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Cerebellar granule cells in culture exhibit a ganglioside-sialidase presumably linked to the plasma membrane

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Cerebellar granule cells differentiated in culture were incubated with ganglioside [^{3}H-Sph]GD1a in order to have it inserted into the plasma membrane, internalized by endocytosis, and metabolized. The metabolites formed included GM1, product of GD1a desialosylation. No GM1 or other metabolites were present in the incubation medium, whereas with the lysosomal apparatus blocked by chloroquine, or GD1a endocytosis prevented at 4°C, the only metabolite formed was GM1. These results suggest that GD1a desialosylation did not occur either extracellularly or intracellularly but likely, at the membrane level. Similar results were obtained with [^{3}H-Gal]GD1b, whereas no degradation of [^{3}H-NeuAc]GM1 took place in the presence of chloroquine or at 4°C. In conclusion, cerebellar granule cells express in vivo a sialidase, presumably located on the cell surface, that affects GD1a and GD1b but not GM1.

Cerebellar granule cell; Ganglioside-sialidase; Metabolic processing; Chloroquine

1. INTRODUCTION

Granule cells from cerebellum of 8-day-old rats undergo full differentiation in vitro [2], showing in parallel a 12-fold increase of the ganglioside content [3,4], and a 100-fold increase of the sialidase active on gangliosides [5]. This suggests a role for sialidase and ganglioside sialosylation in the process of functional synapses formation [6–9].

Cerebellar granule cells in culture are able to metabolize exogenous gangliosides [4–10], following insertion of the ganglioside molecules into the plasma membrane [11] and internalization via endocytosis [12,13]. The metabolic processing consists of the formation of compounds that derive from: (i) ganglioside degradation, (ii) biosynthetic recycling of degradation, and (iii) direct glycosylation of internalized ganglioside [10].

Since sialic acid release by sialidase action is one of the steps of ganglioside metabolism, we used cerebellar granule cells cultured in the presence of exogenous gangliosides for studying the site(s) of ganglioside desialosylation in vivo. For this purpose cerebellar granule cells were incubated with radiolabelled gangliosides GD1a, GD1b and GM1 in the absence or presence of chloroquine, a potent lysosomal inhibitor, and at 4°C, a temperature where endocytosis is blocked. The results obtained showed that sialic acid release can occur at the plasma membrane level.

Abbreviations: this article follows the ganglioside nomenclature of Svennerholm [1]. NeuAc, *N*-Acetylneuraminic acid; Gal, galactose; Sph, sphingosine; *O*-Ac, *O*-acetylated; HPTLC, high performance thin layer chromatography; HPLC, high performance liquid chromatography.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Basal modified Eagle's medium and fetal calf serum were from Flow Laboratories (Irvine, Scotland); poly-L-lysine, 1-α-D-arabinofuranosylcytosine, NeuAc, chloroquine, gentamycin, and crystalline bovine serum albumin were from Sigma (St. Louis, MO, USA); HPTLC plates were from Merck (Darmstadt, Germany); 4-methylumbelliferone was from Fluka (Buchs, Switzerland); 4-methylumbelliferyl-β-D-galactoside was from Koch-Light (Colnbrook, UK); Sephadex G-25 (fine) was from Pharmacia (Uppsala, Sweden); [^{3}H]$NaBH_4$ (6.5 Ci/nmol) and [^{3}H]acetic anhydride (8.1 Ci/nmol) were from Amersham International (Amersham, UK).

2.2. *Preparation of radiolabelled gangliosides*

The gangliosides used (GD1a, GD1b, GM1) were obtained as previously described [14]. GD1a was ^{3}H-labelled at the long chain base ([^{3}H-Sph]GD1a) [15] and the molecular species containing erythro-C18 and C20-sphingosine were separated by reverse-phase HPLC [16]. GD1b was ^{3}H-labelled at the terminal galactose ([^{3}H-Gal]GD1b) [17], and GM1 at the sialic acid acetyl group ([^{3}H-NeuAc]GM1) [18]. The radiochemical purity was better than 99% for all labelled compounds and the specific radioactivity 1.15, 1.05 and 4.05 Ci/mmol for [^{3}H-Sph[GD1a, [^{3}H-Gal]GD1b and [^{3}H-NeuAc]GM1, respectively.

