Activation of UDP-Galactose:Globotriaosylceramide α1–3-Galactosyltransferase during PC12D Cell Differentiation Induced by Galactosylceramide†

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ABSTRACT: We measured the activities of UDP-galactose:globotriaosylceramide α1–3-galactosyltransferase (α-GalTase) and protein kinase C (PKC) in PC12D pheochromocytoma (PC12D) cells which were induced to differentiation by nerve growth factor (NGF), forskolin (FRK), staurosporine (STP), retinoic acid (RA), 2-chloroadenosine (ClAd), and/or galactosylceramide (GalCer). NGF, STP, FRK, and RA were found to be stimulators for the PKC activity, whereas ClAd appeared to be an inhibitor of the enzyme. At the concentration of 25 μM, GalCer having normal fatty acids was found to be a stimulator, whereas GalCer having hydroxy fatty acids was ineffective in modulating the PKC activity. Interestingly, all stimulators of PKC activities, including GalCer having normal fatty acids, appeared to be activators for the α-GalTase activity. On the other hand, GalCer having α-hydroxy fatty acids had no effect and ClAd was found to be a potent inhibitor for the α-GalTase activity. These data suggest that α-GalTase activity during PC12D cell differentiation may be regulated by a PKC-dependent process.

Many extracellular agents regulate cellular differentiation and function through their binding to specific receptors at the cell surface. In the subsequent intracellular signal transduction process, phosphorylation–dephosphorylation of proteins is known to play a major regulatory mechanism (Greengard, 1978; Krebs & Beavo, 1979; Cohen, 1985; Edelman et al., 1987). A number of phosphorylation systems are altered, some increased and some decreased, by treatment of cells with differentiating agents, e.g., nerve growth factor (NGF),[1] forskolin (FRK), staurosporine (STP), and K252a. The activities of the cAMP-dependent protein kinase and protein kinase C (PKC) have also been reported to increase when cells are treated with NGF (Cremins et al., 1986; Schubert et al., 1978; Heasley & Johnson, 1989). Extrapolation from these studies permits the generalization that the biochemical mechanism by which NGF acts on the cell is through a sequence of phosphorylation reactions starting at the membrane. A number of reports indicate that inducers of differentiation such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), dimethyl sulfoxide (DMSO), butyrate, and retinoic acid (RA) have profound effects on glycosyltransferase activities (Xia et al., 1989; Momoi et al., 1986; Moskal et al., 1987; Burczak et al., 1984). We have reported that these differentiating agents are capable of inducing changes in the glycolipid pattern in human squamous SQCC/Y1 cells (Tatsumura et al., 1988) and HL-60 cells (Xia et al., 1989). Since these agents modulate the phosphorylation state of cells, there is a possibility that glycosyltransferase activities may be under the control of protein kinase systems. Thus, GM3, GM2, and GM1 synthases have been suggested to be regulated by this mechanism (Moskal et al., 1987; Burczak et al., 1984; Scheideler et al., 1984; Scheideler & Dawson, 1986). Xia et al. (1989) also reported a correlation between the activities of the PKC and GM3 synthase. However, direct evidence that the activities of glycosyltransferases are under phosphorylation–dephosphorylation control is still lacking.

The PC12 pheochromocytoma cells are derived from a clonal cell line from rat adrenal medullary pheochromocytomas and share properties of normal adrenal chromaffin cells originating from neural crest cells, such as the capacity to store and secrete catecholamines in response to cholinergic agonists or increasing extracellular K^+ ion concentrations (Greene & Tischler, 1976, 1982). In addition, this cell line displays such characteristics as neurite outgrowth, increased cellular adhesion, and increased levels of choline acetyltransferase, acetylcholinesterases, norepinephrine uptake sites, etc., in response to NGF (Greene & Tishler, 1976; Greene & Rein, 1977). Therefore, PC12 cells have been subjected to intensive studies as a model for investigating neuronal function and differentiation (Greene & Rein, 1977). Recently, Katoh-Semba et al. (1987) reported a new subline of PC12 pheochromocytoma cells (PC12D cells) which responded much more quickly to NGF than did PC12 cells. In PC12D cells, the neurites extended quickly within 24 h in response to not only NGF but also cAMP analogues or cAMP-enhancing reagents such as dibutyryl-cAMP. With respect to these characteristics, PC12D cells seem to closely resemble "NGF-primed" PC12 cells; i.e., PC12D cells are a little more differentiated than conventional PC12 cells (Katoh-Semba et al., 1987; Sano et al., 1988, 1990). This suggests that PC12D cells respond to differentiation agents such as NGF much faster than the original PC12 cells.

