Biochemical and Biophysical Research Communications 430 (2013) 179–182

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Role of rhodopsin N-terminus in structure and function of rhodopsin-bitter taste receptor chimeras

Sai Prasad Pydi, Raja Chakraborty, Rajinder Pal Bhullar, Prashen Chelikani *

Department of Oral Biology, University of Manitoba, and The Manitoba Institute of Child Health, Winnipeg MB, Canada R3E 0W4

ARTICLE INFO

Article history:
Received 17 October 2012
Available online 15 November 2012

Keywords:
G protein-coupled receptors (GPCRs)
Bitter taste receptors
T2R4
Rhodopsin
Chimeras
Molecular modeling

ABSTRACT

The bitter taste receptors (T2Rs) belong to the G protein-coupled receptor (GPCR) superfamily. In humans, bitter taste sensation is mediated by 25 T2Rs. Structure–function studies on T2Rs are impeded by the low-level expression of these receptors. Different lengths of rhodopsin N-terminal sequence inserted at the N-terminal region of T2Rs are commonly used to express these receptors in heterologous systems. While the additional sequences were reported, to enhance the expression of the T2Rs, the local structural perturbations caused by these sequences and its effect on receptor function or allosteric ligand binding were not characterized. In this study, we elucidated how different lengths of rhodopsin N-terminal sequence effect the structure and function of the bitter taste receptor, T2R4. Guided by molecular models of T2R4 built using a rhodopsin crystal structure as template, we constructed chimeric T2R4 receptors containing the rhodopsin N-terminal 33 and 38 amino acids. The chimeras were functionally characterized using calcium imaging, and receptor expression was determined by flow cytometry. Our results show that rhodopsin N-terminal 33 amino acids enhance expression of T2R4 by 2.5-fold and do not cause perturbations in the receptor structure.

1. Introduction

G protein-coupled receptors (GPCRs) are cell surface proteins and help in transducing an extracellular signal into an intracellular signal. GPCRs constitute roughly 3% of the human genome and encode more than 700 proteins. The molecules that activate GPCRs include light, peptides, nucleotides, hormones and alkaloids [1]. In native systems, GPCRs except for rhodopsin are expressed at very low levels [2]. Different strategies have been used to overexpress GPCRs in heterologous systems, which include codon optimization, and the use of export tags inserted at N-terminal regions of GPCRs [3,4]. There is a wide variation in the design (the receptor from which the residues were derived, the number of residues etc.) of these export tags, and an amino acid sequence that is commonly used to enhance the expression of low expressing GPCRs is the rhodopsin N-terminal sequence. The rhodopsin N-terminal 20 amino acids were used to express olfactory receptors in HEK293 cells [4], while the N-terminal 38 and 39 residues have been used for expression of bitter taste receptors (T2Rs) in heterologous systems [5–7]. While these additional sequences were reported to enhance the expression of the candidate GPCRs, the local structural perturbations caused by these sequences and its effect on function or allosteric ligand binding were not studied. In addition, the optimum length of the rhodopsin N-terminal sequence and the percentage increase in expression was not reported.

The bitter taste receptors (T2Rs) belong to the GPCR superfamily [8]. In humans, 25 different genes code for 25 T2Rs [9]. These receptors are activated by wide range of bitter compounds including alkaloids, lactones, terpenoids and peptides. Using T2R4 as our experimental model and information derived from the rhodopsin crystal structure, we elucidated how different lengths of rhodopsin N-terminal sequences effect the structure and function of this bitter taste receptor. Guided by molecular models of T2R4 based on a rhodopsin template, we constructed chimeric T2R4 receptors. Receptor expression was determined by flow cytometry analysis, and the chimeras were functionally characterized using calcium imaging. Our results show that rhodopsin N-terminal 33 amino acids are sufficient to enhance expression of T2R4 and do not cause perturbations in the receptor structure.

