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Formation of free sphingosine and ceramide from exogenous ganglioside GM1 by cerebellar granule cells in culture

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Cerebellar granule cells differentiated in culture were incubated with ganglioside [^{3}H-Sph]GM1 in order to have it inserted into the plasma membrane and metabolized. Among the formed metabolites radioactive sphingosine and ceramide were identified. [^{3}H]Ceramide started to be measurable after 10 min of incubation (pulse), and [^{3}H]sphingosine after 15 min. Their concentrations increased with pulse time, and, after a 1-hour pulse, with chase time. After a 1-hour pulse with 2×10^{-6} M [^{3}H-Sph]GM1 followed by a 4-hour chase, the amount of [^{3}H]sphingosine and [^{3}H]ceramide formed were 0.04 and 0.4 pmol/10^6 cells, respectively. Particularly the ability to produce sphingosine was higher in differentiated than in undifferentiated cells. It is concluded that ganglioside turnover contributes to the maintenance of the intracellular levels of free sphingosine and ceramide.

Sphingosine; Ceramide; Ganglioside; Cerebellar granule cells; Second messengers

1. INTRODUCTION

Recent studies have demonstrated that sphingosine and ceramide, together with sphingosine-1-phosphate, *N*-monomethyl-sphingosine, *N,N*-dimethyl-sphingosine, and ceramide-1-phosphate, exert a powerful regulatory effect on enzymes, like protein kinase C, that are fundamental in the control of cellular metabolism [2–5]. This evidence prompted the hypothesis that sphingosine and ceramide serve as metabolic second messengers [4–6], and that sphingolipids may produce them under particular conditions of cell stimulation by external substances. According to this hypothesis, sphingolipids, most of which reside at the level of the plasma membrane, acquire the important role of being directly involved in transmembrane signalling processes [4, 6]. Two questions appear to be crucial in assessing the validity of the above hypothesis: (a) by which pathway are sphingosine and ceramide formed from sphingolipids, and (b) which external stimuli are able to modify the rate of production of sphingosine and ceramide from sphingolipids.

In the present work we approached the first question, using cerebellar granule cells differentiated in culture as the cell model and ganglioside GM1, ^{3}H-radiolabelled at the sphingosine moiety ([^{3}H-Sph]GM1), as the sphingolipid. The cells were pulsed [7] with [^{3}H-Sph]GM1 in order to stably insert radiolabelled GM1 molecules into the external lipid layer of the plasma membrane, and then the radioactive metabolic fragments, produced under basal conditions, were analyzed. Labelled sphingosine and ceramide were recognized to be produced and the kinetics of their formation was established.

Abbreviations: this article follows the ganglioside nomenclature of Svennerholm [1]; Sph, sphingosine; NeuAc, *N*-acetylneuraminic acid; BSA, bovine serum albumin; HPTLC, high-performance thin-layer chromatography

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Commercial chemicals were of the highest purity available. Basal modified Eagle's medium (BME) and fetal calf serum (FCS) were from Flow Laboratories (Irvine, Scotland); crystalline BSA NeuAc and calf thymus DNA (Type 1) were from Sigma (St. Louis, MO, USA); 1-fluoro-2,4-dinitrobenzene and HPTLC silica gel plates were from Merck (Darmstadt, Germany); [^{3}H]$NaBH_4$ (6.5 Ci/mMol), used to prepare radioactive ganglioside, was from Amersham International (Amersham, UK); ceramide glycanase from *Macrobdella decora* was from Boehringer Mannheim (Mannheim, Germany); and *E. Coli* sn-1,2-diacylglycerol kinase from Calbiochem (La Jolla, CA, USA). Ganglioside GM1, obtained as previously described [8], was ^{3}H-labelled at the long chain base ([^{3}H-Sph]GM1 [9] and the molecular species containing erythro-C18 and C20-sphingosine were used [10]. The specific radioactivity was 1.15 Ci/mmol, and the radiochemical purity higher than 99%.

