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Ligand-dependent cholesterol interactions with the human A_{2A} adenosine receptor

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Abstract

We present nearly ten microseconds of all-atom simulation data of a G-protein coupled receptor, the human A_{2A} adenosine receptor, bound to four different ligands. Our focus is on binding of cholesterol to the “cholesterol consensus motif,” a cluster of five amino acids on the second and fourth transmembrane helices, which interact with two cholesterol molecules in the intracellular leaflet of the bilayer. We find evidence for a ligand-specific interaction between the CCM and cholesterol, mediated by the rotameric dynamics and configuration of Trp129. Binding of the synthetic agonist UK432097 disrupts hydrogen bonding between Trp129 and Ser47, which activates the rotameric dynamics of Trp129 and disrupts the interaction with one of the two cholesterol molecules. We also investigate the effect of four thermostabilizing mutations, three of which are located on helix two. The conformational stability of helix two has been proposed to be sensitive to interaction with cholesterol in the CCM, suggesting a mechanism for the thermostabilization. However, our data are instead suggestive of a force-field dependent “straightening” of helix two, and therefore offer no basis for rationalizing the effect of the quadruple mutant.

Keywords

G-protein coupled receptor; Cholesterol; Molecular dynamics

1.0 Introduction

The G-protein coupled receptors (GPCR) are diverse superfamily of integral membrane proteins that share a common seven transmembrane helix topology. (Congreve and Marshall, 2010; Filmore, 2004) Comprising some 800 members in the humane proteome, they transduce chemical signals in an enormous variety of physiological contexts, ranging from smell and taste, to regulating the excitability of brain and heart tissue, to modulating the immune response. (Gilcrest, 2010) Ubiquitous and accessible to pharmacological agents, the GPCRs account for the targets of nearly half the drugs currently on the market.

Being integral membrane proteins, much attention has focused on the role of the lipid matrix in regulating GPCR function. Cholesterol, in particular, is believed to regulate class A GPCR function. (Paila et al., 2009; Pucadyil and Chattopadhyay, 2006) Cholesterol has been

known for some time to regulate the function of Rhodopsin, with increased cholesterol shifting the protein toward the inactive state.(Albert et al., 1996; Bennett and Mitchell, 2008; Mitchell et al., 1990) More recently, extensive simulations of rhodopsin predicted specific cholesterol interaction sites(Khelashvili et al., 2009; Pitman et al., 2005). Specific cholesterol binding was observed in high resolution crystal structures of the β_2 adrenergic receptor, and was found to stabilize the receptor against denaturation.(Cherezov et al., 2007; Hanson et al., 2008) Four residues that interact with the resolved cholesterol are conserved in many class A GPCRs, and therefore proposed to form a “cholesterol consensus motif,” (CCM) which is also found in A_{2A} .(Hanson et al., 2008) The CCM was then investigated by bioinformatic techniques, and expanded to include an additional residue on the basis of purifying selection.(Adamian et al., 2011) A cholesterol consensus motif has also been identified in the serotonin 1A receptor,(Jafurulla et al., 2011) and proposed to mediate observed regulation of that receptor by cholesterol.(Saxena and Chattopadhyay, 2012) Several cholesterol molecules were also observed in a recently published structure of the A_{2A} adenosine receptor.(Liu et al., 2012)

