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Removal of sphingomyelin headgroup inhibits the ligand binding function of hippocampal serotonin$_{1A}$ receptors

Pushpendra Singh, Amitabha Chattopadhyay *

Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, Uppal Road, Hyderabad 500 007, India

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ABSTRACT

Sphingolipids are essential components of eukaryotic cell membranes and are thought to be involved in a variety of cellular functions. Sphingomyelin is the most abundant sphingolipid in the nervous system. In this work, we explored the ligand binding function of the hippocampal serotonin$_{1A}$ receptor upon hydrolyzing sphingomyelin to ceramide and phosphocholine using sphingomyelinase. The serotonin$_{1A}$ receptor is an important neurotransmitter receptor and belongs to the superfamily of G-protein coupled receptors. It is involved in the generation and modulation of various cognitive, behavioral and developmental functions. We show here that specific agonist binding to serotonin$_{1A}$ receptors in native hippocampal membranes is considerably reduced upon sphingomyelinase treatment. Interestingly, the overall membrane order does not exhibit any appreciable change under these conditions. Our results show the importance of sphingomyelin (specifically, the sphingomyelin headgroup) for the function of serotonin$_{1A}$ receptors. These novel results constitute the first report on the effect of enzymatic hydrolysis of sphingomyelin on the ligand binding function of this important neurotransmitter receptor in native hippocampal membranes. Our results assume greater relevance in the broader perspective of the influence of the membrane lipid environment on the function of the serotonin$_{1A}$ receptor in particular, and other G-protein coupled receptors in general.

1. Introduction

Sphingolipids are essential components of eukaryotic cell membranes and constitute ~10–20% of the total membrane lipids [1]. They represent diverse and dynamic regulators of a large number of cellular processes such as cell signaling, growth, differentiation, and neoplastic transformation. Sphingomyelin is the most abundant sphingolipid in the nervous system. For example, it makes up ~25% of total lipids in the myelin sheath [2]. Sphingolipids such as sphingomyelin are regarded as reservoirs for bioactive second messengers such as ceramide and sphingosine 1-phosphate [3,4]. The distribution of sphingomyelin in the cellular plasma membrane appears heterogeneous and patchy [5]. Importantly, sphingomyelins have been shown to be involved in the development and progression of several neurological diseases such as Alzheimer's disease [4,6] which could be due to impaired neurotransmission.

The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [7,8]. GPCRs regulate physiological responses to a diverse array of stimuli, and mediate multiple physiological processes. GPCRs have therefore emerged as major targets for the development of novel drug candidates in all clinical areas [9]. It is estimated that ~50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs [10]. The serotonin$_{1A}$ receptor is an important neurotransmitter receptor and belongs to the GPCR superfamily. It is the most extensively studied among the serotonin receptors [11]. The serotonin$_{1A}$ receptor plays a key role in the generation and modulation of various cognitive, behavioral and developmental functions [11–13]. Agonists and antagonists of the receptor have been shown to possess potential therapeutic effects in anxiety or stress-related disorders [11]. The serotonin$_{1A}$ receptor therefore serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. In the context of increasing pharmacological significance of the serotonin$_{1A}$ receptor [14,15], its interaction with the surrounding lipids such as sphingomyelin assumes relevance. In this overall context and keeping in mind the relevance of sphingolipids in the nervous system [4], we explored the importance of the sphingomyelin headgroup on ligand binding function of the serotonin$_{1A}$ receptor in native hippocampal membranes.

Abbreviations: 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GPCR, G-protein coupled receptor; PMSF, phenylmethylsulfonyl fluoride.

* Corresponding author. Fax: +91 40 2716 0311.
E-mail address: amit@ccmb.res.in (A. Chattopadhyay).

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2. Materials and methods

2.1. Materials

Sphingomyelinase (EC 3.1.4.12) from *Bacillus cereus*, cholesterol, DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). BCA reagent for protein estimation was from Pierce (Rockford, IL). [^{3}H]8-OH-DPAT (sp. activity 106 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, UK). All solvents used were of analytical grade. Pre-coated silica gel 60 thin layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity available. 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 until further use.

