Dynamics of membrane lipid domains in neuronal cells differentiated in culture[1]

Elena Ottico, Alessandro Prinetti,[2] Simona Prioni, Claudia Giannotta, Luisa Basso, Vanna Chigorno, and Sandro Sonnino

Department of Medical Chemistry, Biochemistry, and Biotechnology, Center of Excellence on Neurodegenerative Diseases, University of Milan, Segrate, Italy

Abstract Treatment with methyl-β-cyclodextrin (MCD) induced a time- and dose-dependent efflux of cholesterol, sphingolipids, and phosphatidylcholine (PC) from cerebellar neurons differentiated in culture. With a "mild" treatment, the loss of cell lipids induced a deep reorganization of the remaining membrane lipids. In fact, the amount of PC associated with a Triton X-100-insoluble membrane fraction (highly enriched in sphingolipids and cholesterol in nontreated cells) was lowered by the treatment. This suggested a reduction of the lipid domain area. However, the cholesterol and sphingolipid enrichment of this fraction remained substantially unchanged, suggesting the existence of dynamic processes aimed at preserving the segregation of cholesterol and sphingolipids in membrane domains. Under these conditions, the lipid membrane domains retained the ability to sort signaling proteins, such as Lyn and c-Src, but cells displayed deep alterations in their membrane permeability. However, normal membrane permeability was restored by loading cells with cholesterol. When MCD treatment was more stringent, a large loss of cell lipids occurred, and the lipid domains were much less enriched in cholesterol and lost the ability to sort specific proteins. The loss of the integrity and properties of lipid domains was accompanied by severe changes in the membrane permeability, distress, and eventually cell death.—Ottico, E., A. Prinetti, S. Prioni, C. Giannotta, L. Basso, V. Chigorno, and S. Sonnino. **Dynamics of membrane lipid domains in neuronal cells differentiated in culture. *J. Lipid Res.* 2003.** 44: **2142–2151.**

Supplementary key words cholesterol • sphingomyelin • ganglioside • sphingolipid-enriched membrane domain • cyclodextrin

A number of data deriving from very heterogeneous research areas and experimental approaches, including biochemical analysis, optical and electron immunomicroscopy, atom force microscopy, and single particle tracking [as reviewed in ref. (1)], led to the notion that membrane complex lipids are not randomly distributed within a cell membrane but form lipid domains, where sphingolipids and cholesterol are segregated in dipalmitoylphosphatidylcholine-rich membrane areas. These domains are involved in the trafficking and sorting of specific proteins, as well as in signal transduction processes (2, 3). The lipid domain composition and the machinery of signal transduction were extensively studied in neurons (4–14). In neurons and neuronal cell lines, many lines of evidence suggest that lipid membrane domains or multimolecular complexes localized within such domains are directly involved in the process of neuronal differentiation. As a well-documented example, in cerebellar neurons spontaneously differentiating in culture, neuronal differentiation is accompanied by a marked increase in the membrane area occupied by these domains and by deep changes in their lipid composition (8, 9). In particular, lipid membrane domains from fully differentiated neurons bear the highest sphingolipid density and content with respect to undifferentiated and senescent cells, while cholesterol relative content steadily and markedly decreases with in vitro development (9). Particularly significant seem to be the interactions of sphingolipids with pro-

Manuscript received 9 June 2003 and in revised form 22 July 2003.

Published, JLR Papers in Press, August 1, 2003.
DOI 10.1194/jlr.M300247-JLR200

Abbreviations: BME, basal modified Eagle's medium; HPTLC, high-performance thin-layer chromatography; [1-^{3}H]sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-[1-^{3}H]octadecene; MCD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide; PC, phosphatidylcholine; SM, sphingomyelin.

[1] Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm (Svennerholm, L. 1980. *Adv. Exp. Med. Biol.* **125:** 11) and the IUPAC-IUBMB recommendations (IUPAC-IUBMB Joint Commission on Biochemical Nomenclature. 1997. *Pure Appl. Chem.* **69:** 2475–87; 1998. *Carbohydr. Res.* **312:** 167–17). GM1, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD3, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1a, α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1b, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; *O*-Ac-GT1b α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5,9Ac$_2$-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GT1b, α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GQ1b, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer.

[2] To whom correspondence should be addressed.
e-mail: alessandro.prinetti@unimi.it

tein kinases of the Src family, probably the most typical protein family localized in lipid domains, as well as with other proteins implicated in the Src signaling cassette (4, 7, 8–14). Interestingly, the lipid composition changes were accompanied by changes in the association of signaling molecules with the lipid membrane domain during neuronal cell differentiation (9). Similarly, in the case of Neuro2a, neuroblastoma cell differentiation induced by treatment with exogenous gangliosides (likely able to artificially increase the ganglioside content of lipid membrane domains), activation of c-Src kinase, and dissociation of Csk from the lipid membrane domain were observed (4). These findings suggest a direct link between the lipid composition of lipid membrane domains and their biological and functional properties as signaling units. However, the molecular basis of this link is poorly understood.

A very promising experimental approach to a better understanding of the involvement of lipids in the structural and functional properties of membrane domains is represented by the artificial manipulation of lipid composition and/or organization within these structures. To this purpose, a wide variety of experimental approaches allowing the downregulation of the biosynthesis of membrane lipids (15–21) or the lowering of cholesterol levels in the plasma membrane by external ligands (20–28) has been introduced. Among these tools, the extraction of cholesterol from cells by treatment with cyclodextrins is certainly the most widely used, as evidenced by the hundreds of papers found combining the two key words "cyclodextrin" and "cholesterol" in a Medline search from 1996 to the present. However, although the efflux of cholesterol mediated by cyclodextrin has been studied in detail, only limited information can be found regarding the effect of cyclodextrins on the possible removal of other complex lipids from living cells (24, 25, 29, 30). This is quite surprising, insofar as it is known that cyclodextrins are able to form complexes with sphingolipid monomers (31–33) and to disrupt ganglioside aggregates (32).

In the present study, we analyzed the efflux of cholesterol, sphingolipids, and glycerophospholipids from cells and the changes in the composition of membrane lipid domains after treating rat cerebellar granule cells differentiated in culture with methyl-β-cyclodextrin (MCD). Our data provide a new scenario, in which the membrane lipid domains appear as dynamic structures whose existence strongly influences cell membrane properties.

EXPERIMENTAL PROCEDURES

Materials

Commercial chemicals were the purest available, common solvents were distilled before use, and water was doubly distilled in a glass apparatus. MCD, trypsin, crystalline BSA, and several reagents for cell culture were purchased from Sigma Chemicals. Basal modified Eagle's medium (BME) and fetal calf serum were purchased from EuroClone. Sphingosine was prepared from cerebroside (34). Standard sphingolipids and glycerophospholipids were extracted from rat brain, purified, and characterized (35). [1-^{3}H]sphingosine was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine, followed by reduction with sodium boro[^{3}H]hydride (36) (radiochemical purity over 98%; specific radioactivity 2 Ci/mmol). ^{3}H-labeled lipids were extracted from [1-^{3}H]sphingosine-fed cells, purified, characterized, and used as chromatographic standards. [9,10(n)-^{3}H]palmitic acid, 55.0 Ci/mmol, was from Amersham Pharmacia Biotech. Anti-Lyn, anti-c-Src, anti-Akt rabbit polyclonal IgG, and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology.

