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Membrane Organization of the Serotonin$_{1A}$ Receptor Monitored by a Detergent-Free Approach

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SUMMARY

1. Insolubility of membrane constituents in nonionic detergents such as Triton X-100 has been a widely used biochemical criterion to indicate their localization in membrane domains. However, concerns on the possibility of membrane perturbation in the presence of detergents have led to the development of detergent-free approaches.

2. We have explored the organization of the serotonin$_{1A}$ receptor, an important G-protein coupled receptor, from bovine hippocampus and CHO cells using a detergent-free approach in order to address the points of agreement with our previous results using Triton X-100.

3. A significant fraction of the serotonin$_{1A}$ receptor has been found to be localized in a heavy density fraction obtained using a detergent-free approach to isolate membrane domains. In addition, we have characterized the membrane fractions isolated in terms of their lipid composition and membrane physical properties.

4. The results obtained on the membrane localization of the serotonin$_{1A}$ receptor from the present experiments using a detergent-free approach correlate well with our earlier findings obtained using a detergent-based method (Kalipatnapu, S., and Chattopadhyay, A., *FEBS Lett.* 576:455–460, 2004). These results provide important information on the membrane organization of the hippocampal serotonin$_{1A}$ receptor and are relevant in view of the concerns on the use of detergent in determination of membrane organization of constituent proteins and lipids.

KEY WORDS: serotonin$_{1A}$ receptor; membrane organization; detergent-free method; bovine hippocampus.

INTRODUCTION

The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important G-protein coupled receptor (GPCR) involved in a variety of cognitive, behavioral, and developmental functions (Raymond *et al.*, 1999; Pucadyil *et al.*, 2005). The serotonin$_{1A}$ receptor agonists (Blier *et al.*, 1990) and antagonists (Griebel, 1999) represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mice lacking the serotonin$_{1A}$ receptor, generated a few years back, exhibit enhanced anxiety-related behavior (Julius, 1998) and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders

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and aggression (Toth, 2003). Although pharmacological and behavioral aspects of the serotonin$_{1A}$ receptor have been well explored, membrane organization of the receptor and its functional relevance are only beginning to be addressed (Pucadyil *et al.*, 2005; Kalipatnapu and Chattopadhyay, 2006).

Current understanding of the organization of biological membranes involves the concept of lateral heterogeneities in the membrane, collectively termed membrane domains (Mukherjee and Maxfield, 2004). Several kinds of membrane domains such as rafts, caveolae, and glycolipid-enriched domains, with possible overlapping features have been proposed (Simons and Ikonen, 1997; Hooper, 1999; Pike, 2004). Insolubility of membrane components in nonionic detergents such as Triton X-100 has been a widely used biochemical criterion to identify, isolate, and characterize membrane domains (particularly "rafts") (Brown and Rose, 1992; Hooper, 1999; Chamberlain, 2004). Although detergent insolubility continues to be a principal biochemical tool to isolate membrane domains, the possible membrane perturbing nature of this approach has been a cause of concern (Brown and London, 1998; Edidin, 2003). In addition, weak but essential interactions of proteins with membrane domains may be difficult to identify in the presence of detergents. In order to avoid the limitations of detergent-based methods, biochemical approaches which do not require detergents for the isolation of membrane domains have been proposed (Smart *et al.*, 1995; Song *et al.*, 1996; Luria *et al.*, 2002; Macdonald and Pike, 2005). These approaches involve milder treatments such as mild sonication and/or extraction with sodium carbonate. Interestingly, membrane domains isolated by detergent-based and detergent-free approaches have been shown to possess several overlapping features which include enrichment of lipids such as cholesterol and sphingolipids (Luria *et al.*, 2002).

