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Cholesterol depletion reduces serotonin binding and signaling via human 5-HT$_{7(a)}$ receptors

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Abstract

Lipids, including cholesterol, are critical components of the cell membrane where they are enriched in microdomains, lipid rafts, which organize and concentrate receptors and intracellular proteins involved in signal transduction. The present study examined the effects of cholesterol depletion on serotonin (5-HT) binding and signaling via 5-hydroxytryptamine$_7$ (5-HT$_7$) receptors in stably transfected HeLa cells. Immunohistochemical, ligand-binding and biotinylation experiments demonstrated that the studied cells expressed high levels of 5-HT$_7$ receptors at their surface with a pharmacological profile resembling 5-HT$_7$ receptors in native tissue. Depletion of cholesterol, by combined treatment with mevastatin, fumonisin B$_1$ and mevalonate or methyl-β-cyclodextrin (MβCD), caused highly significant reductions in the B_{max} values of [^{3}H]5-HT- and [^{3}H]-(*R*)-3-(2-(2-(4-methylpiperidin-1-yl)-ethyl)pyrrolidine-1-sulfonyl)phenol ([^{3}H]SB269970)-binding to 5-HT$_7$ receptors. Cholesterol depletion also reduced the total level of 5-HT$_7$ receptor protein determined by Western blot analysis. None of the examined treatments affected the affinity of [^{3}H]5-HT- or [^{3}H]SB269970-binding to 5-HT$_7$ receptors. Treatment with serotonin caused strong inductions in the phosphorylation states of Ser^{63}-ATF-1 and Ser^{133}-CREB. These effects of serotonin on signal transduction were significantly counteracted by pre-treatment with cholesterol synthesis inhibitors. Altogether, the present study demonstrates that cholesterol depletion decreases binding of both agonist and antagonist radioligands to 5-HT$_7$ receptors and counteract 5-HT$_7$ receptor-mediated intracellular signaling.
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Keywords: Lipid raft; G protein coupled receptor; SB269970; Serotonin; 5-HT$_7$ receptor; Cholesterol

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) plays a crucial role in a number of physiological and pathological processes in many organs including the brain, gastrointestinal tract, blood cells and vessels. Serotonin exerts its effects via fourteen different receptors (Barnes and Sharp, 1999). All of them are G protein coupled receptors, with the exception of 5-HT$_3$ receptors, which are ionotropic. They act primarily by means of the following second messenger systems: 5-HT$_{1A,B,D,E,F}$ receptors decrease cyclic AMP (cAMP) formation; 5-HT$_{2A,B,C}$ receptors increase inositol triphosphate and diacylglycerol formation; 5-HT$_3$ receptors increase Na^+ and Ca^{2+} influx; and 5-HT$_{4,6,7}$ receptors increase cAMP formation. The physiological effector system for 5-HT$_{5A,B}$ receptors remains elusive, although inositol triphosphate and diacylglycerol formation have been implicated in transfected cells (Francken et al., 2000).

5-HT$_7$ receptors are widely expressed in different cell types in the central nervous system and in peripheral tissues (Ruat et al., 1993; Shen et al., 1993; Shimizu et al., 1997). Genetic and pharmacological studies have provided evidence that 5-HT$_7$ receptors may be involved in regulation of emotions, thermoregulation, circadian rhythmicity, memory processes and smooth muscle relaxation (Vanhoenacker et al., 2000; Hedlund and Sutcliffe, 2004).

Lipids, including cholesterol and sphingolipids, play important roles to form dynamic liquid ordered microdomains in cellular membranes that regulate the sub-cellular localization of certain membrane receptors and signaling molecules (for review, see Simons and Toomre, 2000). These lipid microdomains are often referred to as "lipid rafts", although the biological substrate for this notion is not clearly defined (Munro, 2003). Nevertheless, there is vast literature showing that membranous lipids

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organize and concentrate intracellular proteins mediating signal transduction. Several studies have also shown the importance of cholesterol-enriched membrane domains in controlling signaling via G protein coupled receptors, such as Neurokinin 1 and β-Adrenergic receptors (Monastyrskaya et al., 2005; Rybin et al., 2000; Chini and Parenti, 2004). Moreover, several proteins involved in G protein coupled receptor-mediated signal transduction, such as G proteins (Oh and Schnitzer, 2001) and adenylyl cyclase subtypes (Rybin et al., 2000) are clustered in cholesterol-enriched membrane domains.

The present study utilized HeLa cells stably transfected with human 5-HT_7 receptors to investigate whether 5-HT_7 receptor function is affected by alterations in cholesterol levels. For this purpose, we studied the effects of cholesterol depletion on [^{3}H]5-HT- and [^{3}H]SB269970-binding to 5-HT_7 receptors and on serotonin-mediated signaling via 5-HT_7 receptors.

2. Materials and methods

2.1. Materials

HeLa cells stably transfected with the human 5-$HT_{7(a)}$ receptor were generated and cultured as previously described (Heidmann et al., 1997; Zhukovskaya and Neumaier, 2000). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Trypsin-EDTA was from Invitrogen (Stockholm, Sweden). Geniticin (G-418) was from Promega (SDS Biosciences, Falkenberg, Sweden). Mevastatin was from Tocris (Bromma, Sweden). NHS-biotin and Neutravidin were from Pierce (Stockholm, Sweden). Protease inhibitor cocktail III was from Calbiochem (Stockholm, Sweden). Bovine serum albumin (BSA), methyl-β-cyclodextrin (MβCD), cholesterol (water-soluble), mevalonate, fumonisin B_1, 5-HT, 5-Metoxytryptamine (5-MeOT), Clozapine, (*R*)-3-(2-(2-(4-Methylpiperidin-1-yl)-ethyl)pyrrolidine-1-sulfonyl)phenol (SB269970), Ketanserin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), Theophylline, Triton X-100, Raclopride and Pargyline were all from Sigma (Stockholm, Sweden). [^{3}H]5-HT (108 Ci/mmol), [^{3}H]5-Carboxytryptamine (5-CT; 109 Ci/mmol) and [^{3}H]SB269970 (36 Ci/mmol) were from GE Healthcare (Uppsala, Sweden). Unless indicated, all fine chemicals were from Sigma (Stockholm, Sweden).

2.2. Culture of HeLa cells stably transfected with the human 5-$HT_{7(a)}$ receptor

Cells were maintained in DMEM, containing 4.5 g/l glucose, 2 mM L-Glutamine, 25 mM HEPES, 10% FBS and 500 μg/ml G-418 at 37 °C in 5% CO_2/95% air. Cells were split by trypsination two times a week at a ratio of 1:10. For binding and biotinylation assays, cells were plated in 100 mm plates in complete medium at a density of 400,000 cells/ml one day before the assay. For cholesterol analysis, MTT viability and protein phosphorylation experiments, cells were plated in 6-well plates in complete medium at a density of 500,000 cells/ml one day before the assay.

