Membrane Cholesterol Modulates Serotonin Transporter Activity†

Siobhan M. Scanlon,‡,§ D. Clive Williams,‡ and Patrick Schloss*,§

Biochemistry Department, Trinity College, Dublin 2, Ireland, and Biochemical Laboratory, Central Institute for Mental Health, 68159 Mannheim, Germany

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ABSTRACT: The synaptic actions of the neurotransmitter serotonin are terminated by a selective high-affinity reuptake mediated by the serotonin transporter (SERT). To gain insight into the modulation of the functional properties of this integral membrane protein by cholesterol, a main component of the lipid bilayer, we stably expressed the rat SERT in human embryonic kidney 293 cells and, upon altering the cholesterol content of these cells by different means, analyzed SERT activity. Depletion of the level of membrane cholesterol by treatment with either the cholesterol chelating agent methyl-β-cyclodextrin (MβCD), cholesterol oxidase, or the cholesterol-binding fluorochrome filipin resulted in a decrease in SERT activity due to both a loss of affinity of substrate and ligand binding and a concomitant reduction of the maximal transport rate. In cholesterol-depleted membranes, cholesterol levels could be restored to those found in untreated membranes by incubation of the membranes with an MβCD−cholesterol complex, which correlated with a reversal of the cholesterol depletion-mediated decrease in the level of high-affinity binding. This was not the case when other steroids, such as ergosterol, 5-cholestene, or pregnenolone, were substituted into cholesterol-depleted membranes. These results suggest that membrane cholesterol modulates the functional properties of the SERT by specific molecular interactions which are needed to stabilize the transporter in its optimally active form.

In the central nervous system, serotonergic neurotransmission is terminated by high-affinity reuptake of serotonin (or 5-hydroxytryptamine, 5-HT)[1] from the synaptic cleft. The serotonin transporter (SERT) is a member of the Na^+- and Cl^--dependent neurotransmitter transporter family which also comprises transporters for norepinephrine, dopamine, γ-aminobutyric acid (GABA), and glycine (*1*, *2*). The serotonergic system is known to modulate numerous physiological and behavioral functions. Among other findings, a reduced activity of serotonergic neurotransmission has been postulated for the pathogenesis of depression (*3*, *4*). Because the SERT clears the synaptic cleft of the neurotransmitter, it plays a crucial role in serotonergic neurotransmission by controlling the concentration of the free, active neurotransmitter in the synaptic cleft. Hence, drugs blocking the serotonin transporter, thereby elevating extracellular serotonin levels and potentiating serotonergic actions, have been successfully used for the treatment of depression. Besides being affected by antidepressants, the SERT also represents the target for amphetamine-induced 5-HT release. Among other psychostimulants, MDMA ("Ecstasy") and *p*-chloramphetamine (PCA) have been found not only to competitively inhibit 5-HT transport but also to stimulate previously accumulated [^{3}H]-5-HT efflux by reversed membrane transport, thus implicating the serotonin transporter as a 5-HT−amphetamine exchange system (*5*−*8*).

In addition to inhibition of SERT activity via exogenous drug binding, endogenous regulation of SERT functional properties by intracellular processes also has been investigated. Several studies reported on the regulation of SERT activity via intracellular Ca^{2+}, nitric oxide, and cGMP (*9*, *10*), and an important means of modulating SERT activity seems to involve phosphorylation and dephosphorylation of this protein by various kinases and phosphatases (*11*−*16*). In particular, modulation by activation of protein kinase C (PKC) has been extensively investigated. It has been shown that activation of PKC results in a marked reduction in serotonin transport activity without affecting substrate affinity. Interestingly, stimulation of PKC has been shown also to downregulate the transport activity of the homologous transporters for GABA (*17*, *18*), glycine (*19*, *20*), and dopamine (*21*). These findings may suggest a common mechanism for PKC modulation of these related transporter proteins. When these effects were studied in more detail using cells expressing recombinant SERT proteins, biotinylation/immunoblot analyses revealed that the PKC-induced reduction of transport activity was due to a decrease in the level of cell surface-expressed serotonin transporter molecules (*13*).

In addition to these protein-mediated regulatory mechanisms, the composition of the lipid bilayer has also been reported to influence the activity of transporter proteins. Being an important constituent of the eukaryotic plasma membrane, cholesterol has been well established as having a profound effect on the physical state of the phospholipid

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* To whom correspondence should be addressed. Telephone: +0049-621-1703-835. Fax: +0049-621-1703-837. E-mail: schloss@as200.zi-mannheim.de.

‡ Trinity College.

§ Central Institute for Mental Health.

[1] Abbreviations: SERT, serotonin transporter; 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; GAT, GABA transporter; MβCD, methyl-β-cyclodextrin; LBB, ligand binding buffer.

