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Metabolism of exogenous ganglioside GM1 in cultured cerebellar granule cells

The fatty acid and sphingosine moieties formed during degradation are re-used for lipid biosynthesis

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Cerebellar granule cells, differentiated in vitro, were parallelly fed with [Sph-^{3}H]GM1 and [stearoyl-^{14}C]GM1, under identical conditions (10^{-6} M ganglioside; pulse, from 1–4 h; chase, up to 24 h after 4 h pulse) and the salvage pathways of sphingosine and stearic acid were investigated. It was observed that both sphingosine and stearic acid, liberated during the intralysosomal degradation of ganglioside, are metabolically recycled, along distinct pathways. Sphingosine is used for the biosynthesis of a number of sphingolipids, particularly ceramide, glucosyl-ceramide, gangliosides and sphingomyelin; stearic acid is utilized for the biosynthesis of sphingolipids, and to a greater extent, glycero-phospholipids, especially those endogenously richer in stearic acid (phosphatidyl-ethanolamine and phosphatidyl-choline). No evidence was provided for a salvage pathway for ceramide.

Cerebellar granule cell; Ganglioside; Sphingosine; Fatty acid; Metabolic salvage pathway; Phospholipid

1. INTRODUCTION

The composition of the cell plasma membrane is maintained constant through a rapid process where the individual components are continuously internalized and brought back to the surface, a portion of them being degraded and substituted by newly synthesized molecules. Gangliosides, characteristic components of the plasma membranes of most vertebrate cells, appear to participate in this flow process [2]. They are internalized by endocytosis [3] and submitted to degradation in the lysosomes, with formation of catabolic fragments that are partially re-utilized for biosynthetic purposes in the endoplasmic reticulum and Golgi apparatus ('salvage pathways') [4]. By the use of gangliosides, radiolabeled in different portions of the molecule and administered to animals or cultured cells, it was demonstrated that different monosaccharides derived from ganglioside (NeuAc, Gal, GalNAc) undergo salvage pathways [5–8]. In rat liver it was also shown that some catabolic fragments from the lipid moiety of gangliosides are metabolically recycled, particularly, glucosyl-ceramide for the biosynthesis of gangliosides, sphingosine for that of sphingomyelin, and fatty acid for that of phospholipids [9].

In the present work we investigated the salvage pathways occurring in cultivated neurons for the sphingosine and fatty acid moieties of gangliosides. To this purpose cerebellar granule cells differentiated in culture were fed with ganglioside GM1, radiolabeled at the level of the sphingosine ([Sph-^{3}H]GM1) or fatty acid ([stearoyl-^{14}C]GM1) moiety, and the formation of radiolabeled metabolites was followed. We observed that the salvage pathways for sphingosine and fatty acid are distinct.

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Abbreviations: gangliosides (GM3, GM2, GM1, GD1a, GD1b, GT1b) are named according to Svennerholm [1]; Sph, sphingosine; NeuAc, *N*-acetylneuraminic acid; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; lac, lactose; O-Ac-GT1b, GT1b carrying 9-*O*-acetyl-*N*-acetylneuraminic acid; HPTLC, high-performance thin-layer chromatography; GLC, gas-liquid chromatography; PE, diacyl-phosphatidylethanolamine; PPE, alkenyl-acyl phosphatidylethanolamine; PC, diacyl-phosphatidylcholine; PPC, alkenyl-acyl phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidyl-serine; SM, sphingomyelin; FCS, fetal calf serum.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Commercial chemicals were of the highest available grade; solvents were redistilled before use. Basal modified Eagle's medium (BME) and FCS (heat-inactivated before use) were from Flow Laboratories (Irvine, UK). Poly-L-lysine, 1-α-D-arabinofuranosylcitosine, NeuAc, gentamycin, crystalline bovine serum albumin, standard phospholipids

(PE, PPE, PC, PPC, PI, PS, SM) and phosphorus standard solution were from Sigma Chemical Co. (St. Louis, MO, USA); standard fatty acid methyl esters from Supelco (Bellefonte); [^{3}H]$NaBH_4$ (6.5 Ci/mmol) and [1-^{14}C]stearic acid (55 mCi/mmol) from Amersham International (Amersham, Bucks, UK).

