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Anti-apoptotic role of phospholipase D isozymes in the glutamate-induced cell death

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Abbreviations: DAG, diacylglycerol; PA, phosphatidic acid; PEt, phosphatidylethanol; PLD, Phospholipase D

Abstract

Phospholipase D (PLD) plays an important role as an effector in a variety of physiological processes that reveal it to be a member of the signal transducing phospholipases. Recently, PLD2 was reported as a necessary intermediate in preventing apoptosis induced by hydrogen peroxide or hypoxia in rat pheochromocytoma (PC12) cells. The data presented here show that both PLD isozymes, PLD1 and PLD2 are also required in attenuating glutamate-induced cell death in PC12 cells. Treatment of PC12 cells with glutamate resulted in induction of apoptosis in these cells, which is accompanied by decreased PLD activity and increased ceramide concentration. Incubation of PC12 cells with exogenous C6-ceramide showed a time-dependent decrease of PLD activity. When cDNAs of PLD1 and PLD2 were transfected into PC12 cells respectively, overexpression of PLD1 or PLD2 resulted in inhibition of glutamate-induced apoptotic cell death. These data indicate that both PLD1 and PLD2 play a protective role against glutamate-induced cell death in PC12 cells.

Keywords: apoptosis; ceramides; glutamic acid; PC12 cells; phospholipase D

Introduction

Glutamate is a major excitatory neurotransmitter in central nervous system. Sometimes, it may become a potent excitotoxin and contribute to the generation of neurodegeneration (Lipton *et al.*, 1994). The excessive activation of glutamate receptor induces pathological membrane permeability changes that result in excitotoxic neuronal cell death (Olney *et al.*, 1995). Receptors that respond to glutamate are classified into two types, ionotropic and metabotropic. The ionotropic glutamate receptors are cation-specific ion channels that mediate fast excitatory glutamate responses and subdivided into α-amino-3-hydroxy-5-methylisokazole-4-propionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA) receptors. In contrast, metabotropic glutamate receptors (mGluRs) mediate slower glutamate responses by coupling to various second messenger cascades via heterotrimeric G proteins. Excessive stimulation of neuronal NMDA and AMPA/kainate classes of glutamate receptors permits excessive sodium and calcium ion entry accompanied by chloride and water leading to early intracellular volume expansion (Choi, 1987). Depending on the intensity and duration of glutamate exposure, necrosis or apoptosis has been occurred in glutamate-stimulated neurons (Ankarcrona *et al.*,1995; Beart *et al.*, 1998). Although Ca^{2+} influx through the NMDA subtype of glutamate receptors is an initial event that plays a central role in glutamate-evoked neuronal excitotoxicity, the exact mechanism of glutamate-induced neuronal death is still not clear.

Ceramide is a product of sphingomyelin breakdown by sphingomyelinase and acts as a lipid second messenger to mediate the effects of extracellular agents on cell differentiation, growth inhibition, and apoptosis (Hannun, 1994). Specifically, substantial evidence has been presented that the generation of endogenous ceramide is mediated by apoptosis inducing agonists such as tumor necrosis factor-α, Fas ligand, and interleukin-1 (Hannun *et al.*, 1994). Several reports show that ceramide has been implicated in the regulation of PLD. In FRTL-5 thyroid cells, ceramide inhibits the PLD activity in an early apoptotic phase and down-regulates PLD mRNA levels (Park *et al.*, 1999). The addition of exogenous C6-ceramide in mouse ovarian granulose cells induced drastic morphological

change including nuclear fragmentation and typical apoptotic DNA degradation. Furthermore, C6-ceramide decreased PLD activity in a time- or a dose-dependent manner (Kim *et al.*, 1999).

