Cholesterol modulates bitter taste receptor function

Sai Prasad Pydia, Md. Jafurullac, Lisa Wai, Rajinder P. Bhullara, Prashen Chelikani, Amitabha Chattopadhayya

1. Introduction

G protein-coupled receptors (GPCRs) are cellular nanomachines that represent the most important class of membrane-embedded receptors in eukaryotes. They act as signaling hubs and mediate diverse cellular responses [1,2]. As a consequence, GPCRs constitute the largest family of clinical drug targets [3]. The human bitter taste receptors (T2Rs) are members of GPCR family. They display similarities and differences with Class A GPCRs [4,5]. T2Rs are chemosensory receptors with important therapeutic potential [6,7]. Bitter taste perception is believed to act as a defense mechanism against ingestion of potential toxic substances. In humans, bitter taste is perceived by 25 distinct T2Rs. They are expressed in the oral cavity, gastrointestinal neuroendocrine cells of the large intestine and in many extraoral tissues [8,9]. T2Rs typically consist of 290–333 amino acids and display considerable degree of evolutionary divergence [10].

The role of membrane lipids in GPCR function is a promising and emerging area of research [11–17]. In this context, membrane cholesterol enjoys a unique position in modulating GPCR organization, dynamics and function [11–16,18]. Most of the work involving GPCR-cholesterol interaction has been carried out with Class A GPCRs, mainly the serotonin1A receptor, β2-adrenergic receptor and rhodopsin. Work from a number of laboratories has shown that the function of these receptors depends on membrane cholesterol content, although the exact mechanism for such cholesterol dependence of receptor function is not clear. The effect of cholesterol on GPCR function and organization is believed to be due to direct and/or indirect effects [19]. On the other hand, it has been shown that membrane cholesterol is not necessary for the function of the neurotensin receptor 1, another member of Class A GPCR family [20]. Clearly, it is still early days for predicting whether the function of a specific GPCR would depend on membrane cholesterol. Keeping in mind this overall context, in this work, we have explored the membrane cholesterol sensitivity of a representative bitter taste receptor, T2R4. A schematic representation of the membrane embedded T2R4 is shown in Fig. 1. Our results show that the signaling of T2R4 is sensitive to membrane cholesterol content. In addition, we have analyzed the basis of the cholesterol sensitivity of receptor function in terms of cholesterol recognition/interaction amino acid consensus (CRAC) motif found in T2R4. In T2R4, Leu110, Tyr114 and Lys117 are the conserved amino acids of the CRAC motif...
(Ballesteros-Weinstein numbering) [5]. Our recent studies showed that the L110A mutation does not affect the expression or function of the receptor [7]. In this study, we mutated the other two CRAC motif residues, Tyr114[3.59] and Lys117[3.62], to alanine in order to understand their role in cholesterol binding and function of T2R4. To the best of our knowledge, this is the first report of cholesterol sensitivity of taste receptor function. Given the importance of cholesterol in human diet, cholesterol sensitivity of bitter taste receptors assumes physiological relevance.

2. Materials and methods

2.1. Materials

Cholesterol, methyl-β-cyclodextrin (MβCD), fetal calf serum, quinine and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Calcium sensitive dye Fluo-4 NW, lipofectamine 2000, D-MEM/F-12 [Dulbecco’s modified Eagle medium: nutrient mixture F-12 (Ham) (1:1)], and Amplex Red cholesterol assay kit were purchased from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals used were of the highest purity available.

2.2. Methods

2.2.1. Cell culture and transient transfection

CHO cells were maintained in D-MEM/F-12 (1:1) supplemented with 1.2 g/l of sodium bicarbonate, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml of streptomycin in a humidified atmosphere with 5% CO2 at 37 °C. Fusion of mammalian expression vectors pcDNA3.1 were plated at a density of ~1 × 10⁵ in clear bottom black walled 96-well plates in D-MEM/F-12 medium 6–8 h after transfection. Cells were grown for 14–16 h, followed by incubation in serum-free medium for 3 h. Cholesterol depletion was carried out by treating cells with 5 mM MβCD for 30 min at 37 °C [24]. After a wash with serum-free medium, cholesterol-depleted cells were used either for cholesterol replenishment or for calcium mobilization measurements.

