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Membrane cholesterol oxidation inhibits ligand binding function of hippocampal serotonin$_{1A}$ receptors ☆

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Abstract

We have monitored the ligand binding function of the bovine hippocampal 5-HT$_{1A}$ receptor following treatment of native membranes with cholesterol oxidase. Cholesterol oxidase is a water soluble enzyme that acts on the membrane interface to catalyze the conversion of cholesterol to cholestenone. Oxidation of membrane cholesterol significantly inhibits the specific binding of the agonist and antagonist to 5-HT$_{1A}$ receptors. Fluorescence polarization measurements of membrane probes incorporated at different locations in the membrane revealed no appreciable effect on membrane order due to the oxidation of cholesterol to cholestenone. These results therefore suggest that the ligand binding function of the 5-HT$_{1A}$ receptor is a cholesterol-dependent phenomenon that is not related to the ability of cholesterol to modulate membrane order. Importantly, these results represent the first report on the effect of a cholesterol-modifying agent on the ligand binding function of this important neurotransmitter receptor.

Keywords: 5-HT$_{1A}$ receptor; Hippocampus; Cholesterol; Cholesterol oxidase; Fluorescence polarization

Lipid–protein interactions play a crucial role in maintaining the structure and function of biological membranes [1]. A large portion of any transmembrane receptor remains in contact with the membrane lipid environment. This raises the possibility that the membrane and its constituent lipids could be important modulators of receptor structure, dynamics, and function. Cholesterol is an essential component of eukaryotic cell membranes and is often found distributed non-randomly in domains in biological and model membranes [2,3]. The effect of cholesterol on the structure and function of integral membrane proteins has been a subject of intense investigation [4]. It has been proposed that such effects could occur either due to a specific local molecular interaction with membrane proteins [5], or due to alterations in the membrane physical properties induced by the presence of cholesterol [1,6], or due to a combination of both factors. In view of the importance of cholesterol in the organization, dynamics, and function of eukaryotic membranes [2,7,8], the interaction of cholesterol with membrane proteins represents an important determinant in the functional studies of such proteins.

The brain is abundant in membranous structures and is enriched in cholesterol. It accounts for only 2% of the body mass, yet contains ~25% of free cholesterol present in the whole body [9,10]. Cholesterol in the brain is synthesized in situ with no evidence for the transfer of cholesterol from blood plasma to brain [9,10]. However, the

☆ *Abbreviations:* BCA, bicinchoninic acid; CO, cholesterol oxidase; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; 5-HT, 5-hydroxytryptamine; MβCD, methyl-β-cyclodextrin; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; *p*-MPPF, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; *p*-MPPI, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine; PMSF, phenylmethylsulfonyl fluoride; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; TLC, thin layer chromatography; Tris, *tris*- (hydroxymethyl)aminomethane.

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distribution and metabolism of brain cholesterol remains poorly understood [11]. The interaction between this exclusive pool of brain cholesterol and other molecular components (such as neuronal receptors) therefore assumes relevance for a comprehensive understanding of the function of such receptors in neuronal tissues.

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent [12], biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems [13]. Serotonergic signaling appears to be crucial to various cognitive and behavioral functions including sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression, and learning [14,15]. Serotonin exerts its diverse actions by binding to distinct membrane receptors which have been characterized based on their pharmacological responses to specific ligands, sequence similarities at the gene and amino acid levels, gene organization, and second messenger coupling pathways [16]. All serotonin receptors, except the 5-HT_3 receptor, belong to the superfamily of seven transmembrane domain G-protein coupled receptors [17]. These receptors couple to and transduce signals via guanine nucleotide binding regulatory proteins (G-proteins) [18]. Among the 14 subtypes of serotonin receptors, the G-protein coupled serotonin$_{1A}$ (5-HT_{1A}) receptor is the best characterized for a number of reasons [19] that include its relevance to neuronal physiology in health and disease [14,15].