2.2. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [16,17]. Native membranes were suspended in a minimum volume of 50 mM Tris, pH 7.4 buffer, homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70 °C. Protein concentration was assayed using the BCA reagent [18].

2.3. Treatment of native membranes with sphingomyelinase

Native membranes were resuspended in 50 mM Tris, pH 7.4 buffer at a protein concentration of 2 mg/ml and treated with sphingomyelinase (aliquoted from a stock solution of 200 U/ml 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.

2.4. Radioligand binding assays

Receptor binding assays were carried out as described earlier [17]. 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 [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at 25 °C. Nonspecific 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 [19]. Filters were then washed three times with 3 ml of cold water (4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

2.5. Estimation of sphingomyelin by thin layer chromatography

Total lipids were extracted from control and sphingomyelinase-treated membranes according to Bligh and Dyer [20]. The lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were dissolved in a chloroform/methanol mixture (1:1, v/v). Total lipid extracts were resolved by thin layer chromatography using a chloroform/methanol/water (65:25:4, v/v/v) as the solvent system [21]. The separated lipids were visualized under ultraviolet light by spraying a fluorescent solution of 0.01% (w/v) primuline prepared in acetone [22]. A sphingomyelin standard was used to identify its position on TLC plates run with total lipid extracts obtained from control and sphingomyelinase-treated membranes. Sphingomyelin bands were scraped from TLC plates, lipids were re-extracted with a chloroform/methanol mixture (1:1, v/v) from samples, and the phosphate content was estimated and normalized to the phosphate content obtained from control samples.

2.6. Estimation of cholesterol content

Cholesterol content in membranes treated with different concentration of sphingomyelinase was estimated using the Amplex Red cholesterol assay kit [23].

2.7. Estimation of phospholipid content

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [24] 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.

2.8. Fluorescence anisotropy measurements

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH as described previously [17,25]. 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 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 [26]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (r) values were calculated from the equation [27]:

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

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 the ratio of the efficiencies of the detection system for vertically and horizontally polarized light 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.

2.9. Statistical analysis

Significance levels were estimated using Student's two-tailed unpaired t-test using Graphpad Prism software version 4.0 (San Diego, CA).

3. Results

Sphingomyelinases are water soluble enzymes that act at the membrane interface and specifically hydrolyze sphingomyelin into ceramide and phosphocholine ([28,29]; see Fig. 1). Phosphocholine, a water soluble moiety, is liberated from sphingomyelin leaving a hydrophobic ceramide backbone of sphingomyelin in the

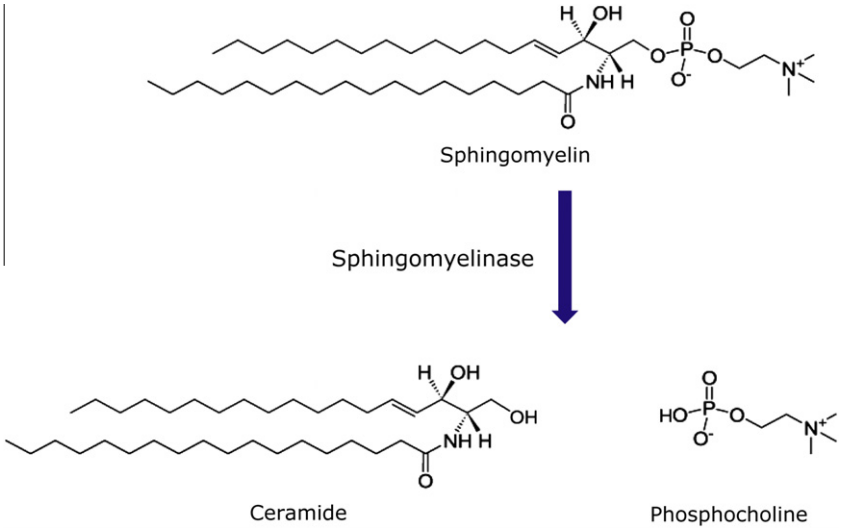

Fig. 1. A schematic representation of sphingomyelin hydrolysis catalyzed by sphingomyelinase, with chemical structures of sphingomyelin (ceramide phosphocholine), ceramide and phosphocholine. Sphingomyelinase, an important hydrolytic enzyme, is involved in sphingolipid metabolism. It catalyzes the hydrolysis of sphingomyelin into ceramide and phosphocholine. See text for details.

