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

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Abstract

The serotonin$_{1A}$ receptor is an important member of the G-protein coupled receptor family, and is involved in the generation and modulation of a variety of cognitive, behavioral, and developmental functions. Solubilization of the hippocampal serotonin$_{1A}$ receptor by 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) is accompanied by loss of membrane cholesterol which results in a reduction in specific agonist binding activity. Replenishment of cholesterol to solubilized membranes restores the cholesterol content of the membrane and significantly enhances specific agonist binding activity. In order to test the stringency of the requirement of cholesterol in this process, we solubilized native hippocampal membranes followed by replenishment with 7-dehydrocholesterol (7-DHC). 7-DHC is an immediate biosynthetic precursor of cholesterol differing only in a double bond at the 7th position in its sterol ring. Our results show, for the first time, that replenishment of solubilized hippocampal membranes with 7-DHC does not restore ligand binding activity of the serotonin$_{1A}$ receptor, in spite of recovery of the overall membrane order. This observation shows that the requirement for restoration of ligand binding activity 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 sterols with this important neuronal receptor under pathogenic conditions such as the Smith–Lemli–Opitz syndrome.
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Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting [1]. Interestingly, the central nervous system which accounts for only 2% of the body mass contains ∼25% of free cholesterol present in the whole body [2]. 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 [3]. For example, in the Smith–Lemli–Opitz syndrome (SLOS), the marked abnormalities in brain development and function leading to serious neurological and mental dysfunctions have their origin in the fact that the major input of brain cholesterol comes from *in situ* synthesis and such synthesis is defective in this syndrome [4]. 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-dehydrocholesterol (7-DHC) and the ratio of 7-DHC to cholesterol contents are representative parameters for the diagnosis of SLOS. Alteration in the cholesterol content and/or presence of abnormal precursor sterols in neuronal membranes on receptor function therefore represents an important determinant in the analysis of neurogenesis and several neuropathologies.

Previous work from our laboratory has shown the requirement of membrane cholesterol in the function of an important neurotransmitter receptor, the serotonin$_{1A}$ receptor (recently reviewed in [5]). Serotonin receptors represent one of the largest, evolutionarily ancient, conserved family of seven transmembrane G-protein coupled receptors (GPCRs) [6,7]. The G-protein coupled serotonin$_{1A}$ (5-HT$_{1A}$) receptor is the best characterized among the 14 subtypes of serotonin receptors [8]. Serotonin$_{1A}$ receptors appear to play a key role in the generation and modulation of various cognitive, behavioral, and developmental functions such as sleep, mood, addiction, depression, anxiety, alcohol abuse, aggression, and learning [9]. This is supported by the fact that agonists and antagonists of the serotonin$_{1A}$ receptor represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for the analysis of complex traits such as anxiety disorders and aggression in higher animals [10,11]. Keeping in mind the pharmacological relevance of the serotonin$_{1A}$ receptor, which is a transmembrane protein, its interaction with the surrounding lipid environment assumes greater significance in modulating its function in healthy and diseased states. Such lipid–protein interactions can be suitably monitored if the membrane protein in question is purified. An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from its native membrane and dispersed in solution. This process, usually achieved using amphiphilic detergents, is termed as solubilization [12,13]. We have earlier partially purified and solubilized the hippocampal serotonin$_{1A}$ receptor in a functionally active form [14] using CHAPS, a mild, non-denaturing, and zwitterionic detergent.

A common problem associated with most solubilization experiments is delipidation, i.e., loss of lipids. This often leads to considerable loss of activity of the solubilized protein or receptor since lipid–protein interactions play a crucial role in maintaining the structure and function of integral membrane proteins and receptors [15]. It has been previously reported that solubilization of the serotonin$_{1A}$ receptor by CHAPS leads to reduction in membrane cholesterol and ligand binding activity [16,17]. Interestingly, we have recently reported that the ligand binding activity of the serotonin$_{1A}$ receptor could be restored upon replenishment of solubilized membranes with cholesterol [17]. In order to further examine the stringency of the requirement of membrane cholesterol in serotonin$_{1A}$ receptor function,

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 solubilized hippocampal serotonin$_{1A}$ receptor.

