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Membrane cholesterol stabilizes the human serotonin$_{1A}$ receptor

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ABSTRACT

A number of recently solved crystal structures of G-protein coupled receptors reveal the presence of closely associated cholesterol molecules in the receptor structure. We have previously shown the requirement of membrane cholesterol in the organization, dynamics and function of the serotonin$_{1A}$ receptor, a representative G-protein coupled receptor. In this work, we explored the role of membrane cholesterol in the stability of the human serotonin$_{1A}$ receptor. Analysis of sensitivity of the receptor to thermal deactivation, pH, and proteolytic digestion in control, cholesterol-depleted and cholesterol-enriched membranes comprehensively demonstrate that membrane cholesterol stabilizes the serotonin$_{1A}$ receptor. We conclude that these results could have potential implications in future efforts toward crystallizing the receptor.

1. Introduction

Cholesterol represents the most abundant and important component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting [1,2]. Cholesterol is the end product of a long, multi-step and exceedingly fine-tuned sterol biosynthetic pathway involving more than 20 enzymes. According to the "Bloch hypothesis", proposed by Konrad Bloch, the sterol biosynthetic pathway parallels sterol evolution [3]. It essentially means that cholesterol is selected over a very long time scale of natural evolution for its ability to optimize certain physical properties of eukaryotic cell membranes with regard to biological functions.

In biological and model membranes, cholesterol is often found distributed non-randomly (thereby resulting in variable patchiness of the membrane) in domains [4–7]. These types of domains (sometimes termed as 'lipid rafts') are believed to be important in cellular physiology since membrane sorting and trafficking [8], signal transduction processes [9], and the entry of pathogens [10,11] have been attributed to these type of domains. Importantly, cholesterol has been shown to play a crucial role in the function and organization of membrane proteins and receptors [12–15]. The exact mechanism of the interaction of cholesterol with membrane proteins and receptors is not clear. It has been proposed that such effects of cholesterol on integral membrane proteins could occur either through specific molecular interaction, or due to alterations in membrane physical properties, or by a combination of both [16,17].

The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [18,19]. GPCRs are typically seven transmembrane domain proteins and include >800 members which are encoded by ~5% of human genes [20]. Since GPCRs regulate multiple physiological processes, they have emerged as major targets for the development of novel drug candidates in all clinical areas [21]. It is estimated that ~50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs [22]. 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 [23–25]. Serotonin$_{1A}$ receptor agonists [26] and antagonists [27] represent major classes of molecules with potential therapeutic applications in anxiety- or stress-related disorders.

Previous work from our laboratory comprehensively demonstrated the requirement of membrane cholesterol in the organization, dynamics and function of the serotonin$_{1A}$ receptor ([28–31]; reviewed in refs. [13,14]). We also showed that the interaction between cholesterol and the serotonin$_{1A}$ receptor is considerably stringent since immediate biosynthetic precursors of cholesterol (differing with cholesterol in a double bond) were not able to maintain receptor function [29,32,33]. An interesting feature from a number of recently solved crystal structures of GPCRs is the close association of cholesterol in the receptor structure. For example, high resolution crystal structures of GPCRs such as rhodopsin [34], the β_1-adrenergic receptor [35], and β_2-adrenergic receptor [36,37] all show closely associated cholesterol molecules. In this context, we recently proposed that cholesterol binding sites in GPCRs could represent

Abbreviations: 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; BSA, bovine serum albumin; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; CCM, cholesterol consensus motif; CRAC, cholesterol recognition/interaction amino acid consensus; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GPCR, G-protein coupled receptor; MβCD, methyl-β-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; Tris, *tris*-(hydroxymethyl)aminomethane

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nonannular binding sites at inter- or intramolecular (interhelical) protein interfaces [38]. Interestingly, cholesterol has been previously reported to improve stability of the β_2-adrenergic receptor [39], and appears to be necessary for crystallization of the receptor [36]. The cholesterol analogue, cholesterol hemisuccinate, has been shown to stabilize the β_2-adrenergic receptor against thermal inactivation [37]. Although cholesterol sensitivity of the serotonin$_{1A}$ receptor constituted one of the early reports in the area of GPCR-cholesterol interaction [28], the effect of membrane cholesterol on the stability of the receptor has not been explored yet. In the present work, we have studied the role of membrane cholesterol in the stability of the human serotonin$_{1A}$ receptor. For this, we monitored the ligand binding function of the receptor in membranes of varying cholesterol content under conditions such as high temperature, extreme pH and proteolytic degradation that could affect receptor stability. Our results show that membrane cholesterol stabilizes ligand binding of the serotonin$_{1A}$ receptor. These results could provide useful insight into future efforts to crystallize the receptor.

2. Materials and methods

2.1. Materials

BCA, cholesterol, DPH, DMPC, EDTA, EGTA, MβCD, $MgCl_2$, $MnCl_2$, Na_2HPO_4, PMSF, penicillin, streptomycin, gentamycin sulfate, serotonin, polyethylenimine, trypsin, acetic acid, CAPS and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin inhibitor was from Roche Applied Science (Indianapolis, IN). DMEM/F-12 (Dulbecco's modified Eagle's medium), fetal calf serum, and geneticin (G 418) were from Invitrogen Life Technologies (Carlsbad, CA). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). BCA reagent for protein estimation was from Pierce (Rockford, IL). [^{3}H] 8-OH-DPAT (sp. activity 106 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). 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.

2.2. Methods

2.2.1. Cells and cell culture

Chinese Hamster Ovary (CHO) cells stably expressing the human serotonin$_{1A}$ receptor (termed as CHO-5-HT$_{1A}$R) were maintained in D-MEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin sulfate, and 200 μg/ml geneticin in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2.2. Cell membrane preparation

Cell membranes were prepared as described earlier [40]. Total protein concentration in isolated cell membranes was determined using the BCA assay [41].

2.2.3. Cholesterol depletion and enrichment of cell membranes

Cholesterol depletion or enrichment of cell membranes was achieved by utilizing either MβCD or cholesterol-MβCD complex [30]. The water soluble cholesterol-MβCD complex was prepared as described previously [28]. Briefly, the required amounts of cholesterol and MβCD in a ratio of 1:10 (mol/mol) were dissolved in 50 mM Tris, pH 7.4 buffer under constant shaking at 25 °C. Stock solutions (typically 4 mM cholesterol:40 mM MβCD) of this complex were freshly prepared prior to each experiment. In order to achieve cholesterol depletion or enrichment, membranes suspended at a protein concentration of 2 mg/ml were treated with either 30 mM MβCD or cholesterol-MβCD (1 mM:10 mM) complex in 50 mM Tris, pH 7.4 buffer at 25 °C under constant shaking for 30 min. Membranes were then spun down at 50,000 ×*g* for 10 min, washed once with 50 mM Tris, pH 7.4 buffer and resuspended in the same buffer.

