



## Membrane cholesterol depletion from live cells enhances the function of human serotonin<sub>1A</sub> receptors

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### ABSTRACT

Work from our laboratory has previously demonstrated the requirement of membrane cholesterol in the function of the serotonin<sub>1A</sub> receptor, a member of the G-protein coupled receptor (GPCR) superfamily. In order to monitor the effect of cellular organization on the function of human serotonin<sub>1A</sub> receptors, we explored receptor function following cholesterol depletion in live cells and membranes isolated from cholesterol-depleted cells. We report here the novel observation that while ligand binding of serotonin<sub>1A</sub> receptors displays an increase in membranes isolated from cholesterol-depleted cells, such trend is absent when binding is performed on cholesterol-depleted intact cells. Importantly, we show here, for the first time, that G-protein coupling of the serotonin<sub>1A</sub> receptor is enhanced in membranes isolated from cholesterol-depleted cells. These results assume pharmacological relevance in view of the recently described structural evidence of specific cholesterol binding sites in GPCRs, and may help in designing better therapeutic strategies for diseases related to GPCRs.

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### Introduction

Cholesterol is an essential lipid in higher eukaryotic cellular membranes and plays a crucial role in the organization, dynamics, function, and sorting of membranes. It is often found distributed non-randomly in domains in biological and model membranes [1–3]. Many of these domains (sometimes termed as 'lipid rafts') are thought to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains has proven to be challenging [3,4]. The idea of such specialized membrane domains gains relevance in cell physiology since important functions such as membrane sorting and trafficking [5], signal transduction processes [6], and the entry of pathogens [7–9] have been implicated to these putative domains.

Previous work from our laboratory has shown the requirement of membrane cholesterol in the function of the neurotransmitter receptor, the serotonin<sub>1A</sub> (5-HT<sub>1A</sub>) receptor (reviewed in [10]). Serotonin<sub>1A</sub> receptors represent one of the largest, evolutionarily

ancient, and highly conserved families of seven transmembrane G-protein coupled receptors (GPCRs) [11,12]. Serotonergic signaling plays a key role in the generation and modulation of various cognitive, behavioral and developmental functions [13]. This is supported by the fact that the agonists and antagonists of the serotonin<sub>1A</sub> receptor represent major classes of molecules with potential therapeutic effects in anxiety- and stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin<sub>1A</sub> receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for genetic vulnerability to complex traits such as anxiety disorders and aggression in higher animals [14,15]. In the context of increasing pharmacological relevance of the serotonin<sub>1A</sub> receptor, a transmembrane protein, its interaction with surrounding lipids assumes significance in modulating the function of the receptor in healthy and diseased conditions.

In this paper, we monitored the function of the human serotonin<sub>1A</sub> receptor upon specific depletion of membrane cholesterol from live cells stably expressing the receptor. Cholesterol depletion from cell membranes was achieved using methyl- $\beta$ -cyclodextrin (M $\beta$ CD). In order to explore the effect of cellular organization on the function of the serotonin<sub>1A</sub> receptor, we examined receptor function subsequent to cholesterol depletion in live cells and membranes isolated from cholesterol-depleted cells. The corresponding changes in membrane dynamics were monitored in both cases by monitoring fluorescence anisotropy of the membrane probe TMA-DPH. We report here the novel observation that while ligand binding of serotonin<sub>1A</sub> receptors displays an increase in membranes isolated from cholesterol-depleted cells, it does not exhibit

*Abbreviations:* 5-HT<sub>1A</sub> receptor, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; GPCR, G-protein coupled receptor; GTP- $\gamma$ -S, guanosine-5'-O-(3-thiotriphosphate); M $\beta$ CD, methyl- $\beta$ -cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene

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such trend when the binding assay is performed on cholesterol-depleted intact cells. Interestingly, the overall membrane order in cholesterol-depleted intact cells and in membranes isolated from cholesterol-depleted cells appears similar. Importantly, our results show that G-protein coupling of serotonin<sub>1A</sub> receptors is enhanced in membranes isolated from cholesterol-depleted cells.

## Materials and methods

**Materials.** Cholesterol, M $\beta$ CD, DMPC, EDTA, MgCl<sub>2</sub>, MnCl<sub>2</sub>, PMSF, serotonin, polyethylenimine, Tris, and Na<sub>2</sub>HPO<sub>4</sub> 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 (Carlsbad, CA). GTP- $\gamma$ -S was from Roche Applied Science (Mannheim, Germany). Amplex Red cholesterol assay kit and TMA-DPH were from Molecular Probes (Eugene, OR). BCA reagent for protein estimation was from Pierce (Rockford, IL). [<sup>3</sup>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). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Solvents used were of analytical grade.