In this study, we present evidence that GalCer having normal fatty acids is a potent activator for the UDP-galactose: globotriaosylceramide α1–3-galactosyltransferase (α-Gal-

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[1] Abbreviations: PKC, protein kinase C; α-GalTase, UDP-galactose: globotriaosylceramide α1–3-galactosyltransferase; HPTLC, high-performance thin-layer chromatography; NGF, nerve growth factor; FRK, forskolin; STP, staurosporine, RA, retinoic acid; ClAd, 2-chloroadenosine; PBS, phosphate-buffered saline; PC12D cells, PC12D pheochromocytoma cells. The nomenclature used for gangliosides and neutral glycosphingolipids is based on that recommended by IUPAC (1976).

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Tase) and that a correlation exists between the activities of this enzyme during PC12D cell differentiation and PKC.

MATERIALS AND METHOD

Galactosylceramide (GalCer) was isolated from human brain, and GalCer with hydroxy and nonhydroxy fatty acids was purified by preparative HPTLC on silica gel (E. Merck, Darmstad, FRG) and Iatrobeads column chromatography as described previously (Ariga et al., 1980). Globotriaosylceramide (Gb3) was isolated from porcine erythrocyte membrane as described previously (Ariga et al., 1980). Galα1–3Gb3 was purified from pheochromocytomas that were produced by transplanting the PC12h cells into New England Deaconess Hospital rats (Ariga et al., 1987, 1988). Forskolin (FRK), staurosporine (STP), retinoic acid (RA), and 2-chloroadenosine (ClAd) were purchased from Sigma Co. (St. Louis, MO). Nerve growth factor (NGF; 2.5S) was obtained from Biomedical Technologies, Inc. (Stoughton, MA). 1,2-Diolein, phosphatidylserine, and histone III-S were obtained from Sigma. All other reagents were of the highest analytical grade and were also obtained from Sigma. [γ-^{32}P]ATP and UDP-[^{14}C]galactose were obtained from New England Nuclear Co. (Boston, MA).

Cell Culture. PC12D pheochromocytoma cells were grown in Dulbecco's modified Eagle medium (DME) supplemented with 10% heat-inactivated horse serum (Bioproducts Inc., Maryland) and 5% fetal bovine serum (Bioproducts) in the presence of insulin (5 mg/L) (Katoh-Semba et al., 1987). For the PKC and α-GalTase assays, the serum concentration was reduced to 3%. The cells were placed in 25 cm^2 plastic culture flasks and grown in 2 mL of the medium with or without differentiating agents, 100 ng of NGF, 50 nM STP, 10 μM FRK, 100 nM RA, or 50 μM ClAd alone or in combination with 25 μM GalCer. They were kept in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C for 2, 4, 8, and 24 h. The cell pellets were washed three times in phosphate-buffered saline (PBS) and used for assaying the PKC and α-GalTase activities. The protein concentration was determined by the method of Bradford (1976). For morphological studies, the cells were viewed with a Nikon diaphot TMS-F inverted phase-contrast microscope.