Cell cultures

Granule cells obtained from the cerebellum of 8-day-old Harlan Sprague-Dawley rats were prepared and cultured as described (37–39). The cells were seeded in 60 mm or 100 mm dishes at a density of 0.155×10^6 cells/cm^2 and cultured with BME containing 10% fetal calf serum. Primary cultures from 8-day-old rat cerebellum are highly enriched in neurons. In these cultures, about 90% of the cells are immature excitatory granule cells. The replication of non-neuronal cells was prevented by adding 10 μM cytosine arabinoside to the culture medium. Rat cerebellar granule cells in culture spontaneously undergo a developmental pattern that resembles that of cerebellar neurons in vivo, reaching a mature state after 8 days. The eighth day in culture corresponds to morphologically and biochemically fully differentiated neurons; the cells are mostly grouped in large aggregates, connected by a complex net of fasciculate fibers characterized by the presence of axo-axonic synapses. From the biochemical point of view, granule cells at this stage of in vitro development are characterized by the expression of voltage-dependent sodium channels, neurotransmitter receptors, neuronal surface sialoglycoproteins, and by the ability to synthesize glutamate and release this neurotransmitter under depolarizing conditions (37, 40). Typical protein content was 700 μg of protein/dish. Cell integrity was assessed by the Trypan blue exclusion method, and cell mitochondrial metabolic activity was determined with the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) test (41).

Treatment of cell cultures with [1-^{3}H]sphingosine

Cells at the sixth day in culture were incubated in the presence of 3×10^{-8} M [1-^{3}H]sphingosine (2 ml/dish, or 5 ml/dish for 60 mm or 100 mm dishes, respectively) in cell-conditioned medium for a 2 h pulse followed by a 48 h chase. Under these conditions, free radioactive sphingosine was barely detectable in the cells, and all cell sphingolipids and phosphatidylethanolamine (obtained by recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H]sphingosine) were metabolically radiolabeled (8).

Treatment of cell cultures with [9,10(n)-^{3}H]palmitic acid

Cells at the fifth day in culture were incubated in the presence of $4{,}5 \times 10^{-7}$ M [9,10(n)-^{3}H]palmitic acid (2 ml/dish) in cell-conditioned medium for a 24 h pulse followed by a 48 h chase. Under these conditions, all cell sphingolipids and glycerolipids were metabolically radiolabeled (42).

Treatment of cell cultures with MCD

After sphingolipid metabolic labeling as described in the previous section, cells at the eighth day in culture were treated with MCD as follows. At the time of the experiments, the chase medium was discarded and dishes (60 mm dishes) were washed three times with 1 ml Locke's balanced saline solution [156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES (pH 7.4)] prewarmed to 37°C. Cells were incubated in the presence of 2 ml Locke's solu-

tion (incubation buffer) containing different concentrations of MCD (1–10 mM) or not (control) at 37°C for different times (5–120 min). Incubation buffer was removed, centrifuged at 1,000 *g* for 10 min to remove detached cells and cell debris, and stored at −80°C until lipid analysis. The number of cells remaining after each treatment was determined by counting the cells in 4–5 random fields in each dish using a phase-contrast microscope. Cells were quickly harvested in ice-cold water, snap frozen, lyophilized, and stored at −80°C until lipid analysis.

Cholesterol repletion

Cells at the eighth day in culture were treated with 1–5 mM MCD or with incubation buffer for 30 min as described in the previous section. The incubation buffer was removed, and cells were further incubated for 1 h in BME or in BME containing 16 μg/ml cholesterol and 0.4% MCD complex. A stock solution of 0.4 mg/ml cholesterol and 10% MCD was prepared by adding 200 μl cholesterol solution (20 mg/ml in ethanol) to 10 ml of 10% MCD in BME and vortexing at ~40°C (23).

Sucrose gradient centrifugation

After metabolic radiolabeling with [1-^{3}H]sphingosine or [9,10(n)-^{3}H]palmitic acid, cells were treated with 5 mM or 10 mM MCD in Locke's solution or Locke's alone (control) for 30 min at 37°C as described above. The number of cells remaining after each treatment was determined as described above. Cells were subjected to ultracentrifugation on discontinuous sucrose gradients as previously described (4, 8). Briefly, cells were harvested, lysed in lysis buffer [1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/ml aprotinin, 5–8 × 10^7 cells/ml], and Dounce homogenized (10 strokes, tight). Cell lysate was centrifuged (5 min, 1,300 *g*) to remove nuclei and cellular debris. The postnuclear fraction was mixed with an equal volume of 85% sucrose (w/v) in 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1 mM Na_3VO_4, placed at the bottom of a discontinuous sucrose concentration gradient (30–5%) in the same buffer, and centrifuged (17 h, 200,000 *g*) at 4°C. After ultracentrifugation, 11 fractions were collected, starting from the top of the tube. The light-scattering band located at the interface between 5% and 30% sucrose and corresponding to fraction 5 (F5) was regarded as the sphingolipid-enriched membrane fraction (8). The entire procedure was performed at 0–4°C in ice immersion. The protein contents of the postnuclear fraction and of the sucrose gradient fractions were determined as described below.

Lipid analyses

The cell samples, incubation buffers, and sucrose gradient fractions obtained from control or MCD-treated cells were analyzed to determine their lipid content. Incubation buffers and gradient fractions were extensively dialyzed, and lyophilized. Lipids from cell samples and incubation buffers were extracted with chloroform-methanol-water (2:1:0.1; v/v/v) (8). In gradient fractions, water was omitted. The delipidized pellets were solubilized in NaOH, and their protein content was determined as described below. The total lipid extracts were analyzed by high-performance thin-layer chromatography (HPTLC), followed by radioactivity imaging for quantification of radioactivity or by chemical detection of cholesterol and phosphatidylcholine (PC) as described below. Identification of lipids separated by HPTLC was accomplished by comigration with standard lipids, the lipid mixture characterization having been previously established (8). [^{3}H]lipids were separated by monodimensional HPTLC carried out with the solvent system chloroform-methanol-0.2% aqueous $CaCl_2$ (50:42:11; v/v/v). Phospholipids were separated by monodimensional HPTLC using the solvent system (8) chloroform-methanol-acetic acid-water (30:20:2:1; v/v/v/v) or chloroform-methanol-acetone-acetic acid-water (10:2:4:2:1; v/v/v/v/v). Phospholipid species were quantified after separation on HPTLC, followed by specific detection with a molybdate reagent (43). The relative amounts of each phospholipid were determined by densitometry.

Cholesterol was separated by monodimensional HPTLC using the solvent system hexane-ethylacetate (3:2; v/v), and was quantified after separation on HPTLC followed by visualization with 15% concentrated sulfuric acid in 1-butanol. The quantity of cholesterol was determined by densitometry and comparison with 0.1–2 μg of standard compound using the Molecular Analyst program (Bio-Rad Laboratories). In the cholesterol, the SD of the procedure was ±20% in the range of 0.1–0.5 μg, and ±10% in the range of 1–2 μg. These values were similar to those already reported for analytical methods based on HPTLC separation and charring of cholesterol (44).

Protein analyses

Protein content was determined according to Lowry et al. (45) using BSA as reference standard. Proteins were separated by SDS-PAGE. After separation, proteins were transferred to polyvinylidene difluoride membranes, and the presence of Lyn and c-Src was assessed by immunoblotting with specific antibodies, followed by reaction with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (Pierce Supersignal® Chemiluminescence Substrate).

Radiochemical analyses

The radioactivity associated with cells, with cell fractions, with lipids, and with delipidized pellets was determined by liquid scintillation counting. Digital autoradiography of the HPTLC plates was performed with a Beta-Imager 2000 instrument (Biospace) using an acquisition time of ~48–65 h. The radioactivity associated with individual lipids was determined using the specific β-Vision software provided by Biospace.

