



Rapid report

Effect of sphingomyelinase treatment on ligand binding activity of human serotonin_{1A} receptorsMd. Jafurulla, Thomas J. Pucadyil¹, Amitabha Chattopadhyay^{*}

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ARTICLE INFO

Article history:

Received 12 March 2008

Received in revised form 7 July 2008

Accepted 9 July 2008

Available online 16 July 2008

Keywords:

Serotonin_{1A} receptor
Sphingomyelin
Sphingomyelinase
Ligand binding activity
Fluorescence anisotropy

ABSTRACT

The serotonin_{1A} receptor is an important member of the G-protein coupled receptor family, and is involved in the generation and modulation of a variety of cognitive, behavioral, and developmental functions. We have monitored the ligand binding of the human serotonin_{1A} receptor stably expressed in CHO cells (termed CHO-5-HT_{1A}R) following treatment with sphingomyelinase (SMase), an enzyme that specifically catalyzes the hydrolysis of sphingomyelin into ceramide and phosphorylcholine. Our results show, for the first time, that the specific ligand binding activity of the serotonin_{1A} receptor in membranes isolated from CHO-5-HT_{1A}R cells is increased upon sphingomyelinase treatment. Saturation binding analysis reveals increase in binding affinity of the receptor under these conditions. This is accompanied by a reduction in membrane order, as monitored by fluorescence anisotropy of the membrane probe 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) in intact cells. These results represent the first report on the effect of sphingomyelinase treatment on the ligand binding activity of this important neurotransmitter receptor.

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Sphingolipids are ubiquitous constituents of eukaryotic cell membranes and constitute ~10–20% of membrane lipids [1]. Sphingolipids are recognized as diverse and dynamic regulators of a multitude of cellular processes. The sphingoid base, ceramide and other intermediates of sphingolipid metabolism are known to act as signaling molecules in mediating cell cycle control, differentiation, stress response and apoptosis. The distribution of sphingolipids in the plasma membrane appears to be heterogeneous, and it has been postulated that sphingolipids and cholesterol localize in laterally segregated lipid domains (sometimes termed as ‘lipid rafts’) [2,3].

Serotonin receptors represent one of the largest, evolutionarily ancient, conserved family of seven transmembrane G-protein coupled receptors (GPCRs) [4,5]. The G-protein coupled serotonin_{1A} (5-HT_{1A}) receptor is the best characterized among the 14 subtypes of serotonin receptors [6]. Serotonin_{1A} receptors appear to play a key role in the generation and modulation of various cognitive, behavioral and developmental functions [6,7]. The serotonin_{1A} receptor agonists [8] and antagonists [9] represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin_{1A} receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for the analysis of complex traits such as anxiety disorders and aggression in higher animals [10,11]. In view of

the pharmacological relevance of the serotonin_{1A} receptor, a transmembrane protein, its interaction with the surrounding lipid environment assumes greater significance in modulating the function of the receptor in healthy and diseased states. Previous work from our laboratory has shown the requirement of membrane cholesterol in the function of this important neurotransmitter receptor (recently reviewed in [12,13]). In the overall context of the role of sphingolipids (along with cholesterol) in the formation and maintenance of membrane domains [2,3], and keeping in mind the relevance of sphingolipids in the nervous system [14,15], we monitored the effect of sphingomyelinase treatment on the ligand binding activity of the human serotonin_{1A} receptor stably expressed in CHO cells (termed CHO-5-HT_{1A}R) (described earlier [16]). It should be mentioned here that the effect of sphingolipids on ligand binding function of the serotonin₇ receptor has recently been reported [17].