Ganglioside GM1, obtained as previously described [10], was isotopically radiolabeled at the level of (a) C-3 of the long chain base ([Sph-^{3}H]GM1) [11], the molecular species containing erythro-C18-sphingosine being separated [12] and used; or (b) C-1 of the stearic acid ([stearoyl-^{14}C]GM1) [13]. The specific radioactivity was 1.15 and 0.055 Ci/mmol respectively, the radiochemical purity being better than 99% in both cases. Standard [^{3}H]gangliosides, [^{3}H]lactosyl-ceramide, [^{3}H]glucosyl-ceramide, [^{3}H]sphingomyelin, [^{3}H]ceramide and [^{3}H]sphingosine were obtained as previously reported [14].

2.2. *Cell cultures*

Granule cells were prepared and cultured as previously described [15]. Treatment with radiolabelled gangliosides was carried out on day 8 in culture, when cells were fully differentiated. Dishes were washed twice with temperature-conditioned BME without FCS and incubated for a given period of time (pulse) in the same medium (2 ml/dish) containing 10^{-6} M [Sph-^{3}H]GM1 or [stearoyl-^{14}C]GM1, carrying 1.15 μCi/ml and 0.055 μCi/ml of radioactivity, respectively. The medium was then removed and the cells were washed (10 min treatment for three times) with BME containing 10% FCS. After different periods of incubation (chase) in the same medium, cells were rinsed twice with phosphate buffered solution, scraped off the plates and lyophilised.

2.3. *Lipid extraction and purification*

Total lipids were extracted and partitioned as previously described [14]. The individual [^{3}H]lipids contained in the aqueous (gangliosides) and organic (non-ganglioside lipids) phases were separated by HPTLC and quantified (see below). The recognition and identification of individual gangliosides, neutral glycolipids, sphingomyelin and ceramide was achieved as previously described [14]. Glycerophospholipids were separated by HPTLC [16,17] and identified by comparison with authentic reference standards.

2.4. *HPTLC procedures*

The separation of the different lipids was performed by one-dimensional HPTLC, using chloroform/methanol/0.2% $CaCl_2$ (50:42:11, v/v) for gangliosides, and chloroform/methanol/water (55:20:3, v/v) for neutral glycolipids, sphingomyelin, ceramide and sphingosine. Individual phospholipids were separated by two-dimensional HPTLC, with intermediate exposure to 12 N HCl fumes [17], using chloroform/methanol/acetic acid/water (30:20:2:1, v/v) and chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, v/v) for the first and the second run, respectively. After HPTLC, the plates were sprayed with a *p*-dimethylaminobenzaldehyde reagent [18] for ganglioside detection, with a diphenylamine reagent [19] for neutral glycolipid detection or with a molybdate reagent for phospholipid detection [20]. Plates were then densitometrically scanned with a Camag TLC densitometer, connected to an IBM personal computer, equipped with data analysis software. Alternatively, the plates were submitted to autoradiography (Digital Autoradiograph, Berthold, Germany).

2.5. *Colorimetric methods*

Gangliosides were determined, as bound NeuAc [21], using NeuAc as standard; total phospholipids after perchloric acid digestion, according to the method of Bartlett [22], as modified by Dodge and Phillips [23]; proteins [24] using bovine serum albumin as the standard.

2.6. *Fatty acid analysis*

Fatty acid analysis was performed on PE and PC, by far the most abundant glycerophospholipids of cultured cerebellar granule cells (see section 3). To this purpose phospholipids were separated by two-dimensional HPTLC (see above) and the spots corresponding to PE and PC were scraped off and eluted from the gel by three washes with 2 ml each of chloroform/methanol (2:1, v/v). Each phospholipid was then hydrolyzed with 0.5 N anhydrous methanolic HCl at 80°C overnight in a sealed tube. Fatty acid methyl esters were extracted into *n*-hexane and submitted to GLC as previously described [25].

