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Differential Effects of Glycosphingolipids on Protein Kinase C Activity in PC12D Pheochromocytoma Cells

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Abstract
Previous studies have shown that certain glycosphingolipids may function as modulators of protein kinase C (PKC) activity. To study the structure-activity relationship, we examined the effects of 17 gangliosides, 10 neutral glycolipids, as well as sulfatide, psychosine and ceramide on PKC activity in PC12D cells. Using an in vitro assay system, we found that all but one (GQ1b) ganglioside inhibited PKC activity at concentrations between 25 and 100 μM, and the potency was proportional to the number of sialic acid residues. However, at lower concentrations several gangliosides, including GM1 and LM1 behaved as mild activators of PKC activity. GQ1b had no effect within the range 0.1–10 μM, but acted as a mild activator of PKC activity at 25 μM. On the other hand, fucosyl-GM1 and GM1 containing blood group B determinant, which are abundant in PC12 cells, were potent inhibitors of PKC activity. Among the neutral glycosphingolipids tested, LacCer, Gb3, GalGb3, and GA1, all of which have a terminal galactose residue, were found to be ineffective or acted as mild activators of PKC activity. In contrast, GA2, Gb4 and Gb5 which have a terminal N-acetylgalactosamine residue, were potent inhibitors of the PKC activity. Thus, the terminal sugar residue may play a pivotal role in determining the effect of glycosphingolipids in modulating PKC activity. In addition, we also found that GalCer containing normal fatty acids acted as potent activators of PKC activity. Ceramide and GlcCer appeared to be ineffective in modulating PKC activity, whereas psychosine and sulfatides appeared to be inhibitory. We conclude that the carbohydrate head groups and the hydrophobic groups of gangliosides and neutral glycolipids may modulate the PKC system in unique manners, which may in turn affect various biological processes in the cell.

The expression of glycosphingolipids (the nomenclature used throughout this paper for gangliosides and neutral glycosphingolipids is based on that recommended by IUPAC-IUB [26]) is regulated during the cell cycle and oncogenic transformation [16, 50]. It is well known that addition of exogenous glycosphingolipids to several cell lines grown in tissue culture causes growth inhibition by extending the length of the G_1 phase of the cell cycle, and blocks cellular proliferation in the presence of growth factors [7]. Thus, glycosphingolipids have been implicated in

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diverse cellular functions including modulation of transmembrane signal transduction, resulting in regulation of cell proliferation and cell-cell or cell-substratum recognition [17]. Despite many reports documenting the neuritogenic and neurotrophic effects of glycosphingolipids [8, 31, 38, 39, 48], the mechanism of action and signal transduction pathways modulated by these complex lipids has largely remained obscure. Gangliosides may mediate these cellular processes by modulating protein kinase activities [10, 14, 20, 30, 35, 47].

PC12 pheochromocytoma cells are derived from a clonal cell line from rat adrenal medullary pheochromocytomas [15]. They share with normal adrenal chromaffin cells originating from sympathetic nerve ganglia such characteristics as neurite outgrowth, increased cellular adhesion and increased levels of choline acetyltransferase, acetylcholine esterases, and norepinephrine uptake sites in response to nerve growth factor (NGF) [15]. Therefore, PC12 cells have been used as a model to study neuronal function and differentiation. Several recent studies have indicated that protein kinase C (PKC) is an important component of the NGF-sensitive phosphorylation system in PC12 cells [13, 19]. Hilbush and Levine [24] reported that GM1 ganglioside modulated NGF signal transduction in PC12 cells. Hall et al. [18] reported that PKC plays a role in mediating the neuritogenic effects of NGF by virtue of a sphingosine-sensitive pathway. There have been several reports indicating that sphingosine acts as an inhibitor of a variety of PKC-dependent processes [21, 22, 34, 52]. In the present study, we describe the effects of exogenous gangliosides and neutral glycosphingolipids on PKC activity in PC12D cells, a subclone of PC12 cells [28]. PC12D cells were chosen because they are morphologically more homogeneous than the parent PC12 cells which are quite heterogeneous [5, 28]. Our results demonstrate that certain gangliosides and neutral glycosphingolipids can modulate PKC-dependent phosphorylation.

Materials and Methods

The glycosphingolipids used were prepared in our laboratory using established methods [1, 2, 3, 27, 37] from the following sources: gangliosides (GM2, GM1, GD1a, GD1b, GD2, GT1a, GT1b, and GQ1b) and asialo glangliosides (GA1 and GA2) from human brain; neutral glycosphingolipids (LacCer, Gb3, Gb4, and Gb5) from bovine or sheep erythrocyte membranes; GD3 and GT3 from bovine buttermilk, GM3(A) and GM3(G) from bovine adrenal medulla; LM1 and paragloboside (nLc4) from human erythrocyte membrane; GQ1c from cod fish brain; fucosyl-GM1 (FGM1), GM1 with blood group B determinant (BGM1), and Gb3 with α1-3 galactosyl residue (GalGb3) from PC12h cells [39, 40]; GlcCer from the spleen of a patient with Gaucher's disease (kindly provided by Dr. Oshima of The Clinical Research Institute, National Medical Center, Tokyo, Japan). The isolation of GalCer and sulfatides with α-hydroxy and normal fatty acids from human brain was achieved by preparative HPTLC on silica gel (Merck, Darmstadt, Germany) and Iatrobeads column chromatography, as described previously [2]. Galactosyl psychosine (Psy) was prepared from GalCer by the procedure of Taketomi and Yamakawa [44]. Ceramide was prepared from GalCer by the method of Carter et al. [9]. The individual glycosphingolipids were found to be chromatographically pure on HPTLC plates in several solvent systems. The structures of glycosphingolipids used in this study are shown in table 1.