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membranes. Changes in the cholesterol content of biological membranes are known to alter the lipid fluidity and thus membrane integrity (*22*, *23*). It has been demonstrated that cholesterol is required for the optimal reconstitution of the GABA transporter (GAT) purified from rat brain, and studies on the specificity and potency of the sterol's effects indicated that this was likely to be due to a direct interaction with the transporter protein (*24*). In our study, we have investigated whether membrane cholesterol also modulates the functional properties of the serotonin transporter stably expressed in HEK-293 cells (HEKrSERT) with respect to substrate transport and antidepressant binding efficacy. To this end, in the first approach we reversibly altered the cholesterol content of HEKrSERT cells by using methyl-β-cyclodextrin (MβCD) as a cholesterol chelator and conversely sterol−MβCD complexes as sterol donors to replenish cholesterol-depleted membranes. In another attempt, we analyzed transporter−ligand interactions after treating the membranes with cholesterol oxidase. Cholesterol oxidase converts cholesterol into the functional inactive steroid 4-cholesten-3-one which does not alter membrane fluidity (*25*). Thus, this allowed us to distinguish between specific cholesterol−transporter interactions and indirect cholesterol effects due to changes in the physical state of the plasma membrane which may in turn modify SERT functional properties. In addition, we also analyzed the effect of the cholesterol-binding fluorochrome filipin on SERT activity. The results obtained in these studies provide evidence that specific binding of cholesterol to the SERT is needed to stabilize the transporter in its fully active form.

MATERIALS AND METHODS

Materials. ^{3}H-labeled citalopram (82 Ci/mmol) and ^{3}H-labeled 5-HT (10.2 Ci/mmol) were purchased from Amersham and NEN, respectively. Cyclodextrins (α-, methyl-β-, and γ-), cholesterol, the cholesterol assay kit filipin complex, and cholesterol oxidase were all purchased from Sigma. All other chemicals were analytical grade.

Membrane Preparation. HEKrSERT membranes were prepared as previously reported. Briefly, cells in 10 cm dishes were washed once with PBS (without Ca^{2+} or Mg^{2+}); 2 mL of PBS was then added to the cell monolayer, and the cells were scraped off with a 32 cm scraper and homogenized using a Polytron PT-10 instrument. This cell lysate was then centrifuged at 357*g* for 3 min, and the supernatant was centrifuged at 37000*g* for a further 20 min. The resulting pellet was resuspended in 120 μL of PBS containing 5% (v/v) glycerol per 10 cm plate using 10 strokes of a Dounce glass homogenizer. The protein concentration was determined using the Markwell assay (*26*). The aliquoted membranes were stored at −80 °C.

[^{3}H]Citalopram Binding Assay. Binding of [^{3}H]citalopram to HEKrSERT membranes was performed as described previously (*27*). Saturation binding was performed at room temperature using 25 μg of membrane protein in a total volume of 200 μL of assay buffer containing 0−24 nM [^{3}H]-citalopram. Nonsaturable binding was assessed in the presence of 50 μM unlabeled citalopram. All binding data were analyzed by nonlinear regression using SigmaPlot (SPSS Science Software).

[^{3}H]-5-HT Transport. 5-HT uptake was performed as described previously (*28*). Briefly, HEK 293 cells were plated into 24-well dishes (2 cm in diameter) which had previously been treated with poly(L-lysine) (0.1 mg/mL) and allowed to grow to confluency. The culture medium was replaced with TB1 buffer (200 μL) containing 120 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.5) with various concentrations of 5-HT (0.25−5.125 μM) using 250 nM [^{3}H]-5-HT as a tracer. After 6 min at room temperature, the medium was removed quickly and the cells were washed twice with ice-cold TB1 before being lysed with 10% (w/v) SDS. The amount of radioactivity was determined by scintillation counting. The specific level of 5-HT uptake is determined as the difference between SERT-mediated and control HEK293 uptake in parallel culture dishes. All transport measurements were analyzed by nonlinear regression analysis using the graphics program SigmaPlot.

Determination of the Cholesterol Content of HEK293 Cells. Cholesterol content of membranes was determined spectrophotometrically using the Sigma cholesterol oxidase-based assay kit. Briefly, membranes (250−1000 μg of protein) were resuspended in 50−100 μL of HEPES buffer, and cholesterol reagent was added (1 mL). Samples were incubated at 37 °C for 10 min, followed by a brief centrifugation to remove particulate material, and the absorbance of the supernant was measured at 500 nm. The cholesterol content was determined by comparison with a cholesterol standard.

Treatment of Cells with MβCD. HEK293 and HEKrSERT cells were grown as described above in 24-well plates. The standard culture medium was replaced with serum-free DMEM containing 1% (v/v) penecillin/streptomycin to which MβCD (5−10 mg/mL) had been added. Cells were then incubated for 30 min at 37 °C, followed by washing with serum-free medium (400 μL), and 5-HT transport activity was measured as described above.