2.3. *Cell cultures*

Granule cells, obtained from the cerebellum of 8-day-old rats, were prepared and cultured as described [2,4,10]. Cell viability was assess-

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ed by the Trypan blue exclusion method and cell morphology inspected by phase contrast microscopy.

Treatment with radiolabelled ganglioside was carried out on day 7 in cultures at 37°C, 16°C and 4°C. Dishes were washed twice with temperature conditioned supplemented basal modified Eagle's medium without fetal calf serum and then incubated for 2 h in the same medium (2 ml/dish) containing 10^{-6} M ganglioside, carrying 1 μCi/ml of radioactivity. The medium was then removed, the cells were washed with supplemented basal modified Eagle's medium containing 10% fetal calf serum to eliminate loosely bound labelled gangliosides [10], and incubated for 4 h (chase) in the same medium. Before analysis the cells were rinsed twice with saline, scraped off the plates, and centrifuged (800 × g for 10 min). The medium removed from the dish, with cell washings added, was collected, lyophilized and stored at −20°C for lipid analysis. In parallel experiments cell treatment with radiolabelled gangliosides was carried out at 37°C in the presence of (10–100 μM) chloroquine. This substance was added 30 min before radiolabelled ganglioside and maintained for all the duration of the experiment.

2.4. *Lipid extraction and purification*

The extraction and partitioning of lipids from pelleted cells were performed as previously described [4,10] an organic phase (containing non-ganglioside lipids), a dialyzed aqueous phase (containing gangliosides) and a delipidized pellet (containing mostly proteins) being obtained. The individual components of the organic and aqueous phases were subjected to HPTLC separation (see below). The extraction and partitioning of lipids from the lyophilized culture medium (and washings) were accomplished by the method of Folch-Pi et al. [19]. Prior to extraction the lyophilized material was dissolved with chloroform/methanol/water (60:30:4.5, by volume) and desalted by Sephadex G-25 column chromatography [4]. Radioactive sphingolipids of granule cells were identified [10], and radioactive saccharides present in the delipidized pellet recognized as described [20].

2.5. *Enzyme assays*

β-D-Galactosidase was fluorimetrically assayed [21], using 4-methylumbelliferyl-β-D-galactoside as substrate, under optimized conditions. The buffer system was 0.05 mol/l sodium acetate–acetic acid, optimal pH 3.3. The enzyme activity was expressed as nmol hydrolyzed substrate/min per mg cell protein (mUnits/mg cell protein). Total proteins were assayed by the method of Lowry et al. [22], using bovine serum albumin as the standard.

2.6. *HPTLC*

HPTLC was performed using the following solvent systems: (a) chloroform/methanol/0.2% aqueous $CaCl_2$ (50:42:11, by volume), for gangliosides; and (b) chloroform/methanol/water (55:20:3, by volume), for the other sphingolipids.

2.7. *Determination of radioactivity*

Radioactivity was determined by liquid scintillation counting, fluorography, or radiochromatoscanning (Digital Autoradiography Berthold, Germany) as previously described [4,10].

3. RESULTS AND DISCUSSION

Cerebellar granule cells, at 7 days in culture displayed the features of fully differentiated cells. When incubated at 37°C in the absence of fetal calf serum for up to 2 h, and for a further 4 h in the presence of fetal calf serum both in the absence and presence of

Table I

Effect of chloroquine and temperature on the total radioactivity incorporated by cerebellar granule cells after 2 h incubation (pulse) in the presence of 10^{-6} M [^{3}H-Sph]GM1, or [^{3}H-Sph]GD1a, or [^{3}H-Gal]GD1b

	Chloroquine (μM)	Temperature (°C)	Chase hours)	Administered ganglioside [^{3}H-NeuAc]GM1	[^{3}H-Sph]GD1a	[^{3}H-Gal]GD1b
(a)	0	37	0	115.0	120.2	127.4
	0	37	4	78.5	104.6	105.2
(b)	10	37	0	111.0	122.4	119.8
	10	37	4	80.4	106.2	102.3
(c)	30	37	0	118.2	113.8	122.5
	30	37	4	79.8	102.2	103.8
(d)	50	37	0	113.4	124.2	130.3
	50	37	4	79.5	105.3	106.1
(e)	100	37	0	119.2	117.3	124.8
	100	37	4	81.2	104.7	100.2
(f)	0	16	0	87.7	83.3	74.6
	%(*)			76.2	69.3	58.6
	0	16	4	63.2	73.1	65.2
	%(**)			80.5	69.9	62.0
(g)	0	4	0	62.2	48.5	49.0
	%(*)			54.1	40.3	38.5
	0	4	4	52.4	43.8	48.4
	%(**)			66.3	44.9	46.0

The pulse with radioactive ganglioside was followed, or not, by a 4 h period of chase. Radioactivity uptake is expressed as nCi/mg cell protein. The data shown are the means of 3 experiments with SD values never exceeding 8% of the means.

