Plasmalopsychosine of Human Brain Mimics the Effect of Nerve Growth Factor by Activating Its Receptor Kinase and Mitogen-activated Protein Kinase in PC12 Cells

INDUCTION OF NEURITE OUTGROWTH AND PREVENTION OF APOPTOSIS*

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Plasmalopsychosine, a characteristic fatty aldehyde conjugate of β-galactosylsphingosine (psychosine) found in brain white matter, enhances p140Ytrk phosphorylation and mitogen-activated protein kinase (MAPK) activity and as a consequence induces neurite outgrowth in PC12 cells. The effect of plasmalopsychosine on neurite outgrowth and its prolonged activation of MAPK was similar to that of nerve growth factor (NGF), and the effect was specific to neuronal cells. Plasmalopsychosine was not capable of competing with cold chase-stable, high affinity binding of NGF to Trk A, indicating that plasmalopsychosine and NGF differ in terms of Trk A-activating mechanism. Tyrosine kinase inhibitors K-252a and staurosporine, known to inhibit the neurotrophic effect of NGF, also inhibited these effects of plasmalopsychosine, suggesting that plasmalopsychosine and NGF share a common signaling cascade. Plasmalopsychosine prevents apoptosis of PC12 cells caused by serum deprivation, indicating that it has "neurotrophic factor-like" activity. Taken together, these findings indicate that plasmalopsychosine may play an important role in development and maintenance of the vertebrate nervous system.

A novel cationic glycosphingolipid (GSL),1 plasmalopsychosine (PLPS), was previously isolated from white matter of human brain. The conjugates through 3,4- and 4,6-cyclic plasmal linkage were identified and termed PLPS A and B, respectively (1), and these compounds were chemically synthesized (2). PLPS has a unique molecular shape in which two aliphatic hydrophobic tails, Sph and fatty aldehyde, are oriented in opposite directions. This is in striking contrast to all other GSLs, which have two hydrophobic tails (Sph and fatty acid) oriented in the same direction (Fig. 1).

Nerve growth factor (NGF) is required for the survival and development of neurons in the sympathetic and sensory nervous systems (3, 4). NGF induces signal transduction through activation of tyrosine kinase associated with its receptor p140Ytrk (Trk A) (5, 6) and causes a phenotypic change of PC12 cells involving neurite outgrowth, which has been regarded as a criterion of neuronal cell differentiation (7, 8). Various gangliosides and sialic acid derivatives have been shown to promote neuritogenesis of Neuro 2A cells in the presence or absence of NGF (7, 9). Signal pathways from cell surface receptors to nuclear events are of central importance for understanding control of cell differentiation and proliferation. It has become obvious that the signal modulatory activity of GSLs as originally described (10, 11) resides not only in the entire complex structures but also in their backbone structures and metabolites, e.g. lyso-GSLs (12) and de-N-acetyl gangliosides (13), Sph (14), and its derivatives (15–18). Psychosine is known to inhibit kinase C (1, 14) and mitochondrial cytochrome c oxidase (19). Based on this background, we studied the effect of PLPS on neuronal differentiation and associated transmembrane signal changes in PC12 cells.

EXPERIMENTAL PROCEDURES

Materials—4,6- and 3,4-PLPS were prepared from lipid extract of human brain white matter through cation-exchange chromatography (1) or synthesized chemically (2). Because of the scarcity of the natural compounds, synthetic compounds were used for most of the experiments described here.

Mouse NGF and anti-MAPK (erk1-CT) antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p140Ytrk (Trk A) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). K-252a was purchased from Kamiya Biochemical Co. (Thousand Oaks, CA). A stock solution (2 mM) was prepared in dimethyl sulfoxide and stored at −20 °C. All other reagents were purchased from Sigma (St. Louis, MO).