Sphingomyelinases are enzymes that specifically catalyze the hydrolysis of sphingomyelin into ceramide and phosphorylcholine [18,19]. CHO-5-HT_{1A}R cells were treated with different concentrations of sphingomyelinase (SMase) (either 25 or 50 mU/ml) for 90 min in D-MEM/F-12 medium under serum free condition. Enzyme treatment was terminated by washing off the medium containing enzyme with phosphate buffered saline (PBS) and the cells were collected for further analysis. Sphingomyelin levels of CHO-5-HT_{1A}R cells with or without sphingomyelinase treatment were quantitated by thin layer chromatography of total lipids (extracted according to Bligh and Dyer [20]), using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. The TLC plate was sprayed with a solution of 0.01% (w/v) primuline prepared in acetone [21] and the lipid bands were

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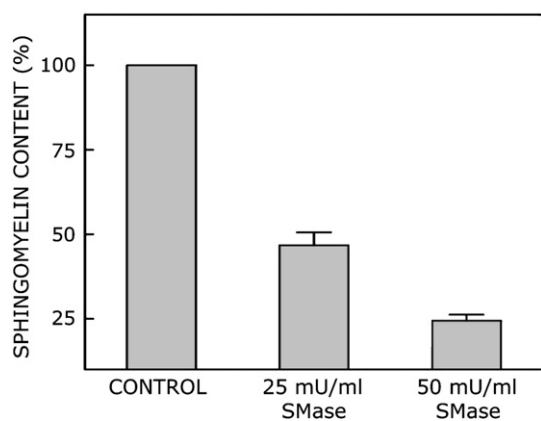


Fig. 1. Estimation of spingomyelin content of control and spingomyelinase treated CHO-5-HT_{1A}R cells. Spingomyelinase treatment was carried out as described in the text. Values are expressed as percentages of spingomyelin content of untreated (control) cells. Data shown are means±S.E. of duplicate points from at least three independent measurements. See text for other details.

visualized under ultraviolet light. The lipid bands corresponding to the location of that of the spingomyelin standard on the TLC plate were scraped out and the lipids were re-extracted. The extract was dried, and the concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [22] using Na₂HPO₄ as standard. Fig. 1 shows the reduction in the spingomyelin content as a result of spingomyelinase treatment. There is ~53% reduction in spingomyelin content when cells were treated with 2 mU/ml of spingomyelinase, which reduces to ~75% when 50 mU/ml of spingomyelinase was used. Similar extents of reduction in spingomyelin content have previously been reported under certain conditions [23,24].

We further monitored the effect of spingomyelinase treatment on the specific binding of the agonist [³H]8-OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin) to the serotonin_{1A} receptor in cell membranes prepared from CHO-5-HT_{1A}R cells with or without treatment. Cell membrane preparation and receptor binding assays were carried out as described earlier [16]. Total protein concentration in cell membranes was determined using Bicinchoninic acid (BCA) reagent [25]. Fig. 2 shows change in the specific binding of the agonist [³H]8-OH-DPAT to the serotonin_{1A} receptor upon treatment with spingomyelinase. Interestingly, the specific agonist binding increases by ~40% upon spingomyelinase treatment, irrespective of the enzyme concentration used. To the best of our knowledge, this is the first report

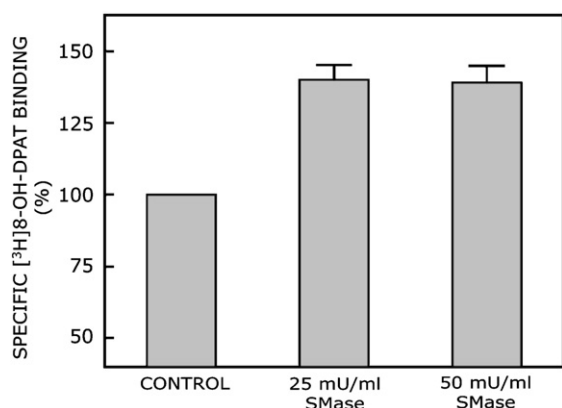


Fig. 2. Specific binding of the agonist [³H]8-OH-DPAT to serotonin_{1A} receptors in membranes isolated from control and spingomyelinase treated CHO-5-HT_{1A}R cells. Spingomyelinase treatment was carried out as described in the text. Values are expressed as percentages of specific agonist binding obtained in membranes isolated from untreated (control) cells. Data represent means±S.E. of duplicate points from at least eight independent measurements. See text for other details.