2.3. Immunofluorescent visualization of 5-HT_7 receptors

5-HT_7 receptor-expressing HeLa cells were fixed with 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 10 min. Nonspecific staining was blocked by 30 min of incubation with 10% BSA in PBS. Cells were thereafter incubated for 1 h with rabbit anti-5-HT_7 receptor antibody diluted in 10% BSA (1:500; Sigma, Stockholm, Sweden), followed by washes and incubation with Alexa Fluor 568-labeled goat anti-rabbit secondary antibody in 10% BSA for 1 h (1:500; Invitrogen, Stockholm, Sweden). After additional washes in PBS, cover slips were mounted on the cells by using Gel/Mount (Biomeda, Foster City, CA, USA). Fluorescent images of 5-HT_7 receptors were acquired by using a laser-scanning microscope (Zeiss, Hamburg, Germany).

2.4. Biotinylation experiments to examine surface expression of 5-HT_7 receptors

Cells were washed twice with ice-cold PBS and thereafter incubated for 30 min with 4 ml biotin solution (1.5 mg/ml in PBS) at room temperature. Cells were washed three times with Tris-buffered saline (TBS) pH 7.4 and twice with PBS and incubated for 20 min in 830 μl Triton X-100-based buffer (10 mM Tris pH 7.5, 120 mM NaCl, 25 mM KCl, 1 μl/ml Protease inhibitor cocktail III, 0.5% v/v Triton X-100) at room temperature. Cells were harvested, briefly sonicated and centrifuged for 10 min at 9000 ×*g* at 4 °C. 75 μl of the supernatant (total fraction) was kept. The remaining supernatant was transferred to new tubes and mixed overnight with 50 μl Neutravidin at 4 °C. Samples were centrifuged for 10 min at 17,000 ×*g* at 4 °C. 75 μl of the supernatant was then removed (cytosolic fraction). The pellet (membrane fraction) was washed five times with the aforementioned Triton X-100-based buffer. The samples were then separated by 15% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred overnight to Immobilon-P membranes (Millipore, Stockholm, Sweden). The membranes were incubated for 1 h with 5% (w/v) dry milk in TBS-Tween20, for 2 h with the primary rabbit antibody against 5-HT_7 receptors (1:500), washed in TBS-Tween20, incubated for 1 h with a secondary horse radish peroxidase (HRP)-coupled anti-rabbit antibody (1:6000; Pierce, Stockholm, Sweden) and finally washed in TBS-Tween20. The immunoreactive bands with a molecular weight corresponding to 5-HT_7 receptors were detected by chemiluminescence, using enhanced chemiluminescence (ECL) reagents (Perkin Elmer, Stockholm, Sweden).

2.5. Radioligand binding in intact cells

Radioligand-binding experiments were performed with [^{3}H]5-HT, [^{3}H]5-CT and [^{3}H]SB269970. Cells were harvested in PBS and centrifuged for 5 min at 2000 ×*g*. They were then resuspended in serum-free DMEM supplemented with 0.1% BSA to a density of 2×10^6 cells/ml, which correspond to ~400 μg total protein. 50 μl cell suspension (100,000 cells/well), 50 μl radioligand and 50 μl vehicle or displacing agents were added to

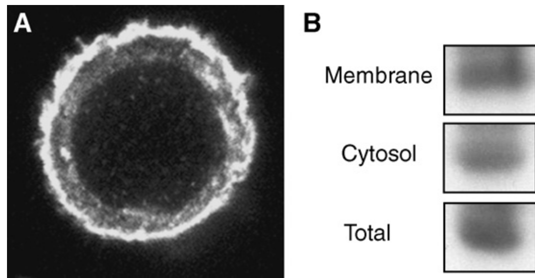

Fig. 1. Localization of 5-HT_7 receptors in the studied cell line. (A) Immunofluorescence image showing 5-HT_7 receptor staining in a representative cell from the stably 5-HT_7 receptor transfected HeLa cells. Note the enriched staining at the cell surface and submembranous compartments. (B) Western blot analysis of 5-HT_7 receptors in membrane, cytosolic and total cell extracts separated in a biotinylation experiment.

wells in a 96-well microtiter plate. All binding assays were performed in the presence of 10 μM Pargyline to inhibit degradation of serotonin. For saturation experiments, nonspecific binding was defined as the binding occurring in the presence of 10 μM 5-MeOT. Except for association/dissociation experiments, the incubations lasted for 1 h at room temperature. Assays were terminated by rapid filtration over glass fiber filters using a Skatron 1719 cell harvester (Skatron AS, Tranby, Norway). Filters were thereafter washed with 5 ml ice-cold PBS before being transferred to scintillation vials (Molecular Devices, Gothenburg, Sweden) and incubated with 3 ml scintillation fluid (Ready Safe, Beckman Coulter, Bromma, Sweden), at room temperature overnight. Vials were counted in a β-counter (1209 Rackbeta; LKB, Stockholm, Sweden) for 2 min/vial. All experiments were performed with duplicate samples.

2.6. Cholesterol depletion

Two different approaches were used to lower cholesterol levels. For cholesterol depletion by synthesis inhibition, cells were treated with mevastatin, fumonisin B_1 and mevalonate in DMEM with 0.1% BSA for 24 h at 37 °C. In some experiments cholesterol levels were restored by replacing mevastatin, fumonisin B_1 and mevalonate with medium containing 10 μg/ml cholesterol for 60 min. (For the phosphorylation experiments, the treatment was done in the absence of fumonisin B_1, as this agent has been reported to affect mitogen activated protein kinase (MAPK) (Wattenberg et al., 1996). Similar results were obtained in binding experiments regardless whether fumonisin B_1 was included in the treatment solution (data not shown). For cholesterol depletion by sequestering cholesterol, cells were treated with methyl-β-cyclodextrin (MβCD) in serum-free DMEM supplemented with 0.1% BSA for 30 min at 37 °C. Restoration of cholesterol levels was performed as described above.