Materials and methods

Materials. 7-DHC, cholesterol, CHAPS, coprostanol, DMPC, DPH, MβCD, EDTA, EGTA, MgCl$_2$, MnCl$_2$, NaCl, Na$_2$HPO$_4$, iodoacetamide, PEG, PMSF, polyethylenimine, serotonin, sodium azide, sucrose, 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). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco (Bellefonte, PA, USA). Solvents used were of analytical grade. 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 [18]. Protein concentration was determined using the BCA reagent with bovine serum albumin as a standard [19].

Solubilization of native membranes. Native hippocampal membranes were solubilized as described earlier using CHAPS [17]. CHAPS-solubilized membrane was precipitated using PEG in order to remove NaCl from the solubilized extract, since the agonist binding of the serotonin$_{1A}$ receptor is inhibited by NaCl [20]. This procedure is also believed to remove the detergent. The PEG-precipitated CHAPS-solubilized membrane was suspended in 50 mM Tris, pH 7.4 buffer and used immediately either for radioligand binding assays or for sterol replenishment.

7-DHC and cholesterol replenishment of PEG-precipitated solubilized membranes. PEG-precipitated solubilized membranes were replenished with 7-dehydrocholesterol (7-DHC) or cholesterol using water soluble 7-DHC–MβCD or cholesterol–MβCD complex. The complex was prepared by dissolving 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 PEG-precipitated solubilized membranes with 1 mM 7-DHC (or cholesterol):10 mM MβCD complex for 30 min in 50 mM Tris, pH 7.4 buffer at 25 °C under constant shaking. Membranes were then spun down at 100,000*g* for 1 h at 4 °C, suspended in the same buffer, and immediately used for radioligand binding assays.

Gas chromatography and mass spectrometry. GC–MS analysis was carried out on an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a model 5973i mass selective detector and 7683 series injector. For membrane sterol measurements, total lipids were extracted in chloroform–methanol [21], after the addition of coprostanol as an internal recovery standard. The chloroform phase obtained was dried under nitrogen. Sterols were identified and quantitated using GC–MS as described previously [22] with some modifications. Briefly, an aliquot of the extract was hydrolyzed in 1 M NaOH in ethanol for 1 h at 70 °C, extracted with *n*-hexane, and converted into trimethylsilyl ether derivatives followed by injection into a capillary column. This was a chemically bonded, fused silica, non-polar CP-Sil 8CB (Varian, Middleburg, The Netherlands) capillary column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness). To achieve optimal separation of sterols, the column oven temperature was maintained at 250 °C with a solvent delay of 5 min from the time of injection. Helium at the rate of
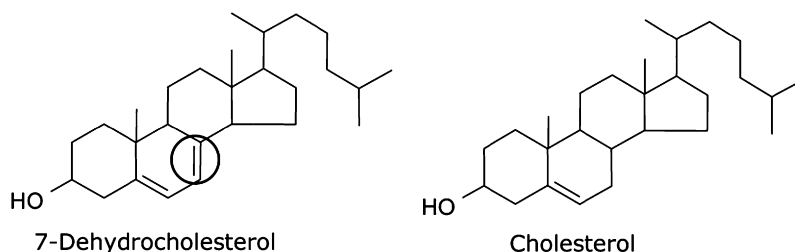
Fig. 1. Chemical structures of cholesterol and 7-dehydrocholesterol. The principal route of cholesterol synthesis in humans is the Kandutsch–Russell pathway [28]. In this pathway, the immediate precursor of cholesterol is 7-DHC which differs only in its unsaturation at 7th position in the sterol ring (highlighted in its chemical structure). Elevated levels of 7-DHC have been characterized as a diagnostic parameter of the Smith–Lemli–Opitz syndrome. See text for more details.