**Cells and cell culture.** Chinese hamster ovary (CHO) cells stably expressing the human serotonin<sub>1A</sub> receptor (termed as CHO-5-HT<sub>1A</sub>R cells) were maintained as described earlier [16,17].

**Cholesterol depletion of cells in culture.** Cells at density of  $1.5 \times 10^6$  in 150 cm<sup>2</sup> flasks were grown for 3 days followed by incubation in serum-free D-MEM/F-12 (1:1) medium for 3 h at 37 °C. Cholesterol depletion was carried out by treating cells with increasing concentrations of M $\beta$ CD in serum-free D-MEM/F-12 (1:1) medium for 30 min at 37 °C, followed by wash with 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 buffer (buffer A).

**Cell membrane preparation.** Cell membranes were prepared as described earlier [16]. Total protein concentration in isolated membranes was determined using the BCA assay [18].

**Radioligand binding assay in isolated membranes.** Receptor binding assays were carried out as described earlier [16] with ~50  $\mu$ g total membrane protein. The concentration of [<sup>3</sup>H]8-OH-DPAT used was 0.29 nM.

**Radioligand binding assay on live cells.** CHO-5-HT<sub>1A</sub>R cells were washed with buffer A and detached from flasks in buffer A with 0.25 mM EDTA. Cells were spun at 2000 rpm for 5 min and suspended in serum-free D-MEM/F-12 (1:1) medium and counted using a hemocytometer. Cells (~10<sup>6</sup>) in 1 ml serum-free D-MEM/F-12 (1:1) medium were incubated in triplicate at 25 °C for 15 min in presence of 1 nM [<sup>3</sup>H]8-OH-DPAT. Non-specific binding was determined by performing the assay in presence of 10  $\mu$ M serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Brandel cell harvester (Gaithersburg, MD) through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0  $\mu$ m pore size) which were presoaked in 0.15% (w/v) polyethylenimine for 3 h. The 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 scintillation fluid.

**Analysis of cholesterol and phospholipid contents.** Cholesterol content in cell membranes was estimated using the Amplex Red cholesterol assay kit [19]. Total phospholipid content of membranes was determined subsequent to digestion with perchloric acid [20] using Na<sub>2</sub>HPO<sub>4</sub> as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

**GTP- $\gamma$ -S sensitivity assay.** In order to estimate the efficiency of G-protein coupling, GTP- $\gamma$ -S sensitivity assays were carried out as described earlier [16]. The concentration of GTP- $\gamma$ -S leading to 50% inhibition of specific agonist binding (IC<sub>50</sub>) were calculated by non-linear regression fitting of the data to a four-parameter logistic function:

$$B = [a/(1 + (x/I)^s)] + b \quad (1)$$

where  $B$  is the specific agonist binding normalized to binding at the lowest concentration of GTP- $\gamma$ -S,  $x$  denotes the concentration of GTP- $\gamma$ -S,  $a$  is the range ( $y_{\max} - y_{\min}$ ) of the fitted curve on the ordinate ( $y$ -axis),  $I$  is the IC<sub>50</sub> concentration,  $b$  is the background of the fitted curve ( $y_{\min}$ ) and  $s$  is the slope factor.

**Fluorescence anisotropy measurements on live cells and isolated cell membranes.** Fluorescence anisotropy measurements on live cells were carried out as described earlier [21]. Briefly, CHO-5-HT<sub>1A</sub>R cells (with or without M $\beta$ CD treatment) were washed twice with buffer A and were lifted from the surface using 0.25 mM EDTA in buffer A. Cells were gently mixed to obtain uniform suspension, and were immediately used for measuring cell density and fluorescence anisotropy. The optical density of the samples measured at 358 nm was ~0.1 which corresponds to cell density of  $\sim 2 \times 10^5$  cells/ml. Cell density was carefully chosen to avoid any depolarization due to scattering of emitted light [22]. Cell suspensions were added to a vigorously pre-mixed TMA-DPH suspension in buffer A, and mixed gently. The final concentration of TMA-DPH in the samples was 0.5  $\mu$ M. Samples were incubated in dark at room temperature (~23 °C) for 5 min prior to anisotropy measurements. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory at ~23 °C. Measurements were completed as soon as possible, after mixing cell suspensions with TMA-DPH. The parameters used for fluorescence anisotropy measurements were same as described earlier [17]. Intensities were found to be stable over time and were averaged over three 5 s readings. Anisotropy values were calculated from the equation [23]:

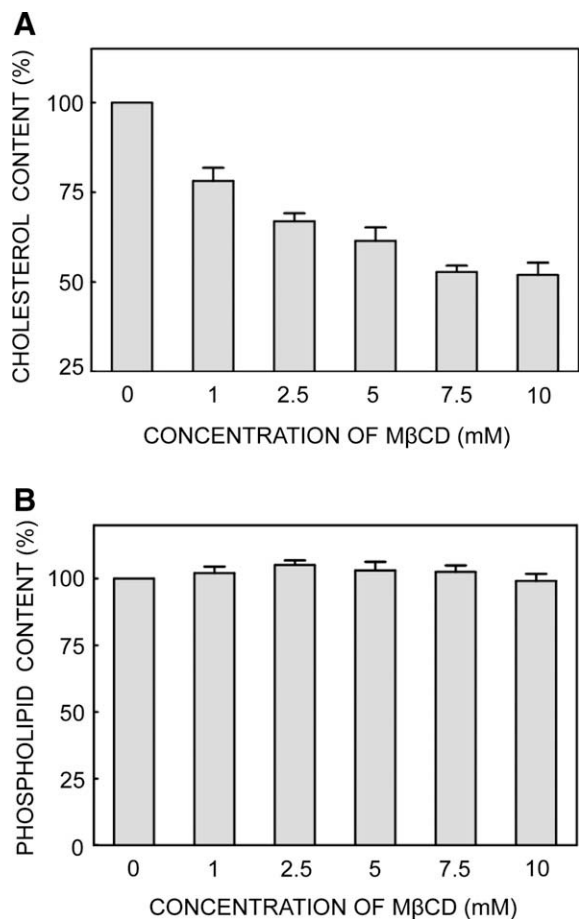
$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

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  $I_{HV}/I_{HH}$ . Experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 4. Fluorescence anisotropy measurements were also performed the same way using membranes isolated from CHO-5-HT<sub>1A</sub>R cells. In these cases, the samples contained 50 nmol of total phospholipids at a probe to phospholipid ratio of 1:100 (mol/mol) suspended in 1.5 ml of 50 mM Tris, pH 7.4 buffer as mentioned earlier [17].

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

## Results and discussion

The water-soluble compound M $\beta$ CD has previously been shown to selectively and efficiently extract cholesterol from membranes by including it in a central nonpolar cavity [24]. Fig. 1A shows the cholesterol content in membranes isolated from cholesterol-depleted CHO-5-HT<sub>1A</sub>R cells. Upon treatment of cells with 1 mM M $\beta$ CD, the membrane cholesterol content was reduced to ~78% of that of control (without treatment). This reduction in membrane cholesterol content appears to plateau with increasing concentrations of M $\beta$ CD above 5 mM. The cholesterol content of cell mem-