Assay of Protein Kinase C (PKC). PKC was partially purified as described by Kreutter et al. (1987) and Xia et al. (1989). Briefly, the cells (approximately 1 × 10^6 cells) were suspended in 0.5 mL of a lysis buffer (2 mM Tris–HCl, 0.1 mM EGTA, 10% sucrose, 50 mM 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 1 mL of DEAE–cellulose equilibrated in lysis buffer was added. After incubation for 15 min, the mixture was centrifuged for 5 min at 1000 rpm and washed twice with 3 mL each of the lysis buffer and the enzyme eluted with 1 mL of 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 mM magnesium acetate, 1 mM EGTA, 1.1 μM $CaCl_2$, 0.01 mM ATP, 20 μg/mL phosphatidylserine, 2 μg/mL 1,2-diolein, 200 μg/mL histone III-S, and 15–40 μg/mL enzyme. 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 (Milipore HA, 0.45 μm) and the filters were washed three times with 4 mL of 5% ice-cold TCA. The filters were placed in scintillation vials with 10 mL of scintillation fluid, and the ^{32}P incorporation into histone III-S was determined using a scintillation counter.

Assay of UDP-Galactose:Globotriaosylceramide (Gb3) α1–3-Galactosyltransferase (α-GalTase). The α-GalTase activity was determined using Gb3 as the acceptor for the galactose of UDP-[^{14}C]galactose, and the corresponding ^{14}C-labeled glycolipid product (Galα1–3Gb3) was identified using an authentic sample (Pal et al., 1992). Briefly, the reaction mixture consisted of 25 μg of Gb3, 50 mM UDP-galactose containing 0.1 μCi of UDP-[^{14}C]galactose, 0.1% Triton CF-54, 5 mM $MnCl_2$, 50 mM HEPES buffer (pH 7.0), and an enzyme preparation (80 μg of protein) in a total volume of 100 μL. CDP-choline and galactonolactone (5 mM each) were also incubated in the reaction mixture to inhibit activities of endogenous nucleotide pyrophoshatase and α-galactosidase, respectively. Blanks were prepared without the enzyme preparation. After incubation of the mixture at 37 °C for 1 h, total lipids were extracted from the mixture with chloroform–methanol (2:1, v/v), followed by base treatment to hydrolyze the contaminating phospholipids in the sample. The lipids were subjected to Folch's partitioning by adding 2.4 mL of chloroform–methanol (2:1, v/v) and 0.5 mL of 0.9% NaCl (Folch et al., 1957). After removing the upper phase, the lower phase was rinsed with theoretical upper phase (chloroform–methanol–water, 3:48:47, v/v). The glycolipids recovered in the lower phase were mixed with authentic Galα1–3Gb3 (0.5–1.0 μg) and were separated by high-performance thin-layer chromatography (HPTLC) with a solvent system of chloroform–methanol–2.5 N NH_4OH (55:50:10, v/v). The lipids were visualized by brief heating of the plate after spraying with the orcinol–sulfuric acid reagent. The silica gel corresponding to the Galα1–3Gb3 band was scraped off the plate and placed in a scintillation vial with 10 mL of scintillation fluid. The radioactivity was determined using a scintillation counter. In a separate experiment, the plate was exposed to an X-ray film. The bands corresponding to the ^{14}C-labeled glycolipid products were determined by a densitometric chromatoscanner at 430 nm.

RESULTS

Figure 1 shows the morphological changes of PC12D cells induced by differentiating agents such as NGF, FRK, STP, RA, ClAd, and/or GalCer having normal fatty acids. A new subline of PC12 cells (PC12D cells) were hexagonal and showed short neurites even in the absence of NGF; they seemed to closely resemble NGF-primed PC12 cells (Katoh-Semba et. al., 1987) (Figure 1A). PC12D cells responded to NGF much faster than the original PC12 cells (Katoh-Semba, 1987) and showed neurite outgrowth within 24 h (Figure 1B). Morphological changes in PC12D cells induced by FRK were similar to those of NGF (Figure 1C). There were no obvious changes in morphology in cells treated by RA (Figure 1E). The cell size seemed to be enlarged by the addition of STP or GalCer (Figures 1D,G). Interestingly, the shapes of PC12D cells induced by ClAd were changed to round; they seemed to closely resemble the original PC12 cells (Greene & Tischler, 1976) (Figure 1F). There were no obvious changes in morphology in cells treated by a combination of various differentiating agents and GalCer in which cell behavior might be reflected mainly by the agents inducing (data not shown).