2.7. Analysis of cholesterol content

Cells were treated with cholesterol depleting agents as described above. Cells were washed twice, harvested in ice-cold

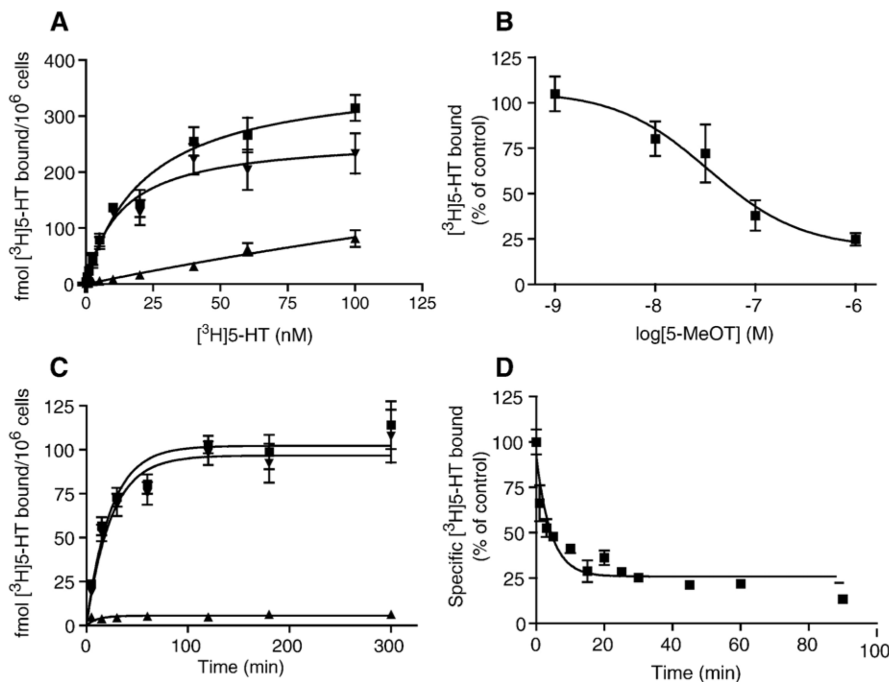

Fig. 2. [^{3}H]5-HT binding to 5-HT_7 receptors. (A) Saturation experiment showing total (■), unspecific (▲) and specific (▼) [^{3}H]5-HT binding. The specific binding is 71% of the total binding with a K_d value of 13.1±3.6 nM and a B_{max} value of 262±21.8 fmol/10^6 cells. (B) Displacement of 10 nM [^{3}H]5-HT binding by 5-MeOT occurs with a K_i value of 73 nM (95% C.I. 47–113 nM) and a Hill coefficient of −0.93 (95% C.I. −2.270–0.3914). (C) 10 nM [^{3}H]5-HT associates to the 5-HT_7 receptor with a k_{+1} of 1.8×10^7 M^{-1} min^{-1} and reaches maximum after 120 min. Total (■), unspecific (▲) and specific (▼) binding is shown. (D) Dissociation of 10 nM [^{3}H]5-HT from 5-HT_7 receptors occurs with a half life of 2.97 min and a k_{-1} of 0.23±0.04 min^{-1}. (A) Results from ten experiments. (B, C, D) Results from three experiments. All experiments were run in duplicates.

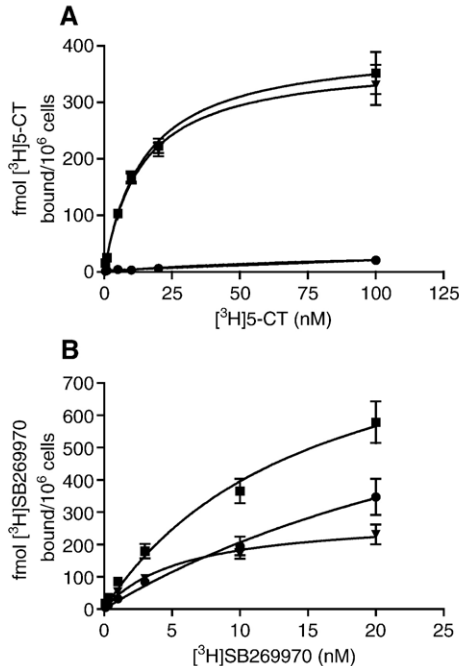

Fig. 3. [^{3}H]5-CT- and [^{3}H]SB269970-binding to 5-HT$_7$ receptors. (A) Saturation experiment showing the total (■), unspecific (●) and specific (▼) [^{3}H]5-CT binding with a K_d value of 13.7±2.3 nM and a B_{max} value of 375±22.2 fmol/10^6 cells. (B) Saturation experiment showing the total (■), unspecific (●) and specific (▼) binding of the 5-HT$_7$ receptor selective antagonist [^{3}H]SB269970 with a K_d value of 5.8±1.8 nM and a B_{max} value of 288±33.0 fmol/10^6 cells. (A) Results from three experiments. (B) Results from two experiments. All experiments were run in duplicates.

PBS and collected by centrifugation for 5 min at 3300 ×*g* at 4 °C. Pellets were re-suspended in PBS and protein concentration of each sample was determined using BSA as a standard, according to the manufacturer's protocol (Pierce, Stockholm, Sweden). Sample volume corresponding to 400 µg of total protein was added to duplicate wells on a 96-well microtiter plate. 200 µl Infinity cholesterol reagent (Thermo Electron, Victoria, Australia) was added to each well. The plate was incubated at 37 °C for 10 min and the colored reaction was analyzed at 490 nm in a Thermo max spectrophotometer (Molecular Devices, Stockholm, Sweden).

2.8. MTT viability assay

The cells were treated with either mevastatin, fumonisin B$_1$ and mevalonate or methyl-β-cyclodextrin for 24 h or 30 min, respectively. To determine appropriate concentrations of each of the treatments, the effects of several different concentrations were initially examined in the standard MTT viability assay. The used concentrations of mevastatin, fumonisin B$_1$ and mevalonate treatment were; 0.4, 2, 4, 8 and 20 µM mevastatin, 1, 5, 10, 20 and 50 µM fumonisin B$_1$ and 25, 125, 250, 500 and 1250 µM mevalonate. The concentrations of methyl-β-cyclodextrin were 0.1, 1, 5, 10, 20 and 50 mM. MTT was dissolved in PBS and added to the media at a final concentration of 0.3 mg/ml for 1 h at 37 °C. Media was then removed, the cells

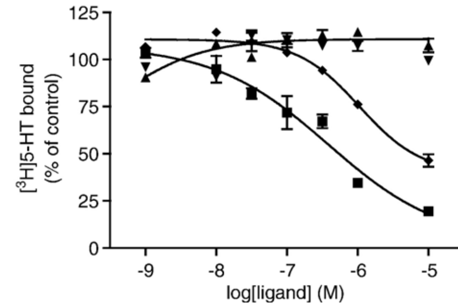

Fig. 4. Displacement of [^{3}H]5-HT binding at the 5-HT$_7$ receptors by various antagonists. The selective 5-HT$_7$ receptor antagonist, SB269970 (■), displaces [^{3}H]5-HT binding with a K_i value 158 nM (95% C.I. 71–620 nM) and a Hill coefficient of −0.54 (95% C.I. −1.06–0.014). The non-selective 5-HT$_7$ receptor antagonist, clozapine (◆) displaces [^{3}H]5-HT with a K_i value of 594 nM (95% C.I. 385–918 nM) and a Hill coefficient of −0.99 (95% C.I. −1.55–0.44), whereas neither ketanserin (▼) nor raclopride (▲) shows any detectable affinity for the 5-HT$_7$ receptors under the present conditions. Results from two experiments run in duplicates.

lysed and harvested in isopropanol with 0.04 M HCl. The MTT-induced color reaction, corresponding to cell viability, was detected at 540 nm. The viability for each sample was expressed as percent of control.