2.2.4. Incubation of cell membranes at high temperature

Control, cholesterol-depleted and -enriched membranes at a protein concentration of 2 mg/ml were incubated at 37 °C for varying time periods ranging from 0 to 2 h. After incubation, membranes were cooled to 25 °C and radioligand binding assays were carried out.

2.2.5. Incubation of cell membranes at varying pH

Control, cholesterol-depleted and -enriched membranes suspended in 50 mM Tris, pH 7.4 buffer were spun down at 50,000 ×*g* for 10 min, resuspended in buffers spanning a pH range 4–12 at a protein concentration of 2 mg/ml and incubated at 25 °C for 30 min. The buffers used were 10 mM acetate (pH 4 and 6), 50 mM Tris (pH 8), 10 mM CAPS (pH 10) and 50 mM CAPS (pH 12). After incubation, membranes were spun down at 50,000 ×*g* for 10 min, washed once with 50 mM Tris, pH 7.4 buffer and resuspended in the same buffer for neutralization. Neutralized membranes were utilized to perform radioligand binding assays.

2.2.6. Trypsin treatment of cell membranes

Proteolytic degradation of the serotonin$_{1A}$ receptor in membranes was achieved utilizing trypsin. Trypsin and trypsin inhibitor stock solutions were prepared in 50 mM Tris, pH 7.4 buffer. Control, cholesterol-depleted and -enriched membranes at a protein concentration of 2 mg/ml were incubated with 0.05 mg/ml (0.33 U/ml) trypsin for 15 min at 25 °C. Trypsin action was terminated after incubation by adding 0.1 mg/ml trypsin inhibitor and radioligand binding assays were carried out immediately.

2.2.7. Radioligand binding assays

Receptor binding assays in control and cholesterol-depleted and -enriched membranes were carried out as described earlier [29,30,42] with ~40 μg total protein. The concentration of [^{3}H]8-OH-DPAT used was 0.29 nM.

2.2.8. Estimation of cholesterol and phospholipid contents

Cholesterol content in cell membranes was estimated using the Amplex Red cholesterol assay kit [43]. The content of lipid phosphate in membranes was determined subsequent to total digestion with perchloric acid [44] using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.9. Fluorescence anisotropy measurements

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH as described previously [29,45]. Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (~23 °C). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal bandpasses of 1.5 and 20 nm were used. The optical density of the samples measured at 358 nm was ~0.12. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (r) values were calculated from the equation [46]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light and is equal to I_{HV}/I_{HH}. All experiments

were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 5.

2.2.10. Statistical analysis

Significance levels were estimated using Student's two-tailed unpaired *t*-test using Graphpad Prism software version 4.0 (San Diego, CA).

3. Results and discussion

3.1. Lipid contents of cell membranes treated with either MβCD or cholesterol–MβCD complex

MβCD is a water-soluble polymer with a nonpolar central cavity. It has previously been shown to selectively and efficiently extract cholesterol from cellular membranes by incorporating it in a central nonpolar cavity [47]. Lipid contents of isolated cell membranes upon cholesterol depletion and enrichment are shown in Fig. 1. Treatment of cell membranes with MβCD alone resulted in reduction in cholesterol content, while treatment with cholesterol-MβCD complex resulted in an increase in cholesterol content (see Fig. 1A). Cholesterol content was reduced to ~18% of control (untreated) membranes upon treatment with MβCD. On the other hand, membranes treated with cholesterol-MβCD complex exhibited ~53% enrichment in cholesterol content, relative to control membranes. Importantly, phospholipid contents are found to be unaltered under these conditions (see Fig. 1A). The ratio of cholesterol to phospholipid contents (C/PL) in membranes provides an estimate of the fraction of cholesterol present. C/PL ratio was found to be ~0.14 (mol/mol) for control membranes (see Fig. 1B). This ratio is altered to ~0.03 and ~0.21 upon cholesterol depletion and enrichment of membranes, respectively.

3.2. Effect of membrane cholesterol on temperature sensitivity of the serotonin$_{1A}$ receptor

Specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors in isolated cell membranes upon cholesterol depletion and enrichment is shown in Fig. 2A. The figure shows that the ligand binding function of the serotonin$_{1A}$ receptor is reduced by ~21% in cholesterol-depleted membranes. On the other hand, cholesterol-enriched membranes exhibited an increase of ~16% in ligand binding relative to control membranes.

In order to explore the effect of cholesterol content on the temperature sensitivity (stability) of the serotonin$_{1A}$ receptor, we monitored ligand binding of the receptor at 25 °C in cholesterol-depleted and -enriched membranes, pre-treated at 37 °C for varying time periods. Fig. 2B and C show that ligand binding exhibits a progressive reduction with an increase in the time of pre-treatment for control and cholesterol-depleted membranes. The reduction in ligand binding under these conditions is found to be more in case of cholesterol-depleted membranes and is not significant for cholesterol-enriched membranes (see Fig. 2B and C). Fig. 2C shows that after 2 h of treatment at 37 °C, ligand binding was reduced to ~74% and ~49% of the activity monitored in the absence of any treatment in control and cholesterol-depleted membranes, respectively. This reduction in ligand binding in these cases could be due to irreversible thermal denaturation of either the receptor or G-proteins during incubation at 37 °C. We have previously shown that incubation at high temperature could cause irreversible inactivation of G-proteins coupled to the serotonin$_{1A}$ receptor [48]. It should be mentioned here that the conformation of the subtype of G-proteins that is coupled to the serotonin$_{1A}$ receptor, namely G_i [49], has previously been reported to be sensitive to temperature [50]. The reduction in ligand binding also 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 [51].

