Chapter 16
Role of Lipid-Mediated Effects in β_2-Adrenergic Receptor Dimerization

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Introduction

G protein-coupled receptors (GPCRs) constitute the largest and most diverse family of the mammalian membrane receptors (Pierce et al. 2002). Members of the GPCR superfamily are involved in mediating several physiological processes and respond to a wide range of ligands (Rosenbaum et al. 2009). As a result, more than half of the current clinical drugs target GPCRs (Heilker et al. 2009). Members of the GPCR family show a low sequence similarity but they share common structural features (Venkatakrishnan et al. 2013). All GPCRs comprise of seven transmembrane helices that traverse the bilayer (Lagerstroem and Schioeth 2008; Katritch et al. 2012). Recent structural characterization of GPCRs have provided insights into their function (Cherezov et al. 2007; Rosenbaum et al. 2007; Rasmussen et al. 2007; Jaakola et al. 2008; Xu et al. 2011; Chien et al. 2010; Haga et al. 2012; Granier et al. 2012), but the dynamics related to their function are still largely unknown.

The β_2-adrenergic receptor, an important GPCR involved in muscle relaxation, has been the focus of several seminal studies. Until recently, the β_2-adrenergic receptor was hypothesized to exist as monomers and the reconstituted monomeric receptor was shown to be functional (Whorton et al. 2007). Previous studies have proposed that β_2-adrenergic receptors dimerize in the membrane (Angers et al. 2000; Hébert et al. 1996). Advances in spectroscopic and imaging techniques have confirmed

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the existence of dimers and higher order oligomers in related receptors (Paila et al. 2011a; Ganguly et al. 2011). Single particle tracking methods have shown that the GPCRs exist in a dynamic equilibrium between the different associated species (Kasai et al. 2011). Recent single molecule studies on the β_2-adrenergic receptor suggested a distinct dynamics and organization of the receptor (Calebiroa et al. 2012). Interestingly, crystal structures of the β_1-adrenergic receptor, revealed two distinct dimer interfaces, corroborating the existence of associated GPCR states (Huang et al. 2013).

The spatio-temporal organization of GPCRs has been shown to be affected by changes in membrane composition, especially cholesterol concentrations (Paila et al. 2011a; Ganguly et al. 2011). Further, lipid composition has been shown to influence organization, stability and function of several GPCRs (Soubias and Gawrisch 2012; Yao and Kobilka 2005; Paila et al. 2011b; Pucadyil and Chattopadhyay 2004, 2007; Gibson and Brown 1993; Brown 1994; Saxena and Chattopadhyay 2012). The effect of the membrane composition on GPCR dynamics has been suggested to be due to either specific direct interactions (such as binding sites) or indirect effects (such as the changes in membrane physical properties) (Paila and Chattopadhyay 2009). The lipid and cholesterol binding sites that have been resolved in several GPCR crystal structures (Cherezov et al. 2007; Liu et al. 2012), point toward a direct effect. In contrast, spectroscopic studies have suggested a modulation of receptor organization by indirect effects such as hydrophobic mismatch and curvature changes (Botelho et al. 2006; Oates and Watts 2011; Alves et al. 2005).

The molecular details of receptor-lipid interactions are being increasingly probed at the atomistic resolution by computational methods, such as molecular dynamics simulations. With increase in computational power, longer timescale atomistic simulations of GPCR monomers have been performed (Grossfield et al. 2006, 2008; Dror et al. 2009; Lyman et al. 2009). The simulations have probed detailed GPCR dynamics and identified several important protein-lipid interactions. Specific cholesterol binding sites have also been proposed based on μs timescale simulations (Sengupta 2012a; Cang et al. 2013; Lee and Lyman 2012). To understand the more complex higher-order organization of GPCRs, coarse-grain methods have been used (Periole et al. 2007; Mondal et al. 2009) that suggest hydrophobic mismatch as an important driving force. Additionally, we have recently shown that membrane composition can directly modulate the dimer interface of the β_2-adrenergic receptor (Prasanna et al. 2014). Dimerization profiles have also been calculated by biased molecular dynamics for a few interfaces, but focused mainly on the protein energetics (Periole et al. 2012; Johnston et al. 2012). It is becoming increasingly clear that GPCR association is modulated by the membrane environment, although a more detailed investigation of the receptor-lipid interactions is still missing. Importantly, the significance of cholesterol in modulating receptor organization has been established, but the role of the phospholipids in modulating organization remains largely unexplored.