Here we focus on the A_{2A} adenosine receptor. The first published long timescale simulations of A_{2A} already investigated the role of specific cholesterol binding(Lyman et al., 2009) based on the crystal structure of A_{2A} cocrystallized with an antagonist.(Jaakola et al., 2008) In that work, the authors placed two cholesterol molecules at the location observed in the β_2 AR structure, interacting with the CCM in a cleft in the intracellular leaflet in between transmembrane helices 1 and 3.(Hanson et al., 2008) It was observed that (i) two cholesterol molecules bind in the same location as observed in the β_2 AR crystal structure on the simulation timescale of 1 microsecond, and (ii) when bound, the cholesterol molecules stabilize the kinked structure of transmembrane helix 2. Simultaneously, and unbeknownst to those authors, O'Malley, et al. were characterizing human A_{2A} in micelle environments. Using a combination of small angle neutron diffraction, biochemical, and biophysical characterization, O'Malley, et al. observed that native like ligand binding is only obtained in micelles that contain mol fractions of cholesterol derivatives which recapitulate native membrane environments.(O'Malley et al., 2011a; O'Malley et al., 2011b) At these mol fractions, small angle neutron diffraction data indicated that the hydrophobic thickness of the micelle is similar to what one would expect in a native membrane. And very recently, a new high resolution structure of A_{2A} was published in which several specifically bound cholesterol molecules were observed.(Liu et al., 2012) None of those cholesterol molecules are in the pocket between helices 1 and 3 observed in the β_2 AR structure and studied by Lyman, et al., although electron density was observed in that region. If the density corresponds to a cholesterol, it is apparently too disordered to be resolved. Recently published simulation results suggest three interaction sites for cholesterol on the A_{2A} receptor, one of which overlaps with one of the crystallographically observed sites, and two which await experimental confirmation.(Lee and Lyman, 2012b)

In this paper, we follow up on the work of Lyman, et al., leveraging several new high-resolution structures of A_{2A} published in the interim in which the protein is cocrystallized with three different agonists.(Lebon et al., 2011; Xu et al., 2011) One of these structures was solved by the T4 lysozyme fusion strategy, and two were solved by a quadruple mutation that stabilizes the agonist bound form. The quadruple mutant is intriguing in the context of the hypothesis that cholesterol binding stabilizes helix 2, as three of the four mutations are located on helix 2, and the fourth is situated to interact with helix 2. We therefore performed a series of molecular dynamics simulations based on each of these four structures, in each case including two cholesterol molecules aligned to those in the β_2 AR structure(Hanson et al., 2008) at the CCM site in the pocket between helices 1 and 3. We also performed calculations based on the adenosine bound structure—which was solved by the quadruple mutant strategy—but with the wild type sequence of the receptor, in order to examine the impact of

the mutations on the structure and dynamics of helix 2. We present three observations based on an analysis of helix 2 and the interactions with cholesterol in each receptor state. (1) In most cases, the cholesterol maintains interactions with the protein in the neighborhood of their initial positions, but that they are overall very dynamic—the binding is not “tight”, as in receptor-drug interactions. This is consistent with recently published blind predictions of cholesterol interaction sites. (Lee and Lyman, 2012b) (2) There are reproducible differences in the cholesterol interactions among the receptor states, but surprisingly these differences are not between agonist and antagonist forms, or between the T4L structures and the mutant structures, but rather between the UK432097 bound structure and the rest. (3) In several trajectories, helix 2 was observed to undergo a noticeable conformational change, in which a “bulging” turn of helix in the neighborhood of a proline kink became more helical.

2.0 Simulation details

For each of the four ligation states, initial structures were built starting from the coordinates deposited for each in the protein data bank: 3EML(Jaakola et al., 2008) (ZM241385 bound), 3QAK(Xu et al., 2011) (UK432097 bound), 2YDO(Lebon et al., 2011) (adenosine bound), and 2YDV(Lebon et al., 2011) (NECA bound). For the T4L fusion constructs (3EML and 3QAK) the T4L was excised from the third intracellular loop, and the wild type sequence inserted with coordinates taken from a well-equilibrated, previously published structure. (Lyman et al., 2009) The missing region of the 2YDO and 2YDV structures (residues 214–223) were from our other previously published simulations of the adenosine bound receptor. (Lee and Lyman, 2012a) Native receptor initial coordinates were generated by replacing the four thermostabilizing mutations with their wild type counterparts. The four new sidechains were then minimized with the backbone fixed. The membrane-protein system was then subjected to the same minimization and heating protocol as the other structures, described below. Table 1 lists all the simulations, their duration, ligation states, and the details of the structure preparation.