2. Materials and methods

2.1. Materials

Cell culture media DMEM-F12 and DMEM High glucose, and the dye Fluo-4NW were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and Quinine HCl were purchased from Sigma. The monoclonal antibody rho-1D4 was a gift from

* Corresponding author. Address: D319, Department of Oral Biology, 780 Bannatyne Avenue, University of Manitoba, Winnipeg MB, Canada R3E 0W4. Fax: +1 204 789 3913.
E-mail address: chelikan@cc.umanitoba.ca (P. Chelikani).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.11.029

Dr. H.G. Khorana, MIT, USA and the Gα16/44 chimera was a gift from Dr. Takashi Ueda, Nagoya City University, Japan. Common chemicals were purchased from Fisher or Sigma.

2.2. Molecular biology and cell culture

A codon-optimized TAS2R4 gene with a bovine rhodopsin C8 peptide tag (ETSQVAPA) immediately 5′ to the natural stop codon of TAS2R4 was designed, and into this gene at the N-terminus, the DNA sequence(s) encoding the first 33 and 38 amino acids of bovine rhodopsin were inserted in frame at the 5′ end. The three genes, codon-optimized for expression in mammalian cells were synthesized commercially (GenScript Inc., USA). The genes in pcDNA3.1 were transiently expressed in HEK293T cells using lipofectamine 2000 (Invitrogen) according to previously published methods [10].

2.3. Flow cytometry

Analysis of receptor expression was carried out by flow cytometry as described below. HEK293T cells were transfected with 6 μg of DNA per 5×10^6 cells using lipofectamine 2000. 24 h after transfections 1×10^5 viable cells were taken into flow cytometry tube, washed twice with FACS buffer (Phosphate buffer saline containing 0.5% BSA) by centrifugation for 4 min at 1500 rpm. The cells were fixed with 2% paraformaldehyde and incubated for 10 min with vortexing after every 3 min. For permeabilizing, the cells were treated with 0.05% saponin for 15 min. The cells were then incubated with 1 μl of (3.2 mg/ml) of monoclonal rho-1D4 antibody diluted (1:200) in PBS and incubated for 1 h on ice. The cells were then washed twice with FACS buffer by centrifugation for 4 min at 1500 rpm. The secondary antibody Alexa-488 diluted (1:500) in PBS was used, and incubated with the cells in darkness for 1 h on ice. The cells were washed twice with FACS buffer by centrifugation for 4 min at 1500 rpm. The cells were suspended in 500 μl of FACS buffer, the fluorescence signals of at least 1×10^4 cells/tube were measured using single color analysis by BD Canto FACS analyzer. The users settings of Forward scatter (FSC) 159 volts, Side scatter (SSC) 379 volts, and voltage for Alexa-488 was set to 385. The results are analyzed using FACS Diva and FlowJo softwares and represented in mean fluorescence intensities (MFI). All data shown are mean values ±SE for n (3–5) determinations.

2.4. Functional assay

Determination of calcium mobilized was as described before [11]. Briefly, HEK293T cells were cultured in 10 cm dishes at 37 °C, 5% CO_2 in DMEM-F12 and 10% FBS. Once the cells were 70–80% confluent, they were transfected with either T2R4, Rho33-T2R4 or Rho38-T2R4 and co-transfected with Gα16/44 using lipofectamine 2000. Data presented was from two to five independent transfections in triplicate. Dose response curves were generated by normalizing the relative fluorescence units (RFU) to wild type receptor expression and EC_{50} values were calculated by nonlinear regression analysis using PRISM software version 4.0 (GraphPad Software Inc., San Diego, CA).

2.5. Molecular modeling

The T2R4, Rho33-T2R4 and Rho38-T2R4 models were generated by submitting the amino acid sequence to the I TASSER server using opsin (PDB ID: 3DQB) as template. The models were analyzed and the images generated using PyMol, and as described before [11].

3. Results and discussion

Fig. 1 shows a secondary structure representation of the human bitter taste receptor T2R4 amino acid sequence. Previously, T2R4 was shown to be activated by multiple agonists [12]. There is diversity in the structure of the agonists that activate T2R4, which include sesquiterpene lactones (arborescin, artemorin), amine derivatives (denatonium benzoate, chlorpheniramine), purine analogs (azathioprine) and aromatic quinolines (quinine hydrochloride). Quinine is one of the most intense bitter tasting compound [13], and activates T2R4 with the highest efficacy [11].