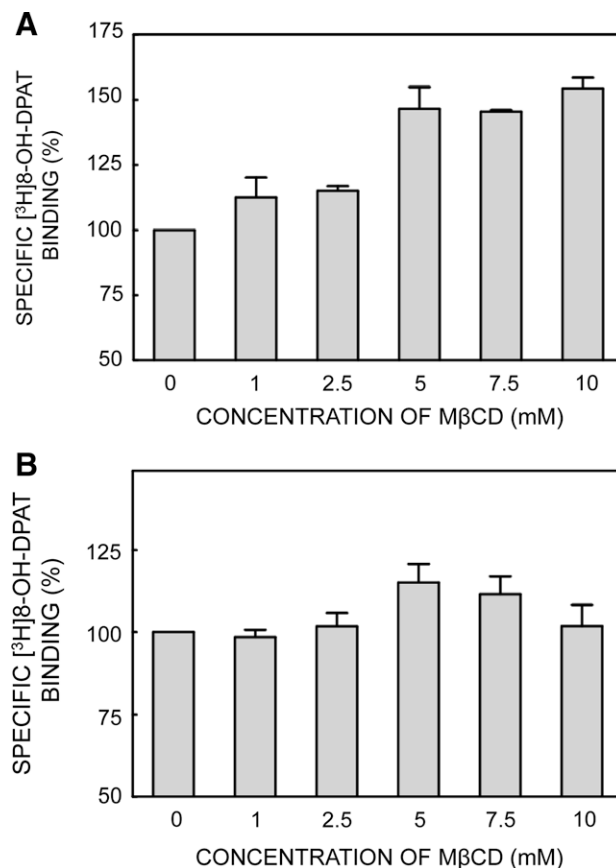


**Fig. 1.** Lipid contents of membranes isolated from CHO-5-HT<sub>1A</sub>R cells upon cholesterol depletion. (A) Cholesterol and (B) total phospholipid contents in membranes isolated from cells treated with increasing concentrations of MβCD. Values are expressed as percentages of the respective lipid content in control (without MβCD treatment) cell membranes. Data represent means ± SE from four independent measurements. See Materials and methods for other details.

branes was reduced to ~52% of the control, when cells were treated with 10 mM MβCD (Fig. 1A). The concentration range of MβCD was carefully chosen to minimize any possible change in membrane phospholipid content. Fig. 1B shows that the phospholipid content remains invariant under these conditions.

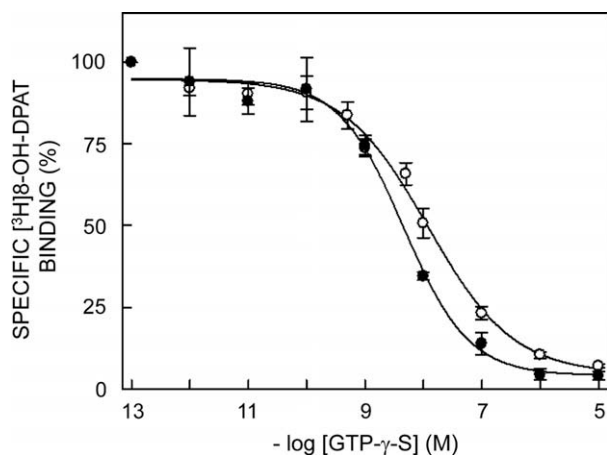
Fig. 2A shows the increase in specific [<sup>3</sup>H]8-OH-DPAT binding in membranes isolated from CHO-5-HT<sub>1A</sub>R cells treated with increasing concentrations of MβCD. For example, specific agonist binding is enhanced by ~54% of the control (in the absence of MβCD treatment), when 10 mM MβCD was used. Fig. 1A shows that the reduction in cholesterol content with increasing concentrations of MβCD is not linear. Interestingly, the increase in ligand binding activity of the serotonin<sub>1A</sub> receptor levels off at MβCD concentrations above 5 mM, possibly reflecting the relatively stationary levels of cholesterol content. Fig. 2B shows the effect of cholesterol depletion of CHO-5-HT<sub>1A</sub>R cells on specific [<sup>3</sup>H]8-OH-DPAT binding when the assay was carried out on intact cells. No appreciable increase in ligand binding is observed under this condition. The difference in results in these two cases could possibly be due to the differential membrane organization surrounding the immediate vicinity of the receptor. In other words, the immediate membrane environment around the receptor could be different in cholesterol-depleted intact cells and membranes isolated from them (but see later).

Guanine nucleotides are known to regulate ligand binding of GPCRs. The serotonin<sub>1A</sub> receptor agonists such as 8-OH-DPAT are



**Fig. 2.** Specific binding of the agonist [<sup>3</sup>H]8-OH-DPAT to the human serotonin<sub>1A</sub> receptor upon cholesterol depletion from CHO-5-HT<sub>1A</sub>R cells. (A) Specific [<sup>3</sup>H]8-OH-DPAT binding to receptors in membranes isolated from cells treated with increasing concentrations of MβCD. (B) Specific [<sup>3</sup>H]8-OH-DPAT binding to serotonin<sub>1A</sub> receptors in intact cells treated with increasing concentrations of MβCD. Values are expressed as percentages of specific binding obtained in control (without MβCD treatment) cells. Data represent means ± SE from at least four independent measurements. See Materials and methods for other details.