2.9. Western blot analysis to determine the phosphorylation states of CREB and ATF-1

Cells were pre-treated with DMEM that included 10 µM Pargyline, to inhibit metabolism of serotonin, 5 mM Theophylline, to inhibit cAMP phosphodiesterases, and 0.1% BSA for 15 min at 37 °C. Stimulation of phosphorylation events was done by the addition of 10 µM 5-HT for 30 min at 37 °C. After several washes, cells were harvested in ice-cold PBS, containing a cocktail of protease inhibitors and collected by centrifugation for 5 min at 3300 ×*g* at 4 °C. The PBS was then removed and the pellets frozen at −80 °C. The pellets were sonicated in 1% sodium dodecyl sulfate (SDS) and boiled for 10 min. Protein concentrations of each sample were

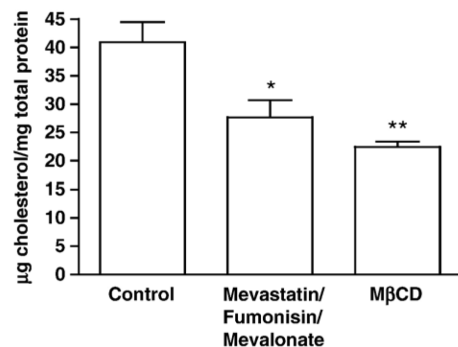

Fig. 5. Effects of treatments with mevastatin, fumonisin B$_1$ and mevalonate or methyl-β-cyclodextrin on cholesterol levels. Histogram showing the effects of 8 µM mevastatin/20 µM fumonisin B$_1$ /500 µM mevalonate or 10 mM of methyl-β-cyclodextrin on cholesterol levels. Results from three experiments. *$p<0.05$; **$p<0.01$ by Student's *t*-test.

determined, using BSA as a standard, according to the manufacturer's description (Pierce, Stockholm, Sweden). 25 μg of each sample was subjected to SDS-PAGE using a 12% running gel and transferred overnight to Immobilon-P membranes (Millipore, Stockholm, Sweden). The membranes were incubated for 1 h with 5% (w/v) dry milk in TBS-Tween20, for 2 h with the primary rabbit antibody against either Ser^{133}-CREB/Ser^{63}-ATF-1 (1:500; Cell Signaling, Danvers, MA, USA) or total CREB (1:500; Upstate Biochemicals, Malmo, Sweden), washed in TBS-Tween20, incubated for 1 h with a secondary HRP-coupled anti-rabbit antibody (1:6000; Pierce, Stockholm, Sweden) and finally washed in TBS-Tween20. The immunoreactive bands with molecular weights corresponding to CREB or ATF-1 were detected by chemiluminescence, using ECL reagents (Perkin Elmer, Stockholm, Sweden).

2.10. Data analyses and statistics

Results from radioligand experiments were analyzed using non-linear curve fitting in the GraphPad Prism 4 software (GraphPad Prism, San Diego, CA, USA). For determining the association constant (k_{+1}) the following formula was used: $k_{+1}=k_{obs}\ /(L^*+K_d)$, where k_{obs} is the slope of the first-order plot, L^* is the concentration of [^{3}H]5-HT used and K_d is the dissociation constant determined from saturation binding experiments. From the dissociation binding experiments the dissociation rate constant k_{-1} is given directly in the non-linear regression analysis (GraphPad Prism, San Diego, CA, USA). Autoradiograms from Western blot experiments were quantified with the NIH Image 1.63 software (NIH, USA). For studies on protein phosphorylation, levels of phosphorylated proteins were