3.1. Molecular models of T2R4

Previous structure–function analysis of the intradiscal (extracellular) domain of bovine rhodopsin showed that deletion in the N-terminal tail produced mutants with poor chromophore regeneration and abnormal glycosylation [14]. The first 30 amino acid residues in the N-terminal tail were shown to play an important role in rhodopsin structure and function [14]. The crystal structure of opsin (PDB ID: 3DQB) shows that the first transmembrane (TM) helix starts from residue number 34 [15]. We built homology models of T2R4 containing the first 33 (Rho33-T2R4) and 38 (Rho38-T2R4) amino acids of rhodopsin (Fig. 2). Analyses of our molecular models show that presence of the additional five amino acids in the Rho38-T2R4 model causes changes in the tertiary structure with extension of the TM1 helix in T2R4 by one turn (Fig. 2). As expected, this was not observed in the Rho33-T2R4 model. To elucidate the impact of this change on T2R4 structure and function we carried out functional assays.

3.2. Functional characterization of the chimeras

All three genes used in this study were codon-optimized for expression in mammalian cells. Our previous studies with β_2-AR showed that codon-optimization increases the expression of these GPCRs [16]. Functional analysis of the wild-type and chimeric T2R4 receptors was carried out by measuring changes in the intracellular calcium in HEK293T cells transiently expressing these receptors, after application of different concentrations of quinine. The Rho33-T2R4 and Rho38-T2R4 displayed EC_{50} values of 1.31 ± 0.5 mM and 1.23 ± 0.4 mM, which are comparable to the EC_{50} value of 1.04 ± 0.4 mM for WT-T2R4 (Fig. 3A). However, there are significant changes in receptor activity as shown by an upward shift in the response curves, with the Rho33-T2R4 chimera showing the maximum upward shift. Increased potency was observed, when intrinsic activity was characterized for these receptors using a single saturating concentration of quinine (data not shown). To elucidate whether the hyperactivity observed (Fig. 3A), is due to increased receptor expression or increased coupling to Gα16/44, expression of the chimeras and WT-T2R4 was determined by flow cytometry analysis.

3.3. Expression and pharmacological characterization of the chimeras

From the flow cytometry analysis, the expression of the receptors as quantified by MFI (after subtracting the MFI from mock transfected cells) are 1683 ± 560, 3977 ± 436 and 1726 ± 122, for T2R4, Rho33-T2R4, and Rho38-T2R4, respectively. The MFI of the chimeras were normalized to WT-T2R4 and expressed as percentage (Fig. 3B). Interestingly, Rho33-T2R4 showed more than a 2-fold increase in expression compared to WT-T2R4, whereas there was no statistically significant difference in expression levels between Rho38-T2R4 and WT-T2R4 (Fig. 3B). To analyze the effect of receptor expression on function, we normalized the calcium responses