We have recently shown the requirement of membrane cholesterol in ligand binding function of the 5-HT_{1A} receptor from bovine hippocampus [20]. This was achieved by the use of methyl-β-cyclodextrin (MβCD) which physically depletes cholesterol from membranes. Treatment of bovine hippocampal membranes with MβCD results in a reduction in ligand binding function of the 5-HT_{1A} receptor. In addition, cholesterol depletion of hippocampal membranes is associated with a reduction in membrane order as reported by fluorescence polarization of the membrane-phase insensitive fluorescent probe DPH and its derivative TMA-DPH [20]. We reasoned that if cholesterol is necessary for ligand binding function of the 5-HT_{1A} receptor, modulating the cholesterol content by other means could affect receptor function. In this paper, we tested this proposal by treating hippocampal membranes with cholesterol oxidase (CO) which catalyzes the oxidation of cholesterol resulting in the formation of cholestenone (cholest-4-en-3-one) [21]. The oxidation of cholesterol perturbs the functionally important hydroxyl group in the molecule thereby altering its potential to participate in specific molecular interactions with sphingomyelin or lipids with saturated fatty acyl chains [22–24]. In the process, the ability of cholesterol to form domains that exist in the liquid-ordered state has been reported to be affected [23,24]. Importantly, compared to physical depletion of cholesterol from membranes, cholesterol oxidation appears to be milder to the membrane, i.e., induces relatively less perturbation to membrane physical properties. The analysis of the ligand binding function of the 5-HT_{1A} receptor under conditions of cholesterol oxidation would therefore provide useful information regarding the specificity of the receptor/cholesterol interaction in a native-like, yet cholesterol-deficient, membrane environment. In order to monitor whether receptor function is affected by cholesterol oxidation, we report here the effect of treatment of hippocampal membranes with CO on the binding of the specific agonist 8-OH-DPAT and antagonist *p*-MPPF to 5-HT_{1A} receptors in these membranes.

Materials and methods

Materials. BCA, cholesterol, cholesterol oxidase (EC 1.1.3.6 from *Cellulomonas* sp.), DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, *p*-MPPI, PMSF, 5-HT, sucrose, polyethylenimine, sodium azide, TMA-DPH, and Tris were obtained from Sigma Chemical (St. Louis, MO, USA). DOPC was obtained from Avanti Polar Lipids (Alabaster, AL, USA). BCA reagent kit for protein estimation was from Pierce (Rockford, IL, USA). The radiolabeled agonist [^{3}H]8-OH-DPAT (sp. activity = 123.0 Ci/mmol) and antagonist [^{3}H]*p*-MPPF (sp. activity = 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). All other chemicals were of the highest purity available. GF/B glass microfiber filters were from Whatman International (Kent, UK). Pre-coated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). The solvents used were of analytical grade and 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 until further use.

Preparation of native hippocampal membranes. Native hippocampal membranes were prepared as described earlier [20]. Bovine hippocampal tissue (~100 g) was homogenized as 10%(w/v) with a polytron 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, and 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 the pellet was discarded. The supernatant was further centrifuged at 50,000*g* for 20 min at 4 °C. The resulting pellet was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, and 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 membrane) was resuspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash-frozen in liquid nitrogen, and stored at −70 °C until further use. Protein concentration was determined using the BCA reagent with bovine serum albumin as standard [25].

Treatment of native hippocampal membranes with cholesterol oxidase. Native membranes were resuspended in buffer C at a protein concentration of 2 mg/ml and treated with cholesterol oxidase (CO) (aliquoted from a stock solution of 200 U/ml prepared in 10 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 10 min at 4 °C and resuspended in the same buffer.