Figure 2 shows the time course for induction of the PKC activity using the various differentiating agents or GalCer containing normal fatty acids. The PKC activities of PC12D cells treated with NGF, FRK, or STP increased within 2 h, and the activity showed a pronounced increase until 24 h.

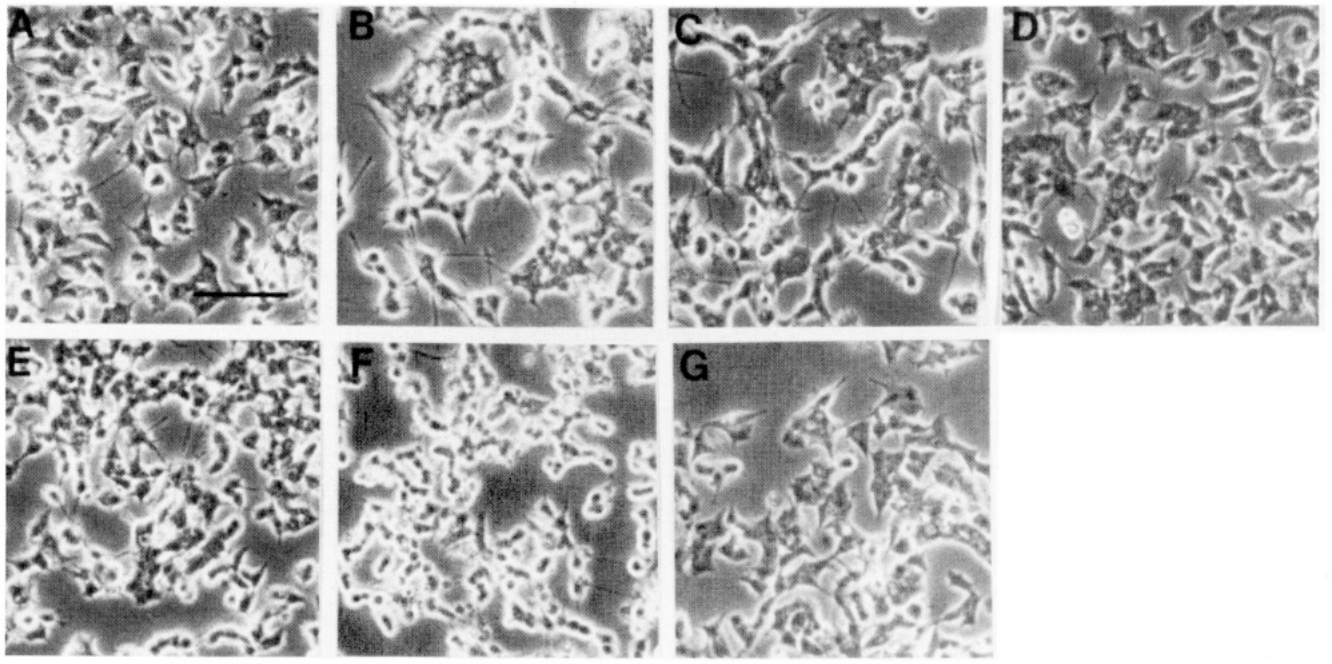

FIGURE 1: Phase-contrast microscopic photographs of PC12D cells. PC12D cells were planted in DME medium containing 3% fetal bovine serum (A), with 100 ng of NGF (B), 10 μM FRK (C), 50 nM STP (D), 100 nM RA (E), 50 μM ClAd (F), or 25 μM GalCer containing normal fatty acids (G) incubated for 24 h under 95% air and 5% CO_2 gas at 37 °C (bar = 50 μm).