known to specifically activate pertussis toxin-sensitive G<sub>i</sub>/G<sub>o</sub> class of G-proteins in CHO cells [25]. Agonist binding to such receptors therefore displays sensitivity to agents such as GTP-γ-S, a non-hydrolyzable analog of GTP, that uncouple the normal cycle of guanine nucleotide exchange at the Gα subunit caused by receptor activation. We have previously shown that in the presence of GTP-γ-S, serotonin<sub>1A</sub> receptors undergo an affinity transition, from a high affinity G-protein coupled to a low-affinity G-protein uncoupled state [26]. Fig. 3 shows a characteristic reduction in binding of the agonist [<sup>3</sup>H]8-OH-DPAT in the presence of a range of concentrations of GTP-γ-S, in membranes isolated from cholesterol-depleted CHO-5-HT<sub>1A</sub>R cells. Table 1 shows that the half maximal inhibition concentration (IC<sub>50</sub>) of specific [<sup>3</sup>H]8-OH-DPAT binding by GTP-γ-S is ~15 nM for membranes isolated from control cells. The inhibition curve in case of membranes isolated from cells treated with 10 mM MβCD displays a significant (~3-fold, *p* < 0.05) shift toward lower concentrations of GTP-γ-S with a reduced IC<sub>50</sub> value of ~4.6 nM. This implies that the agonist binding to the serotonin<sub>1A</sub> receptor upon MβCD treatment is more sensitive to GTP-γ-S, indicating that the G-protein coupling efficiency is enhanced in membranes from cholesterol-depleted cells. Interestingly, the cholesterol content in membranes has previously been reported to modulate the function of G-proteins [27,28]. These experiments could not be performed in intact cells since GTP-γ-S is impermeable to cells.



**Fig. 3.** Enhanced G-protein coupling of the serotonin<sub>1A</sub> receptor upon cholesterol depletion. The figure shows the effect of increasing concentrations of GTP- $\gamma$ -S on specific binding of [<sup>3</sup>H]8-OH-DPAT to serotonin<sub>1A</sub> receptors in membranes isolated from CHO-5-HT<sub>1A</sub>R cells treated with (●) or without (○) M $\beta$ CD. Values are expressed as percentages of specific binding obtained at the lowest concentration of GTP- $\gamma$ -S. The curves are non-linear regression fits to the experimental data using Eq. (1). Data points represent means  $\pm$  SE of duplicate points from at least three independent measurements. See Materials and methods for other details.

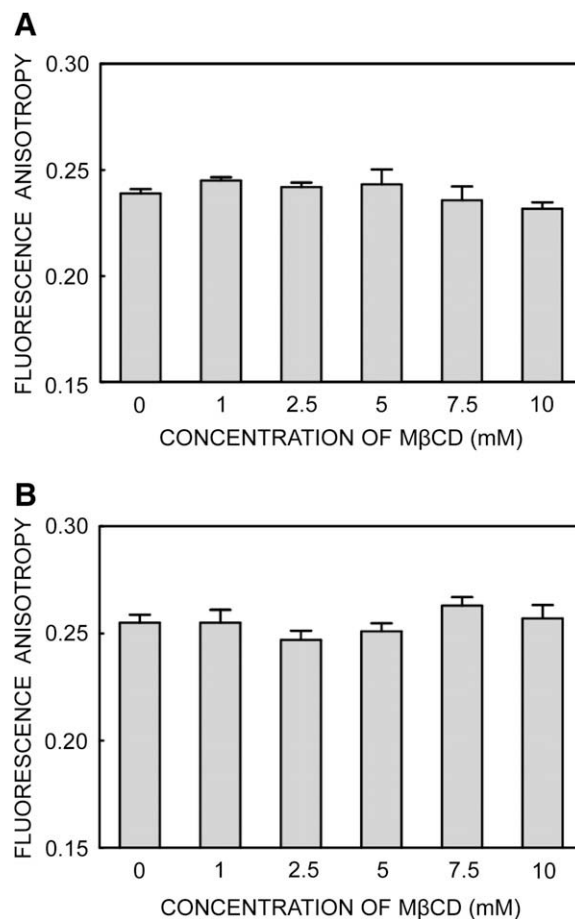
**Table 1**  
Effect of membrane cholesterol depletion from live cells on the efficiency of G-protein coupling.<sup>a</sup>

Condition	IC <sub>50</sub> (nM)
Control	15.01 $\pm$ 2.90
M $\beta$ CD treated	4.61 $\pm$ 0.35

<sup>a</sup> Sensitivity of specific [<sup>3</sup>H]8-OH-DPAT binding to GTP- $\gamma$ -S in the assay was measured by calculating the IC<sub>50</sub> for inhibition of [<sup>3</sup>H]8-OH-DPAT binding in the presence of a range of concentration of GTP- $\gamma$ -S. Inhibition curves were analyzed using the four-parameter logistic function. Data represent means  $\pm$  SE from at least three independent measurements. See Materials and methods for other details.