Gangliosides were quantitated by measuring the lipid-bound sialic acid using the resorcinol-hydrochloric acid method [43]. Other glycosphingolipids were estimated by measuring their sugar content using gas-liquid chromatography [46]. Ceramide was estimated by measuring the sphingosine bases [23]. [γ-^{32}P]ATP was purchased from DuPont-New England Nuclear (Boston, Mass., USA). All other reagents were of the highest grade available and were obtained from Sigma (St. Louis, Mo., USA). PC12D pheochromocytoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (Bioproducts Md., USA) and 5% fetal bovine serum (Bioproducts) in the presence of insulin (5 mg/l; Gibco N.Y., USA) as described previously [28].

PKC was partially purified from PC12D cells as described by Kreutter et al. [30] and Xia et al. [47]. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and resuspended in a lysis buffer (2 m*M* Tris-HCl, 0.1 m*M* EGTA, 10% sucrose, 50 m*M* 2-mercaptoethanol, pH 7.5) and disrupted by sonication for 3 s. Triton X-100 was added to a final concentration of 0.3%. The mixture was incubated on ice for 15 min to solubilize membrane-bound PKC, and then 2 ml of DEAE-cellulose equilibrated in lysis buffer were added. After incubation for 15 min, the mixture was centrifuged for 5 min at 1,000 rpm and washed twice with the lysis buffer and the enzyme was eluted with 0.2 *M* NaCl in lysis buffer. The activity of PKC was measured by the incorporation of [^{32}P] from [γ-^{32}P]ATP into histone III-S. The reaction mixture for assaying PKC activity contained 10 *M* Mg acetate, 1 m*M* EGTA, 1.1 μ*M* $CaCl_2$, 0.01 m*M* ATP (0.1 μCi of [γ-^{32}P]ATP), 20 μg/ml of phosphatidyl serine, 2 μg/ml of 1,2-diolein, 200 μg/ml of histone III-S, 0–100 μ*M* of glycosphingolipids, and 15–40 μg/tube of PKC, in a final volume of 250 μl. Reactions were carried out at 37 °C for 10 min, and terminated by addition of 1.0 ml of 25% ice-cold trichloroacetic acid (TCA). After 5 min, the reaction mixture was filtered (Millipore HA, 0.45 μm), the filters were washed three times with 4 ml 5% ice-cold TCA, placed in scintillation vials with 10 ml scintillation fluid and the [^{32}P] incorporation into histone III-S was determined using a scintillation counter.

Protein concentration was determined by the method of Bradford [6]. The data are expressed as values ± SD. (%) relative to the value of control conditions (100.0%) including 20 μg/ml of phosphatidyl serine and 2 μg/ml of 1,2-diolein, but without glycosphingolipids. Statistical analyses were performed using Student's t test (significance at $p < 0.05$).

Table 1. Structures of neutral glycosphingolipids and gangliosides

Ceramide	Cer
Psychosine (Psy)	Galβ1-1′sphingosine
Glucosyl ceramide	Glcβ1-1′Cer
Gala series	
Galactosyl ceramide	Galβ1-1′Cer
Sulfatide	Gal(SO_3)β1-1′Cer
Lacto series	
LacCer	Galβ1-4Glcβ1-1′Cer
GM3(A)	Gal(NeuAc)β1-4Glcβ1-1′Cer
GM3(G)	Gal(NeuGc)β1-4Glcβ1-1′Cer
GD3	Gal($NeuAc_2$)β1-4Glcβ1-1′Cer
GT3	Gal($NeuAc_3$)β1-4GLcβ1-1′Cer
Globo series	
Gb3	Galα1-4Galβ1-4Glcβ1-1′Cer
Gb4	GalNAcβ1-3Galα1-4Galβ1-4GLcβ1-1′Cer
GalGb3	Galα1-3Galα1-4Galβ1-4Glcβ1-1′Cer
Gb5	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
Gangliotriaosyl series	
GA2	GalNAcβ1-4Galβ1-4Glcβ1-1′Cer
GM2(A)	GalNAcβ1-4Gal(NeuAc)β1-4Glcβ1-1′Cer
GM2(G)	GalNAcβ1-4Gal(NeuGc)β1-4Glcβ1-1′Cer
GD2	GalNAcβ1-4Gal($NeuAc_2$)β1-4Glcβ1-1′Cer
Gangliotetraosyl series	
GA1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer
GM1	Galβ1-3GalNAcβ1-4Gal(NeuAc)β1-4Glcβ1-1′Cer
GD1a	Gal(NeuAc)β1-3GalNAcβ1-4Gal(NeuAc)β1-4Glcβ1-1′Cer
GD1b	Galβ1-3GalNAcβ1-4Gal($NeuAc_2$)β1-4Glcβ1-1′Cer
GT1a	Gal($NeuAc_2$)β1-3GalNAcβ1-4Gal(NeuAc)β1-4Glcβ1-1′Cer
GT1b	Gal(NeuAc)β1-3GalNAcβ1-4Gal($NeuAc_2$)β1-4Glcβ1-1′Cer
GQ1b	Gal($NeuAc_2$)β1-3GalNAcβ1-4Gal($NeuAc_2$)β1-4Glcβ1-1′Cer
GQ1c	Gal(NeuAc)β1-3GalNAcβ1-4Gal($NeuAc_3$)β1-4Glcβ1-1′Cer
Neolacto series	
nLc4	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer
LM1	Gal(NeuAc)β1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer
Other gangliosides	
FGM1	Gal(Fuc)β1-3GalNAcβ1-4Gal(NeuAc)β1-4Glcβ1-1′Cer
BGM1	Galα1-3Gal(Fuc)β1-3GalNAcβ1-4Gal(NeuAc)β1-4Glcβ1-1′Cer

Cer = Ceramide; Glc = glucose; Gal = galactose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; NeuAc = N-acetylneuraminic acid; NeuGc = N-glycolylneuraminic acid; Fuc = fucose.