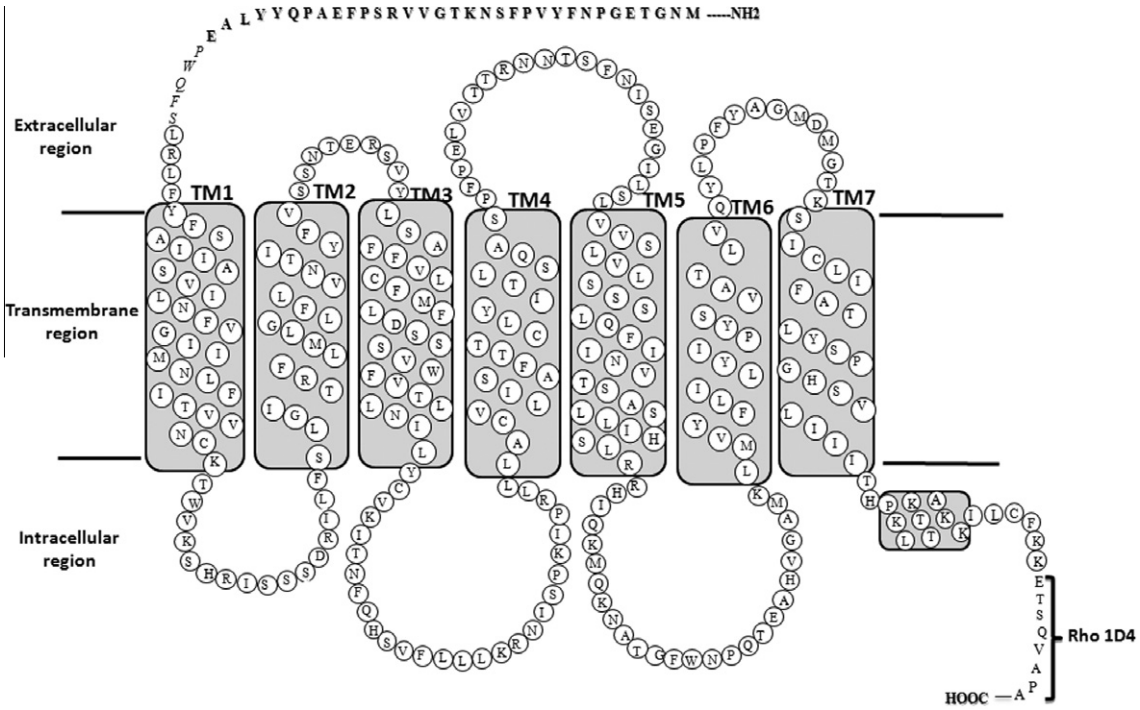

Fig. 1. Two-dimensional representation of the T2R4 amino acid sequence. The amino acids of the native T2R4 are represented in circles. The receptor consists of seven transmembrane (TM) helices, a short N-terminus, three extracellular and three intracellular loops. Bovine rhodopsin N terminal 33 amino acids (represented in bold) and 38 amino acids (represented in bold and italics) were introduced at the N-terminus of the native T2R4 sequence. The rhodopsin C-terminal octapeptide sequence (rho-1D4) was introduced at the C terminal tail of T2R4 to facilitate detection of the protein using the monoclonal antibody rho-1D4.

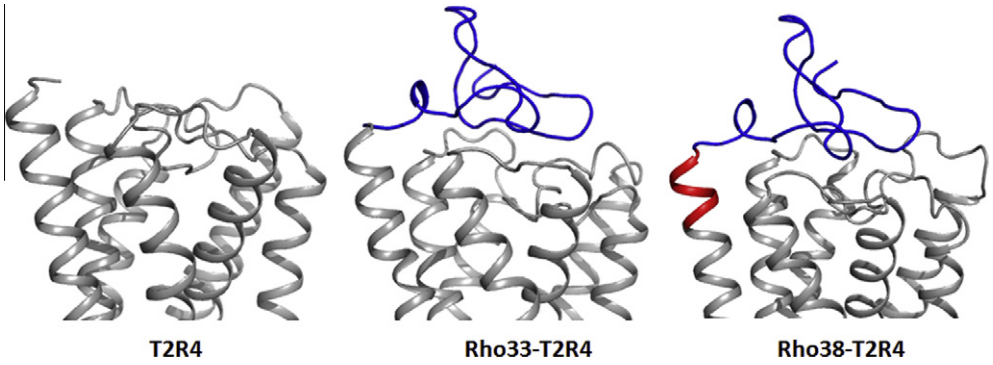

Fig. 2. Three-dimensional representation of the different T2R4 models built using opsin (PDB ID: 3DQB) crystal structure as template. The N-terminus, extracellular loops and the seven transmembrane helices are shown. T2R4 represents the native T2R4 model. Rho33-T2R4 represents the chimeric receptor model with N terminal 33 residues of bovine rhodopsin. Rhodopsin N terminal 33 residues were shown in blue color. Rho38-T2R4 represents the chimeric receptor model with bovine rhodopsin N terminal 38 residues. Based on the molecular model amino acid residues from 34 to 38 are part of TM1 and these residues were represented in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to receptor expression levels as determined by flow cytometry analysis (Fig. 3B). As expected there was not much of a difference between the chimeras and WT-T2R4 (Fig. 3C). This confirms that the differences in potency observed for quinine for the different chimeras was not due to allosteric effects and/or enhanced G-protein interactions but due to increased receptor expression.

