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The cholesterol-complexing agent digitonin modulates ligand binding of the bovine hippocampal serotonin$_{1A}$ receptor

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Abstract
The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important member of the superfamily of seven transmembrane domain G-protein-coupled receptors. We have examined the modulatory role of cholesterol on the ligand binding of the bovine hippocampal 5-HT$_{1A}$ receptor by cholesterol complexation in native membranes using digitonin. Complexation of cholesterol from bovine hippocampal membranes using digitonin results in a concentration-dependent reduction in specific binding of the agonist 8-OH-DPAT and antagonist *p*-MPPF to 5-HT$_{1A}$ receptors. The corresponding changes in membrane order were monitored by analysis of fluorescence polarization data of the membrane depth-specific probes, DPH and TMA-DPH. Taken together, our results point out the important role of membrane cholesterol in maintaining the function of the 5-HT$_{1A}$ receptor. An important aspect of these results is that non-availability of free cholesterol in the membrane due to complexation with digitonin rather than physical depletion is sufficient to significantly reduce the 5-HT$_{1A}$ receptor function. These results provide a comprehensive understanding of the effects of the sterol-complexing agent digitonin in particular, and the role of membrane cholesterol in general, on the 5-HT$_{1A}$ receptor function.

Keywords: *5-HT$_{1A}$ receptor, cholesterol, digitonin, fluorescence polarization*

Abbreviations: *BCA, bicinchoninic acid, DMPC, dimyristoyl-sn-glycero-3-phosphocholine, DPH, 1,6-diphenyl-1,3,5-hexatriene, 5-HT, 5-hydroxytryptamine, 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.*

Introduction

G-protein-coupled receptors constitute a superfamily of transmembrane proteins whose function is to transmit information across the cell membrane from the extracellular environment to the interior of the cells, thus providing a mechanism of communication between the exterior and the interior of the cell [1,2]. They represent major targets for the development of novel drug candidates in all clinical areas [3,4]. It is estimated that 50% of clinically prescribed drugs act as either agonists or antagonists of G-protein-coupled receptors which points to their immense therapeutic potential [5]. The membrane organization of G-protein-coupled receptors assumes significance in the light of their role in health and disease.

Interestingly, the efficiency of signal transduction processes carried out by G-protein-coupled receptors appears to be influenced by the local composition and organization of lipids within the plasma membrane [6]. It has been proposed that the G-protein-coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains [7,8]. It has been shown that some of these domains are enriched in cholesterol [6]. For example, it has recently been reported that serotonin$_{2A}$ receptors are localized in cholesterol-enriched membrane microdomains (caveolae) and serotonergic signaling induced by serotonin$_{2A}$ receptors depends on the membrane cholesterol content [9] and on caveolin-1, a scaffolding protein found in caveolae [10]. Localization of G-protein-coupled receptors into domains has given rise to new challenges and complexities in receptor signaling since signaling has to be understood in context of the three dimensional organization of various signal transduction components which include receptors and G-proteins [7].

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in mem-

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brane organization, dynamics, function, and sorting [11,12]. It is often found distributed non-randomly in domains or pools in biological and model membranes [13–15]. Many of these domains are believed to be important for the maintenance of membrane structure and function. Cholesterol in the plasma membrane plays a key role in regulating the activity of membrane proteins including receptors [reviewed in ref. 16]. The interaction between cholesterol and other membrane components (such as receptors) in the brain therefore assumes relevance for a comprehensive understanding of brain function.

Serotonin receptors represent one of the largest, evolutionarily ancient, and highly conserved families of G-protein-coupled receptors [17]. Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups [18]. Serotonin receptors are members of a superfamily of seven transmembrane domain receptors [1,2] that couple to and transduce signals via GTP-binding regulatory proteins (G-proteins) [19]. Among the 14 subtypes of serotonin receptors, the G-protein coupled 5-HT_{1A} receptor is the best characterized for a variety of reasons [20]. We have earlier partially purified and solubilized 5-HT_{1A} receptors from bovine hippocampus in a functionally active form [21,22].