Radioligand binding assays. Radioligand binding assays were performed as described earlier [20,26]. Briefly, tubes in duplicate

containing 0.5 mg total protein in a volume of 1 ml buffer D (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, and 5 mM $MnCl_2$, pH 7.4) for agonist binding or in 1 ml buffer E (50 mM Tris, 1 mM EDTA, pH 7.4) for antagonist binding assays were used. Tubes were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) or antagonist [^{3}H]*p*-MPPF (final concentration in assay tube being 0.5 nM) for 1 h at 25 °C. Non-specific binding was determined by performing the assay either in the presence of 10 μM 5-HT (for agonist binding assays) or in the presence of 10 μM *p*-MPPI (for antagonist binding assays). 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%(w/v) polyethylenimine for 1 h [27]. The filters were then washed three times with 3 ml cold water (4 °C), dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml scintillation fluid.

Estimation of inorganic phosphate. The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [28] 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.

Thin layer chromatography of lipid extracts. Lipid extracts from native and CO-treated membranes were prepared according to Bligh and Dyer [29]. The extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were then resuspended in a mixture of chloroform/methanol (1:1, v/v). As described earlier [30], cholesterol and cholestenone were resolved by thin layer chromatography of lipid extracts using chloroform/methanol (100:2, v/v) as the solvent system. The separated lipids were visualized on the TLC plate by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at 150 °C for ~5 min. As a control, small unilamellar sonicated vesicles composed of DOPC and cholesterol (1:1, mol/mol) in buffer C were treated overnight with CO at 37 °C. Importantly, thin layer chromatography of lipid extracts prepared from these vesicles did not suggest any effect of the enzyme treatment on phospholipids. Moreover, these samples acted as standards to identify cholesterol and cholestenone on the thin layer chromatogram run with lipid extracts from native and CO-treated hippocampal membranes. The TLC plates were scanned and lipid spot intensities were analyzed using the Adobe Photoshop software version 5.0 (Adobe Systems, San Jose, CA, USA). Intensities of the cholesterol and cholestenone spots on the TLC were normalized to the intensity of the phospholipid spot obtained from the same sample to correct for any inadvertent errors during lipid extraction and loading the extracts on TLC plates.

Fluorescence polarization measurements. Fluorescence polarization experiments were carried out with membranes containing 50 nmol of total phospholipids suspended in 1.5 ml buffer C as described earlier [20]. Stock solutions of the fluorescent probes (DPH and TMA-DPH) were prepared in methanol. The final probe concentration was 1 mol% with respect to the total phospholipid content. This ensures optimal fluorescence intensity with negligible membrane perturbation. Membranes were vortexed for 1 min after addition of the probe and kept in the dark for 1 h before measurements at room temperature. Background samples were prepared the same way except that the probe was omitted. The final probe concentration was 0.33 μM in all cases and the methanol content was low (0.03%, v/v). 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 bandpasses of 1.5 and 20 nm were used. The excitation slit was kept low to avoid any photoisomerization of DPH. In addition, fluorescence was measured with a 30 s interval between successive openings of the excitation shutter (when the sample was in the dark in the fluorimeter) to reverse any photoisomerization of DPH and TMA-DPH [31]. The optical density of the samples measured at 358 nm was ~0.15. Scattering artifacts, if any, in these measurements were checked by dilution of the membrane samples with buffer C [32]. Fluorescence polarization values did not show significant differences upon dilution indicating the absence of scattering artifacts in these measurements. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation [33]:

$$P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$$

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}.

Results

Cholesterol oxidase (CO) is a water soluble enzyme that acts at the membrane interface to catalyze the oxidation of cholesterol to cholestenone (cholest-4-en-3-one) [21]. The reaction catalyzed by CO appears to be influenced by the cholesterol organization in the membrane [34], lipid composition [22,35], and physical state [36] of the membrane. Fig. 1 shows that treatment of hippocampal membranes with CO results in the production of cholestenone in the membrane. Interestingly, the extent of cholesterol oxidation does not appear to be linearly proportional to the concentration of CO in the reaction. Thus, treatment of native membranes with 2 U/ml CO for 1 h results in the oxidation of ~5% of cholesterol whereas the use of 4 U/ml of the enzyme for the same duration catalyzes oxidation of ~28% of cholesterol. The phospholipid content of these samples remained unaltered during this treatment (data not shown).