In order to monitor membrane organization of the serotonin$_{1A}$ receptor, we have earlier developed a novel GFP-based approach to directly determine detergent insolubility of membrane proteins, and showed a small but significant fraction of the serotonin$_{1A}$ receptor to be insoluble in Triton X-100 (Kalipatnapu and Chattopadhyay, 2004). In addition, we examined the membrane organization of the serotonin$_{1A}$ receptor tagged to enhanced yellow fluorescent protein under conditions of reduced membrane cholesterol and agonist stimulation using this approach (Kalipatnapu and Chattopadhyay, 2004, 2005). In order to compliment these results and to check the possible effects of detergent, we have now used a detergent-free method to assess the membrane organization of the serotonin$_{1A}$ receptor. For this purpose, we have employed the detergent-free approach proposed by Luria *et al.* (2002), which involves mild sonication of the membranes before the separation of membrane fractions on a sucrose density gradient. The isolated membrane fractions are designated as light, heavy, and extra heavy fractions based on their density. It has been shown that the light membrane fraction isolated from *Xenopus* oocytes exhibits similar physical and biochemical characteristics as that of the detergent-resistant membrane (DRM) fraction isolated from the same system (Luria *et al.*, 2002). In particular, the light fraction isolated in the absence of detergent and the DRMs have been found to display a comparable protein composition, enrichment of lipids like cholesterol and sphingolipids, and a similar thermotropic phase behavior. The heavy membrane fraction was found to display significant differences

from both the light membrane fraction as well as DRMs (Luria *et al.*, 2002). We have utilized this detergent-free method to explore membrane organization of the serotonin$_{1A}$ receptor from the bovine hippocampus which is a relatively rich natural source of the receptor, and from CHO cells. We have determined the relative levels of the serotonin$_{1A}$ receptor in the membrane fractions isolated by detergent-free approach by performing receptor–ligand binding assays of the fractions. Further, the membrane fractions isolated by the detergent-free method have been characterized in terms of their lipid content and membrane order.

MATERIALS AND METHODS

Materials

5-HT (5-hydroxytryptamine), DMPC (dimyristoyl-*sn*-glycero-3-phosphocholine), DPH (1,6-diphenyl-1,3,5-hexatriene), EDTA, EGTA, gentamycin sulfate, iodoacetamide, $MgCl_2$, *p*-MPPI (4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine), PMSF (phenylmethylsulfonyl fluoride), penicillin, polyethylenimine, serotonin, sodium azide, streptomycin, sucrose, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). D-MEM/F-12 (Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) (1:1)), fetal calf serum, and geneticin (G 418) were from Invitrogen Life Technologies (Grand Island, NY). Sphingomyelin was obtained from Avanti Polar Lipids (Alabaster, AL). [^{3}H]8-OH-DPAT (8-hydroxy-2(di-*N*-propylamino)tetralin; sp. activity 135.0 Ci/mmol) and [^{3}H]*p*-MPPF (4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; sp. activity 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). BCA (bicinchoninic acid) reagent kit for protein estimation was obtained from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was obtained from Molecular Probes (Eugene, OR). Precoated silica gel 60 TLC (thin layer chromatography) plates were obtained from Merck (Merck KGaA, Germany). Solvents used for TLC were of analytical grade. Water was purified through a Millipore (Bedford, MA) MilliQ system and used throughout. All other chemicals used were of the highest purity available. GF/B glass microfiber filters were from Whatman International (Kent, UK). Concentration of stock solution of DPH in methanol was estimated from its molar absorption coefficient (ε) of 88,000 M^{-1} cm^{-1} at 350 nm (Haugland, 1996). 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 (Harikumar and Chattopadhyay, 1999). Protein concentration was determined using the BCA reagent with bovine serum albumin as standard (Smith *et al.*, 1985).

Cells and Cell Culture

CHO-K1 (Chinese hamster ovary) cells stably expressing the serotonin$_{1A}$ receptor tagged to enhanced yellow fluorescent protein (referred to as CHO-5-HT$_{1A}$R-EYFP) were used. Cells were grown in D-MEM/F-12 (1:1) supplemented with 2.4 g/L of sodium bicarbonate, 10% fetal calf serum, 60 μg/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamycin sulfate in a humidified atmosphere with 5% CO_2 at 37°C. CHO-5-HT$_{1A}$R-EYFP cells were maintained in the above-mentioned conditions with 300 μg/mL geneticin.