Fig. 2B and C show that membrane cholesterol provides considerable protection against thermal deactivation since receptors in cholesterol-enriched membranes do not show significant reduction in ligand binding activity even after 2 h of treatment at 37 °C. This is also apparent from the different extents of reduction in ligand binding for control (~26%) and cholesterol-depleted (~51%) membranes after 2 h of treatment at 37 °C. Membrane cholesterol therefore appears to provide stability to the receptor against thermal deactivation. In other words, receptors in membranes with relatively high cholesterol content appear to enjoy less sensitivity to thermal deactivation relative to receptors in a membrane with lower cholesterol content. Interestingly, cholesterol has been shown to increase the thermal stability of membrane proteins such as the Ca^{2+}/Mg^{2+}-ATPase [52], the nicotinic acetylcholine receptor [53] and the oxytocin receptor [54].

3.3. Effect of membrane cholesterol on pH sensitivity of the serotonin$_{1A}$ receptor

Side chains of acidic or basic amino acid residues of proteins are sensitive to pH of the environment. The charge status of these pH-sensitive residues could contribute to conformation of the protein and in turn could govern its function. Fig. 3A depicts ligand binding activity of the serotonin$_{1A}$ receptor in control membranes pre-treated at various pH. Ligand binding activity of the receptor was drastically reduced in case of highly acidic (pH 4) or basic (pH 12) conditions. This could be due to pH-induced irreversible denaturation of the receptor and/or G-protein. Such pH-dependent activity has previously been reported for GPCRs such as the β_2-adrenergic receptor [55].

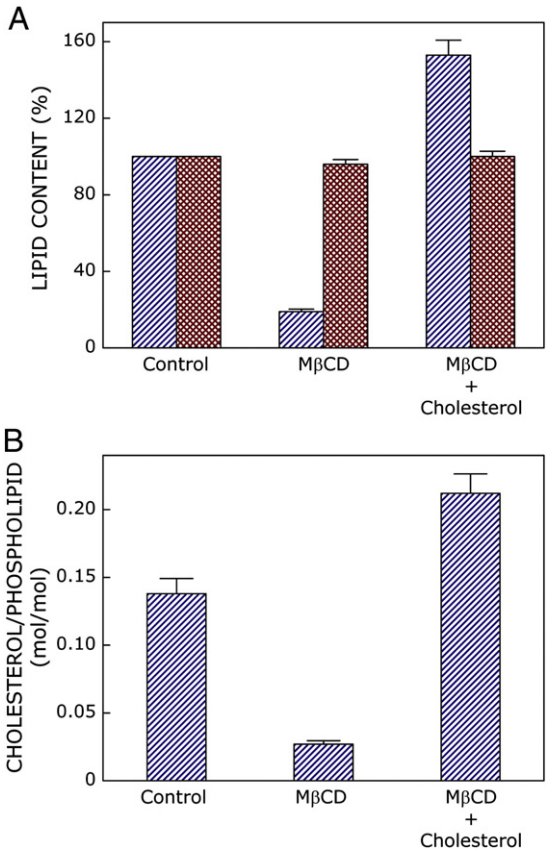

Fig. 1. Lipid contents of membranes isolated from CHO-5-HT$_{1A}$R cells upon cholesterol depletion and enrichment. Panel A shows cholesterol (hatched bar) and phospholipid contents (crisscrossed bar) in cholesterol-depleted (MβCD treated) and -enriched (cholesterol–MβCD complex treated) membranes. Values are expressed as percentages of respective lipid contents in control (untreated) membranes. Panel B represents the molar ratio of cholesterol and phospholipid contents in cholesterol-depleted and -enriched membranes. Data represent means ± S.E. of five independent experiments. See Materials and methods for more details.