Results

To study the structure-activity relationship between various glycosphingolipids and PKC activity in PC12D cells, we examined the effects of 17 gangliosides and 10 neutral glycosphingolipids as well as ceramide, psychosine, sulfatides, and N-acetylneuraminic acid between the concentrations of 2.5 and 100 μ*M* in an in vitro assay system (tables 2, 3). Among gangliosides with a ganglioteraose core structure, GM1 ganglioside acted as a mild activator of PKC activity within the range 2.5–10 μ*M*. At 10 μ*M*, GD1b also appeared to be a mild activator of PKC activity. All but one gangliotetraosyl series ganglioside inhibited PKC activity at concentrations between 25 and 100 μ*M*. GQ1b had no effect within the range 0.1–10 μ*M*; however, at 25 μ*M* this glanglioside appeared to be a mild activator of PKC activity. GQ1c was only mildly inhibitory of PKC activity. At 50 μ*M*, both GQ1b and

Table 2. The effects on gangliosides, sulfatides and sialic acid on PKC activity

	0.1 μM	1.0 μM	2.5 μM	5.0 μM	10 μM	25 μM	50 μM	100 μM
GM1	104.8±5.6 (3)	100.1±3.6 (3)	107.1±2.4 (3)*	117.7±5.0 (3)*	116.8±5.5 (9)**	74.8±7.1 (9)***	51.4±3.0 (9)***	22.7±0.2 (3)***
GD1a	95.6±6.4 (3)	100.9±2.0 (3)	n.d.	n.d.	106.6±4.8 (9)	61.9±7.6 (6)***	31.4±7.3 (6)***	n.d.
GD1b	96.7±2.9 (6)	98.3±2.0 (3)	n.d.	n.d.	112.4±5.0 (6)*	47.3±4.9 (3)**	27.5±2.6 (3)***	n.d.
GT1a	114.9±8.4 (3)	90.8±6.3 (3)	n.d.	n.d.	93.2±6.1 (3)	62.2±5.3 (3)**	23.0±5.8 (3)**	n.d.
GT1b	92.3±5.2 (6)	106.8±6.1 (6)	n.d.	n.d.	97.9±8.7 (6)	51.7±8.8 (6)***	4.5±3.9 (3)***	n.d.
GQ1b	103.3±2.7 (9)	98.4±7.2 (9)	n.d.	n.d.	110.3±7.3 (9)	113.1±5.1 (6)**	77.8±3.5 (3)**	n.d.
GQ1c	92.5±5.3 (3)	91.4±3.1 (6)	101.3±6.3 (3)	102.2±1.8 (3)	92.6±5.5 (9)*	80.9±5.3 (9)***	76.3±5.5 (3)*	n.d.
LM1	103.9±5.0 (3)	114.9±6.8 (3)*	n.d.	n.d.	109.8±4.5 (3)*	40.7±1.7 (3)***	17.5±5.2 (3)**	n.d.
FGM1	76.9±3.5 (3)**	78.7±4.3 (6)*	85.0±5.0 (3)*	85.6±0.8 (3)***	74.5±2.0 (6)***	60.4±1.7 (6)***	39.1±5.6 (3)**	n.d.
BGM1	89.1±5.0 (3)	85.2±1.8 (6)***	85.6±7.4 (3)	75.4±4.5 (3)*	69.3±1.3 (6)***	56.9±4.9 (6)***	30.0±8.1 (3)**	n.d.
GM3(A)	111.3±12.9 (3)	108.0±5.4 (3)	n.d.	n.d.	80.0±6.6 (9)**	57.4±7.0 (9)***	35.6±2.1 (9)***	11.8±4.5 (6)***
GM3(G)	113.7±11.5 (3)	83.9±11.4 (3)	n.d.	n.d.	68.8±8.7 (6)*	38.0±2.3 (3)***	18.5±2.9 (3)***	10.4±5.0 (3)***
GM2(A)	88.7±12.3 (3)	84.1±5.8 (3)	n.d.	n.d.	72.4±7.0 (9)**	57.2±6.8 (6)***	36.3±4.3 (6)***	n.d.
GM2(G)	96.1±4.0 (3)	87.6±8.6 (3)	n.d.	n.d.	70.7±3.6 (6)***	40.2±3.0 (3)**	20.0±0.6 (3)***	n.d.
GD3	94.1±1.1 (3)*	86.4±5.9 (3)	n.d.	72.5±4.9 (3)*	77.1±11.9 (6)*	39.7±5.1 (6)***	12.5±5.6 (6)***	n.d.
GD2	100.5±3.3 (3)	88.5±10.9 (6)	86.0±6.9 (3)	86.5±10.8 (3)	90.8±11.9 (9)	61.1±7.8 (6)***	26.0±1.7 (3)**	n.d.
GT3	76.0±0.8 (3)***	75.5±7.1 (3)*	n.d.	n.d.	59.1±2.6 (3)**	19.3±6.2 (3)**	n.d.	n.d.
Sulf(N)	n.d.	n.d.	60.0±1.4 (3)**	67.4±5.4 (3)*	72.3±3.4 (3)**	60.4±0.2 (3)***	n.d.	n.d.
Sulf(H)	n.d.	n.d.	55.8±6.9 (3)**	44.6±2.0 (3)	65.5±1.9 (3)**	54.7±4.7 (3)**	n.d.	n.d.
NeuAC	107.2±5.7 (3)	109.4±5.1 (3)	n.d.	n.d.	108.7±6.7 (3)	105.5±7.0 (3)	106.8±2.0 (3)	n.d.