Standard [^{3}H]glucosylceramide, [^{3}H]lactosylceramide, [^{3}H]sphyngomyelin were obtained as described [7,23].

2.2. *Preparation of standard [^{3}H]sphingosine and [^{3}H]ceramide*

[^{3}H]Ceramide was prepared from [^{3}H-Sph]GM1 by treatment with ceramide glycanase [11]. Briefly, the incubation mixture, containing, in a volume of 1 ml, 0.15 mCi of [^{3}H-Sph]GM1, 2 mU enzyme, 50 mM acetic acid-sodium acetate buffer, pH 5.0, and 2 mM sodium cholate, was incubated at 37°C for 24 h. The mixture, after adding 5 vols. of chloroform/methanol, 2:1 (v/v), was vortexed for 2 min and centrifuged (3000 × g, 15 min). The formed [^{3}H]ceramide, recovered in the organic phase, was stored at −20°C. The yield of the reaction was over

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99%. A portion of the [^{3}H]ceramide solution, carrying 0.05 mCi of radioactivity, was evaporated to dryness, suspended with 0.2 ml of 10 N aqueous KOH/*N*-butanol, 1:9 (v/v), and heated at 100°C for 3 h, according to Taketomi and Kawamura [12]. Under these conditions alkaline hydrolysis of [^{3}H]ceramide was virtually complete. The reaction mixture was then dried and solubilized in 2 ml of distilled water. [^{3}H]Sphingosine was extracted with 2 ml of diethylether, and stored at −20°C.

2.3. *Cell cultures*

Granule cells were prepared and cultured as described [13]. Treatment with radiolabelled ganglioside was carried out on day 2, 4 or 8 in culture at 37°C. Dishes were washed twice with temperature-conditioned BME without FCS and incubated for a given period of time in the same medium (2 ml/dish) containing 10^{-5} M or 2×10^{-6} M [^{3}H-Sph]GM1, carrying 10 μCi/ml and 2μCi/ml of radioactivity, respectively. The medium was then removed, the cells were washed with BME containing 10% FCS to eliminate loosely bound labeled gangliosides and incubated for 1, 2, or 4 h in the same medium. Before analysis the cells were rinsed twice with saline, scraped off the plates and lyophilized.

2.4. *Lipid extraction and purification*

Lyophilized cells (5×10^6–10^7 cells) were suspended with 0.5 ml methanol, stirred for 15 min at room temperature, then added with 1 ml chloroform and stirred for a further 15 min. After centrifugation the pellet was re-extracted for 30 min with 0.5 ml chloroform/methanol (2:1, v/v) and the two supernatants were combined. A two phase partitioning according to Folch [14] was then applied. The formed organic phase was submitted to mild alkaline hydrolysis (0.2 M methanolic NaOH at 37°C for 1 h) to remove glycerophospholipids [15]. [^{3}H]Lipids contained in the final preparation were quantified and separated by two- or one-dimensional HPTLC (see below), the recovery of standard [^{3}H]ceramide and [^{3}H]sphingosine being over 95% and 90%, respectively.

2.5. *Isolation and identification of metabolically formed [^{3}H]sphingosine and [^{3}H]ceramide*

[^{3}H]Sphingosine and [^{3}H]ceramide were isolated from cerebellar granule cells, pulsed with 2×10^{-6} M [^{3}H-Sph]GM1 for 4 h and submitted to a chase of 4 h in the absence of [^{3}H-Sph]GM1. The final lipid preparation was spotted on HPTLC plates, and developed two-dimensionally (see below). The spots corresponding to [^{3}H]sphingosine and [^{3}H]ceramide were scraped from the plates and eluted from the gel by treatments with 1 ml of chloroform/methanol, 2:1 (v/v) (twice), and 1 ml of chloroform/methanol, 1:1 (v/v) (twice). The pooled extracts of each compound were evaporated to dryness. An aliquot of the [^{3}H]sphingosine residue was incubated with acetic anhydride in methanol [16] in order to obtain the *N*-acetyl-derivative. A second aliquot was incubated with 1-fluoro-2,4-dinitrobenzene [17], in order to obtain the 2,4-dinitrophenyl derivative. Samples of standard [^{3}H]sphingosine were submitted in parallel to the same processes. An aliquot of the [^{3}H]ceramide residue was submitted to alkaline hydrolysis (see above) in order to liberate the long chain base. A second aliquot was treated with sn-1,2-diacylglycerol kinase [18, 19], in order to obtain ceramide-1-phosphate. Samples of standard [^{3}H]ceramide were submitted to the same treatments. [^{3}H]ceramide-1-phosphate, produced from standard [^{3}H]ceramide, was separated by one-dimensional HPTLC with chloroform/methanol/acetic acid (65:15:5 (v/v)), eluted from the gel with chloroform/methanol (1:1 (v/v)), stored at −20°C, and used as a standard.