Preparation of Cell Membranes

Cell membranes were prepared as described earlier (Kalipatnapu *et al.*, 2004). Cells were harvested by treatment with ice-cold buffer containing 10 mM Tris, 5 mM EDTA, 0.1 mM PMSF, pH 7.4. Cells were then homogenized for 10 s at 4°C at maximum speed with a Polytron homogenizer. The cell lysate was centrifuged at 500 $\times$ *g* for 10 min at 4°C and the resultant postnuclear supernatant was centrifuged at 40,000 $\times$ *g* for 30 min at 4°C. The pellet thus obtained (cell membranes) was suspended in 50 mM Tris, pH 7.4 buffer. Total protein concentration in cell membranes thus isolated was determined using the BCA reagent (Smith *et al.*, 1985).

Detergent-Free Method to Isolate Various Membrane Fractions

Membrane fractions were isolated on a sucrose density gradient as described earlier (Luria *et al.*, 2002; Monneron and d'Alayer, 1978) with a few modifications. Native bovine hippocampal membranes or cell membranes were suspended at a protein concentration of $\sim$2 mg/mL in 50 mM Tris, pH 7.4 buffer. The membranes were briefly sonicated on ice using a Branson model 250 sonifier fitted with a microtip, and mixed with a sucrose solution (prepared in 50 mM Tris, pH 7.4) such that the final sucrose concentration would be 40% (w/v). This sample was then placed at the bottom of a centrifuge tube and overlaid with $\sim$3 mL each of 35, 22.5, and 10% (w/v) sucrose solutions prepared in 50 mM Tris, pH 7.4 buffer. Gradients were centrifuged for 3 h at a speed of 100,000 $\times$ *g* at 4°C using a Beckman SW-41 rotor. Diffuse bands were obtained at 10/22.5% and 22.5/35% sucrose interfaces of the sucrose density gradient which are termed light and heavy membrane fractions, respectively (Luria *et al.*, 2002). A faint band visible at the 35/40% sucrose interface was termed the extra heavy membrane fraction. All three bands were collected separately and diluted approximately threefold with cold 50 mM Tris, pH 7.4 buffer. The diluted membrane fractions were then centrifuged at $\sim$140,000 $\times$ *g* for 30 min at 4°C. The resulting pellets were suspended in 50 mM Tris, pH 7.4 buffer and used for further analysis.

Radioligand-Binding Assays

Receptor-binding assays for agonist and antagonist were carried out as described earlier (Harikumar and Chattopadhyay, 1999) with a few modifications.

Briefly, tubes in duplicate containing 0.2–0.4 mg of total protein in a volume of 1 mL of buffer D (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4) for agonist-binding assays, and 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 the assay tube being 0.29 nM) or antagonist [^{3}H]*p*-MPPF (final concentration in the assay tube being 0.5 nM) for 1 h at 25°C. Nonspecific binding was determined by performing the assay in the presence of 10 μM unlabeled 5-HT in the case of agonist binding or 10 μM unlabeled *p*-MPPI in the case of antagonist binding. The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter (1.0 μm pore size) glass microfiber filters which were presoaked in 0.15% (w/v) polyethylenimine for 3 h (Bruns *et al.*, 1983). The filters were then washed three times with 3 mL of ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 mL of scintillation fluid.

Estimation of Inorganic Phosphate and Cholesterol

The concentration of lipid phosphate of membranes and membrane fractions was determined subsequent to total digestion by perchloric acid (McClare, 1971) 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. Cholesterol content was estimated using the Amplex Red cholesterol assay kit (Amundson and Zhou, 1999).

Estimation of Sphingomyelin Content of Membranes

Analysis of sphingomyelin content of membranes and membrane fractions involved lipid extraction of the membrane samples followed by the separation of lipids using TLC. Lipid extraction of membranes and membrane fractions was carried out according to Bligh and Dyer (1959). The extracts were dried under a stream of nitrogen at 45°C and resuspended in a mixture of chloroform/methanol (1:1, v/v). The extracted lipids were then separated on a TLC plate using chloroform/methanol/water (65:25:4, v/v/v). A sphingomyelin standard was run along with the lipid extracts. The TLC plate was sprayed with a solution of 0.01% (w/v) primuline prepared in acetone (van Echten-Deckert, 2000) and the lipid bands were visualized under ultraviolet light. The lipid bands corresponding to the location of that of the sphingomyelin standard on the TLC plate were scraped out and the lipids were re-extracted. The extract was dried, and the concentration of lipid phosphate of the extract was assessed as described above, in order to determine the sphingomyelin content.