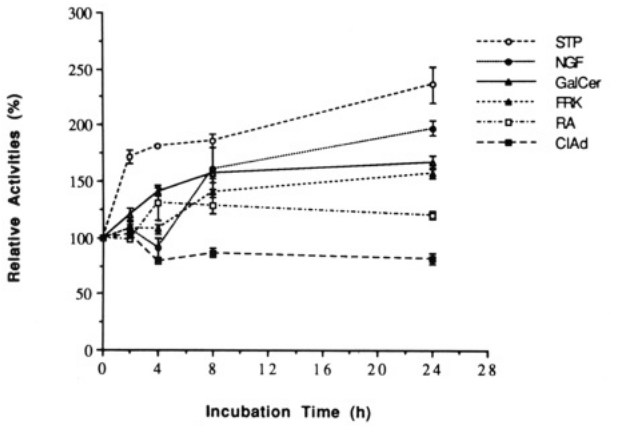

FIGURE 2: Time course for induction of the PKC activity at 2, 4, 8, and 24 h using NGF, FRK, STP, RA, ClAd, or GalCer having normal fatty acids. The values were expressed as the mean percentages of normal control (untreated cells) ± standard deviation (N = 4 for 2, 4, and 8 h and N = 6 for 24 h).

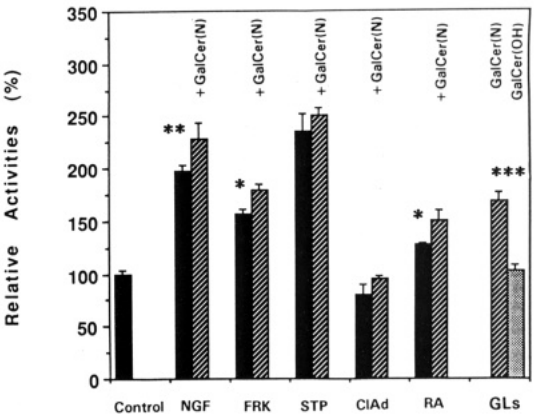

FIGURE 3: Induction of the PKC activity at 24 h using the various differentiating agents and/or GalCer: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The values were expressed as the mean percentages of normal control (untreated cells) ± standard deviation (N = 6).

Interestingly, STP was found to be an activator for PKC activity, and the activity increased by 1.7-fold over that of the untreated cells within 2 h and to 2.5-fold by 24 h. In the case of RA, the PKC activity appeared to increase slightly at 4 h. The PKC activity for cells treated with ClAd appeared to decrease slightly within 2 h, and by 24 h the activity decreased to 75% of that of the untreated cells. Interestingly, GalCer with normal fatty acid was found to be a potent activator for the PKC activity, and by 24 h the effect increased to 1.6-fold of that of the untreated cells as shown in Figure 3. However, GalCer with hydroxy fatty acids appeared to have no effect on the PKC activity. The combined use with the differentiating agent and GalCer with normal fatty acids appeared to have a synergistic effect; the PKC activity during the time course of induction increased 5–20% over ($P < 0.05$) that of the cells treated with the agents alone (Figure 3).

Figure 4 shows the effects of GalCer containing normal fatty acids on α-GalTase activity at 24-h incubation. NGF, FRK, STP, and RA had no effect or served as mild activators for α-GalTase activity. However, ClAd was found to be a potent inhibitor for α-GalTase activity; the activity decreased to 52% of that of the untreated cells. Interestingly, GalCer containing normal fatty acids was found to be an activator for the α-GalTase activity; the activity increased by 1.8-fold over that of the untreated cells. However, GalCer containing α-hydroxy fatty acids appeared to have no effect on the α-GalTase activity. Thus, GalCer containing normal fatty acids seemed to be a stimulator of α-GalTase even in the presence of differentiating agents.

DISCUSSION

PC12D cells chosen for this study appeared to be homogeneous; all the cells were hexagonal whereas the original cells were round as well as hexagonal (Figure 1A). In this study, we found neurite outgrowth induced by FRK was similar to that induced by NGF (Figures 1B,C). Katoh-Semba et al. (1987) reported that the neurites for PC12D cells in the FRK-treated culture appeared to be similar to those in the NGF-treated culture with respect to general morphological observations and neuritic behavior. They also reported the effects