2.2.2. Cholesterol depletion of cells in culture

Depletion of membrane cholesterol from the cells in culture was carried out using MβCD as described earlier [24] with some modifications. CHO cells transfected with wild type, Y114A or K117A receptor genes in pcDNA3.1 were plated at a density of ~1 × 10⁵ in clear bottom black walled 96-well plates in D-MEM/F-12 medium 6–8 h after transfection. Cells were grown for 14–16 h, followed by incubation in serum-free medium for 3 h. Cholesterol depletion was carried out by treating cells with 5 mM MβCD for 30 min at 37 °C [24]. After a wash with serum-free medium, cholesterol-depleted cells were used either for cholesterol replenishment or for calcium mobilization measurements.

2.2.3. Cholesterol replenishment in cholesterol-depleted cells

Cholesterol replenishment in cholesterol-depleted cells was carried out using water-soluble cholesterol-MβCD complex as described earlier [25]. Briefly, cholesterol-depleted CHO cells were replenished with cholesterol by incubating with cholesterol-MβCD complex for 10 min in a humidified atmosphere with 5% CO2 at 37 °C. The complex was prepared by dissolving the required amount of cholesterol and MβCD in a ratio of 1:10 (mol/mol) in sterile water by constant shaking at room temperature (~23 °C). Stock solutions (typically 2:20 mM of cholesterol:MβCD) of this complex were freshly prepared and were added to 2 × serum-free medium to yield final solution containing 1:10 mM of cholesterol:MβCD complex [25]. After a wash with serum-free medium, these cells were used for calcium mobilization measurements.

2.2.4. Receptor expression and calcium mobilization assays

Cell surface receptor expression was analyzed by ELISA using anti-FLAG antibody. The mutants Y114A and K117A were properly expressed on the cell surface with expression levels of 110 ± 12% and 80 ± 5%, respectively relative to that of wild type T2R4 (taken as 100%). Calcium mobilization measurements were carried out as described earlier [21,26]. Briefly, calcium sensitive dye Fluo-4 NW was loaded into cells by incubating at 37 °C for 45 min followed by 45 min at ~22 °C. Receptor activation was determined by measuring changes in intracellular calcium (monitored by measuring increase in fluorescence) upon stimulation with increasing concentration of quinine (0.078 to 5.0 mM) or buffer alone (for measuring basal activity) using Flexstation-3 fluorescence plate reader (Molecular Devices; Sunnyvale, CA) at 525 nm following excitation at 494 nm. Calcium mobilized was expressed as change in relative fluorescence unit (ΔRFU) after subtracting the responses of cells transfected with the empty vector.
2.2.5. Cell membrane preparation and cholesterol quantification

Cell membranes were prepared from untreated, cholesterol-depleted, and cholesterol-replenished cells as previously described [27]. Briefly, cells were harvested by treatment with 10 mM Tris, 5 mM EDTA, pH 7.4 buffer and homogenized with a Polytron homogenizer. The cell lysate was centrifuged at 500 × g for 10 min and the resulting post-nuclear supernatant was centrifuged at 40,000 × g for 30 min. The pellet was suspended in 50 mM Tris, pH 7.4 buffer. The amount of cholesterol present in these membranes was quantified using Amplex Red cholesterol assay kit [28].

2.2.6. Amino acid sequence analysis and identification of CRAC motif in human T2Rs

All the amino acid sequences of human T2Rs were retrieved from the NCBI database. Multiple sequence alignment was performed using ClustalW multiple sequence alignment program, as described previously [7]. The CRAC motif in human T2Rs (with the conserved tyrosine (Y) along with leucine (L)/valine (V) toward its amino terminus and lysine (K)/arginine (R) toward carboxy terminus, within five residues on either side) were manually identified.

