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BRIEF REPORT

Lysine 101 in the CRAC Motif in Transmembrane Helix 2 Confers Cholesterol-Induced Thermal Stability to the Serotonin$_{1A}$ Receptor

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Abstract

G protein-coupled receptors (GPCRs) constitute the largest class of membrane proteins that transduce signals across the plasma membrane and orchestrate a multitude of physiological processes within cells. The serotonin$_{1A}$ receptor is a crucial neurotransmitter receptor in the GPCR family involved in a multitude of neurological, behavioral and cognitive functions. We have previously shown, using a combination of experimental and simulation approaches, that membrane cholesterol acts as a key regulator of organization, dynamics, signaling and endocytosis of the serotonin$_{1A}$ receptor. In addition, we showed that membrane cholesterol stabilizes the serotonin$_{1A}$ receptor against thermal deactivation. In the present work, we explored the molecular basis of cholesterol-induced thermal stability of the serotonin$_{1A}$ receptor. For this, we explored the possible role of the K101 residue in a cholesterol recognition/interaction amino acid consensus (CRAC) motif in transmembrane helix 2 in conferring the thermal stability of the serotonin$_{1A}$ receptor. Our results show that a mutation in the K101 residue leads to loss in thermal stability of the serotonin$_{1A}$ receptor imparted by cholesterol, independent of membrane cholesterol content. We envision that our results could have potential implications in structural biological advancements of GPCRs and design of thermally stabilized receptors for drug development.

Keywords GPCR · Thermal stability · Serotonin$_{1A}$ receptors · Cholesterol · CRAC · MβCD

Abbreviations

BCA	Bicinchoninic acid
CRAC	Cholesterol recognition/interaction amino acid consensus
GPCR	G protein-coupled receptor
MβCD	Methyl-β-cyclodextrin
FCS	Fetal calf serum
PMSF	Phenylmethylsulfonyl fluoride

Introduction

G protein-coupled receptors (GPCRs) are the largest and the most diverse class of proteins in higher eukaryotic plasma membranes that facilitate a gamut of signal transduction processes across the membrane (Katritch et al. 2013; Chattopadhyay 2014; Sakmar 2017). A common mechanism of signal transduction by GPCRs requires their activation by extracellular ligands followed by relay of signals to the cellular interior through coordinated structural changes in their transmembrane (or extramembranous) regions (Erlandson et al. 2018; Weis and Kobilka 2018; Pal and Chattopadhyay 2019; Wingler and Lefkowitz 2020). Since GPCRs regulate numerous crucial physiological processes that include cellular metabolism, neurotransmission, growth, immune response and cellular differentiation, they have emerged as major therapeutic targets (Jacobson 2015; Sriram and Insel 2018) and account for ~ 40% of current drug targets across all clinical areas (Chan et al. 2019; Insel et al. 2019). The serotonin$_{1A}$ receptor, an important neurotransmitter receptor which belongs to the GPCR family, is extensively studied among the serotonin receptors and mediates a multitude of neurological, behavioral and cognitive functions (Pucadyil et al. 2005; Kalipatnapu and Chattopadhyay 2007; Müller et al. 2007; Glikmann-Johnston et al. 2015; Sarkar et al. 2018, 2021). Due to the essential role of the serotonin$_{1A}$ receptor in human physiology, it represents a major therapeutic target in developing drugs against neuropsychiatric disorders such as depression, anxiety and even cancer (Lacivita et al. 2008; Fiorino et al. 2014).