The palmitoyl-oleoyl phosphatidylcholine (POPC) bilayer was built around the protein (residues 3–310 in each case) using Maestro v. 9.2 by aligning the proteins to the 3EML structure in the orientations of proteins in membranes database. (Lomize et al., 2006) Two cholesterol molecules were situated in the CCM as they are observed in the β 2AR structure (Hanson et al., 2008) in each case. The simulation cell was sized to ensure at least 10 Å between periodic images of the protein, resulting in approximately 90 POPC, 9,000 TIP3P water, and a simulation cell with dimensions 80 Å × 60 Å × 100 Å. The protein was modeled with the CHARMM22 forcefield with CMAP correction, (MacKerell et al., 1998) the lipids with the CHARMM36 parameter set, (Klauda et al., 2010) and the ligands were typed from the CGenff (Vanommeslaeghe et al., 2010) using the paramchem webserver.

Initial relaxation and heating followed a 7 stage minimization and heating protocol distributed with the Maestro 2009 suite, designed specifically for membrane protein systems. The protocol includes 4000 steps of steepest descent minimization, followed by heating and equilibration totaling 2 nsec while backbone restraints are gradually removed. The equations of motion were integrated with Desmond v30110 (Bowers et al., 2006) under conditions of constant pressure and temperature (1 atm and 310 K) using the Martyna-Tobias-Klein coupling method. (Martyna et al., 1994) Electrostatic interactions were computed every timestep with the particle mesh Ewald method; (Darden et al., 1993) Lennard-Jones interactions were cutoff at 10 Å and both the potential and force were switched smoothly to zero from 10 to 12 Å, as demanded by the CHARMM36 parameter set. Full details can be found in the simulation configuration file included as a supplement. All trajectories were run for approximately 1 μ sec, requiring roughly 25 days per trajectory on 150 cores of our local cluster. (2 quad core Intel Xeon E5630 chips per node, internode

communication via Mellanox infiniband switch.) All molecular images were rendered with VMD.(Humphrey et al., 1996)

3.0 TM helix 2 stability

Given that three of the four agonist-bound stabilizing mutations are found on helix 2, we were interested to compare the stability and conformational dynamics of helix 2 between the mutant and wild type sequences. The locations of the four mutations are shown in Figure 1. Two of the mutations—L48A and Q89A—are thought to be involved in transitions between active and inactive conformations. No obvious explanation exists for the stabilizing effect of the other two mutations. Might they function by stabilizing helix 2, the structure of which has been observed to be sensitive to cholesterol binding in the CCM?(Lyman et al., 2009) To this end, in Figure 2 the alpha carbon root mean squared deviation of helix 2 from the initial structure is plotted for each of the ten trajectories.

The strongest feature in the conformational dynamics, indicated by a vertical arrow on the plots, is present in one copy of each receptor. At the arrows, a sudden and significant jump in the RMSD of helix 2 is observed. Inspection of the trajectories shows that this increase corresponds to a conformational change in a region that, in the crystal structures, is not a canonical α -helix. The region appears as a “bulge” at the location of a proline kink—the wild type sequence, starting with residue 56, is Gly-Val-Leu-Ala-Ile-Pro, with the bulge most prominent at the positions of the Val and Leu, which face the interior of the membrane. The conformational change observed in the RMSD timeseries corresponds to this bulge region becoming more like a canonical helix, as shown in detail in Figure 3. The fact that this shift is observed in both the wild type and mutant sequences would seem to rule out any specific sequence dependence. Indeed, the same sudden shift is observed in several other trajectories, some of which are mutant and some of which are wild type, each indicated in Figure 2 by an arrow. Furthermore, several other trajectories—in which a sudden jump in the RMSD is not observed—still evolve over time into a similar conformation. These trajectories are the other two adenosine trajectories and the other NECA trajectory. The UK432097 trajectories also have a similar bulge in the crystal structure, but these trajectories evolve toward a *different* conformation, in which the kink in helix 2 becomes *more* pronounced. Taken together, these data seem to indicate that the preferred conformation under the force field used here is one in which the bulge at residues 56 and 57 is relieved. This result may reflect the structural dynamics of the protein in its native environment, but it is also conceivable that it is simply an artifact of the forcefield. It will therefore be essential in future work to repeat several of our calculations with a different empirical forcefield.