Measurement of Fluorescence Polarization

Fluorescence polarization measurements were carried out with membranes containing 50 nmol of total phospholipid suspended in 50 mM Tris, pH 7.4 buffer. Stock solution of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was

prepared in methanol. The final probe concentration was 1 mol% with respect to the total phospholipid content, so that optimal fluorescence intensity could be obtained with negligible membrane perturbation. The final probe concentration was 0.33 μM and the methanol content was low (0.03% v/v). The samples were incubated at room temperature (23°C) for 30 min in dark. Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. 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. To reverse any photoisomerization of DPH, samples were kept in dark in the fluorimeter for 30 s before the excitation shutter was opened and fluorescence was measured (Chattopadhyay and London, 1984). Background intensities of samples in which fluorophores were omitted were negligible and were subtracted from sample intensities to cancel out any scattering artifacts. Fluorescence polarization measurements were performed at 23°C using a Hitachi polarization accessory. Polarization values were calculated from the following equation (Chen and Bowman, 1965):

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

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 equal to I_{HV}/I_{HH}. The optical density of the samples was kept low to avoid possible scattering artifacts. All experiments were done with multiple sets of samples and average values of fluorescence polarization are shown in Fig. 2. Significance levels were estimated by one-way ANOVA using Microcal Origin software version 5.0 (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

Membrane organization of the serotonin$_{1A}$ receptor represents an important aspect in the understanding of the function of this pharmacologically relevant receptor (Pucadyil *et al.*, 2005; Kalipatnapu and Chattopadhyay, 2006). We have recently addressed the membrane organization of the serotonin$_{1A}$ receptor through the biochemical criterion of detergent insolubility (Kalipatnapu and Chattopadhyay, 2004, 2005). Although detergent insolubility continues to be of great practical value for the study of membrane domains, the issue of whether use of detergent merely helps in isolation of membrane domains, or induces their formation remains a cause for concern (Heerklotz, 2002; Edidin, 2003). We therefore used a detergent-free method to isolate membrane fractions, which have earlier been shown to correspond to those isolated employing detergents such as Triton X-100 (Luria *et al.*, 2002), to further explore the membrane organization of the serotonin$_{1A}$ receptor. Membranes prepared from bovine hippocampus were subjected to a detergent-free method, originally described by Luria *et al.* (2002), to isolate various membrane fractions. A typical isolation pattern of membrane fractions on a sucrose density gradient is shown in Fig. 1A. These fractions are designated as light, heavy, and

