CGS15943, ZM241385	[45]
		Agonist	Decreases	CADO, DPMA, NECA, CGS21680	[45]
Gln89 (3.37)	Ala, Asp	Antagonist	Increases	SCH58261, CGS15943, XAC, ZM241385	[39,45]
		Agonist	Increases	CADO, DPMA, NECA, R-PIA, IB-MECA, ADAC	[39,45]
Ser90 (3.38)	Ala	Antagonist	No effect	SCH58261, CGS15943, XAC, ZM241385	[45]
		Agonist	Increases	CADO, DPMA, NECA, R-PIA, IB-MECA, ADAC	[45]
Ser91 (3.39)	Ala	Antagonist	No effect	SCH58261, CGS15943, XAC, ZM241385	[45]
		Agonist	No effect	CADO, DPMA, NECA, R-PIA, IB-MECA, ADAC	[45]
Glu151 (4.72)	Ala, Asp, Gln	Antagonist	No binding	XAC	[46]
		Agonist	No binding	CGS21680	[46]
Glu161 (5.22)	Ala	Antagonist	Increases	CGS15243, XAC	[46]
		Agonist	No effect	CADO, DPMA, R-PIA, IB-MACA, NECA	[46]
Glu169 (5.30)	Ala	Antagonist	No binding	XAC	[46]
		Agonist	No binding	CGS21680	[46]
	Gln	Antagonist	No effect	XAC	[46]
		Agonist	CADO and NECA, increases, for other minor decreases	CADO, DPMA, R-PIA, IB-MECA, NECA	[46]
Asp170 (5.31)	Lys	Antagonist	No effect	XAC	[46]
		Agonist	No effect	CGS21680	[46]
Pro173 (5.34)	Arg	Antagonist	No effect	XAC	[46]
		Agonist	No effect	CGS21680	[46]
Phe180 (5.41)	Ala	Antagonist	No effect	CGS15943, XAC	[47]
		Agonist	No effect	DPMA, NECA	[47]
Asn181 (5.42)	Ser	Antagonist	No effect	CGS15943, XAC	[47]
		Agonist	No effect	CADO, DPMA, NECA, R-PIA	[47]
Phe182 (5.43)	Ala	Antagonist	No binding	CGS15943, XAC	[47]
		Agonist	No binding	CGS21680	[40]
	Tyr, Trp	Antagonist	No effect	CGS15943, XAC	[47]
		Agonist	Decreases	CADO, DPMA, NECA, R-PIA	[47]
His250 (6.52)	Ala	Antagonist	No binding	XAC	[47]
		Agonist	No binding	CGS21680	[47]
His250 (6.52)	Phe, Tyr, Asn	Antagonist	No effect	CGS15943, XAC	[44,47]
	Phe, Tyr	Agonist	Decreases	CADO, DPMA, NECA, R-PIA	[47]
	Asn	Agonist	Minor increase	DPMA, NECA, IB-MECA	[44]
Asn 253 (6.55)	Ala	Antagonist	No binding	XAC	[47]
		Agonist	No binding	CGS21680	[47]
Cys254 (6.56)	Ala	Antagonist	No effect	XAC	[47]
		Agonist	No effect	DPMA, NECA	[47]
Phe257 (6.59)	Ala	Antagonist	No binding	XAC	[47]
		Agonist	No binding	CGS21680	[47]
Cys262 (6.64)	Gly	Antagonist	No effect	XAC	[46]
		Agonist	No effect	CGS21680	[46]
Ile274 (7.39)	Ala	Antagonist	No binding	XAC	[47]
		Agonist	No binding	CGS21680	[47]
Ser277 (7.42)	Asn, Thr, Ala, Cys	Antagonist	No effect	SCH58261, CGS15943, XAC, ZM241385	[45,47]
	Ala	Agonist	No effect	CGS21680, CADO, DPMA, NECA, R-PIA, IB-MECA	[40]
	Cys	Agonist	Decreases	CADO, DPMA, NECA, R-PIA, IB-MECA, ADEC	[45]
	Glu	Antagonist	No effect	CGS15943	[39]
	Glu, Asn, Thr	Agonist	No effect	CADO, DPMA, NECA, R-PIA, IB-MECA	[39,47]
His278 (7.43)	Asp, Glu	Antagonist	No effect	CGS15943	[39]
	Asp, Glu	Agonist	No effect	NECA	[39]
His278 (7.43)	Tyr	Antagonist	Decrease	SCH58261, CGS15943, Theo, XAC	[48]
		Agonist	Decrease	DPMA, CGS21680, CPA, NECA	[48]
His278 (7.43)	Ala	Antagonist	No binding	XAC	[47]
		Agonist	No binding	CGS21680	[47]
Ser281 (7.46)	Ala	Antagonist	No binding	XAC	[47]
		Agonist	No binding	CGS21680	[47]
Ser281 (7.46)	Thr	Antagonist	Increase for XAC	XAC, CGS159261	[47]
		Agonist	Increases	CADO, DPMA, NECA, R-PIA, IB-MECA, ADAC	[47]
Ser281 (7.46)	Asn	Antagonist	Decrease	SCH58261, CGS15943, XAC, ZM241385	[45]
		Agonist	Decrease	SCH58261, CGS15943, XAC, ZM241385	[45]

