THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

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Identification of Cholesterol Binding Sites in the Serotonin$_{1A}$ Receptor

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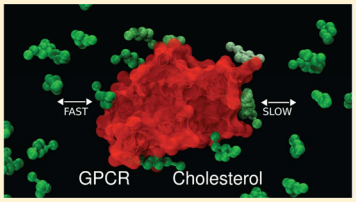
Supporting Information

ABSTRACT: The serotonin$_{1A}$ receptor is a representative member of the G protein-coupled receptor (GPCR) superfamily and serves as an important drug target in the development of therapeutic agents for neuropsychiatric disorders. Previous work has shown the requirement of membrane cholesterol in the organization, dynamics, and function of the serotonin$_{1A}$ receptor. We show here that membrane cholesterol binds preferentially to certain sites on the serotonin$_{1A}$ receptor by performing multiple, long time scale MARTINI coarse-grain molecular dynamics simulations. Interestingly, our results identify the highly conserved cholesterol recognition/interaction amino acid consensus (CRAC) motif on transmembrane helix V as one of the sites with high cholesterol occupancy, thereby confirming its role as a putative cholesterol binding motif. These results represent the first direct evidence for membrane cholesterol binding to specific sites on the serotonin$_{1A}$ receptor and represent an important step in our overall understanding of GPCR function in health and disease.



INTRODUCTION

The G protein-coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes.[1,2] It is estimated that ~50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs.[3] Cholesterol is an essential and representative lipid in higher eukaryotic cellular membranes.[4] Importantly, membrane cholesterol has been shown to play a crucial role in the organization and function of GPCRs.[5−8] These effects of cholesterol on GPCRs have been attributed either to specific interactions of lipids with amino acids in proteins or to bulk properties of membranes.[9] An interesting feature observed in recently solved high resolution crystal structures of GPCRs (such as β_1-adrenergic receptor,[10] β_2-adrenergic receptor,[11,12] and the adenosine$_{2A}$ receptor[13]) is the close association of cholesterol molecules to the receptor. We have recently identified cholesterol recognition/interaction amino acid consensus (CRAC) motifs in GPCRs.[14] The CRAC motif represents a characteristic structural feature of proteins that are believed to result in preferential association with cholesterol.[15] The CRAC sequence is defined by the presence of the pattern -L/V-(X)$_{1-5}$-Y-(X)$_{1-5}$-R/K-, in which (X)$_{1-5}$ represents between one and five residues of any amino acid. However, whether these motifs are directly involved in preferential cholesterol binding in GPCRs is not known.

The serotonin$_{1A}$ receptor is an important neurotransmitter receptor of the GPCR family and is implicated in the generation and modulation of various cognitive, behavioral, and developmental functions.[16−18] Serotonin$_{1A}$ receptor agonists[19] and antagonists[20] represent major classes of molecules with potential therapeutic applications in anxiety- or stress-related disorders. The requirement of membrane cholesterol in the organization, dynamics, and function of the serotonin$_{1A}$ receptor has been comprehensively demonstrated earlier.[21−24]

In this work, we have carried out coarse-grain molecular dynamics simulations to analyze the molecular nature of GPCR−cholesterol interaction for the serotonin$_{1A}$ receptor. Toward this goal, we have simulated the serotonin$_{1A}$ receptor in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membrane bilayers in the presence of varying concentrations of cholesterol, chosen to mimic the biological environment of the serotonin$_{1A}$ receptor. Our results show that cholesterol binds to all transmembrane helices, although with different propensities. A high probability for cholesterol binding is observed at the CRAC motif corresponding to transmembrane helix V. Our results constitute one of the first reports using molecular dynamics on the interaction of membrane cholesterol with the serotonin$_{1A}$ receptor, in particular, and GPCRs, in general.