2.7. *Determination of radioactivity*

Radioactivity was determined by liquid scintillation counting, fluorography or radiochromatoscanning (Digital Autoradiograph, Berthold, Germany). Since the two administered gangliosides had different specific radioactivity, and in order to facilitate comparative evaluations, all data of radioactivity determinations were expressed as pmol of radioactive compound/mg protein.

3. RESULTS AND DISCUSSION

Previous investigations [6,8,14,16] showed that cerebellar granule cells in culture are able to take up and internalize exogenous ganglioside GM1 and to submit it to metabolic processing. This process consisted of: (a) degradation, with formation of GM2, GM3, lactosyl-ceramide, glucosyl-ceramide, ceramide and single components like monosaccharides and sphingosine; (b) formation of anabolic products originated by either recycling of catabolic fragment(s) – salvage pathways – (GD1a, GD1b, GT1b, O-Ac-GT1b, sphingomyelin, etc.) and direct glycosylation of internalized GM1 (GD1a). Both degradative and salvage pathways were

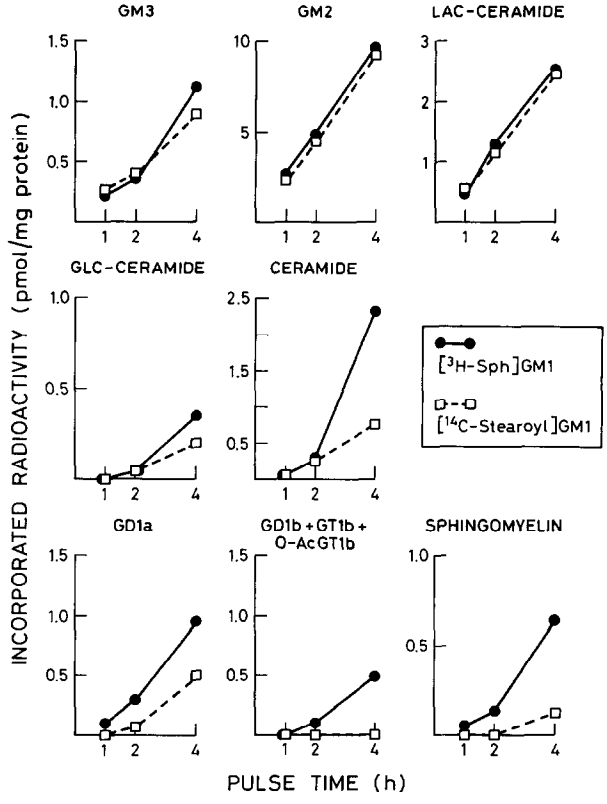

Fig. 1. Incorporation of radioactivity into different sphingolipids after different pulse times with 10^{-6} M [Sph-^{3}H]GM1 and [stearoyl-^{14}C]GM1 in cerebellar granule cells differentiated in culture. Data are the mean of three experiments and are expressed as pmol/mg protein. S.D. never exceeded 15% of the mean.