present in 21% of rhodopsin-like GPCRs. The motif specificity for cholesterol and other lipids/steroids and its pharmacological significance needs to be further verified by mutagenesis and functional studies [35–37].

Steroids/lipids, protein modulators and allosteric compounds might offer opportunities for the design of novel drugs that act by binding at locations other than orthosteric ligand binding site, potentially overcoming subtype selectivity and cross-reactivity

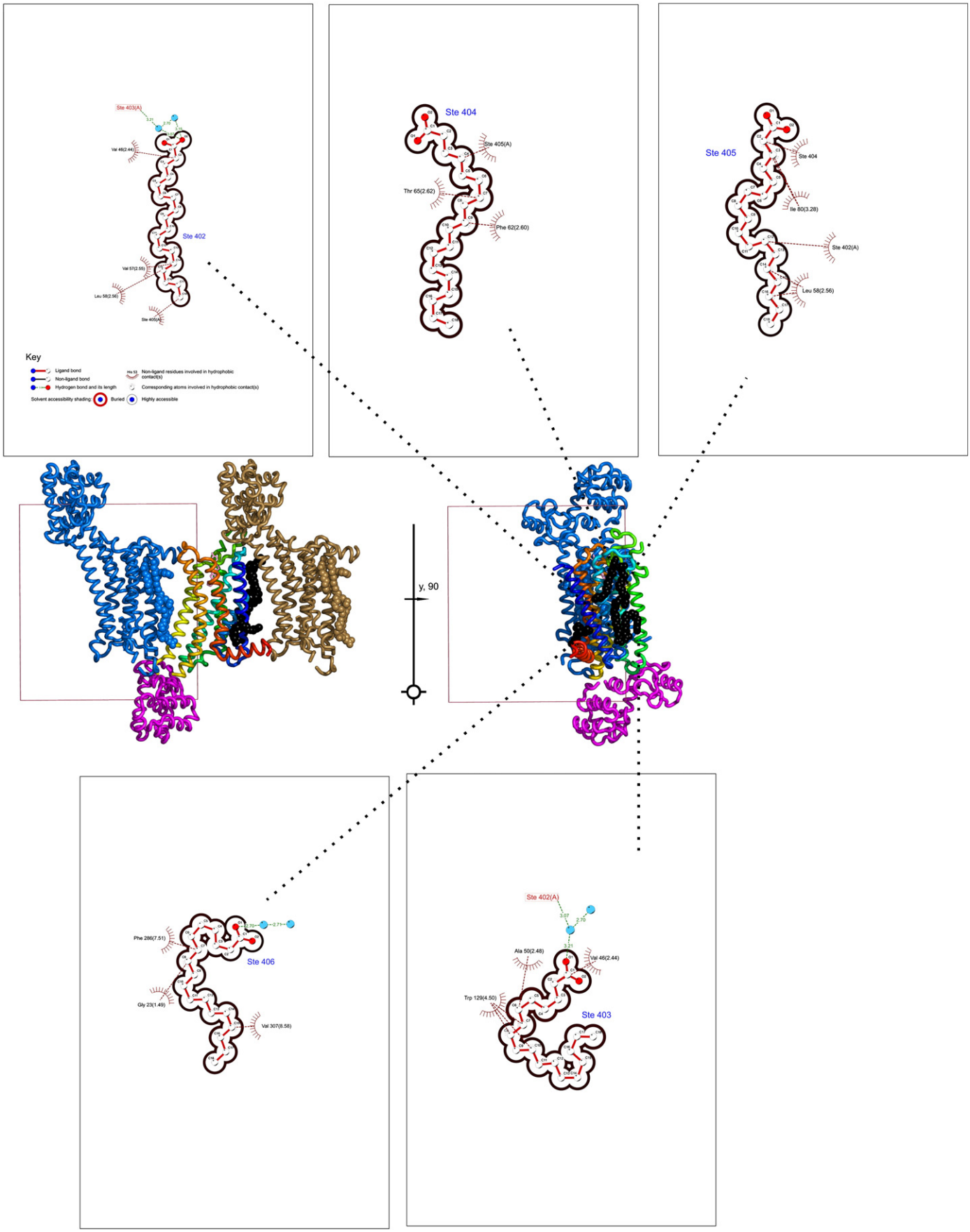

Fig. 4. Lipid binding to the human adenosine A_{2A} receptor. Phospholipids (#402–406) bound to the receptor are shown as ball-and-stick models. Surrounding solvent accessible area and receptor residues or other phospholipid molecules contributing to specific phospholipid binding are shown in smaller boxes surrounding the receptor (middle), and two symmetry related molecules shown in blue and brown. The unit cell is indicated as a purple line.

problems associated with compounds that target the highly conserved orthosteric ligand binding site.

6. Concluding remarks

The crystallographic model of the human adenosine A_{2A} receptor with bound antagonist ZM241385 provides new insight from which a number of previous binding and functional studies can be re-examined and new studies conceptualized. Nevertheless, like any other scientific journey, the crystallographic model of the adenosine receptor and its counterparts the β-adrenoceptor and rhodopsin/opsin have raised more questions than answers. Further research must be targeted to answer these questions: What is the molecular mechanism of ligand selectivity between adenosine receptor subtypes or between β-adrenoceptor subtypes? How different is the agonist-bound state(s) from the antagonist-bound state? Are these states conformationally different and stable or do they merely alter the binding kinetics of the receptor with its intracellular binding partners, and their different populations? There are also more demanding questions around the corner such as the activation mechanism and the receptor:agonist:G protein complexes. Based on several conference presentations and public web-sites this will be an exciting and interesting year for new GPCRs structures; several new GPCRs structures have been solved but not yet deposited, including new β-adrenoceptors and adenosine receptor complexes, the chemokine CXCR4 receptor, the dopamine D_3 receptor and the Sphingosine-1-phosphate S1P1 receptor (http://cmpd.scripps.edu; http://www.receptos.com; Stevens, unpublished). It is our hope that some of these structures might answer some of these questions.

The recent and new crystallographic models with various mutations, fusions and truncations may easily lead to the "*drunks under the lamp-post*" problem – "*you know the keys are not there but the light is bright so that is where you look for them*". The static crystallographic models need to be complemented with biophysical, biochemical and pharmacological approaches to fully appreciate the dynamic nature of heptahelical receptors and their signaling partners.