Recently published results demonstrate that adenosine, when bound to the mutant receptor, is in fact very dynamic, sampling a variety of poses not observed in the crystal structure.(Lee and Lyman, 2012a) As the mutant receptor was selected for its ability to stabilize the agonist bound form, it is worth considering our new data for the mutant and wild type receptors when bound to adenosine in this context. In light of our previous data, it is interesting that we observe spontaneous unbinding of adenosine in one each of the mutant and wild type receptor trajectories. In the other trajectories, the ligand leaves the native pose within the first 100 nsec, but remains within the receptor, albeit subject to qualitatively the same dynamics previously reported. We note, however, that the wild type receptor trajectories shown in Figure 2 are only two out of five wild type receptor trajectories that were obtained. In every one of those other three wild type trajectories, the adenosine unbound within the first 300 nsec. It has to be stressed that these are anecdotal data—in order to quantify the unbinding rate, significantly more trajectories would need to be

obtained. However, they are consistent with the experimental observation that the mutant receptor stabilizes the bound state of the receptor.

3.1 Cholesterol interactions with A_{2A} and the CCM

Each of our trajectories reported here incorporates, in the initial structure, a pair of cholesterol molecules positioned to interact with the CCM. (Hanson et al., 2008) The putative binding site is located in a shallow cleft in the intracellular leaflet between helices 1, 3, and 4 and directly in front of helix 2. The CCM is depicted in Figure 4, and consists in A_{2A} of the following residues (Ballesteros-Weinstein numbering given in parentheses (Ballesteros and Weinstein, 1995)): Tyr43(2.41), Ser47(2.45), Lys122(4.43), Ile125(4.46), Trp129(4.50). The Tyr and Lys are positioned to form hydrogen bonds with the hydroxyl group of cholesterol, the Ile to form hydrophobic contacts, and the Trp—conserved in all class A GPCRs—could form a ring stacking interaction with a cholesterol ring system. The Ser was discovered by Adamian, Naveed, and Liang by identifying residues that are subjected to purifying selection pressure. (Adamian et al., 2011) In the crystal structures the hydroxyl of the Ser does not make contact with cholesterol, but is positioned to form a hydrogen bond with the indole ring of the Trp, holding it in position and forming a flat surface in the CCM groove. In previously published work it was observed that in the apo receptor without cholesterol, helix 2 becomes significantly more kinked, and the bulge discussed earlier becomes more pronounced, significantly distorting the binding pocket. (Lyman et al., 2009) The presence of cholesterol in the CCM, on the other hand, maintains helix 2 in a structure more like the crystal structure. It was suggested that the CCM may thus serve as a cholesterol sensor via the conformation of helix 2. We have already seen in Section 3.0 that there appears to be no obvious ligand or sequence dependence of the conformation of helix 2. Here we consider whether cholesterol binding at this location shows any dependence on the ligation state of the receptor, with a keen interest in the role of the specific residues of the CCM.

3.1.1 Ligand dependent cholesterol interactions—Figures 5 and 6 present timeseries of contacts between the two cholesterol molecules and the receptor for each trajectory. The most apparent ligand-dependent difference pertains to cholesterol R in the UK432097-bound receptor. In both UK432097 trajectories, cholesterol R remains bound for the duration of the trajectory, whereas in every other trajectory save one (ADNmut_1), cholesterol R unbinds in relatively short order. In the sole exception, cholesterol R unbinds after approximately 600 nsec. The most stable contact formed by cholesterol R is a hydrogen bond formed with Tyr43 of the CCM, with the rest of the cholesterol forming only transient contacts with the receptor.