(*) Referred to the corresponding value at 37°C 0 h chase

(**) Referred to the corresponding value at 37°C 4 h chase

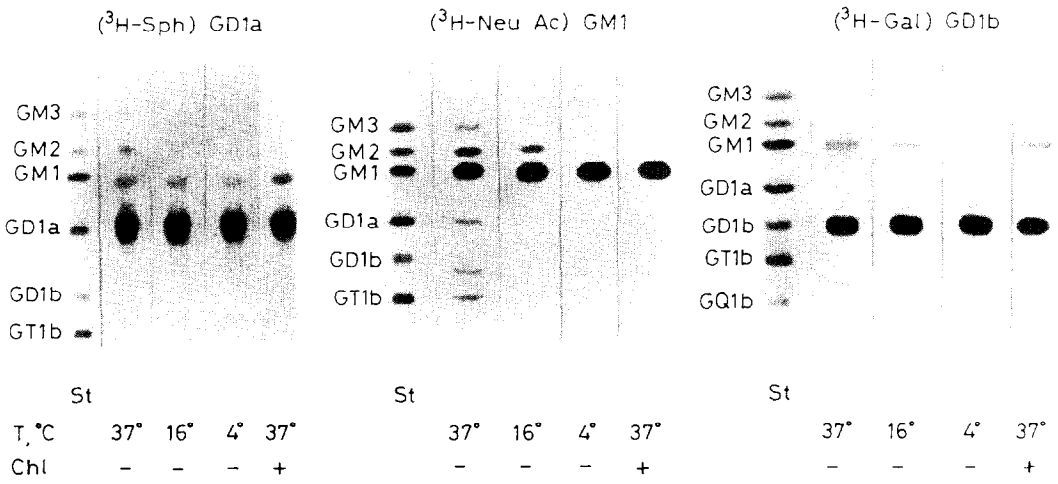

Fig. 1. Effect of temperature (T,°C) and 100 μM chloroquine (Chl) on the metabolic processing of exogenous [^{3}H-Sph]GD1a, [^{3}H-NeuAc) GM1, and [^{3}H-Gal]GD1b by cerebellar granule cells in culture. The gangliosides (GM3, GM2, GM1, GD1a, GD1b, *O*-Ac-GT1b, GT1b), contained in the dialyzed aqueous phase obtained from the total lipid extracts of the cells, were separated by HPTLC with solvent system (a). Ganglioside-bound radioactivity was detected by fluorography (see section 2 for details). The radioactive spot between GD1b and GT1b obtained with [^{3}H-NeuAc]GM1 corresponds to *O*-Ac-GT1b.

gangliosides, no changes in viability and morphology were observed. The presence of 10–100 μM chloroquine as well as incubation at 16°C and 4°C did not produce any adverse effects on cell viability; a modest degree of vacuolisation (presumably enlarged lysosomes) was observed at the highest chloroquine concentration. As shown in Table I the total radioactivity carried by cerebellar granule cells after 2 h incubation (pulse) in the presence of 10^{-6} M [^{3}H-Sph]GD1a, [^{3}H-NeuAc]GM1, or [^{3}H-Gal]GD1b was 115.0 ± 7.5, 120.2 ± 8.5 and 127.4 ± 9.2 nCi/mg cell protein, respectively, and underwent, in all cases, about 20% decrease