RESULTS

Complex lipids were metabolically labeled in cultured neuronal cells with [^{3}H]sphingosine and [^{3}H]palmitic acid. **Figure 1** (lane 1a), shows the pattern of radioactive lipids separated by HPTLC after metabolic labeling with [1-^{3}H]sphingosine. As expected (8), sphingomyelin (SM) and all glycosphingolipids were radioactive. **Figure 2** (lane 1a) shows that the metabolic labeling with [9,10(n)-^{3}H]palmitic acid led to the biosynthesis of radioactive sphingolipids and glycerophospholipids. Because glycerophospholipids are the main cell complex lipids and each molecule contains two fatty acid chains, they contained much more radioactivity than sphingolipids. After pulse-chase metabolic labeling, the culture medium was removed and cells were incubated in the presence of Locke's balanced saline solution alone or containing 1–10 mM MCD (incubation buffer) for different times (5–120 min). Radioactive lipids released in the incubation buffer under these experimental conditions were qualitatively and quantitatively analyzed. HPTLC patterns detected after [^{3}H]sphingosine and [^{3}H]palmitic acid metabolic labeling are shown in Figs. 1 and 2, respectively. Independent of the labeling procedure, small amounts of radioactive lipids (from 0.2% to 6.2% of total cell lipid, de-

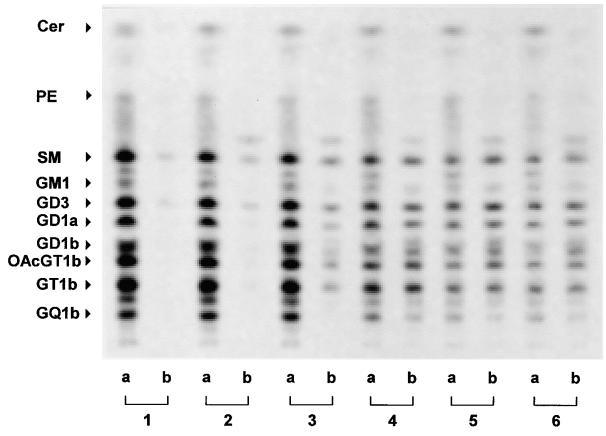

Fig. 1. Effect of methyl-β-cyclodextrin (MCD) on the release of sphingolipids from cultured rat cerebellar granule cells. Cells were metabolically labeled with [1-^{3}H]sphingosine for a 2 h pulse followed by a 48 h chase and treated with vehicle (controls) or 5 mM MCD as described under Experimental Procedures. Radioactive lipids were extracted, separated by high-performance thin-layer chromatography (HPTLC), and detected by digital autoradiography (200–1,000 dpm applied on a 3 mm line; time of acquisition was 48 h). Lanes a: radioactive cell lipids; lanes b: radioactive cell medium lipids. Lane 1: control; lanes 2 to 6: 5, 10, 30, 60, and 120 min treatment, respectively. Patterns are representative of those obtained in three different experiments. Cer, ceramide; PE, phosphatidylethanolamine; SM, sphingomyelin.

pending on the time of treatment and the lipid species) were detected in negative controls (Figs. 1, 2, lanes 1b). This probably reflected a natural shedding process. The amount of radioactive lipids released in the incubation buffer increased in a time- and dose-dependent manner during treatment with MCD. Starting from 30 min treatment with 5 mM MCD, a complex [^{3}H]sphingosine-labeled sphingolipid pattern was released in the incubation buffer that closely resembled that of the cell homogenate (Fig. 1). The only notable exception was represented by ceramide. Radioactive ceramide accounted for about 3% of [^{3}H]sphingosine-labeled cell lipids, but it was either not detectable or detectable only in traces in the incubation buffer, even at the highest MCD concentration and at the longest time of treatment. On the other hand, in the [^{3}H]palmitic acid-labeled glycerophospholipids, PC was the main compound released from cells after MCD treatment (Fig. 2). The other glycerophospholipids were present in the incubation buffer only as traces, even at high MCD concentration and a long treatment time. As expected, MCD treatment resulted in a marked reduction in the cell cholesterol content (**Fig. 3**). Figure 3 shows the quantitative data describing the MCD-induced time- and dose-dependent release of sphingolipids (SM and glycosphingolipids) and cholesterol from cells. In all cases, the release was sustained (50.2%, 14.5%, and 17.1% of total cell component for cholesterol, SM, and glycosphingolipids, respectively, were released after 30 min treatment with 5 mM MCD) and reached a plateau between 30 and 60 min of treatment. The glycerophospholipids were released from cells to a much lesser extent (about 2% of total cell component after 30 min treatment with 5 mM MCD).

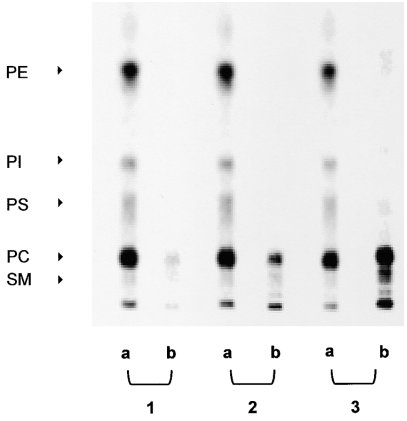

Fig. 2. Effect of MCD on the release of glycerolipids from cultured rat cerebellar granule cells. Cells were metabolically labeled with [9,10(n)-^{3}H]palmitic acid for a 24 h pulse followed by a 48 h chase and treated for 30 min with vehicle (controls) or MCD as described under Experimental Procedures. Radioactive lipids were extracted, separated by HPTLC, and detected by digital autoradiography (200–1,000 dpm applied on a 3 mm line; time of acquisition was 48 h). Lanes a: radioactive cell lipids; lanes b: radioactive lipids released in the incubation buffer. Lane 1: control; lane 2: 5 mM MCD; lane 3: 10 mM MCD. Patterns are representative of those obtained in three different experiments. PI, phosphatidylinositol; PS, phosphatidyl serine; PC, phosphatidylcholine.

The effects of MCD treatment on cell morphology, cell membrane integrity, and cell mitochondrial function were determined. Optical microscopy analysis showed that the morphology of the majority of cells remained substantially unchanged up to 2 h and up to 5 mM MCD treatments. Under these experimental conditions, only a few cells showed morphologically distinguishable signs of damage. On the other hand, treatment with 10 mM MCD led to a clearly distinguishable time-dependent progressive degeneration of neurites. After 2 h treatment with 10 mM MCD, almost all cells bore no neurites and appeared round and translucent but still well-attached to the matrix. A 30 min treatment with 5 mM MCD led to Trypan blue incorporation into 80% of cells (**Table 1**), and this value became 100% when MCD concentration was increased up to 10 mM. Nevertheless, when cells were treated with 5 or 10 mM MCD and then repleted with cholesterol by incubation in the presence of a cholesterol/MCD complex, only 20% and 40% of cells, respectively, incorporated the dye (Table 1). These results would suggest that MCD treatment heavily modified the ability of cells to incorporate Trypan blue, but only a limited number of cells were actually killed by the treatments. In fact, when cell viability was assessed by determining the mitochondrial metabolic activity of viable cells using the MTT reduction assay (**Table 2**), cell viability was only reduced to about 50%, even after a 2 h treatment with 10 mM MCD.