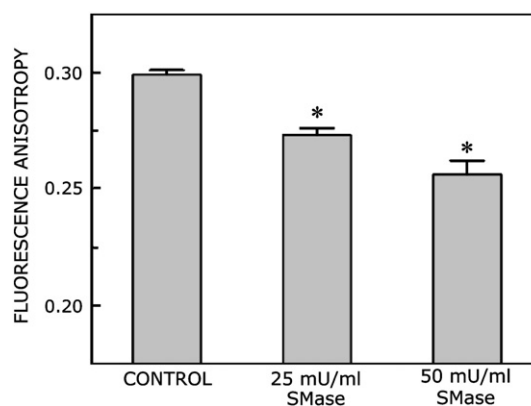


Fig. 3. Fluorescence anisotropy measurements of the membrane probe TMA-DPH in control and spingomyelinase treated CHO-5-HT_{1A}R cells. Spingomyelinase treatment was carried out as described in the text. Data shown are means±S.E. of duplicate points from three independent measurements (* corresponds to $p < 0.05$; the change in fluorescence anisotropy was tested against the corresponding value obtained with untreated (control) cells). See text for other details.

describing change in specific agonist binding activity of the serotonin_{1A} receptor upon spingomyelinase treatment. The specific [³H]8-OH-DPAT binding (%) is normalized to the amount of total protein used in the assay. Although the total protein content varied in different assays, control experiments reported before showed specific binding to be linear with protein content [16].

The observed increase in specific ligand binding activity due to modulation in spingomyelin (or ceramide) level could be because of an alteration in overall membrane organization (order). In order to monitor whether there is a change in membrane order upon spingomyelinase treatment, fluorescence anisotropy measurements were carried out on cell suspensions using the membrane probe TMA-DPH, which is a derivative of DPH with a cationic moiety attached to the *para* position of one of the phenyl rings [26]. TMA-DPH has previously been shown to specifically label plasma membranes of intact living cells [27]. Fluorescence anisotropy measurements were carried out as described earlier [28] with some modifications. Fluorescence anisotropy measured using probes such as TMA-DPH, is correlated to its rotational diffusion [29], which is sensitive to the packing in the membrane interfacial region. Excitation and emission wavelengths were set at 358 and 430nm. Excitation and emission slits

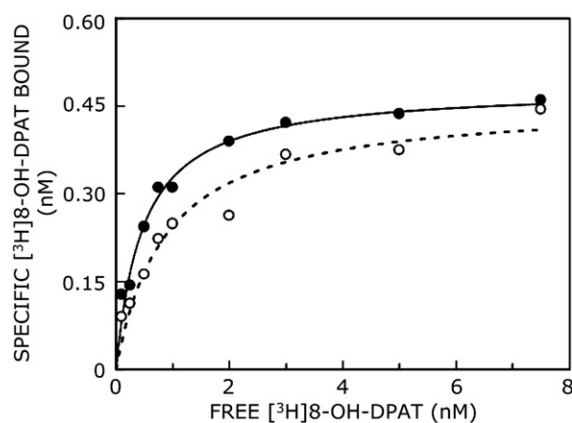


Fig. 4. Saturation binding analysis of specific [³H]8-OH-DPAT binding to serotonin_{1A} receptors in membranes isolated from untreated (control) and spingomyelinase treated CHO-5-HT_{1A}R cells. Representative plots are shown for specific [³H]8-OH-DPAT binding with increasing concentrations of free [³H]8-OH-DPAT under control (---○---) and 25 mU/ml (—●—) spingomyelinase treated conditions. The curves are non-linear regression fits to the experimental data using Graphpad Prism software. See text and Table 1 for other details.

Table 1
Effect of sphingomyelinase treatment on specific [³H]8-OH-DPAT binding parameters^a

Treatment	K_d (nM)	B_{max} (pmol/mg)
Control	0.61 ± 0.04	0.42 ± 0.03
25 mU/ml	0.47 ± 0.01	0.45 ± 0.02
50 mU/ml	0.43 ± 0.05	0.39 ± 0.02

^a Binding parameters were calculated by analyzing saturation binding isotherms with a range (0.1–7.5 nM) of radiolabeled [³H]8-OH-DPAT using Graphpad Prism software. The data represent the means ± S.E. of duplicate points from three independent measurements. See text for other details.

