Biochimica et Biophysica Acta 1818 (2012) 2936–2942

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

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

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ARTICLE INFO

Article history:
Received 9 June 2012
Received in revised form 20 July 2012
Accepted 23 July 2012
Available online 8 August 2012

Keywords:
Human serotonin$_{1A}$ receptor
Cholesterol
Ligand binding
Thermal deactivation
Fluorescence anisotropy

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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http://dx.doi.org/10.1016/j.bbamem.2012.07.032

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