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Involvement of ganglioside GT1b in glutamate release from neuroblastoma cells

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ABSTRACT

Since gangliosides play many important roles in neural systems, we investigated whether gangliosides are involved in glutamate release from neural cells. Differentiated neruro2a cells were treated with gangliosides, including GM3, GM1, GD1a, GD3, GD1b, or GT1b, for 30 min, and glutamate concentration in the culture media was measured using *o*-phthalaldehyde derivatization. Among the tested gangliosides, GT1b significantly increased the glutamate concentration when compared with untreated cells. Moreover, GT1b increased the glutamate concentration in the culture media of neuroblastoma × dorsal root ganglion neuron hybrid F11 cells. These results suggested that gangliosides are important in regulating extracellular glutamate concentration in the nervous system.

1. Introduction

Gangliosides are sialic acid containing glycosphingolipids synthesized from sphinganine/sphingosine. These compounds can be divided into four groups according to their biosynthetic pathway: the asialo-, a-, b-, and c-series gangliosides. Gangliosides are abundant in the nervous system, and are thought to be involved in axonal elongation [4,17]; synaptic transmission [26]; and neuron–glia interactions [16,24]. Gangliosides have also been reported to be components of lipid rafts, acting as key assemblers and regulators of the sorting of other molecules into lipid rafts that regulate cell–cell interactions and signal transduction complexes [20].

Gangliosides also function in synaptic transmission and long-term potentiation (LTP). For example, the accumulation of exogenous or endogenous GM1 by sialidase treatment has been found to enhance synaptic transmission and LTP [8,27], and exogenous addition of the b-series ganglioside GQ1b has been found to enhance ATP-induced LTP [6]. In addition, LTP was attenuated in β1,4 GalNAc-T transgenic mice, which have an increase in a-series and a decrease in b-series gangliosides [9].

All of these synaptic transmission pathways utilize glutamate a major excitatory neurotransmitter. Moreover, the extracellular concentration of glutamate is regulated by its release and uptake via several pathways, such as those involving synaptic vesicles and transporters in neuron and glial cells [21]. In contrast, membrane proteins involved in synaptic transmission, including glutamate signaling, have been localized into lipid rafts [1,15], which consist of cholesterol and sphingolipids including gangliosides. We have reported that exogenous GT1b enhances extracellular glutamate concentrations in subdermal tissues [25], in which nerve fibers are regarded as the major source of glutamate [2,5,12]. To determine whether gangliosides induce glutamate release from neuronal cells, we examined the effects of exogenously added gangliosides on glutamate concentration in the supernatants of differentiated neuroblastoma cell cultures. We found that the b-series ganglioside GT1b, not the a-series ganglioside GM1, enhanced extracellular glutamate release from these cells.

2. Materials and methods

2.1. Cell cultures

Neuro2a cells, the kind gift of Dr. Masami Takahashi (Mitsubishi Kagaku Institute of Life Sciences, Machida, Tokyo, Japan) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37 °C in an atmosphere of 5% CO_2 and 95% air. For differentiation, 1.0×10^4 cells/cm^2 were plated onto poly-D-lysine coated 24 well plates in DMEM plus 10% FBS and incubated overnight, after which they were grown in DMEM plus 1% FBS, 2 mM glutamine, and 20 μM retinoic acid for 72 h.

Neuroblastoma × dorsal root ganglion neuron hybrid F-11 cells [19] were grown in DMEM plus 10% FBS and 2 mM glutamine at 37 °C in 5% CO_2 and 95% air. For differentiation, 0.25×10^4 cells/cm^2 were plated onto poly-D-lysine coated 24 well plates in DMEM plus 10% FBS and incubated overnight, after which they were cultured

Abbreviations: D-PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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for 7–8 days in DMEM plus 0.5% FBS, 2 mM glutamine, and 10 μM forskolin.

To measure glutamate released by cells, each plate was washed with 500 μl HEPES-buffered saline (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 4.5 mM glucose, 20 mM HEPES–NaOH pH 7.4), and the cells were incubated at 37 °C in the same buffer containing 5–50 μM various gangliosides for 10–60 min. To inhibit ganglioside biosynthesis, cells were incubated for 24 h with 4 μM D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), the kind gift of Prof. Jin-ichi Inokuchi (Tohoku Pharmaceutical University, Sendai, Miyagi), before glutamate was assayed. To assess their viability, the D-PDMP-treated cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 90 min at 37 °C. The resultant formazan crystals were dissolved in 100 μl DMSO, and absorbance at 570 nm was determined with a microplate reader.

2.2. Amino acid analysis

Amino acids in the HEPES-buffered saline (50 μl) were analyzed by high-performance liquid chromatography (HPLC) following *o*-phthalaldehyde derivatization and fluorescence detection. Amino acids were quantified by reverse-phase chromatography using a ZORBAX Eclipse AAA column (5 μm, 75 mm × 4.6 mm I.D., Agilent Technology, Santa Clara, CA) attached to an HPLC system consisting of a pump connected to a degasser, a fluorescence HPLC monitor, and a chromato-integrator. The samples were eluted using the following program: 0–1.0 min with solvent A (40 mM NaH_2PO_4, pH 7.8); 1.0–9.8 min with a linear gradient of 0–57% solvent B (methanol:acetonitrile:water, 45:45:10); 9.8–10.0 min with a linear gradient of 57–100% solvent B; 10.0–12.0 min with 100% solvent B; 12.0–12.5 min linear gradient with 100–0% solvent B; and 12.5–14.0 min with solvent A. The flow rate and temperature were kept constant at 2.0 ml/min and 40 °C, respectively.