In this work, we study the interaction of the β_2-adrenergic receptor with the membrane lipids and explore the direct and indirect membrane effects that could be important in receptor dimerization. We have recently carried out coarse-grain molecular dynamics simulations of the β_2-adrenergic receptor in membranes of varying

cholesterol composition (Prasanna et al. 2014). We have shown that cholesterol "hot-spots" present on the receptor surface could modulate the receptor dimerization. Here, we examine the role of the membrane lipids in driving and modulating receptor dimerization. We first calculate the hydrophobic mismatch in the monomeric and dimeric regimes, and compare its role in driving association. We also explore direct lipid binding sites in the receptor monomers and dimers. We have identified a putative lipid-binding site between transmembrane helices I and VII that shows a high occupancy by a lipid molecule. Our results show that both direct and indirect membrane effects contribute toward the dimerization of the receptor.

Methods

System Setup: Molecular dynamics simulations of the membrane embedded β_2-adrenergic receptor were carried out in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers in the absence and presence of 50 % cholesterol. The systems were represented using the MARTINI coarse-grain force-field (version 2.1) (Marrink et al. 2007; Monticelli et al. 2008). We have used the MARTINI force-field in our study since it has been shown to be suitable for applications such as membrane protein association (Periole et al. 2012; Sengupta et al. 2009; Sengupta and Marrink 2010; Prasanna et al. 2013) and partitioning of membrane proteins between membrane domains of varying compositions (Schäfer et al. 2011; Sengupta 2012b). A homology model of β_2-adrenergic receptor (amino acid residues 29–342) was generated from crystal structure (PDB: 2RH1) using the software SWISS-MODEL (Arnold et al. 2006). Bilayers containing POPC and with 50 % cholesterol concentration were generated from an initial conformation of randomly placed POPC, cholesterol and water molecules. For the simulations of the monomeric receptor, a single copy of the receptor in its coarse-grain representation was embedded at the middle of an equilibrated bilayer and simulated for 5 μs. For the dimer simulations, two copies of the receptor, in its coarse-grain representation, were embedded into the equilibrated membrane, such that the inter-receptor distance (centre of mass) was at least 6 nm (minimum distance at least 3 nm). After the receptors associated, the dimer regime was simulated for a further 20 μs. Further details of the simulations are described in our previous study (Prasanna et al. 2014).

Simulation parameters: All simulations were performed using the GROMACS simulation package, version 4.5.4 (Van Der Spoel et al. 2005). The cut-off for non-bonded interactions was 1.2 nm with electrostatic interactions shifted to 0 in the range 0–1.2 nm and Lennard-Jones interactions shifted to 0 in the range 0.9–1.2 nm. A relative electrostatic screening of 15 was used. The temperature for each group was weakly coupled using Berendsen thermostat algorithm with a coupling constant of 0.1 ps to maintain a constant temperature of 300 K during simulation (Berendsen et al. 1984). Semi-isotropic pressure was maintained using Berendsen barostat algorithm with a pressure of 1 bar independently in the plane of the membrane and perpendicular to the membrane with a coupling constant of 0.5 ps and a compressibility

of 3×10^{-5} bar^{-1}. The time step used in the simulations was 20 fs. Simulations were rendered using VMD software (Humphrey et al. 1996).

Analysis: Local Membrane Thickness: The local membrane thickness was calculated from the difference in the *z*-position of the PO_4 bead of the POPC molecule. The values were calculated by binning the bilayer into 0.5 nm bins and averaging over the trajectory. To compare between different membrane compositions, a normalized membrane thickness has been defined: $X_{norm} = X/X_{av}$, where X is the local bilayer thickness and X_{av} the average bilayer thickness in the bulk membrane. Correspondingly, the bilayer thickness far from the receptor is 1, and local thickening or thinning will be denoted by a value greater than or less than 1, respectively. A snapshot of the receptor has been superimposed on the 2D local thickness profile.

Spatial density function (*SDF*): The spatial distribution of the phospholipid molecules around the β_2-adrenergic receptor was calculated as the 3D spatial distribution function of the lipid beads. The SDF was calculated from the last 5 μs trajectory by using the g_spatial program in the Gromacs package. The voxel element was set to 0.1 nm in each direction. In general, the SDF reflects the average 3D density distribution of the lipids and therefore points toward the locations where lipid molecules reside with higher probability. The calculated 3D SDFs were averaged over the extracellular and intracellular leaflets by projecting onto the upper and lower membrane planes, respectively. A snapshot of the receptor has been superimposed on the projected SDFs.