2.2.7. Molecular modeling and docking studies

Three-dimensional molecular model of T2R4 was built using Sybyl-X 2.0 molecular modeling suite (Tripos Inc., USA). Rhodopsin crystal structure (PDB ID: 3DQ8) was used as a template. First stage minimization was performed using the steepest descent and conjugate gradient algorithms. MD simulation for 10 ns was carried out with a time-step of 2 fs, collecting trajectory data every 500 ps. The SHAKE algorithm, which constrains the hydrogen-heavy atom bonds was applied. Simulations were carried out using Sybyl-X 2.0 at constant temperature (300 K), and pressure (1 atm). Periodic boundary conditions were used to treat long-term electrostatics. T2R4 model obtained this way was used for cholesterol docking. Cholesterol was docked to the identified CRAC motif of T2R4 using the SurfexDock docking program within Sybyl-X 2.0. This complex was further energy minimized. PyMol molecular visualizer software was used to analyze the T2R4-cholesterol complex.

2.2.8. Analysis of data

Nonlinear curve fitting of the dose response data was carried out using Graphpad Prism version 4.0 (San Diego, CA). EC50 values were calculated from nonlinear regression analysis.

3. Results

3.1. Membrane cholesterol sensitivity of human T2R4

In order to examine the sensitivity of T2R4 function to membrane cholesterol, we monitored the phospholipase C mediated increase in calcium mobilization upon activation of T2R4 with increasing concentration of the specific agonist quinine, under conditions of varying cholesterol content. Quinine is the only pharmacologically characterized agonist for CRAC motif in human T2Rs (with the conserved tyrosine (Y) along with leucine (L)/valine (V) toward its amino terminus and lysine (K)/arginine (R) toward carboxy terminus, within five residues on either side) were manually identified.

![Fig. 2. Cholesterol-dependent T2R4 signaling](image)

(a) Membrane cholesterol content of CHO cells transfected with T2R4 upon cholesterol depletion and replenishment. Values are expressed as nmol of cholesterol per mg protein. (b) Agonist-stimulated calcium signaling of wild type receptors as a function of membrane cholesterol. Calcium signaling in control (●, olive), cholesterol-depleted (▲, blue), and cholesterol-replenished (■, maroon) CHO cells expressing wild type receptors is shown upon stimulation with increasing concentrations of T2R4 agonist (quinine). CHO cells expressing wild type receptors were treated with MβCD to deplete membrane cholesterol and subsequently treated with cholesterol-MβCD complex to replenish membrane cholesterol. Cells transfected with T2R4 (or the empty vector, pcDNA) were preloaded with the calcium-sensitive dye Fluo-4 NW were stimulated with increasing concentrations of quinine and the resultant calcium mobilization was measured. The difference in relative fluorescence units (RFU) of T2R4 expressing cells with cells transfected with pcDNA is shown as ΔRFU. Nonlinear curve fitting of the dose-response data was carried out using Graphpad Prism version 4.0 (San Diego, CA). Data shown are means ± S.E. of at least three independent experiments (**corresponds to significant (p < 0.01) difference in cholesterol content of cholesterol-depleted cells relative to control cells). See Materials and methods for other details.
**Table 1** EC₅₀ values for calcium signaling of T2R4 receptors as a function of membrane cholesterol.

<table>
<thead>
<tr>
<th>Condition</th>
<th>EC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Y114A</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>K117A</td>
<td>–</td>
</tr>
<tr>
<td>Cholesterol-depleted cells</td>
<td>–</td>
</tr>
<tr>
<td>Cholesterol-replenished</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>K117A</td>
<td>1.8 ± 0.6</td>
</tr>
</tbody>
</table>

*Note: EC₅₀ values (in mM) were calculated from nonlinear regression analysis of the dose-response data using Graphpad Prism version 4.0.*

3.2. Cholesterol binding motif in T2Rs

An increasingly emerging feature in recently solved high resolution crystal structures of GPCRs is the presence of closely associated cholesterol molecules ([31–34]; recently reviewed in [15]). Several structural motifs of proteins and receptors have been postulated to induce preferential association with cholesterol [13,15]. A major motif in this category is the CRAC (cholesterol recognition interaction amino acid consensus) motif [35,36]. The CRAC motif is characterized by the sequence -L/V-(X)₁₋₅-Y-(X)₁₋₅-R/K-, in which (X)₁₋₅ represents one and five residues of any amino acid. Importantly, we recently reported the presence of CRAC motifs in representative GPCRs such as rhodopsin, the β₂-adrenergic receptor, and the serotonin1A receptor [37], that have been shown to exhibit membrane cholesterol sensitivity for their function. High occupancy of cholesterol at some of the CRAC sites in the serotonin1A receptor has been reported by molecular dynamics simulations using the MARTINI coarse-grain approach [38].