We monitored the effect of cholesterol oxidation on the ligand binding function of 5-HT_{1A} receptors in hippocampal membranes. The agonist 8-OH-DPAT and antagonist *p*-MPPF have earlier been reported to specifically bind to the hippocampal 5-HT_{1A} receptor with high affinity [20,26]. The effect of CO treatment of hippocampal membranes on the ligand binding function of 5-HT_{1A} receptors is shown in Fig. 2. The oxidation of ~28% of membrane cholesterol (achieved using 4 U/ml CO, see Fig. 1) inhibits the specific radiolabeled agonist [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors by ~25% (Fig. 2). Interestingly, the specific radiolabeled antagonist [^{3}H]*p*-MPPF binding appears to be more sensitive to the oxidation of membrane cholesterol. Thus, under similar conditions (using 4 U/ml CO), the specific antagonist [^{3}H]*p*-MPPF binding reduces by ~46% (Fig. 2). To the best of our knowledge, this result represents the first observation of inhibition in specific ligand binding to the 5-HT_{1A} receptor due to oxidation of membrane cholesterol. We have earlier reported that the physical depletion of cholesterol from hippocampal membranes using MβCD reduces the ligand binding function of the 5-HT_{1A} receptor [20]. The present results show that a mere

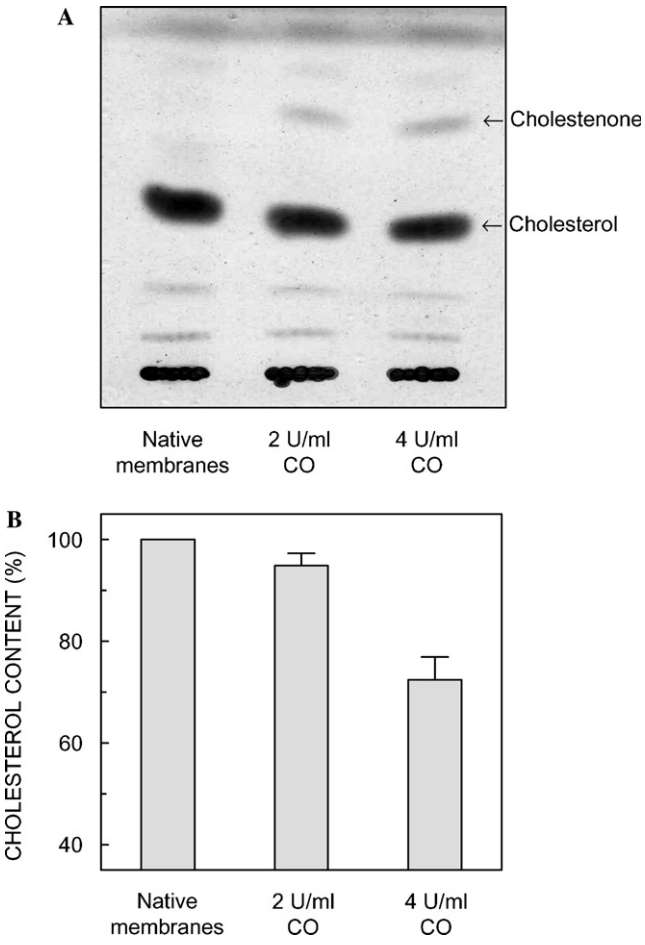

Fig. 1. Estimation of cholesterol content of hippocampal membranes treated with cholesterol oxidase (CO). Total lipids extracted from CO-treated membranes were separated by thin layer chromatography as shown in (A). The amount of cholesterol in lipid extracts was determined by a densitometric analysis of the thin layer chromatogram and is shown in (B). Values are expressed as percentages of the cholesterol content in native membranes in the absence of any treatment. Data represent means ± SE of four independent experiments. See Materials and methods for other details.