Energetics: The protein-protein, protein-lipid, and lipid-lipid interaction energies were calculated by summing the Lennard Jones and Coulomb terms. For POPC-cholesterol bilayers, the contributions from POPC and cholesterol were summed. The values were binned at a bin size of 0.1 nm inter-receptor distance.

Results

Coarse-grain molecular dynamics simulations of β_2-adrenergic receptors were performed in POPC bilayers in the absence and presence (50 %) of cholesterol. We have previously shown that cholesterol modulates the dimer interfaces of the β_2-adrenergic receptor via cholesterol "hot-spots" (Prasanna et al. 2014). Here, we extend our previous work to analyze the effects mediated by the lipids, both the direct and indirect effects, and probe their relation to receptor association.

Indirect Effects: Hydrophobic Mismatch Around the Receptor

We analyzed the local membrane thickness around a β_2-adrenergic receptor, embedded in POPC bilayers containing 0 and 50 % cholesterol (see Fig. 16.1). Since the thickness of the POPC-cholesterol bilayer is larger than the POPC bilayer (Nezil and Bloom 1992), we compare a normalized thickness between the two

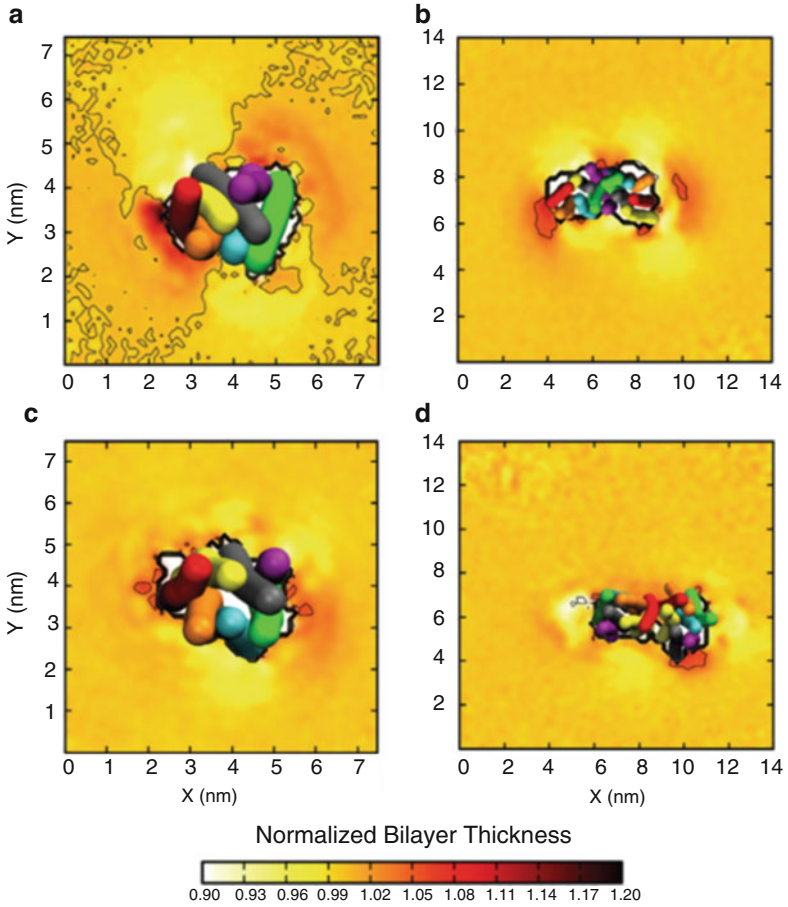

Fig. 16.1 Bilayer thickness profile around the β_2-adrenergic receptor in POPC bilayers with 0 % (**a** and **b**) and 50 % (**c** and **d**) cholesterol concentration. The profiles correspond to the monomer (*left*) and dimer (*right*) regimes of the receptor. To compare the local variations in the bilayer thickness, a normalized bilayer thickness is plotted. The top views of the receptor monomer and dimers are superimposed on the plots and transmembrane helices I and IV are labeled. The transmembrane helices have been color coded as follows: I (red), II (yellow), III (gray), IV (purple), V (green), VI (cyan), VII (orange). Thickness profiles were generated with previously developed tools (Castillo et al. 2013). For further details see Methods

bilayers. The normalized thickness is defined as $X_{norm} = X/X_{av}$, where X is the local bilayer thickness and X_{av} the average bilayer thickness in the bulk membrane. This measure allows us to directly compare the local variations in the thickness compared to the bulk membrane. In POPC bilayers, the thickness profile shows two distinct areas of increased thickness (Fig. 16.1a). The first site is localized around helix I and VII and the second site is located at helices IV and V. At the other faces of the receptor, membrane thinning was observed around the grooves formed by helices I, II and helices VI, VII. The same two sites show a positive mismatch in POPC-cholesterol bilayers (Fig. 16.1c), but the magnitude of the mismatch is less. The decrease in the