Treatment of Membranes with MβCD and Membrane Modulators. HEK membranes were incubated with various concentrations of MβCD. The samples were agitated gently at room temperature for 30 min. The mixture was centrifuged at 20000*g* for 20 min. The resulting pellets were washed in 500 μL of assay buffer followed by centrifugation. The resulting pellets were resuspended in LBB. The treatment of membranes with α- and γ-cyclodextrins was carried out in the same manner as it was for MβCD.

Pretreatment of the membranes with filipin was similarly performed. Filipin (stock prepared in DMSO) at concentrations ranging from 0 to 250 μM (prepared in LBB) was added to the membranes and the mixture incubated at room temperature for 10 min. The membranes were washed by centrifugation and resuspended in LBB.

Incorporation of Cholesterol into Cholesterol-Depleted Membranes. Membranes were treated with methyl-β-cyclodextrin as described above to remove endogenous cholesterol. Membranes were then incubated with various amounts of the methyl-β-cyclodextrin−cholesterol complex with gentle agitation for 30 min at room temperature. The membranes were centrifuged for 20 min at 20000*g* and resuspended in LBB. This treatment was followed by ligand binding analysis.

Complexing of Steroids with MβCD. Complexing of steroids (ergosterol, cholesterol, pregnenolone, and 5-cholestene) with MβCD was carried out as follows. MβCD (1

g) was dissolved in distilled water (19 mL) and the mixture heated to 80 °C. Steroid (15 mg of pregenonlone, 40 mg of cholesterol, 15 mg of 5-cholestene, or 25 mg of ergosterol) in chloroform and methanol (2:1, v/v) was added in small aliquots; the solutions were stirred, and the temperature was maintained at 80 °C until the solutions were clear. The solutions were then freezed-dried, and the resulting powder complexes were stored at −80 °C until they were used.

Incorporation of Steroids into Cholesterol-Depleted Membranes. Incorporation of steroids into the cholesterol-depleted membranes was performed as described above. Complexes of cyclodextrin containing various sterols were prepared as described above, and cholesterol-depleted membranes were treated with these complexes in the same manner described for membrane treatment with MβCD.

Treatment of Membranes with Cholesterol Oxidase. Cholesterol oxidase treatment was carried out as described previously (*29*). HEK293 membranes expressing the rat serotonin transporter were pretreated at 37 °C for 30 min with cholesterol oxidase [200 units/mL in a buffer containing 100 mM MES (pH 6.0) and 3 M NaCl] at various concentrations in a buffer containing 20 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 10 mM mannitol, and 1 milliunit/mL sphingomyelinase [0.04 unit/mL stock in a buffer containing PBS (pH 7.4) and 50% (v/v) glycerol].

RESULTS

To investigate the interaction between membrane cholesterol and the functioning of the SERT, we analyzed SERT-mediated transport of [^{3}H]-5-HT into HEKrSERT cells at various membrane cholesterol concentrations. In the first set of experiments, depletion of membrane cholesterol was achieved by extraction of membranes with MβCD and the remaining cholesterol content in the membranes was determined using a cholesterol oxidase-based assay as described in Materials and Methods. HEKrSERT cell membranes contained cholesterol at a concentration of approximately 115 μg/mg of protein, and this was reduced in a dose-dependent manner by treatment with MβCD with 50% depletion of membrane cholesterol being achieved at MβCD concentrations between 10 and 20 mg/mL (Figure 1). It appeared that MβCD removed cholesterol selectively from the membranes, and alteration of the level of membrane cholesterol was not achieved when membranes were treated with αCD and γCD.

Before analyzing the influence of membrane cholesterol content on SERT-mediated [^{3}H]-5-HT uptake, we tested the effect of MβCD incubation on the viability of HEKrSERT cells. Viability and cytotoxicity assays of cells treated with various concentrations of MβCD revealed that MβCD up to 10 mg/mL did not affect the integrity of the cells (Figure 2). After MβCD treatment under nontoxic conditions, [^{3}H]-5-HT transport assays with HEKrSERT cells showed a decrease in the level of SERT-mediated transport of [^{3}H]-5-HT in proportion to the depletion of membrane cholesterol (Figure 3A). Following saturation, analysis of [^{3}H]-5-HT uptake after treatment with 10 mg/mL MβCD showed that the reduction of transporter function was due to both a loss in the apparent affinity [K_m(untreated) = 429 ± 74 nM; K_m(MβCD-treated) = 740 ± 118 nM] and a concomitant reduction in the maximal transport rate of ~25% (Figure 3B and Table 1). To investigate whether the loss of 5-HT uptake after cholesterol depletion was reversible, we attempted to replenish the cholesterol level in the MβCD-treated cells by incubation with an MβCD−cholesterol complex. However, after treatment of the cells at nontoxic concentrations of MβCD followed by careful removal of this compound by washing, a second treatment of the cells with the MβCD−cholesterol complex resulted in partial cell death, and therefore, these experiments could not be evaluated.