To study the effect of MCD on lipid membrane organization, cells were lysed in the presence of Triton X-100, and a low-density detergent-resistant fraction was prepared by sucrose gradient centrifugation (4, 8). The light-

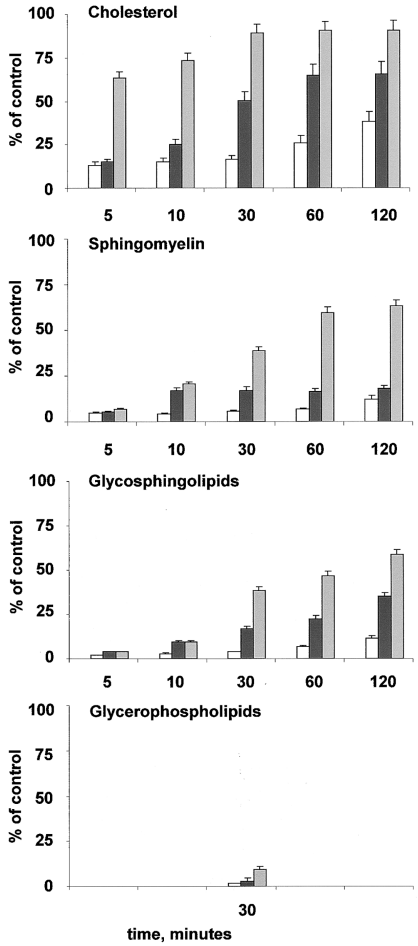

Fig. 3. Time- and dose-dependent release of cholesterol, sphingomyelin, glycosphingolipids, and glycerophospholipids induced by MCD treatment. Cells were metabolically labeled with [1-^{3}H]sphingosine or with [9,10(n)-^{3}H]palmitic acid as described in the legends of Figs. 1 and 2, for the detection of sphingolipids and glycerolipids, respectively, and treated with 1 mM (white), 5 mM (dark gray), and 10 mM (light gray) MCD for 5–120 min (in the case of glycerophospholipids, 30 min only). Lipids were extracted and separated by HPTLC. Radioactive lipids were detected by digital autoradiography (200–1,000 dpm applied on a 3 mm line; time of acquisition was 48 h). Cholesterol was colorimetrically detected as described under Experimental Procedures. The amounts of each lipid or lipid class released in the incubation buffer are expressed as percentages of lipid in cells before exposure to MCD (control). Data are the means ± SD of three different experiments.

scattering band located at the interface between 5% and 30% sucrose (collected as F5 as described under Experimental Procedures), corresponding to the sphingolipid- and cholesterol-enriched membrane fraction in untreated cells, was still clearly observed after a 30 min treatment with 5 mM MCD, but became very faint after a 30 min treatment with 10 mM MCD. **Figure 4** shows the amounts of proteins, glycosphingolipids, SM, PC, and cholesterol associated with each gradient fraction prepared from cells treated with 5 and 10 mM MCD or vehicle (controls) for 30 min. **Figure 5** compares the changes in the contents of sphingolipids, cholesterol, and PC in F5 and in the high-density (HD) region of the gradient (corresponding to

TABLE 1. Effect of 30 min MCD treatment on Trypan blue exclusion in rat cerebellar granule cells

	Cells Permeable to Trypan Blue (% of Total)	
MCD (mM)	MCD Treatment	MCD Treatment Followed by Cholesterol Repletion
0	4.0 ± 0.2	4.5 ± 0.1
5	82.0 ± 7.7	20.0 ± 1.6
10	99.6 ± 8.9	40.0 ± 3.2

MCD, methyl-β-cyclodextrin. Data are the means ± SD of three different experiments.

fractions 9 to 11 of the gradient, which accounted for the bulk of the cell proteins) after MCD treatment. The loss of cell sphingolipids caused by MCD treatment was almost entirely associated with F5, while the amount of sphingolipids found in the HD region was not significantly changed by MCD treatments (Fig. 5, middle panel). **Figure 6** shows the patterns of radioactive sphingolipids associated with the cell lysate, F5, and the HD region in control cells and in cells treated with 5 mM MCD for 30 min. The sphingolipid patterns in control and treated cells are very similar, indicating that MCD treatment does not modify the sphingolipid pattern in F5. As shown in Fig. 5 (lower panel), the loss of PC from cells was also almost entirely associated with F5. After 30 min treatment, the amount of cholesterol lost by cells was higher than the amount of cholesterol belonging to F5 from untreated cells (Fig. 5, upper panel). Thus, after MCD treatment the loss of cholesterol was associated with both F5 and HD fractions.

On the basis of these results, we calculated the molar lipid composition of F5 after MCD treatments. As shown in **Fig. 7**, the amount of PC, the main lipid of the lipid membrane domains in control cells, progressively decreased with the increase of the MCD concentration, indicating a progressive reduction of the total cell surface area occupied by the lipid domains. Nevertheless, after a 30 min treatment with 5 mM MCD, the detergent-resistant fraction was also depleted of cholesterol and sphingolipids; thus the molar ratio between PC, cholesterol, and sphingolipids of F5 from treated cells was not so different from that of the fraction prepared from control cells. As derived from the data reported in Fig. 6, the molar ratio between PC, cholesterol, and sphingolipids, which in F5

TABLE 2. Effect of MCD treatment on rat cerebellar granule cell viability, as mitochondrial metabolic activity measured by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide reduction assay

	Time (Min)			
MCD (mM)	10	30	60	120
5	100 ± 9	58 ± 6	61 ± 6	60 ± 4
10	89 ± 8	38 ± 3	40 ± 2	50 ± 3

Data are expressed as percent of negative controls. Data are the means ± SD of three different experiments.

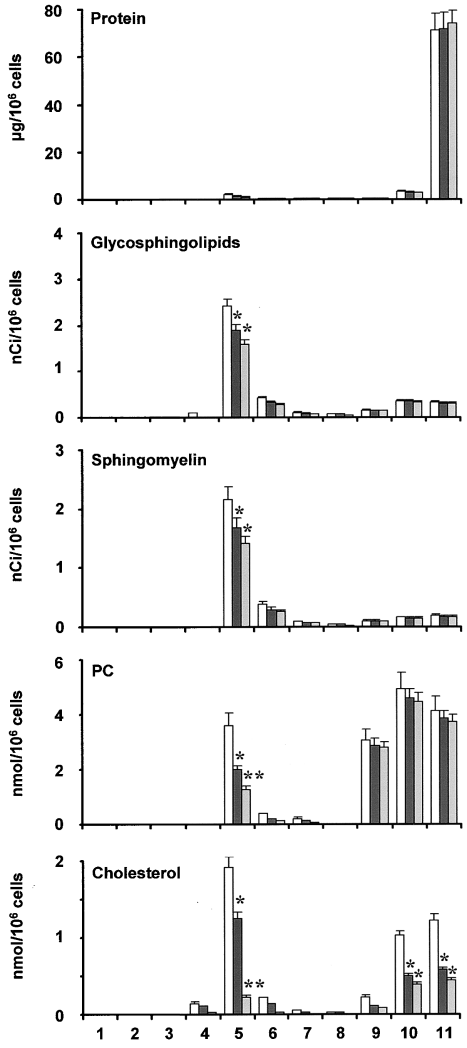

Fig. 4. Effect of MCD treatment on protein and lipid distribution in sucrose gradient fractions prepared from cultured rat cerebellar granule cells. Protein content of each fraction was determined by the method of Lowry et al. (45). Cell sphingolipids and cholesterol were analyzed as described in the legend of Fig. 3. PC was colorimetrically detected as described under Experimental Procedures. Cells were treated with vehicle (controls, white), 5 mM MCD (dark gray), and 10 mM (light gray) MCD for 30 min, lysed in the presence of 1% Triton X-100, and subjected to sucrose gradient fractionation as described under Experimental Procedures. After ultracentrifugation, 11 fractions were collected, starting from the top of the tube. The protein and lipid contents of each fraction are expressed as absolute amounts (as mass for protein, PC, and cholesterol, and as radioactivity for sphingolipids), normalized for the number of cells loaded on the gradient for each treatment. Data are the means ± SD of three different experiments. Statistical significance of differences was determined by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ versus control.

