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Efflux of sphingolipids metabolically labeled with [1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid from normal cells in culture

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Abstract The membrane complex lipids of human fibroblasts and differentiated rat cerebellar granule cells in culture were metabolically radiolabeled with [1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid. A relevant efflux of radioactive sphingolipids and phosphatidylcholine was observed from cells to the culture medium in the presence of fetal calf serum. This event was independent of the concentration and structure of the metabolic precursor administered to cells, and it was linearly time-dependent. The radioactive lipid patterns present in the medium were different from those present in the cells. Radioactive sphingomyelin and ganglioside GM3 containing short acyl chains were the main species present in the medium from human fibroblasts, while sphingomyelin and GD3 ganglioside in that from neuronal cells. In the absence of proteins in the culture medium, the efflux of complex lipids was much lower than in the presence of serum, and the patterns of released molecules were again different from those of cells.

Keywords Sphingolipids · Metabolic labeling · Fibroblasts · Neuronal cells · Shedding

Abbreviations

Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm [1], and the IUPAC-IUBMB recommendations [2].

GlcCer	ß-Glc-(1-1)-Cer
LacCer	ß-Gal-(1-4)-ß-Glc-(1-1)-Cer GM3, II^{3}Neu5Ac-LacCer, α-Neu5Ac-(2-3)-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
GM1	II^{3}Neu5AcGg$_4$Cer, ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
GD3	II^3(Neu5Ac)$_2$LacCer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
GD1a	IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$Cer, α-Neu5Ac-(2-3)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
GD1b	II^3(Neu5Ac)$_2$GgOse$_4$Cer, ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; *O*-Ac-GT1b, sIV^{3}Neu5AcII^3[α-Neu5, 9Ac$_2$-(2-8)-α-Neu5Ac-(2-3)]GgOse$_4$Cer
GT1b	IV^{3}Neu5AcII^3(Neu5Ac)$_2$GgOse$_4$Cer, α-Neu5Ac-(2-3)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
GQ1b	IV^3(Neu5Ac)$_2$II^3(Neu5Ac)$_2$GgOse$_4$Cer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer
Neu5Ac	*N*-acetylneuraminic acid
Cer	ceramide, *N*-acyl-sphingosine
Sph	sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene; [1-^{3}H]sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-[1-^{3}H]octadecene
PE	phosphatidylethanolamine
SM	sphingomyelin
BME	Basal modified Eagle's medium
EMEM	Eagle's minimum essential medium
FCS	fetal calf serum
HPTLC	high-performance thin-layer chromatography

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PMSF	phenyl-methylsulfonyl fluoride
SDS	sodium dodecylsulfate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

Introduction

The strong amphiphilic nature of sphingolipids and glycerophospholipids [3–5] raises the possibility that multiple equilibrium exists between monomers inserted in the plasma membrane (where they can be present in different states of segregation) and molecules in the aqueous extracellular environment, where they might exist as monomers and as part of aggregates of different nature, thus generating a lipid flux between cell and extracellular milieu. This possible exchange was poorly studied in the past, but some evidence indicates it is actually taking place in living organisms, at least under certain circumstances. Sphingolipids are detectable in serum and cerebrospinal fluid from humans and animals [6–9], and the release of gangliosides from tumors and tumor cells has been described [10–14]. Ganglioside molecular species containing less hydrophobic ceramides [9,13,15], are usually preferred for shedding, while no significant differences were observed in the shedding of gangliosides on the basis of their oligosaccharide chains. A detailed biochemical study on the fate of sphingolipids released from normal and tumoral cells reporting the suggestion that a part of the released sphingolipids is taken up by the cells and catabolised has recently been presented [16].

In this paper, we compare the efflux process of sphingolipids from normal cells, cultured in the presence or not of proteins in the medium, after their metabolic radiolabeling using three sensitive procedures. Moreover, for the first time we report on the efflux of phosphatidylcholine.