PC12 Cell Culture and Measurement of Neurite Outgrowth—PC12 rat pheochromocytoma cells were maintained at 37 °C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum. For studies of differentiation in the presence of 4,6-PLPS, NGF, and other reagents, cells were plated at 3 × 105/35 mm culture well and allowed to attach before the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 2% horse serum, 1% fetal bovine serum, and experimental reagents. For quantitation of neurite outgrowth, three to four random photographs were made per well, and processes longer than two cell body diameters were counted as neurites. Three separate experiments were performed in triplicate for each data point. Neurites were identified and counted from 100 cells/photograph.

In order to estimate dose dependence of PLPS and the additive effect of NGF and PLPS, PC12 cells were treated with a half-saturating dose of NGF (20 ng/ml) and 0.5–20 μg/ml PLPS, and neurite outgrowth was measured and statistically analyzed.

Immune Complex MAPK Assay—PC12 cells were lysed with lysis
buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM p-nitrophenylphosphate, 25 mM β-glycerophosphate, 0.5 mM EGTA, 1 mM phenylmethylsulfonfyl fluoride, and 1% aprotinin and centrifuged at 10,000 g for 30 min. Supernatants were subjected to immunoprecipitation with anti-MAPK antibody, and immune complexes were recovered with protein A-Sepharose. Immunoprecipitates were suspended in kinase buffer (50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM EGTA, 10% glycerol), with 20 μM ATP, 1 μCi of γ³²PATP, and 0.5 mg/ml myelin basic protein. After 30 min at 30°C, reaction was terminated by adding 10 μl of 5× Laemmli’s sample buffer. Proteins were resolved by electrophoresis on SDS-polyacrylamide gels (12.5%) followed by autoradiography to visualize the phosphorylated myelin basic protein. The radioactivity associated with bands of phosphorylated proteins was determined with a Beckman liquid scintillation spectrometer.

In order to examine MAPK activation by PLPS in nonneuronal cell lines, murine fibroblast BALB/c 3T3 A31 and human fibroblast WI.38 cells were treated with PLPS, and MAPK activity was assayed as described above.

Immunoprecipitation and Immunoblots of p140 Trk (TrkA)—For assay of PLPS-induced tyrosine phosphorylation, cells were preincubated with or without 100 nM K-252a or staurosporine for 10 min and then incubated for 5 min with PLPS or NGF. After incubation, cells were washed with cold PBS and lysed with ice-cold lysis buffer (100 mM sodium phosphate, pH 7.0, 100 mM NaCl, 1% Triton X-100, 5 mM EDTA, 100 μM sodium vanadate, 2 mM phenylmethylsulfonfyl fluoride, 1% aprotinin). After immunoprecipitation with anti-p140 Trk (TrkA-specific) antibody, immunoprecipitates were subjected to 7.5% SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-phosphotyrosine antibody PY20 and rabbit anti-mouse IgG and visualized by ECL kit (Amersham Corp.).

Cold Chase-Stable NGF Binding to PC12 Cells—A cold chase experiment (20) was performed to differentiate slow (stable) from fast (labile) binding. Various concentrations (0.01 nM to 10 μM) of NGF, PLPS, or Gαs were incubated with PC12 cells (1 × 10⁶ cells/ml) in the presence of 100 μM rp1251-NGF for 60 min at 37°C followed by cold chase at 0.5°C for 30 min with an excess of unlabeled NGF. Amount of [³²P]NGF remaining bound to cells was determined in triplicate. After incubation of cells with [³²P]NGF and each agent, three 100-μl aliquots of cell suspension were layered over a 0.15 M sucrose solution and centrifuged. Nonspecific binding was measured by parallel incubation with or without 100 nM K-252a or staurosporine for 10 min and then centrifuged. Supernatants were subjected to immunoprecipitation with anti-p140 Trk (TrkA-specific) antibody, immunoprecipitates were subjected to 7.5% SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-phosphotyrosine antibody PY20 and rabbit anti-mouse IgG and visualized by ECL kit (Amersham Corp.).

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Measurement of Apoptosis by Flow Cytometry—Apoptosis of cells treated with various agents in serum-free medium was measured by flow cytometry as described previously (21). The cell pellet (1 × 10⁶ cells) obtained by 200 × g centrifugation was suspended in 1 ml of fluorochrome solution (50 μg/ml propidium iodine, 0.1% sodium citrate, 0.1% Triton X-100). Samples were plated overnight in the dark at 4°C, and fluorescence of individual nuclei was measured using an EPICS flow cytometer (Coulter Electronics, Hialeah, FL). Data were analyzed using Coulter flow cytometry software.