Table 16.1 Length of the hydrophobic segment of the transmembrane helices

Transmembrane Helix	Helix length (nm)	Helix length along the bilayer normal (nm)
I	3.7	2.6
II	3.6	2.9
III	3.8	3.4
IV	3.1	3.1
V	3.5	3.3
VI	3.7	3.5
VII	3.8	3.7

The hydrophobic segment of each transmembrane helix was calculated by measuring the average distance between the terminal backbone beads. To account for helix tilting, a second measure of the length component of transmembrane helix parallel to the bilayer normal was also calculated. The hydrophobic region of the bilayer, calculated as the average distance between the beads representing Sn2 glycerol ester carbon of POPC, is 3.25 nm

magnitude of the mismatch is due to the increased membrane thickness in the presence of cholesterol. It is difficult to correlate residue-based hydrophobic length of the helices to the bilayer perturbations due to helix tilting, apolar flanking residues and asymmetric distribution of apolar residues on different helix faces (Table 16.1).

To analyze the membrane profile around the receptor in the dimer regime, two dimer states corresponding to the most sampled states in our simulations were considered (see Rasmussen et al. 2007). The dimer structure most populated in POPC bilayers with 0 % cholesterol is defined by helices IV and V at the interface. At 50 % cholesterol concentration, the most populated dimer structure comprised of helices I and II at the interface. A distinct relationship is seen between the helices that define the dimer interface and those that show a positive hydrophobic mismatch with the bilayer. The differences in the membrane thickness were less pronounced in the dimer regime, for both dimer structures. Interestingly, in the dimers observed in POPC bilayers, the thickness changes around helix I were reduced (Fig. 16.1b), although the helix was not in the dimer interface. In the dimer states in POPC bilayers with 50 % cholesterol, an asymmetric thickening around helix I persisted, though it was involved at the dimer interface (Fig. 16.1d). Although there appears to be a distinct connection between hydrophobic mismatch and dimer interfaces, the relationship between the two is not straightforward. The decreased populations of helix I in the dimer interface in POPC bilayers, despite a high hydrophobic mismatch in the monomer regime, coupled with a complex mismatch pattern in the dimer states, points toward more complex dimerization behavior of the receptor.

Direct Effects: Specific Protein-Lipid Interactions

The spatial distribution function (SDF) of lipid molecules around the monomer was calculated for a representative simulation set and is plotted in Fig. 16.2. The density profiles of the POPC molecules was calculated over several z-slices and averaged