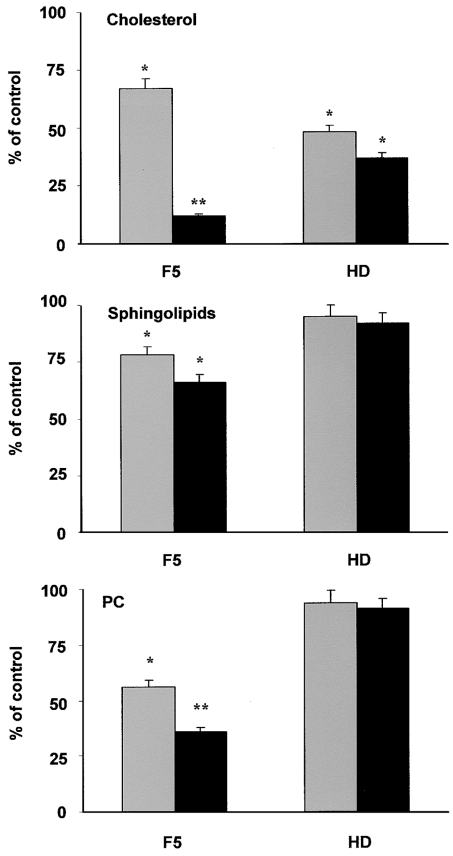

Fig. 5. Cholesterol, sphingolipid, and PC changes induced by MCD treatment in a low-density, detergent-insoluble membrane fraction prepared from cultured rat cerebellar granule cells. Cell lipids were analyzed as described in the legend of Fig. 3. Cells were treated with vehicle (controls), 5 mM MCD (gray), and 10 mM (black) MCD for 30 min and subjected to lysis and sucrose gradient fractionation as described in the legend of Fig. 4. The composition of the low-density, detergent-insoluble membrane fraction [fraction 5 (F5)] and of the high-density (HD) fractions (9 to 11) are compared. The amounts of each lipid or lipid class associated with each fraction after MCD treatment are expressed as percentages of the amounts of lipid present in fractions prepared from control cells. Data are the means ± SD of three different experiments. Statistical significance of differences was determined by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ versus control.

from control cells was 10:5.5:1.7, changed to 10:7.5:2.5. Lyn and c-Src, typical protein components of the lipid membrane domains prepared from neuronal cells (7–9), were still associated with F5 after a 30 min treatment with 5 mM MCD (**Fig. 8**).

After a 30 min treatment with 10 mM MCD, a deep change in the supramolecular organization of the lipid domains of F5 occurred. In fact, under this condition, F5 was strongly impoverished in cholesterol while still highly enriched in sphingolipids. Thus, the molar ratio between PC, cholesterol, and sphingolipids became 10:1.7:3.3. Lyn and c-Src were no longer present in this fraction, being exclusively associated with the HD fraction. The distribution of Akt, which in these cells was exclusively associated with the HD fraction (9), was unaltered by MCD treatment.

DISCUSSION

The addition of cyclodextrins to cultured cells induces the release of cholesterol from the cells themselves. The

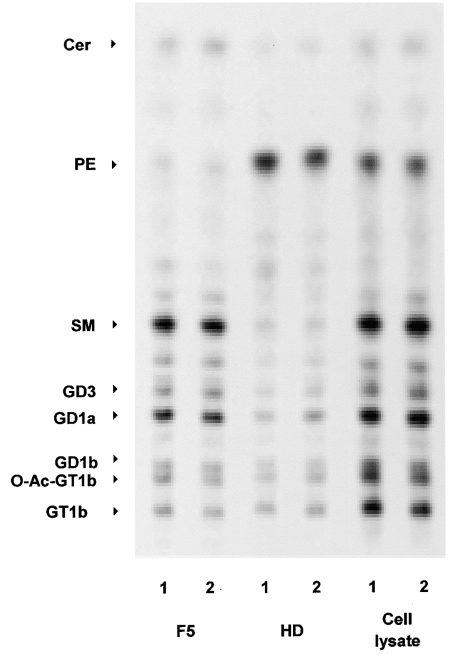

Fig. 6. Effect of MCD treatment on the sphingolipid pattern of a low-density, detergent-insoluble membrane fraction prepared from cultured rat cerebellar granule cells. Cell lipids were metabolically labeled with [1-^{3}H]sphingosine as described in the legend of Fig. 1. Cells were treated with vehicle (controls, lanes 1) or 5 mM MCD (lanes 2) for 30 min and a low-density, detergent-insoluble membrane fraction (F5) was prepared by sucrose gradient fractionation as described under Experimental Procedures. HD: high-density fractions (9 to 11). Cell lysate: radioactive lipids from a total cell lysate before gradient fractionation. Lipids were extracted and separated by HPTLC. Radioactive lipids were detected by digital autoradiography (200–1,000 dpm applied on a 3 mm line; time of acquisition was 48 h). Patterns are representative of those obtained in three different experiments.

process has been shown to be dependent on cyclodextrin concentration, time, and cell type (20–30). The mechanism by which cholesterol is lost by the cells in the presence of cyclodextrins is not clear; this process probably involves different plasma membrane cholesterol pools (22, 24) but not the intracellular cholesterol pools (22). The knowledge of the effects of cyclodextrins on the release of other membrane lipids from cell cultures is very scant and merely qualitative (24, 25, 29, 30). To the best of our knowledge, in the case of gangliosides, only qualitative information on the efflux of GM1 ganglioside from lymphocytes (22) is available. However, the ability of cyclodextrins to disrupt micellar aggregates of GM1 or mixed aggregates of glycerophospholipids and GM1, and their ability to form complexes with ganglioside monomers have been studied in detail by NMR spectroscopy and biochemical procedures (31–33, 46). Both cholesterol and sphingolipids are segregated in membrane lipid domains that are able to sort proteins involved in the modulation of several aspects of cell function. Thus, treatment of cells with cyclodextrins became a tool widely used to study the properties of cholesterol- and sphingolipid-enriched membrane domains. In this study, we investigated the ability of MCD

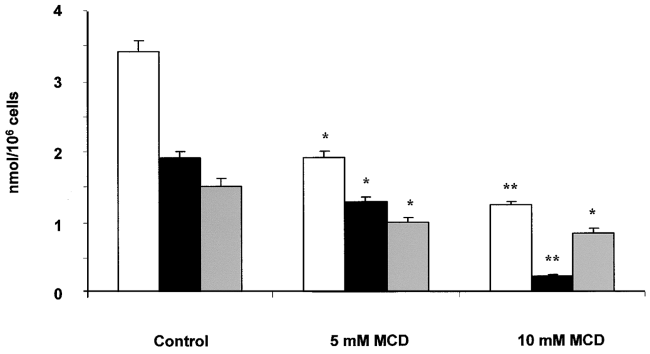

Fig. 7. Effect of MCD treatment on the PC, cholesterol, and sphingolipid composition of a low-density, detergent-insoluble membrane fraction prepared from cultured rat cerebellar granule cells. The PC (white), cholesterol (black), and sphingolipid (gray) molar contents of the low-density, detergent-insoluble membrane fractions prepared from cerebellar granule cells treated with vehicle (control), 5 mM MCD, and 10 mM MCD for 30 min were calculated as described under Experimental Procedures. Data are expressed as absolute mass amounts, normalized for the number of cells loaded on the gradient for each treatment. Data are the means ± SD of three different experiments. Statistical significance of differences was determined by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ versus control.

to induce the release of sphingolipids, cholesterol, and glycerophospholipids from rat cerebellar granule cells in culture, and the effect of this release on the existence and properties of lipid membrane domains. In a few experiments, we obtained qualitative data suggesting that other cyclodextrins, such as (2-hydroxypropyl)-β-cyclodextrin, had properties very similar to those of MCD.