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References

[1] T.W. Stone, S. Ceruti, M.P. Abbracchio, Adenosine receptors and neurological disease: neuroprotection and neurodegeneration, Handb. Exp. Pharmacol. (2009) 535–587.
[2] G.S. Thomas, R.C. Thompson, M.I. Miyamoto, T.K. Ip, D.L. Rice, D. Milikien, H.D. Lieu, V.S. Mathur, The RegEx trial: a randomized, double-blind, placebo- and active-controlled pilot study combining regadenoson, a selective A(2A) adenosine agonist, with low-level exercise, in patients undergoing myocardial perfusion imaging, J. Nucl. Cardiol. 16 (2009) 63–72.
[3] J.W. Daly, R.F. Bruns, S.H. Snyder, Adenosine receptors in the central nervous system: relationship to the central actions of methylxanthines, Life Sci. 28 (1981) 2083–2097.
[4] M.A. Hernan, B. Takkouche, F. Caamano-Isorna, J.J. Gestal-Otero, A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease, Ann. Neurol. 52 (2002) 276–284.
[5] S. Ferre, C. Quiroz, A.S. Woods, R. Cunha, P. Popoli, F. Ciruela, C. Lluis, R. Franco, K. Azdad, S.N. Schiffmann, An update on adenosine A2A-dopamine D2 receptor interactions: implications for the function of G protein-coupled receptors, Curr. Pharm. Des. 14 (2008) 1468–1474.
[6] S. Ferre, F. Ciruela, J. Borycz, M. Solinas, D. Quarta, K. Antoniou, C. Quiroz, Z. Justinova, C. Lluis, R. Franco, S.R. Goldberg, Adenosine A1-A2A receptor heteromers: new targets for caffeine in the brain, Front. Biosci. 13 (2008) 2391–2399.
[7] K. Golembiowska, A. Dziubina, M. Kowalska, K. Kaminska, Effect of adenosine A(2A) receptor antagonists on L: -DOPA-induced hydroxyl radical formation in rat striatum, Neurotox. Res. 15 (2009) 155–166.
[8] K. Golembiowska, A. Dziubina, Striatal adenosine A(2A) receptor blockade increases extracellular dopamine release following l-DOPA administration in intact and dopamine-denervated rats, Neuropharmacology 47 (2004) 414–426.
[9] Z. Gao, Y. Ni, G. Szabo, J. Linden, Palmitoylation of the recombinant human A1 adenosine receptor: enhanced proteolysis of palmitoylation-deficient mutant receptors, Biochem. J. 342 (Pt 2) (1999) 387–395.
[10] Z. Gao, A.S. Robeva, J. Linden, Purification of A1 adenosine receptor-G-protein complexes: effects of receptor down-regulation and phosphorylation on coupling, Biochem. J. 338 (Pt 3) (1999) 729–736.
[11] T.M. Palmer, G.L. Stiles, Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor, Mol. Pharmacol. 57 (2000) 539–545.
[12] T. Hirai, S. Subramaniam, J.K. Lanyi, Structural snapshots of conformational changes in a seven-helix membrane protein: lessons from bacteriorhodopsin, Curr. Opin. Struct. Biol. 19 (2009) 433–439.
[13] M.A. Hanson, R.C. Stevens, Discovery of new GPCR biology: one receptor structure at a time, Structure 17 (2009) 8–14.
[14] J.A. Ballesteros, H. Weinstein, Integrated methods for modeling G-protein coupled receptors, Methods Neurosci. 25 (1995).
[15] P. Scheerer, J.H. Park, P.W. Hildebrand, Y.J. Kim, N. Krauss, H.W. Choe, K.P. Hofmann, O.P. Ernst, Crystal structure of opsin in its G-protein-interacting conformation, Nature 455 (2008) 497–502.
[16] C. Altenbach, A.K. Kusnetzow, O.P. Ernst, K.P. Hofmann, W.L. Hubbell, High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7439–7444.
[17] J.A. Ballesteros, A.D. Jensen, G. Liapakis, S.G. Rasmussen, L. Shi, U. Gether, J.A. Javitch, Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6, J. Biol. Chem. 276 (2001) 29171–29177.