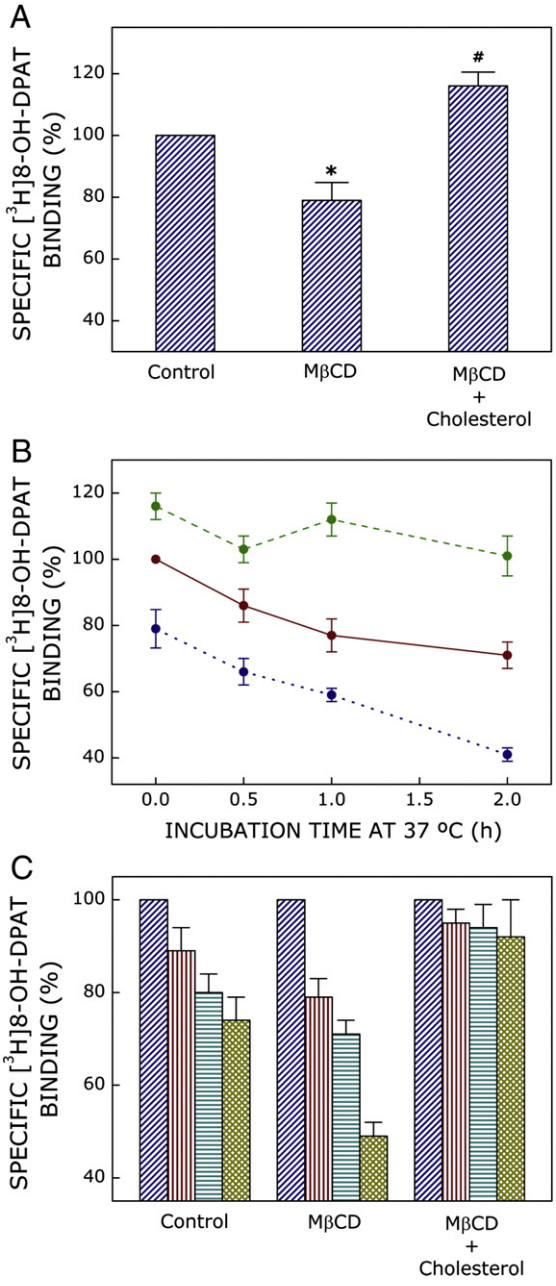

Fig. 2. Effect of membrane cholesterol content on temperature sensitivity of ligand binding of the serotonin$_{1A}$ receptor. Panel A shows specific [^{3}H]8-OH-DPAT binding to receptors in cholesterol-depleted and -enriched membranes. Values are expressed as percentages of specific binding obtained in control membranes. Panels B and C show the effect of cholesterol on stability of the receptor incubated at 37 °C for varying time periods. Control, cholesterol-depleted and -enriched membranes were incubated at 37 °C for varying time periods ranging from 0 to 2 h. After incubation, membranes were cooled to 25 °C and ligand binding was carried out. Panel B shows specific [^{3}H]8-OH-DPAT binding to receptors in control (——), cholesterol-depleted (·····) and -enriched (— — — —) membranes, pre-incubated at 37 °C for increasing time periods. Values are expressed as percentages of specific binding obtained in control membranes without incubation at 37 °C. Panel C shows specific [^{3}H]8-OH-DPAT binding to receptors in control, cholesterol-depleted and -enriched membranes, pre-incubated at 37 °C for 0 h (hatched bar), 1 h (vertical bar), 1.5 h (horizontal bar) and 2 h (crisscrossed bar). Values are expressed as percentages of specific binding obtained in membranes in absence of incubation at 37 °C for respective conditions. Data represent means ± S.E. of four independent experiments [* and # correspond to significant ($p = 0.006$ and 0.005, respectively) difference in specific [^{3}H]8-OH-DPAT binding to cholesterol-depleted and -enriched membranes relative to control membranes]. See Materials and methods for more details.

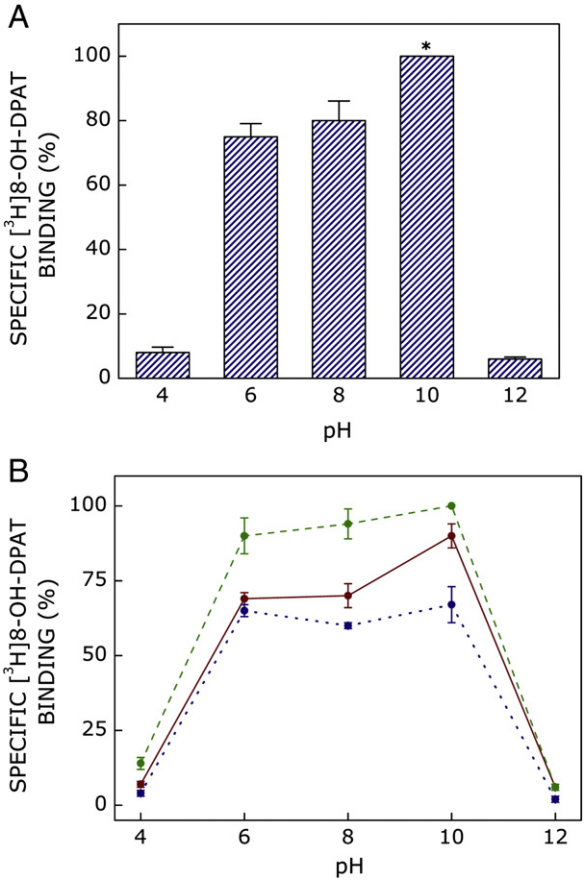

Fig. 3. Effect of membrane cholesterol content on pH sensitivity of ligand binding of the serotonin$_{1A}$ receptor. Panel A shows specific [^{3}H]8-OH-DPAT binding to receptors in control membranes pre-incubated in buffers of varying pH. Values are expressed as percentages of specific binding obtained in membranes pre-incubated in pH 10 buffer. Panel B shows the effect of membrane cholesterol content on pH-sensitivity of the receptor. Control, cholesterol-depleted and -enriched membranes were suspended in buffer, spanning a pH range 4–12, and incubated at 25 °C for 30 min. Membranes were neutralized following incubation, and [^{3}H]8-OH-DPAT binding assays were performed. Panel B represents specific [^{3}H]8-OH-DPAT binding to receptors in control (——), cholesterol-depleted (·····) and -enriched (— — — —) membranes, pre-incubated in buffers of varying pH. Values are expressed as percentages of specific binding obtained in cholesterol-enriched membranes pre-incubated in pH 10 buffer. Data shown are means ± S.E. of four independent experiments [*corresponds to significant ($p = 0.007$) difference in specific [^{3}H]8-OH-DPAT binding to receptors in membranes pre-treated at pH 10 relative to membranes pre-treated at pH 8]. See Materials and methods for more details.

In order to gain insight into the role of membrane cholesterol on pH-sensitivity of the serotonin$_{1A}$ receptor, we monitored ligand binding in cholesterol-depleted and -enriched membranes, pre-treated at varying pH ranging from 4 to 12. The ligand binding activity of receptors in membranes of different cholesterol content, pre-treated at various pH, is shown in Fig. 3B. Interestingly, ligand binding of the receptor is found to be correlated with the cholesterol content of the membrane at each pH. Ligand binding was found to be maximum in cholesterol-enriched membranes and minimum in cholesterol-depleted membranes, with control membranes displaying ligand binding in the intermediate range at each pH. Our results therefore show that membrane cholesterol provides stability to the receptor against pH-induced denaturation.

3.4. Effect of membrane cholesterol on proteolytic degradation of the serotonin$_{1A}$ receptor

Proteases are enzymes that hydrolyze proteins and are abundantly present in cells. We monitored the effect of cholesterol depletion and enrichment on the proteolytic degradation of the serotonin$_{1A}$ receptor in order to explore whether membrane cholesterol could protect the receptor from proteolytic degradation. For this, ligand binding was measured in membranes of different cholesterol levels

after trypsin treatment. Trypsin is a serine protease which cleaves proteins predominantly at the carboxyl side of the amino acids lysine or arginine [56]. The serotonin$_{1A}$ receptor contains 44 sites (including two in putative transmembrane domains) susceptible to cleavage by trypsin (see Fig. 4A). Importantly, one of the sites is located in the conserved DRY motif which is known to be crucial for G-protein coupling of GPCRs [23,57]. In addition, membrane anchored G-proteins could represent one of the prime targets of trypsin as proposed earlier [58]. We initially confirmed the efficacy of trypsin treatment of the serotonin$_{1A}$ receptor (see Supplementary Fig. 1). The serotonin$_{1A}$ receptor exhibits a decrease in ligand binding upon trypsin treatment in both control and cholesterol-depleted membranes (see Fig. 4B). Trypsin treatment caused ~54% and ~42% reduction in the ligand binding activity in control and cholesterol-depleted membranes, respectively. On the other hand, receptors in cholesterol-enriched membranes retained ligand binding ability to a considerable extent even after trypsin treatment. Receptors in these membranes exhibited only ~24% reduction in ligand binding upon trypsin treatment. The reduction in ligand binding could partly be due to loss of G-protein coupling upon cleavage of either the receptor at DRY region or G-proteins [57], since agonist binding is sensitive to the extent of G-protein coupling of receptors [59]. These results indicate that receptors in cholesterol enriched-membranes are less susceptible to proteolytic degradation probably due to the less accessibility of the receptor to trypsin. Alternatively, almost similar extent of reduction in ligand binding in both control and cholesterol-depleted membranes points out that receptors in these membranes are equally accessible to trypsin. It is possible that there could be a threshold cholesterol content in cell membranes which determines the susceptibility to proteolytic degradation. Taken together, our results highlight the protective role of membrane cholesterol against proteolytic degradation.