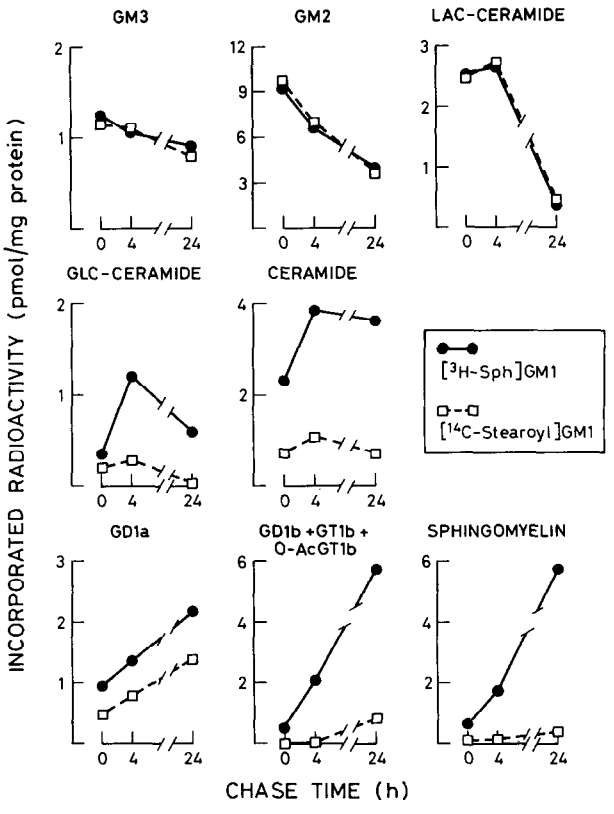

Fig. 2. Incorporation of radioactivity into sphingolipid metabolites at different chase times after four hour pulse with 10^{-6} M [Sph-^{3}H]GM1 and [stearoyl-^{14}C]GM1 in cerebellar granule cells differentiated in culture. Data are the mean of three experiments and are expressed as pmol/mg protein. S.D. never exceeded 15% of the mean.

completely stopped by blockage of the lysosomal function, indicating the central role of lysosomes in the processes [8].

With the present work the salvage pathways of the lipid components of GM1, sphingosine and fatty acid, were explored. To this purpose cerebellar granule cells differentiated in culture were parallelly fed with [Sph-^{3}H]GM1 and [stearoyl-^{14}C]GM1, under identical conditions (10^{-6} M ganglioside; pulse: 1–4 h; chase, up to 24 h after 4 h pulse). At all the investigated times the amounts of cell associated GM1, and formed labeled metabolites, were very similar, regardless of the labeling position of administered GM1.

Out of the radiolabeled molecules produced from exogenous GM1 in the pulse and pulse-chase experiments, some (mainly sphingolipids) were common to both labelings, and others were detected only with [stearoyl-^{14}C]GM1 (glycero-phospholipids) or with [Sph-^{3}H]GM1 (sphingosine). With reference to metabolites that are common to the two differently labeled gangliosides (Figs. 1 and 2), the amounts of GM2, GM3 and lactosyl-ceramide were practically identical in the two cases, under both pulse and chase conditions. This indicates that these metabolites are products of the sequential hydrolysis of GM1, as expected. In the case of glucosyl-ceramide and ceramide their amounts were similar only until 2 h of pulse (Fig. 1); for longer incubation times a much higher formation was obtained with the sphingosine than the stearoyl label (Figs. 1 and 2). Presumably, until 2 h of incubation degradation is the main process, salvage pathways of catabolic fragments (especially sphingosine) becoming predominant thereafter. Other metabolites of sphingolipid nature (gangliosides GD1a, GD1b, O-Ac-GT1b, GT1b and sphingomyelin) underwent a progressive increase during the pulse and chase periods. However, at all investigated times, they were in much higher amounts after [Sph-^{3}H]- than [stearoyl-^{14}C]GM1 administration (Figs. 1 and 2). All this means that in cultured cerebellar granule cells sphingosine and stearic acid, and not a bigger fragment (like ceramide) liberated from the degradation of GM1, are re-utilized for the biosynthesis of sphingolipids. Moreover, they are metabolically recycled in different proportion, presumably reflecting the difference in the corresponding endogenous pools.

The salvage pathway of sphingosine to sphingomyelin biosynthesis was already reported to occur in other cultured cells and tissues [9,26]. On the contrary, the evidence that free sphingosine is re-cycled to gangliosides contrasts with data obtained on rat liver [9], showing glucosyl-ceramide as the major lipid fragment reused for ganglioside biosynthesis. This means that metabolic salvage processes imply different metabolites and follow different trends in different cells or tissues.