1.2 ml/min was used as the carrier gas under constant flow mode. The inlet and interface temperatures were kept at 250 and 280 °C, respectively. The ion source and quadrupole temperatures were kept at 230 and 150 °C, respectively. Mass spectra were scanned from 30 to 600 Da under scan mode. The ions at m/z 329, 351, and 370 were used under selected ion monitoring (SIM) mode for the estimation of cholesterol, 7-DHC, and coprostanol, respectively. Sterols were identified using authentic standards. Peak areas were obtained individually for each compound under SIM mode in the concentration range of 100–1200 ppm for both cholesterol and 7-DHC, 100–500 ppm for coprostanol, and the calibration curves were constructed. The absolute concentrations of various sterols in the samples were calculated from the calibration curves. The concentration of coprostanol is used to calculate the recovery of the sterols. Membrane sterol contents are expressed as µg of sterol/mg of protein.

Estimation of inorganic phosphate. Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [23] 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.

Radioligand binding assays. Receptor binding assays were carried out as described earlier [18]. 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 [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 (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.

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 [24]. 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 0.15 ± 0.01. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [25]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation [26]:

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

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 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. 4.

Results and discussion

Fig. 2 shows the change in sterol contents due to solubilization of native hippocampal membranes and subsequent replenishment of solubilized membranes with either 7-DHC or cholesterol. Sterols were separated, identified and quantitated using GC–MS. Fig. 2A shows a representative selected ion chromatogram of 7-DHC, cholesterol, and coprostanol (used as an internal recovery standard). These sterols were identified and quantitated using authentic standards. Fig. 2B shows that there is ∼50% reduction in the cholesterol content of hippocampal membranes upon solubilization. This is accompanied by a corresponding reduction (∼34%) in the specific [³H]8-OH-DPAT binding (see Fig. 3). Subsequent treatment of solubilized membranes with cholesterol–MβCD complex resulted in almost complete replenishment of cholesterol to the original cholesterol content (see Fig. 2B). This resulted in recovery of specific agonist binding to ∼94% of native membranes (Fig. 3). In order to monitor whether replenishment with 7-DHC could restore specific [³H]8-OH-DPAT binding, solubilized membranes were replenished with 7-DHC–MβCD complex. Interestingly, although the conditions of replenishment (sterol–MβCD complex, 1:10 (mol/mol)) were comparable with conditions used for cholesterol replenishment, specific agonist binding could not be restored (Fig. 3), in spite of the fact that the extent of loading of 7-DHC was even higher to what was obtained with cholesterol replenishment (Fig. 2B). This clearly shows the inability of 7-DHC in supporting specific agonist binding activity of the solubilized hippocampal serotonin$_{1A}$ receptor.