RESULTS

Induction of Neurite Outgrowth by PLPS—Significant neuritogenesis was induced either by NGF or 4,6-PLPS (Fig. 2, D and E). The neuritogenic effect of 3,4-plasmopsychosine was weaker than that of 4,6-PLPS (Fig. 2F). Neuritogenesis was not observed after treatment with EGF, psychosine (Fig. 2, B and C), Gαs, ganglioside, lysophosphatidic acid, and galactocerebroside (data not shown). Most psychosine-treated cells were swollen and detached (Fig. 2B). Neurite length per cell and percentage of neurite-positive cells after treatment with PLPS or other GSLs in the presence or absence of NGF were statistically analyzed (Table I). Induction of neurite outgrowth by 4,6-PLPS was dose-dependent at concentrations of 1–20 μM (Fig. 3), and there was no difference in neuritogenesis between natural and synthetic 4,6-PLPS (data not shown). Addition of a high (saturating) dose of NGF to PLPS-treated PC12 cells produced a weak additional increase of neurite outgrowth (Table I). Under our experimental conditions, 100 ng/ml of NGF was the saturating dose, and 20–25 ng/ml was the half-saturating dose, in agreement with Lyons et al. (22). The half-saturating dose and saturating dose of 4,6-PLPS were 2.5 and 10 μg/ml, respectively, under our experimental conditions (Fig. 3). Mixing ex-
cells preincubated with 100 nM K-252a or staurosporine (Trk A) was phosphorylated when PC12 cells were incubated with various concentrations of 4,6-PLPS (0.5–20 ng/ml) up to half-saturating dose produced significant increases in percent of cells showing neurite outgrowth, and neurite length per cell (Fig. 3). This indicates that a combination of low concentration of both NGF and 4,6-PLPS showed significant additive effect. The additive effect of both agents at saturating doses was minimal (Table I) as compared with results shown in Fig. 3.

Prompt Induction of Prolonged Activation of MAPK by PLPS—Within 10 min after addition of 4,6-PLPS to PC12 cells, MAPK activity was increased to maximal value and sustained for a prolonged period (Fig. 4 and Table I). A nearly identical MAPK activation curve was observed following NGF stimulation and by costimulation with NGF and 4,6-PLPS. Sustained MAPK activation by 3,4-PLPS was also observed, but it was much smaller in degree than that by 4,6-PLPS. Only a transient activation of MAPK was induced by lysophosphatidic acid or EGF, neither of which elicited neurite outgrowth (Figs. 2 and 4 and Table I). MAPK response studies as above revealed a remarkable similarity of neuronal differentiation and MAPK activation in PC12 cells induced by PLPS and NGF.

Trk A Phosphorylation Response Induced by PLPS—p140

(Trk A) was phosphorylated when PC12 cells were incubated with PLPS or NGF. No such phosphorylation was detectable in cells preincubated with 100 nM K-252a or staurosporine (Fig. 5), both of which are known tyrosine kinase inhibitors as well as neurotrophic effect blockers (23–25).

Effect of Tyrosine Kinase Inhibitors on MAPK Activation and Neurite Outgrowth Induced by PLPS—MAPK activation induced by 4,6-PLPS or NGF was inhibited by K-252a in association with its inhibition of neurite outgrowth (Fig. 6). Similar inhibition of MAPK activation and neurite outgrowth was produced by staurosporine at low concentrations (2.5–5 nM). In contrast, high concentrations (50–200 nM) of staurosporine inhibited MAPK activation by 4,6-PLPS or NGF, but induced neurite outgrowth (of short processes) in the absence of NGF or 4,6-PLPS (data not shown). These results support previous observations that staurosporine by itself induces neurite outgrowth in the absence of NGF at high concentration but inhibits NGF-induced neurite outgrowth at low concentration (24, 25).