2.6. *Separation by thin layer chromatography and determination of [^{3}H]sphingosine and [^{3}H]ceramide*

The separation of [^{3}H]sphingosine and [^{3}H]ceramide, formed during the degradation of [^{3}H-Sph]GM1 and recovered in the final lipid preparation, was attained by two-dimensional HPTLC, using the solvent system I: chloroform/methanol/water, 55:20:3 (v/v), for the 1st run, and II: chloroform/methanol/32% NH_4OH, 40:10:1 (v/v), for the 2nd run. In some cases mono-dimensional TLC was accomplished using one of the above solvent systems.

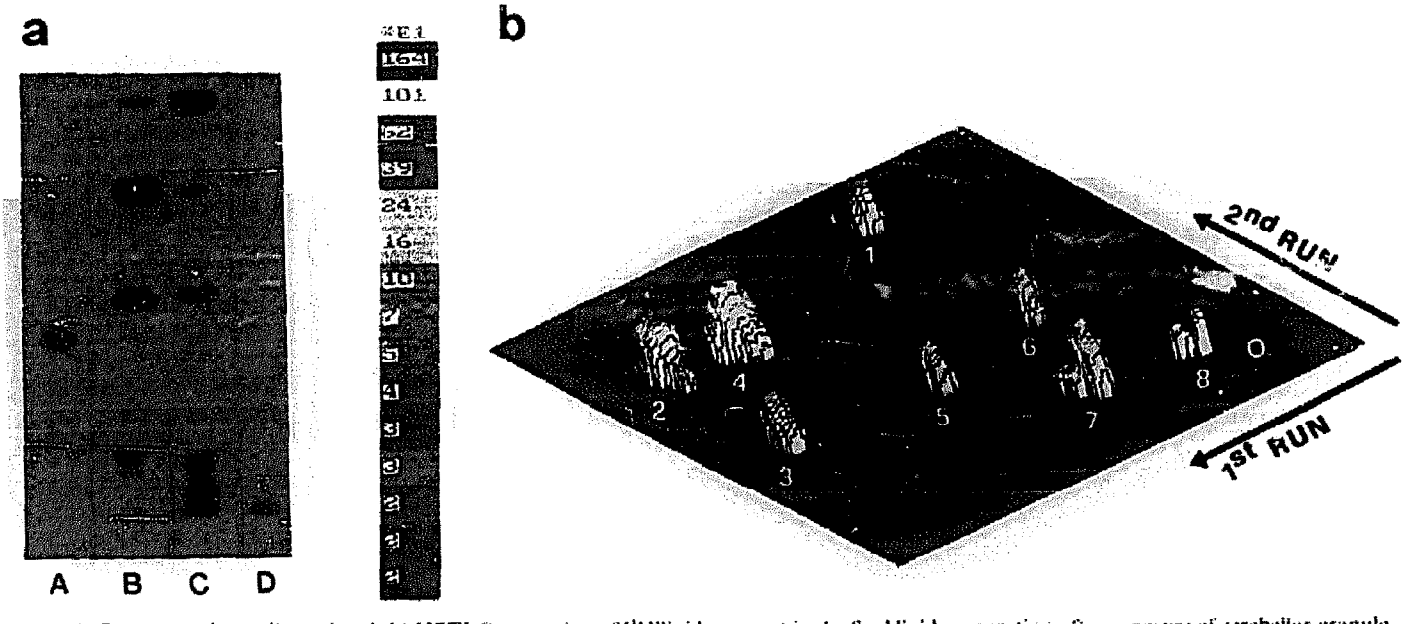

Fig. 1. One- (a) and two-dimensional (b) HPTLC separation of [^{3}H]lipids present in the final lipid preparation after exposure of cerebellar granule cells to 2×10^{-6} M [^{3}H-Sph]GM1 for 4 h, followed by a 4-h chase. (a) Monodimensional HPTLC; solvent system I; detection by fluorography. A, standard [^{3}H]sphingosine; B, from top to bottom standard [^{3}H]ceramide, [^{3}H]glucosyl-ceramide, [^{3}H]lactosyl-ceramide and [^{3}H]sphingomyelin; C, [^{3}H]lipids from cerebellar granule cells; D, [^{3}H]lipids from cerebellar granule cells treated with 5×10^{-5} M chloroquine. (b) Three-dimensional view of the [^{3}H]lipids separated by two-dimensional HPTLC. First run, solvent system I; second run, solvent system II; detection by digital autoradiography. O, origin; 1, standard [^{3}H]sphingosine submitted only the first run; 2 and 3, standard [^{3}H]ceramide and [^{3}H]sphingosine, respectively, submitted only to the second run; 4, 5, 6, 7 and 8, [^{3}H]ceramide, [^{3}H]glucosyl-ceramide, [^{3}H]sphingosine, [^{3}H]lactosyl-ceramide and [^{3}H]sphyngomyelin from granule cells, respectively.