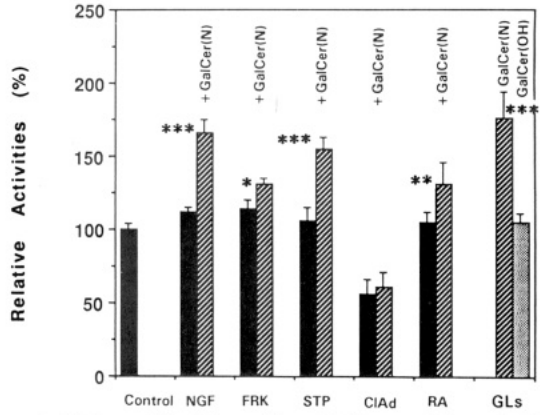

FIGURE 4: Effects of GalCer on the α-GalTase activity at 24 h using the various differentiating agents and/or GalCer: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The values were expressed as the mean percentages of normal control (untreated cells) ± standard deviation ($N = 6$).

of FRK on such biochemical parameters as the activities of tyrosine hydroxylase, ornithine decarboxylase, acetylcholinesterase, and neuron-specific enolase being almost the same as those of NGF (Katoh-Semba et al., 1987). In the case of staurosporine (STP), the cells appeared to be expanded as compared to the untreated cells. Hashimoto and Hagino (1990) reported that STP alone promoted rapid generation of neurites in a dose-dependent manner. They showed STP blocked NGF-induced neurite outgrowth at low doses, whereas at high doses PC12h cells responded to STP by rapid generation of neurites. Tischler et al. (1990) also reported that STP promoted the outgrowth of short processes from PC12 cells, which resemble processes in the early stages of the NGF response. In this study, the neurites in STP-treated cells appeared to be similar to those in the untreated cells. This discrepancy might reflect differences in the culture systems or the subclones. The morphological changes induced by ClAd were quite interesting in that the cells appeared to be round, thus resembling the original PC12 cells (Greene & Tischler, 1976). In the case of RA, the size of the cells appeared to be similar to that of the untreated cells.

The specific transduction mechanisms that mediate many NGF-induced changes including biochemical and morphological changes characteristic of sympathetic neuronal differentiation in PC12 cells are not clearly understood. It has been reported that PKC and cAMP second messenger systems are activated in response to NGF (Chan et al., 1989; Contreras & Guroff, 1987; Cremins et al., 1986; Hama et al., 1986; Heasley & Johnson, 1989). In this study, NGF appeared to be an activator for the PKC activity which was widely accepted in the PC12 cell system. FRK and ClAd were known to be cAMP-stimulating agents (Seamon et al., 1981; Roth et al., 1991). In this study, the PKC activity induced by FRK was found to be similar to that induced by NGF. However, ClAd was also found to be a mild inhibitor for the PKC activity. In comparing the PKC activities in PC12 cells and the two subcloned PC12D and PC12h cells, it was found that the activity of this enzyme was significantly elevated in the subclones, with the highest activity in the PC12D cells (T. Ariga et al., unpublished data). The PC12D cells induced by ClAd had a round morphology, and resembled the parent PC12 cells. It is possible that an increase in the PKC activity may participate in PC12 cell differentiation.

STP, an alkaloid isolated from the culture broth of *Streptomyces staurosporeus*, is structurally similar to K252a and is reported to be a potent inhibitor of protein kinases (Tamaoki et al., 1986). Hashimoto and Hagino (1989) reported that STP promoted the rapid generation of neurites in a dose-dependent manner at concentrations higher than 10 nM, with the maximal effect seen at 100 nM. They also reported the inhibitory effect of STP on tyrosine hydroxylase phosphorylation (Hashimoto & Hagino, 1990). In this study, we used the concentration of 50 nM for the cell culture. Surprisingly, STP was found to be a potent activator for the PKC activity, and by 2 h of initial addition the activity was elevated substantially (Figure 2). Since we used washed cells for our source of PKC, we attribute this finding to a net increase in PKC levels in the treated cells. RA appeared to be a mild stimulator for the PKC activity at 4 h, and by 24 h there were no obvious changes in the activity. Interestingly, GalCer containing normal fatty acid at the concentration of 25 μM was found to be a stimulator of the PKC activity, but GalCer having hydroxy fatty acids was not (Figure 3).