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The sensitivity of GPCRs to membrane cholesterol constitutes an exciting area of research in GPCR biology (Pucadyil and Chattopadhyay 2006; Paila and Chattopadhyay 2010; Oates and Watts 2011; Jafurulla and Chattopadhyay 2013; Gimpl 2016; Jafurulla et al. 2019). Work from our laboratory, using a judicious combination of experimental and simulation approaches, has previously established that the serotonin$_{1A}$ receptor exhibits sensitivity toward membrane cholesterol in terms of its organization, dynamics, signaling and endocytosis (Pucadyil and Chattopadhyay 2004, 2007; Paila et al. 2008, 2011; Shrivastava et al. 2010; Jafurulla et al. 2014; Ganguly et al. 2011; Prasanna et al. 2016; Ganguly and Chattopadhyay 2010; Chakraborty et al. 2018; Kumar and Chattopadhyay 2020; Sarkar et al. 2020; Kumar et al. 2021). It has been proposed that the sensitivity of GPCRs toward membrane cholesterol could occur either through specific molecular interactions via structural motifs present in the receptor, or due to cholesterol-induced alterations in membrane physical properties, or by a combination of both these mechanisms (Paila and Chattopadhyay 2009; Jafurulla et al. 2019). In this backdrop, cholesterol interaction motifs such as the cholesterol recognition/interaction amino acid consensus (CRAC) motif offer putative interaction/binding sites on GPCRs that could promote the cholesterol-dependent function of these receptors.

We previously reported the presence of CRAC motifs in transmembrane helices 2, 5 and 7 of the serotonin$_{1A}$ receptor that are conserved over natural evolution (Jafurulla et al. 2011; Sarkar and Chattopadhyay 2020; Fatakia et al. 2019, 2020). Notably, we recently attributed cholesterol dependence of serotonin$_{1A}$ receptor signaling to a CRAC motif present in the transmembrane helix 2 of the receptor (CRAC motif I, see Fig. 1) that facilitates a preferential association with membrane cholesterol (Kumar et al. 2021). We showed that a key lysine residue (K101) in this CRAC motif of the serotonin$_{1A}$ receptor establishes polar interaction with the hydroxyl headgroup of cholesterol and thereby acts as a molecular sensor of membrane cholesterol (Kumar et al. 2021). In addition to modulating organization, dynamics and function of the serotonin$_{1A}$ receptor, we previously showed that membrane cholesterol stabilizes the receptor against thermal deactivation (Saxena and Chattopadhyay 2012). Although cholesterol sensitivity of the serotonin$_{1A}$ receptor has been previously studied, the molecular basis of cholesterol-induced stability of the serotonin$_{1A}$ receptor has not been explored yet. In this work, we addressed the possible role of the K101 residue in cholesterol-induced thermal stability of the serotonin$_{1A}$ receptor.

Materials and Methods

Materials

Bovine serum albumin (BSA), EDTA, $MgCl_2$, $MnCl_2$, doxycycline, hygromycin B solution, methyl-β-cyclodextrin