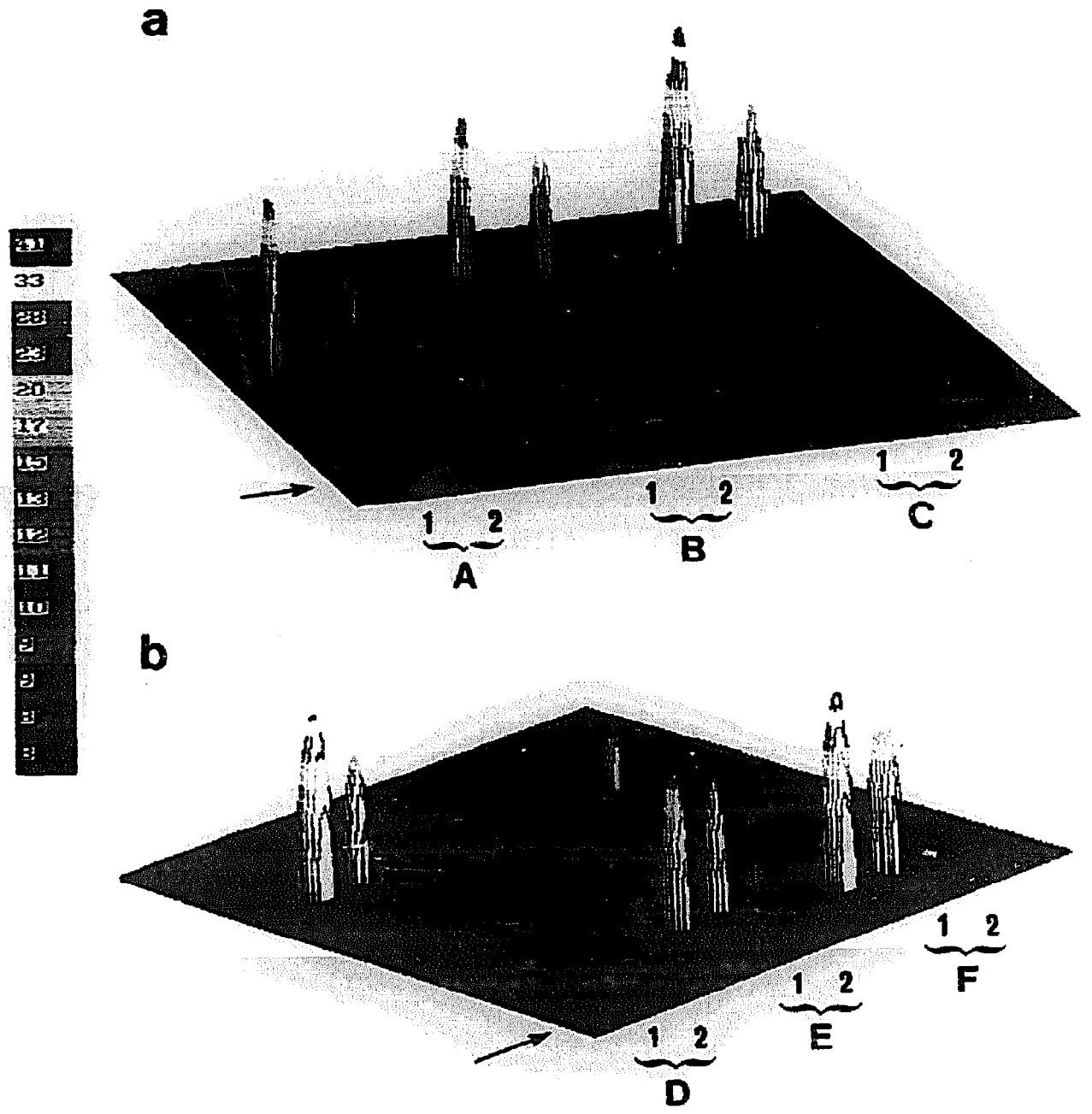

Fig. 2. One-dimensional HPTLC analysis of [^{3}H]sphingosine, [^{3}H]ceramide and their derivatives; solvent system II. (a) [^{3}H]Sphingosine and its derivatives. 1, standard [^{3}H]sphingosine; 2, [^{3}H]sphingosine isolated from the lipid extract of cerebellar granule cells after a 4-h pulse with 2×10^{-6} M [^{3}H-Sph]GM1, followed by a 4-h chase. Both 1 and 2 before (A), and after treatment with methanolic acetic anhydride (*N*-acetyl-derivatives) (B), or 2,4-dinitrofluorobenzene (dinitrophenyl-derivatives) (C). (b) [^{3}H]Ceramide and its derivatives; solvent system I. 1, standard [^{3}H]ceramide; 2, [^{3}H]ceramide isolated from the lipid extract of cerebellar granule cells after a 4-h pulse with 2×10^{-6} M [^{3}H-Sph]GM1 followed by 4 h chase. Both 1 and 2 before (D), and after alkaline hydrolysis (formation of sphingosine) (E), or treatment with sn-1,2-diacylglycerol kinase (formation of ceramide-1-phosphate) (F); arrow, origin.

2.7. *Other methods*