METHODS

System Setup. Molecular dynamics simulations of the serotonin$_{1A}$ receptor embedded in POPC membranes were carried out in the absence and presence of cholesterol. The systems were represented using the MARTINI coarse-grain force-field (version 2.1).[25,26] In the MARTINI force-field, there are four basic interaction sites: polar (P), nonpolar (N), apolar (C), and charged (Q). POPC is modeled with a 13 bead model with two Q beads representing the zwitterionic headgroup, two N beads modeling the ester groups, and four to five C beads in the two fatty acyl chains. Cholesterol is modeled with eight beads with a single P bead representing the sole hydroxyl group

Received: October 6, 2012
Published: October 15, 2012

   dx.doi.org/10.1021/jp309888u | J. Phys. Chem. B 2012, 116, 12991−12996

and seven C type beads modeling the steroid ring and the hydrocarbon tail. The homology model of the serotonin$_{1A}$ receptor previously developed by us[27] was mapped to its coarse-grain representation and inserted into a pre-equilibrated bilayer with approximately 3000 surrounding water beads. In the absence of cholesterol, the receptor was simulated in a POPC bilayer containing 200 lipid molecules. Three different concentrations of cholesterol were tested: 10, 30, and 50% cholesterol, comprising of 114/12, 114/48, and 114/114 POPC and cholesterol molecules, respectively. A control simulation was carried out with 207/21 POPC and cholesterol molecules so as to keep the total number of POPC and cholesterol molecules constant. At the highest cholesterol concentration used, additional simulations were performed with serotonin bound to the receptor. The serotonin parameters were derived from the MARTINI parameters of tryptophan and optimized. A comprehensive list of all simulations performed is shown in Supporting Information, Table 1.

Simulation Details. Simulations were performed using the GROMACS program package (version 4.0.2)[28] with the scheme developed for coarse-grain simulations, under periodic boundary conditions. Temperature was weakly coupled (coupling time 0.1 ps) to a thermostat at T = 300 K using the Berendsen algorithm.[29] Pressure was weakly coupled (coupling time, 1.0 ps; compressibility, 5×10^{-5} bar^{-1}) using a semi-isotropic coupling scheme in which the lateral and perpendicular pressures are coupled independently at 1 bar. The nonbonded interactions were treated with a switch function from 0 to 1.2 nm for the Coulomb interactions and 0.9−1.2 nm for Lennard-Jones interactions (pair-list update frequency of once per 10 steps). A time step of 25 fs was used.

Analysis. A cholesterol molecule was defined to be bound to a particular amino acid residue (site) if it were within 0.6 nm of that site. A distance based cutoff criteria to define association has been used previously with the MARTINI model.[30] The value of the cutoff is dependent on the distance of the first minimum of radial distribution function. It should be noted here that in the coarse-grain model, the minimum distance of approach between two beads is ∼0.5 nm (except for the smaller ring particle beads).[25] *Total occupancy time* was calculated as the total time during which any cholesterol molecule was bound at a particular site. The value was normalized for all simulation lengths, such that the largest value of total occupancy time is 1 in all cases. *Maximum occupancy time* was defined as the longest time a given cholesterol molecule was bound at a particular site. The value was normalized for all simulation lengths, such that the largest value of maximum occupancy time is 1 in all cases. Since the occupancy times were stochastic, the normalizing factor was dependent on the length of the simulation and cholesterol occupancy at different sites. The total and maximum occupancy times for POPC were calculated in a similar fashion.

RESULTS

Cholesterol Occupancy Times at the Transmembrane Helices. To probe the interaction of membrane cholesterol with the serotonin$_{1A}$ receptor, we performed multiple simulations (see Supporting Information, Table 1) with the receptor embedded in either a POPC bilayer or a POPC/cholesterol bilayer with varying cholesterol concentrations (10, 30, or 50%). A representative snapshot of the membrane embedded serotonin$_{1A}$ receptor is shown in Figure 1. In general, high cholesterol density was observed on the surfaces