MCD mediated the efflux of cholesterol, sphingolipids, and glycerolipids. It is noteworthy that a quantitative analysis of this phenomenon was only possible thanks to cell

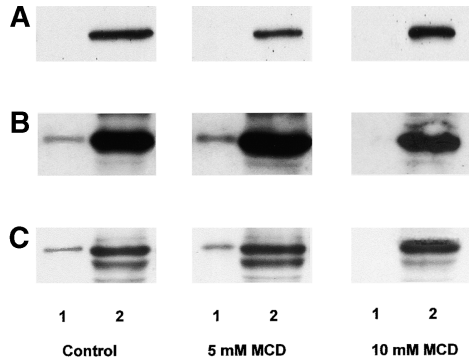

Fig. 8. Effect of MCD treatment on the association of Lyn and c-Src with a low-density, detergent-insoluble membrane fraction prepared from cultured rat cerebellar granule cells. The same percentage of the total from the low-density, detergent-insoluble membrane fractions (F5, lanes 1) and from the HD fractions (9 to 11) (lanes 2) prepared from cerebellar granule cells treated with vehicle (control), 5 mM MCD, and 10 mM MCD for 30 min were analyzed by SDS-PAGE followed by detection by Western blotting using specific anti-Akt (A), anti-Lyn (B), and anti-c-Src (C) antibodies. Patterns are representative of those obtained in two different experiments.

lipid metabolic labeling procedures using tritiated sphingosine and palmitic acid as precursors for sphingolipids and glycerophospholipids (8, 42). This allowed us to follow simultaneously the loss of lipids from cells and their parallel increase in the incubation buffer. In the case of cholesterol, the analytical interference of MCD did not allow for the determination of the amount of cholesterol present in the incubation buffer, and its release from cells was determined as decrease of cell content. MCD mediated the efflux of all sphingolipid species to a similar extent, including gangliosides, neutral glycosphingolipids, and SM. The only sphingolipid we did not find in the incubation buffer was ceramide. This is probably related to the strong hydrophobic character of ceramide, together with its stable association with the membrane as a result of the hydrogen bond network promoted by its amide linkage (47). Among glycerophospholipids, PC was by far the major compound released in the incubation buffer, the other species being present only in traces. The amounts of sphingolipids, glycerophospholipids, and cholesterol released by a 30 min treatment with 5 mM MCD were 16%, 2%, and 50%, respectively. Recalling that the half-life of complex lipids in differentiated neurons is on the order of a few hours (48), in the case of a 30 min MCD treatment, it is very likely that there is not enough time to neobiosynthesize all the lipids lost by the cells and to reconstitute the plasma membrane integrity. In fact, under these experimental conditions, the plasma membrane of the majority of cells was deeply altered, as proven by the incorporation of Trypan blue dye, and a large number of them eventually died, as shown by the low level of mitochondrial metabolic activity measured by the MTT reduction assay.

As mentioned above, it has been demonstrated that both cholesterol and sphingolipids are segregated in specific areas of the plasma membrane, where they participate in the functional modulation of signaling proteins involved in inter- and intracellular communications. Cyclodextrin treatment was shown to alter or to abolish the segregation of some proteins within the lipid membrane domains, suggesting that altering the lipid composition of the domain has profound influences on its function. However, the mechanisms linking the changes occurring in the lipid composition of the domains with those occurring in their protein composition are not clear.

A low-density, detergent-insoluble membrane fraction, F5, that in cultured cerebellar granule cells was shown to contain the sphingolipid-enriched membrane domains, was prepared after MCD treatment and analyzed for its lipid content. Our findings suggest that the quantity and composition of the membrane regions organized as lipid domains are strongly affected by MCD treatments and are dependent on the experimental conditions used. The increase of the MCD concentration led to a progressive reduction of PC in F5. PC is the main cell glycerophospholipid, and it is the main lipid component of lipid membrane domains from differentiated cerebellar neurons (8). Thus, the reduction in the content of PC can be considered a good indication of a general reduction of the surface area occupied by the lipid domains. According to this hypothesis, a 30 min treatment with 5 mM MCD led to a reduction of the lipid membrane domain area of about 40%. Previously, we showed that in differentiated cerebellar neurons, about 20% of the total cell PC belongs to F5 (8, 9). Thus, the amount of PC lost by the cells, 2% of the total cell PC (see Fig. 3), corresponding to about 0.7 nmol/10^6 cells, was much less than the amount of PC missing from F5 (about 40% of total PC in F5, corresponding to about 1.4 nmol/10^6 cells). This suggests that exchanges of lipids might occur between lipid membrane

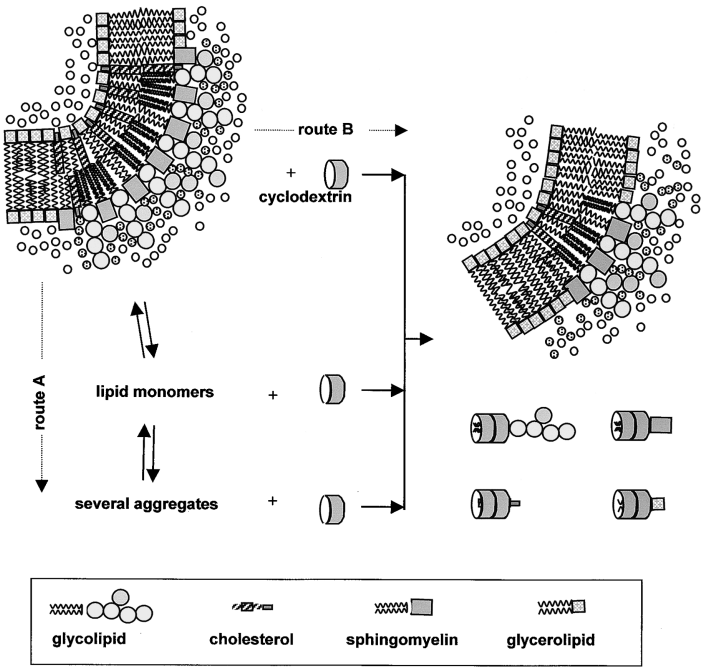

Fig. 9. Scheme depicting possible molecular models for the efflux of membrane lipids mediated by MCD. The scheme is hypothetical, but some parts of it are supported by experimental evidence, such as the natural shedding of membrane lipids (49), the equilibrium between monomers and aggregates (50), the formation of ganglioside-MCD (32, 33) and of cholesterol-MCD aggregates (22), and the changes of membrane curvature as a function of the hydrophilic size of the membrane components (50).

domains and areas of the membrane not organized as lipid domains. The amounts of cholesterol and sphingolipids were also reduced in F5, so that the final molar ratio between PC, cholesterol, and sphingolipids remained approximately constant in the lipid domains. About 50% of total cell cholesterol was released under these experimental conditions (Fig. 3). This quantity (corresponding to about 2.4 nmol/10^6 cells) exceeded the cholesterol content of lipid membrane domains from untreated cells (1.9 nmol/10^6 cells) (8). Thus, although our data are not sufficient to establish the subcellular origin of the large amount of cholesterol released by the cells, it is clear that a very dynamic process is occurring, possibly involving an exchange of cholesterol between the membrane lipid domains and other membrane and/or intracellular sites.