Phospholipase D (PLD) hydrolyzes phosphatidylcholine into phosphatidic acid (PA) and choline. PA acts as a second messenger and further metabolized to diacylglycerol (DAG) by phosphatidate phosphohydrolase. PLD has been involved in physiological processes including exocytosis, Golgi function, respiratory burst, proliferation, differentiation and apoptosis (Exton, 1997; Cockcroft *et al.*, 1999; Daniel *et al.*, 1999; Nakashima *et al.*, 1999). Two distinct PLD isoforms, PLD1 and PLD2, have been cloned (Morris *et al.*, 1996). PLD1 has low basal activity and is activated by Arf1, RalA, RhoA, Cdc42 and PKC in the presence of phosphatidylinositol bisphosphate *in vitro* (Morris *et al.*, 1996; Hammond *et al.*, 1997; Han *et al.*, 1998; Kim *et al.*, 1998). In contrast to PLD1, PLD2 is constitutively active *in vitro*, and its activity is not affected by PLD1-activating factors (Colley *et al.*, 1997). In rat pheochromocytoma (PC12) cells, hypoxic incubation (1% O_2) induces a transient increment of PLD activity and pretreatment with PLD of *S. chromofuscus* inhibits hypoxia-induced apoptotic cell death (Yamakawa *et al.*, 2000). Treatment of hydrogen peroxide (H_2O_2) induces apoptosis in PC12 cells which is accompanied by the activation of PLD (Oh *et al.*, 2000) and increased PLD2 activity suppresses H_2O_2-induced apoptosis in these cells (Lee *et al.*, 2000). However, the exact role and regulation of PLD activity in each cell response have not yet been fully elucidated. In this study, we examined the change of PLD activity during glutamate-induced PC12 cell death. Possible anti-apoptotic roles of PLD1 and PLD2 in the glutamate-induced cell death will be discussed

Materials and Methods

Materials

L-glutamic acid (monosodium salt) was purchased from Sigma (St. Louis, MO). C6-ceramide (*N*-hexanoylsphingosine) was purchased from BioMol Research Lab (Plymouth Meeting, PA). [^{3}H]palmitic acid, and [^{3}H]serine were purchased from Du Pont-New England Nuclear (Boston, MA). Fetal bovine serum (FBS), penicillin/streptomycin solution, and RPMI-1640 medium were from Gibco-BRL (Gaithersburg, MD). Polyclonal antibody that recognized both PLD1 and PLD2 was generously provided by Dr. Do Sik Min (Department of Physiology, College of Medicine, The Catholic University of Korea, Seoul, Korea).

Cell culture

PC12 cells were grown on polystyrene tissue culture dishes in RPMI-1640 supplemented with heat-inactivated 10% horse serum, 5% fetal bovine serum, and 100 units/ml penicillin, 100 μg/ml streptomycin in 37°C incubator in 5% CO_2 in a humidified atmosphere. Maintenance cultures of PC12 cells were routinely subcultured at a cell density of 2×10^6 cells/dish at least once a week, and the medium was changed every 2-3 days. PC12 cells were incubated in serum-free RPMI-1640 medium for 24 h before treatment with glutamate.

MTT [3-(4,5-dimethy thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay

Cells were plated onto 96-well plate (1×10^5 cells/ml) and treated with 200 ng/ml of anti-Fas monoclonal antibody for predetermined time. MTT solution (0.4 mg/ml) was added and incubated for 4 h at 37°C and centrifuged at 400 *g* for 5 min. The resulting supernatant was discarded and 150 μl of DMSO was added. After shaking the plates for 10 min, the optical density was immediately measured at 540 nm.

Sphingomyelin hydrolysis assay

Sphingomyelin and its hydrolysis product, ceramide levels were monitored by the method of Estevez *et al.* (1995) with slight modifications. PC12 cells (1.5×10^6 cells/well) were labeled with 5 μCi/ml of [^{3}H]-L-serine in the minimum essential medium for 18 h and treated with 10 mM glutamate for various time periods indicated. After stimulation, the cells were quickly washed with ice-cold PBS, and suspended in ice-cold methanol. After the lipid extraction according to the method of Bligh and Dyer (1959), lipids in the lower organic phase were applied to a thin layer chromatography (TLC) silica gel plate. The plates were then developed using two sequential developments with ethyl acetate/iso-octane/acetic acid/water (130:20:30:100, by vol.) as the first solvent and chloroform/methanol/acetic acid /water (100:60:20:5, by vol.) as the second. Fractions that co-migrated with the same retention factors such as authentic sphingomyelin and ceramide were scraped into scintillation vials and the associated radioactivity was determined by liquid scintillation counting.