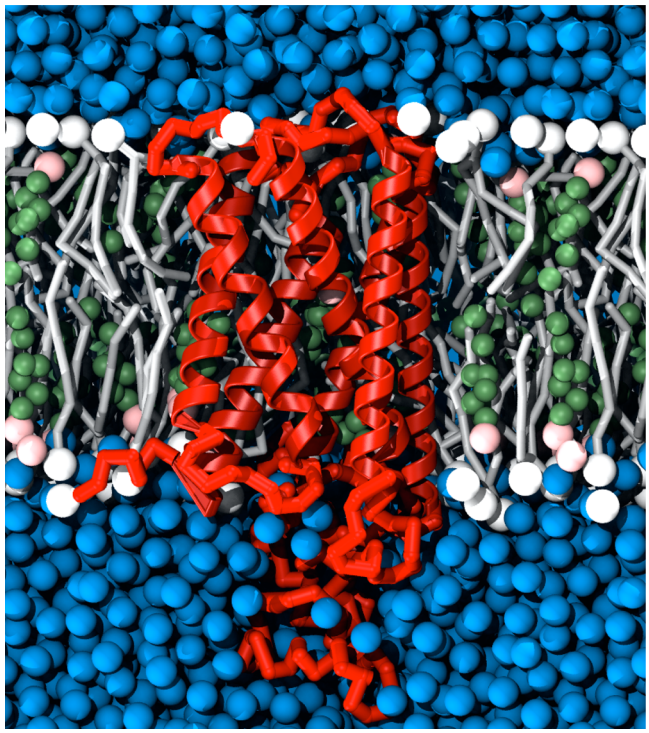


Figure 1. Representative snapshot of the serotonin$_{1A}$ receptor in a membrane bilayer of POPC and 30% cholesterol. The backbone of the receptor is shown in red; cholesterol molecules are shown in green (with the terminal polar bead, representing the hydroxyl group, in pink). POPC molecules are shown in gray (with its phosphate bead in white) and the surrounding water molecules in blue. See Methods for other details.

of all transmembrane helices and visual examination revealed the presence of a few sites with higher cholesterol density.

To further quantitate receptor-cholesterol interaction, we calculated the total occupancy time, that is, the time during which any cholesterol molecule was bound to it. The total occupancy time of cholesterol around each of the transmembrane helices (subclassified as outer or inner leaflet) was of the order of μs (shown in Figure 2a). The values shown in the figure represent averages of five simulations performed with membrane bilayer of POPC and 30% cholesterol. The time scale of the maximum occupancy time was comparable to the total simulation length, thereby implying that cholesterol was almost permanently present at these sites. Due to the comparable accessible surface area of the transmembrane helices and the occurrence of several short-lived binding events, the total occupancy time was comparable for most helices. To exclusively account for the specific binding events, we calculated the maximum occupancy time, that is, the maximum time a given cholesterol molecule was bound to each transmembrane helix (shown in Figure 2b). The maximum occupancy time at these sites was also of the order of μs, although multiple binding−unbinding events were observed. The sites with high maximum occupancy time of cholesterol were transmembrane helix VII (outer leaflet), helix I (inner leaflet), and helix V (inner leaflet). The radial distribution functions correlate well with the maximum occupancy time data (Supporting Information, Figure 1).

Occupancy per Residue. To characterize the amino acid residues involved in cholesterol binding, the total occupancy time of any cholesterol molecule at each residue was calculated.

dx.doi.org/10.1021/jp309888u | J. Phys. Chem. B 2012, 116, 12991−12996

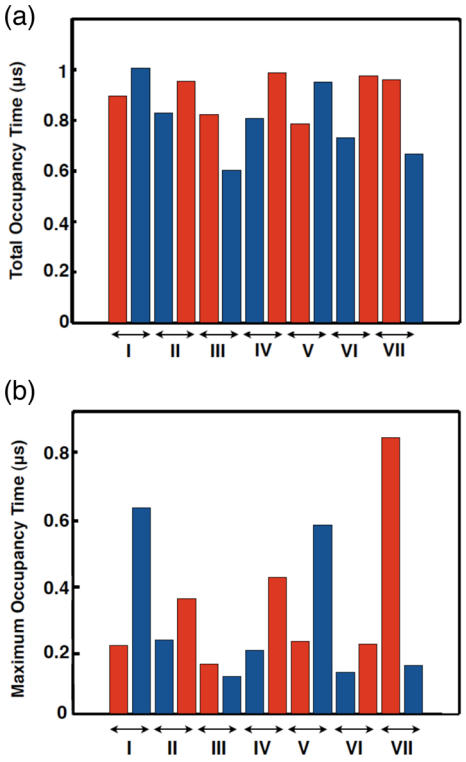


Figure 2. Cholesterol binding at the transmembrane helices of the serotonin$_{1A}$ receptor. (a) Total occupancy time of cholesterol at each of the transmembrane helices (further subclassified by whether they reside in the outer (red) or inner (blue) leaflet of the membrane bilayer). (b) Maximum occupancy time of a given cholesterol molecule at the transmembrane helices (subclassified again by whether they reside in the outer (red) or inner (blue) leaflet of the membrane bilayer). The values shown represent averages of five simulations performed with membrane bilayer of POPC and 30% cholesterol. See Methods for other details.