2.3. Drugs

GM1, GD1b, GD1a, and GT1b were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and GM3 and GD3 were purchased from ENZO Life Sciences (Farmingdale, NY, USA). All gangliosides were derived from bovine brain.

2.4. Statistical analysis

Results are presented as means ± S.E.M. The statistical significance between groups was assessed by two-tailed unpaired Student's *t* tests, two-way repeated-measures ANOVA followed by Bonferroni post-tests, and means of one-way analysis of variance followed by Dunnett's multiple comparison test, as appropriate. All statistical analyses were performed using GraphPad software (San Diego, CA, USA), with *P*-values less than 0.05 ($P < 0.05$) considered statistically significant.

3. Results

To identify which gangliosides enhance extracellular glutamate concentration in the supernatants of differentiated neuroblastoma cells, we incubated cells with 50 μM of each ganglioside and measured glutamate concentrations in the culture media (Fig. 1). We found that GT1b significantly enhanced glutamate concentration, whereas the other gangliosides (GM3, GM1, GD1a, GD3 and GD1b) did not. Assessment of the dose–response relationship showed that 50 μM GT1b maximally and significantly enhanced glutamate concentration (Fig. 2). Moreover, this increase was time-dependent, becoming significant after incubation for 30 min (Fig. 3).

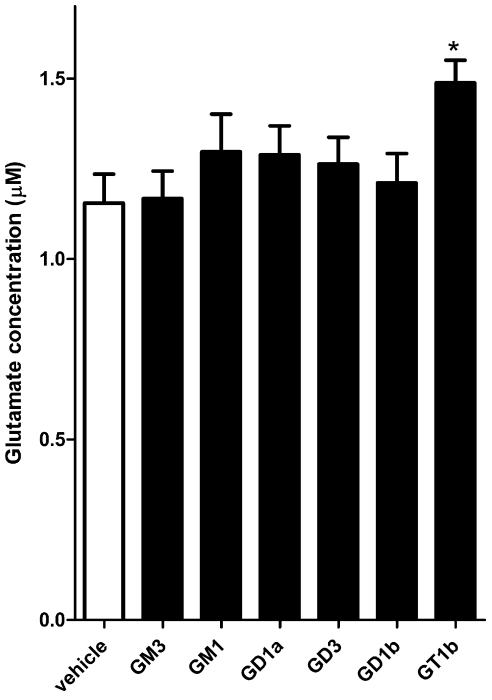

Fig. 1. The effects of gangliosides of different structures on glutamate release by differentiated neuro2a cells. Cells were treated with 50 μM gangliosides for 30 min at 37 °C and glutamate concentrations in the culture supernatants were measured using HPLC. Results are expressed as means ± S.E.M. of 5 experiments. *$P < 0.05$ compared with vehicle by one-way ANOVA followed by Dunnett's test.

During nociception, glutamate acts as a nociceptive agent in the epidermis, and as an important neurotransmitter between dorsal root ganglion cells and dorsal horn neurons in the spinal cord. Since the free nerve endings of dorsal root ganglion cells are thought to be the primary sources of glutamate in subdermal tissues [12,13], we assessed the effects of GT1b on glutamate release from neuroblastoma × dorsal root ganglion F-11 neuron hybrid cells. GT1b also increased glutamate concentration in the supernatants of differentiated F-11 cells (Fig. 4), suggesting that gangliosides influence not only the central but the peripheral nervous system. To

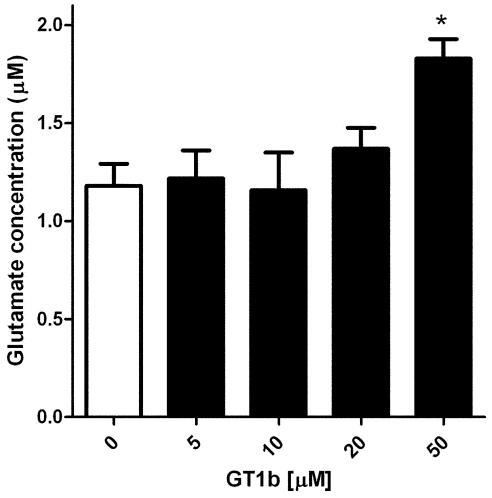

Fig. 2. Dose–response relationship between GT1b concentration and glutamate concentration in supernatants of differentiated neuro2a cells. Cells were treated with various concentrations of gangliosides for 30 min at 37 °C and glutamate concentrations in the culture supernatants were measured using HPLC. Data are expressed as means ± S.E.M. of 4 experiments. *$P < 0.05$ compared with vehicle by one-way ANOVA followed by Dunnett's test.