Experimental procedures

Materials

The commercial chemicals were the purest available; common solvents were distilled before use and deionized water, obtained by a MilliQ system (Millipore), was distilled in a glass apparatus. High performance silica gel precoated thin-layer plates (TLC Kieselgel 60, 10×10 cm) were purchased from Merck. Basal modified Eagle's medium (BME), Eagle's minimum essential medium (EMEM) and fetal calf serum (FCS) were from Hy-clone. Other reagents for cell cultures and bovine serum albumin were from Sigma Chemical Co.

L-[3-^{3}H]serine, 30 Ci/mmole, and [9,10-^{3}H]palmitic acid, 55 Ci/mmole, were from Amersham International. Sphingosine was prepared from cerebroside [17]. [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 [18] (radiochemical purity over 98%; specific radioactivity 2.2 Ci/mmol). Radioactive sphingolipids were extracted from cells fed with [1-^{3}H]sphingosine, purified, characterized as previously described [19–20], and used as chromatographic standards.

Normal human skin fibroblasts were obtained by the punch technique, cultured and propagated as described [21] using EMEM supplemented with 10% FCS, and used for the experiments when confluent. Granule cells were obtained from cerebellum of 8-day-old Harlan Sprague-Dawley rats and cultured as described [22–24]. Cells were plated at a density of 9×10^6 cells/dish in 100 mm dishes, cultured with BME supplemented with 10% FCS and used for the experiments between the 6^{th} and 8^{th} day in culture. The contents, patterns and characterizations of cell sphingolipids were previously reported [20,25].

Treatment of cell cultures with [1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid

[1-^{3}H]sphingosine dissolved in methanol was transferred into a sterile glass tube and dried under a nitrogen stream; the residue was then dissolved in an appropriate volume of pre-warmed (37°C) 2% FCS-EMEM (fibroblasts) or in cell-conditioned medium (granule cells) to obtain the desired final concentration (3×10^{-9} M, 3×10^{-8} M and 3×10^{-7} M). After a 2 h incubation (pulse), the medium was removed, cells were washed with EMEM or BME, and incubated for variable time (chase) with EMEM or BME not containing radioactive precursors in the presence or in the absence of FCS.

L-[3-^{3}H]serine dissolved in sterile water was diluted into a pre-warmed (37°C) EMEM to obtain a final concentration of 4.0×10^{-7} M and incubated for 20 h with cultured human fibroblasts. After removal of the pulse medium and washing, a 48 h chase was performed in 10% FCS-containing medium.

[9,10-^{3}H]palmitic acid dissolved in ethanol was transferred into a sterile glass tube and dried under a nitrogen stream; the residue was then dissolved in 5 μL of dimethylsulfoxide and the solution added to pre-warmed (37°C) EMEM to obtain a final concentration of 4.5×10^{-7} M. Cultured human fibroblasts were incubated for 20 h with the radioactive palmitic acid solution; then the medium was removed, cells were washed and further incubated for 48 h in fresh medium containing 10% FCS.

Cell viability was determined by the Trypan Blue exclusion method.

Extraction and analysis of radioactive lipids

In all the radiolabeling experiments described above, at the end of chase the cell medium was carefully collected, and cells were harvested in ice-cold water (2 mL) by scraping with a rubber policeman.

The cell suspensions were snap frozen, lyophilized, and the residues subjected to lipid extraction. Fibroblast lipids were extracted according to the tetrahydrofuran procedure adapted to cell cultures [25–26], and neuronal cell lipids were extracted with chloroform/methanol as previously described [19]. The cell medium from cells fed with radioactive precursors ([1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid) were dialyzed for 2 days, snap frozen, lyophilized, and the residues subjected to lipid extraction with tetrahydrofuran/water [26]. The resulting total lipid extracts were dried under nitrogen stream and dissolved in chloroform-methanol, 2:1 by vol. In some experiments the lipid extracts were treated with 0.5 M NaOH in methanol to remove glycerophospholipids [20].

Lipids were analyzed by HPTLC using the solvent systems chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 by vol, to separate fibroblast lipids and products formed by the enzymatic treatments described above, and chloroform/methanol/0.2% aqueous $CaCl_2$ 50:42:11 by vol, to separate granule cell lipids. After separation, radioactive lipids were visualized with a Beta-Imager 2000 (Biospace). The radioactivity associated with individual lipids was determined with the specific β-Vision software (Biospace).