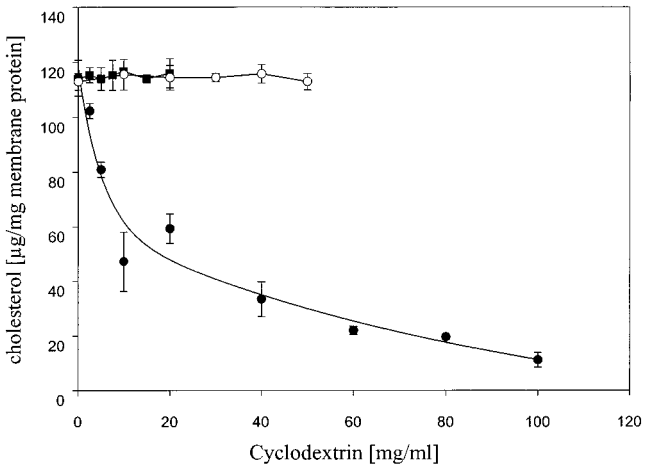

FIGURE 1: MβCD specifically reduces the cholesterol content of HEKrSERT membranes. For cholesterol depletion, HEKSERT membranes were treated with various concentrations of αCD (■), MβCD (●), and γCD (○) for 30 min at room temperature. Following removal of the cyclodextrins by washing, the membranes were harvested by centrifugation and their cholesterol content was determined using a cholesterol assay kit as described in Materials and Methods. The assays were performed three times with similar results, and results from a representative experiment are shown.

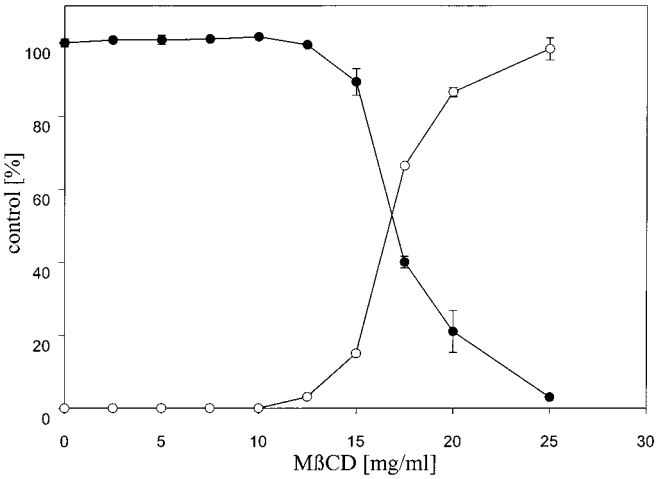

FIGURE 2: Effect of cholesterol depletion of HEKrSERT cells. HEKrSERT cells were incubated with MβCD at the indicated concentrations for 30 min at room temperature. The cells were assayed for cytotoxic effects using MTT (●) and LDH (○) assays as described in Materials and Methods.

To further investigate the effect of cholesterol removal on the functional properties of the SERT, we then analyzed the binding of [^{3}H]citalopram to membranes prepared from HEKrSERT cells before and after treatment with various concentrations of the different cyclodextrins. As shown in Figure 4, preincubation of HEKrSERT membranes with MβCD, but not with αCD or γCD, dose-dependently reduced the level of binding of [^{3}H]citalopram. Saturation binding data of untreated membranes from HEKrSERT cells for [^{3}H]-