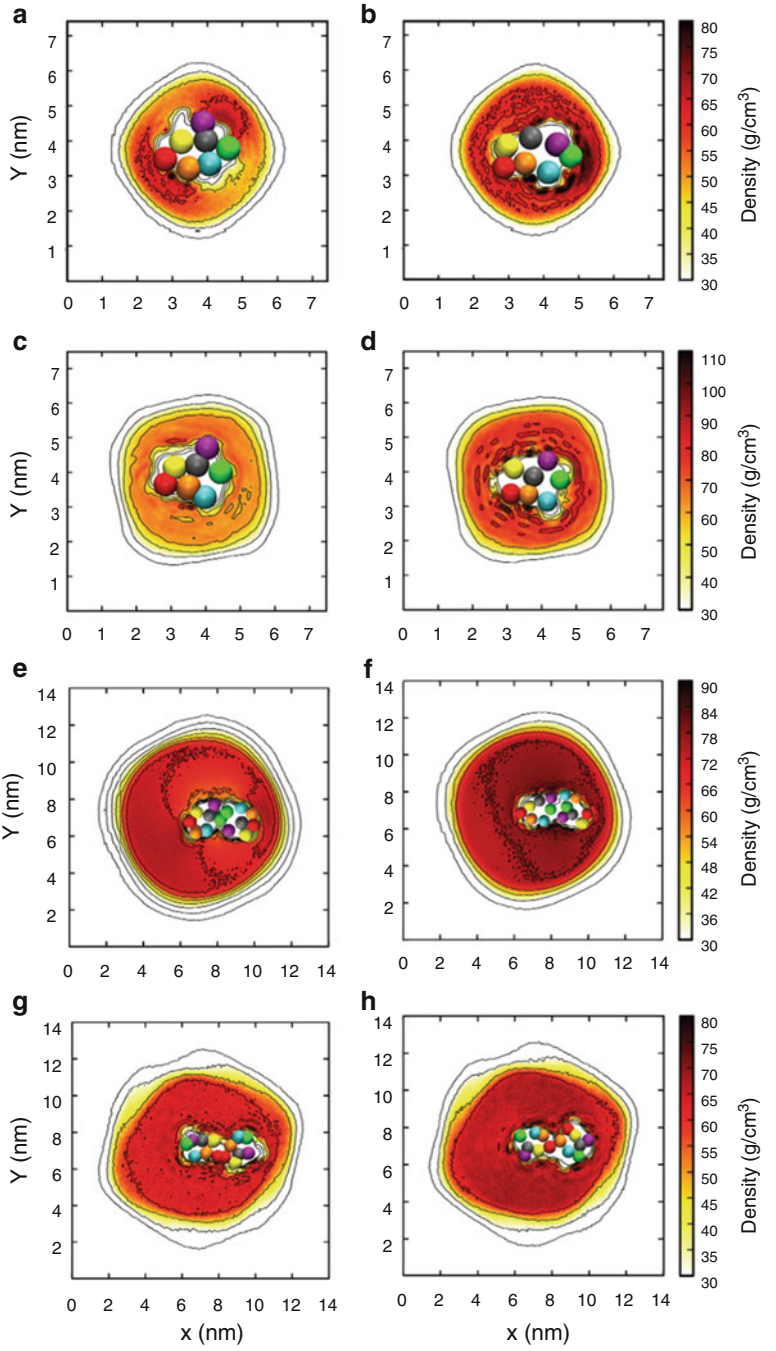

Fig. 16.2 The spatial distribution function (SDF) of POPC around the β_2-adrenergic receptor monomer in POPC bilayers with 0 % (**a**, **b**) and 50 % (**c**, **d**) cholesterol. The SDF is represented for the extracellular (**a**, **c**) and intracellular (**b**, **d**) leaflets separately. The SDF around the receptor dimer is shown for the extracellular (**e**, **g**) and intracellular (**f**, **h**) leaflets in POPC bilayers with 0 % (**e**, **f**) and 50 % (**g**, **h**) cholesterol concentration. The top views of the receptor monomer and dimers are superimposed on the plots. For further details see Methods

over the extracellular and the intracellular leaflets separately. The regions of high density correspond to sites with the highest probability of finding a POPC molecule. The SDF profile shows one distinct site between helices I and VII in the extracellular leaflet (Fig. 16.2a). In the inner leaflet, a more spread-out density was observed (Fig. 16.2b). The first site was centered around helix IV, and was close to the putative cholesterol binding site (CCM). The remaining sites were spread out over helices V, VI and VII. In the presence of cholesterol, a competition was observed between POPC and cholesterol molecules and at most sites POPC binding persisted. On the extracellular site, the SDF is high in the same groove, i.e. between I and VII, although the location appears to be shifted (Fig. 16.2c). The magnitude of the SDF in this groove is similar to that in POPC bilayers. In the intracellular site, the main region of high lipid distribution is located around helix IV, although the magnitude is reduced (Fig. 16.2d). The remaining regions of high density observed in POPC bilayers are further reduced in magnitude. Upon dimerization, the sites of high lipid density persist (Fig. 16.2e–h). Taken together, these results suggest a competition between cholesterol and POPC molecules at the putative cholesterol binding site at helix IV. In contrast, the high SDF site at helix VII appears to be stable and we propose it to be a putative lipid binding site.

To test the robustness of the density data and to exclusively account for the specific binding events, we calculated the maximum occupancy time of POPC around each of the transmembrane helices during the simulation (Fig. 16.3). We defined the maximum occupancy time as the maximum time a given PO_4 bead of the POPC molecule was continuously bound to a given site, normalized to the simulation length. A value of 1 implies that the lipid molecule was present at the given site throughout the entire simulation time and 0 implies it was never present at that site. The values were averaged over ten simulations and the error bars denote the standard deviation between the simulations. A similar trend to the density profiles is observed in maximum occupancies. The maximum occupancy of POPC was the highest at helix VII at the extracellular leaflet and the highest at helix IV on the intracellular leaflet. In presence of cholesterol, the occupancy at both the sites decreases although the trend persists. Combining our data on the SDFs and occupancies, it is clear that POPC binding to some of these sites is specific.