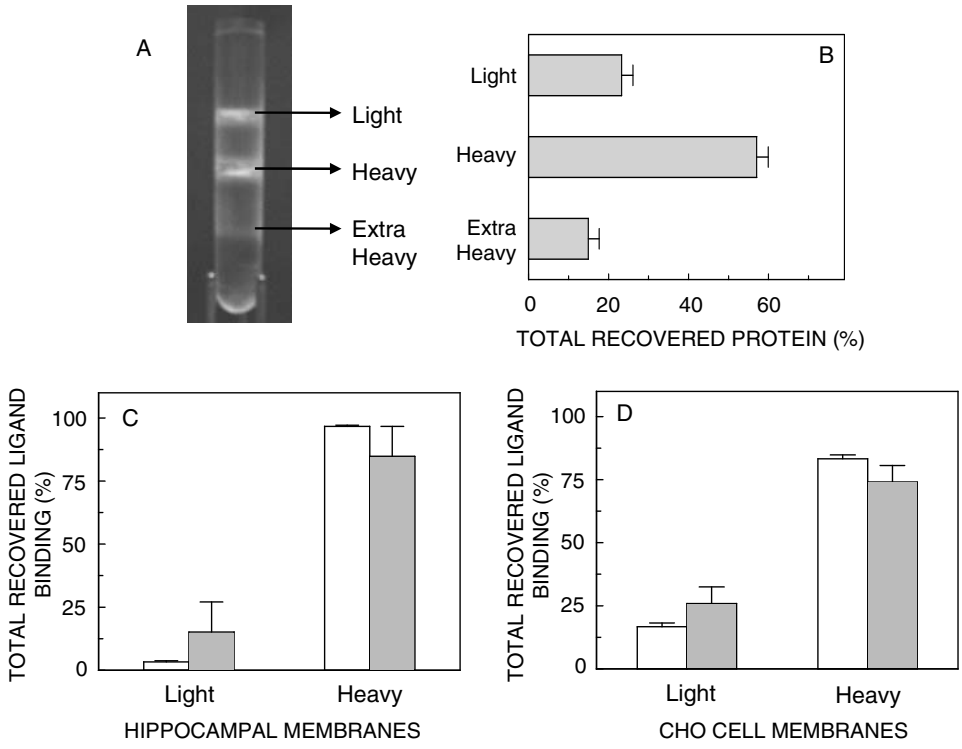

Fig. 1. (A) Typical pattern of isolation of light, heavy, and extra heavy membrane fractions from hippocampal membranes on a sucrose density gradient using the detergent-free method of Luria *et al.* (2002). The membrane fraction at 10–22.5% sucrose interface is designated as "light," at 22.5–35% sucrose interface as "heavy," and a faintly visible fraction at 35–40% sucrose interface as "extra heavy." (B) Comparison of total protein content of light, heavy, and extra heavy membrane fractions. Values are expressed as a percentage of the total protein content recovered from all the three membrane fractions isolated. The data points represent means ± SD of duplicate points from three independent experiments. See Materials and Methods section for other details. (C) Comparison of ligand binding to serotonin$_{1A}$ receptors from the light and heavy membrane fractions isolated using a detergent-free method from native hippocampal membranes. The *white bars* represent the binding of the agonist [^{3}H]8-OH-DPAT and the *shaded bars* that of the antagonist [^{3}H]*p*-MPPF. Values are expressed as a percentage of the total recovered ligand binding obtained from the light and heavy membrane fractions. The data points represent means ± SD of duplicate points from three independent experiments. See Materials and Methods section for other details. (D) Comparison of ligand binding to serotonin$_{1A}$ receptors from the light and heavy membrane fractions isolated using a detergent-free method from CHO-5-HT$_{1A}$R-EYFP cell membranes. The *white bars* represent the binding of the agonist [^{3}H]8-OH-DPAT and the *shaded bars* that of the antagonist [^{3}H]*p*-MPPF. Values are expressed as a percentage of the total recovered ligand binding obtained from the light and heavy membrane fractions. The data points represent means ± SD of duplicate points from two independent experiments. See Materials and Methods section for other details.

extra heavy based on their density. Figure 1B shows a comparison of the protein content recovered from each of the membrane fractions. The heavy membrane fraction, with ~57% of the total recovered protein, contains the highest amount of protein. The light and extra heavy membrane fractions are found to contain ~23 and 15% of the total recovered protein, respectively (see Fig. 1B). The light and

heavy membrane fractions have been earlier shown to be derived primarily from the plasma membrane, whereas the extra heavy fraction is shown to be mainly from intracellular components (Luria *et al.*, 2002; Monneron and d'Alayer, 1978). We therefore chose the light and heavy membrane fractions for further analysis.

