Differential effects of cholesterol and desmosterol on the ligand binding function of the hippocampal serotonin1A receptor: Implications in desmosterolosis

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Abstract

Cholesterol is a unique molecule in terms of high level of in-built stringency, fine tuned by natural evolution for its ability to optimize physical properties of higher eukaryotic cell membranes in relation to biological functions. We previously demonstrated the requirement of membrane cholesterol in maintaining the ligand binding activity of the hippocampal serotonin1A receptor. In order to test the molecular stringency of the requirement of cholesterol, we depleted cholesterol from native hippocampal membranes followed by replenishment with desmosterol. Desmosterol is an immediate biosynthetic precursor of cholesterol in the Bloch pathway differing only in a double bond at the 24th position in the alkyl side chain. Our results show that replenishment with desmosterol does not restore ligand binding activity of the serotonin1A receptor although replenishment with cholesterol led to significant recovery of ligand binding. This is in spite of similar membrane organization (order) in these membranes, as monitored by fluorescence anisotropy measurements. The requirement for restoration of ligand binding activity therefore appears to be 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 in diseases such as desmosterolosis.

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1. Introduction

G-protein coupled receptors (GPCRs) are prototypical members of the family of seven transmembrane domain proteins involved in signal transduction across the plasma membrane [1]. GPCRs constitute one of the largest family of proteins in mammals and account for 1–2% of the total proteins coded by the human genome [2]. The serotonin1A (5-HT1A) receptor is an important GPCR and is implicated in the generation and modulation of various cognitive, behavioral and developmental functions [3,4]. The serotonin1A receptor agonists [5] and antagonists [6] represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mice lacking the serotonin1A receptor exhibit enhanced anxiety-related behavior [7] and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders and aggression [8].

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting [9]. It is often found distributed non-randomly in domains in biological and model membranes and has a wide variety of effects on the physical properties of membranes [10,11]. Cholesterol is the end product of the long and multistep sterol biosynthetic pathway. Konrad Bloch speculated that the sterol biosynthetic pathway parallels sterol evolution (the “Bloch hypothesis”). According to the Bloch hypothesis, cholesterol has been 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 [12]. Defects in the cholesterol biosynthetic pathway have been identified with several inherited metabolic disorders such as desmosterolosis [13–17]. Desmosterolosis is an autosomal, recessive congenital disease and is characterized by multiple anomalies. It is caused by mutations in 3β-hydroxy-steroid-Δ24-reductase (DHCR24), an enzyme required in the final step of the Bloch pathway of cholesterol biosynthesis. Desmosterolosis is clinically diagnosed with elevated levels of desmosterol and reduced levels of cholesterol in plasma, cells and tissues. The disease is characterized by distinct facial anomalies, underdeveloped genital organs and abnormalities in brain development and function, leading to serious developmental and neurological dysfunctions. Desmosterol, an immediate biosynthetic precursor of cholesterol in the Bloch pathway of cholesterol biosynthesis, differs with cholesterol only in a double bond at the 24th position in the flexible alkyl side chain (see Fig. 1). Desmosterol is converted to cholesterol in the final step of the Bloch pathway by the enzyme DHCR24. Importantly, it has been recently demonstrated that...
2.1. Materials and methods

2.1.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [21]. Bovine hippocampal tissue (−50 g) was homogenized as 10% (w/v) in a polycoton homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900×g for 10 min at 4 °C. The supernatant was filtered through four layers of cheesecloth and centrifuged at 50,000×g for 20 min at 4 °C. The pellet obtained was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000×g for 20 min at 4 °C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was resuspended in the same buffer.

2.2. Methods

2.2.1. Radioligand binding assays

Receptor binding assays were carried out as described earlier [19] with some modifications. Tubes in duplicate with 0.5 mg protein in a total volume of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, 10 mM MgCl₂, 5 mM MnCl₂, pH 7.4) were incubated with the radiolabeled agonist [³H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at 25 °C. Non-specific 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 (10 μm pore size), which were pre-soaked in 0.15% polyethyleneimine for 1 h [23]. 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.

2.2.2. Cholesterol depletion of native membranes

Native hippocampal membranes were depleted of cholesterol using Mj/CD as described previously [19]. Briefly, membranes resuspended at a protein concentration of 2 mg/ml were treated with 40 mM Mj/CD in buffer C at 25 °C with constant shaking for 1 h. Membranes were then spun down at 50,000×g for 10 min, washed once with buffer C and resuspended in the same buffer.