Earlier studies have indicated that there are metabolic changes of glycolipids during NGF treatment of PC12 cells (Margolis et al., 1983, Schwarting et al., 1986; Katoh-Semba et al., 1984). Schwarting et al. (1986, 1990) reported that the overall incorporation of [^{14}C]galactose into both neutral glycolipids and gangliosides increased during PC12 cell differentiation induced by NGF. Proportionately higher incorporation of [^{14}C]galactose was found in complex neutral glycolipids than in gangliosides (Schwarting et al., 1990). We also reported the accumulation of a number of globo series neutral glycolipids containing galactose residues in the α1–3 linkage to Gb3 in the subcloned PC12h cells and hypothesized that the α-GalTase activity might be elevated in PC12h cells as compared to that in the original PC12 cells (Ariga et al., 1988, 1989). This hypothesis received support from our own study that the α-GalTase activity increased in PC12h cells which had more extensive neurite outgrowth than the original cells (Pal et al., 1992), although a causal correlation between the state of differentiation and the α-GalTase activity is still elusive. However, Kojima et al. (1991) reported the alteration in the α-GalTase level of PC12 cells associated with neuronal differentiation. Cummings and Mattox (1988) and Joziasse et al. (1992) also reported that the activity of glycoprotein α-GalTase increased during differentiation in F9 cells. With the exception of ClAd which inhibited the α-GalTase activity, all other differentiating agents had variable effects on this enzyme. PC12D cells have sometimes short processes and are hexagonal in shape. They resemble NGF-primed PC12 cells and are considered to be a little more differentiated than conventional PC12 cells since they respond to cAMP-enhancing agents as well as NGF by extending neurites (Katoh-Semba et al., 1987). Thus, the down regulation of α-GalTase activity in ClAd-treated cells may be closely associated with the undifferentiation process. This is supported by the fact that ClAd-treated cells show round morphology as in the case of conventional PC12 cells (Figure 1F).

Our most interesting finding is that GalCer containing normal fatty acids appeared to be a potent activator for the α-GalTase activity in the presence or absence of various inducers of differentiation (Figure 4). The fact that this glycolipid alone can stimulate the PKC activity (Figure 3) suggests that the increase in α-GalTase activity in GalCer-treated cells is likely mediated directly or indirectly through a PKC-dependent process. Several lines of evidence have indicated that glycosyltransferase activities can be altered by inducers of differentiation (Xia et al., 1989; Yu, 1990; Chen et al., 1989; Roth et al., 1991). For example, treatment of HL-60 cells with GM3 or phorbol esters (TPA) induced differen-

tiation, and these agents caused an increase in sialyltransferase activity for GM3 synthesis (Xia et al., 1989; Yu, 1990). These effects are accompanied by more than a 2-fold increase in PKC activity, suggesting that the activation of sialyltransferase is mediated through a PKC-dependent mechanism (Yu, 1990). Chan et al. (1989) reported that in RA-induced differentiation of TERA-2 derived human embryonal carcinoma cells the activities of α1–4GalTase and β1–3GalTase were found to be reduced 3–4-fold. Begovac and Shur (1990) reported that the glycoprotein β1–4-galactosyltransferase (β-GalTase) activity is mediated by the initiation of neurite outgrowth on laminin in PC12 cell differentiation. Roth et al. (1991) recently reported an increased glycoprotein β-GalTase activity in PC12 cells induced by either FRK or ClAd and that the induction of activity is dependent on protein kinase A. In our own findings, ClAd appeared to be a potent inhibitor for the glycolipid α-GalTase activity. This discrepancy might reflect differences between the GalTases, the kinase systems, and/or the cell lines.

In conclusion, the presence of α-linked galactose residues on glycoconjugates of the cell surface is found to be associated with cellular differentiation. GalCer with normal fatty acids is capable of stimulating the activities of PKC and α-GalTase. Thus, the induction of the α-GalTase activity may participate in PC12 cell differentiation which may be associated with a PKC-dependent phosphorylation process.

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