Cold Chase-Stable NGF Binding to PC12 Cells—Under these conditions, 0.1 nM NGF was enough to inhibit >60% of cold

| Agents | 32P Incorporation in myelin basic protein | Neurite outgrowth |
|--------|-----------------------------------------|------------------|
|        | cpm (%) | Neurite length per cell | Percent of cells with neurite |
| Control | 350 ± 28.1 | 1.1 ± 0.3 | 0.6 |
| EGF (30 nM) | 2,115.3 ± 351.4 | (604.4 ± 10.0) | 4.8 ± 2.1 | 2.4 |
| NGF (100 ng/ml) | 2,423.2 ± 427.3 | (640.9 ± 122.3) | 42.1 ± 7.9 | 221.2 |
| 4,6-plasmalopsychosine (5 μg/ml) + NGF (100ng/ml) | 2,308.2 ± 770.0 | (659.4 ± 220.0) | 56.7 ± 10.5 | 251.4 |
| 4,6-plasmalopsychosine (5 μg/ml) | 2,315.4 ± 386.1 | (661.4 ± 110.3) | 52.4 ± 11.3 | 244.5 |
| 3,4-plasmalopsychosine (5 μg/ml) | 1,735.3 ± 351.0 | (358.0 ± 91.7) | 20.3 ± 4.7 | 48.8 |
| Psychosine (5 μg/ml) | 283.3 ± 22.9 | (80.6 ± 6.5) | 0 | 0 |
| (20 μg/ml) | 255.3 ± 22.6 | (72.9 ± 6.5) | 0 | 0 |
| GalCer (5 μg/ml) | 374.6 ± 91.5 | (107.0 ± 26.1) | 1.8 ± 0.4 | 0.9 |
| (20 μg/ml) | 516.1 ± 185.8 | (147.4 ± 53.1) | 0.9 ± 0.2 | 0.5 |
| LPA (5 μg/ml) | 1,546.3 ± 697.6 | (441.7 ± 197.9) | 1.9 ± 0.3 | 1.0 |
| (20 μg/ml) | 2,079.2 ± 501.0 | (594.0 ± 143.1) | 2.0 ± 0.5 | 1.0 |
| GM1 (5 μg/ml) | 358.0 ± 73.9 | (102.3 ± 21.1) | 1.7 ± 0.2 | 0.9 |
| (20 μg/ml) | 390.1 ± 156.2 | (111.4 ± 44.6) | 0.8 ± 0.1 | 0.4 |

a p < 0.001 with Student's t test in comparison with nontreated cells.

b p < 0.05 with Student's t test in comparison with nontreated cells.
chasestablebinding, and 1.0 mM NGF completely inhibited stable binding (Fig. 7). These conditions exclude NGF binding to low affinity binding site p75. In contrast, 3,4- and 4,6-PLPS and GM1 did not significantly inhibit cold chase stable binding even at 10 μM (Fig. 7). These results indicate that PLPS does not compete with binding of NGF to Trk A or p75. The results do not absolutely exclude the possibility that PLPS binds to Trk A or p75. Nevertheless, if PLPS binds to Trk A or p75, it may differ from NGF in terms of binding domain within the receptor. The mechanism of PLPS-induced Trk A activation remains to be studied.

Long-Term Survival of PC12 Cells in the Presence of PLPS Resulting from Inhibition of Apoptosis—Under our experimental conditions, 100% of PC12 cells died within 7 days of serum withdrawal because of apoptosis. In the presence of PLPS, apoptosis of PC12 cells was prevented in 60–80% of cells for a 7-day period (Fig. 8A). This preventive effect of PLPS is similar to that observed for NGF. Agarose gel electrophoresis revealed extensive intranucleosomal DNA fragmentation pattern in cells exposed to serum-free medium for 72 h (Fig. 8B, lane 2). DNA fragmentation was not observed in cells in serum-free medium with NGF or PLPS added (Fig. 8B, lanes 3–5). The survival-enhancing effect of 4,6-PLPS (lane 4) was stronger than that of 3,4-PLPS (lane 5), consistent with their relative degree of neurite outgrowth induction.