2.2.3. Desmosterol and cholesterol replenishment of cholesterol-depleted membranes

Cholesterol-depleted hippocampal membranes were replenished with desmosterol or cholesterol using either desmosterol–Mj/CD or cholesterol–Mj/CD complex which is soluble in water. The sterol–Mj/CD complex was prepared as described previously [24]. The complex was prepared by dissolving the required amounts of desmosterol or cholesterol and Mj/CD in a ratio of 1:10 (mol/mol) in buffer C under constant shaking at 25 °C. Stock solutions (typically 2 mM desmosterol (or cholesterol):20 mM Mj/CD) of this complex were freshly prepared before each experiment. Desmosterol and cholesterol replenishments were carried out at a protein concentration of 2 mg/ml by incubating the cholesterol-depleted membranes with 1 mM desmosterol (or cholesterol):10 mM Mj/CD complex for 1 h in buffer C at 25 °C under constant shaking. Membranes were then spun down at 50,000×g for 10 min at 4 °C, washed with buffer C and resuspended in the same buffer.

2.2.4. Estimation of desmosterol and cholesterol by thin layer chromatography

Total lipids were extracted from cholesterol-depleted and sterol-replenished membranes according to Bligh and Dyer [25]. The lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were then dissolved in a mixture of chloroform/methanol (1:1, v/v). Sterol contents of membranes under various treatment.
conditions were estimated by TLC. Precoated silica gel TLC plates were impregnated with a 3% (w/v) silver nitrate in methanol, allowed to dry briefly and activated at 120 °C for 15 min. Sterols were resolved using chloroform/diethyl ether (95:5, v/v) as the solvent system. In order to achieve maximum separation, TLC was run three times in the same solvent and the plate was dried after each run. 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. Desmosterol and cholesterol bands were identified with the help of standards (Fig. 2A). TLC plates were scanned and sterol band intensities were analyzed as described earlier [24].

2.2.6. Estimation of inorganic phosphate

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [26] using Na2HPO4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.7. Fluorescence anisotropy measurements

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH as described previously [27]. 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 bandwidths of 1.5 and 20 nm were used. The optical density of the samples measured at 358 nm was 0.15 ± 0.01. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [28]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (r) values were calculated from the following equation [29]:

\[ r = \frac{I_{VV} - G_{GH}}{I_{VV} + 2G_{HH}} \]

where \( I_{VV} \) and \( I_{HH} \) 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 \( I_{HH}/I_{HH} \). All experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 4.

2.2.8. 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

The cholesterol content in hippocampal membranes exhibits ~70% reduction upon treatment with MβCD (see Fig. 2B). This is accompanied by a corresponding reduction (~51%) in specific \([^{3}H]8\)-OH-DPAT binding (see Fig. 3). Replenishment with cholesterol resulted in recovery of specific \([^{3}H]8\)-OH-DPAT binding to ~70% of native membranes when the sterol (cholesterol) content was ~87% of control membranes (Fig. 2B). In order to examine whether replenishment with desmosterol could restore specific ligand binding, cholesterol-depleted membranes were replenished with desmosterol. Interestingly, Fig. 3 shows that specific \([^{3}H]8\)-OH-DPAT binding could not be restored significantly when replenishment was carried out with desmosterol. This is in spite of the fact that the extent of replenishment of desmosterol was comparable such that the total sterol (cholesterol + desmosterol) was ~85% (Fig. 2B). Therefore, we conclude that desmosterol is not capable of restoring specific ligand binding activity of the hippocampal serotonin1A receptor. That is, the total sterol content is similar in cholesterol- and desmosterol-replenished membranes (Fig. 2B). Importantly, the phospholipid content remains unaltered under these conditions (data not shown).

The above difference between cholesterol and desmosterol, in terms of their ability to restore specific ligand binding, could be due to alteration in overall membrane organization (order). In order to monitor the overall membrane order, fluorescence anisotropy measurements were carried out with the membrane probe, diphenyl-hexatriene (DPH). DPH is a rod-like molecule and partitions into the
interior of the bilayer. Fluorescence anisotropy is correlated to the rotational diffusion of membrane embedded probes such as DPH [29], which is sensitive to the packing of lipid acyl chains. Fig. 4 shows that the fluorescence anisotropy of DPH exhibits a significant reduction upon cholesterol depletion from native membranes. Interestingly, fluorescence anisotropy is restored to the same extent upon cholesterol and desmosterol replenishments. This points out that the differential effect of sterols on specific ligand binding is not due to a change in membrane order.