We have recently shown the requirement of membrane cholesterol in modulating ligand binding activity of the 5-HT_{1A} receptor from the bovine hippocampus [23]. This was achieved by the use of methyl-β-cyclodextrin which physically depletes cholesterol from membranes. Treatment of bovine hippocampal membranes with methyl-β-cyclodextrin therefore resulted in specific removal of membrane cholesterol without any change in phospholipid content. Removal of cholesterol from bovine hippocampal membranes in this manner resulted in a reduction in ligand binding and G-protein coupling to the 5-HT_{1A} receptor [23]. If cholesterol is necessary for ligand binding of the 5-HT_{1A} receptor, modulating cholesterol availability by other means could lead to similar effects on the 5-HT_{1A} receptor function. In this paper, we have tested this proposal by treating the membranes with the sterol-complexing agent digitonin which does not physically deplete membrane cholesterol, yet would effectively reduce cholesterol-receptor interactions in the membrane due to complexation [24,25]. Digitonin is a plant glycoalkaloid saponin detergent (see Figure 1) obtained from *Digitalis purpurea*. It forms water-insoluble 1:1 complexes (termed 'digitonides') with cholesterol and other steroids, which possess a planar sterol nucleus, a 3β-hydroxy-Δ^5 configuration and a hydrophobic side chain at C_{17} [26]. Digitonin treatment has been

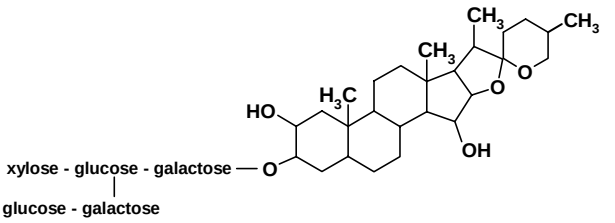

Figure 1. Chemical structure of digitonin.

shown to result in the formation of cholesterol-digitonin rich domains in the membrane [27], thus reducing the freely available cholesterol capable of interacting with other membrane constituents such as receptors. This property of digitonin has resulted in its use as an agent to distinguish between cholesterol-rich and -poor membranes in the ultrastructure analysis of cell membranes [28].

In this paper, we have monitored the modulatory role of cholesterol on the function (specific agonist and antagonist binding) of the bovine hippocampal 5-HT_{1A} receptor by cholesterol complexation in native membranes using digitonin. The corresponding changes in membrane dynamics were monitored by analysis of fluorescence polarization data of the membrane depth-specific probes, DPH and TMA-DPH.

Materials and methods

Materials

BCA, DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, Na_2HPO_4, *p*-MPPI, PMSF, TMA-DPH, Tris, iodoacetamide, polyethylenimine, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO). Digitonin was from Research Organics (Cleveland, OH). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). [^{3}H]8-OH-DPAT (specific activity = 135.0 Ci/mmol) and [^{3}H]*p*-MPPF (specific activity = 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). BCA reagent kit for protein estimation was obtained from Pierce (Rockford, IL). All other chemicals used were of the highest available quality. GF/B glass microfiber filters were from Whatman International (Kent, UK). Pre-coated silica gel 60 thin layer chromatography plates were from Merck (Merck KGaA, Germany). All solvents used were of analytical grade. Water was purified through a Millipore (Bedford, MA) 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 [23]. Protein concentration was determined using the BCA reagent with bovine serum albumin as a standard [29].

Preparation of digitonin stock solutions and treatment of hippocampal membranes

Stock solutions of digitonin in water were prepared as described previously [30]. Briefly, digitonin was dissolved in ethanol by mild shaking in a water bath at 37°C and the solution was evaporated to dryness under a gentle stream of N_2. This procedure results in the deposition of a fine film of digitonin on the walls of the glass tube that dissolves instantly upon addition of water. Aliquots of this aqueous stock solution were immediately added to hippocampal membranes at a total protein concentration of 0.5 mg/ml in 50 mM Tris, pH 7.4 buffer and incubated for 1 h at 25°C with constant shaking.

Membrane solubilization mediated by digitonin was assayed as described previously [31]. Briefly, membrane samples treated with increasing concentrations of digitonin for 1 h at 25°C were spun down at 100,000 × *g* for 45 min at 4°C. The lipid phosphate and cholesterol content of the membrane pellet dissolved in 50 mM Tris, pH 7.4 buffer was assayed as described below.