The observed difference between 7-DHC and cholesterol, in terms of being able to restore the ligand binding activity of the solubilized serotonin$_{1A}$ receptor (Fig. 3), could be due to an alteration in overall membrane organization (order). In order to monitor whether there is a change in overall membrane order, fluorescence anisotropy measurements were carried out with the membrane probe, DPH. Fluorescence anisotropy measured using probes
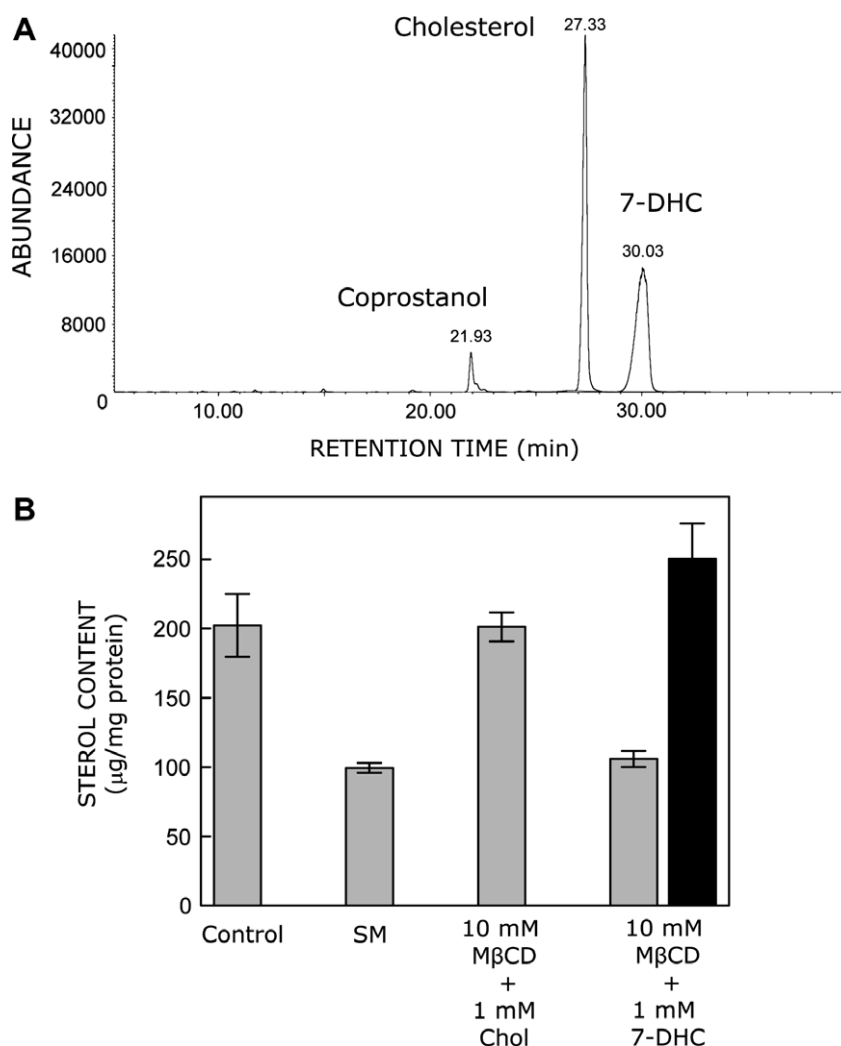
Fig. 2. Quantitation of sterols in native hippocampal membranes (control), PEG-precipitated CHAPS-solubilized hippocampal membranes (SM), and solubilized membranes replenished with either 7-DHC or cholesterol. PEG-precipitated solubilized membranes were replenished with 7-DHC or cholesterol, using 1 mM 7-DHC (or cholesterol):10 mM MβCD complex. Sterols were separated and quantitated using GC–MS. 7-DHC, cholesterol, and coprostanol were identified using authentic standards. (A) selected ion chromatogram for coprostanol, cholesterol and 7-DHC obtained from 7-DHC loaded solubilized membranes. (B) The contents of membrane cholesterol (gray bars), 7-DHC (black bar) from native (control), solubilized membranes (SM), and solubilized membranes replenished with either 7-DHC or cholesterol. The sterol contents are normalized to protein and are represented as μg/mg of protein. Data represent means ± SE of at least three independent experiments. See Materials and methods for other details.

such as DPH is correlated to the rotational diffusion of membrane embedded probes [26], which is sensitive to the packing of lipid chains. Fig. 4 shows that the fluorescence anisotropy of DPH exhibits a significant reduction upon solubilization, possibly due to loss of cholesterol from native membranes. Interestingly, when solubilized membranes were replenished with either 7-DHC or cholesterol, the fluorescence anisotropy was found to increase and was similar to that of native (control) membranes (within 10%). This indicates that the overall membrane order is recovered upon sterol replenishment. This observation brings out the important point that the requirement for restoration of ligand binding activity is more stringent than the requirement for the recovery of overall membrane order.