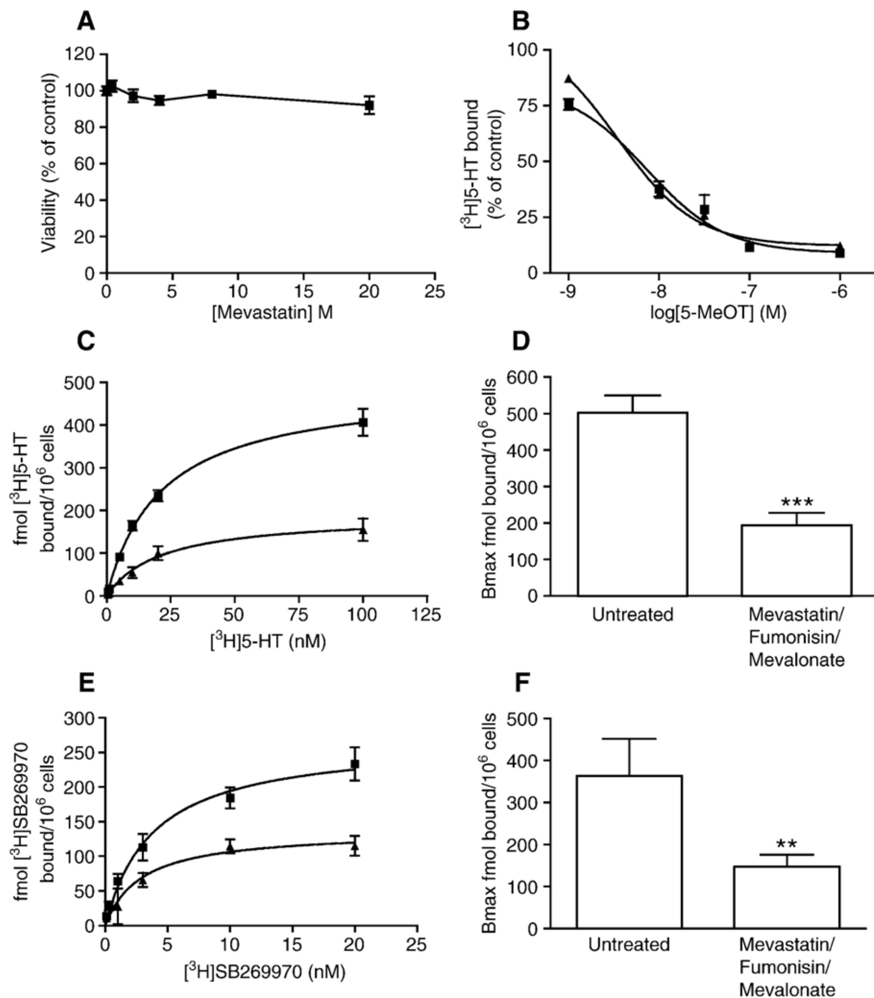

Fig. 6. Effects of mevastatin, fumonisin B_1 and mevalonate on [^{3}H]5-HT- and [^{3}H]SB269970-binding to 5-HT_7 receptors. (A) Results from an MTT assay showing that cholesterol depletion after mevastatin/fumonisin B_1/mevalonate treatment has no significant effects of cell viability. The used concentrations were: 0.4/1/25, 2/5/125, 4/10/250, 8/20/500 and 20/50/1250 μM (mevastatin/fumonisin B_1/mevalonate). (B) Displacement experiments showing that the treatment does not affect the ability of 5-MeOT to displace [^{3}H]5-HT from 5-HT_7 receptors. (C, D) Saturation experiments showing that cholesterol depletion decreases the B_{max} value of [^{3}H]5-HT binding. Untreated cells (■) have a B_{max} value of 492.6±25.5 fmol/10^6 cells and mevastatin/fumonisin B_1/mevalonate treated cells (▲) have a B_{max} value of 189.7±24.2 fmol/10^6 cells, a reduction by 61 %. (E, F) Saturation experiments showing that cholesterol depletion decreases the B_{max} value of [^{3}H]SB269970-binding. Untreated cells (■) have a B_{max} value of 268.2±22.8 fmol/10^6 cells and cells treated with mevastatin, fumonisin B_1 and mevalonate (▲) have a B_{max} value of 137.0±17.4 fmol/10^6 cells, a reduction by 51%. (A) Results from one experiment run in triplicates. (B) Results from two experiments run in duplicates. (C, D) Results from three experiments run in duplicates. (E, F) Results from two experiments run in duplicates. $^{**}p<0.01$; $^{***}p<0.001$ by Student's t-test.

normalized to total protein levels. The data were analyzed with Student's *t*-test to evaluate statistical differences (GraphPad Prism 4).

3. Results

3.1. Characterization of HeLa cells stably transfected with the human 5-HT$_{7(a)}$ receptor

The localization of G protein coupled receptors is dynamically regulated between the cell surface and sub-membranous compartments. To examine whether the studied 5-HT$_7$ receptors were localized at the cell surface, three distinct experimental approaches were used. Immunofluorescence experiments demonstrated that most of the studied cells exhibited staining for 5-HT$_7$ receptors enriched at the cell surface (Fig. 1A). Biotinylation experiments showed that membranous 5-HT$_7$ receptors were biotinylated when exposed to biotin in the extracellular medium (Fig. 1B). Radioligand-binding experiments showed that [^{3}H]5-HT binding to 5-HT$_7$ receptors occurred in a saturating manner (Fig. 2A). The specific binding of [^{3}H]5-HT to 5-HT$_7$ receptors is 71% of the total binding, with a B_{max} value of 262±21.8 fmol/10^6 cells and a K_d value of 13.1±3.6 nM (Fig. 2A). No specific binding of [^{3}H]5-HT was found in untransfected HeLa cells and free [^{3}H]5-HT ligand did not bind to the glass fiber filters (data not shown). 10 nM [^{3}H]5-HT was displaced by 5-MeOT with a K_i value of 73 nM (95% C.I. 47–113 nM) (Fig. 2B). The specific binding of [^{3}H]5-HT reached maximum after 120 min with an observed association rate constant (k_{obs}) of 0.41±0.009 min^{-1} and a corresponding association rate constant (k_{+1}) of 1.8×10^7 M^{-1} min^{-1} (Fig. 2C). The dissociation occurred with a half-life of 2.97 min, giving a corresponding dissociation rate constant (k_{-1}) of 0.23±0.04 min^{-1} (Fig. 2D).

To further confirm that the 5-HT$_7$ receptors are expressed at high levels at the cell surface, we also performed saturation experiments with an additional agonist, [^{3}H]5-CT, and with an antagonist, [^{3}H]SB269970. Both these ligands have high affinity for 5-HT$_7$ receptors (Ruat et al., 1993; Shen et al., 1993; Lovell et al., 2000). The specific binding of [^{3}H]5-CT to 5-HT$_7$ receptors is 94% of the total binding, with a B_{max} value of 375±22.2 fmol/10^6 cells (Fig. 3A) and a K_d value of 13.7±2.3 nM. The specific binding of [^{3}H]SB269970 to 5-HT$_7$ receptors is 29% of the total binding, with a B_{max} value of 288±33.0 fmol/10^6 cells (Fig. 3B) and a K_d value of 5.8±1.8 nM.

We also examined the ability of a set of ligands to displace [^{3}H]5-HT binding to 5-HT$_7$ receptors. As shown in Fig. 4, SB269970 displaced 10 nM [^{3}H]5-HT with a K_i value of 158 nM (95% C.I. 71–620 nM). Clozapine also displaced [^{3}H] 5-HT binding to 5-HT$_7$ receptors (K_i value 594 nM; 95% C.I. 385–918 nM), whereas ketanserin and raclopride caused no displacement under the present conditions. Altogether, these data indicate that the examined 5-HT$_7$ receptors have a similar pharmacology as other cloned 5-HT$_7$ receptors (Shen et al., 1993; Krobert et al., 2001), as well as 5-HT$_7$ receptors expressed in native brain tissue (To et al., 1995).

3.2. Effect of cholesterol depletion on radioligand binding to human 5-HT$_{7(a)}$ receptors

To lower the cholesterol levels in cells, a combined treatment with fumonisin B$_1$, a sphingolipid synthesis inhibitor, and mevastatin and mevalonate, two distinct Hydroxymethyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors (Hering et al., 2003), or treatment with methyl-β-cyclodextrin (MβCD), an agent that binds and sequesters cholesterol (Ohtani et al., 1989), were used. We confirmed that mevastatin/fumonisin B$_1$/ mevalonate treatment, at the concentrations of 8 μM mevastatin, 20 μM fumonisin B$_1$ and 500 μM mevalonate, as well as 10 mM of methyl-β-cyclodextrin significantly reduced cholesterol in the studied cells (Fig. 5).

The combined mevastatin/fumonisin B$_1$/mevalonate treatment has not previously been widely used, so we initially examined the effect of mevastatin/fumonisin B$_1$/mevalonate on cell viability, using the MTT assay. As shown in Fig. 6A, none of the examined concentrations of mevastatin/fumonisin B$_1$/mevalonate had any impact on cell viability. In saturation experiments, it was found that mevastatin/fumonisin B$_1$/mevalonate treatment caused a strong reduction of [^{3}H]5-HT binding to 5-HT$_7$ receptors (Fig. 6C and D; B_{max} values, 492.6±25.5 fmol/10^6 cells (untreated) vs. 189.7±24.2 fmol/10^6 cells (mevastatin/fumonisin B$_1$/mevalonate). Since treatment with mevastatin, fumonisin B$_1$ and mevalonate could be associated with nonspecific effects unrelated to cholesterol depletion, cholesterol (10 μg/ml) was added back to the cells for 1 h. Such cholesterol restoration caused a significant (p<0.05 vs. untreated cells) reversal of the