Estimation of inorganic phosphate and cholesterol

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [32] 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. Membrane cholesterol was estimated using the Amplex Red cholesterol assay kit [33].

Thin layer chromatography of lipids extracted from native membranes

Lipid extraction was carried out according to Bligh and Dyer [34]. The 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). Thin layer chromatography of the extracted lipids was carried out using chloroform/methanol/water (65:25:4, v/v/v). 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. The TLC plates were scanned and lipids band intensities were analyzed using the Adobe Photoshop software version 5.0 (Adobe Systems Inc., San Jose, CA).

Radioligand binding assays

Receptor binding assays were carried out as described earlier [35,36] in presence of increasing concentrations of digitonin using 0.5 mg total protein.

Fluorescence polarization measurements

Fluorescence polarization experiments were carried out with membranes containing 50 nmol of total phospholipids suspended in 1.5 ml of 50 mM Tris, pH 7.4 buffer with increasing concentrations of digitonin as described earlier [23,31]. Stock solutions of the fluorescent probes (DPH and TMA-DPH) were prepared in methanol. The amount of probe added was such that 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. 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). Control experiments showed that at this concentration of methanol, the ligand binding properties of the receptor are not altered.

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 nm and 20 nm were used. The excitation slit was kept low to avoid any photoisomerization of DPH and TMA-DPH. In addition, fluorescence was measured with a 30 sec 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 [37]. Fluorescence polarization experiments were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation [38]:

$$P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH}) \quad (1)$$

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 instrumental 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}. The optical density (measured with a Hitachi U-2000 spectrophotometer using 1 cm path length quartz cuvettes) of the membrane samples in the absence of digitonin measured at 358 nm was ~0.2 which increased in presence of digitonin. To avoid any scattering artifacts due to the presence of digitonin, the fluorescence polarization values reported in Figure 7 were corrected for scattering by diluting the samples with 50 mM Tris, pH 7.4 buffer as described earlier [39]. Fluorescence polarization values of samples (control and digitonin-treated, undiluted and diluted with the same buffer) were plotted against their corresponding optical densities as shown in Figure 6. The intercept on the ordinate (y-axis) from linear fits of this data was considered as the corrected fluorescence polarization value.

Results

Digitonin complexes membrane cholesterol without altering the cholesterol and phospholipid contents of hippocampal membranes

Digitonin is a widely used detergent and has been used to solubilize specific membrane components such as receptors [40]. It is therefore important to choose a range of concentration where digitonin would be present predominantly as a monomer and exert its cholesterol-complexing activity without causing any significant loss of membrane lipids (cholesterol and/or phospholipids). We estimated the loss of membrane lipids in the presence of digitonin by assaying the amount of total cholesterol and phospholipids present in the insoluble pellet obtained by high speed centrifugation following digitonin treatment [31]. The total cholesterol content was analyzed by TLC of lipids extracted from digitonin-treated membranes. Figure 2a shows a thin layer chromatogram of lipids extracted from native membranes, and membranes treated with 0.6 and 1 mM digitonin. Quantitative densitometric analysis of the cholesterol content on the TLC plate (Figure 2b) indicates that the treatment with digitonin does not deplete membrane cholesterol. However, analysis of the cholesterol content of digitonin-treated membranes using the cholesterol oxidase enzyme based Amplex Red cholesterol assay kit [33] reveals a reduction in the free (available) cholesterol content (Figure 2b). Thus, treatment of membranes with 1 mM digitonin reduces the available cholesterol content by ~50%. These results suggest