Our results using different rhodopsin-T2R chimeras show that the sequence and length of the rhodopsin N-terminus plays an important role in expression of T2Rs. However, we could not find a sequence element within the five amino acid residues PWQFS in the N-terminus of Rho38-T2R4 (shown in italics, Fig. 1) that would increase the retention of this chimeric construct in the intracellular milieu. Based on our molecular models, the helix generated by PWQFS in the Rho38-T2R4, negatively influences the structure and as a result the expression of this chimeric construct. This is in agreement with previous studies on rhodopsin, where it was shown that short deletions of the N-terminal tail or any of the extracellular loops effect the assembly of functional rhodopsin [14]. Previous studies on T2Rs have used the rhodopsin N-terminal 38 or 39 amino acids for heterologous expression; however, none of them quantified the increase in expression [5]. We did not observe any statistically significant difference in expression between the Rho38-T2R4 and WT-T2R4 (Fig. 3B). In our studies it is possible that codon-optimization of the genes might have played a role in enhancing the expression of both Rho38-T2R4 and WT-T2R4, such that no difference in expression was observed between them. Thus far, in our structure–function studies on T2Rs, we have always used a codon-optimized T2R and without an expression tag, and we never encountered problems with receptor expression [11,13]. The Rho33-T2R4 chimera reported here, results in a robust 2.5-fold

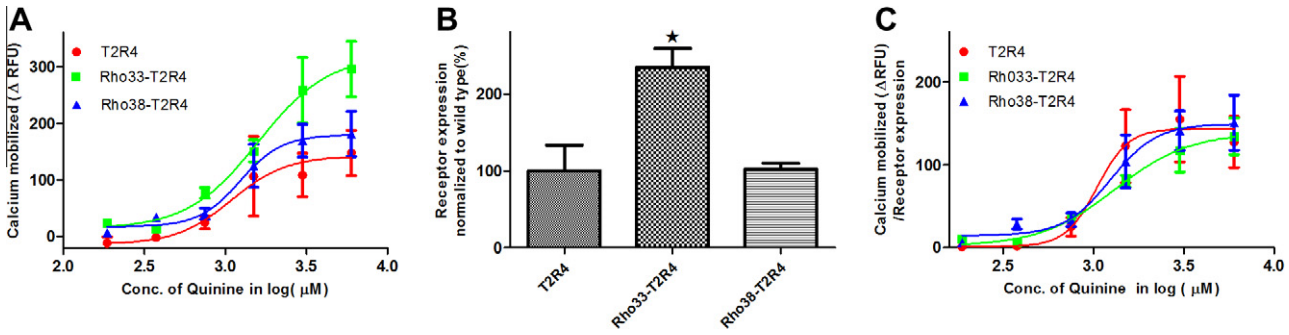

Fig. 3. (A) Calcium dose response curves of T2R4, Rho33-T2R4 and Rho38-T2R4 after treating with different concentration of the agonist, quinine. The calcium mobilized (ΔRFUs) were represented after deducting the response from control (cells transfected with pcDNA and Gα16/44 chimera). (B) Mean fluorescence intensity of T2R4 and chimeric receptors were measured using flow cytometry and normalized to the wild type T2R4. The results were analyzed using one way ANOVA with *Tukeys* post hoc test, at significance level $p < 0.05$. (C) Calcium dose response curves of T2R4, Rho33-T2R4 and Rho38-T2R4 after normalizing to receptor expression.

increase in functional expression of T2R4. This is the highest increase in expression reported for any T2R, and would now allow the production of large amounts of functional receptors needed for high-resolution structural studies.

It was proposed by Dr. Khorana and colleagues that the first step in the formation of a three-dimensional structure in the rhodopsin molecule, starts with the formation of a folded structure in the extracellular domain, that is stabilized by a disulphide bond between Cys-110 and Cys-187. This leads to the correct alignment of the membrane-embedded helices, and consequently the correct structure at the cytoplasmic surface [14]. Given the low sequence conservation between Class A GPCRs and T2Rs, the short N-terminal tail observed in native T2Rs, and the absence of a conserved disulphide bond that might stabilize the extracellular domain in T2Rs [13], it is quite possible that T2Rs might have a different folding pathway that remains to be investigated.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant (RGPIN 356285) and a New Investigator Award from Heart and Stroke Foundation of Canada (HSFC) to PC. A University of Manitoba Graduate Fellowship (UMGF) to SP, and Manitoba Health Research Council (MHRC) and Manitoba Institute of Child Health (MICH) Graduate Fellowship to RC.