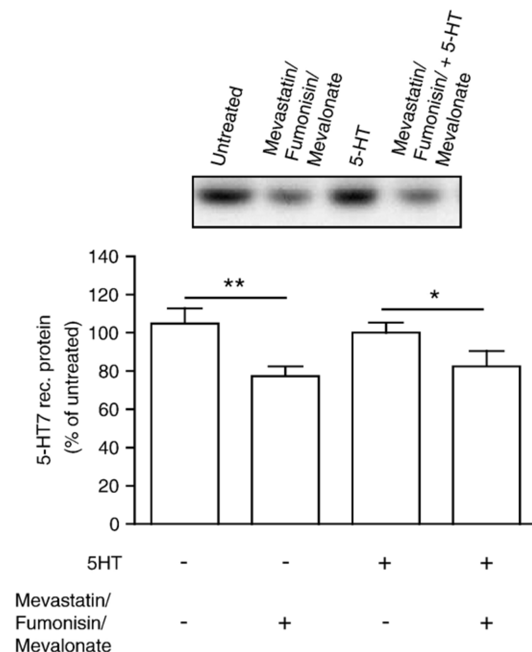

Fig. 7. The effect of treatment with mevastatin, fumonisin B$_1$ and mevalonate on 5-HT$_7$ receptor levels in whole cell extracts. Results from Western blot experiments showing that treatment with 8 μM mevastatin, 20 μM fumonisin B$_1$ and 500 μM mevalonate reduces the levels of total 5-HT$_7$ receptor proteins both under basal conditions and following stimulation with 10 μM 5-HT. Results from six experiments run in triplicates. *p<0.05; **p<0.01 by Student's *t*-test.

effect of mevastatin/fumonisin B_1/mevalonate on [^{3}H]5-HT binding to 5-HT_7 receptors (B_{max} value, 290.7±24.7 fmol/10^6 cells (mevastatin/fumonisin B_1/mevalonate+cholesterol)). These data provide evidence that the effects of mevastatin/fumonisin B_1/mevalonate treatment are due to cholesterol depletion. Neither mevastatin/fumonisin B_1/mevalonate nor mevastatin/fumonisin B_1/mevalonate+cholesterol had any effects on the affinity of [^{3}H]5-HT binding to 5-HT_7 receptors (K_d values, 21.4±2.9 nM (untreated), 21.2±7.0 nM (mevastatin/fumonisin B_1/mevalonate), 24.7±5.3 nM (mevastatin/fumonisin B_1/mevalonate+cholesterol)) or on the ability of 5-MeOT to displace bound [^{3}H]5-HT (Fig. 6B). The K_i value for untreated cells was 4.97 nM (95% C.I. 2.66–9.29 nM). For treated cells the K_i values were 2.36 nM (95% C.I 1.71–3.24 nM) in the absence of cholesterol and 2.61 nM (95% C.I. 1.95–3.51 nM) in the presence of cholesterol. The differences between these K_i values were not significant.

Cholesterol depletion by mevastatin/fumonisin B_1/mevalonate treatment also reduced the binding of [^{3}H]SB269970 to 5-HT_7 receptors (Fig. 6E and F; B_{max} values: 268.2±22.8 fmol/10^6 cells (untreated) vs. 137.0±17.4 fmol/10^6 cells (mevastatin/fumonisin B_1/mevalonate).

The possibility that the reduced binding of [^{3}H]5-HT to 5-HT_7 receptors could involve a decrease in the absolute number of 5-HT_7 receptors was examined using Western blot analysis. Indeed, such experiments demonstrated that there was a reduction of 5-HT_7 receptor immunoreactivity in cells following mevastatin/fumonisin B_1/mevalonate treatment (Fig. 7).

To further examine the role of cholesterol in regulating [^{3}H]5-HT binding to 5-HT_7 receptors, cells were treated with methyl-β-cyclodextrin. The MTT assay showed that methyl-β-cyclodextrin treatment caused a dose-dependent reduction in cell viability (Fig. 8A). However, at 10 mM, methyl-β-cyclodextrin caused no significant toxic actions. Treatment

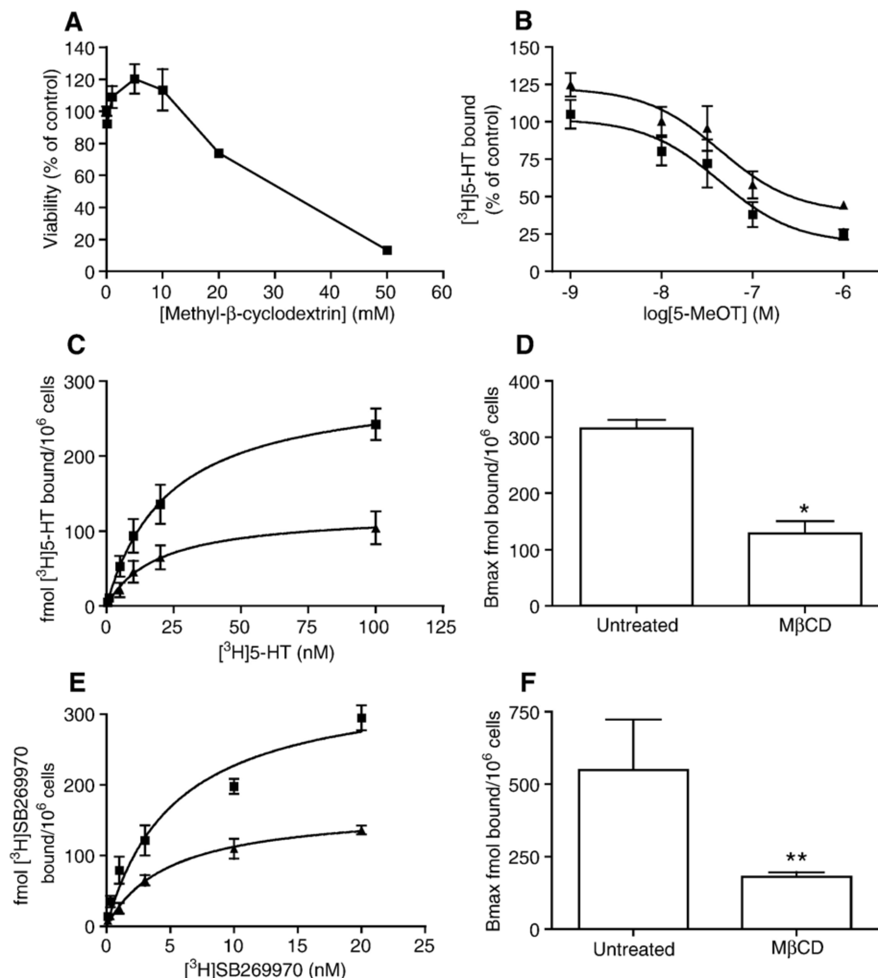

Fig. 8. Effect of treatment with methyl-β-cyclodextrin (MβCD) on [^{3}H]5-HT and [^{3}H]SB269970-binding at 5-HT_7 receptors. (A) MTT assay showing that methyl-β-cyclodextrin treatment significantly decreased cell viability at concentrations exceeding 10 mM. (B) Displacement experiments showing that cholesterol depletion with 10 mM methyl-β-cyclodextrin has no effect on the ability of 5-MeOT to displace [^{3}H]5-HT from 5-HT_7 receptors. (C, D) Saturation experiments showing that cholesterol sequestration decreases the B_{max} value of [^{3}H]5-HT binding. Untreated cells (■) have a B_{max} value of 297.5±30.7 fmol/10^6 cells. In the methyl-β-cyclodextrin treated cells (▲), B_{max} is reduced to 124.9±21.2 fmol/10^6 cells, a reduction by 58%. (E, F) Saturation experiments showing that cholesterol depletion decreases the B_{max} value of [^{3}H]SB269970-binding. Untreated cells (■) have a B_{max} value of 349.2±35.0 fmol/10^6 cells. In the methyl-β-cyclodextrin treated cells (▲), B_{max} is reduced to 167.6±14.3 fmol/10^6 cells, a reduction by 48%. (A) Results from two experiments run in triplicates. (B) Results from two experiments run in duplicates. (C, D) Results from three experiments run in duplicates. (E, F) Results from two experiments run in duplicates. *$p<0.05$; **$p<0.01$ by Student's t-test.