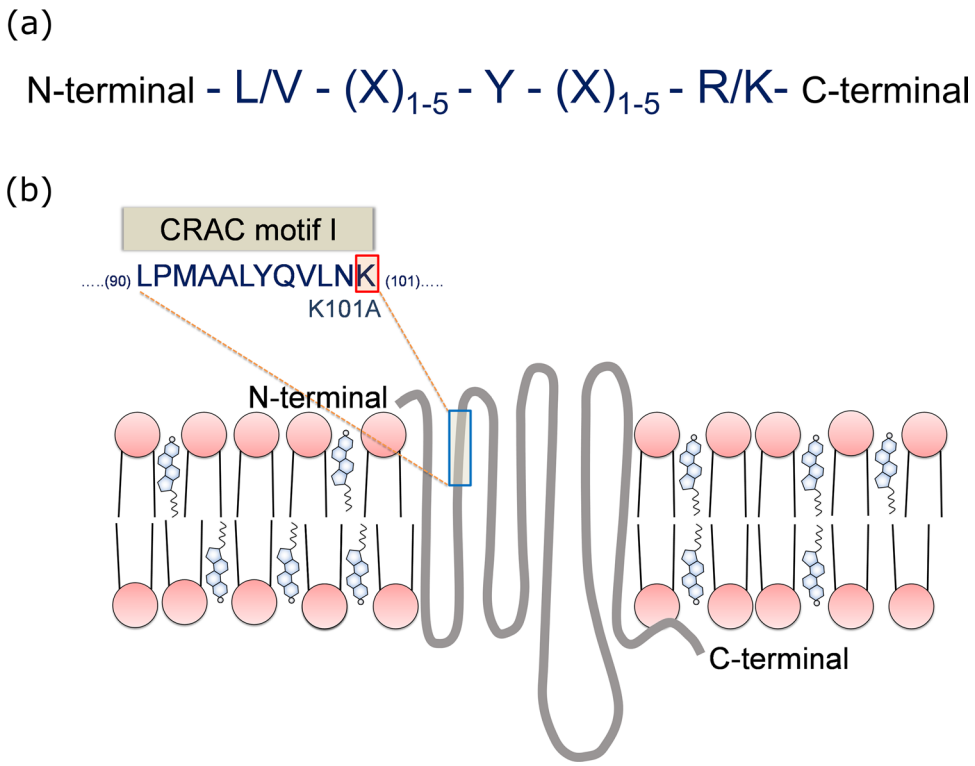

Fig. 1 **a** The linear sequence of amino acids constituting the CRAC motif shown in one letter amino acid code from N-terminus to C-terminus, where $(X)_{1-5}$ represents between one and five residues of any amino acid. **b** A schematic representation of the serotonin$_{1A}$ receptor embedded in a membrane bilayer showing CRAC motif I (highlighted as a blue box) in transmembrane helix 2. The sequence and positions of the amino acids corresponding to CRAC motif I of the serotonin$_{1A}$ receptor is indicated. The residue K101 is highlighted as a red box

(MβCD), phenylmethylsulfonyl fluoride (PMSF), penicillin, streptomysin, gentamicin sulphate, serotonin and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM/F-12 [Dulbecco's modified Eagle's medium:nutrient mixture F-12 (Ham) (1:1)] and fetal calf serum (FCS) were obtained from Invitrogen/Life technologies (Grand Island, NY). Bicinchoninic acid (BCA) assay reagent was from Pierce (Rockford, IL). Amplex red cholesterol assay kit was purchased from Molecular Probes/Invitrogen (Eugene, OR). [^{3}H]8-OH-DPAT (specific activity 141.1 Ci/mmol) was purchased from MP Biomedicals (Santa Ana, CA). GF/B glass microfiber filters were from Whatman international (Kent, U.K.). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cells and Cell Culture

Human embryonic kidney (HEK-293) cells stably expressing N-terminal myc-tagged wild-type human serotonin$_{1A}$ receptors (termed HEK-5-HT$_{1A}$R cells) or K101A mutant were maintained in DMEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% (v/v) FCS, 60 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin sulfate and 250 µg/ml hygromycin B (complete media). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cell culture medium was supplemented with 1 µg/ml doxycycline for 24 h for induction of receptor expression prior to experiments.

Membrane Cholesterol Depletion and Estimation

Cholesterol was depleted from HEK-5-HT$_{1A}$R wild-type and K101A mutant cells in an acute fashion using MβCD (Pucadyil and Chattopadhyay 2007). For this, cells were treated with 10 mM MβCD in serum-free medium for 30 min at 37 °C. Subsequently, to remove the serum-free media containing MβCD, cells were washed with PBS and harvested using ice-cold hypotonic buffer [10 mM Tris, 5 mM EDTA and 0.1 mM PMSF (pH 7.4)]. Cholesterol content of cell membranes was estimated using the Amplex Red cholesterol assay kit (Amundson and Zhou 1999).

Cell Membrane Preparation

Cell membranes were isolated as described previously (Kalipatnapu et al. 2004). Briefly, cells were harvested in ice-cold hypotonic buffer and were homogenized for ~ 15 s with a polytron homogenizer at maximum speed. Following this, the cell lysate was centrifuged at 500×*g* for 10 min at 4 °C. The post-nuclear supernatant was further centrifuged at 40,000×*g* for 30 min at 4 °C and the final pellet containing membranes was resuspended in 50 mM Tris buffer (pH 7.4). The total protein concentration in isolated membranes was measured using the BCA assay (Smith et al. 1985).