with nominal bandpasses of 1.5 and 20 nm were used. Cell monolayers, treated with sphingomyelinase, were lifted from the surface using 0.25 mM EDTA in phosphate buffered saline. The optical density of the samples measured at 358 nm was ~0.1 corresponding to cell density of ~3 × 10⁵ cells/ml. Higher cell concentrations were avoided as they could induce depolarization effects due to the scattering of the emitted light [28]. The concentration of TMA-DPH in the samples was 0.5 μM. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory at room temperature (~23°C). Anisotropy values were calculated from the equation [29]:

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

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH} . Fig. 3 shows the change in fluorescence anisotropy of TMA-DPH in untreated (control) and sphingomyelinase treated CHO-5-HT_{1A}R cells. Since the rate of internalization of TMA-DPH is relatively slow [27], these anisotropy values represent organization in the cellular plasma membranes. The fluorescence anisotropy of TMA-DPH appears to decrease significantly upon sphingomyelinase treatment. The apparent reduction in anisotropy was ~14% upon treatment with 50 mU/ml sphingomyelinase.

Saturation binding assays were carried out with increasing concentrations (0.1–7.5 nM) of the radiolabeled agonist [³H]8-OH-DPAT as described previously [16]. The protein content for the saturation binding assays was in the range of 40–50 μg. Binding data was analyzed as described previously [30]. The dissociation constant (K_d) and maximum binding sites (B_{max}) were calculated by non-linear regression analysis of binding data using Graphpad Prism software version 4.00 (San Diego, CA). Data obtained after regression analysis were used to plot graphs with GRAFIT program version 3.09b (Erithacus Software, Surrey, U.K.). Saturation binding analysis, shown in Fig. 4 and Table 1, with the agonist [³H]8-OH-DPAT binding carried out with membranes isolated from control and sphingomyelinase treated CHO-5-HT_{1A}R cells reveals that the increase in ligand binding can primarily be attributed to an increase in the affinity of ligand binding (up to ~30%, $p < 0.05$ using Graphpad Prism software) with no significant change in total binding sites.

Taken together, our results show that the specific ligand binding activity of the serotonin_{1A} receptor in membranes isolated from CHO-5-HT_{1A}R cells increases upon sphingomyelinase treatment due to an increase in the affinity of ligand binding. This could be due to the reduction in the cellular sphingomyelin content or the resultant increase in ceramide content, or both. The increase in ligand binding activity is accompanied by a reduction in membrane order, as monitored by fluorescence anisotropy of TMA-DPH in intact cells. We have earlier reported that membrane cholesterol is required in maintaining ligand binding activity of the serotonin_{1A} receptor [12,30]. We report here, for the first time, that sphingolipids (sphingomyelin and ceramide) are important for ligand binding activity of the serotonin_{1A} receptor. These results assume relevance

in the backdrop of the role of sphingolipids, in conjunction with cholesterol, in inducing membrane domains [2,3]. We have previously shown that the specific ligand binding activity of the serotonin_{1A} receptor displays an increase in membranes isolated from cholesterol-depleted CHO-5-HT_{1A}R cells [31]. Interestingly, we observe a similar increase in ligand binding activity in the present work, in which binding was monitored in membranes isolated from sphingomyelinase treated cells. It remains to be explored whether the underlying mechanism behind these effects is similar. For example, we have previously shown that cholesterol depletion induces dynamic confinement of the serotonin_{1A} receptor in the plasma membrane of living cells [31,32]. It would be interesting to speculate whether such dynamic rearrangement of the receptor could take place upon change in cellular sphingomyelin/ceramide contents.

Acknowledgements

This work was supported by the Council of Scientific and Industrial Research, Government of India. T.J.P. thanks the Council of Scientific and Industrial Research for the award of a Senior Research Fellowship. A.C. is an Adjunct Professor at the Special Centre for Molecular Medicine of Jawaharlal Nehru University (New Delhi, India), and Honorary Professor of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India). We gratefully acknowledge Sourav Halder for his help with fluorescence anisotropy measurements and members of our laboratory for critically reading the manuscript.

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