The values are expressed as the percentages of normal control ± SD. The number in parentheses indicates the number of determinations.
n.d. = Not determined; Sulf = sulfatide; NeuAc = N-acetylneuraminic acid.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. The effects of neutral glycolipids, ceramide and psychosine on PKC activity

	0.1 μM	1.0 μM	2.5 μM	5.0 μM	10 μM	25 μM	0 μM	100 μM
Psy	n.d.	n.d.	90.4±1.8 (3)*	n.d.	98.8±3.0 (3)	84.9±2.8 (3)**	84.6±3.5 (3)*	72.0±3.8 (3)**
Cer	n.d.	n.d.	89.0±6.3 (3)	80.2±5.1 (3)*	83.0±6.6 (3)*	95.6±1.9 (3)*	95.6±2.5 (3)	96.4±1.3 (3)
GlcCer	n.d.	n.d.	96.3±3.0 (3)	106.7±3.0 (3)	102.8±4.0 (3)	87.8±5.0 (3)*	n.d.	n.d.
GalCer(N)	n.d.	n.d.	98.9±2.3 (9)	100.9±3.4 (9)	110.9±5.0 (9)*	155.1±4.3 (9)***	132.6±3.1 (6)***	121.5±3.0 (3)***
GalCer(H)	n.d.	n.d.	91.6±6.7 (9)	87.2±4.1 (6)*	93.0±6.9 (6)	94.3±5.1 (9)	81.5±3.7 (6)***	66.5±6.4 (3)*
LacCer	99.3±3.0 (3)	104.6±2.7 (3)	n.d.	n.d.	111.7±5.0 (6)*	109.2±7.7 (6)	113.9±4.3 (6)**	n.d.
Gb3	n.d.	n.d.	102.8±2.1 (6)	95.7±3.6 (6)*	100.3±5.1 (6)	90.9±5.2 (3)**	n.d.	n.d.
Gb4	n.d.	n.d.	88.1±2.4 (6)**	88.6±3.3 (6)*	55.7±4.5 (6)***	26.7±2.2 (6)***	n.d.	n.d.
Gb5	n.d.	n.d.	86.6±3.7 (6)***	71.3±5.1 (6)***	51.2±0.5 (6)***	19.7±0.5 (6)***	n.d.	n.d.
GA2	79.3±4.3 (3)*	74.0±8.6 (6)**	72.6±2.0 (3)**	70.3±3.5 (3)**	60.2±9.1 (9)***	51.4±4.5 (7)***	36.0±4.9 (3)**	n.d.
GA1	106.0±7.3 (3)	115.9±8.5 (6)	110.2±10.2 (3)	n.d.	101.0±7.4 (6)	99.4±10.7 (3)	114.0±8.0 (3)	93.7±5.5 (3)
nLc4	n.d.	n.d.	106.7±7.6 (3)	101.2±2.8 (3)	95.7±3.6 (3)*	n.d.	n.d.	n.d.
GalGb3	n.d.	n.d.	107.8±0.6 (6)**	98.7±2.8 (6)	103.6±0.8 (6)*	91.3±7.9 (6)*	n.d.	n.d.

The values are expressed as the percentages of normal control ± SD. The number in parentheses indicates the number of determinations.
n.d. = Not determined.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

GQ1c appeared to possess inhibitory potency, with the PKC activity remaining at approximately 80% of the control. However, GA1 was found to have no effect on PKC activity (table 3). N-Acetylneuraminic acid itself had no effect within the range of 0.1–100 μM (table 2). Lacto and gangliotriaosyl series gangliosides were also found to be potent inhibitors of PKC activity at concentrations between 25 and 100 μM (table 2). The corresponding asialo compounds of the two series of gangliosides had different effects on PKC: LacCer appeared to be ineffective at concentrations less than 1.0 μM or was a mild activator between 10 and 50 μM, while GA2 strongly inhibited PKC activity.

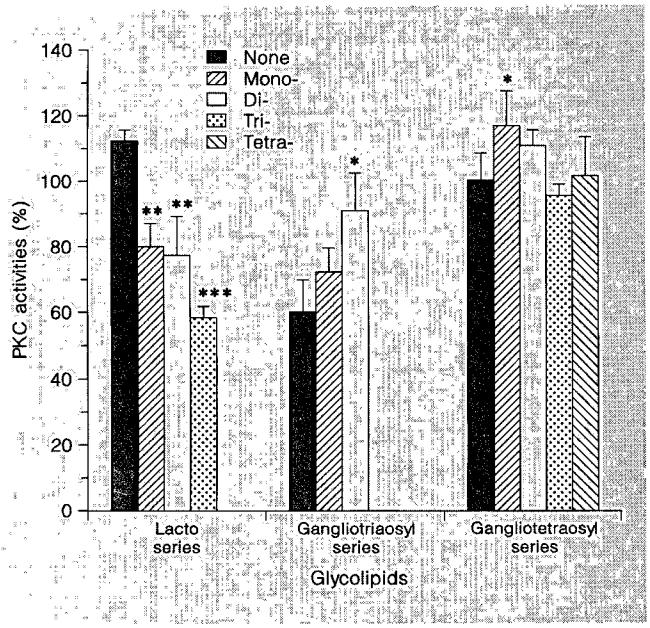

Fig. 1. Effects of the number of sialic acid residues on PKC activity at the ganglioside concentration of 10 μ*M*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (for asialo-gangliosides).