Incubation of Cell Membranes at Varying Time Periods

Membranes isolated from control and cholesterol-depleted cells expressing the wild-type or K101A mutant serotonin$_{1A}$ receptors were incubated at 37 °C for different time points ranging from 1 to 4 h. After incubation, radioligand binding assays were carried out at 25 °C.

Radioligand Binding Assay

Tubes in duplicate containing ~ 100 µg total membrane protein in a volume of 1 ml of buffer [50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$ (pH 7.4)] were incubated with the radiolabeled specific agonist [^{3}H]8-OH-DPAT for 1 h at 25 °C. Binding assays were performed using 1 nM radiolabeled agonist [^{3}H]8-OH-DPAT. Nonspecific binding was measured by carrying out the binding assay in the presence of 10 µM unlabeled serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B filters (1.0 µm pore size) which were pre-soaked in 0.3% (w/v) polyethylenimine for 1 h (Bruns et al. 1983). Following this, filters were washed three times with 5 ml of ice-cold water, dried and the retained radioactivity was measured in a Packard Tri-Carb 2900 liquid scintillation counter (Perkin Elmer, Waltham, MA) using 5 ml of scintillation fluid.

Statistical Analysis

Significance levels were evaluated using a Student's two-tailed unpaired *t*-test using GraphPad Prism software, version 4.0. Plots were generated using OriginPro 2022, version 9.9 (OriginLab, Northampton, MA).

Results and Discussion

Membrane Cholesterol Depletion from HEK-5-HT$_{1A}$R Cells Using MβCD

Membrane cholesterol depletion offers a convenient strategy to monitor cholesterol-dependent functions of GPCRs (Jafurulla et al. 2019). Physical depletion of cholesterol from the plasma membrane is achieved using sterol carriers such as MβCD, a water-soluble carbohydrate polymer with seven residues of methylated-glucose (Kilsdonk et al. 1995; Christian et al. 1997). MβCD selectively and efficiently extracts membrane cholesterol under carefully controlled conditions by including it in a central nonpolar

cavity (Zidovetzki and Levitan 2007; Mahammad and Parmryd 2015; Vahedi and Farnoud 2020). We depleted membrane cholesterol using 10 mM MβCD in HEK-293 cells stably expressing the wild-type or K101A mutant human serotonin$_{1A}$ receptors. We previously showed that the human serotonin$_{1A}$ receptor heterologously expressed in HEK-5-HT$_{1A}$R cells harbors functional and pharmacological characteristics similar to the native receptor expressed in the hippocampus and can, therefore, be used to reliably explore aspects of receptor biology (Kumar et al. 2019). Figure 2 shows membrane cholesterol content in HEK-5-HT$_{1A}$R cells expressing the wild-type or K101A mutant serotonin$_{1A}$ receptors upon treatment with 10 mM MβCD for 30 min. We observed ~ 45% reduction in membrane cholesterol content in cells expressing the wild-type serotonin$_{1A}$ receptor upon treatment with MβCD (Fig. 2). Cells expressing the K101A mutant serotonin$_{1A}$ receptor showed similar (~ 51%) reduction in membrane cholesterol content upon treatment with 10 mM MβCD. Importantly, we previously showed that membrane cholesterol depletion using 10 mM MβCD did not result in a notable effect on the plasma membrane localization of the wild-type and K101A mutant serotonin$_{1A}$ receptors in HEK-5-HT$_{1A}$R cells (Kumar et al. 2021).