We have determined the binding of the receptor to its ligands, 8-OH-DPAT and *p*-MPPF, which act as the agonist and antagonist, respectively, in order to assess the distribution of serotonin$_{1A}$ receptors among the light and heavy membrane fractions. Fig. 1C shows a comparison of ligand binding to hippocampal serotonin$_{1A}$ receptors from the light and heavy membrane fractions. Data obtained from ligand-binding assays has been represented as a percentage of the total recovered ligand binding in order to appreciate the distribution of active serotonin$_{1A}$ receptors among the light and heavy membrane fractions. As seen from Fig. 1C, the heavy membrane fraction represents $\sim$96 and 85% of the total recovered agonist and antagonist binding, respectively, of the serotonin$_{1A}$ receptor. In contrast, the light membranes show significantly lower ligand binding. In terms of the specific ligand-binding activity (ligand binding per milligram total protein) of the serotonin$_{1A}$ receptor, the heavy membrane fraction has been found to show $\sim$1.6- and 1.7-fold higher agonist- and antagonist-binding activity, respectively, as compared to native membranes (data not shown). We further monitored ligand binding of receptors from membrane fractions isolated by detergent-free method from cells expressing serotonin$_{1A}$ receptor fused to EYFP. The light and heavy membrane fractions were isolated from cell membranes using a sucrose density gradient, and relative agonist and antagonist binding of 5-HT$_{1A}$R-EYFP were determined (Fig. 1D). As seen from the figure, the heavy membrane fraction displays $\sim$83 and 74% of the total recovered agonist and antagonist binding, respectively. These results, obtained from both native and heterologous expression systems, indicate a distinct enrichment of the serotonin$_{1A}$ receptor in the heavy membrane fraction over that of the light membrane fraction as monitored by ligand-binding assays. The light fraction isolated by this detergent-free method has previously been shown to resemble detergent-resistant membranes in terms of its lipid and protein composition (Luria *et al.*, 2002). Our earlier findings on detergent insolubility of the serotonin$_{1A}$ receptor fused to EYFP suggest a small fraction of the receptor to be insoluble in Triton X-100 (Kalipatnapu and Chattopadhyay, 2004), indicating a relatively large fraction to be soluble in the detergent. This is probably reflected in the higher ligand binding in case of the heavy membrane fraction compared to the light fraction isolated by the detergent-free method.

A comparison of cholesterol, phospholipid, and sphingomyelin contents of the native hippocampal membranes and the membrane fractions isolated by the detergent-free method is shown in Table I. This analysis indicates a higher lipid content of the light membranes compared to the heavy membrane fractions. The light membrane fraction displays $\sim$1.6-fold higher cholesterol content over the heavy membrane fraction. The corresponding cholesterol to phospholipid ratios indicate an enrichment of cholesterol in the light membrane fraction. Analysis of the sphingomyelin content of the membrane fractions by TLC shows that the light fraction contains $\sim$1.5-fold higher levels of sphingomyelin than does the heavy membrane fraction (Table I). In order to gain insights into the physical characteristics of the

Table I. Analysis of Cholesterol, Phospholipid, and Sphingomyelin Contents of Native Hippocampal Membranes, Light and Heavy Membrane Fractions Isolated on a Sucrose Density Gradient

Membranes/ membrane fractions	Cholesterol[a] (nmol/mg protein)	Phospholipid[b] (nmol/mg protein)	C/P ratio[c] (mol/mol)	Sphingomyelin[d] (nmol/mg protein)
Native	390 ± 25	1007 ± 62	0.39 ± 0.02	60.6 ± 6.2
Light	766 ± 86	1537 ± 96	0.51 ± 0.02	91.4 ± 4.9
Heavy	470 ± 14	1146 ± 67	0.42 ± 0.04	59.5 ± 5.3

[a] Cholesterol content was assessed using the Amplex Red cholesterol oxidase-based assay and was normalized to the total protein. Data shown represent the means ± SE of at least three independent experiments.

[b] Phospholipid content has been determined as described in Materials and Methods section. Values have been normalized to the total protein. Data shown represent the means ± SE of at least three independent experiments.

[c] Cholesterol to phospholipid ratio. Data shown represent means ± SE of at least three independent experiments.

[d] Analysis of sphingomyelin content. Total lipids extracted from native membranes, light and heavy membrane fractions were separated by TLC. The total sphingomyelin content in the lipid extracts was assessed by determining the phosphate content of the bands corresponding to sphingomyelin in each case. Values are normalized with respect to the total protein content. Data shown represent the means ± SE of four independent experiments.