with 10 mM methyl-β-cyclodextrin for 30 min caused a significant reduction in the B_{max} value of [^{3}H]5-HT binding to 5-HT$_7$ receptors (Fig. 8C and D; 297.5±30.7 fmol/10^6 cells (untreated) vs. 124.9±21.2 fmol/10^6 cells (MβCD)) that could be significantly ($p<0.05$ vs. untreated cells) reversed by cholesterol replacement (B_{max} value: 238.0±46.3 fmol/10^6 cells). Neither methyl-β-cyclodextrin nor methyl-β-cyclodextrin+cholesterol had any effects on the affinity of [^{3}H]5-HT binding to 5-HT$_7$ receptors (K_d values, 23.0±6.1 nM (untreated), 19.0±8.5 nM (MβCD), 31.0±14.6 nM (MβCD+cholesterol)).

Like the mevastatin/fumonisin B$_1$/mevalonate treatment, methyl-β-cyclodextrin did not affect the ability of 5-MeOT to displace bound [^{3}H]5-HT (Fig. 8B). The K_i value for untreated cells was 23.7 nM (95% C.I. 6.08–92.4 nM). For treated cells the K_i value was 27.6 nM (95% C.I. 8.1–93.9 nM) and for cholesterol-restored cells the K_i value was 40.8 nM (95% C.I. 15.6–106.9 nM). The differences between these K_i values were not significant.

The effects of methyl-β-cyclodextrin on saturating [^{3}H]SB269970-binding to 5-HT$_7$ receptors showed that methyl-β-cyclodextrin treatment reduced [^{3}H]SB269970-binding (Fig. 8E and F; B_{max} values: 349.2±35.0 fmol/10^6 cells (untreated) vs. 167.6±14.3 fmol/10^6 cells (MβCD)).

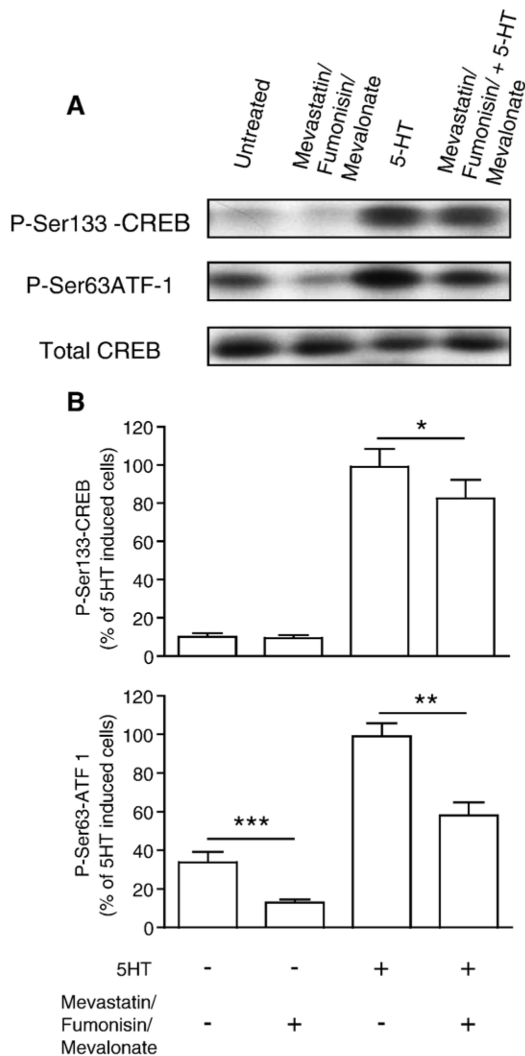

Fig. 9. Effects of treatment with mevastatin, fumonisin B$_1$ and mevalonate on 5-HT-induced phosphorylation of Ser^{133}-CREB and Ser^{63}-ATF-1. (A) Representative Western blots illustrating the effects of 8 µM mevastatin, 20 µM fumonisin B$_1$ and 500 µM mevalonate and 5-HT (10 µM) on P-Ser^{133}-CREB, P-Ser^{63}-ATF-1 and total CREB levels. The total CREB levels are not significantly affected by any of the treatments. (B) Histograms showing the quantifications of P-Ser^{133}-CREB and P-Ser^{63}-ATF-1 levels, respectively. Treatment significantly counteracts the 5-HT-induced phosphorylation of both the studied proteins. In the case of P-Ser^{63}-ATF-1, the mevastatin/fumonisin B$_1$/mevalonate treatment also causes a reduction of basal phosphorylation levels. Results from five experiments run in triplicates. *$p<0.05$; **$p<0.01$; ***$p<0.001$ by Student's t-test.

3.3. The effect of cholesterol depletion on signaling via the human 5-HT$_{7(a)}$ receptor

5-HT$_7$ receptors are primarily G$_s$-coupled receptors that activate adenylyl cyclase/cAMP/protein kinase A (PKA)/phospho-Ser^{133}-CREB signaling (Bard et al., 1993; Lovenberg et al., 1993; Tsou et al., 1994; Adham et al., 1998). However, recent reports have also shown that stimulation of 5-HT$_7$ receptors activates additional G proteins, including G$_{\alpha12}$ (Kvachnina et al., 2005), and increases intracellular calcium (Norum et al., 2005). Moreover, it is known that G$_{\alpha s}$-coupled receptors often activate Phospholipase C (PLC)/Protein kinase C (PKC) signaling in transfected cell lines (e.g. Zhu et al., 1994). Phosphorylation of CREB at Ser^{133} is known to be a point of convergence for several signaling pathways (e.g. Mayr and Montminy, 2001) and we therefore thought that studies on the phosphorylation of CREB would mirror the efficacy of 5-HT$_7$ receptor-mediated signaling rather well. To determine whether cholesterol depletion would affect 5-HT$_7$ receptor-mediated signal transduction, we studied effects of treatment with mevastatin, fumonisin B$_1$ and mevalonate on the ability of 5-HT (10 µM) to increase the phosphorylation state of two phospho-substrates for PKA, Ser^{133}-CREB and Ser^{63}-ATF-1. It was found that 5-HT caused very strong increases in the phosphorylation states of Ser^{133}-CREB and Ser^{63}-ATF-1, which were significantly counteracted by mevastatin/fumonisin B$_1$/mevalonate treatment (Fig. 9). In the case of phospho-Ser^{63}-ATF-1, but not phospho-Ser^{133}-CREB, treatment with mevastatin, fumonisin B$_1$ and mevalonate also caused a reduction of basal phosphorylation levels.