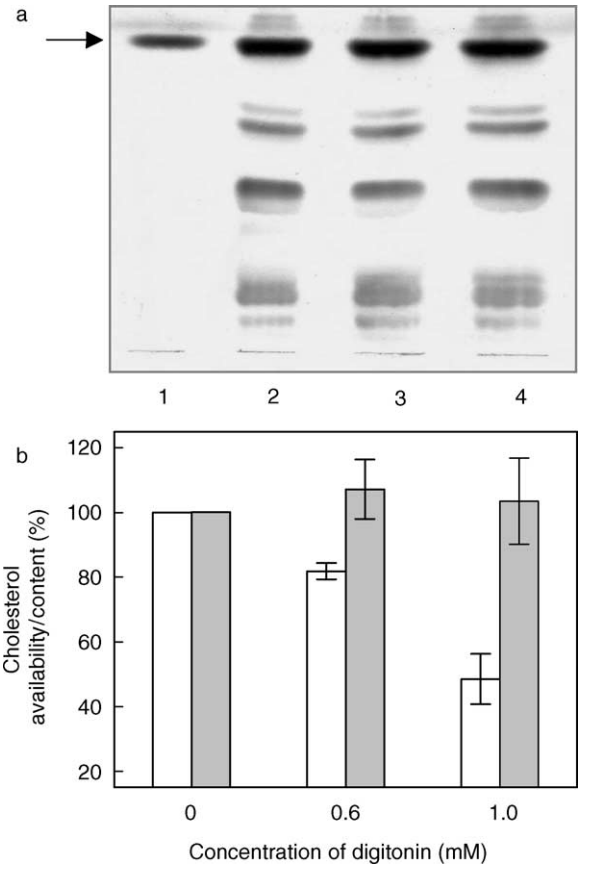

Figure 2. Cholesterol-complexing effect of digitonin. Total lipids extracted from digitonin-treated membranes were separated by thin layer chromatography as shown in (a). The lanes represent lipids extracted from native membranes (lane 2), and membranes treated with 0.6 mM (lane 3) and 1 mM (lane 4) digitonin. The arrow represents position of cholesterol on the thin layer chromatogram identified using a standard in lane 1. The total and freely available cholesterol content in digitonin-treated membranes were distinguished as shown in (b). The total cholesterol content (grey bars) in the lipid extract was determined by a densitometric analysis of the thin layer chromatogram. Values are expressed as a percentage of the cholesterol content in native membranes in the absence of digitonin. The amount of freely available cholesterol content (white bars) in membranes treated with digitonin was determined by the Amplex Red cholesterol assay kit. Values are expressed as a percentage of available cholesterol in native membranes in the absence of digitonin. Data represents the mean ± SD of two independent experiments. See Materials and methods for further details.

that the presence of digitonin reduces the ability of the enzyme cholesterol oxidase to catalyze the oxidation of cholesterol thereby demonstrating the cholesterol-complexing ability of digitonin. Importantly, Figure 3 shows that in the concentration range of digitonin used (up to 1 mM), there is minimal loss of phospholipid content. All experiments were therefore carried out with this range (up to 1 mM) of digitonin concentration that complexes membrane cholesterol to a significant extent without physically depleting membrane lipids.

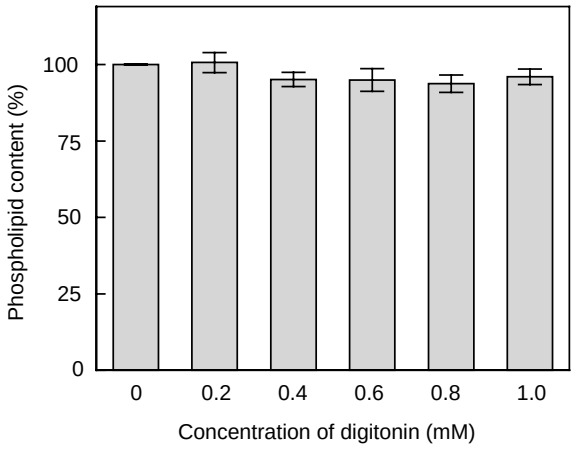

Figure 3. Effect of increasing concentrations of digitonin on the phospholipid content of bovine hippocampal membranes. Phospholipid contents were assayed as described in Materials and methods. Values are expressed as a percentage of the phospholipid content in native membranes in the absence of digitonin. Data represent the means ± SE of three independent experiments.

Ligand binding to 5-HT$_{1A}$ receptors is differentially affected in presence of digitonin

Figure 4 shows the reduction in the specific binding of the selective 5-HT$_{1A}$ receptor agonist [^{3}H]8-OH-DPAT with increasing concentrations of digitonin, which results in progressive complexation of membrane cholesterol. This shows that complexation of cholesterol from hippocampal membranes results in loss of specific agonist binding to the 5-HT$_{1A}$ receptor. Thus, the specific agonist binding reduces by 86% in the presence of 1 mM digitonin. To the best of our knowledge, this result represents the first observation of reduction in specific ligand binding to the 5-HT$_{1A}$ receptor due to complexation of membrane cholesterol by digitonin.