membrane fractions isolated by the detergent-free method, we monitored fluorescence polarization of the fluorescent membrane probe DPH, incorporated in these membranes. Fluorescence polarization monitors rotational diffusion of the probe, which is sensitive to the packing of the fatty acyl chains (Lakowicz, 1983). A higher polarization value would indicate higher motional restriction (more order) experienced by the probe. Fluorescence polarization values of DPH incorporated into native membranes, and light and heavy membrane fractions are shown in Fig. 2. The light membrane fraction shows the highest polarization value indicating a relatively rigid environment in these membranes compared to the heavy membrane fraction as well as the native membranes (Fig. 2). It is interesting to note that the overall trend observed in cholesterol to phospholipid ratios for different membrane fractions is in agreement with the corresponding fluorescence polarization values. Thus, an enrichment of cholesterol in the light membrane fraction is accompanied by higher fluorescence polarization values (indicative of greater order) of this membrane fraction. These results are relevant in the context of the role of cholesterol in modulating physical properties of biological membranes (Yeagle, 1985) and in its proposed involvement in the formation of membrane domains (Silvius, 2003; Mukherjee and Maxfield, 2004). The preferential interaction of cholesterol with phospholipids containing saturated acyl chains, such as sphingomyelin, resulting in tight packing could make the membrane regions enriched in these lipids more ordered (Brown and London, 1998; London, 2002). This phenomenon is also thought to confer detergent resistance to such regions of the membrane and to the proteins residing in them.

The main objective of this paper is to identify the points of agreement between the results obtained using detergent-based and detergent-free approaches in relation to the membrane organization of the serotonin$_{1A}$ receptor. Results from

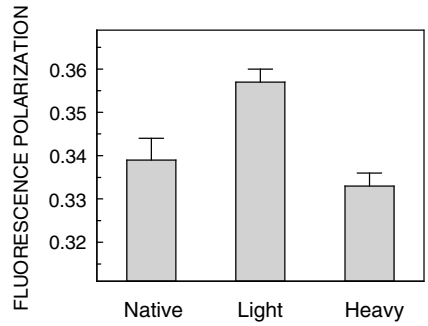

Fig. 2. Fluorescence polarization of DPH incorporated into native hippocampal membranes (shown as native), light and heavy membrane fractions. Fluorescence polarization experiments were carried out with membranes containing 50 nmol of total phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23°C). Values represent means ± SE of three independent experiments. Fluorescence polarization values for the light and heavy membrane fractions are significantly different ($P < 0.05$). See Materials and Methods section for other details.

the present experiments indicate that the heavy membrane fraction isolated by the detergent-free method represents a much larger pool of serotonin$_{1A}$ receptors compared to the light membrane fraction, as monitored by ligand-binding assays. These results correlate well with our earlier data on the detergent insolubility of the serotonin$_{1A}$ receptor fused to EYFP (Kalipatnapu and Chattopadhyay, 2004). It should be mentioned here that the possibility of physical presence of receptor with altered ligand-binding activity in any of the membrane fractions could affect our analysis of the present results. This aspect could be dealt with by employing a method of detection of the receptor which is independent of modulation by membrane environment. Nevertheless, the distribution of the active receptor among various membrane fractions merits to be addressed. Importantly, the fact that a very similar overall trend was observed whether hippocampal membranes, or a heterologous expression system has been used for the analysis strengthens our analysis.

Detergent insolubility of membrane components has been a crucial biochemical method available to study membrane domains in spite of concerns on whether lipids and proteins isolated as an insoluble membrane fraction upon treatment with detergents would correspond to those residing in domains in intact cells. In this context, detergent-free methods provide an additional biochemical means of understanding membrane organization. Detergent-based and detergent-free methods of membrane domain isolation have been compared earlier in the literature, and an overall analysis of these reports presents a somewhat varied picture. Domains isolated by these two approaches have been shown to display overlapping characteristics involving lipid and protein composition, and physical properties

(Luria *et al.*, 2002; Gaus *et al.*, 2005). On the other hand, there are reports suggesting membrane domains prepared in the presence or absence of detergents could have different constituent lipids and proteins. For example, the epidermal growth factor receptor is found to be soluble in Triton X-100, but found to localize in membrane domains when assessed using a detergent-free method (Pike *et al.*, 2005). Against this backdrop, it would be prudent to monitor the membrane organization of a given protein and its functional significance by multiple approaches in order to gain useful insights into the role of membrane environment in protein function. The present work on the serotonin$_{1A}$ receptor represents an attempt in this direction. Taken together, our results on the membrane organization of the serotonin$_{1A}$ receptor obtained from a detergent-free approach correlate well with our earlier observations from a detergent-based method. It would be interesting to compare such results for other G-protein coupled receptors.

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