There is also a reproducible similarity in the pattern of cholesterol contacts among different ligation states, in particular in the contacts formed between cholesterol Q and the receptor in its NECA bound and ZM241385 bound forms. These ligands have different functionality—NECA is an agonist and ZM241385 is an antagonist—and different sequence—the NECA trajectories are of the quadruple mutant, and the ZM241385 are of the wild type sequence. Nonetheless, cholesterol Q forms many contacts with helices 2 and 4 that are maintained throughout the simulations. (Although cholesterol Q does unbind from ZM_Nat1 at the end the trajectory.) Inspection of the trajectories in which cholesterol Q remains in contact with the receptor reveals some interesting trends, when considered in the light of the CCM.

3.1.2 Cholesterol Q in the context of the CCM—In four trajectories (ADN_Nat1, NECA_Mut1, ZM_1, ZM_2), we observe that cholesterol Q remains associated with the protein for an extended period of time, with a set of contacts that are consistent with the CCM. In every single case, the hydrogen bond between Ser47 and Trp129 is formed a majority of the time, and Trp129 remains in the rotamer observed in the crystal structure.

Cholesterol Q is oriented the majority of the time with the bumpy face toward the CCM—i.e., it does not form an aromatic stacking interaction with Trp129. In ZM_1, this h-bond is broken at approximately 760 nsec, Trp129 rotates so that the indole ring points away from Ser47, and cholesterol Q unbinds. It is not clear which event precedes the other (and such an observation would be anecdotal at best). In ADN_Nat2, we observe that the Ser47-Trp129 h-bond is stable for an extended period of time (up to 600 nsec), but that cholesterol Q unbinds and is briefly replaced by cholesterol R in the CCM, before it too unbinds. In three other trajectories (ADN_mut1, ADN_mut2, NECA_mut1) the Ser47-Trp129 h-bond is formed only very transiently, and the conformation of Trp129 is correspondingly dynamic, sampling an alternate rotamer with the indole hydrogen pointed away from Ser47. In ADN_mut2 there are several events during which Trp129 and cholesterol Q are both dynamic, but Q does not unbind, before cholesterol Q finally unbinds at about 550 nsec. The UK432097 trajectories are quite distinct from the rest as regards the CCM motif. Despite the fact that the CCM looks “as it should” in the UK432097 crystal structure, and after our initial relaxation and heating protocol, it very quickly adopts a different shape in both trajectories. The Ser47-Trp129 h-bond is broken, leading to a disruption of the geometry of the shape of the CCM depicted in Figure 4, and resulting in generally more dynamic residues of the CCM.

4.1 Discussion

We have presented data intended to address several questions regarding the interplay of the structure of the A_{2A} adenosine receptor and the local density of cholesterol in the neighborhood of the protein, as mediated by the cholesterol consensus motif, a five amino acid sequence on TM helices 2 and 4 that mediates cholesterol binding.(Adamian et al., 2011; Hanson et al., 2008; Lyman et al., 2009) Comparison of simulation data for wild type A_{2A} and a thermostabilized quadruple mutant—three of the four mutations are on helix 2, and the fourth interacts with helix 2— were inconclusive on the question of whether the mutant helix 2 is stabilized relative to the wild type. Significant structural changes were observed for helix 2, but at this stage we cannot rule out the possibility that helix 2 is straightened by a force field bias toward structures that are too helical.(Cino et al., 2012)

We also studied in detail the interactions between the CCM and bound cholesterol, for four different ligands and both wild type and thermostabilized mutant sequences. We find a ligand-specific interaction, in which the protein when bound to UK432097 interacts more strongly with cholesterol “R” rather than cholesterol “Q”—in contradistinction to the rest of our data. Cholesterol R is more distally bound, while cholesterol Q is in contact with the CCM. Interestingly, in the UK432097 trajectories, we observe that the CCM motif is disrupted by the breaking of the Ser47-Trp129 hydrogen bond, which leads to unbinding of cholesterol Q. In the other trajectories we find that the dynamics of cholesterol Q are related to the dynamics of the Trp129 rotamer, with cholesterol Q dynamics (and in some cases unbinding) following the dynamics of Trp129. Our data therefore support the identification of Ser47 as a part of the CCM.(Adamian et al., 2011)