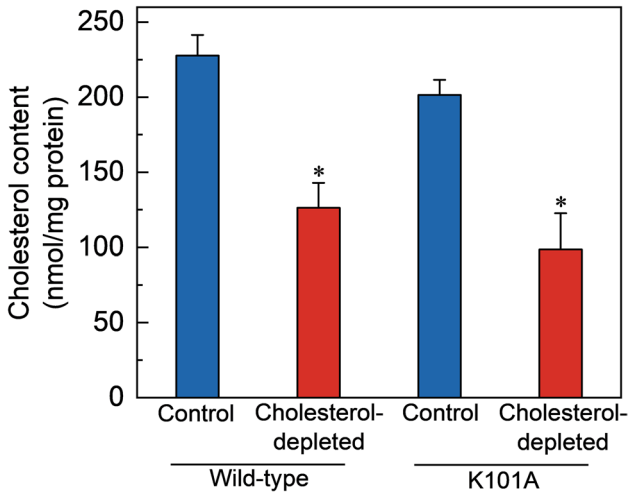

Fig. 2 Cholesterol content of HEK-293 cells stably expressing wild-type or K101A mutant human serotonin$_{1A}$ receptors. Cholesterol was depleted from cells using 10 mM MβCD for 30 min in serum-free medium. Values are normalized to the membrane cholesterol content of cells without MβCD treatment (shown as blue bars); red bars represent cholesterol-depleted (MβCD-treated) cells. Data represent means ± SE of three independent experiments (* represents significant ($p < 0.05$) difference in cholesterol content values in cholesterol-depleted wild-type and K101A mutant cells relative to respective cells without MβCD treatment). See "Materials and Methods" for more details

Cholesterol Stabilizes the Serotonin$_{1A}$ Receptor

We monitored the agonist binding activity of the serotonin$_{1A}$ receptor as a readout of receptor stability in membranes of varying cholesterol under high temperature that could affect receptor stability. To explore the effect of cholesterol depletion on the thermal stability of the serotonin$_{1A}$ receptor, we monitored ligand binding of the receptor at 25 °C in control and cholesterol-depleted membranes pre-treated at 37 °C for varying time periods. Figure 3a shows that in cells expressing the wild-type serotonin$_{1A}$ receptors, ligand binding exhibited a progressive reduction with an increase in the time of pre-treatment for both control (blue) and cholesterol-depleted (red) membranes. The reduction in ligand binding in these cases could be due to inactivation of the serotonin$_{1A}$ receptor since it is known that membrane proteins could exhibit a loss of structure and function due to thermal deactivation when exposed to high temperature (Moore and Wetlaufer 1973; González Flecha 2017; Ponleitner et al. 2022). Notably, the extent of reduction in ligand binding was found to be significantly more in case of cholesterol-depleted membranes relative to control membranes (see Fig. 3a, b). For example, after 4 h of pre-treatment at 37 °C, ligand binding was reduced to ~ 61% and ~ 30% relative to ligand binding values monitored in the absence of any pre-treatment (0 h) in control and cholesterol-depleted membranes, respectively. These results suggest that membrane cholesterol could provide considerable stability to the serotonin$_{1A}$ receptor against thermal deactivation since receptors in cholesterol-depleted membranes show a significant reduction in ligand binding activity relative to control membranes with normal cholesterol content. This is also apparent from different extents of reduction in ligand binding for control and cholesterol-depleted membranes when incubated for varying time points at 37 °C. These results are in agreement with our previous work where we showed that the serotonin$_{1A}$ receptor enjoys less sensitivity to thermal deactivation in membranes with relatively high cholesterol content relative to receptors in cholesterol-depleted membranes (Saxena and Chattopadhyay 2012).

Role of CRAC Motif in Cholesterol-Induced Thermal Stability

What are the molecular factors that allow the serotonin$_{1A}$ receptor to respond to changes in membrane cholesterol levels in terms of altered thermal stability? To address this, we explored whether mutations in the CRAC motif I of the serotonin$_{1A}$ receptor could make the thermal stability of the mutant receptor insensitive to cholesterol. For this, we monitored the role of K101 residue in CRAC motif I conferring such stability and selectively mutated the lysine residue in the 101 position in this motif to an alanine residue. The rationale behind our approach was that if the K101 residue is involved in providing cholesterol-dependent thermal