Previous work from our laboratory demonstrated the necessity of membrane cholesterol in maintaining the ligand binding function of the hippocampal serotonin1A receptor [19,30]. Interestingly, the function of serotonin1A receptors has subsequently been shown to depend on membrane cholesterol, see [32]). In this paper, we have replaced cholesterol with desmosterol to test the stringency of the requirement of membrane cholesterol in maintaining the function of the hippocampal serotonin1A receptor. Desmosterol differs with cholesterol only in a double bond at the 7th position.

Our present results are reminiscent of our earlier work in which we showed that 7-dehydrocholesterol (7-DHC, an immediate biosynthetic precursor of cholesterol in the Kandutsch–Russell pathway, differing with cholesterol only in a double bond at the 7th position) does not support the function of the serotonin1A receptor [24,37]. This is in support of the proposition that cholesterol represents a molecule with high level of in-built stringency, fine tuned by millions of years of natural evolution for its ability to optimize physical properties of eukaryotic cell membranes in relation to biological functions [12,34].

The molecular mechanism underlying the effect of cholesterol on the structure and function of integral membrane proteins is not always clear [20,38]. It has been proposed that such effects could occur either due to a specific molecular interaction with membrane proteins leading to a conformational change in the receptor [39,40], or due to alterations in the membrane physical properties induced by the presence of cholesterol [41,42], or due to a combination of both factors. We previously reported that oxidation of membrane cholesterol results in inhibition of the ligand binding function of the hippocampal serotonin1A receptor without any alteration in overall membrane order [43]. As mentioned earlier, we recently showed that 7-DHC, an immediate biosynthetic precursor of cholesterol with an extra double bond, does not support the function of the serotonin1A receptor, even when there is no change in overall membrane order [24,37]. Taken together, these results show that the requirement of membrane cholesterol in maintaining the ligand binding function of serotonin1A receptors could be attributed to specific interaction, although global bilayer effects may not be completely ruled out [44].

We have recently proposed that membrane cholesterol could occupy “nonanular” binding sites in GPCRs [32,45]. Nonanular sites are characterized by lack of accessibility to the annular lipids, i.e., lipid in these sites cannot be displaced by competition with annular lipids [46]. The binding to the nonanular sites is believed to be more specific compared to binding to annular sites [47]. The possible locations for the nonanular sites have been postulated to be either inter- or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface [46,48]. It is therefore possible that the inability of desmosterol to support the ligand binding activity of the serotonin1A receptor could be due to its relative inability in comparison to cholesterol to be accommodated at the nonanular sites. It is not possible at this point in time to predict the relative ease of accommodating closely related sterols to the nonanular binding sites of membrane receptors. A combination of X-ray crystallographic structures and molecular modeling could provide useful insight in addressing the issue of lipid shape and binding to nonanular sites. In summary, our results have potential implications in understanding the interaction of membrane cholesterol with the serotonin1A

Fig. 3. Effect of replenishment of cholesterol and desmosterol into cholesterol-depleted membranes on specific binding of [3H]8-OH-DPAT to the hippocampal serotonin1A receptor. Native membranes were treated with M/CD and were replenished with either cholesterol or desmosterol. Values are expressed as percentages of specific binding obtained in native membranes. Data shown are means±S.E. of five independent experiments *correspond to significant (p=0.001) and * represents not significant (p=0.127) difference in specific [3H]8-OH-DPAT binding of cholesterol- and desmosterol replenished membranes, respectively, in comparison to M/CD treated membranes (cholesterol-depleted membranes). See Section 2 for more details.

Fig. 4. Effect of replenishment of cholesterol and desmosterol into cholesterol-depleted membranes on fluorescence anisotropy of the membrane probe DPH. Cholesterol depletion was achieved using M/CD. Fluorescence anisotropy experiments were performed with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (∼23 °C). The values represent means±S.E. of duplicate points from three independent experiments. See Section 2 for more details.
receptor, in general and under desmosterolosis-like condition in particular. Our future work will focus on generating desmosterolosis-like condition in a cellular model and monitoring serotonin_{1A} receptor signaling under such condition.

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