Characterization of the Lipid Site at Helix I

To explore the molecular details of the POPC occupancy site at transmembrane helix VII, we calculated a residue-based distance map between the bound POPC molecule and the amino acid residues on transmembrane helix I and VII. A representative snapshot is shown in Fig. 16.4. The headgroup beads are observed to interact with a charged residue, Glu306. Several aromatic and apolar residues line the lipid occupancy groove. Interestingly, the same site has been identified as a putative lipid binding site in a recent high resolution A_{2A}-adenosine receptor structure (Liu et al. 2012). The lipid binding groove between helices I and VII from the crystal structure of the A_{2A}-adenosine receptor is also depicted in Fig. 16.4.

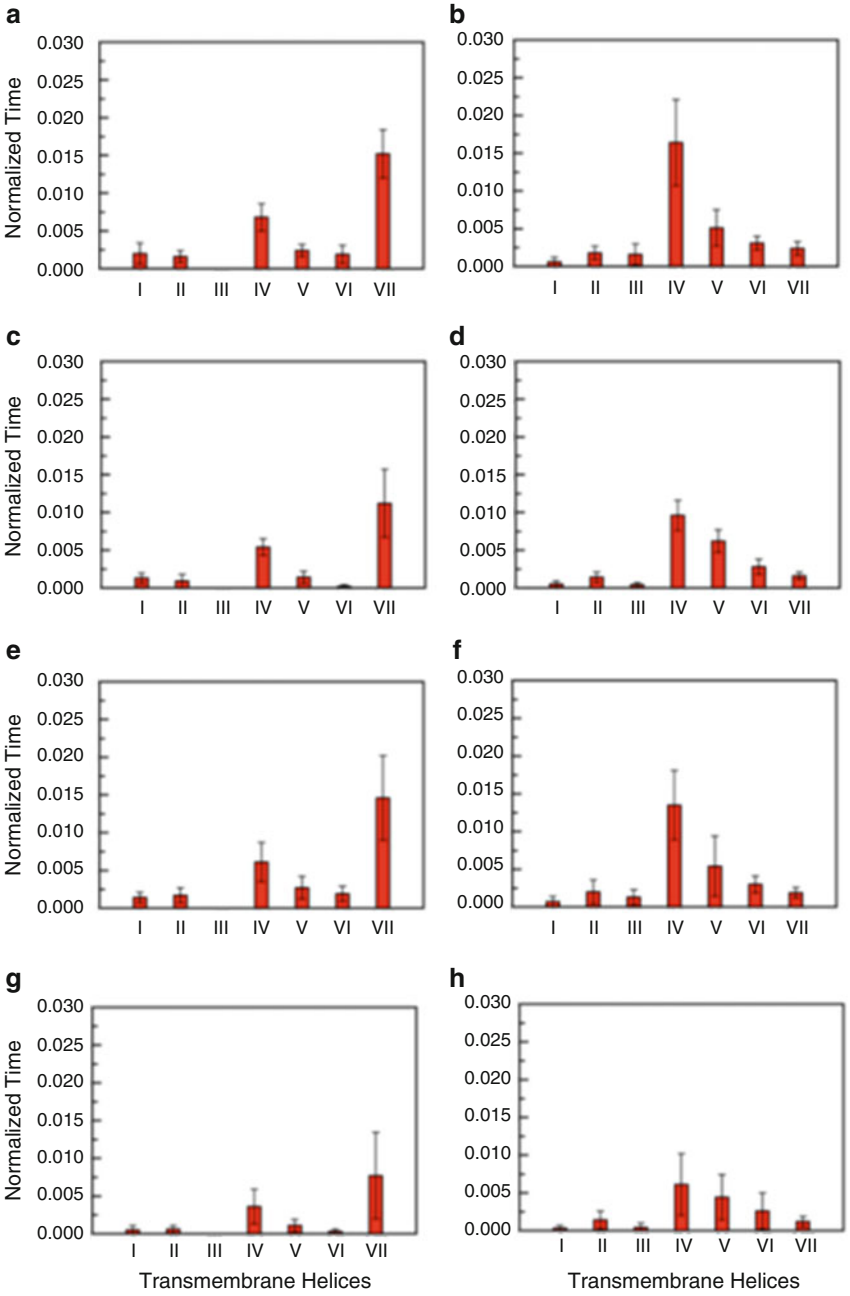

Fig. 16.3 Maximum occupancy of POPC molecules around the β_2-adrenergic receptor monomer in POPC bilayers with 0 % (**a**, **b**) and 50 % (**c**, **d**) cholesterol. The occupancy is shown for the extracellular (**a**, **c**) and intracellular (**b**, **d**) leaflets separately. The occupancy around the receptor dimer is shown for the extracellular (**e**, **g**) and intracellular (**f**, **h**) leaflets in POPC bilayers with 0 % (**e**, **f**) and 50 % (**g**, **h**) cholesterol concentration. For further details see Methods