In order to explore molecular details of cholesterol sensitivity of T2R4 function (Fig. 2b), we examined whether bitter taste receptors in general, and T2R4 in particular, possess CRAC sequence. We found that the interface of transmembrane helix III and intracellular loop 2 of T2R4 has a CRAC motif (see Fig. 1). Amino acid sequence alignment of human T2Rs showed that the CRAC motif is conserved in 22 out of 25 human T2Rs (see Fig. S1). This implies that CRAC motif is an inherent characteristic feature of majority of T2Rs. Interestingly, we have previously shown that CRAC motifs are inherent characteristic features of the serotonin1A receptor and are conserved over natural evolution [37].

3.3. CRAC motif in T2R4: importance of crucial residues in signaling

In order to understand the importance of conserved CRAC motif residues in T2R4 function, we mutated specific amino acid residues in the CRAC sequence and monitored agonist-stimulated calcium mobilization signal upon stimulation with increasing concentrations of quinine. Data represent means ± S.E. of at least three independent experiments. See Materials and methods Figs. 2–4 for more details.

![Fig. 3. Role of crucial amino acid residues of the CRAC sequence in agonist-stimulated calcium signaling of T2R4 receptors. Calcium mobilization in CHO cells expressing wild type (●, olive), Y114A (●, blue), and K117A (●, maroon) receptors, is shown upon stimulation with increasing concentrations of quinine. All other conditions are as in Fig. 2. Data shown are means ± S.E. of at least three independent experiments.](image)

3.4. Cholesterol sensitivity of mutant receptors: importance of key CRAC residues

The significance of the key amino acid residues of the CRAC sequence could be further explored by monitoring the sensitivity of the function of the mutant receptors under conditions of varying cholesterol content. We therefore modulated membrane cholesterol content of cells expressing the mutant Y114A and K117A receptors and monitored agonist-stimulated calcium signaling for these receptors. Fig. 4a shows that membrane cholesterol content of cells expressing Y114A and K117A receptors was reduced to ~68% and ~38% of control levels upon treatment with Me3CD. Cholesterol replenishment of cholesterol-depleted cells using cholesterol-Me3CD complex resulted in recovery of cholesterol to ~90% and ~108% of control, respectively. The effect of such modulation of membrane cholesterol content on the signaling of Y114A and K117A receptors is shown in Fig. 4b and c. Fig. 4b shows that the mutant Y114A receptor exhibits concentration-dependent quinine-stimulated calcium signaling with EC₅₀ value of 1.9 mM under control conditions (in the absence of cholesterol modulation), similar to wild type receptor (see Fig. 3 and Table 1). Interestingly, upon depletion of membrane cholesterol, Y114A receptor was found to exhibit enhanced signaling, as evident from the agonist dose response curve (see Fig. 4b). Since the dose response curve did not display saturation, we could not determine EC₅₀ in cholesterol-depleted cells. The figure also shows that agonist mediated calcium signaling was restored to control levels (EC₅₀ value ~1.8 mM, see Table 1) when cholesterol was replenished in cholesterol-depleted cells. On the other hand, the mutant K117A receptor was found to exhibit enhanced signaling relative to the wild type receptor (see Fig. 3 and Table 1). Importantly, the K117A receptor did not exhibit any appreciable change in its signaling upon modulation of membrane cholesterol content (Fig. 4c). Representative raw calcium traces for CHO cells mock-transfected or transfected with Y114A and K117A mutants upon stimulation with quinine under control, cholesterol-depleted and cholesterol-replenished conditions are shown in Fig. S3. Taken together, these results point out that the Lys117 residue of CRAC motif is not only important in normal functioning of the T2R4 receptor but also plays a significant role in sensitivity of the receptor to membrane cholesterol content.
3.5. Molecular docking of cholesterol to the T2R4 CRAC motif