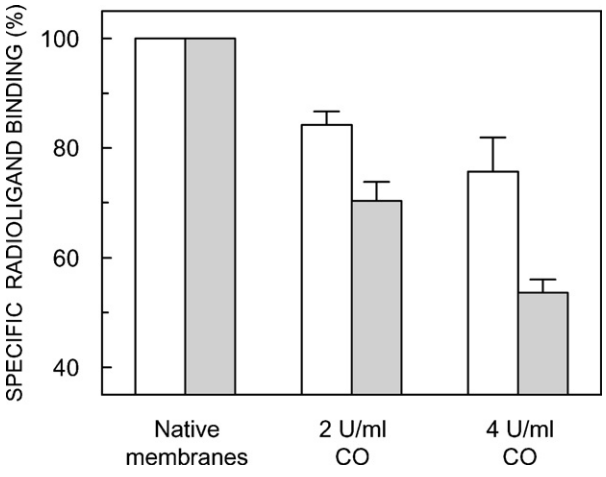

Fig. 2. Effect of treatment of hippocampal membranes with increasing concentrations of CO on the specific binding of the agonist [^{3}H]8-OH-DPAT (white bars) and antagonist [^{3}H]*p*-MPPF (gray bars) to the 5-HT_{1A} receptor. Values are expressed as percentages of the specific binding obtained in control (native) membranes. The data shown are means ± SE of duplicate points from three independent experiments. See Materials and methods for other details.

reduction in the functionality of cholesterol in hippocampal membranes by its conversion to cholestenone is sufficient to inhibit the ligand binding function of hippocampal 5-HT_{1A} receptors. These results thus further refine the role of membrane cholesterol in modulating the ligand binding function of 5-HT_{1A} receptors.

Treatment of hippocampal membranes with CO merely results in the oxidation of the hydroxyl group of cholesterol to a keto group. Since the bulk of the cholesterol molecule would still be present in the membrane, alteration in the membrane physical properties due to the formation of cholestenone would be considerably less in magnitude compared to physical depletion with agents such as MβCD. In order to monitor any change in the overall order of the membrane due to cholesterol oxidation, we measured the steady state fluorescence polarization of two membrane probes, DPH and TMA-DPH, in CO-treated membranes. DPH and its derivatives represent popular membrane probes for monitoring organization and dynamics in membranes [37]. Fluorescence polarization is correlated to the rotational diffusion [33] of membrane embedded probes which is sensitive to the packing of fatty acyl chains and cholesterol. Since the membrane is considered to be a two-dimensional anisotropic fluid, any possible change in membrane order may not be uniform or restricted to a unique location in the membrane. It is therefore important to monitor the change in membrane order at multiple regions in the membrane to obtain a comprehensive understanding of any change in membrane (lipid) dynamics. DPH and TMA-DPH differ in their orientation and location in the membrane. While DPH is known to partition into the hydrophobic core of the membrane [38], the amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid–water interface [39]. The effect of cholesterol oxidation on the fluorescence polarization of membrane embedded DPH and TMA-DPH probes is shown in Fig. 3. The higher polarization of TMA-DPH (0.367) compared to that of DPH (0.333) in native membranes is indicative of a shallower location of TMA-DPH and hence a greater restriction on its rotation mobility as has been observed earlier [20]. Importantly, the treatment of hippocampal membranes with CO appears to affect the fluorescence polarization of both probes to a minimal extent, even when ~28% of membrane cholesterol is oxidized (see Figs. 1 and 3). Previous reports on the effect of CO treatment on the physical properties of natural membranes as monitored using fluorescence polarization of DPH have indicated a reduction in membrane order due to the formation of cholestenone [40,41]. However, a significant reduction