References

[1] W.K. Kroeze, D.J. Sheffler, B.L. Roth, G-protein-coupled receptors at a glance, J. Cell Sci. 116 (2003) 4867–4869.
[2] K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Trong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, M. Miyano, Crystal structure of rhodopsin: a G protein-coupled receptor, Science 289 (2000) 739–745.
[3] A.N. Pronin, H. Tang, J. Connor, W. Keung, Identification of ligands for two human bitter T2R receptors, Chem. Senses 29 (2004) 583–593.
[4] D. Krautwurst, K.W. Yau, R.R. Reed, Identification of ligands for olfactory receptors by functional expression of a receptor library, Cell 95 (1998) 917–926.
[5] J. Chandrashekar, K.L. Mueller, M.A. Hoon, E. Adler, L. Feng, W. Guo, C.S. Zuker, N.J. Ryba, T2Rs function as bitter taste receptors, Cell 100 (2000) 703–711.
[6] K. Maehashi, M. Matano, H. Wang, L.A. Vo, Y. Yamamoto, L. Huang, Bitter peptides activate hTAS2Rs, the human bitter receptors, Biochem. Biophys. Res. Commun. 365 (2008) 851–855.
[7] T. Ueda, S. Ugawa, H. Yamamura, Y. Imaizumi, S. Shimada, Functional interaction between T2R taste receptors and G-protein alpha subunits expressed in taste receptor cells, J. Neurosci. 23 (2003) 7376–7380.
[8] S.P. Pydi, J. Upadhyaya, N. Singh, R.P. Bhullar, P. Chelikani, Recent advances in structure and function studies on human bitter taste receptors, Curr. Protein Pept. Sci. 13 (2012) 501–508.
[9] M. Behrens, S. Foerster, F. Staehler, J.D. Raguse, W. Meyerhof, Gustatory expression pattern of the human TAS2R bitter receptor gene family reveals a heterogenous population of bitter responsive taste receptor cells, J. Neurosci. 27 (2007) 12630–12640.
[10] J. Upadhyaya, S.P. Pydi, N. Singh, R.E. Aluko, P. Chelikani, Bitter taste receptor T2R1 is activated by dipeptides and tripeptides, Biochem. Biophys. Res. Commun. 398 (2010) 331–335.
[11] S.P. Pydi, R.P. Bhullar, P. Chelikani, Constitutively active mutant gives novel insights into the mechanism of bitter taste receptor activation, J. Neurochem. 122 (2012) 537–544.
[12] W. Meyerhof, C. Batram, C. Kuhn, A. Brockhoff, E. Chudoba, B. Bufe, G. Appendino, M. Behrens, The molecular receptive ranges of human TAS2R bitter taste receptors, Chem. Senses 35 (2010) 157–170.
[13] N. Singh, S.P. Pydi, J. Upadhyaya, P. Chelikani, Structural basis of activation of bitter taste receptor T2R1 and comparison with class a G-protein-coupled receptors (GPCRs), J. Biol. Chem. 286 (2011) 36032–36041.
[14] T. Doi, R.S. Molday, H.G. Khorana, Role of the intradiscal domain in rhodopsin assembly and function, Proc. Natl. Acad. Sci. USA 87 (1990) 4991–4995.
[15] P. Scheerer, J.H. Park, P.W. Hildebrand, Y.J. Kim, N. Krauss, H.W. Choe, K.P. Hofmann, O.P. Ernst, Crystal structure of opsin in its G-protein-interacting conformation, Nature 455 (2008) 497–502.
[16] P. Chelikani, P.J. Reeves, U.L. Rajbhandary, H.G. Khorana, The synthesis and high-level expression of a beta2-adrenergic receptor gene in a tetracycline-inducible stable mammalian cell line, Protein Sci. 15 (2006) 1433–1440.