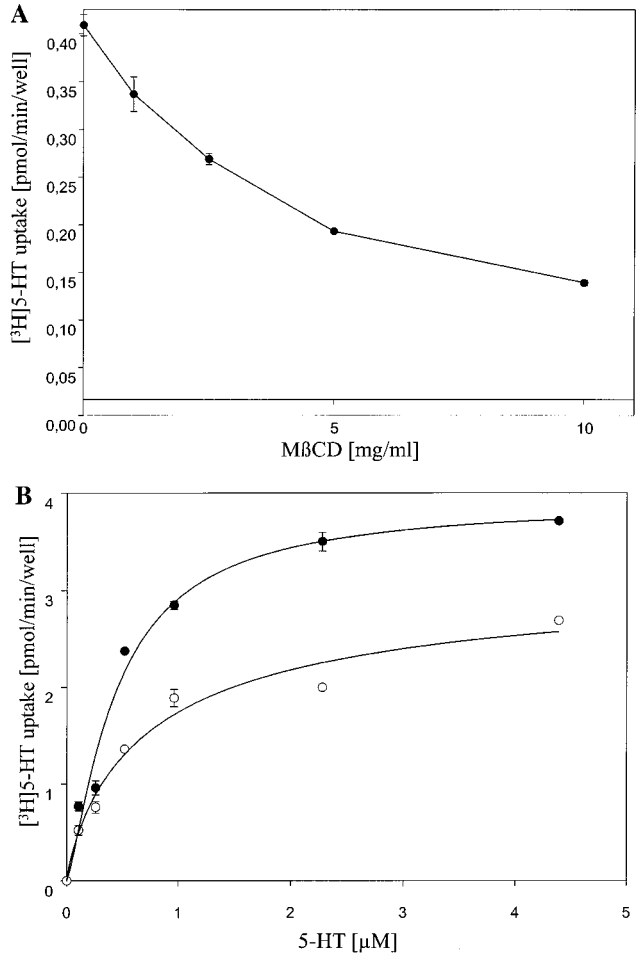

FIGURE 3: MβCD reduces the level of [^{3}H]-5-HT uptake into SERT-expressing HEK cells. HEKrSERT cells were plated out in 24-well plates and treated for 30 min at room temperature with MβCD at the indicated concentrations. After removal of MβCD, the cells were washed twice with serum-free DMEM, and [^{3}H]-5-HT (0.23 μM) uptake was followed for 6 min (A). In another set of experiments (B), a saturation analysis was performed of 5-HT transport into control cells (●) and cells treated with MβCD at 10 mg/mL (○). Each point is the mean of four replicates ± standard error of the mean (error bars are shown where they are larger than the symbol). This experiment was carried out three times, and the data that are shown are from one representative experiment. K_M and V_{max} values in this particular experiment were 410 ± 110 nM and 3.91 ± 0.34 pmol min^{-1} well^{-1}, respectively, for control cells and 800 ± 60 nM and 3.19 ± 0.02 pmol min^{-1} well^{-1}, respectively, for MβCD-treated cells.

Table 1: Comparison of 5-HT Uptake Parameters in Control and MβCD-Treated HEKrSERT Cells[a]

treatment	V_{max} (pmol min^{-1} well^{-1})	K_m (μM)
none	3.71 ± 0.45	0.43 ± 0.07
MβCD (10 mg/mL)	2.80 ± 0.21	0.74 ± 0.12

[a] MβCD treatment and 5-HT transport were performed as described in Materials and Methods. Data represent the mean ± standard error of the mean of three independent experiments performed in quadruplicate.

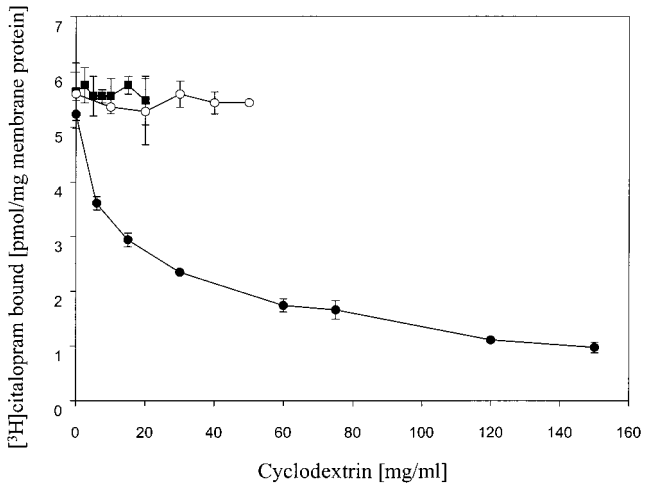

FIGURE 4: Cholesterol removal by MβCD treatment specifically reduces the level of [^{3}H]citalopram binding to HEKrSERT membranes. For cholesterol depletion, membranes were treated with various concentrations of αCD (■), MβCD (●), and γCD (○) for 30 min at room temperature. Following removal of the cyclodextrins by washing, the membranes were harvested by centrifugation (14 000 rpm for 15 min) and analyzed for [^{3}H]citalopram binding at 2.2 nM as described in Materials and Methods. Each point is the mean of three replicates, and the error bars are shown where they are larger than the symbol.