Other analytical methods

Radioactivity associated with cells, with cell fractions, with medium, with medium fractions, and with lipid extracts was determined by liquid scintillation counting. The protein content was determined according to Lowry [27] with bovine serum albumin as the reference standard.

Results

[1-^{3}H]sphingosine administered to human fibroblasts and differentiated rat cerebellar granule cells in culture, in agreement with previous data [20,25,28–30], was very rapidly taken up by both cell types, and entered into the complex sphingolipid biosynthetic pathway. Within 2 h, free sphingosine could no longer be observed in the cells, and stable radioactive lipid patterns were obtained. A part of [1-^{3}H]sphingosine was catabolized by the cells, the catabolic radioactive ethanolamine being recycled for the biosynthesis of phosphatidylethanolamine [20,25]. Figures 1 and 2 show the radioactive sphingolipid patterns obtained under these conditions from human fibroblasts and differentiated rat cerebellar granule cells, respectively.

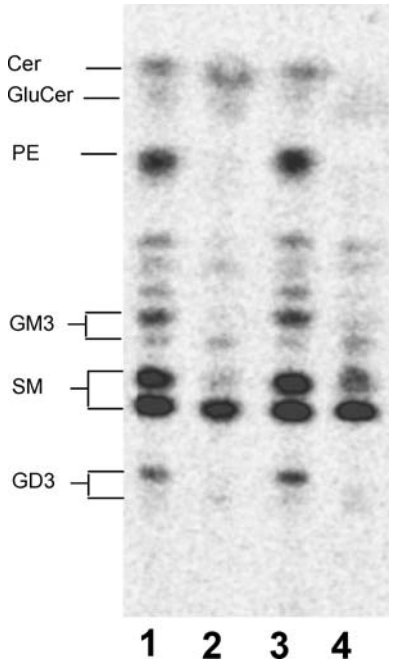

Fig. 1 Patterns of radioactive lipids present in human fibroblasts and their cell-conditioned medium after feeding 3×10^{-8} M [1-^{3}H]sphingosine, (2 hrs pulse followed by 48 h-chase). Lipids from the cells (lanes 1 and 3) and from the cell-conditioned medium (lanes 2 and 4) were extracted and separated by HPTLC as described under "Experimental procedures". The chase was performed in the presence, (lanes 1 and 2), or in the absence (lanes 3 and 4), of 10% FCS in the medium. Solvent system: chloroform-methanol-0.2% aqueous $CaCl_2$, 55/45/10 by vol. Radioactive lipids were detected by digital autoradiography; 100–200 dpm/lane; time of acquisition: 48 hrs

Along the chase time, an efflux of radioactivity from both cell types to the culture medium was observed. The efflux was quite linear, and after 5 days about 40% of the radioactive sphingolipids synthesized by the cells was released into the medium in the presence of 10% FCS. In the absence of FCS in the chase medium, the magnitude of the efflux process was reduced to about 8 to 10%. SM was the main radioactive sphingolipid released from human fibroblasts into the medium, while GM3 ganglioside could be observed in lower amount (Fig. 1). Both SM and GM3 have been shown to be present in the fibroblast cell homogenates as heterogeneous molecular species differing in the ceramide structures [25,31]. Thus, they were resolved by HPTLC in two spots corresponding to the molecular species having longer (upper spot) and shorter (lower spot) acyl chains, respectively. For both SM and GM3, the molecular species having shorter acyl chains were the major species released into the medium. Molecular species bearing longer acyl chains were hardly detectable (Fig. 1). Radioactive PE, the only radiolabeled glycerophospholipid present in the cells under these experimental conditions, was not detectable in the cell-conditioned medium.