[18] X. Yao, C. Parnot, X. Deupi, V.R. Ratnala, G. Swaminath, D. Farrens, B. Kobilka, Coupling ligand structure to specific conformational switches in the beta2-adrenoceptor, Nat. Chem. Biol. 2 (2006) 417–422.
[19] S. Ye, E. Zaitseva, G. Caltabiano, G.F. Schertler, T.P. Sakmar, X. Deupi, R. Vogel, Tracking G-protein-coupled receptor activation using genetically encoded infrared probes, Nature 464 (2010) 1386–1389.
[20] R.O. Dror, D.H. Arlow, D.W. Borhani, M.O. Jensen, S. Piana, D.E. Shaw, Identification of two distinct inactive conformations of the beta2-adrenergic receptor reconciles structural and biochemical observations, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 4689–4694.
[21] S. Vanni, M. Neri, I. Tavernelli, U. Rothlisberger, A conserved protonation-induced switch can trigger "ionic-lock" formation in adrenergic receptors, J. Mol. Biol. 397 (5) (2010) 1339–1349.
[22] T.E. Angel, S. Gupta, B. Jastrzebska, K. Palczewski, M.R. Chance, Structural waters define a functional channel mediating activation of the GPCR, rhodopsin, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14367–14372.
[23] T.E. Angel, M.R. Chance, K. Palczewski, Conserved waters mediate structural and functional activation of family A (rhodopsin-like) G protein-coupled receptors, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 8555–8560.
[24] V.P. Jaakola, M.T. Griffith, M.A. Hanson, V. Cherezov, E.Y. Chien, J.R. Lane, A.P. Ijzerman, R.C. Stevens, The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist, Science 322 (2008) 1211–1217.
[25] L. Shi, G. Liapakis, R. Xu, F. Guarnieri, J.A. Ballesteros, J.A. Javitch, Beta2 adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch, J. Biol. Chem. 277 (2002) 40989–40996.
[26] B. Holst, R. Nygaard, L. Valentin-Hansen, A. Bach, M.S. Engelstoft, P.S. Petersen, T.M. Frimurer, T.W. Schwartz, A conserved aromatic lock for the tryptophan rotameric switch in TM-VI of seven-transmembrane receptors, J. Biol. Chem. 285 (2010) 3973–3985.
[27] V.P. Jaakola, J.R. Lane, J.Y. Lin, V. Katritch, A.P. Ijzerman, R.C. Stevens, Identification and characterization of amino acid residues essential for human A2A adenosine receptor:ZM241385 binding and subtype selectivity, J. Biol. Chem. (2010).
[28] V. Katritch, V.P. Jaakola, J.R. Lane, J. Lin, A.P. Ijzerman, M. Yeager, I. Kufareva, R.C. Stevens, R. Abagyan, Structure-based discovery of novel chemotypes for adenosine A(2A) receptor antagonists, J. Med. Chem. 53 (2010) 1799–1809.
[29] P. Kolb, D.M. Rosenbaum, J.J. Irwin, J.J. Fung, B.K. Kobilka, B.K. Shoichet, Structure-based discovery of beta2-adrenergic receptor ligands, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 6843–6848.
[30] F. Ciruela, C. Albergaria, A. Soriano, L. Cuffi, L. Carbonell, S. Sanchez, J. Gandia, V. Fernandez-Duenas, Adenosine receptors interacting proteins (ARIPs): behind the biology of adenosine signaling, Bba-Biomembranes 1798 (2010) 9–20.
[31] G. Navarro, M.S. Aymerich, D. Marcellino, A. Cortes, V. Casado, J. Mallol, E.I. Canela, L. Agnati, A.S. Woods, K. Fuxe, C. Lluis, J.L. Lanciego, S. Ferre, R. Franco, Interactions between calmodulin, adenosine A2A, and dopamine D2 receptors, J. Biol. Chem. 284 (2009) 28058–28068.
[32] M.A. Hanson, V. Cherezov, M.T. Griffith, C.B. Roth, V.P. Jaakola, E.Y. Chien, J. Velasquez, P. Kuhn, R.C. Stevens, A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor, Structure 16 (2008) 897–905.
[33] V. Cherezov, D.M. Rosenbaum, M.A. Hanson, S.G. Rasmussen, F.S. Thian, T.S. Kobilka, H.J. Choi, P. Kuhn, W.I. Weis, B.K. Kobilka, R.C. Stevens, High-resolution

crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor, Science 318 (2007) 1258–1265.
[34] D.M. Rosenbaum, V. Cherezov, M.A. Hanson, S.G. Rasmussen, F.S. Thian, T.S. Kobilka, H.J. Choi, X.J. Yao, W.I. Weis, R.C. Stevens, B.K. Kobilka, GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function, Science 318 (2007) 1266–1273.
[35] R.S. Lam, D. Nahirney, M. Duszyk, Cholesterol-dependent regulation of adenosine A(2A) receptor-mediated anion secretion in colon epithelial cells, Exp. Cell Res. 315 (2009) 3028–3035.
[36] E. Lyman, C. Higgs, B. Kim, D. Lupyan, J.C. Shelleys, R. Farid, G.A. Voth, A role for a specific cholesterol interaction in stabilizing the Apo configuration of the human A(2A) adenosine receptor, Structure 17 (2009) 1660–1668.
[37] M.A. O'Malley, T. Lazarova, Z.T. Britton, A.S. Robinson, High-level expression in *Saccharomyces cerevisiae* enables isolation and spectroscopic characterization of functional human adenosine A2a receptor, J. Struct. Biol. 159 (2007) 166–178.
[38] A. Martinelli, T. Tuccinardi, Molecular modeling of adenosine receptors: new results and trends, Med. Res. Rev. 28 (2008) 247–277.
[39] S.K. Kim, Z.G. Gao, P. Van Rompaey, A.S. Gross, A. Chen, S. Van Calenbergh, K.A. Jacobson, Modeling the adenosine receptors: comparison of the binding domains of A2A agonists and antagonists, J. Med. Chem. 46 (2003) 4847–4859.
[40] K.A. Jacobson, Z.G. Gao, Adenosine receptors as therapeutic targets, Nat. Rev. 5 (2006) 247–264.
[41] B.B. Fredholm, P.A. Ijzerman, K.A. Jacobson, K.N. Klotz, J. Linden, International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors, Pharmacol. Rev. 53 (2001) 527–552.
[42] Z.G. Gao, Q. Jiang, K.A. Jacobson, A.P. Ijzerman, Site-directed mutagenesis studies of human A(2A) adenosine receptors: involvement of glu(13) and his(278) in ligand binding and sodium modulation, Biochem. Pharmacol. 60 (2000) 661–668.
[43] A.P. Ijzerman, J.K. Von Frijtag Drabbe, Kunzel, J. Kim, Q. Jiang, K.A. Jacobson, Site-directed mutagenesis of the human adenosine A2A receptor. Critical involvement of Glu13 in agonist recognition, Eur. J. Pharmacol. 310 (1996) 269–272.
[44] Q. Jiang, B.X. Lee, M. Glashofer, A.M. van Rhee, K.A. Jacobson, Mutagenesis reveals structure-activity parallels between human A2A adenosine receptors and biogenic amine G protein-coupled receptors, J. Med. Chem. 40 (1997) 2588–2595.
[45] Q. Jiang, A.M. Van Rhee, J. Kim, S. Yehle, J. Wess, K.A. Jacobson, Hydrophilic side chains in the third and seventh transmembrane helical domains of human A2A adenosine receptors are required for ligand recognition, Mol. Pharmacol. 50 (1996) 512–521.
[46] J. Kim, Q. Jiang, M. Glashofer, S. Yehle, J. Wess, K.A. Jacobson, Glutamate residues in the second extracellular loop of the human A2a adenosine receptor are required for ligand recognition, Mol. Pharmacol. 49 (1996) 683–691.
[47] J. Kim, J. Wess, A.M. van Rhee, T. Schoneberg, K.A. Jacobson, Site-directed mutagenesis identifies residues involved in ligand recognition in the human A2a adenosine receptor, J. Biol. Chem. 270 (1995) 13987–13997.
[48] Z.G. Gao, A.P. Ijzerman, Allosteric modulation of A(2A) adenosine receptors by amiloride analogues and sodium ions, Biochem. Pharmacol. 60 (2000) 669–676.