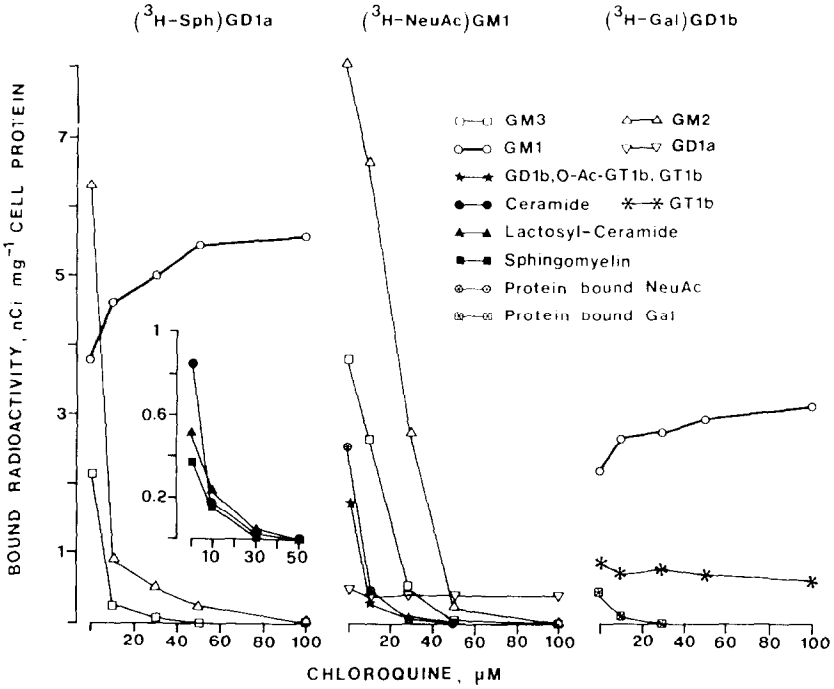

Fig. 2. Influence of different concentrations of chloroquine on the pattern of metabolic processing of exogenous [^{3}H-Sph]GD1a, [^{3}H-NeuAc]GM1, and [^{3}H-Gal]GD1b by cerebellar granule cells in culture. The gangliosides (GM3, GM2, GM1, GD1a, GD1b, *O*-Ac-GT1b, GT1b), contained in the dialyzed aqueous phase obtained from the total lipid extracts of the cells, were separated by HPTLC with solvent system (a) (see section 2). The non-ganglioside sphingolipids (lactosylceramide, ceramide, sphingomyelin) contained in the organic phase obtained from the total lipid extract of the cells, were separated by HPTLC with solvent system (*b*). Protein-bound NeuAc and protein-bound Gal were contained in the delipidized pellet, in both cases bound radioactivity was almost completely carried by isolated NeuAc and Gal, respectively. The radioactivity carried by the individual lipids was measured by autoradioscanning; protein-linked radioactivity was measured by liquid scintillation counting.

after 4 h of chase. The predominant portion of bound radioactivity (over 90%) was constituted, in all cases, by the ganglioside used for pulsing and the remainder by non volatile metabolites.

The presence of chloroquine did not significantly modify the total amount of bound radioactivity after a 2 h pulse, whether followed or not, by a 4 h chase. This indicates that chloroquine does not affect the binding and internalization of exogenous ganglioside. Instead, lowering the temperature to 4°C caused a substantial decrease in the amount of radioactivity linked to cells, presumably as the result of a decrease of both binding and internalization of exogenous ganglioside. As shown in Figs 1 and 2, after a 2 h pulse with exogenous ganglioside followed by 4 hours of chase, GM1, GM2, GM3, lactosylceramide, ceramide (all of them degradation products) and sphingomyelin (biosynthesized by recycling of liberated sphingosine) were produced from [^{3}H-Sph]GD1a; GM2 and GM3 (degradation products), GD1b, *O*-Ac-GT1b, GT1b and protein bound NeuAc (biosynthesized by recycling of liberated NeuAc), and GD1a (obtained by direct glycosylation of GM1) from [^{3}H-NeuAc]GM1; GM1 (degradation product), protein-bound Gal (obtained by recycling of released Gal), and GT1b (obtained by direct glycosylation of GD1b) from [^{3}H-Gal]GD1b. All these results are in agreement with previous findings [4,10].