Production of recombinant retrovirus

The complete coding sequences of the human full-length PLD1 and PLD2 cDNAs were cloned LXSN retroviral vector whose promoter is the long terminal repeat (Clontech Laboratories), respectively. To generate the amphotropic producer cell lines, the GP+ envAm 12 packaging cell line was transfected with 10

μg of LXSN-PLD1 or LXSN-PLD2 using a standard calcium phosphate method (Markowitz *et al.*, 1988). After geneticin (800 μg/ml) selection for 2 weeks, individual clones were collected and their titer was assessed by infecting NIH 3T3 cells with a serial dilution of the different viral stocks. Selected clones were maintained in the presence of geneticin (800 μg/ml). The supernatant containing retroviruses were collected 48 h after subculture from the selected clones.

Construction of PLD1-PC12 and PLD2-PC12 cell lines

For cell line infection, PC12 cells were plated on 10-well plates, and the filtered retroviral supernatants of high-titer cloned packaging cell lines were added overnight in the presence of polybrene (8 μg/ml). Two days after gene transfer, geneticin (500 μg/ml) was added to the culture medium to select clonal lines expressing PLD1 or PLD2. After cells had grown for 2 weeks, we picked each clone, placed them first in 96-well plates, gradually expanded to 10 cm plates, and maintained them in the presence of geneticin (500 μg/ml) (Zocchi *et al.*, 1998)

Determination of PLD activity

PLD activity was determined as previously described by measuring [^{3}H]-phosphatidylethanol (PEt) produced via PLD-catalyzed transphosphatidylation in [^{3}H]-palmitic acid-labeled cells (Lim *et al.*, 2002). Briefly, cells (1.5×10^6 cells/well) cultured on 6-well plates were metabolically labeled with 5 μCi/ml of [^{3}H]-palmitic acid in the serum free medium for 18 h. The cells were then pretreated with 1% (v/v) ethanol for 15 min before stimulation with agonists. After stimulation, the cells were quickly washed with ice-cold PBS and suspended in 0.5 ml of ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer (1959). PEt was separated from other phospholipids by TLC on silica gel 60A plates using a solvent system of ethyl acetate/iso-octane/acetic acid/water (110:50:20:100, v/v). The regions corresponding to the authentic PEt band were identified with 0.002% (w/v) primulin in 80% (v/v) acetone, scraped and counted using a scintillation counter, respectively.

Immunoprecipitation and Western blotting of PLD

Serum-starved cells on 100-mm dishes (1×10^6 cells/ml) were incubated with agonists for each indicated times, scraped in PBS and harvested by microcentrifugation. The cells were then resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1% Triton X-100, 0.5% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin) on ice and disrupted by sonication. Cell lysates were centrifuged at $10,000\times g$ at 4°C for 10 min. Lysates from control or stimulated cells were pooled, and 1.5 ml of supernatants was used for each immunoprecipitation. Immunocomplexes were collected using Protein G-Agarose (Roche Diagnostics, Germany) and anti-PLD antibody. Incubation was carried out overnight at 4°C. Samples were centrifuged $1,500\times g$ and washed 3 times with lysis buffer. After boiling for 5 min in a mixture 10% glycerol, 100 mM DTT, 2% SDS, and 50 mM Tris-HCl, pH 6.8, immunoprecipitated proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes using a Bio-Rad semi-dry transfer system. The membranes were blocked for 1 h with 5% (w/v) BSA in TTBS (Tris-buffered saline containing 0.01% Tween-20) and then incubated for another hour with diluted specific anti-PLD antibody (1 μg/ml). Unbound primary antibodies were removed by three washes (10 min each) with TTBS. Detection was performed with the ECL system (Amersham Corp.) according to the manufacturer's protocol.

Results

Dose- and time-dependent glutamate toxicity

The dose-dependent glutamate toxicity was examined by determining the cell viability using MTT assay test upon incubation of PC12 cells for 6 h with different glutamate concentrations in the range of 10 μM-10