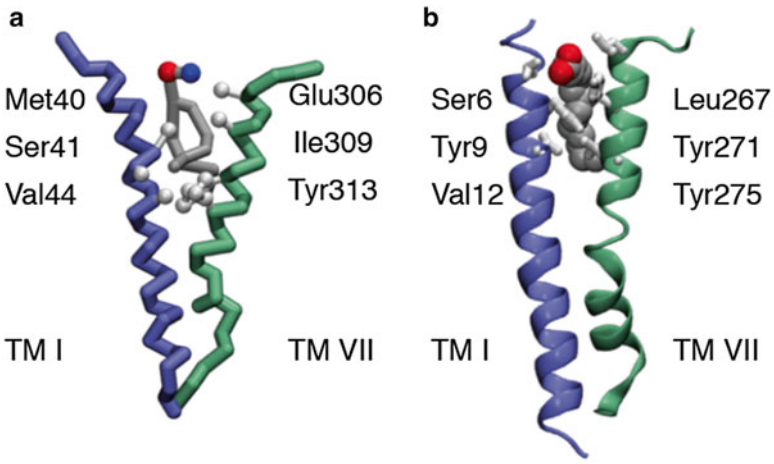

Fig. 16.4 A schematic representation of (**a**) the high lipid occupancy site in seen in coarse-grain simulations and (**b**) the putative lipid binding site in the crystal structure of A_{2A}-adenosine receptor. Only transmembrane helices I and VII are shown for clarity. The side chains that are involved at the site are shown in light grey. The POPC molecule is represented in dark grey and the head-group PO_4 and NC3 beads are depicted as spheres

Exploring the Energetics of Association

The membrane effects described above are a consequence of the interplay between the energetics of the protein and the lipid. We have calculated the interaction energies as a function of inter-receptor separation (shown in Fig. 16.5). The values shown are averaged over ten simulations, totaling about 130 μs of total simulation time and are distinct from the free energy of dimerization. In POPC and POPC-cholesterol bilayers, the protein-protein interaction energy decreases expectedly as the receptors approach each other (Fig. 16.5a, b). A minimum could not be discerned at low inter-receptor distances, possibly due to the lack of sampling of unfavorable close contacts. Multiple smaller minima could be discerned along the pathway that could correspond to meta-stable states along the dimerization pathway. The protein-lipid interaction energy increased as the receptors approach each other due to the de-lipidation of the protein (Fig. 16.5c, d). The lipid-lipid interaction energy decreases correspondingly as the receptors associate and interact with each other (Fig. 16.5e, f). To fully understand the energetics of receptor association, free-energy calculations sampling over all possible receptor interfaces is required, that still remains a challenge within current computational methods.

Discussion

GPCR organization is a critical factor in cellular signaling (Saxena and Chattopadhyay 2011) and understanding these processes within heterogeneous membrane compositions gives rise to new challenges and complexities. It is becoming clear that cellular