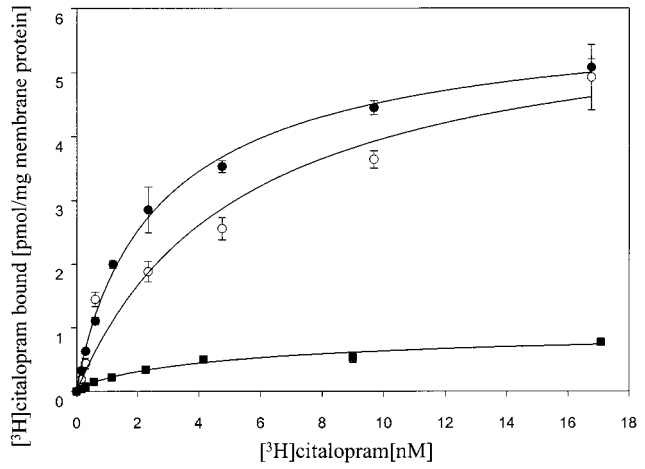

FIGURE 5: Saturation analysis of [^{3}H]citalopram binding to MβCD-treated HEKrSERT membrane preparations. HEKrSERT membranes were incubated in LBB buffer (●) or LBB buffer containing MβCD at 15 mg/mL (○) and 60 mg/mL (■) for 30 min at room temperature. Following removal of the cyclodextrins by washing, the membranes were harvested by centrifugation (14 000 rpm for 15 min) and analyzed for [^{3}H]citalopram binding. Each point is the mean of three replicates, and standard error of the mean error bars are given where they are larger than the symbol. This experiment was carried out three times, and the data that are shown are from one representative experiment. K_D and B_{max} values in this particular experiment were 2.46 ± 0.23 nM and 5.77 ± 0.15 pmol/mg of protein, respectively, for control membranes, 5.40 ± 1.03 nM and 6.12 ± 0.93 pmol/mg of protein, respectively, for membranes treated with 15 mg/mL MβCD, and 5.83 ± 4.1 nM and 1.04 ± 0.09 pmol/mg of protein, respectively, for membranes treated with 60 mg/mL MβCD.

citalopram binding were best fitted with the following equilibrium binding parameters: K_d = 1.98 ± 0.43 nM and B_{max} = 5.61 ± 0.56 pmol/mg of membrane protein. Treatment of the membrane preparations with MβCD (15 mg/mL) resulted in a loss of affinity of the SERT for [^{3}H]-citalopram (K_d = 4.34 ± 0.63 nM) with no significant change in the maximal number of binding sites (B_{max} = 5.49 ± 0.40 pmol/mg of protein; Figure 5 and Table 2). With further depletion of membrane cholesterol using higher MβCD concentrations (60 mg/mL), the maximal number of binding sites also decreased (K_d = 5.68 ± 0.13 nM; B_{max} = 1.19 ± 0.21 pmol/mg of protein; Figure 5 and Table 2).

In cholesterol-depleted membranes, cholesterol levels could be restored to those found in untreated membranes by

Table 2: Comparison of the Properties of [^{3}H]Citalopram Binding to Membrane Preparations from Control and MβCD-Treated HEKrSERT Cells[a]

treatment	B_{max} (pmol/mg of protein)	K_d (nM)
none	5.61 ± 0.56	1.98 ± 0.43
MβCD (15 mg/mL)	5.49 ± 0.40	4.34 ± 0.63
MβCD (60 mg/mL)	1.19 ± 0.21	5.68 ± 0.13

[a] MβCD treatment and [^{3}H]citalopram binding were performed as described in Materials and Methods. Data represent the mean ± standard error of the mean of three independent experiments performed in triplicate.

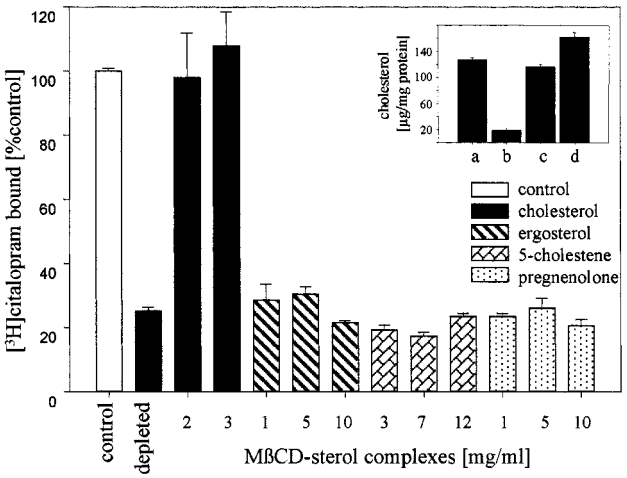

FIGURE 6: Cholesterol rescues [^{3}H]citalopram binding in cholesterol-depleted HEKrSERT membranes. HEKrSERT membranes were treated with MβCD (60 mg/mL) for 30 min at room temperature. After removal of MβCD and two washing steps, the membranes were incubated with MβCD−steroid complexes of cholesterol, 5-cholestene, pregnenolone, and ergosterol for 30 min at room temperature as indicated by the different symbols in the figure. The treatment concentrations of the MβCD−sterol complexes are given below. After centrifugation at 14 000 rpm for 15 min, the membranes were washed twice in LBB and analyzed for [^{3}H]-citalopram binding at 2.3 nM. Control membranes were subjected to the same incubation and centrifugation procedures in LBB buffer devoid of MβCD and MβCD−steroid complexes. When refilling the MβCD-treated membranes with cholesterol, in a parallel assay we determined the cholesterol content using a cholesterol assay kit as described in Materials and Methods. The successful reloading of cholesterol is shown in the inset of this figure (a, control; b, cholesterol-depleted; c and d, replenishment with 2 and 3 mg/mL MβCD−cholesterol complex, respectively; each bar represents the mean of triplicate determinations, and the particular results that are shown are representative of two such experiments with similar results).