4. Discussion

The present study demonstrates that cholesterol depletion reduces binding of [^{3}H]5-HT and [^{3}H]SB269970 to 5-HT$_7$ receptors and counteracts 5-HT$_7$-mediated intracellular signaling.

An initial characterization of the examined 5-HT$_7$ receptors showed saturating radioligand binding that was displaced by 5-MeOT>SB269970>clozapine⋙ketanserin, raclopride. The studied 5-HT$_7$ receptors have a pharmacology reminiscent of other cloned 5-HT$_7$ receptors (Shen et al., 1993; Krobert et al., 2001), as well as 5-HT$_7$ receptors expressed in native brain tissue (To et al., 1995). These data indicate that the data on the effect of cholesterol depletion on 5-HT7 receptor function obtained in the present study may be relevant also in other cell systems.

Two distinct approaches were used to deplete membranous cholesterol: 1) Combined treatment with mevastatin, fumonisin B_1 and mevalonate to deplete cholesterol by synthesis inhibition (Hering et al., 2003). 2) Treatment with methyl-β-cyclodextrin, which binds and sequesters membranous cholesterol (Ohtani et al., 1989). Whereas mevastatin/fumonisin B_1/mevalonate treatment is not commonly used to study effects of cholesterol depletion on G protein coupled receptor function, several reports (e.g. Becher et al., 2001; Monastyrskaya et al., 2005; Rybin et al., 2000) have used methyl-β-cyclodextrin treatment. Both treatments significantly lowered cholesterol levels. The combined treatment with mevastatin, fumonisin B_1 and mevalonate caused no detrimental effects on cell viability over a wide range of concentrations. However, in agreement with previous reports (e.g. Hering et al., 2003; Magnani et al., 2004) we found that treatment with methyl-β-cyclodextrin can decrease cell viability. Using the MTT viability assay, we found that concentrations above 10 mM decreased cell viability. It appears, thus, that it is favorable to deplete cholesterol by synthesis inhibition in our experimental setting and we therefore used this treatment strategy in most experiments. Another advantage of using cholesterol synthesis inhibition is that this treatment utilizes compounds that have medical relevance. In fact, mevastatin (also named, compactin and ML-236B) was the first potent, specific inhibitor of HMG-CoA reductase and its derivatives, lovastatin and simvastatin, were subsequently approved for clinical use. The cholesterol-lowering effect of mevastatin, fumonisin B_1 and mevalonate treatment observed here is the same range as observed in some clinical studies with HMG-CoA reductase inhibitors (e.g. Baigent et al., 2005).

The most prominent finding in the present study is that cholesterol depletion, in a reversible manner, reduces the binding of $[^3H]$5-HT to 5-HT_7 receptors. This reduction could involve an altered G protein coupling of 5-HT_7 receptors. Indeed, it is well-known that agonists bind to receptors present in the high-affinity state, i.e. coupled with holotrimeric G proteins that have not bound any guanine nucleotide, whereas the antagonists bind to the receptor also when it is not associated with G proteins (Kent et al., 1980). To investigate this possibility, we studied the effects of cholesterol depletion on the antagonist radioligand $[^3H]$SB269970 to 5-HT_7 receptors. An unexpected observation was that $[^3H]$SB269970 did not exhibit a significantly higher B_{max} value than the agonist radioligands $[^3H]$5-HT and $[^3H]$5-CT. A possible explanation is that $[^3H]$SB269970 is a poorer radioligand in our cell system, since the nonspecific binding of $[^3H]$SB269970 was higher than that of $[^3H]$5-HT and $[^3H]$5-CT. Nonetheless, cholesterol depletion also reduced the binding of the antagonist radioligand $[^3H]$SB269970 to 5-HT_7 receptors. This suggests that cholesterol depletion decreases the number of 5-HT_7 receptors at the cell surface rather than altering their G protein coupling. A part of this reduction could actually be explained by decreased levels of the absolute number of 5-HT_7 receptors, as we found a reduction in its immunoreactivity after cholesterol depletion. Furthermore, cholesterol depletion could affect cell functions in additional ways, such as increase the amount of 5-HT_7 receptors in recycling endosomes, which would affect binding properties of the studied 5-HT_7 receptor radioligands.

These data also show that the levels and organization of cholesterol regulate intracellular signaling via 5-HT_7 receptors. Indeed, the ability of serotonin to phosphorylate Ser^{133}-CREB and Ser^{63}-ATF-1 was reduced in cells depleted of cholesterol. As mentioned above, we decided to study the effects of 5-HT_7 receptor-mediated signaling by measuring the phosphorylation state of CREB at Ser^{133} as this phospho-site is known to be a point of convergence for several signaling pathways, i.e. $G_{\alpha s}$/adenylyl cyclase/cAMP/PKA, calcium influx, PLC/PKC and $G_{\alpha 12}$ activation pathways, that are likely to be activated via 5-HT_7 receptors in transfected cells (Bard et al., 1993; Lovenberg et al., 1993; Tsou et al., 1994; Zhu et al., 1994; Adham et al., 1998; Mayr and Montminy, 2001; Kvachnina et al., 2005; Norum et al., 2005). It would be interesting, in future studies, to examine the effect of cholesterol depletion on the ability of 5-HT_7 receptors to activate each of these upstream signaling pathways. Nonetheless, we would like to point out that the effect of cholesterol depletion on 5-HT_7 receptor signaling was less pronounced than the effect of this treatment on $[^3H]$5-HT binding to 5-HT_7 receptors. This discrepancy could perhaps be explained by the cell system utilized in this study. The 5-HT_7 receptors in the studied HeLa cells showed a pharmacological profile reminiscent of cloned and native 5-HT_7 receptors (Shen et al., 1993; To et al., 1995; Krobert et al., 2001). However, the high levels of $[^3H]$5-HT binding to 5-HT_7 receptors could indicate the presence of spare receptors that would explain the discrepancy between the effects of cholesterol depletion on $[^3H]$5-HT binding and 5-HT-mediated signaling. Indeed, it is well-known that some recombinant cell lines have a substantial amount of spare receptors (Kenakin, 1997). It will be important to study effects of cholesterol depletion on 5-HT_7 receptor function in additional cell lines and in intact tissue preparations.

In conclusion, our data provide evidence that 5-HT_7 receptors can be added to the growing list of G protein coupled receptors that are functionally regulated by membranous cholesterol.

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