We have earlier reported that membrane cholesterol is required in maintaining ligand binding activity of the solu-bilized hippocampal serotonin$_{1A}$ receptor [17]. In order to examine the stringency of the requirement of membrane cholesterol in maintaining the function of the solubilized serotonin$_{1A}$ receptor, we have replaced cholesterol with 7-DHC in this work. 7-DHC differs with cholesterol only in a double bond at the 7th position in the sterol ring (highlighted in Fig. 1). Our results show that 7-DHC does not support the ligand binding activity of the solubilized serotonin$_{1A}$ receptor. This is in spite of the fact that replenishment of the solubilized membrane with 7-DHC restores overall membrane order to that of native membranes. In this context, it is important to mention here that we have previously shown that membrane cholesterol oxidation leads to inhibition of the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor without any change in membrane order [27]. This previous result along with our present results, demonstrate that the molecular basis for
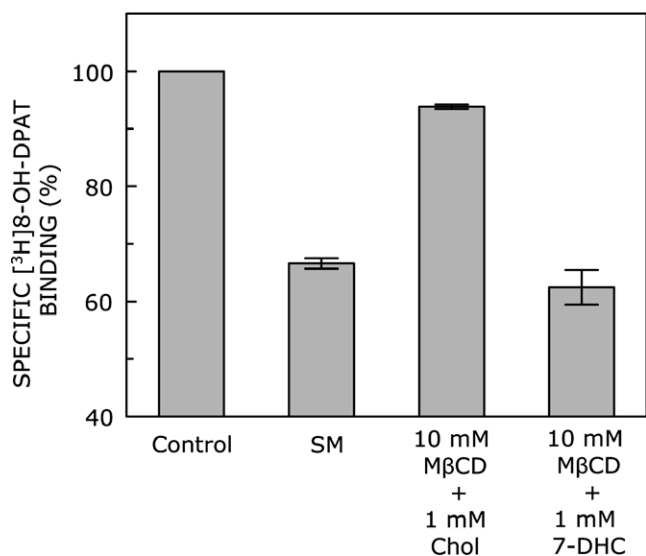
Fig. 3. Effect of replenishment of 7-DHC or cholesterol into PEG-precipitated CHAPS-solubilized membranes on specific binding of the [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor. Solubilized membranes were replenished with 7-DHC or cholesterol, using 1 mM 7-DHC (or cholesterol):10 mM MβCD complex. Values are expressed as percentages of specific binding obtained in native membranes. Data shown are means ± SE from at least five independent experiments. See Materials and methods for other details.
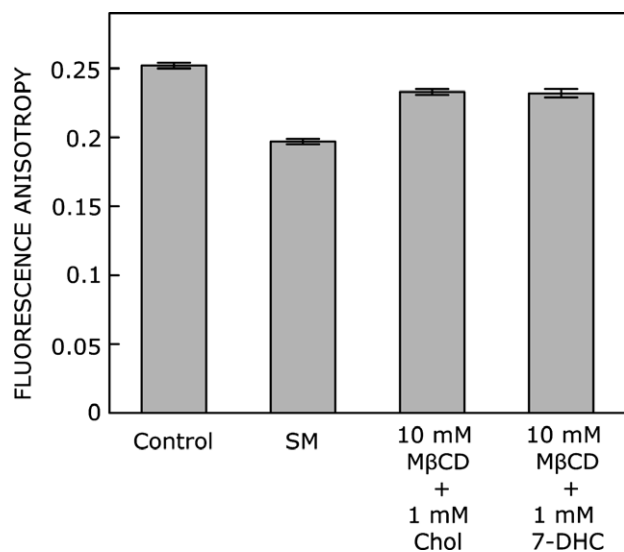

Fig. 4. Effect of replenishment of 7-DHC or cholesterol into PEG-precipitated CHAPS-solubilized membranes on fluorescence anisotropy of the membrane probe DPH. Solubilized membranes were replenished with 7-DHC or cholesterol, using 1 mM 7-DHC (or cholesterol):10 mM MβCD complex. Fluorescence anisotropy 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 ± SE of duplicate points from at least four independent experiments. See Materials and methods for other details.

the requirement of membrane cholesterol in maintaining the ligand binding activity of serotonin$_{1A}$ receptors could be specific interaction, although global bilayer effects may not be ruled out. In addition, our results have potential

implications in understanding the interaction of membrane lipids with this important neuronal receptor under SLOS-like condition.

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