Radioactivity was determined by liquid scintillation counting, fluorography, or radiochromatoscanning (Digital Autoradiograph Berthold, Germany) as previously described [7]. Gangliosides were determined as bound NeuAc [20], using NeuAc as the standard, proteins [21] using BSA as the standard, and DNA [22] using calf thymus DNA as the standard.

3. RESULTS AND DISCUSSION

Previous studies [7,23] showed that cerebellar granule cells in culture are able to incorporate exogenous gangliosides into their plasma membranes and rapidly metabolize them with the formation of catabolic fragments, and these are in part recycled for biosynthet-

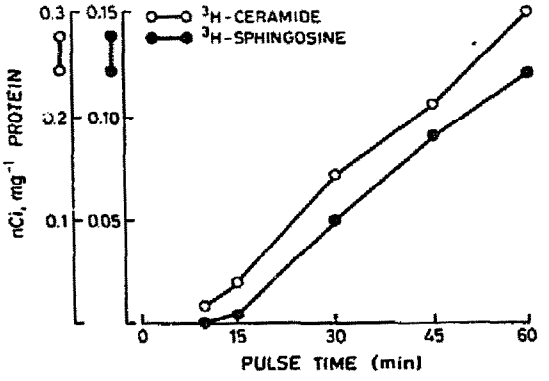

Fig. 3. Time course of radioactivity incorporation into sphingosine and ceramide after exposure of cerebellar granule cells to 10^{-5} M [^{3}H-Sph]GM1. The data exposed are the mean values of three experiments with a standard deviation never exceeding 15% of the mean values. Radiolabelled sphingosine and ceramide were separated and quantitated as described in section 2.