membrane. The phosphocholine moiety appears to act as an anchor for sphingomyelin at the membrane interface. Importantly, sphingomyelinases act as regulators of cell signaling by modulating cellular ceramide levels [5,30], and can be stimulated with naturally occurring ligands or under stress conditions [4,31].

Fig. 2A shows that treatment of hippocampal membranes with sphingomyelinase results in the reduction of sphingomyelin content. Treatment of hippocampal membranes with 1 U/ml sphingomyelinase hydrolyzes ~65% sphingomyelin, whereas increasing the concentration of the enzyme to 2 U/ml resulted in ~70% hydrolysis of sphingomyelin. The extent of sphingomyelin hydrolysis therefore appears to level off under these conditions. Since sphingomyelin contains phosphocholine headgroup, it contributes to the total phospholipid content of hippocampal membranes. Fig. 2B shows that the total phospholipid content in sphingomyelinase-treated membranes shows a reduction by ~19% corresponding to ~70% reduction in sphingomyelin content due to hydrolysis with 2 U/ml sphingomyelinase (Fig. 2A). We have previously reported that bovine hippocampal membranes contain ~68% phospholipids and ~32% cholesterol [32]. Sphingomyelin therefore accounts for ~18% of the total lipids in hippocampal membranes. These values are in very good agreement with literature reports of sphingomyelin content in neuronal membranes [1,2]. Sphingomyelin is anchored through phosphocholine moiety toward the membrane interface and is believed to interact with cholesterol through hydrogen-bonding between the hydroxyl group of cholesterol and the amide group of sphingomyelin [33]. Ceramide, generated by the action of sphingomyelinase, tends to form ceramide-enriched microdomains [30]. To examine the effect of sphingomyelinase on membrane cholesterol content, we estimated cholesterol content in sphingomyelinase-treated membranes. Fig. 2C shows that cholesterol content does not exhibit any significant change upon sphingomyelinase treatment.

In order to explore the effect of sphingomyelinase on ligand binding function of serotonin$_{1A}$ receptors in hippocampal membranes, we monitored specific binding of the agonist [^{3}H]8-OH-DPAT to the receptor. Fig. 3 shows the change in specific binding of the agonist [^{3}H]8-OH-DPAT to the serotonin$_{1A}$ receptor upon sphingomyelinase treatment. Interestingly, specific agonist binding shows a reduction with increasing concentration of sphingomyelinase. Fig. 3 shows that specific agonist binding is reduced by ~13% when 1 U/ml of the enzyme was used. The reduction in specific agonist binding increases to ~43% when an enzyme concentration of 2 U/ml was used. These results show that removal of the phosphocholine headgroup from sphingomyelin (resulting in the formation of ceramide) inhibits the ligand binding function of hippocampal serotonin$_{1A}$ receptors. These observations are relevant in the light of our recent observations that metabolic depletion of sphingolipids impairs the serotonin$_{1A}$ receptor function [34]. Our present results show the structural importance of sphingomyelin for serotonin$_{1A}$ receptor function.