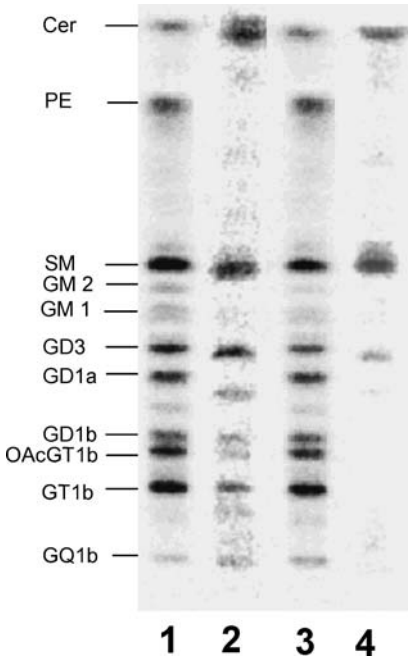

Fig. 2 Patterns of radioactive lipids present in differentiated rat cerebellar granule cells and their cell-conditioned medium after feeding 3×10^{-8} M [1-^{3}H]sphingosine, (2 h pulse followed by 48 h-chase). Lipids from the cells (lanes 1 and 3) and from the cell-conditioned medium (lanes 2 and 4) were extracted and separated by HPTLC as described under "Experimental procedures". The chase was performed in the presence, (lanes 1 and 2), or in the absence (lanes 3 and 4), of 10% FCS in the medium. Solvent system: chloroform-methanol-0.2% aqueous $CaCl_2$, 50/42/11 by vol. Radioactive lipids were detected by digital autoradiography; 100–200 dpm/lane; time of acquisition: 48 h

Efflux of sphingolipids to the medium was also observed from differentiated rat cerebellar granule cells in culture (Fig. 2). The radioactive sphingolipids synthesized by these cells after 2 h pulse with 3×10^{-8} M [1-^{3}H]sphingosine, followed by a 48 h chase, were released into the cell medium, but in relative proportions different from those of the cell homogenate. As already observed in the case of human fibroblasts, no radioactive PE was released in the medium.

Some differences in the radioactive sphingolipid pattern released into the cell medium were observed upon removal of FCS. In the case of fibroblasts, a higher quantity of the more hydrophobic molecular species of GM3 and SM was observed in the medium.

Feeding experiments using three different concentrations of sphingosine were performed to determine any possible influence of this experimental condition on the efflux process. Figure 3 shows that, for [1-^{3}H]sphingosine concentrations ranging between 3×10^{-9} M and 3×10^{-7} M, the distribution of radioactive lipids between cell homogenate and cell-conditioned medium in human fibroblasts was remarkably constant.

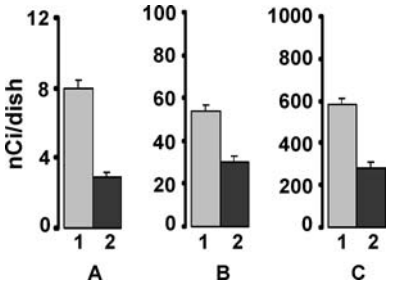

Fig. 3 Efflux of metabolically radiolabeled sphingolipids from human fibroblasts in culture. Radioactive sphingolipids associated with the cell homogenate (1) and with the cell-conditioned medium (2) after incubation of cells with 3×10^{-9} M [1-^{3}H]sphingosine, 2.2 Ci/mmole (Panel A), 3×10^{-8} M [1-^{3}H]sphingosine, 2.2 Ci/mmole (Panel B) 3×10^{-7} M [1-^{3}H]sphingosine, 2.2 Ci/mmole (Panel C) for 2 h followed by 48 h chase

Efflux of radioactive sphingolipids from cells to the medium was also observed after sphingolipid metabolic radiolabeling with L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid. Independently by the sphingolipid precursor used, the pattern of radioactive sphingolipids released in the medium was very similar to that found after sphingolipid labeling with [1-^{3}H]sphingosine. Nevertheless, feeding tritium-labeled serine and palmitic acid allowed to recognition also of some radioactive glicerophospholipids that were released in the cell-conditioned medium, PC being the most abundant. Figure 4 shows the radioactive lipid patterns from human fibroblasts and from the corresponding cell-conditioned medium obtained after metabolic labeling of cell lipids with L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid. The quantitative analysis of the radioactivity associated with cells, and with cell-conditioned medium after cell feeding with [1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid, showed that, independently of the sphingolipid precursor used, the relative amounts of sphingolipids released in the medium were very similar. Figure 5 shows data on the SM efflux. After lipid labeling using [1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid, 23%, 18% and 22% of radioactive SM synthesized by cells, respectively, was released in the medium under comparable experimental conditions.