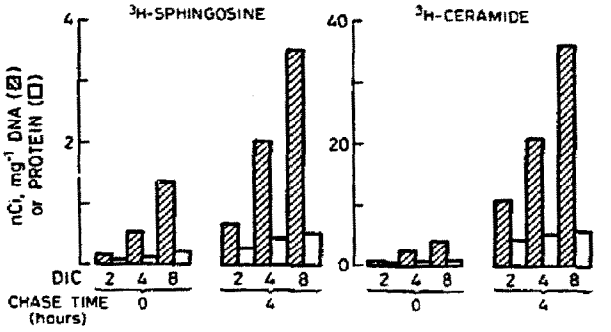

Fig. 5. Incorporation of radioactivity into [^{3}H]sphingosine and [^{3}H]ceramide by cerebellar granule cells after exposure to 2×10^{-6} M [^{3}H-Sph]GM1 for 1 h followed by a chase period of 4 h, as a function of cell differentiation in culture (2, 4, 8 days in culture, DIC). The data, expressed as nCi·mg^{-1} DNA or protein, are the mean values of three experiments, with a standard deviation never exceeding 15% of the mean values.

ic purposes. The increasing interest [2–6] on sphingosine and ceramide, as potential second messengers arising from sphingolipid turnover, prompted us to better investigate the above situation with specific attention to the formation of these two substances. As shown in Fig. 1 (a and b), sphingosine and ceramide appear to be produced by cerebellar granule cells after a pulse (4 h)-chase (4h) with 2×10^{-6} M [^{3}H-Sph]GM1. Formed [^{3}H]sphingosine, scraped from the plate and chemically transformed into the *N*-acetyl-, and 2,4-dinitro-phenyl-derivatives, had an identical chromatographic behaviour as standard [^{3}H]sphingosine, submitted to the same treatments (Fig. 2a). Also, formed [^{3}H]ceramide produced [^{3}H]sphingosine after alkaline hydrolysis and was transformed into ceramide-1-phosphate by sn-1,2-diacylglycerol kinase, exactly as standard [^{3}H]ceramide

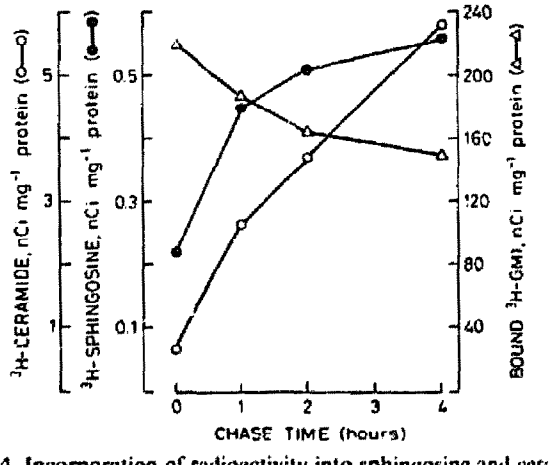

Fig. 4. Incorporation of radioactivity into sphingosine and ceramide after exposure of cerebellar granule cells to 2×10^{-6} M [^{3}H-Sph]GM1 for 1 h followed by chase for different periods of time. The values of bound [^{3}H-Sph]GM1 under the same conditions are also shown. The data are the mean values of three experiments, with a standard deviation never exceeding 15% of the mean values. For details see the section 2.

(Fig. 2b). All this indicates that both sphingosine and ceramide are authentically produced by cerebellar granule cells during GM1 turnover.