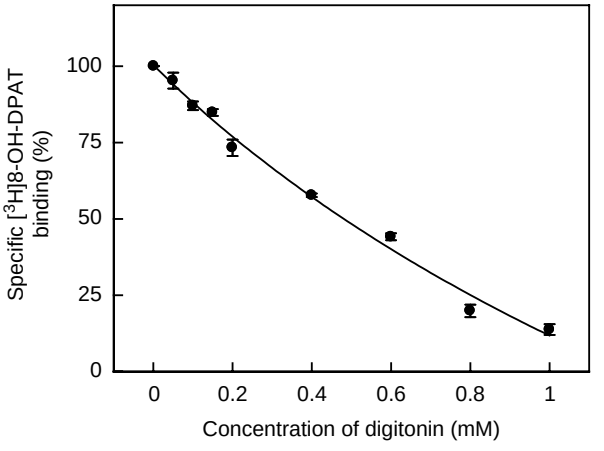

Figure 4. Effect of increasing concentrations of digitonin on specific [^{3}H]8-OH-DPAT binding to the 5-HT$_{1A}$ receptor in hippocampal membranes. Values are expressed as a percentage of specific binding for native membranes in the absence of digitonin. Data shown are the means ± SE of at least three independent experiments. See Materials and methods for other details.

Figure 5 shows the decrease in specific binding of the 5-HT$_{1A}$ selective antagonist [^{3}H]*p*-MPPF in the presence of increasing concentrations of digitonin. In contrast to the observed reduction in specific agonist binding (Figure 4), the reduction in specific antagonist binding appears to be more drastic at lower digitonin concentrations used. For example, the reduction in specific antagonist binding is ~83% in the presence of 0.4 mM digitonin. The corresponding reduction in specific agonist binding is ~42% (see Figure 4). Taken together, these results show that the complexation of free membrane cholesterol in hippocampal membranes results in the loss of the 5-HT$_{1A}$ receptor ligand binding ability. Although the requirement of membrane cholesterol for ligand binding to 5-HT$_{1A}$ receptors [23], and other G-protein-coupled receptors [41] has been demonstrated earlier, the present results constitute the first observation where the availability of free cholesterol rather than its mere physical presence in the membrane is essential for receptor function.

Change in hippocampal membrane dynamics due to digitonin treatment

The overall membrane order and dynamics could be an important determinant for the function of a transmembrane receptor, a large portion of which remains in contact with membrane lipids. In order to explore the possible changes in membrane order induced by digitonin treatment, we measured the fluorescence polarization of membrane depth-specific fluorescence probes DPH and TMA-DPH. In general, fluorescence polarization is correlated to the rotational diffusion [38] of membrane embedded probes, which is sensitive to the packing of fatty acyl

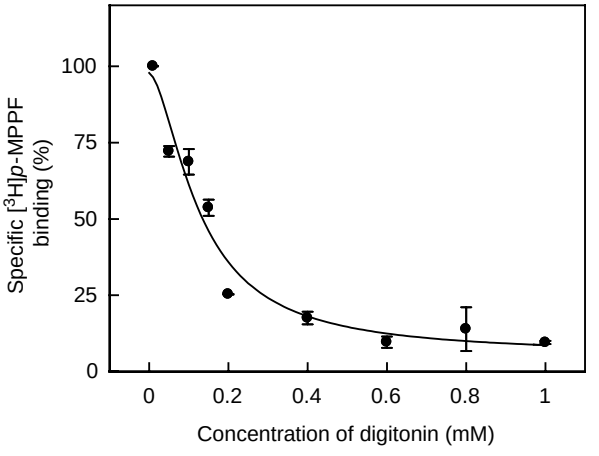

Figure 5. Effect of increasing concentrations of digitonin on specific [^{3}H]*p*-MPPF binding to the 5-HT$_{1A}$ receptor in hippocampal membranes. Values are expressed as a percentage of specific binding for native membranes in the absence of digitonin. Data shown are the means ± SE of at least three independent experiments. See Materials and methods for other details.