Measurement of Apoptosis by Flow Cytometry—The fragmented DNA content of apoptotic cells resulted in an unequivocal hypodiploid DNA peak, which was distinguishable from the diploid DNA peak after 72 h of serum deprivation (Fig. 8C, panel a). Treatment of cells with NGF or PLPS in serum-free medium prevented apoptosis induced by serum deprivation.

![Fig. 4. Time course of MAPK activation in PC12 cells by PLPS.](image1)

PC12 cells were treated for the indicated times with NGF plus 4,6-PLPS, NGF, 4,6-PLPS, EGF, or 3,4-PLPS. Panel A, time course of MAPK activation. Data points as in Fig. 3. Panel B, phosphorylated myelin basic protein.

![Fig. 5. PLPS induces phosphorylation of p140Trk (Trk A) in PC12 cells, and both K-252a and staurosporine block PLPS-dependent tyrosine phosphorylation of p140Trk.](image2)

After preincubation for 10 min with or without 100 nM K-252a (lanes 3, 6, and 9) or staurosporine (lanes 4, 7, and 10), cells were treated for 5 min with 100 ng/ml NGF or 5 μg/ml 4,6- or 3,4-PLPS (lanes 2-4, 5-7, and 8-10, respectively). Lysates (containing 30 μg of protein) from PC12 cells were immunoprecipitated with anti-p140Trk antibody. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-phosphotyrosine antibody PY20 or rabbit anti-mouse IgG, and visualized by autoradiography with ECL kit for 24 h.

![Fig. 6. Effect of K-252a and staurosporine on MAPK activation and neurite outgrowth by PLPS.](image3)

Cells were incubated in the presence or absence of K-252a (100–200 nM) or staurosporine (2.5–200 nM) in culture medium, and were further incubated with or without NGF or PLPS for 5 min. MAPK activation (panel A) and neurite outgrowth (panel B) were examined. Data points were as in Fig. 3, except that each experiment was performed in duplicate. Panel B, a, 4,6-PLPS (5 μg/ml); b, 4,6-PLPS (5 μg/ml) plus K-252a (100 nM); c, 4,6-PLPS (5 μg/ml) plus staurosporine (5 nM); d, untreated (control). PC12 cells were treated with these agents for 48 h, and neurite outgrowth was examined.
Neuritogenesis of neuronal cell lines such as neuroblastoma, glialoma, and pheochromocytoma has been used often as a model of neuronal differentiation and its biochemical mechanism. Mouse neuroblastoma cell line Neuro2a shows strong neuritogenic response to exogenous addition of various gangliosides and synthetic sialosyl compounds in the absence of NGF. Gangliosides and NGF function synergistically to affect neuritogenesis in Neuro2a cells (7, 26). Various gangliosides, including GM1, are mitogens for astroglial cells, and induce astroglial maturation factor (27, 28). Neuritogenesis in primary culture of normal rat astroglial cells, rat astroglialoma cell line GA-1, and rat Schwannian glialoma line 354-A is strongly promoted by various synthetic sialosyl derivatives, including α- and β-sialosylcholesterol (29). Neuritogenesis of two human neuroblastoma lines, GOTO and NB-1, was strongly promoted when nM order concentrations of the specific ganglioside GQ1b were added to culture medium (30). This effect was ascribed to activation of a GQ1b-sensitive cell surface protein kinase, and claimed to be present on the neuroblastoma cells (31). Neurite outgrowth and cholinergic differentiation in Neuro2a cells were recently shown to be induced by transfection and expression of sialyltransferase gene for synthesis of b-series gangliosides, i.e. CMP-sialic acid: NeuAcα2→3Galα2→8-sialyltransferase (32).

However, none of the above studies included attempts to clarify the mechanism of transmembrane signaling and its possible modulation by exogenous addition of gangliosides or their analogs, or endogenous stimulation of b-series ganglioside synthesis. Transmembrane signaling in neuronal cells is currently thought to be initiated by activation of tyrosine kinase associated with neurotrophin receptors (Trk family) (33).