incubation of the membranes with an MβCD−cholesterol complex (Figure 6, inset). As also shown in Figure 6, reincorporation of cholesterol correlated with a reversal of the cholesterol depletion-mediated inhibition of [^{3}H]citalopram binding. This was not the case when other steroids such as ergosterol, 5-cholestene, or pregnenolone were substituted into cholesterol-depleted membranes. Removal of ~80% of the initial cholesterol content of HEKrSERT membrane preparations reversibly led to an ~80% loss of binding.

To assess whether it is the influence of cholesterol on the physical state of the plasma membrane that modifies SERT functional properties, HEKrSERT membrane preparations were incubated with various concentrations of cholesterol oxidase before determining the level of [^{3}H]citalopram binding to the SERT. Cholesterol oxidase converts cholesterol into the functionally inactive steroid 4-cholesten-3-one which does not alter the physical state of plasma membranes (*28*). As shown in Figure 7A, treatment with cholesterol oxidase dose-dependently reduced the level of [^{3}H]citalopram binding to the membranes. This effect was absent when the membranes were treated with the heat-inactivated enzyme.

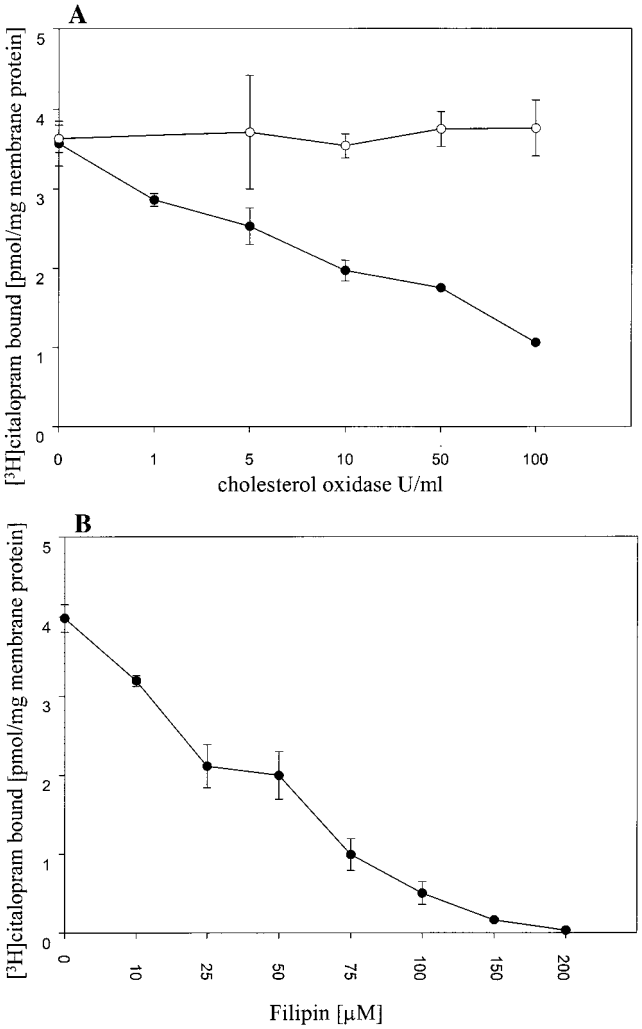

FIGURE 7: Treatment of HEKrSERT membranes with cholesterol oxidase (A) and filipin (B) reduces the level of [^{3}H]citalopram binding. (A) HEKrSERT membranes were treated with active (●) and heat-inactivated cholesterol oxidase (○) at the indicated concentrations for 30 min at 37 °C. Thereafter, the level of binding of [^{3}H]citalopram (2.3 nM) was determined by filtration. Each point of measurement is the mean of three replicates, and standard error of the mean error bars are given where they are larger than the symbol. This experiment was carried out three times, and the data that are shown are from one representative experiment. (B) HEKrSERT membranes were treated with increasing concentrations of filipin for 15 min at room temperature in the dark. Following washing and resuspension of the membranes, the level of binding of [^{3}H]citalopram (2.7 nM) was determined by filtration. Each point of measurement is the mean of three replicates, and standard error of the mean error bars are given where they are larger than the symbol. This experiment was carried out twice with similar results, and the data that are shown are from one representative experiment.