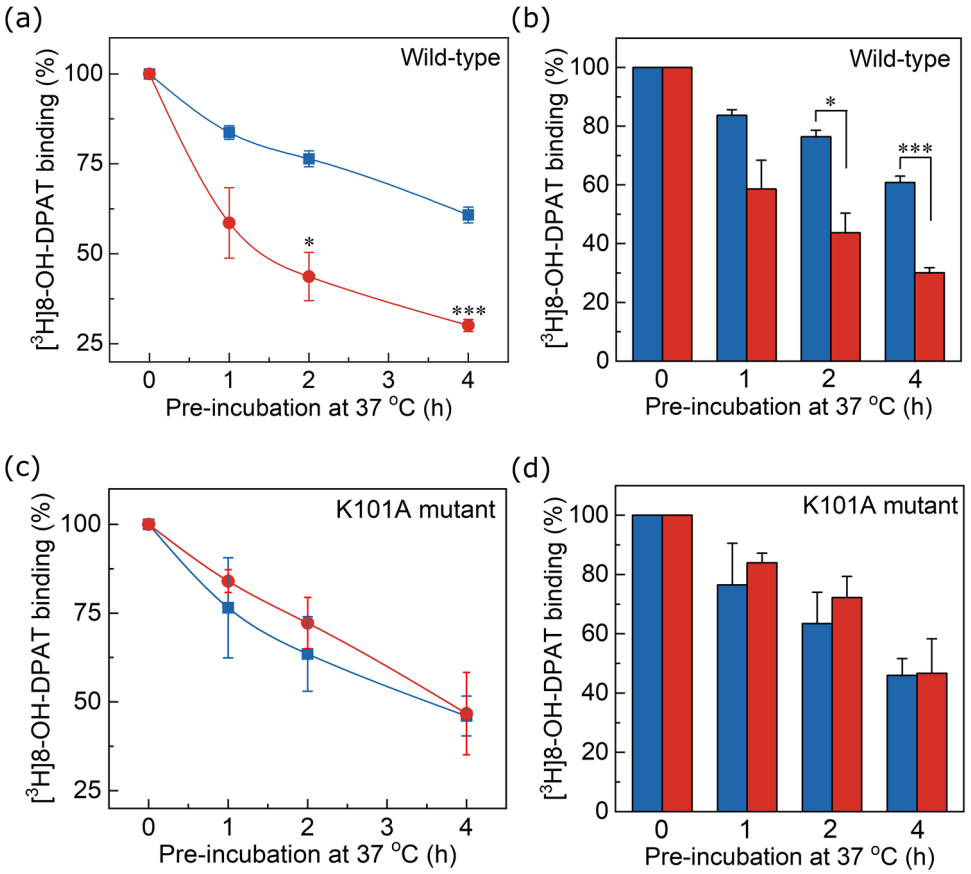

Fig. 3 Effect of membrane cholesterol content on temperature sensitivity of ligand binding of the human serotonin$_{1A}$ receptor. The effect on the stability of the serotonin$_{1A}$ receptor due to change in membrane cholesterol content in **a**, **b** wild-type and **c**, **d** K101A mutant HEK-293 cells, respectively. Membranes isolated following MβCD treatment were incubated at 37 °C for varying times (1, 2 and 4 h) and radioligand binding assay was performed at 25 °C. Values are expressed as percentages of specific binding obtained in membranes in the absence of pre-treatment at 37 °C (0 h) in respective conditions. As shown in the figure, the sensitivity of receptor thermal stability to membrane cholesterol content is lost in the case of the K101A mutant, suggesting a possible role of K101 (in CRAC motif I) in conferring cholesterol-induced stability to the serotonin$_{1A}$ receptor. Data represent means ± SE of three independent experiments (* and *** correspond to significant ($p < 0.05$ and $p < 0.001$, respectively) difference in specific [^{3}H]8-OH-DPAT binding to wild-type serotonin$_{1A}$ receptors in cholesterol-depleted membranes relative to wild-type receptors in membranes without MβCD and pre-incubated for the same time). Blue (■) and red (●) symbols represent radioligand binding data for control (without MβCD treatment) and cholesterol-depleted membranes, respectively (the lines are provided merely as viewing guides). See "Materials and Methods" for more details

stability to the serotonin$_{1A}$ receptor, a mutant receptor harboring a different residue in place of K101 should be thermally stable, irrespective of membrane cholesterol content. In other words, mutations in amino acids that are involved in providing cholesterol-induced stability by sensing membrane cholesterol, should not support cholesterol-dependent changes in receptor thermal stability (as observed with the wild-type receptor). Importantly, we previously showed that the K101A mutation does not affect receptor expression and downstream signaling (Kumar et al. 2021).