Overall, our data continue to make the case for molecular dynamics as a powerful complement to the study of GPCR function by experimental and bioinformatic approaches. Future work will focus on the stability of helix 2 (and proline kinks in general) under different force fields—an essential area of inquiry if we wish to claim predictive validity for molecular dynamics of GPCRs. We are also exploring molecular dynamics as a tool for targeting experimental study of cholesterol interaction hotspots, as detailed in a recent publication.(Lee and Lyman, 2012b)

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Highlights

- cholesterol interacts with A2A via the cholesterol consensus motif
- cholesterol-A2A interactions are ligand specific
- helix 2 conformation may depend on sequence

Figure 1. Relaxed structure of adenosine bound A_{2A} , wild type sequence, detailing the locations of the four thermostabilizing mutations. The initial coordinates were taken from the crystal structure of the quadruple mutant (PDB ID 2YDO). TM helix 2 is in green, and TM helix 3 is in blue.

Figure 2

Figure 2.

C α RMSD of TM helix 2 from the initial structure as a function of simulation time for all ten trajectories. Black arrows indicate times at which the "bulge" region in the neighborhood of Pro61 is observed to obtain a more canonical alpha helix conformation.

Figure 3.

Detail of the bulge region at the kink in TM helix 2. In gray is the structure of TM helix 2 as observed at 50 nsec in trajectory ADN Nat_1, in green the same trajectory at 100 nsec, after the helix has straightened. These configurations are representative of the conformational change observed at the arrows in Figure 2.

Figure 4. Cholesterol consensus motif detail. The left panel shows the crystal structures of the ZM241385 bound structure (green, PDB ID 3EML) UK432097 (purple, PDB ID 3QAK), and adenosine (blue, PDB ID 2YDO). The five residues that constitute the CCM are indicated. The middle panel shows the same three structures following the heating and relaxation protocol. No rotameric transition is observed in Trp129 during the relaxation stages. The right panel shows the disposition of the two cholesterol.

Figure 5.

Contact timeseries between cholesterol and A_{2A} for the first series of trajectories (i.e., those with a “1” suffix). A vertical black mark indicates a contact (interatomic distance less than 4 Å) between any atom of the cholesterol and the indicated residue. The TM helices are indicated by black bars along the vertical axis, from TM 1 (bottom) to TM 4 (top). Contacts are only observed between TM helices 1 to 4. The vertical dashed line indicates the duration of the trajectory. Both cholesterol molecules are shown for each trajectory, as indicated by the “Q” or “R” designation.

Figure 6. Contact timeseries between cholesterol and A_{2A} for the second series of trajectories (i.e., those with a "2" suffix). See Figure 5 caption for an explanation of the graphs.

Table 1

Simulation	Initial Struct	Modifications	Ligand	Duration (μsec)
ADN nat_1	2YDO	1,4	adenosine	1.25
ADN nat_2	2YDO	1,4	adenosine	1.28
ADN mut_1	2YDO	1	adenosine	1.08
ADN mut_2	2YDO	1	adenosine	1.11
NECA mut_1	2YDV	1	NECA	1.16
NECA mut_2	2YDV	1	NECA	1.03
UK nat_1	3QAK	2,3	UK432097	1.15
UK nat_2	3QAK	2,3	UK432097	1.14
ZM nat_1	3EML	2,5	ZM241385	1.04
ZM nat_2	3EML	2,5	ZM241385	1.03

Simulation summary. Key to modifications: (1) Residues 214–233 added, (2) Residues 149–157 added, (3) T4L excised and residues 208–221 added, (4) Quadruple mutant changed to wild type sequence (5) T4L excised and residues 209–221 added