3.5. Effect of membrane cholesterol on cell membrane organization and dynamics: results from DPH anisotropy measurements

Membrane order and dynamics could be an important determinant of receptor structure and function [12,60]. In order to gain insight into the changes in membrane order induced upon modulation of cholesterol levels in cell membranes, we monitored the steady-state fluorescence anisotropy of the membrane probe, DPH. DPH, a rod-like molecule, partitions into the interior of the bilayer and represents a popular membrane probe for monitoring organization and dynamics in membranes [61]. Fluorescence anisotropy is correlated to the rotational diffusion of membrane-embedded probes [46], which is sensitive to the packing of fatty acyl chains and cholesterol. This is due to the fact that fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing. The change in fluorescence anisotropy of DPH incorporated in cholesterol-depleted and -enriched membranes is shown in Fig. 5. The fluorescence anisotropy of DPH in control membranes is found to be ~0.23. Interestingly, the fluorescence anisotropy of DPH shows a decrease of ~17% (to ~0.19) upon cholesterol depletion (see Fig. 5). Cholesterol-enriched membranes, on the other hand, exhibit an increase of ~13% in DPH anisotropy (to ~0.26) suggesting that DPH experiences an overall more ordered environment in cholesterol-enriched membranes. The fluorescence anisotropy of DPH in cell membranes appears to correlate well with cholesterol/phospholipid ratio of membranes with varying cholesterol contents (see inset in Fig. 5).

Taken together, our results show that membrane cholesterol stabilizes the serotonin$_{1A}$ receptor as evident from the sensitivity of the receptor to thermal deactivation, pH, and proteolytic digestion. Recent efforts in crystallizing GPCRs have shown that the addition of cholesterol often turns out to be crucial for maintaining receptor stability, an essential criterion for obtaining good crystals for structural biological work [62]. It is important to mention in this context that we have recently reported from receptor modeling studies that

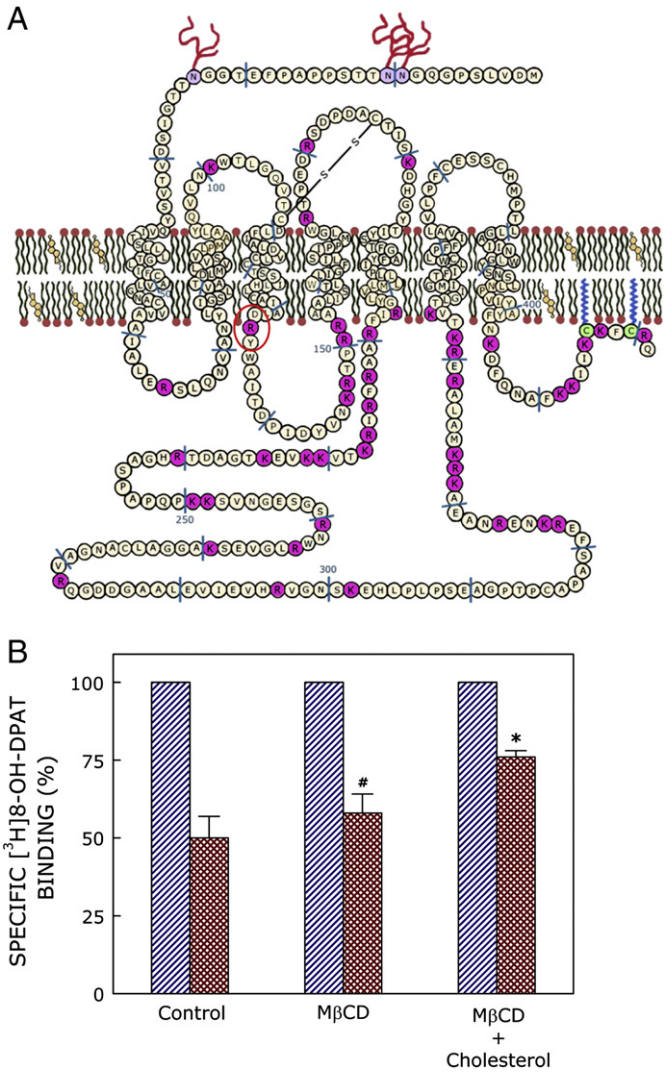

Fig. 4. Effect of membrane cholesterol content on proteolytic degradation of the serotonin$_{1A}$ receptor. Panel A shows a schematic representation of the membrane embedded human serotonin$_{1A}$ receptor in a membrane bilayer composed of phospholipids and cholesterol, representative of eukaryotic membranes. The transmembrane helices of the receptor were previously predicted using TMHMM2 [38]. Transmembrane stretches, composed of ~22 amino acids, are depicted as putative α-helices. The amino acids in the receptor sequence are shown as circles. There are 44 sites (shown in magenta) susceptible to cleavage by trypsin. Trypsin cleaves proteins predominantly at the carboxyl side of the amino acids lysine (K) or arginine (R) [56]. Importantly, one of the cleavage sites is present in DRY motif (highlighted as a circle) of the receptor, crucial for G-protein coupling [57]. Further structural details of the receptor are available in [63]. Adapted and modified from ref. [38]. Panel B shows the effect of membrane cholesterol content on proteolytic degradation of the receptor. Control, cholesterol-depleted and -enriched membranes were treated with trypsin at 25 °C for 15 min. The action of trypsin was terminated by adding trypsin inhibitor and ligand binding assays were immediately carried out. Specific [^{3}H]8-OH-DPAT binding to receptors are shown in the presence (crisscrossed bar) and absence (hatched bar) of trypsin treatment in control, cholesterol-depleted and -enriched membranes. Values are expressed as percentages of specific binding obtained in membranes in absence of trypsin treatment for each condition. Values are normalized this way to eliminate any effect due to alteration in ligand binding associated with modulation of membrane cholesterol content. Data shown are means ± S.E. of four independent experiments (*corresponds to significant ($p = 0.013$) and $^{\#}$represents not significant ($p = 0.256$) difference in specific [^{3}H]8-OH-DPAT binding to receptors in cholesterol-enriched and -depleted membranes relative to control membranes in each case). See Materials and methods for more details.