We observed that the K101A mutant did not exhibit any appreciable difference in ligand binding activity upon cholesterol depletion relative to control (normal cholesterol) membranes pre-treated at 37 °C for different time points, thereby implying that the stability of the mutant receptor is independent of membrane cholesterol content (Fig. 3c, d). Although ligand binding values exhibited a progressive reduction with increasing time of pre-treatment for both control (without MβCD, shown in blue) and cholesterol-depleted (red) membranes harboring the K101A mutant serotonin$_{1A}$ receptor, we did not observe any significant difference in ligand binding values for control and cholesterol-depleted membranes even after 4 h at 37 °C (see Fig. 3c, d). For example, after 4 h of pre-treatment at 37 °C, ligand binding was reduced to ~ 46% and ~ 47% relative to ligand binding values monitored in the absence of any pre-treatment (0 h) in control and cholesterol-depleted membranes for the K101A mutant serotonin$_{1A}$ receptors, respectively.

Taken together, these results suggest that the K101 residue in CRAC motif I of the receptor is responsible for the cholesterol-induced thermal stability observed in wild-type serotonin$_{1A}$ receptors.

Cholesterol has been previously shown to increase the thermal stability of membrane proteins such as the Ca^{2+}/Mg^{2+}-ATPase (Ortega et al. 1996), the nicotinic acetylcholine receptor (Perez-Ramirez 1994), the oxytocin receptor (Gimpl and Fahrenholz 2002) and the β_2 adrenergic receptor (Yao and Kobilka 2005; Zocher et al. 2012). However, the molecular basis underlying the cholesterol-induced stability of membrane receptors remains elusive. In this work, we showed that a lysine residue (K101) in the CRAC motif in transmembrane helix 2 is crucial for providing cholesterol-mediated thermal stability to the serotonin$_{1A}$ receptor. Interestingly, it should be noted here that the conformation of the subtype Gi-proteins that couple to the serotonin$_{1A}$ receptor (Emerit et al. 1990; Harikumar and Chattopadhyay 1999; Rao et al. 2020), has previously been reported to be sensitive to temperature (Wong et al. 1985). The reduction in agonist binding observed by us could be due to irreversible thermal denaturation of G-proteins during incubation at 37 °C (Javadekar-Subhedar and Chattopadhyay 2004). We have previously shown using molecular dynamics simulations that the presence of physiologically relevant concentration of membrane cholesterol alters conformational dynamics of the serotonin$_{1A}$ receptor and lowers conformational fluctuations (Patra et al. 2015). Interestingly, molecular dynamics simulations have shown that membrane cholesterol specifically interacts with transmembrane helices of GPCRs (Genheden et al. 2017) such as rhodopsin (Khelashvili et al. 2009), human A_{2A} adenosine receptor (Lyman et al. 2009; Lee and Lyman 2012) and β_2 adrenergic receptor (Manna et al. 2016), thereby stabilizing different helices of the receptor. Cholesterol was previously reported to improve the thermal stability of the β_2-adrenergic receptor (Yao and Kobilka 2005), and is necessary for crystallization of the receptor (Cherezov et al. 2007). In addition, cholesterol hemisuccinate, a soluble analog of cholesterol, was shown to stabilize the β_2-adrenergic receptor and improve thermal stability (Hanson et al. 2008). In this overall context, our results could have potential implications in future efforts in structural biology of GPCRs and design of thermally stabilized receptors for drug development.

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Author Contributions AC and PS conceptualized the project and designed experiments; PS and AB performed experiments and analyzed data; PS, AB and AC wrote the manuscript; AC edited the manuscript, organized access to research facilities and funding, and provided overall supervision and mentoring.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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