In order to further understand the possible molecular interactions of the amino acid residues of the CRAC sequence of T2R4 and membrane cholesterol, we performed molecular docking of cholesterol with the CRAC motif. Cholesterol was docked to the CRAC motif of T2R4 (highlighted in Fig. 1), and the T2R4-cholesterol complex was energy minimized. Amino acid residues present within 4 Å of the cholesterol-binding pocket were analyzed for possible interactions. Cholesterol was docked toward the intracellular side of transmembrane helices III and IV. The polar hydroxyl group of cholesterol was pointed toward the cellular interior and the flexible isooyctyl hydrocarbon tail facing the membrane. Fig. 5 shows the T2R4 CRAC motif residues that are involved in interaction with cholesterol. The nitrogen atom of Lys117 side chain was found to be involved in hydrogen bond interaction with the 3β-hydroxyl group of cholesterol. According to this model, Tyr114 does not interact with cholesterol, and its side chain appears to face transmembrane helices of T2R4. The hydrophobic region of cholesterol was flanked in the hydrophobic pocket formed by aliphatic amino acids Leu109, Iso112, Leu113, and Val116 of transmembrane helix III and Iso137 and Leu140 of transmembrane helix IV.

4. Discussion

As mentioned above, the role of membrane cholesterol in GPCR function constitutes an active and emerging area. Recent reports describing the presence of closely associated cholesterol molecules [31–34] in high resolution crystal structures of GPCRs have provided additional impetus in this area. In spite of a growing number of reports on cholesterol sensitivity of GPCR function, a general consensus on the mechanism of GPCR-cholesterol interaction remains elusive.

The human T2Rs belong to the GPCR superfamily. Their importance stems from the fact that a large number of structurally diverse and naturally occurring bitter compounds are recognized by them. Failure in detecting these compounds could be lethal since some of the bitter compounds are extremely toxic [10]. The perception of taste represents a crucial element because it acts as a sensor in the context of ingestion of food. Since cholesterol is an important part of human food, and cholesterol content in human tissues is contributed by food intake and metabolism [41], interaction of membrane cholesterol with bitter taste receptors could be relevant. In this work, we show that T2R4, a representative member of the bitter taste receptor family, exhibits sensitivity to membrane cholesterol in its function.

Fig. 4. Effect of membrane cholesterol content on agonist-stimulated calcium signaling in CHO cells expressing Y114A and K117A receptors. (a) Membrane cholesterol content of CHO cells transfected with Y114A (blue) and K117A (maroon) receptors upon cholesterol depletion and replenishment. Values are expressed as nmol of cholesterol per mg protein. Panels (b) and (c) show calcium signaling in control (●, olive), cholesterol-depleted (▲, blue), and cholesterol-replenished (■, maroon) CHO cells expressing (b) Y114A and (c) K117A receptors upon stimulation with increasing concentrations of quinine. All other conditions are as in Fig. 2. Data shown are means ± S.E. of at least three independent experiments (* and *** correspond to significant (p < 0.05 and p < 0.001) difference in cholesterol content of cholesterol-depleted cells to control cells). See Materials and methods for other details.

