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Differential effects of cholesterol and 7-dehydrocholesterol on the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor: Implications in SLOS

Pushpendra Singh, Yamuna Devi Paila, Amitabha Chattopadhyay *

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

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Abstract

The requirement of membrane cholesterol in maintaining ligand binding activity of the hippocampal serotonin$_{1A}$ receptor has previously been demonstrated. In order to test the stringency of the requirement of cholesterol, we depleted cholesterol from native hippocampal membranes followed by replenishment with 7-dehydrocholesterol. The latter sterol is an immediate biosynthetic precursor of cholesterol differing only in a double bond at the 7th position in the sterol ring. Our results show, for the first time, that replenishment with 7-dehydrocholesterol does not restore ligand binding activity of the serotonin$_{1A}$ receptor, in spite of recovery of the overall membrane order. The requirement for restoration of ligand binding activity therefore is more stringent than the requirement for the recovery of overall membrane order. These novel results have potential implications in understanding the interaction of membrane lipids with this important neuronal receptor under pathogenic conditions such as the Smith-Lemli-Opitz syndrome.

Keywords: Smith-Lemli-Opitz syndrome; Cholesterol; 7-Dehydrocholesterol; Ligand binding activity; Serotonin$_{1A}$ receptor; Fluorescence anisotropy

Seven transmembrane domain G-protein coupled receptors (GPCRs) constitute one of the largest family of proteins in mammals and account for ~2% of the total proteins coded by the human genome [1]. GPCRs represent major targets for the development of novel drug candidates in all clinical areas [2]. The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important GPCR and is the most extensively studied among the serotonin receptors. The serotonin$_{1A}$ receptor is involved in a variety of cognitive, behavioral, and developmental functions (for a recent review, see [3]). The serotonin$_{1A}$ receptor agonists and antagonists represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior [4] and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders and aggression [5].

Cholesterol is an abundant and essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting [6]. Interestingly, the central nervous system which accounts for only 2% of the body mass contains ~25% of free cholesterol present in the whole body [7]. Brain cholesterol is synthesized *in situ* and is developmentally regulated. As a result, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain [8]. In the Smith-Lemli-Opitz syndrome (SLOS), for example, the marked abnormalities in brain development and function leading to serious neurological and mental dys-

Abbreviations: 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; 7-DHC, 7-dehydrocholesterol; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; 7-DHCR, 3β-hydroxy-steroid-Δ^7-reductase; 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; SLOS, Smith-Lemli-Opitz syndrome.

* Corresponding author. Fax: +91 40 2716 0311.
E-mail address: amit@ccmb.res.in (A. Chattopadhyay).

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functions have their origin in the fact that the major input of brain cholesterol comes from the *in situ* synthesis and such synthesis is defective in this syndrome [9]. SLOS is caused by mutations in 3β-hydroxy-steroid-Δ^7-reductase (7-DHCR), an enzyme required in the final step of cholesterol biosynthesis. Elevated plasma levels of 7- and 8-dehydrocholesterol and the ratio of their contents to that of cholesterol are representative parameters for diagnosis of SLOS. The effect of alteration in the cholesterol content of neuronal membranes on membrane dynamics and protein/receptor function therefore represents an important determinant in the analysis of neurogenesis and several neuropathologies.

We have previously shown the requirement of membrane cholesterol in maintaining ligand binding activity of the hippocampal serotonin$_{1A}$ receptor [10,11]. In order to further examine the stringency of cholesterol requirement, we have tested whether 7-DHC, an immediate biosynthetic precursor of cholesterol differing only in its unsaturation at 7th position in the sterol ring (see Fig. 1), can support the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor.

Materials and methods

Materials. Cholesterol, 7-DHC, MβCD, DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BCA reagent for protein estimation was from Pierce (Rockford, IL, USA). [^{3}H]8-OH-DPAT (sp. activity 135 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA, USA). GF/B glass microfiber filters were from Whatman International (Kent, UK). All solvents used were of analytical grade. Pre-coated silica gel 60 thin-layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −70 °C till further use.

Preparation of native hippocampal membranes. Native hippocampal membranes were prepared as described earlier [10]. Protein concentration was determined using the BCA reagent with bovine serum albumin as a standard [12].