Regardless of the ganglioside used for pulsing, all the compounds originated from biosynthetic recycling of degradation fragments (sphingomyelin, protein bound-NeuAc, protein-bound Gal, etc) diminished with increasing concentration of chloroquine and completely

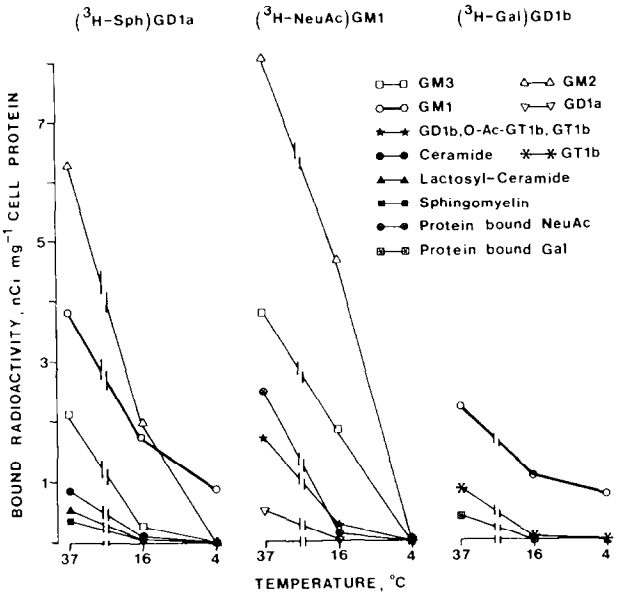

Fig. 3. Effect of temperature (37°C, 16°C, 4°C) on the metabolic processing of [^{3}H-Sph]GD1a, [^{3}H-NeuAc]GM1 and [^{3}H-Gal]GD1b by cerebellar granule cells in culture. For procedural details see section 2 and the legend to Fig. 2.

disappeared at 100 μM drug (Figs 1 and 2), indicating that these fragments are normally formed in the lysosomes. Of course this also means that one site of sialic acid release from gangliosides is the lysosomal apparatus, and implicates the occurrence of a lysosomal sialidase. Instead, the process of direct glycosylation of taken-up ganglioside (GD1a from [^{3}H-NeuAc]GM1; GT1b from [^{3}H-Gal]GD1b) was not affected by chloroquine, meaning that this process does not occur in the lysosomes, as expected [4,10]. All the compounds produced by degradation of taken-up ganglioside disappeared at 100 μM chloroquine (GM2 and GM3 from [^{3}H-NeuAc]GM1; GM2, GM3, lactosylceramide, ceramide from [^{3}H-Sph]GD1a), with the exception of GM1, only when the cells were fed with [^{3}H-Sph]GD1a and [^{3}H-Gal]GD1b. In these cases GM1 formation increased with increasing concentrations of chloroquine, indicating that the release of sialic acid from GD1a and GD1b can be accomplished outside the lysosomes. Analysis of the radioactive compounds present in the culture medium showed that, regardless of the presence of chloroquine, the only detectable radioactive substance was the ganglioside used for pulsing, presumably released from the cell surface. This excludes the possibility for GM1 to be produced extracellularly. As shown in Fig. 3 the products of metabolic processing of exogenous ganglioside were present in lower amounts at 16°C, and completely disappeared at 4°C. The only exception was, again, GM1 that was present in measurable amounts at 4°C after exposure of cells to [^{3}H-Sph]GD1a and [^{3}H-Gal]GD1b. A noteworthy point is that the activity of the lysosomal enzyme β-D-galactosidase diminished from 1.69 mU/mg protein at 37°C to 0.11 at 4°C, indicating that the lysosomal apparatus is somewhat active also at 4°C and potentially capable of degrading ganglioside, if present in the lysosome. Since at 4°C exogenous GD1a and GD1b do bind to the plasma membrane but do not enter into the cells, a plausible explanation for the formation of GM1 is that the hydrolysis of both GD1a and GD1b takes place at the plasma membrane level by the action of a membrane-bound sialidase.

On this basis, the enhanced formation of GM1 from GD1a and GD1b in the presence of chloroquine may be explained assuming that GD1a and GD1b are normally degraded to GM1 by the action of membrane-bound sialidase; formed GM1, which cannot be further degraded, accumulates.

In conclusion, cerebellar granule cells in vivo are able to remove sialic acid from GD1a and GD1b (with formation of GM1), by a sialidase that is presumably linked to the plasma membrane. An additional site of sialidase action is the lysosome, where sialic acid is released along with the degradation of GM1. A proof for the dual location of sialidase in cerebellar granule cells, by conventional subcellular fractionation studies,

is still lacking. However, the mammalian brain is known to carry both a plasma membrane bound [23] and a lysosomal [24] sialidase. Moreover, our findings are in perfect agreement with the observation [25] that in vitro the sialidase present in neural plasma membrane is active on the portion of added GD1a that is inserted into the membrane.

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