In another approach, we used filipin, a cholesterol-binding fluorochrome, to assess the dependence of SERT activity on plasma cholesterol. Preincubation of plasma membranes with filipin neither leads to removal of cholesterol from the membranes nor causes any chemical modification of cholesterol but forms filipin−cholesterol complexes in the

membranes, thereby altering the physical distribution of cholesterol (*29*). HEKrSERT membrane preparations treated with various concentrations of filipin exhibited a dose-dependent decrease in the level of [^{3}H]citalopram binding with an IC_{50} value of ~30 μM filipin (Figure 7B).

DISCUSSION

The membrane composition of the lipid bilayer, in which integral membrane proteins are embedded, is known to modulate the activity of various receptor proteins such as the transferrin receptor (*30*), the nicotinic acetylcholine receptor (*31*, *32*), the oxytocin receptor (*25*, *33*), rhodopsin (*34*), and the galanin receptor (*29*). It is thought there are different mechanisms by which cholesterol affects receptor function: (i) changes of the membrane fluidity as shown for the cholecystokinin receptor or (ii) specific receptor−cholesterol interactions as demonstrated for the oxytocin and galanin receptors (*25*, *29*). In the latter cases, it has been suggested that high-affinity states of the receptors are induced by direct receptor−cholesterol interactions.

A previous study from one of our laboratories has shown that ligand binding to the heterologously expressed SERT in insect Sf9 cells was reversibly dependent on membrane cholesterol levels (*35*). To gain further insight into whether cholesterol can also modulate the functional properties of the serotonin transporter, we measured substrate transport activity and antagonist binding properties at various membrane cholesterol concentrations for the SERT expressed in HEK cells. To distinguish between specific transporter−cholesterol interactions and the effects of membrane fluidity, we employed various methodical approaches to deplete membranes of cholesterol. In a first approach, we altered the cholesterol content of membranes by treatment with MβCD, which is known to extract cholesterol (*33*). This treatment can be reversible because MβCD−sterol complexes can also be used to replenish cholesterol-depleted membranes with cholesterol or analogues thereof. In a second approach, we altered the cholesterol concentration of the membranes by incubation with the cholesterol-converting enzyme cholesterol oxidase. This allowed us to analyze transporter functions at a constant membrane fluidity (*25*). In another approach, we assessed the dependence of SERT activity on plasma cholesterol after treatment of plasma membranes with the cholesterol-binding fluorochrome filipin. Filipin forms filipin−cholesterol complexes in the membranes, thereby altering the physical distribution of cholesterol and, thus, neither removing cholesterol from the membranes nor causing its chemical modification.

All three treatments, reducing cholesterol to concentrations which are nontoxic to cells, resulted in a loss of the apparent affinity (K_m) for substrate and for antagonist binding (K_d), in both cases reflected by a 2-fold increase in K_m and K_d. Similar changes in affinities have been observed for the galanin receptor, where a reduction in affinity is also given by a 3-fold increase in the K_d value after cholesterol depletion (*29*). Comparably, addition of cholesterol to the human oxytocin receptor, heterologously expressed in insect SF9 cells, which have a constitutively lower cholesterol concentration, has been shown to induce high-affinity binding (*36*). These results indicate that there are membrane proteins whose conformational states are dependent on the direct interaction of cholesterol with the polypeptide. Also in the case of the SERT, it appears that both substrate and antagonist binding are of a higher affinity when cholesterol is bound.

Depletion of cholesterol to nontoxic concentrations also gives a reduction in the V_{max} of 5-HT transport. This again is consistent with cholesterol interaction with the transporter inducing a conformational state with an optimal conformation for transport, i.e., transporter function. Similar effects for function with the galanin receptor was also reported whereby not only ligand binding but also signal transduction is affected upon cholesterol reduction, as revealed by a decrease in the level of galanin-stimulated receptor-mediated inositol phosphate production. These observations must be distinguished from the reduction in B_{max} values for antagonist binding at higher MβCD concentrations in the study presented here. This is consistent with a reduction in the amount of transporter molecules capable of binding antagonist, suggesting that the transporter protein may unfold or denature at high levels of cholesterol.

The specificity of cholesterol in the regulation of transporter activity has been demonstrated by the fact that only β-versions (not α- and γ-) of cyclodextrins successfully depleted membranes of cholesterol with a concomitant loss of antidepressant binding. In addition, this loss of binding could only be restored by reincorporation of cholesterol, but not other sterols, into previously cholesterol-depleted membranes. These results suggest that cholesterol specifically modifies the function of the serotonin transporter via direct sterol−protein interaction and extend the observations of Shouffani and Kanner (*24*), who showed that addition of small amounts of cholesterol optimizes GABA transporter reconstitution. Further studies are needed to reveal the molecular mechanisms underlying this modulation of the SERT and GAT by cholesterol, any physiological or pharmacological relevance, and whether these effects can be extended to other neurotransmitter transporters.

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