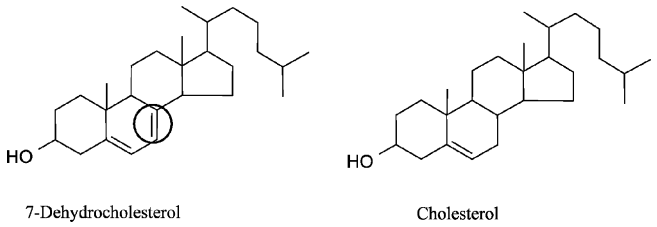

Fig. 1. Chemical structures of cholesterol and 7-dehydrocholesterol. The principal route of cholesterol synthesis in humans is the Kandutsch-Russell pathway [20]. In this pathway, the immediate precursor of cholesterol is 7-dehydrocholesterol which differs only in its unsaturation at 7th position in the sterol ring (highlighted in its chemical structure). Elevated levels of 7-dehydrocholesterol have been characterized as a diagnostic parameter of the Smith-Lemli-Opitz syndrome. See text for more details.

Radioligand binding assays. Receptor binding assays were carried out as described earlier [10] using 0.5 mg total protein. Briefly, tubes in duplicate with 0.5 mg protein in a total 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 agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at 25 °C. Nonspecific binding was determined by performing the assay in the presence of 10 μM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0 μm pore size), which were presoaked in 0.15% polyethylenimine for 1 h. Filters were then washed three times with 3 ml of cold water (4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

Cholesterol depletion of native membranes. Native hippocampal membranes were depleted of cholesterol using MβCD as described previously [10]. Briefly, membranes resuspended at a protein concentration of 2 mg/ml were treated with 40 mM MβCD in 50 mM Tris, pH 7.4, buffer at 25 °C with constant shaking for 1 h. Membranes were then spun down at 50,000*g* for 5 min at 4 °C, washed with 50 mM Tris, pH 7.4, buffer and resuspended in the same buffer.

7-Dehydrocholesterol and cholesterol replenishment of cholesterol-depleted membranes. Cholesterol-depleted hippocampal membranes were replenished with 7-dehydrocholesterol (7-DHC) or cholesterol using either 7-DHC-MβCD or cholesterol-MβCD complex which are soluble in water. The complex was prepared by dissolving the required amounts of 7-DHC or cholesterol and MβCD in a ratio of 1:10 (mol/mol) in 50 mM Tris, pH 7.4, buffer by constant shaking at 25 °C. Stock solutions (typically 2 mM 7-DHC (or cholesterol):20 mM MβCD) of this complex were freshly prepared before each experiment. 7-DHC and cholesterol replenishment were carried out at a protein concentration of 2 mg/ml by incubating the cholesterol-depleted membranes with 1 mM 7-DHC (or cholesterol): 10 mM MβCD complex for 1 h in 50 mM Tris, pH 7.4, buffer at 25 °C under constant shaking. Membranes were then spun down at 50,000*g* for 5 min at 4 °C, washed with 50 mM Tris, pH 7.4, buffer and resuspended in the same buffer.

Estimation of 7-DHC and cholesterol by thin-layer chromatography. Lipid extraction from native, cholesterol-depleted, and membranes replenished with 7-DHC or cholesterol after cholesterol depletion using 40 mM MβCD was carried out according to Bligh and Dyer [13]. The lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were resuspended in a mixture of chloroform/methanol (1:1, v/v). 7-DHC and cholesterol were resolved by thin-layer chromatography (TLC). TLC plates were impregnated with a 3% (w/v) silver nitrate solution in 97% methanol, allowed to dry briefly and activated at 120 °C for 15 min. Total lipid extracts were separated using *n*-heptane/ethylacetate (2:1, v/v) as the solvent system [14]. The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at 150 °C. 7-DHC and cholesterol were used as standards to identify 7-DHC and cholesterol bands on the thin-layer chromatogram run with lipid extracts from native, cholesterol-depleted, and 7-DHC or cholesterol replenished hippocampal membranes. The TLC plates were scanned and lipid band intensities were analyzed using the Adobe Photoshop software version 5.0 (Adobe Systems, San Jose, CA, USA). Intensities of the sterols (7-DHC and cholesterol) from all samples on the TLC plate were normalized to the intensity of the cholesterol band obtained from the native membrane.

Estimation of inorganic phosphate. Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [15] 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.

Fluorescence anisotropy measurements. Fluorescence anisotropy measurements were carried out with fluorescent membrane probe DPH with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (23 °C) as described earlier [16]. 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