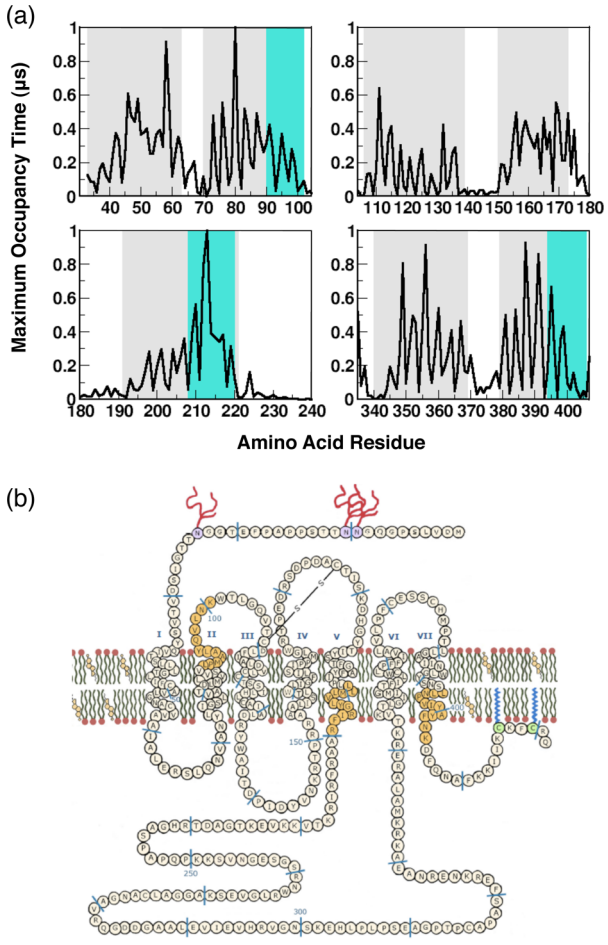


Figure 3. (a) Maximum occupancy time of cholesterol bound to the serotonin$_{1A}$ receptor. The maximum occupancy time of a given cholesterol molecule at each residue averaged over all simulations carried out at three different concentrations of cholesterol (see Supporting Information, Table 1). The gray bands depict the transmembrane helices and the turquoise bands denote the CRAC motifs. See Methods for other details. (b) Schematic representation of the serotonin$_{1A}$ receptor. The positions of the CRAC motifs (in yellow) and the transmembrane helices (I–VII) are shown. Adapted and modified from ref 14.

Values of the total occupancy time for all five simulations at 50% cholesterol concentration is shown in Supporting Information, Figure 2. As apparent from the figure, at such high cholesterol concentration, the total occupancy times of cholesterol at every site along most transmembrane helices are comparable. As mentioned above, this is due to the fact that the total occupancy time also accounts for nonspecific cholesterol binding and multiple binding/unbinding events with low residency times, especially at such high cholesterol concentrations. We, therefore, chose to analyze the maximum occupancy time, that is, the time a given cholesterol molecule was bound to a particular site. Because only specific binding is accounted for this way, the variability in cholesterol binding at different sites was considerably higher. At several sites, cholesterol molecules were found to be bound on a ns time scale. A few sites were identified as high cholesterol binding sites at which the occupancy increased to the order of μs. The occupancy of cholesterol at different sites was stochastic, and therefore, the value of the maximum occupancy time at different sites was variable between different simulations. However, the pattern of maximum occupancy time at different sites (amino acids) was similar, irrespective of cholesterol concentration used. The average maximum occupancy times for all simulations (Supporting Information, Table 1) are shown in Figure 3a (for errors in maximum occupancy times, see Supporting Information, Figure 3). Residues 58, 80, and 213 corresponding to transmembrane helices I, II, and V displayed the maximum occupancy time. As a control, simulations at the highest cholesterol concentration (50%) were performed in the presence of serotonin at its binding site[27] and a similar pattern of cholesterol occupancy time was observed.