After [stearoyl-^{14}C]GM1 administration, under both pulse and chase conditions, radioactivity was incorporated also into glycero-phospholipids, namely PE, PC, PI and PS (Fig. 3). Although PC is the main endogenous phospholipid of differentiated granule cells, and in developing rat cerebellum [27] accounting for more than 50% of total phospholipids (Fig. 4A and B), PE was the

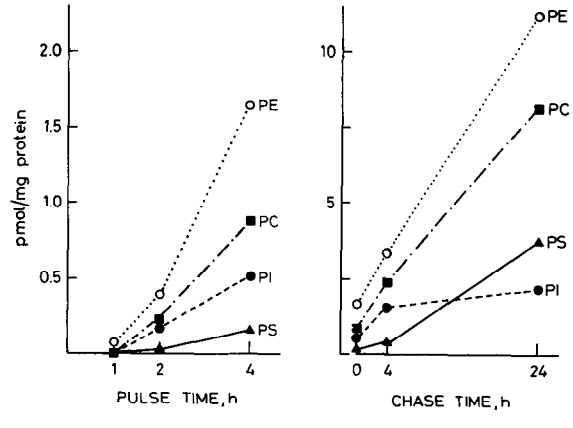

Fig. 3. Incorporation of radioactivity into different glycerophospholipids at different pulse times, and chase times after four hours pulse, with 10^{-6} M [stearoyl-^{14}C]GM1 in cerebellar granule cells differentiated in culture. Data are the mean of three experiments and are expressed as pmol/mg protein. S.D. never exceeded 15% of the mean.

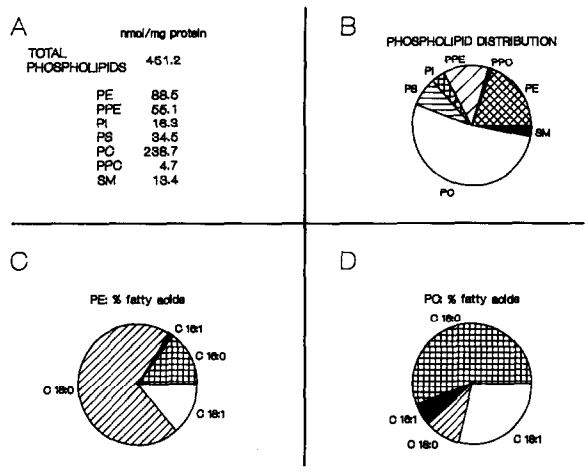

Fig. 4. Phospholipid content and composition (A and B) and fatty acid % composition of PE (C) and PC (D) in cerebellar granule cells differentiated in culture. Data are expressed as nmol of phospholipid/mg protein (A), percent of total phospholipids (B) and percent of main fatty acids in PE (C) and PC (D). Data are the mean of four different determinations, S.D. never exceeding 10% of the mean.

glycero-phospholipid with the highest radioactivity incorporation. It should, however, be noted that stearic acid accounted for 70% of the total fatty acids in PE, and only for 9.7% in PC, where the most abundant fatty acid (55.6%) was palmitic acid (Figs. 4C and D). Thus the metabolic re-utilization of stearic acid, formed from ganglioside degradation, seems to reflect the distribution of stearic acid within the phospholipids present in cerebellar granule cells. Notwithstanding plasmalogens (especially PPE) are present in cerebellar granule cells (Fig. 4B), no measurable radioactivity was found in PPE and PPC. This finding is expected since long-chain alcohol is the substrate in the synthesis of ether-linked glycero-phospholipids [28]. Remarkably, after [stearoyl-^{14}C]GM1 administration the amount of label incorporated into glycero-phospholipids was appreciably higher than that incorporated into sphingolipids.

Concluding, in cerebellar granule cells differentiated in culture salvage pathways are operating that metabolically re-cycle sphingosine and fatty acid, liberated during the degradation of ganglioside. Sphingosine appears to be used for the biosynthesis of a number of sphingolipids, including ceramide, glucosyl-ceramide, gangliosides and sphingomyelin; stearic acid is utilized for the biosynthesis of both sphingolipids and, at a greater extent, glycero-phospholipids. Since in the experimental protocol used sphingosine and stearic acid are formed in the lysosomes, whereas their use for biosynthetic purposes occurs in the endoplasmic reticulum and Golgi apparatus, exit of both compounds from the lysosomal compartment has to be postulated. It is not known whether this process is a protein mediated one as in the case of some monosaccharides (GalNAc, NeuAc) [29], that are produced in the lysosomes during ganglioside degradation. Such a process could be of crucial importance also in view of the suggested involvement of sphingosine as a metabolic bioregulator [30,31].

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