the serotonin$_{1A}$ receptor is more compact in the presence of tightly bound cholesterol [63]. Such a compact conformation could contribute to receptor stability. Interestingly, we have very recently identified cholesterol recognition/interaction amino acid consensus (CRAC) motifs

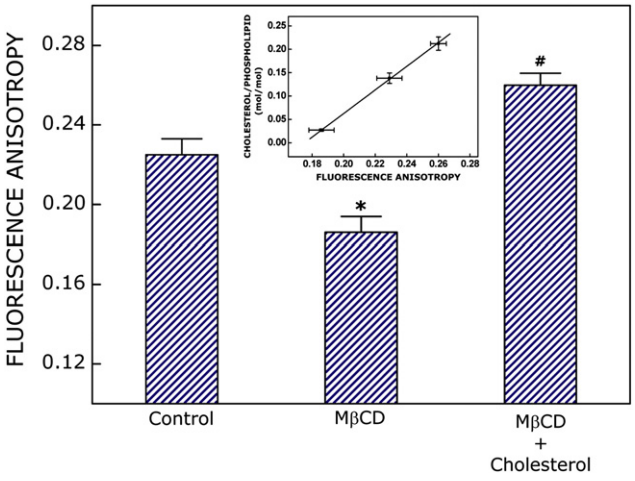

Fig. 5. Effect of membrane cholesterol content on fluorescence anisotropy of the membrane probe DPH. Fluorescence anisotropy experiments were performed with control, cholesterol-depleted and -enriched membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). Values represent means ± S.E. of duplicate points from four independent measurements (* and # correspond to significant ($p = 0.001$ and 0.006, respectively) difference in cholesterol-depleted and -enriched membranes relative to control membranes). The inset shows a plot between fluorescence anisotropy of DPH and cholesterol/phospholipid molar ratio in membranes with varying cholesterol content. See Materials and methods for more details.

in the serotonin$_{1A}$ receptor [64]. The CRAC motif represents a characteristic structural feature of proteins that are believed to result in preferential association with cholesterol [65,66]. In addition, in the crystal structure of the β_2-adrenergic receptor, a cholesterol consensus motif (CCM) has been identified consisting of four amino acids [37]. We have previously shown that CCM is present in the serotonin$_{1A}$ receptor [38]. Interestingly, recent molecular dynamics simulations have shown that membrane cholesterol specifically interacts with transmembrane domains of GPCRs such as rhodopsin [67] and human A_{2A} adenosine receptor [68], thereby stabilizing helix II of the human A_{2A} adenosine receptor. A consequence of this interaction is that the receptor couples to G-proteins only in presence of cholesterol. Membrane ordering induced by cholesterol (see Fig. 5) could also contribute to receptor stability.

We have used the specific binding of the agonist 8-OH-DPAT in monitoring the stability of the serotonin$_{1A}$ receptor. A major reason for the popularity of the serotonin$_{1A}$ receptor subtype is the early availability of this selective agonist that led to extensive biochemical, physiological, and pharmacological characterization of the receptor [23]. Results obtained from point mutations have suggested that Asp^{82} and Asp^{116} in transmembrane helices II and III are crucial for binding agonist binding to the receptor [69]. In addition, residues such as Ser^{393} and Asn^{396} have been shown to be crucial for agonist binding [70].

This stabilizing effect of cholesterol on membrane proteins could be considered analogous to the well documented role of trehalose as a stabilizer of membrane and soluble proteins [71–73]. In addition, it has recently been shown that cholesterol stabilizes less stable discoidal high density lipoproteins, apparently by facilitating favorable packing interactions [74]. In this overall context, our results could have potential implications in future crystallization efforts of the serotonin$_{1A}$ receptor in particular, and the storage and transportation of biomaterials in general.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.07.032.