Discussion

Sphingolipids are released from normal and tumoral cells in culture into the medium; the extent of the process is quite relevant in the presence of fetal calf serum in the cell culture medium, but also occurs in the absence of proteins, even if to a minor extent. The sphingolipid pattern in the medium is different from the cell sphingolipid pattern. All this has been recently reported [16]. Such a study had a great advantage of the availability of radiochemical

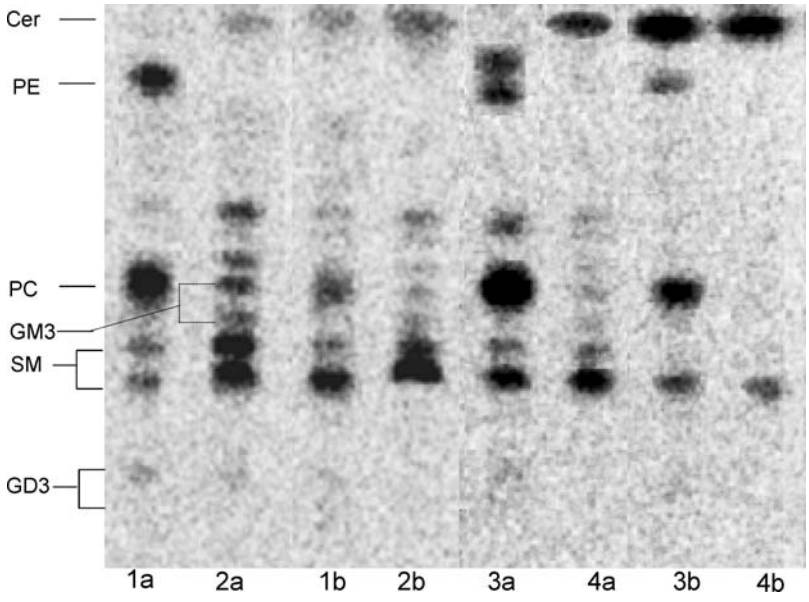

Fig. 4 Patterns of radioactive lipids present in human fibroblasts and their cell-conditioned medium after feeding with L-[3-^{3}H]serine (lanes 1 and 2) and [9,10-^{3}H]palmitic acid (lanes 3 and 4). Lipids from the cells (lanes 1 and 3) and from the cell-conditioned medium (lanes 2 and 4) were extracted and separated by HPTLC as described under "Experimental procedures". Lanes 1a and 3a, cell lipid extract; lanes 2a and 4a, cell lipid extract after alkaline treatment; lanes 1b and 3b, cell-conditioned medium lipid extract; lanes 2b and 4b, cell-conditioned medium lipid extract after alkaline treatment. The radioactive spot at the top of lanes 4a, 3b and 4b is free or released palmitic acid. Solvent system: chloroform-methanol-0.2% aqueous $CaCl_2$, 55/45/10 by vol. Radioactive lipids were detected by digital autoradiography; 100–200 dpm/lane; time of acquisition: 48 h

metabolic labeling procedures and of the availability of radioimaging procedures able to detect very minor quantities of tritiated compounds applied on and separated by TLC.

In this paper we report on a study aimed to verify the possible influence of concentration and structure of the precursor used to metabolically labeled sphingolipids. To do this, we used as precursor [1-^{3}H]sphingosine, L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid. With the use of L-[3-^{3}H]serine and [9,10-^{3}H]palmitic acid it was possible to have information also on the efflux of glycerophospholipids from cells to the medium.