Neuritogenesis of rat pheochromocytoma PC12 cells has been employed as a model of neuronal differentiation induced by NGF. NGF causes PC12 cells to undergo neuritogenesis through activation of its receptor-associated tyrosine kinase, Trk A. Although various gangliosides enhance the neuritogenic effect of NGF in PC12 cells, gangliosides or other GSLs alone in the absence of NGF do not induce neuritogenesis (9). The present study indicates that one novel GSL, PLPS, a peculiar fatty aldehyde conjugate of β-galactosyl-Sph (psychosine) (1), is capable of inducing not only neuritogenesis but also prolonged survival of PC12 cells in the absence of serum and NGF, through prevention of apoptosis. PLPS is present in brain white matter, but not gray matter, in humans and some animals. Of two types of PLPS, i.e. 4,6- and 3,4-cyclic acetal derivatives, 4,6-PLPS shows strong NGF-like activity, and 3,4-PLPS has weaker activity. This is the first case in which a GSL species induces NGF-like activity in PC12 cells in the absence of NGF. The effect of PLPS in inducing differentiation of PC12 cells provides an opportunity to study PLPS-dependent transmembrane signaling mechanism in these cells.

Mixing experiments with NGF and PLPS revealed an additive effect on neuritogenesis when half-saturating doses of both agents were used. When saturating doses were used, there was no clear additive effect. These findings suggest that PLPS may interact with cell surface membranes in a different way than NGF, and indirectly activate NGF receptor. Consequently, PLPS shares the common pathway induced by NGF, leading to...
activation of specific genes required for the differentiation process.

The established signal transduction pathway associated with NGF-dependent differentiation is initiated by activation of NGF receptor kinase (p140[k], Trk A) (5, 6) and leads to prolonged activation of MAPK (34, 35). We studied these two processes in relation to differentiation induction of PC12 cells by NGF and/or PLPS. Similar degrees of p140[k] (Trk A) phosphorylation were induced when PC12 cells were incubated with NGF alone or PLPS alone. No phosphorylated band was detected in cells stimulated in the presence of 100 mM K-252a or staurosporine, both of which are known tyrosine kinase inhibitors and neuritogenesis blockers (23–25). The degree of Trk A activation was much stronger in the presence of 4,6- than 3,4-PLPS, consistent with the relative degree of neuritogenesis induction. Prompt and prolonged MAPK activation was also observed when PC12 cells were treated with 4,6-PLPS, NGF, or a combination. The degree of MAPK activation by 3,4-PLPS was considerably smaller, again comparable with the degree of neuritogenesis induction.

Neuritogenic effect and prolonged MAPK activation by PLPS were also observed in glioma cell line NeuroA2A. PLPS did not activate MAPK in fibroblast line BALB/c 3T3 A31 or human foreskin fibroblast line WI 38 and did not initiate EGFR receptor phosphorylation in PC12 cells (data not shown). Therefore, its effect is specific to NGF-susceptible neuronal cells and may require the presence of NGF receptor. Whether PLPS affects activation of other types of neuronal growth factor receptors (Trk B, Trk C, etc.) remains to be studied.

Data accumulated so far indicate that PLPS mimics the activity of NGF through activation of NGF receptor kinase to the same extent as NGF, triggering a common signal transduction cascade that leads to activation of MAPK. MAPK switches on various transcription factors necessary for activation of genes required for induction of neuronal differentiation (including neuritogenesis, cholinergic activity, and cell survival through prevention of apoptosis).

The major question of how PLPS activates Trk A remains to be elucidated. PLPS, in contrast to unlabeled NGF, does not inhibit binding of 125I-labeled NGF to the NGF receptor (Trk A). This suggests that PLPS does not bind to the NGF binding site on Trk A.