The large loss of cholesterol determined by MCD treatment could be the driving force that leads to the overall reduction of the surface area occupied by the lipid domains. This could be a way to maintain a constant PC-cholesterol molar ratio in the membrane lipid domains. A similar behavior can be described for the sphingolipids. Thus, under these experimental conditions, the final effect induced by MCD is an overall loss of membrane lipid domains without alteration of their original properties, such as their lipid molar composition and the ability to segregate Lyn and c-Src (Fig. 8).

When the concentration of MCD was increased up to 10 mM, we had a further reduction of the lipid domain area, but the dramatic loss of the membrane lipids, particularly of cholesterol, did not allow the maintenance of a constant molar ratio between PC, cholesterol, and sphingolipids within the lipid membrane domain. Thus, the resulting low-density, detergent-resistant membrane fraction was highly impoverished in cholesterol. The enrichment in sphingolipids seems to be increased in respect to untreated cells, but this was not enough to balance the loss of cholesterol. The lipid membrane domain, as a consequence of this dramatic change in its lipid composition, lost the ability to segregate Lyn and c-Src (Fig. 8). This eventually led to severe cell distress, degeneration, and death.

In conclusion, in this paper we show that MCD, together with the release of cholesterol, induced the release of large amounts of sphingolipids from cells. Small amounts of complex lipids were found in the incubation buffer medium in the absence of cyclodextrin, probably reflecting a natural equilibrium between membrane lipids and free lipids (see the route A in the scheme shown in **Fig. 9**). This raises the possibility that MCD increases the natural rate of efflux by modifying this equilibrium, forming complexes with the released lipids (as monomers or aggregates). Of course, a direct interaction of MCD with membrane lipids (route B in Fig. 9) must also be considered. At any rate, independent of the modalities of the efflux process mediated by MCD, the rapid loss of lipids requires a corresponding rapid reorganization of the remaining membrane lipids, suggesting the existence of dynamic processes aimed at preserving the segregation of cholesterol and sphingolipids in membrane domains. When the loss of lipids is relatively limited, as in the case of a "mild" MCD treatment, the membrane lipids change their membrane organization, reducing the total surface area of membrane lipid domains. This can be achieved by reducing the total number of domains or their sizes. But a large loss of lipids, as that induced by a "strong" MCD treatment, very rapidly induces the loss of the integrity and properties of lipid membrane domains, and, as a final consequence, severe changes in the membrane permeability, accompanied by morphological changes and signs of distress in the cells.

This research was supported by a grant from the Consiglio Nazionale delle Ricerche (target project Biotechnology), Cofin-Prin 2001/2002, and the Mizutani Foundation for Glycoscience to S.S.

REFERENCES

1. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1:** 31–39.
2. Hakomori, S., K. Handa, K. Iwabuchi, S. Yamamura, and A. Prinetti. 1998. New insights in glycosphingolipid function: "glycosignaling domain," a cell surface assembly of glycosphingolipids with signal transducer molecules, involved in cell adhesion coupled with signaling. *Glycobiology.* **8:** xi–xix.
3. Brown, D. A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14:** 111–136.
4. Prinetti, A., K. Iwabuchi, and S. Hakomori. 1999. Glycosphingolipid-enriched signaling domain in mouse neuroblastoma Neuro2a cells. Mechanism of ganglioside-dependent neuritogenesis. *J. Biol. Chem.* **274:** 20916–20924.
5. Madore, N., K. L. Smith, C. H. Graham, A. Jen, K. Brady, S. Hall, and R. Morris. 1999. Functionally different GPI proteins are organized in different domains on the neuronal surface. *EMBO J.* **18:** 6917–6926.
6. Bilderback, T. R., V. R. Gazula, M. P. Lisanti, and R. T. Dobrowsky. 1999. Caveolin interacts with Trk A and $p75^{NTR}$ and regulates neurotrophin signaling pathways. *J. Biol. Chem.* **274:** 257–263.
7. Prinetti, A., N. Marano, S. Prioni, V. Chigorno, L. Mauri, R. Caselato, G. Tettamanti, and S. Sonnino. 2000. Association of Src-family protein tyrosine kinases with sphingolipids in rat cerebellar granule cells differentiated in culture. *Glycoconj. J.* **17:** 223–232.
8. Prinetti, A., V. Chigorno, G. Tettamanti, and S. Sonnino. 2000. Sphingolipid-enriched membrane domains from rat cerebellar granule cells differentiated in culture. A compositional study. *J. Biol. Chem.* **275:** 11658–11665.
9. Prinetti, A., V. Chigorno, S. Prioni, N. Loberto, N. Marano, G. Tettamanti, and S. Sonnino. 2001. Changes in the lipid turnover, composition, and organization, as sphingolipid-enriched membrane domains, in rat cerebellar granule cells developing in vitro. *J. Biol. Chem.* **276:** 21136–21145.
10. Prinetti, A., S. Prioni, S. Chigorno, D. Karagogeos, G. Tettamanti, and S. Sonnino. 2001. Immunoseparation of sphingolipid-enriched membrane domains enriched in Src family protein tyrosine kinases and in the neuronal adhesion molecule TAG-1 by anti-GD3 ganglioside monoclonal antibody. *J. Neurochem.* **78:** 1162–1167.
11. Loberto, N., S. Prioni, A. Prinetti, E. Ottico, S. Chigorno, D. Karagogeos, and S. Sonnino. 2003. The adhesion protein TAG-1 has a ganglioside environment in the sphingolipid-enriched membrane domains of neuronal cells in culture. *J. Neurochem.* **85:** 224–233.
12. Prioni, S., N. Loberto, A. Prinetti, S. Chigorno, F. Guzzi, R. Maggi, M. Parenti, and S. Sonnino. 2002. Sphingolipid metabolism and caveolin expression in gonadotropin-releasing hormone-expressing GN11 and gonadotropin-releasing hormone-secreting GT1–7 neuronal cells. *Neurochem. Res.* **27:** 831–840.
13. Kasahara, K., Y. Watanabe, T. Yamamoto, and Y. Sanai. 1997. Cave-