Fig. 5. Energy-minimized model of T2R4 receptor docked to cholesterol. Cholesterol was docked between the transmembrane helices III and IV in the CRAC motif of the receptor. Cholesterol is shown in pink and residues within 4 Å are shown in green. A hydrogen bond between K117 and 3β-OH group of cholesterol is shown as a broken blue line.
Membrane cholesterol has been shown to be crucial for the organization and function of a number of GPCRs [11,13,14,16]. As mentioned above, the effect of membrane cholesterol on GPCR organization and function could be due to direct and/or indirect effects [19]. In this context, a number of structural features of proteins have been suggested to be involved in preferential association with cholesterol [36,42]. Among these, one of the most studied motifs in membrane proteins, that exhibit sensitivity to cholesterol content, is the CRAC motif [35,36]. Subsequent to their identification in peripheral-type benzodiazepine receptors [35], after more than a decade, CRAC motifs were identified in GPCRs such as rhodopsin, the β2-adrenergic receptor, the serotonin1A receptor [37] and the human type 1 cannabinoid receptor [43]. In addition, membrane proteins such as caveolin-1 [44] and the HIV-1 transmembrane protein gp41 [45] have also been shown to possess CRAC motifs. In order to decipher the basis of the observed cholesterol sensitivity of T2R4 function, we examined whether bitter taste receptors in general, and T2R4 in particular, possess CRAC sequence. We report here the presence of CRAC motif in T2R4 and demonstrate that Lys117 plays an important role in its cholesterol sensitivity. To the best of our knowledge, our results represent the first report of the molecular basis of cholesterol sensitivity in the function of taste receptors. It is interesting to note that it has been recently proposed that cholesterol binding motif should contain a positively charged residue capable of participating in electrostatic interactions with 3′-hydroxyl group of cholesterol [39,40]. Our present results with T2R4 therefore support this prediction and demonstrate that lysine in the CRAC sequence of T2R4 plays a crucial role in sensitivity of the receptor signaling to membrane cholesterol.

As stated above, the Y114A mutant did not exhibit any significant difference from the wild type receptor in terms of calcium mobilization (Fig. 3 and Table 1) and sensitivity to membrane cholesterol (Fig. 4b). Analysis of the energy-minimized model of T2R4 (Fig. 5) shows that the side chain of Tyr114 appears to face the transmembrane helices, away from the cholesterol. Our results therefore suggest that the Tyr114 does not appear to play a significant role in signaling by the receptor. However, we cannot exclude the possibility of either direct or indirect influence of other amino acids in T2R4-cholesterol interactions. It is important to note here that several amino acids at the interface of transmembrane helix III and intracellular loop 2, at which T2R4 has CRAC motif, have been shown to play a crucial role in G-protein coupling and signaling by GPCRs.

It is important to note here that the mere presence of CRAC motif(s) does not necessarily mean that this site preferentially associates with cholesterol. In other words, the presence of CRAC motif is only indicative of possible interaction of cholesterol with receptors and it would therefore be prudent to rely on further experimental validation of such interaction. For example, it has been shown that although the neurotensin receptor 1, a member of the GPCR family, possess two CRAC motifs, it does not display any dependence on membrane cholesterol for its function [20]. In this context, it is important to decipher molecular details of the nature of cholesterol sensitivity in case of bitter taste receptors, which would lead to better insight in our overall understanding of the functioning of these receptors in healthy and diseased states. Future analysis of the frequency of occurrence of mutant taste receptors in various populations could provide novel information on the development of gastronomic preferences across the world over the evolution of human civilization.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported by a Discovery grant (RGPIN-2014-04099) from the Natural Sciences and Engineering Research Council of Canada and a MMSF (# 8-2013-00) Allen Rouse Career Award (P.C.) and by the Council of Scientific and Industrial Research (Govt. of India) Network project BSC0115 (A.C.). S.P. was supported by a Graduate Studentship from Manitoba Health Research Council. A.C. gratefully acknowledges support from J.C. Bose Fellowship (Department of Science and Technology, Govt. of India). A.C. is an Adjunct Professor of Tata Institute of Fundamental Research (Mumbai), RMIT University (Melbourne, Australia), Indian Institute of Technology (Kanpur), and Indian Institute of Science Education and Research (Mohali). We thank G. Aditya Kumar for help in making figures, and members of the Chattopadhyay laboratory for their comments and discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2016.06.005.

References


M.A. Hanson, V. Cherezov, M.T. Griffith, C.B. Roth, V.-P. Jaakola, E.Y.T. Chien, J. Velasquez, P. Kuhn, R.C. Stevens, A specific cholesterol binding site is established by the 2.8 Å structure of the human β2-adrenergic receptor, Structure 16 (2008) 897–905.