The time course of radioactivity incorporation into sphingosine and ceramide after exposure of cerebellar granule cells to 10^{-5} M [^{3}H-Sph]GM1 is reported in Fig. 3. Radioactivity is present in ceramide already after 10 min and starts to be detectable in sphingosine after 15 min; it markedly increases with time in both compounds, [^{3}H]ceramide always present in substantially higher amounts than [^{3}H]sphingosine. A similar behaviour was observed in chase experiments where cerebellar granule cells were first pulsed for 1 h with 2×10^{-6} M [^{3}H-Sph]GM1 and then submitted to chase up to 4 h (Fig. 4). At the end of the chase period the newly formed [^{3}H]ceramide increased by about 10-fold and [^{3}H]sphingosine 2.5-fold.

The amount of [^{3}H-Sph]GM1 bound to the cell surface after a 1 h pulse in the presence of 2×10^{-6} M ganglioside was 220 pmol/mg cell protein, which is about 1/50 the specific concentration of endogenous gangliosides in granule cells in culture [7]. The portion of the total bound [^{3}H-Sph]GM1, that underwent turnover during the 4-h period of pulse, was about 10%, which is 22 pmol/mg cell protein. Of course, only a portion of this is present as ceramide or sphingosine; precisely, the amount of formed [^{3}H]sphingosine and [^{3}H]ceramide after a 1 h pulse – 4 h chase was calculated to be 0.04 pmol and 0.4 pmol/10^6 cells, respectively. Keeping in mind that the metabolism of bound gangliosides follows their internalization presumably via endocytosis [7, 23, 24], and that this process likely involves concomitantly the exogenous and endogenous gangliosides, the total amount of sphingosine and ceramide formed under the above conditions should be 2.0 pmol and 20 pmol/10^6 cells, respectively. Assuming that the levels of free sphingosine and ceramide in cerebellar granule cells (these analyses are in progress) are

in the range of those determined in a number of cells (3–20 pmol and 130–230 pmol/10^6 cells, respectively [25–27]. it should be inferred that gangliosides contribute to the maintenance of free sphingosine and ceramide levels under basal conditions. As shown in Fig. 5 the capacity of cerebellar granule cells to produce sphingosine and ceramide, when exposed to exogenous GM1, is dependent on the degree of differentiation of the cell. From the 2nd day in culture, when the cells are undifferentiated, to the 8th day, when they are fully differentiated, the amount of formed [^{3}H]sphingosine in pulse (1 h) and pulse (1 h)-chase (up to 4 h) experiments underwent a 5- to 8-fold and 2- to 3-fold increase, when referred to mg DNA and mg protein, respectively. Under the same conditions the increase of [^{3}H]ceramide was only 3- to 5-fold and 1.5- to 2-fold, respectively. This indicates that differentiated cerebellar granule cells produce specially more sphingosine from the turnover of plasma membrane bound ganglioside than undifferentiated cells. This evidence agrees with the concept [7] that differentiated cerebellar granule cells possess a more rapid turnover of gangliosides than undifferentiated ones.

When the pulse experiments were performed in the presence of 5×10^{-5} M chloroquine, a potent inhibitor of lysosomal function, no formation of sphingosine and ceramide was observed (see Fig. 1a). These results confirm the previous report [23] that most of the metabolic processing of exogenously taken up gangliosides by cultured cells is blocked by impairment of lysosomal function. On this basis any route of formation of sphingosine and ceramide from ganglioside GM1, alternative to the lysosome-assisted degradation, seems to be excluded.

In conclusion, the present work provides clear indication that cerebellar granule cells in culture are able to produce sphingosine and ceramide from exogenously taken up and internalized GM1. Although it is generally accepted [24] that the exogenous lipids which insert into the cell surface are functionally equivalent to the endogenous ones, it cannot be excluded that insertion of exogenous lipids may affect in some manner the normal flow of plasma membrane into the cell interior. Keeping in mind this precaution, the experimental model presented here appears to be suitable to inspect the possible novel process of transmembrane signalling based on the formation of second messengers of sphingoid nature.

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