Binding of Cholesterol at the CRAC Motif in Transmembrane Helix V. The serotonin$_{1A}$ receptor contains three putative CRAC motifs in transmembrane helices II, V, and VII (see Figure 3b) that have been implicated in cholesterol binding. The residues that comprise these motifs are represented in Figure 3a with a turquoise band. The site of the highest maximum occupancy time in the transmembrane helix V correlates well with the CRAC motif in this helix. Interestingly, the aromatic residue Y215 is conserved among serotonin receptor subtypes.[27] This residue has been suggested to be involved in cholesterol binding since there could be an interaction between the planar aromatic ring of tyrosine and the near planar tetracyclic fused ring of cholesterol.[14] Two different binding sites were observed at this motif and are shown in Figure 4. The first interaction site (labeled a in Figure 4) is on

dx.doi.org/10.1021/jp309888u | J. Phys. Chem. B 2012, 116, 12991–12996

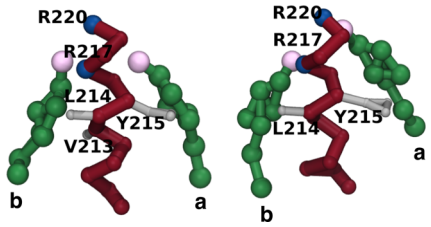


Figure 4. Binding modes of cholesterol at the interaction site of transmembrane helix V. Representative snapshots showing two cholesterol molecules bound at the residues comprising the CRAC motif in the transmembrane helix V. The two binding sites of cholesterol are shown as (a) and (b). The backbone of the helix is shown in maroon. The cholesterol molecules are depicted in green and the terminal polar bead (representing the hydroxyl group) in pink; the two charged arginine backbone beads are represented in blue and the residues V213, L214, and Y215 are depicted in gray.

the interface comprising the residue Y215 and the other site (labeled b in Figure 4) faces the preceding residues V213 and L214. Both interaction sites (a and b) have multiple binding modes as shown in Figure 4 (left and right). The nonpolar beads of the coarse-grain cholesterol molecules (see Methods for a description of the cholesterol model) interact mainly with the hydrophobic residues such as V213, L214, and Y215 along the transmembrane helix V. The polar bead of the cholesterol molecules, representing the hydroxyl groups, interacts with residues R217 as well as R220. At the first binding site, interactions of the hydroxyl bead with the aromatic ring of Y215 are also observed. The cholesterol molecule at the first binding site also interacts with the adjacent transmembrane helix VI (not shown), leading to an improved packing of the hydrophobic beads. In transmembrane helices II and VII, the CRAC motifs are present at the helix termini (Figure 3b) and the terminal charged residue extends into the aqueous phase. As a consequence, the cholesterol occupancy time is lower at these two sites.

Occupancy Times of POPC. The occupancy times of POPC were calculated similar to that of cholesterol. The total occupancy times for POPC molecules at the high occupancy sites were of the order of the simulation length. At 50% cholesterol concentration, the highest values of occupancy times (total and maximum) of POPC and cholesterol were comparable, although the sites with maximum POPC occupancy were different from the highest cholesterol occupancy sites (not shown). A comparison of the maximum occupancy times of POPC and cholesterol (averaged over all simulations) is shown in Supporting Information, Figure 4. At the high cholesterol occupancy sites, the occupancy of POPC was generally lower than that of cholesterol. A visual inspection of the trajectories showed that, under such conditions, POPC was bound adjacent to cholesterol molecules, and POPC binding did not exclude cholesterol binding.

DISCUSSION

Because GPCRs are integral membrane proteins with multiple transmembrane helices, the interaction of surrounding membrane lipids with GPCRs is a crucial determinant in their structure, dynamics, and function. Lipid−protein interactions are particularly relevant in case of GPCRs because they undergo conformational changes for carrying out their function giving rise to structural plasticity.[31,32] These cooperative conformational changes involve participation of surrounding lipid molecules and various conformations are stabilized by binding of different lipids. The functional relevance of such lipid−protein interactions has recently been highlighted by the report that the interaction between GPCRs and G proteins are modulated by membrane lipids.[33] Importantly, the membrane lipid environment of GPCRs has been implicated in disease progression during aging.[34] This is crucial because membrane cholesterol is known to be developmentally regulated and cellular cholesterol content increases with aging.[35] In this emerging scenario, the interaction of the serotonin$_{1A}$ receptor with membrane cholesterol assumes further relevance. Interestingly, recent molecular dynamics simulations reviewed in ref 36 have shown that membrane cholesterol specifically interacts with transmembrane domains of GPCRs, such as rhodopsin[37] and human A_{2A} adenosine receptor.[38]