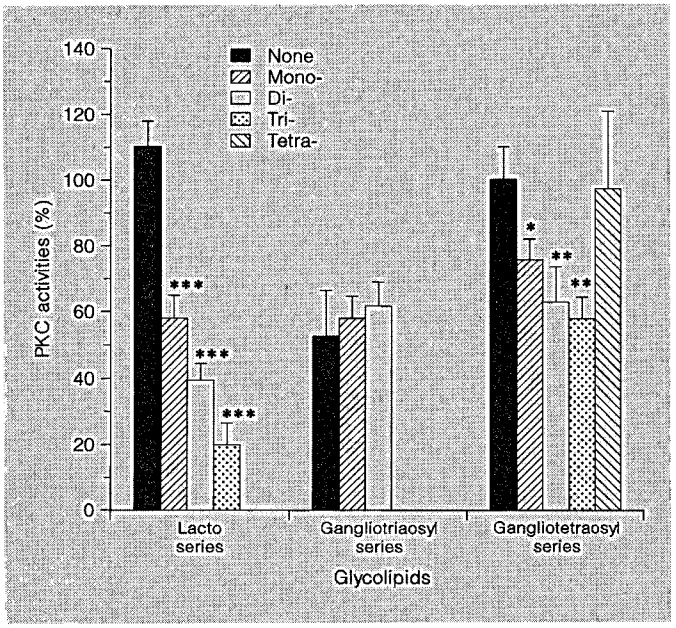

Fig. 2. Effects of the number of sialic acid residues on PKC activity at the ganglioside concentration of 25 μ*M*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (for asialo-gangliosides).

Figures 1 and 2 show the effect of the number of sialic acid residues on PKC activities at the concentrations of 10 and 25 μ*M*, respectively. There appears to be a direct correlation between the number of sialic acid residues and inhibitory potency. This correlation is particularly apparent at the concentration of 25 μ*M* and the rank order for inhibitory potency was trisialogangliosides > disialogangliosides > monosialogangliosides for both the gangliotetraosyl- and lacto-series gangliosides. In the case of gangliotriaose-series gangliosides, however, the number of sialic acid residue(s) appeared to render the ganglioside stimulatory at the concentration of 10 μ*M*.

FGM1 and GM1 containing blood group B determinant (BGM1), which are major gangliosides in PC12 cells [3], acted as potent inhibitors of PKC activity (table 2). LM1 seemed to be a mild activator of PKC activity within the range of 1–10 μ*M*, but suppressed PKC activity above 25 μ*M* (table 2).

Table 3 shows the effects of fatty acids in cerebrosides on PKC activity. GalCer containing normal fatty acids [GalCer (N)] was a potent activator of PKC. At the concentration of 25 μ*M*, the activity increased to approximately 155% of the control value. However, GalCer containing α-hydroxy fatty acids [GalCer (H)] and psychosine appeared to be mildly inhibitory. Sulfatides behaved as potent inhibitors of kinase and differences in fatty acid residues in sulfatides apparently had no effect on PKC activity (table 2). Ceramide and GlcCer were ineffective in modulating PKC activity (table 3).

Among the other neutral glycosphingolipids tested, LacCer, Gb3, GalGb3, and GA1, which have a terminal galactose residue, were ineffective as PKC inhibitors or activators. On the other hand, GA2, Gb4, and Gb5, which have a terminal N-acetylgalactosamine residue, were potent inhibitors of PKC activity (table 3). Gb5 appeared to be more inhibitory than Gb4, suggesting a direct correlation between the number of N-acetylgalactosamine residues and inhibitory potency.

Discussion

Gangliosides are known to play a crucial role in many cellular processes perhaps by serving as membrane transducers of both positive and negative signals that regulate cell growth and differentiation. Recently several lines of evidence have emerged suggesting that gangliosides may mediate many dynamic cellular processes by modulating protein kinase systems [for review see ref. 49]. Yu et al. [51] first demonstrated that gangliosides added to a rat brain membrane preparation had profound effects, both stimulatory and inhibitory, in modulating several protein

kinase systems. Thus, Ca^{2+}-gangliosides may stimulate the calmodulin-dependent kinase II system, while inhibiting the calcium-phospholipid-dependent kinase (PKC) [49]. Such a modulatory role for gangliosides has been shown to operate in a number of systems involving growth factor receptors [17]. Little is known, however, about the mechanism(s) by which the ganglioside-mediated protein phosphorylation exerts its biological and physiological effects. We recently reported that several ganglio-series gangliosides are potent inhibitors of rat brain PKC activity in vitro in the range of 10–200 μM and that the rank order for the inhibitory potency is GT1b > GD1b = GD1a > GM1 = GM3 [30]. In the present study, we have extended our previous work to include lacto-series gangliosides and confirmed our previous finding that most gangliosides suppressed the activity of PKC at high concentrations and that the rank order for inhibitory potency was trisialogangliosides > disialogangliosides > monosialogangliosides (fig. 1, 2). Interestingly, fucosylated gangliosides, FGM1 and BGM1, were found to be potent inhibitors of PKC activity within the range 0.1–50 μM (table 2). We have reported that fucosylated gangliosides, such as FGM1, BGM1, FGD1b, and BGD1b, are major components in the acidic lipid fractions of PC12 cells, but other gangliotetraose-series gangliosides are minor components [3, 4]. It is apparent that glycosphingolipids, even in minute amounts, may have profound effects on protein kinase systems. Thus, de-N-acetyl GM3, GQ1b, and polysialogangliosides, which are present at negligible or undetectable levels in human carcinoma A431 cells [20], human neuroblastoma cells [45], and rat brain [10, 11], have been reported as stimulators of certain kinase systems. In the present context, the endogenous concentrations of glycosphingolipids in PC12 cells are low compared with the exogenously added glycosphingolipids. However, it is not certain how much of the added glycosphingolipids was actually incorporated into the plasma membranes of living cells.