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References

[1] K. Simons, E. Ikonen, How cells handle cholesterol, Science 290 (2000) 1721–1726.
[2] O.G. Mouritsen, M.J. Zuckermann, What's so special about cholesterol? Lipids 39 (2004) 1101–1113.
[3] K.E. Bloch, Sterol structure and membrane function, CRC Crit. Rev. Biochem. 14 (1983) 47–92.
[4] X. Xu, E. London, The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation, Biochemistry 39 (2000) 843–849.
[5] S. Mukherjee, F.R. Maxfield, Membrane domains, Annu. Rev. Cell Dev. Biol. 20 (2004) 839–866.
[6] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science 27 (2010) 46–50.
[7] A. Chaudhuri, A. Chattopadhyay, Transbilayer organization of membrane cholesterol at low concentrations: implications in health and disease, Biochim. Biophys. Acta 1808 (2011) 19–25.
[8] K. Simons, G. van Meer, Lipid sorting in epithelial cells, Biochemistry 27 (1988) 6197–6202.
[9] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[10] J. Riethmüller, A. Riehle, H. Grassmé, E. Gulbins, Membrane rafts in host–pathogen interactions, Biochim. Biophys. Acta 1758 (2006) 2139–2147.
[11] T.J. Pucadyil, A. Chattopadhyay, Cholesterol: a potential therapeutic target in *Leishmania* infection? Trends Parasitol. 23 (2007) 49–53.
[12] K. Burger, G. Gimpl, F. Fahrenholz, Regulation of receptor function by cholesterol, Cell. Mol. Life Sci. 57 (2000) 1577–1592.
[13] T.J. Pucadyil, A. Chattopadhyay, Role of cholesterol in the function and organization of G-protein coupled receptors, Prog. Lipid Res. 45 (2006) 295–333.
[14] Y.D. Paila, A. Chattopadhyay, Membrane cholesterol in the function and organization of G-protein coupled receptors, Subcell. Biochem. 51 (2010) 439–466.
[15] J. Oates, A. Watts, Uncovering the intimate relationship between lipids, cholesterol and GPCR activation, Curr. Opin. Struct. Biol. 21 (2011) 802–807.
[16] Y.D. Paila, A. Chattopadhyay, The function of G-protein coupled receptors and membrane cholesterol: specific or general interaction? Glycoconj. J. 26 (2009) 711–720.
[17] A.G. Lee, Biological membranes: the importance of molecular detail, Trends Biochem. Sci. 36 (2011) 493–500.
[18] K.L. Pierce, R.T. Premont, R.J. Lefkowitz, Seven-transmembrane receptors, Nat. Rev. Mol. Cell Biol. 3 (2002) 639–650.
[19] D.M. Rosenbaum, S.G.F. Rasmussen, B.K. Kobilka, The structure and function of G-protein-coupled receptors, Nature 459 (2009) 356–363.
[20] Y. Zhang, M.E. DeVries, J. Skolnick, Structure modeling of all identified G protein-coupled receptors in the human genome, PLoS Comput. Biol. 2 (2006) 88–99.
[21] R. Heilker, M. Wolff, C.S. Tautermann, M. Bieler, G-protein-coupled receptor-focused drug discovery using a target class platform approach, Drug Discov. Today 14 (2009) 231–240.
[22] S. Schlyer, R. Horuk, I want a new drug: G-protein-coupled receptors in drug development, Drug Discov. Today 11 (2006) 481–493.
[23] T.J. Pucadyil, S. Kalipatnapu, A. Chattopadhyay, The serotonin$_{1A}$ receptor: a representative member of the serotonin receptor family, Cell. Mol. Neurobiol. 25 (2005) 553–580.
[24] S. Kalipatnapu, A. Chattopadhyay, Membrane organization and function of the serotonin$_{1A}$ receptor, Cell. Mol. Neurobiol. 27 (2007) 1097–1116.
[25] C.P. Müller, R.J. Carey, J.P. Huston, M.A. De Souza Silva, Serotonin and psychostimulant addiction: focus on 5-HT$_{1A}$-receptors, Prog. Neurobiol. 81 (2007) 133–178.
[26] P. Blier, N.M. Ward, Is there a role for 5-HT$_{1A}$ agonists in the treatment of depression? Biol. Psychiatry 53 (2003) 193–203.
[27] G. Griebel, 5-HT$_{1A}$ receptor blockers as potential drug candidates for the treatment of anxiety disorders, Drug News Perspect. 12 (1999) 484–490.
[28] T.J. Pucadyil, A. Chattopadhyay, Cholesterol modulates the ligand binding and G-protein coupling to serotonin$_{1A}$ receptors from bovine hippocampus, Biochim. Biophys. Acta 1663 (2004) 188–200.
[29] Y.D. Paila, M.R.V.S. Murty, M. Vairamani, A. Chattopadhyay, Signaling by the human serotonin$_{1A}$ receptor is impaired in cellular model of Smith–Lemli–Opitz syndrome, Biochim. Biophys. Acta 1778 (2008) 1508–1516.
[30] T.J. Pucadyil, A. Chattopadhyay, Cholesterol depletion induces dynamic confinement of the G-protein coupled serotonin$_{1A}$ receptor in the plasma membrane of living cells, Biochim. Biophys. Acta 1768 (2007) 655–668.
[31] S. Shrivastava, T.J. Pucadyil, Y.D. Paila, S. Ganguly, A. Chattopadhyay, Chronic cholesterol depletion using statin impairs the function and dynamics of human serotonin$_{1A}$ receptors, Biochemistry 49 (2010) 5426–5435.