The observed reduction in specific agonist binding induced by change in sphingomyelin (or ceramide) level could possibly be due to an alteration in membrane order. This possibility arises since treatment of hippocampal membranes with sphingomyelinase would generate ceramide that could alter the packing of lipid acyl chain in the membrane [30]. In order to examine whether there is a change in membrane order upon sphingomyelinase treatment, we carried out fluorescence anisotropy measurements using the membrane probe DPH. Fluorescence anisotropy of probes such as DPH is correlated to the rotational diffusion of membrane embedded probes [27], which is sensitive to the packing of lipid acyl chains. This is due to the fact that fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing. DPH, a rod-like hydrophobic molecule, partitions into the interior (fatty acyl chain region) of the bilayer. Fig. 4 shows fluorescence anisotropy of the membrane probe DPH incorporated in hippocampal membranes upon increasing concentrations of sphingomyelinase. Fluorescence anisotropy appears to increase slightly (~7%) upon treatment with 2 U/ml sphingomyelinase, compared to the corresponding anisotropy in control (untreated) hippocampal membranes. These results therefore suggest that removal of the sphingomyelin headgroup does not significantly alter the overall membrane order. Taken together, the reduction in the ligand binding function of the serotonin$_{1A}$ receptor upon sphingomyelin hydrolysis is not accompanied by appreciable change in membrane order. These results indicate that phosphocholine headgroup of sphingomyelin could interact with the serotonin$_{1A}$ receptor, thereby influencing ligand binding function of the receptor.

4. Discussion

In order to examine the structural importance of sphingomyelin in serotonin$_{1A}$ receptor function, we monitored the ligand (agonist)

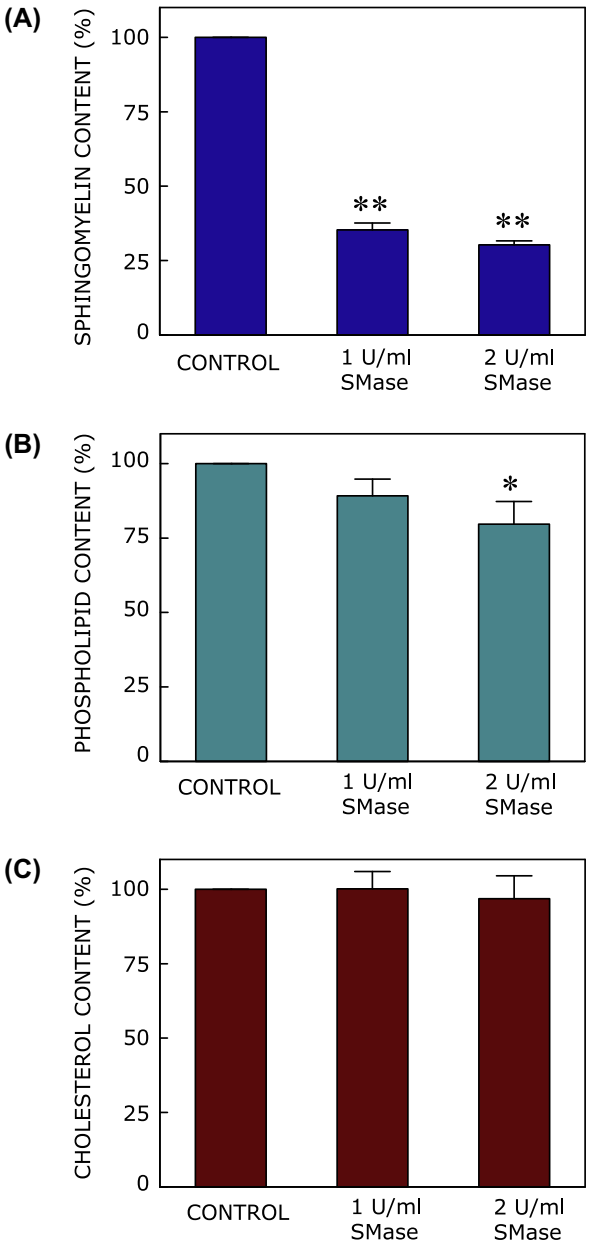

Fig. 2. Estimation of lipid content of control and sphingomyelinase-treated hippocampal membranes. Total lipids extracted from control and sphingomyelinase-treated membranes were separated by thin layer chromatography and sphingomyelin was recovered from the chromatogram. (A) Sphingomyelin and (B) total phospholipid contents were determined by phosphate assay subsequent to total digestion by perchloric acid using Na_2HPO_4 as standard. (C) Cholesterol content from control and sphingomyelinase-treated membranes was estimated using Amplex red assay kit. Values are expressed as percentages of the corresponding lipid content in control (untreated) membranes. Data shown are means ± SE of four independent experiments (*corresponds to a p-value <0.05, **corresponds to a p-value <0.0001). See Section 2 for other details.