GM1 and LM1 gangliosides seemed to be mild activators of PKC activity within the range of 2.5–10 μM; at higher concentrations, they acted as inhibitors (table 2). Such a concentration dependency may reflect the physical state of gangliosides in solution as demonstrated by Maggio et al. [32]. GM1 has been shown to be a stimulator for a Ca^{2+}-dependent protein kinase associated with rat brain membranes [10, 12]. Recently, Hilbush and Levine [24] reported that micromolar concentrations of exogenous GM1, together with NGF, stimulated a Ca^{2+}-dependent protein kinase activity in PC12 cells. Additionally, endogenous GM1 ganglioside can mediate proliferation of lymphocytes [41].

Tsuji et al. [45] reported that GQ1b at nanomolar concentrations stimulated cellular proliferation and neurite outgrowth, perhaps by stimulating a novel ecto-protein kinase system in certain neuroblastoma cell lines. Chan [11] reported that polysialogangliosides appeared to be mild stimulators for certain kinases. In the present study, we demonstrated that GQ1b at concentrations of 0.1–10 μM was ineffective in modulating PKC activity, but this ganglioside was a mild activator at concentrations of 25 μM (table 2). On the other hand, GQ1c, which is an isomer of GQ1b, was found to be a mild inhibitor of PKC activity. PKC activity remained at approximately 80% of the control level even at a concentration as high as 50 μM for these two highly sialylated gangliosides. Since sialic acid itself is ineffective in modulating PKC activity, these polysialogangliosides may play an important modulatory role in PKC activity. It is therefore possible that GM1 and GQ1b, which are only minor gangliosides in PC12 cells [4], act as stimulators for PKC activity within a limited range of concentrations.

There are numerous instances demonstrating that sphingosine plays a crucial role in signal transduction and cellular proliferation in vitro and in vivo [21, 22, 25, 34, 52]. Merrill et al. [33] reported the effects of sphingosine with different stereospecific structures and fatty acids on PKC activity and found that most sphingosines were inhibitors. Variable effects of synthetic sphingosines with different stereospecific structures on PKC activity have also been reported [25]. Okazaki et al. [36] reported that ceramide itself may serve as a lipid mediator of second-messenger transduction for HL-60 cell differentiation.

Concerning the effect of neutral glycosphingolipids on PKC activity, studies from various laboratories are inconclusive; this may reflect differences in the glycosphingolipids used, variations in lipid concentrations, and the kinase systems studied. Several neutral glycosphingolipids, such as paragloboside, and asialo-gangliosides were found to be ineffective on certain protein kinase systems [29, 35]. Recently, Shayman et al. [40] reported that endogenous GlcCer enhanced hormone-stimulated inositol triphosphate formation in MDCK cells, perhaps by modulating the phospholipase C activity. In the present investigation, GalCer having normal fatty acids was found to be a stimulator of PKC activity, but GalCer with hydroxy fatty acid was not. GalCer with normal fatty acids contained large proportions of fatty acids C24:1 and C24:0 [2]. Furthermore, we found that addition to the culture medium of exogenous GalCer having normal fatty acids enhanced neurite outgrowth and exhibited an activatory effect on PKC phosphorylation [5]. This may influence

the receptor activity of the carbohydrate head groups of glycosphingolipids, as exposure of the carbohydrate head group of GalCer has been shown to increase with increasing fatty acid chain length and decrease by hydroxylation of the fatty acid chain [42]. GlcCer and ceramide were ineffective, but psychosine is found to be a mild inhibitor of PKC activity. LacCer, Gb3, GA1, and GalGb3, which have a terminal galactose residue, were completely ineffective as PKC inhibitors, but GA2, Gb4, and Gb5, which have terminal N-acetylgalactosamine residue(s), exhibited an inhibitory effect on kinase-catalyzed phosphorylation. It is remarkable that the terminal sugar residue can have such a profound effect on PKC activity. On the other hand, sulfatides behaved as suppressors of the PKC activity and there were no significant differences with respect to the N-acyl moieties, suggesting that the sulfate residue may be responsible for suppressing the PKC activity. The overall data indicate that the terminal N-acetylgalactosamine residue as well as negatively charged functional groups, such as the sialic acid or sulfate residue, may be structurally important for suppressing PKC activity. Additionally, the terminal N-acetylgalactosamine and sialic acid residues may act as inhibitory structural elements. There have been several reports indicating that modification or substitution of the functional groups of glycosphingolipids as well as their fatty acid chains may modify their effects on protein kinase systems. For example, de-N-acetyl GM3, which is a minor ganglioside in human epidermoid carcinoma A431 cells and mouse B16 melanoma cells, stimulated tyrosine phosphokinase activity associated with the epidermal growth factor or insulin receptor and enhanced proliferation of various cell lines in culture; this is in striking contrast to GM3 and lyso-GM3 which exhibited an inhibitory effect on the receptor-associated tyrosine kinase and on cell growth [20, 35]. Kim et al. [29] reported that asialo-ganglioside GA1 is completely ineffective and that at least one sialic acid residue is required for the modulation of PKC activity. GA2, however, is a potent inhibitor of the PKC activity (fig. 1, 2). Neolacto-series gangliosides having a NeuAcα2-3Gal terminus showed a strong inhibitory effect on insulin receptor phosphorylation, but branched neolacto-series gangliosides with a NeuAcα2-3Gal terminus or neolacto-series gangliosides with a NeuAcα2-6Gal terminus were reported to be ineffective [35]. Additionally, lysogangliosides and psychosine strongly inhibited the PKC activity [21, 22, 25].

In conclusion, we have demonstrated several structural features in glycosphingolipids that may play a dominant role in regulating PKC activity. An understanding of the structural requirement for the modulatory effect of glycosphingolipids should enhance our understanding of their mechanisms of action on the signal transduction systems and their biological functions.