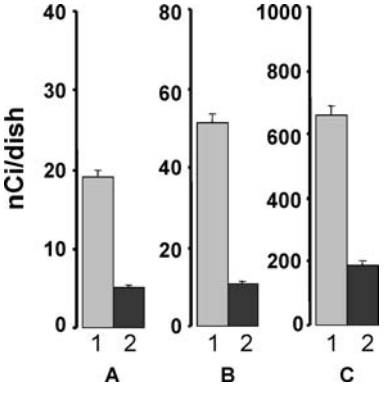

Fig. 5 Efflux of radioactive sphingomyelin from human fibroblasts in culture. Radioactive sphingomyelin associated with the cell homogenate (1) and with the cell-conditioned medium (2) after cell feeding with 3×10^{-8} M [1-^{3}H]sphingosine, 2.2 Ci/mmole (Panel A), 4×10^{-7} M L-[3-^{3}H]serine, 30 Ci/mmole (Panel B) or 4.5×10^{-7} M [9,10-^{3}H] palmitic acid, 55 Ci/mmole (Panel C) as described under "Experimental procedures"

Sphingosine was rapidly taken up by the cells. However, the process of sphingolipid release was not associated with an anomalously high availability of sphingosine inside the cells. In fact, in the range of concentrations between 10^{-9} M and 10^{-7} M, where 50 to 60% of the total administered sphingosine was taken up by the cells, we found very similar extents of sphingolipid efflux from cells.

Sphingosine is a sphingolipid catabolic fragment that is largely recycled for the biosynthesis of complex sphingolipids [25,32–35]. To enter the recycling pathway, the catabolic sphingosine must leave the lysosomes to reach the endoplasmic reticulum. On the other hand, it has been suggested that exogenous sphingosine enters the cells across the membrane, skips the lysosomes, and directly reaches the biosynthetic apparatus [34]. No information is available on protein carriers possibly involved in these two pathways. Thus, to confirm that the efflux of sphingolipids we observed is not simply due to the anomalous cell trafficking of exogenous sphingosine, we also performed sphingolipid metabolic labeling experiments using two other different precursors of sphingolipids, radioactive serine and palmitic acid, which enter the pathway at the very first steps of sphingolipid biosynthesis. Using both these precursors, we observed an efflux of sphingolipids from cultured cells to the culture medium

very similar to that observed after labeling cell sphingolipids with sphingosine (Figs. 4 and 5). As very important additional information, using radioactive serine and palmitic acid we also could observe some efflux of radioactive glycerophospholipids. As observed after feeding cells with [1-^{3}H]sphingosine, we did not find any PE in the cell medium; and by feeding cells with [9,10-^{3}H]palmitic acid we determined that PC was largely the main glycerophospholipid released in the cell medium.

Quite all the cell sphingolipids were released into the medium, even though some in trace amounts while others in larger quantities. Thus, the resulting radioactive sphingolipid pattern present in the cell-conditioned medium was significantly different from that of the cell homogenate (Figs. 1 and 2). Sphingomyelin was the main sphingolipid released into the medium from human fibroblasts, and SM and GD3 ganglioside from differentiated rat cerebellar granule cells. Very interestingly, the sphingolipid efflux from human fibroblasts mainly occurred for the sphingolipid of the more hydrophilic molecular species, those containing shorter acyl chains, both in the cases of sphingomyelin and of GM3 ganglioside. In the case of sphingomyelin, these more hydrophilic species containing shorter acyl chains were only slightly more abundant than the others, and in the case of GM3 they were the less abundant in the cell homogenate. This excludes that the efflux process involves molecules that for some reasons are synthesized in abnormally high amounts, thus becoming abnormally abundant inside the membrane, while it suggests that this process specifically involves only some selected sphingolipid molecules (this selection of molecular species could not be observed by HPTLC analysis in neuronal cells, due to the much less heterogeneity of sphingolipid ceramide moieties in these cells). We could observe that the presence of proteins in the cell medium enhanced the release of species containing the more hydrophilic ceramides. This supports the direct involvement of proteins in determining the extent of the efflux process. The property of proteins which facilitates the release of more hydrophilic complex lipids from the plasma membranes has been largely used to study the sphingolipid trafficking after cell feeding with fluorescent sphingolipid derivatives [36].

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