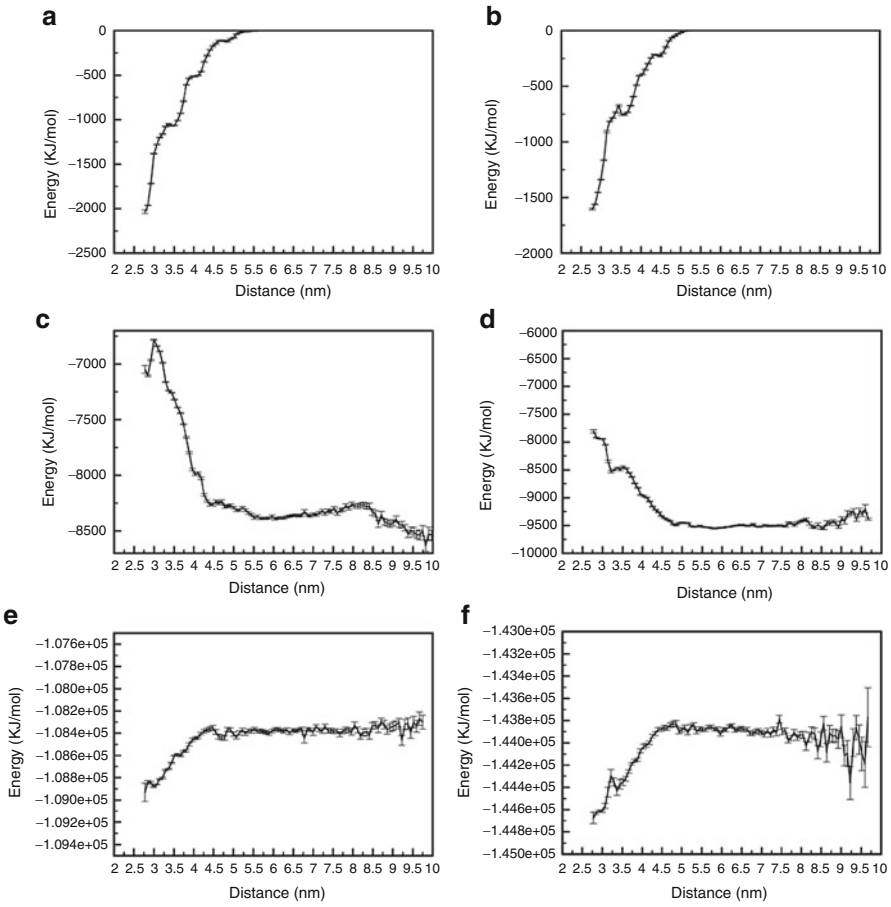

Fig. 16.5 The interaction energies as a function of inter-helical distance calculated for the protein-protein contacts in (**a**) POPC and (**b**) POPC-cholesterol bilayers; the protein-membrane interaction energies in (**c**) POPC and (**d**) POPC-cholesterol bilayers and the membrane-membrane interactions in (**e**) POPC and (**f**) POPC-cholesterol bilayers. The values for each membrane were calculated from ten simulations totaling about 130 μs of simulation time

signaling in general and GPCR function in particular has to be considered in the context of membrane organization and composition. In specific, the interplay between the receptor and the constituent membrane molecules needs to be probed. In this work, we have analyzed the membrane effects around the β_2-adrenergic receptor. Both direct and indirect effects have been probed, together with the estimation of the membrane-protein energetics. We have analyzed multiple μs time scale coarse-grain simulations that allows us to explore the membrane effects in the presence and absence of cholesterol.

Hydrophobic mismatch as a driving force for GPCR association has been proposed previously for β_2-adrenergic receptor (Mondal et al. 2009), rhodopsin (Botelho et al.

2006; Periole et al. 2007) and the opioid receptor (Alves et al. 2005). Although there appears to be a distinct connection between the helices with the maximum hydrophobic mismatch and those present subsequently at the dimer interfaces, the relationship between the two is not straightforward. The decreased populations of helix I in the dimer interface is observed in POPC bilayers, despite a high hydrophobic mismatch in the monomer regime. This, coupled with a complex mismatch pattern in the dimer states, points toward more complex driving forces for receptor association. A more direct effect of the membrane environment on GPCR stability and organization is suggested based on the lipid and cholesterol binding sites that have been resolved in several GPCR crystal structures (Cherezov et al. 2007; Liu et al. 2012). Although cholesterol binding sites have been probed in detail (Sengupta 2012a; Cang et al. 2013; Lee and Lyman 2012), the lipid binding sites remain less explored. Here, using multiple μs time scale coarse-grain simulations we propose a high occupancy site of POPC at helix VII of the β_2-adrenergic receptor. A similar site has been observed in previous atomistic simulations of the β_2-adrenergic receptor, but was not further characterized (Cang et al. 2013). Interestingly, the same site is also proposed to be a lipid binding site based on the recent high-resolution crystal structure of the A_{2A}-adenosine receptor (Liu et al. 2012). We suggest that this site could play a role in the subsequent organization of the receptor and modulate the population of the dimer structures with helices I/VII at the interface.

In conclusion, using multiple coarse-grain simulations of the β_2-adrenergic receptor in POPC bilayers in the absence and presence (50 %) of cholesterol, we have characterized lipid-protein interactions that could play an important role in the stabilization and organization of the receptor. We show the presence of hydrophobic mismatch at helices I/VII and IV/V, that is reduced in the presence of cholesterol. Although there is a relation between the helices with maximum hydrophobic mismatch and its subsequent presence in the dimer interface, the relationship is not straightforward. We have further suggested a putative lipid binding site at helix VII that could play an important role in modulating the dimer interfaces. Based on our results, it appears that lipid-protein interactions play an important role in receptor dimerization. Understanding the underlying membrane-protein interactions will help us appreciate the complex nature of GPCR organization and its link to GPCR function in health and disease.

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