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References

1 Ando S, Yu RK. Isolation and characterization of a novel trisialoganglioside, GT1a, from human brain. J Biol Chem 252:6247–6250;1977.
2 Ariga T, Murata T, Oshima M, Maezawa M, Miyatake T. Characterization of glycosphingolipids by direct inlet chemical ionization mass spectrometry. J Lipid Res 21:879–887;1980.
3 Ariga T, Kobayashi K, Kuroda Y, Yu RK, Suzuki M, Kitagawa H, Inagaki F, Miyatake T. Characterization of tumor-associated fucogangliosides from PC12 pheochromocytoma cells. J Biol Chem 262:14146–14153;1987.
4 Ariga T, Macala LJ, Saito M, Margolis RK, Greene LA, Margolis RU, Yu RK. Lipid composition of PC12 pheochromocytoma cells: Characterization of globoside as the major neutral glycolipids. Biochemistry 27;52–58;1988.
5 Ariga T, Yoshino H, Ren SL, Pal S, Katoh-Semba R, Yu RK. Activation of UDP-galactose:globotriasylceramide a 1-3 galactosyltransferase during PC12D cell differentiation induced by galactosylceramide. Biochemistry 32: 7904–7908;1993.
6 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254;1976.
7 Bremer EG, Hakomori SI. GM3 ganglioside induces hamster fibroblast growth inhibition in chemically defined medium: Gangliosides may regulate growth factor receptor function. Biochem Biophys Res Commun 106:711–718; 1982.
8 Cannella MS, Archer AJ, Ledeen RW. Stimulation of neurite outgrowth in vitro by a glycero-ganglioside. Int J Dev Neurosci 6:319–326; 1988.
9 Carter HE, Rothfus JA, Gigg RJ. Biochemistry of the sphingolipids. XIII: Determination of the structure of cerebrosides from wheat flour. J Lipid Res 2:228–234;1961.
10 Chan KFJ. Ganglioside-modulated protein phosphorylation: Partial purification and characterization of a ganglioside stimulated protein kinase in brain. J Biol Chem 262:5248–5255; 1987.
11 Chan KFJ. Ganglioside-modulated protein phosphorylation. J Biol Chem 263:568–574; 1988.