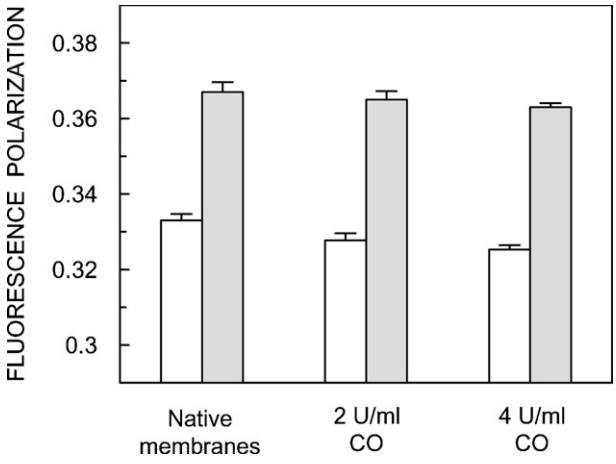

Fig. 3. Effect of treatment of hippocampal membranes with increasing concentrations of CO on fluorescence polarization of membrane embedded probes DPH (white bars) and TMA-DPH (gray bars). Fluorescence polarization experiments were carried out with membranes containing 50 nmol total phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23 °C). The data shown are means ± SE of at least seven independent experiments. See Materials and methods for other details.

in membrane order was observed only upon oxidation of a large fraction (>35%) of membrane cholesterol. Since the extent of oxidation in the present case never exceeded ~28% (see Fig. 1), our results are consistent with these previous reports. Importantly, the oxidation of this relatively small fraction of cholesterol significantly affects the ligand binding function of the 5-HT_{1A} receptor. Taken together, the reduction in the ligand binding function of the 5-HT_{1A} receptor by cholesterol oxidation is not correlated with an alteration in the membrane order.

Discussion

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting [2,7,8]. It is often found distributed non-randomly in domains or pools in biological and model membranes [3,11]. Interestingly, the restricted ability of CO to oxidize cholesterol in natural membranes has been correlated to the presence of domains or pools of cholesterol in such membranes [42]. The integrity of some of these domains is maintained by the presence of cholesterol [43]. Such domains in model membranes have previously been shown to be disrupted by the oxidation of cholesterol [23,24]. The membrane organization of G-protein coupled receptors, such as 5-HT_{1A} receptors, in relation to these domains, assumes significance from the lipid–protein interaction perspective [1] and in light of the role played by the 5-HT_{1A} receptor in health and disease [44]. Interestingly, several clinical studies have established a correlation between symptoms arising due to alterations in the serum cholesterol content to those involving a disruption in the serotonergic signaling pathway [45]. Recent evidence indicates that a spatiotemporally organized system rather than a freely diffusible system of receptors and G-proteins in the membrane is responsible for rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [46]. It has been proposed that G-protein coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains, some of which are presumably enriched in cholesterol [47]. In this regard, the analysis of membrane protein function under conditions that affect membrane cholesterol content, availability, and distribution assumes greater significance.

This report represents one of the first studies on the effect of enzymatic oxidation of cholesterol on the ligand binding function of this important neurotransmitter receptor. Our present results indicate that a reduction in the functionality of cholesterol by its oxidation to cholestenone is sufficient to inhibit the 5-HT_{1A} receptor ligand binding function. In combination with our earlier results on the effect of physical depletion of cholesterol by MβCD on the 5-HT_{1A} receptor ligand binding function [20], these results further refine the role of membrane cholesterol in modulating ligand binding to this receptor. Importantly, the present results suggest that the ligand binding function of the 5-HT_{1A} receptor is a cholesterol-dependent phenomenon that is not related to the ability of cholesterol to modulate membrane order as monitored by fluorescence polarization of membrane embedded probes. In summary, these results are relevant in the overall context of the influence of the membrane lipid environment on the function of the 5-HT_{1A} receptor in particular, and other G-protein coupled transmembrane receptors in general.

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