olin interacts with Trk A and p75^{NTR} and regulates neurotrophin signaling pathways. *J. Biol. Chem.* **272:** 29947–29953.
14. Kasahara, K., Y. Watanabe, K. Takeuchi, H. Kaneko, A. Ooohira, T. Yamamoto, and Y. Sanai. 2000. Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. *J. Biol. Chem.* **275:** 34701–34709.
15. Stevens, V. L., and J. Tang. 1997. Fumonisin B_1-induced sphingolipid depletion inhibits vitamin uptake via the glycosylphosphatidylinositol-anchored folate receptor. *J. Biol. Chem.* **272:** 18020–18024.
16. Naslavsky, N., H. Shmeeda, G. Friedlander, A. Yanai, A. H. Futerman, Y. Barenholz, and A. Taraboulos. 1999. Sphingolipid depletion increases formation of the scrapie prion protein in neuroblastoma cells infected with prions. *J. Biol. Chem.* **274:** 20763–20771.
17. Sheets, E. D., G. M. Lee, R. Simson, and K. Jacobson. 1997. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. *Biochemistry.* **36:** 12449–12458.
18. Inokuchi, J., S. Uemura, K. Kabayama, and Y. Igarashi. 2000. Glycosphingolipid deficiency affects functional microdomain formation in Lewis lung carcinoma cells. *Glycoconj. J.* **17:** 239–246.
19. Taraboulos, A., M. Scott, A. Semenov, D. Avraham, L. Laszlo, and S. B. Prusiner. 1995. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J. Cell Biol.* **129:** 121–132.
20. Ledesma, M. D., K. Simons, and C. G. Dotti. 1998. Neuronal polarity: essential role of protein-lipid complexes in axonal sorting. *Proc. Natl. Acad. Sci. USA.* **95:** 3966–3971.
21. Simons, M., P. Keller, B. De Strooper, K. Beyreuther, C. G. Dotti, and K. Simons. 1998. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. *Proc. Natl. Acad. Sci. USA.* **95:** 6460–6464.
22. Neufeld, E. B., A. M. Cooney, J. Pitha, E. A. Dawidowicz, N. K. Dwyer, P. G. Pentchev, and E. J. Blanchette-Mackie. 1996. Intracellular trafficking of cholesterol monitored with a cyclodextrin. *J. Biol. Chem.* **271:** 21604–21613.
23. Furuchi, T., and R. G. W. Anderson. 1998. Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase ERK. *J. Biol. Chem.* **273:** 21099–21104.
24. Ilangumaran, S., and D. C. Hoessli. 1998. Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane. *Biochem. J.* **335:** 433–440.
25. Sheets, E. D., D. Holowka, and B. Baird. 1999. Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. *J. Cell Biol.* **145:** 877–887.
26. Fukasawa, M., M. Nishijima, H. Itabe, T. Takano, and K. Hanada. 2000. Reduction of sphingomyelin level without accumulation of ceramide in Chinese hamster ovary cells affects detergent-resistant membrane domains and enhances cellular cholesterol efflux to methyl-beta -cyclodextrin. *J. Biol. Chem.* **275:** 34028–34034.
27. Shogomori, H., and A. H. Futerman. 2001. Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. *J. Biol. Chem.* **276:** 9182–9188.
28. Mendez, A. J., G. Lin, D. P. Wade, R. M. Lawn, and J. F. Oram. 2001. Membrane lipid domains distinct from cholesterol/sphingomyelin-rich rafts are involved in the ABCA1-mediated lipid secretory pathway. *J. Biol. Chem.* **276:** 3158–3166.
29. Kilsdonk, E. P. C., P. G. Yancey, G. W. Stoudt, F. W. Bangerter, W. J. Johnson, M. C. Phillips, and G. H. Rothblat. 1995. Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270:** 17250–17256.
30. Hansen, G. H., L. Immerdal, E. Thorsen, L-L. Niels-Christiansen, B. T. Nystrøm, E. J. F. Demant, and E. M. Danielsen. 2001. Lipid rafts exist as stable cholesterol-independent microdomains in the brush border membrane of enterocytes. *J. Biol. Chem.* **276:** 32338–32344.
31. Singh, I., and Y. Kishimoto. 1983. Effect of cyclodextrins on the solubilization of lignoceric acid, ceramide, and cerebroside, and on the enzymatic reactions involving these compounds. *J. Lipid Res.* **24:** 662–665.
32. Casu, B., A. Grenni, A. Naggi, G. Torri, M. Virtuani, and B. Focher. 1990. Interaction of cyclodextrinscyclomalto-oligosaccharides with glycolipids: N.M.R. studies of aqueous system of cyclomaltohexaose and alkyl glycosides. *Carbohydr. Res.* **200:** 101–109.
33. Shiraishi, T., M. Hiraiwa, and Y. Uda. 1993. Effects of cyclodextrins on the hydrolysis of ganglioside GM1 by acid beta-galactosidases. *Glycoconj. J.* **10:** 170–174.
34. Carter, H. E., J. A. Rothfus, and R. Gigg. 1961. Biochemistry of the sphingolipids: XII. Conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside. *J. Lipid Res.* **2:** 228–234.
35. Tettamanti, G., F. Bonali, S. Marchesini, and V. Zambotti. 1973. Parallelism of subcellular location of major particulate neuraminidase and gangliosides in rabbit brain cortex. *Biochim. Biophys. Acta.* **296:** 160–170.
36. Toyokuni, T., M. Nisar, B. Dean, and S. Hakomori. 1991. A facile and regiospecific titration of sphingosine: synthesis of 2S,3R,4E.-2-amino-4-octadecene-1,3-diol-1-^{3}H. *J. Label. Compd. Radiopharm.* **29:** 567–574.
37. Gallo, V., M. Ciotti, A. Coletti, F. Aloisi, and G. Levi. 1982. Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. USA.* **79:** 7919–7923.
38. Thangnipon, W., A. Kingsbury, M. Webb, and R. Balazs. 1983. Observations on rat cerebellar cells in vitro: influence of substratum, potassium concentration and relationship between neurones and astrocytes. *Brain Res.* **11:** 177–189.
39. Kingsbury, A. E., V. Gallo, P. L. Woodhams, and R. Balazs. 1985. Survival, morphology and adhesion properties of cerebellar interneurones cultured in chemically defined and serum-supplemented medium. *Brain Res.* **17:** 17–25.
40. Balasz, R., V. Gallo, and A. Kingsbury. 1988. Effect of depolarization on the maturation of cerebellar granule cells in culture. *Brain Res.* **468:** 269–276.
41. Rizzardini, M., M. Lupi, S. Bernasconi, A. Mangolini, and L. Cantoni. 2002. Mitochondrial dysfunction and death in motor neurons exposed to the glutathione-depleting agent ethacrynic acid. *J. Neurol. Sci.* **207:** 51–58.
42. van Echten, G., H. Iber, H. Stotz, A. Takatsuki, and K. Sandhoff. 1990. Uncoupling of ganglioside biosynthesis by Brefeldin A. *Eur. J. Cell Biol.* **51:** 135–139.
43. Vaskovsky, V. E., and E. Y. Kostetsky. 1968. Modified spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **9:** 396.
44. Klein, T. R., D. Kirsch, R. Kaufmann, and D. Riesner. 1998. Prion rods contain small amounts of two host sphingolipids as revealed by thin-layer chromatography and mass spectrometry. *Biol. Chem.* **379:** 655–666.
45. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
46. Ahmed, S. M., B. Casu, A. Cedro, M. Guerrini, E. Lanzarotti, D. Moltrasio, A. Naggi, and G. Torri. 1994. Disruption of micellar aggregates of ganglioside GM-1 by complexation with α-cyclodextrin. *Int. J. Pharm.* **109:** 99–106.
47. Pascher, I. 1976. Molecular arrangements in sphingolipids. Conformation and hydrogen bonding of ceramide and their implication on membrane stability and permeability. *Biochim. Biophys. Acta.* **455:** 433–451.
48. Riboni, L., R. Bassi, A. Prinetti, and G. Tettamanti. 1996. Salvage of catabolic products in ganglioside metabolism: a study on rat cerebellar granule cells in culture. *FEBS Lett.* **391:** 336–340.
49. Ladisch, S., R. Li, and E. Olson. 1994. Ceramide structure predicts tumor ganglioside immunosuppressive activity. *Proc. Natl. Acad. Sci. USA.* **91:** 1974–1978.
50. Sonnino, S., L. Cantù, M. Corti, D. Acquotti, and B. Venerando. 1994. Aggregative properties of gangliosides in solution. *Chem. Phys. Lipids.* **71:** 21–45.