chains and cholesterol. Since membranes display a considerable degree of anisotropy in terms of motional properties of constituent lipids and proteins [42], a comprehensive understanding of membrane dynamics can be achieved by the use of more than one probe which localize at different depths in the membrane. DPH and its derivatives represent popular probes for monitoring organization and dynamics in membranes [43]. On account of its hydrophobicity, DPH readily partitions into the membrane and localizes at the fatty acyl chain region in the membrane. TMA-DPH is a derivative of DPH with a cationic moiety attached to the para position of one of the phenyl rings [44]. The amphipathic TMA-DPH is oriented in the membrane with its positive charge localized at the membrane interface [45]. The DPH moiety in TMA-DPH is localized at ~11 Å from the center of the membrane and reports the interfacial region of the membrane [46]. In contrast, the average location of DPH has been shown to be ~8 Å from the center of the membrane [46].

We observed an increase in the optical density of samples upon increasing the concentration of digitonin which could complicate fluorescence polarization measurements using DPH and TMA-DPH. The fluorescence polarization data obtained under these conditions were corrected for scattering artifacts according to the formalism previously developed by Lentz et al. [39]. Representative plots (at 0.6 mM digitonin) of experimentally-measured fluorescence polarization as a function of optical density at the excitation wavelength are shown in Figure 6. The intercept on the ordinate (y-axis) from linear fits of this data gives the corrected fluorescence polarization value [39]. The correction significantly altered the polarization values of samples containing digitonin while the polarization values of control samples (without digitonin) remained unaltered.

Corrected fluorescence polarization values of DPH and TMA-DPH obtained this way as a function of digitonin concentration are shown in Figure 7. As apparent from the figure, the corrected fluorescence polarization of DPH (~0.35) in hippocampal membranes in the absence of digitonin is lower than that of TMA-DPH (~0.37). The higher polarization of TMA-DPH compared to DPH is indicative of the shallower interfacial location of TMA-DPH in the membrane [23]. The fluorescence polarization of both the probes shows an overall decrease with increasing concentrations of digitonin. Interestingly, the extent of reduction in fluorescence polarization is higher for DPH (11%) compared to TMA-DPH (7%) up to 1 mM digitonin used. The presence of digitonin (at concentrations that do not deplete membrane lipids, see Figures 2 and 3) therefore induces asymmetric reduction in the membrane order in different regions of the membrane. Importantly, the relatively modest alterations in fluorescence polarization values of DPH and TMA-DPH in hippocampal membranes treated with digitonin ensure that the overall morphology of the membrane remains intact and rule out any possibility of gross changes in membrane architecture.

Discussion

Lipid-protein interactions play a crucial role in maintaining the structure and function of integral membrane proteins and receptors [47,48]. Monitoring lipid-receptor interactions is of particular importance because a cell has the ability of varying the lipid composition of its membrane in response to a