[32] P. Singh, Y.D. Paila, A. Chattopadhyay, Differential effects of cholesterol and 7-dehydrocholesterol on the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor: implications in SLOS, Biochem. Biophys. Res. Commun. 358 (2007) 495–499.
[33] P. Singh, R. Saxena, Y.D. Paila, M. Jafurulla, A. Chattopadhyay, Differential effects of cholesterol and desmosterol on the ligand binding function of the hippocampal serotonin$_{1A}$ receptor: implications in desmosterolosis, Biochim. Biophys. Acta 1788 (2009) 2169–2173.
[34] J.J. Ruprecht, T. Mielke, R. Vogel, C. Villa, G.F. Schertler, Electron crystallography reveals the structure of metarhodopsin I, EMBO J. 23 (2004) 3609–3620.
[35] T. Warne, R. Moukhametzianov, J.G. Baker, R. Nehmé, P.C. Edwards, A.G.W. Leslie, G.F.X. Schertler, C.G. Tate, The structural basis for agonist and partial agonist action on a β_1-adrenergic receptor, Nature 469 (2011) 241–244.
[36] V. Cherezov, D.M. Rosenbaum, M.A. Hanson, S.G.F. Rasmussen, F.S. Thian, T.S. Kobilka, H.-J. Choi, P. Kuhn, W.I. Weis, B.K. Kobilka, R.C. Stevens, High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor, Science 318 (2007) 1258–1265.
[37] 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 β_2-adrenergic receptor, Structure 16 (2008) 897–905.
[38] Y.D. Paila, S. Tiwari, A. Chattopadhyay, Are specific nonannular cholesterol binding sites present in G-protein coupled receptors? Biochim. Biophys. Acta 1788 (2009) 295–302.
[39] Z. Yao, B. Kobilka, Using synthetic lipids to stabilize purified β_2 adrenoceptor in detergent micelles, Anal. Biochem. 343 (2005) 344–346.
[40] S. Kalipatnapu, T.J. Pucadyil, K.G. Harikumar, A. Chattopadhyay, Ligand binding characteristics of the human serotonin$_{1A}$ receptor heterologously expressed in CHO cells, Biosci. Rep. 24 (2004) 101–115.
[41] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[42] R. Prasad, Y.D. Paila, M. Jafurulla, A. Chattopadhyay, Membrane cholesterol depletion from live cells enhances the function of human serotonin$_{1A}$ receptors, Biochem. Biophys. Res. Commun. 389 (2009) 333–337.
[43] D.M. Amundson, M. Zhou, Fluorometric method for the enzymatic determination of cholesterol, J. Biochem. Biophys. Methods 38 (1999) 43–52.
[44] C.W.F. McClare, An accurate and convenient organic phosphorus assay, Anal. Biochem. 39 (1971) 527–530.
[45] R. Prasad, Y.D. Paila, A. Chattopadhyay, Membrane cholesterol depletion enhances ligand binding function of human serotonin$_{1A}$ receptors in neuronal cells, Biochem. Biophys. Res. Commun. 390 (2009) 93–96.
[46] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 3rd ed. Springer, New York, 2006..
[47] R. Zidovetzki, I. Levitan, Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies, Biochim. Biophys. Acta 1768 (2007) 1311–1324.
[48] V. Javadekar-Subhedar, A. Chattopadhyay, Temperature-dependent interaction of the bovine hippocampal serotonin$_{1A}$ receptor with G-proteins, Mol. Membr. Biol. 21 (2004) 119–123.
[49] M.B. Emerit, S. El Mestikawy, S.H. Gozlan, B. Rouot, M. Hamon, Physical evidence of the coupling of solubilized 5-HT$_{1A}$ binding sites with G regulatory proteins, Biochem. Pharmacol. 39 (1990) 7–18.
[50] S.K.F. Wong, B.R. Martin, A.M. Tolkovsky, Pertussis toxin substrate is a guanosine 5′-[β-thio]diphosphate-, *N*-ethylmaleimide-, Mg^{2+}- and temperature-sensitive GTP-binding protein, Biochem. J. 232 (1985) 191–197.
[51] W.V. Moore, D.B. Wetlaufer, Circular dichroism of nerve membrane fractions: effects of temperature, pH and electrolytes, J. Neurochem. 20 (1973) 135–149.
[52] A. Ortega, J. Santiago-García, J. Mas-Oliva, J.R. Lepock, Cholesterol increases the thermal stability of the Ca^{2+}/Mg^{2+}-ATPase of cardiac microsomes, Biochim. Biophys. Acta 1283 (1996) 45–50.
[53] B. Perez-Ramirez, Thermal stability of *Torpedo californica* acetylcholine receptor in a cholesterol lipid environment, Mol. Cell. Biochem. 132 (1994) 91–99.
[54] G. Gimpl, F. Fahrenholz, Cholesterol as stabilizer of the oxytocin receptor, Biochim. Biophys. Acta 1564 (2002) 384–392.
[55] P. Ghanouni, H. Schambye, R. Seifert, T.W. Lee, S.G.F. Rasmussen, U. Gether, B.K. Kobilka, The effect of pH on β_2 adrenoceptor function. Evidence for protonation-dependent activation, J. Biol. Chem. 275 (2000) 3121–3127.
[56] J.V. Olsen, S.-E. Ong, M. Mann, Trypsin cleaves exclusively C-terminal to arginine and lysine residues, Mol. Cell. Proteomics 3 (2004) 608–614.
[57] G.E. Rovati, V. Capra, R.R. Neubig, The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state, Mol. Pharmacol. 71 (2007) 959–964.
[58] N. Ferry, S. Adnot, A. Borsodi, M.-L. Lacombe, G. Guellaën, J. Hanoune, Uncoupling by proteolysis of alpha-adrenergic receptor-mediated inhibition of adenylate cyclase in human platelets, Biochem. Biophys. Res. Commun. 108 (1982) 708–714.
[59] K.G. Harikumar, A. Chattopadhyay, Differential discrimination of G-protein coupling of serotonin$_{1A}$ receptors from bovine hippocampus by an agonist and an antagonist, FEBS Lett. 457 (1999) 389–392.
[60] A.G. Lee, How lipids affect the activities of integral membrane proteins, Biochim. Biophys. Acta 1666 (2004) 62–87.
[61] B.R. Lentz, Membrane fluidity as detected by diphenylhexatriene probes, Chem. Phys. Lipids 50 (1989) 171–190.
[62] A.A. Thompson, J.J. Liu, E. Chun, D. Wacker, H. Wu, V. Cherezov, R.C. Stevens, GPCR stabilization using the bicelle-like architecture of mixed sterol-detergent micelles, Methods 55 (2011) 310–317.
[63] Y.D. Paila, S. Tiwari, D. Sengupta, A. Chattopadhyay, Molecular modeling of the human serotonin$_{1A}$ receptor: role of membrane cholesterol in ligand binding of the receptor, Mol. Biosyst. 7 (2011) 224–234.
[64] M. Jafurulla, S. Tiwari, A. Chattopadhyay, Identification of cholesterol recognition amino acid consensus (CRAC) motif in G-protein coupled receptors, Biochem. Biophys. Res. Commun. 404 (2011) 569–573.
[65] H. Li, V. Papadopoulos, Peripheral-type benzodiazepine receptor function in cholesterol transport: identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern, Endocrinology 139 (1998) 4991–4997.
[66] R.M. Epand, Cholesterol and the interaction of proteins with membrane domains, Prog. Lipid Res. 45 (2006) 279–294.
[67] G. Khelashvili, A. Grossfield, S.E. Feller, M.C. Pitman, H. Weinstein, Structural and dynamic effects of cholesterol at preferred sites of interaction with rhodopsin identified from microsecond length molecular dynamics simulations, Proteins 76 (2009) 403–417.
[68] E. Lyman, C. Higgs, B. Kim, D. Lupyan, J.C. Shelley, R. Farid, G.A. Voth, A role for a specific cholesterol interaction in stabilizing the Apo configuration of the human A_{2A} adenosine receptor, Structure 17 (2009) 1660–1668.
[69] B.Y. Ho, A. Karschin, T. Brancheck, N. Davidson, H.A. Lester, Role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT$_{1A}$ receptor: a site-directed mutation study, FEBS Lett. 312 (1992) 259–262.
[70] P.K. Chanda, M.C. Minchin, A.R. Davis, L. Greenberg, Y. Reilly, W.H. McGregor, R. Bhat, M.D. Lubeck, S. Mizutani, P.P. Hung, Identification of residues important for ligand binding to the human 5-hydroxytryptamine$_{1A}$ serotonin receptor, Mol. Pharmacol. 43 (1993) 516–520.
[71] L.M. Crowe, D.S. Reid, J.H. Crowe, Is trehalose special for preserving dry biomaterials? Biophys. J. 71 (1996) 2087–2093.
[72] J.K. Kaushik, R. Bhat, Why is trehalose an exceptional protein stabilizer? J. Biol. Chem. 278 (2003) 26458–26465.
[73] J.H. Crowe, Trehalose as a "chemical chaperone": fact and fantasy, Adv. Exp. Med. Biol. 594 (2007) 143–158.
[74] S. Jayaraman, S. Benjwal, D.L. Gantz, O. Gursky, Effects of cholesterol on thermal stability of discoidal high density lipoproteins, J. Lipid Res. 51 (2010) 324–333.