The co-presence of Ga1 ganglioside potentiates the effect of NGF in terms of neuritogenesis in PC12 cells. Unlike PLPS, Ga1 in the absence of NGF has no neuritogenic effect (9). It was reported recently that Ga1 at very high concentration (0.5–2 mM) incubated with PC12 cells had only a weak enhancing effect on Trk A phosphorylation (36). This weak stimulatory effect by Ga1 alone, even at mM order concentration, was not sufficient to induce neuritogenic differentiation. Ga1 at mM order concentration may induce membrane perturbation, which somehow indirectly induces weak Trk A phosphorylation. In contrast, PLPS at much lower concentration (1–20 mM) strongly induces Trk A phosphorylation and consequent differentiation of PC12 cells.

Our results indicate that PLPS does not compete with binding of NGF to Trk A (Fig. 7). It is possible that PLPS does not bind to the same binding site as NGF on Trk A within the receptor. Although the exact mechanism for phosphorylation of Trk A by PLPS remains to be elucidated, it was reported recently that Ga1 phosphorylates Trk A and binds to Trk A in SDS-resistant fashion (37). However, these authors did not perform a binding assay, and they state that the exact site for binding of Ga1 to Trk protein is not known at present. They also report that treatment with tunicamycin, a potent N-glycosylation inhibitor, results in loss of association of Ga1 and Trk A protein, and disappearance of tyrosine kinase activity of fully glycosylated 140-kDa Trk A. This suggests that different mechanisms are involved in binding of Ga1 to Trk A and phosphorylation of Trk A by Ga1. We found that Ga1 did not affect binding of 125I-NGF to Trk A (Fig. 7). Based on all of these results, we conclude that the mechanism of Trk A phosphorylation by PLPS or Ga1 differs from that by NGF and that PLPS and NGF share a common signaling cascade from NGF receptor Trk A to MAPK. Further study is necessary to determine the precise mechanism by which PLPS activates Trk A.

Possible explanations for the observed effects of PLPS are as follows. (a) PLPS has a novel structure with two aliphatic chains oriented in opposite directions, and this morphology may cause membrane reorganization and local perturbation, inducing NGF receptor activation via some yet unknown mechanism. (b) The novel structure of PLPS may bind one NGF receptor to another, accelerating receptor-receptor interaction without binding of NGF, although the binding site of PLPS on NGF receptor may well be different from that of NGF. This could enhance Trk A dimerization and consequent phosphorylation. (c) PLPS may affect modulators of Trk A such as P75 (33, 38). In order to distinguish between these possibilities, we are in the process of synthesizing radioactive PLPS.

Apoptosis of PC12 cells results within a few hours from serum withdrawal in culture and can be prevented by the addition of NGF (39). We demonstrated clearly that PLPS has an apoptosis-preventing (neurotrophic) effect similar to that of NGF in serum-deprived cells for a prolonged period (7 days). In other words, PLPS has “neurotrophic factor-like activity.” Psychosine is absent in normal neuronal tissue, but it is known to accumulate in association with a genetic deficiency of ceramide β-galactosidase in brain white matter (Krabbe’s disease) (1, 40). Psychosine is strongly cytotoxic, and its accumulation is regarded as the cause of neuronal dysfunction in Krabbe’s disease. As shown in this study, psychosine inhibits endogenous MAPK activity in PC12 cells (Table I). Most psychosine-treated cells were swollen and detached. Psychosine inhibits neurite outgrowth and MAPK activation by NGF (data not shown). Plasmal conjugation of psychosine results in a product (PLPS) showing a completely opposite physiologic effect from psychosine in the sense that it shows strong neurotrophic factor-like activity and enhanced Trk A phosphorylation leading to prolonged MAPK activation. The process of cyclic aldehyde formation is biochemically unknown, yet it is crucial for conversion of neurotrophic psychosine into neurotrophic PLPS in normal neuronal cells. This process is presumably highly active in normal human brain, since PLPS is present in significant quantity in this tissue, whereas psychosine is completely absent. It is possible that plasmal conjugation reaction is defective in Krabbe’s disease, along with enhanced ceramidase activity that converts cerebroside to psychosine (40, 41).

The presence of a novel lipid factor in normal brain that mimics NGF activity through activation of Trk A and MAPK may be important in development and maintenance of the vertebrate nervous system.

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