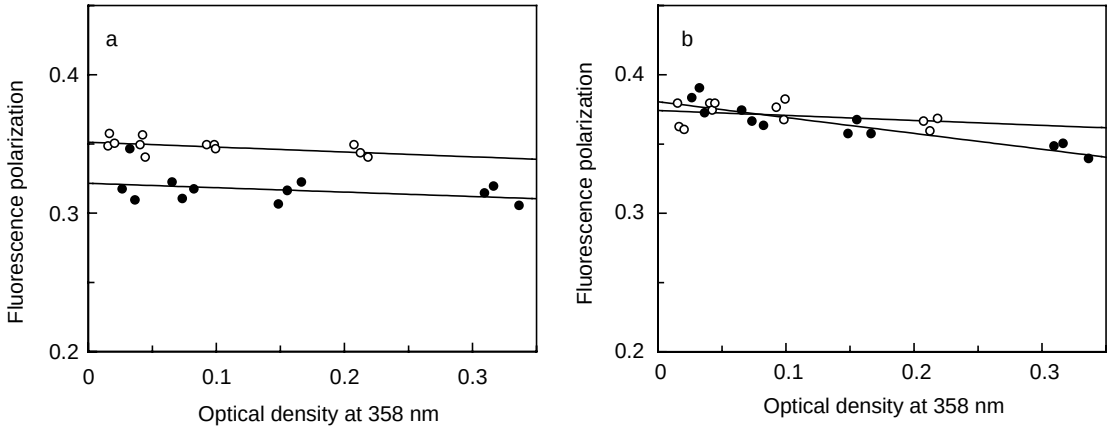

Figure 6. Representative plots of fluorescence polarization as a function of optical density. The plots represent the fluorescence polarization of (a) DPH and (b) TMA-DPH for native membranes (○) which serve as control and 0.6 mM digitonin-treated membranes (●) upon dilution with 50 mM Tris, pH 7.4 buffer. Fluorescence polarization was calculated as described in Materials and methods at 23°C. The solid lines are linear fits to the data with the intercept on the ordinate (y-axis) considered as the corrected fluorescence polarization [39]. The plots are data from at least three independent experiments. See Materials and methods for other details.

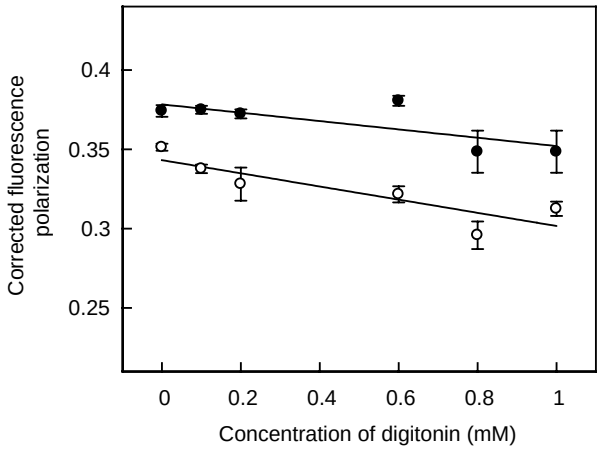

Figure 7. Effect of increasing concentrations of digitonin on fluorescence polarization of membrane probes DPH (○) and TMA-DPH (●) in hippocampal membranes. Fluorescence polarization experiments were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23°C). The data show corrected fluorescence polarization values (as described in Materials and methods, and representative plots shown in Figure 6) and represent the means±SE of at least three independent experiments. The solid lines are linear fits of the data. See Materials and methods for other details.

variety of stress and stimuli, thus changing the environment and the activity of the receptors in its membrane. In view of the importance of cholesterol in relation to membrane domains [11,15], the interaction of cholesterol with membrane receptors [16] represents an important determinant in functional studies of such receptors, especially in the nervous system.

We show here that complexation of cholesterol in hippocampal membranes using digitonin results in a concentration-dependent reduction in specific agonist and antagonist binding to 5-HT$_{1A}$ receptors. Complexation of membrane cholesterol with digitonin is believed to reduce the availability of free cholesterol (present data and [26]) essential to support important membrane functions. This is supported by the observation that the presence of digitonin restores the otherwise suppressed phase transition [49] of dipalmitoylphosphatidylcholine membranes containing cholesterol [25]. The accompanying changes in membrane order were reported by corresponding changes in fluorescence polarization of membrane probes, such as DPH and TMA-DPH, which are localized at different positions (depths) in the membrane. These results point out the important role of membrane cholesterol in maintaining the function of the 5-HT$_{1A}$ receptor. This result assumes significance in the light of a tightly bound cholesterol molecule in the recently reported crystallographic structure of metarhodopsin I, an important photointermediate of rhodopsin which is a representative member of the G-protein-coupled receptor family [50].

Our results on the inhibition of ligand binding to 5-HT$_{1A}$ receptors in the presence of the cholesterol-complexing agent digitonin confirm and extend our previous observation on the requirement of membrane cholesterol for efficient 5-HT$_{1A}$ receptor function [23]. Importantly, since digitonin does not physically deplete cholesterol from membranes unlike agents such as methyl-β-cyclodextrin, our results serve to delineate the mechanism by which cholesterol exerts its influence on the 5-HT$_{1A}$ receptor function. We conclude that the mere non-availability of free cholesterol in the membrane is sufficient to significantly reduce the 5-HT$_{1A}$ receptor function. These results provide a comprehensive understanding of the effects of the sterol-complexing agent digitonin in particular, and the role of membrane cholesterol in general, on the 5-HT$_{1A}$ receptor function from a lipid-protein interaction perspective.

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