In this work, we show that membrane cholesterol binds preferentially to certain sites on the serotonin$_{1A}$ receptor using multiple long time scale coarse-grain simulations. It should be noted here that the binding of cholesterol to membrane proteins in general and GPCRs in particular is currently being extensively explored and it appears that no consensus model has emerged yet. It is believed that the surface of the membrane protein does not consist of a series of deep energy wells into which the lipid molecules (in this case, cholesterol) fall to give rise to a single energetically favored conformation.[39] Instead of this, the total interaction energy between a lipid and protein molecule in the membrane is composed of the sum of many weak, van der Waals, hydrogen bonding, and electrostatic interactions and, therefore, will exhibit extensive fluctuations (in other words, lipid molecules are not frozen in a single, long-lived conformation on the surface of membrane proteins). The energetics of cholesterol−GPCR interaction at different sites could therefore be represented by an undulating energy landscape characterized by shallow energy minima with small energy difference. For this reason, transmembrane helices, such as I and VII, also show high cholesterol occupancy (Figure 2). Interestingly, when we calculate a residue-based occupancy, the relative occupancies at these transmembrane helices are lowered, implying that the binding of cholesterol molecules at these helices are dynamic (see Figure 3). As seen from Figure 4, the cholesterol binding site on transmembrane helix V itself has at least two binding modes, and those on transmembrane helices I/VII could have several more. It emerges from our simulations of monomeric serotonin$_{1A}$ receptor, carried out at a wide range of cholesterol concentrations, that the binding of cholesterol on transmembrane helix V is more stable than the remaining transmembrane helices.

Coarse-grain force fields have been successful in describing the energetics of protein−lipid interactions, including GPCR−lipid interaction.[40−42] An advantage of coarse-grain simulations is the increased sampling achieved in the simulation due to long time scale probed. This is important in membrane simulations because membrane dynamics is slow and, therefore, longer times are often necessary to achieve complete sampling. For example, due to the slow dynamics on several sites, we need to simulate for hundreds of microseconds to be able to sample cholesterol binding/unbinding events in independent simulations (see Supporting Information, Figure 5; the figure shows representative sampling of the cholesterol molecules and confirms that the high cholesterol occupancy seen at transmembrane helix V is independent of the initial lipid distribution). Our future efforts will focus on a more detailed atomistic description, especially for the charged residues and

the aromatic ring. Our results show that cholesterol occupancy at specific receptor sites is at a μs time scale. This is an important observation, particularly keeping in mind the relatively slow diffusion exhibited by the serotonin$_{1A}$ receptor in the membrane.[43,44] In spite of considerable experimental evidence demonstrating membrane cholesterol sensitivity of the serotonin$_{1A}$ receptor, recently reviewed in ref 7, our present results represent the first direct evidence that membrane cholesterol enjoys high occupancy to certain sites on the receptor. These results are in overall agreement with our earlier proposal that membrane cholesterol may occupy "nonannular" binding sites on the serotonin$_{1A}$ receptor.[45] Nonannular sites are characterized by lack of accessibility to the surrounding annular lipids, that is, these sites cannot be displaced by competition with annular lipids. Binding to the nonannular sites is considered to be more specific compared to annular binding sites.[46] It has been suggested that the possible locations for the nonannular sites could be either inter- or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface. Interestingly, the highly conserved CRAC motif on transmembrane helix V^{14} appears to have high cholesterol occupancy, thereby confirming its role as a putative cholesterol binding motif. In summary, we have shown that specific cholesterol binding sites are present in the serotonin$_{1A}$ receptor that correspond to the previously identified CRAC motif. Our results contribute to a molecular level understanding of membrane−GPCR interactions and is an important step in our overall understanding of GPCR function in health and disease.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional analytical information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Council of Scientific and Industrial Research, Govt. of India. D.S. is the recipient of the Ramalingaswami Fellowship from the Department of Biotechnology, Govt. of India. A.C. gratefully acknowledges support from J.C. Bose Fellowship (Department of Science and Technology, Govt. of India). A.C. is an Adjunct Professor at the Special Centre for Molecular Medicine of Jawaharlal Nehru University (New Delhi, India), Indian Institute of Science Education and Research (Mohali, India), and Honorary Professor of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India). We thank Yamuna Devi Paila, Alex H. de Vries, Prabal Maiti, Shrish Tiwari, Sourav Haldar, and Md. Jafurulla for helpful discussions. We thank members of A.C.'s research group for critically reading the manuscript.

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■ NOTE ADDED IN PROOF

We came across two recent articles during the final stages of the proofs confirming the crucial, but relatively weak lipid-protein energetics in GPCRs,[47] especially in cholesterol-GPCR interactions.[48]