The observed increase in specific ligand binding activity upon reduction in membrane cholesterol content could be due to an alteration in overall membrane organization. In order to monitor the change in overall membrane order, we monitored the steady state fluorescence anisotropy of 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 [29]. Fluorescence anisotropy measurements using TMA-DPH were carried out on cholesterol-depleted cell suspensions and membranes isolated from cholesterol-depleted cells. TMA-DPH has previously been shown to specifically label plasma membranes of live cells [30]. Fluorescence anisotropy measured using probes such as TMA-DPH, is correlated to its rotational diffusion [23], which is sensitive to the packing (order) in the membrane interfacial region. The amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid-water interface [31]. The average location of DPH in TMA-DPH has been shown to be  $\sim$ 11 Å from the center of the bilayer [32].

Fig. 4A shows the fluorescence anisotropy of TMA-DPH in membranes isolated from control and cholesterol-depleted CHO-5-HT<sub>1A</sub>R cells. Fig. 4B, on the other hand, shows the fluorescence anisotropy of TMA-DPH in intact CHO-5-HT<sub>1A</sub>R cells (control and cholesterol-depleted). Since the rate of internalization of TMA-DPH is relatively slow [30], these anisotropy values represent organization in the cellular plasma membranes. Interestingly, the fluorescence anisotropy does not exhibit any appreciable change in cholesterol-depleted intact cells or in membranes isolated from them. This implies that while the overall membrane order of intact



**Fig. 4.** Steady state fluorescence anisotropy of the membrane probe TMA-DPH in CHO-5-HT<sub>1A</sub>R cells treated with increasing concentrations of M $\beta$ CD. Fluorescence anisotropy in (A) isolated membranes and (B) intact cells are shown. The excitation wavelength was 358 nm and emission was monitored at 430 nm. Data shown are means  $\pm$  SE of samples from at least four (isolated membranes) and six (live cells) independent measurements. See Materials and methods for other details.

cells and isolated membranes is comparable, there is no significant ( $p > 0.05$ ) change in overall membrane order upon membrane cholesterol depletion in both cases. This suggests that there is no appreciable overall reorganization during the isolation of membranes from cells, as monitored utilizing TMA-DPH anisotropy.

Previous work from our laboratory has shown that depletion of cholesterol from isolated hippocampal membranes using M $\beta$ CD leads to the reduction in ligand binding and G-protein coupling of the serotonin<sub>1A</sub> receptor [33]. In this context, our present observation of increase in agonist binding (Fig. 2A) and G-protein coupling (Fig. 3) of serotonin<sub>1A</sub> receptors in membranes isolated from cholesterol-depleted CHO-5-HT<sub>1A</sub>R cells appears contrary. A possible reason for this discrepancy could be a unique rearrangement of the membrane environment in the vicinity of the receptor, which occurs only upon cholesterol depletion of intact cells in culture and not while depletion is carried out from isolated membranes. Interestingly, we report here that the ligand binding does not exhibit appreciable change when the assay was performed on intact cells. This is in spite of the observation that the overall membrane order of intact cells and membranes isolated from them appears comparable, and it does not display significant change upon cholesterol depletion. In addition, we have previously shown that cholesterol depletion of CHO-5-HT<sub>1A</sub>R cells does not result in significant alteration in the overall distribution of serotonin<sub>1A</sub> receptors [34]. Taken together, these results imply that specific ligand binding to serotonin<sub>1A</sub> receptors upon cholesterol depletion is

influenced by a number of factors depending on whether (i) depletion is carried out in isolated membranes or intact cells and (ii) the binding assay is performed in isolated membranes or intact cells following cholesterol depletion. The differences in ligand binding observed in these cases appear to originate predominantly due to subtle changes in local environment around the receptor rather than from global changes in membrane organization (Fig. 4). Another novel aspect of our present results is that the G-protein coupling of the receptor is enhanced in membranes isolated from cholesterol-depleted cells. Overall, our results are relevant in the context of interaction of membrane cholesterol with GPCRs, particularly in view of the recently described structural evidence of specific cholesterol binding site(s) in GPCRs [35].

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