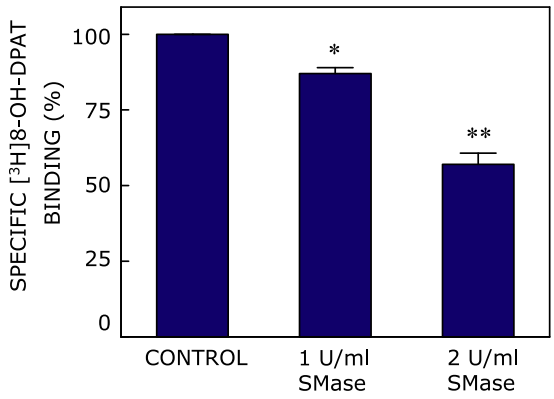

Fig. 3. Specific binding of the agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors in control and sphingomyelinase-treated hippocampal membranes. Values are expressed as percentages of specific agonist binding obtained in control membranes. Data represent means ± SE of duplicate points from five independent measurements (*corresponds to a p-value <0.001, **corresponds to a p-value <0.0001). See Section 2 for other details.

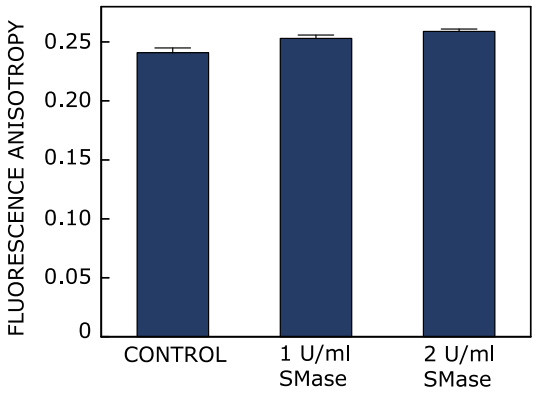

Fig. 4. Fluorescence anisotropy of the membrane probe DPH in control and sphingomyelinase-treated hippocampal membranes. Fluorescence anisotropy measurements were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). The excitation wavelength was 358 nm and emission was monitored at 430 nm. Data shown are means ± SE of duplicate points from three independent experiments. See Section 2 for other details.

binding function of the hippocampal serotonin$_{1A}$ receptor upon hydrolyzing sphingomyelin to ceramide and phosphocholine with sphingomyelinase. Our results show that specific agonist binding to the serotonin$_{1A}$ receptor is reduced upon sphingomyelinase treatment without any appreciable change in overall membrane order. This could be due to the reduction in membrane sphingomyelin content or the resultant increase in ceramide content, or both. We have recently shown that metabolic depletion of sphingolipids impairs the serotonin$_{1A}$ receptor function and leads to an enhancement of receptor mobility [34,35]. Our present results, along with these earlier observations, comprehensively demonstrate the importance of sphingolipids in the function of the serotonin$_{1A}$ receptor. In our future work, we plan to address this issue using specific inhibitors of the sphingolipid biosynthetic pathway and utilizing cell lines which are conditional mutants in sphingolipid biosynthesis [36].

It has been previously 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 sphingomyelin and cholesterol [37]. In this context, analysis of membrane protein function under conditions that affect sphingomyelin and cholesterol distribution in membranes assumes significance. Importantly, we earlier demonstrated that physical depletion of cholesterol from hippocampal membranes using methyl-β-cyclodextrin resulted in loss of ligand binding of the serotonin$_{1A}$ receptor [38]. Removal of phosphocholine headgroup of sphingomyelin could disrupt sphingomyelin-cholesterol interactions [33], leading to membrane reorganization. Our results constitute the first report on the effect of enzymatic hydrolysis of sphingomyelin on the ligand binding function of this important neurotransmitter receptor in native hippocampal

membranes. These results assume relevance in the overall context of the influence of the membrane lipid environment on the function of the serotonin$_{1A}$ receptor in particular, and other G-protein coupled receptors in general [39,40].

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