12 Cimino M, Benfenati F, Farabegoli C, Cattabeni F, Fuxe K, Agnati LF, Toffano G. Differential effect of ganglioside GM1 on rat brain phosphoproteins: Potentiation and inhibition of protein phosphorylation regulated by calcium/calmodulin- and calcium/phospholipid-dependent protein kinases. Acta Physiol Scand 130:317–535;1987.
13 Cremins J, Wagner JA, Halegoua S. Nerve growth factor is mediated by cyclic AMP- and Ca2+/phospholipid dependent kinases. J Cell Biol 103:887–893;1986.
14 Goldenring JR, Otis LC, Yu RK, DeLorenzo RJ. Calcium/ganglioside-dependent protein kinase activity in rat brain membrane. J Neurochem 44:1229–1234;1985.
15 Greene LA, Tischler AS. PC12 pheochromocytoma cultures in neurobiological research. In: Fedoroff S, Hertz L, eds. Cellular Neurobiology. New York, Academic Press, 373–414; 1982.
16 Hakomori SI. Glycosphingolipids as differentiation-dependent, tumor-associated markers and as regulators of cell proliferation. Trends Biochem Sci 9:453–456;1984.
17 Hakomori SI. Bifunctional role of glycosphingolipids: Modulators for transmembrane signalling and mediators for cellular function. J Biol Chem 265:18713–18716;1990.
18 Hall FL, Fernyhough P, Ishi DN, Vulliet PR. Suppression of nerve growth factor-directed neurite outgrowth in PC12 cells by sphingosine, an inhibitor of protein kinase C. J Biol Chem 263:4460–4466;1988.
19 Hama T, Huang K, Guroff G. Protein kinase C as a component of a nerve growth factor-sensitive phosphorylation system in PC12 cells. Proc Natl Acad Sci USA 83:2353–2357;1986.
20 Hanai N, Dohi T, Nores GA, Hakomori SI. A novel ganglioside, de-N-acetyl-GM3 (II^2NeuNH_2LacCer), acting as a strong promoter for epidermal growth factor receptor kinase and as a stimulator for cell growth. J Biol Chem 263: 6296–6301;1988.
21 Hannun YA, Greenberg CS, Bell RM. Sphingosine inhibition of agonist-dependent secretion and activation of human platelets implies that protein kinase C is a necessary and common event of the signal transduction pathways. J Biol Chem 262:13620–13626;1987.
22 Hannun YA, Bell RM. Functions of sphingolipids and sphingolipid breakdown products in generating lipid second messengers in cellular regulation. Science 243:500–506;1989.
23 Higgins TJ. Simplified fluorometric assay for sphingosine bases. J Lipid Res 25:1007–1009; 1984.
24 Hilbush B, Levine JM. Stimulation of a Ca^{2+}-dependent protein kinase by GM1 ganglioside in nerve growth factor-treated PC12 cells. Proc Natl Acad Sci USA 88:5616–5620;1991.
25 Igarashi Y, Hakomori SI, Toyokuni T, Dean B, Fujita S, Sugimoto M, Ogawa T, El-Ghendy K, Racker E. Effect of chemically well-defined sphingosine and its N-methyl derivatives on protein kinase C and src kinase activity. Biochemistry 28:6796–6800;1989.
26 IUPAC-IUB Commission on Biochemical Nomenclature. The nomenclature of lipids. Lipids 12:455–468;1976.
27 Kasai N, Sillerud LO, Yu RK. A convenient method for the preparation of asialo-GM1. Lipids 17:107–110;1982.
28 Katoh-Semba R, Kitajima Y, Yamazaki Y, Sano M. Neuritic growth from a subline of PC12 pheochromocytoma cells: Cyclic AMP mimics the action of nerve growth factor. J Neurosci Res 17:36–44;1987.
29 Kim JYH, Goldenring JR, DeLorenzo RJ, Yu RK. Gangliosides inhibit phospholipid-sensitive Ca^{2+}-dependent kinase phosphorylation of rat myelin basic proteins. J Neurosci Res 15: 159–166;1986.
30 Kreutter D, Kim JYH, Goldenring JR, Rasmussen U, Ukomadu C, DeLorenzo RJ, Yu RK. Regulation of protein kinase C activity by gangliosides. J Biol Chem 262:1633–1637; 1987.
31 Ledeen RW. Biosynthesis, metabolism, and biological effects of gangliosides. In: Margolis RU, Margolis RK, eds. Neurobiology of Glycoconjugates. New York, Plenum, 43–83;1989.
32 Maggio B, Fidelio GD, Cumar FA, Yu RK. Molecular interactions and thermotrophic behavior of glycosphingolipids in model membrane systems. Chem Phys Lipids 42:27–48; 1986.
33 Merrill AH, Nimkar S, Menaldino D, Hannun YA, Loomis C, Bell RM, Tygi SR, Lambeth JD, Stevens VL, Hunter R, Liotta DC. Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C in vitro and for the cellular effects of these compounds. Biochemistry 23:3138–3148;1989.
34 Merrill AH, Stevens VL. Modulation of protein kinase C and diverse cell functions by sphingosine – A pharmacologically interesting compound linking sphingolipids and signal transduction. Biochim Biophys Acta 1010:131–139; 1989.
35 Nojiri H, Stroud M, Hakomori SI. A specific type of ganglioside as a modulator of insulin-dependent cell growth and insulin receptor tyrosine kinase activity: Possible association of ganglioside-induced inhibition of insulin receptor function and monocytic differentiation induction in HL-60 cells. J Biol Chem 266: 4531–4537;1991.
36 Okazaki T, Bielawska A, Bell RM, Hannun YA. Role of ceramides as a lipid mediator of 1,25-dihydroxy vitamin D3-induced HL-60 cell differentiation. J Biol Chem 265:15823–15831;1990.
37 Ren S, Scarsdale JN, Ariga T, Zhang Y, Klein RA, Hartmann R, Kushi Y, Egge H, Yu RK. O-Acetylated gangliosides in bovine buttermilk: Characterization of 7-O-acetyl, 9-O-acetyl, and 7,9-O-diacetyl GD3. J Biol Chem 267: 12632–12638;1992.
38 Roisen FJ, Bartfeld H, Nagele R, Yorke G. Ganglioside stimulation of axonal sprouting in vivo. Science 214:577–578;1981.
39 Rybak S, Ginzburg I, Yavin E. Gangliosides stimulate neurite outgrowth and induce tubulin mRNA accumulation in neural cells. Biochem Biophys Res Commun 116:974–980;1983.
40 Shayman JA, Mahdiyoun S, Deshmukh G, Barcelon F, Inokuchi JI, Radin NS. Glucosphingolipid dependence of hormone-stimulated inositol triphosphate formation. J Biol Chem 265:12135–12138;1990.
41 Spiegel S, Fishman PH, Weber RJ. Direct evidence that endogenous GM1 ganglioside can modulate thymocytic proliferation. Science 230:1285–1287;1985.
42 Stewart R, Boggs JM. Adhesion mediated by the carbohydrate head groups of myelin glycolipids. Trans Am Soc Neurochem 24:207; 1993.
43 Svennerholm L. Quantitative estimation of sialic acid. Biochim Biophys Acta 24:604–611; 1957.
44 Taketomi T, Yamakawa T. Immunochemical studies of lipids. I: Preparation and immunological properties of synthetic psychosine-protein antigens. J Biochem (Tokyo) 54:444–451; 1963.
45 Tsuji S, Nakajima J, Sasaki T, Nagai Y. Bioactive gangliosides. IV. Gangliosides GQ1b/Ca dependent protein kinase activity exists in the plasma membrane fraction of neuroblastoma cell line, GOTO. J Biochem (Tokyo) 97:967–972;1985.
46 Vance DE, Sweeley CC. Quantitative determination of the neutral glycosyl ceramides in human blood. J Lipid Res 8:621–627;1967.
47 Xia XJ, Gu XB, Sartorelli AC, Yu RK. Effects of inducers of differentiation on protein kinase C and CMP-N-acetylneuraminic acid: Lactosylceramide sialyltransferase activities on HL-60 leukemia cells. J Lipid Res 30:181–188; 1989.
48 Yates AJ, Wood CJ, Halterman RK, Stock SM, Walter JD, Johnson JD. Effects of gangliosides, calmodulin, protein kinase C and copper on phosphorylation of proteins in membranes of normal and transected sciatic nerve. In: Ledeen R, Hogan E, Tettamanti G, Yu RK, eds. New Trends in Ganglioside Research, Padua, Liviana, 495–511;1988.
49 Yu RK. Effects of gangliosides on the growth and differentiation of HL-60 human leukemia cells. In: Horrocks L, Neff NH, Yates AJ, Hadjiconstantinou M, eds. Trophic Factors and the Nervous System. New York, Raven, 175–193; 1990.
50 Yu RK. Developmental regulation of ganglioside metabolism. Prog Brain Res 101:33–44; 1994.
51 Yu RK, Goldenring JR, DeLorenzo RJ. Effects of gangliosides on rat brain membrane protein phosphorylation. ISERM 126:335–354;1984.
52 Zhang H, Desai ND, Murphy JM, Spiegel S. Increases in phosphatidic acid levels accompany sphingosine-stimulated proliferation of quiescent Swiss 3T3 cells. J Biol Chem 265: 21309–21316;1990.