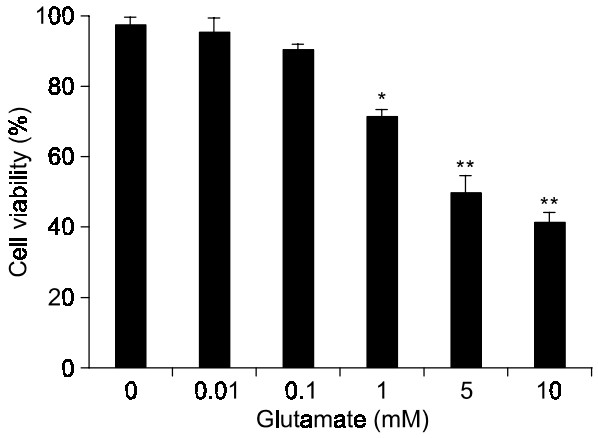

Figure 1. Dose-dependent cytotoxic effect of glutamate on PC12 cells. PC12 cells grown on 96-well plate (1×10^5 cells/ml) were serum starved for 18 h, then were switched to fresh medium without fetal bovine serum in the absence or in the presence of increasing glutamate concentrations (10 μM-10 mM) for 6 h. Cell viability was measured using MTT assay as described in Materials and Methods. Data are means ± SEM from triplicate experiments. *$P < 0.05$; **$P < 0.01$, significantly different compared to control conditions in the absence of glutamate.

mM. As shown in Figure 1, increasing glutamate concentrations induced a decrease in cell survival in a dose-dependent manner. The time-dependent glutamate toxicity was analyzed after incubation of cells during 1, 3, 6, and 12 h with 10 mM glutamate. Glutamate induced a pronounced cell death in PC12 cells in a time-dependent manner, with a significant reduction in cellular viability after 3 h incubation with 10 mM glutamate ($P < 0.01$), which decreased further upto 12 h incubation. After 6 h incubation with 10 mM glutamate, cellular viability was reached to a minimum level (Figure 2).

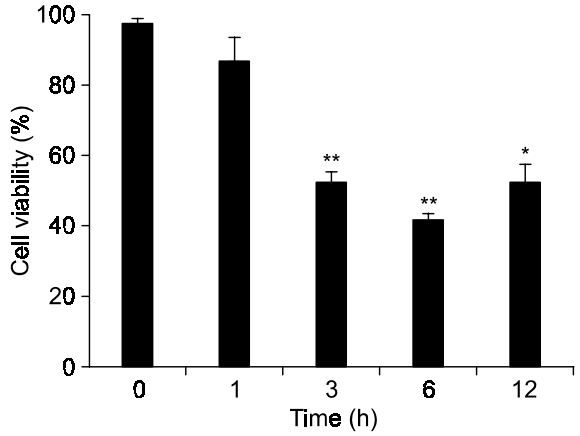

Figure 2. Time course of cell viability of PC12 cells induced by 10 mM glutamate. PC12 cells grown on 96-well plate (1×10^5 cells/ml) were serum starved for 18 h, and medium was removed and replaced by fresh medium without fetal bovine serum containing 10 mM glutamate. After 1, 3, 6, and 12 h incubation, control or glutamate-treated cells were analyzed for cell viability using MTT assay as described in Materials and Methods. Data are means ± SEM from triplicate experiments. *$P < 0.05$; **$P < 0.01$, significantly different compared to control conditions in the absence of glutamate.

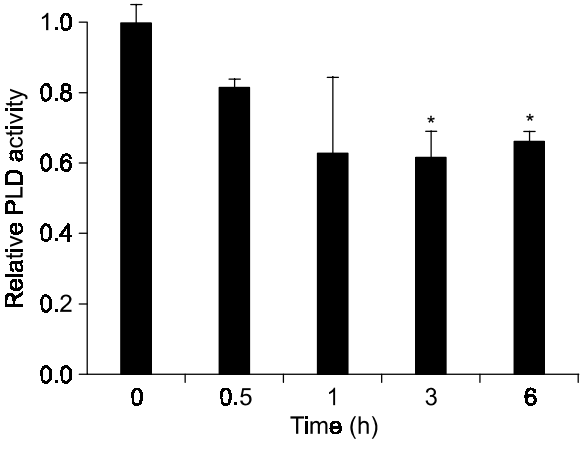

Figure 3. Effect of glutamate on PLD activity of PC12 cells. PLD activity was measured using the transphosphatidylation reaction. PC12 cells grown on 6-well plates at the density of 1×10^6 cells/ml were metabolically labeled with 5 μCi/ml of [^{3}H]-palmitic acid in a serum free medium for 18 h. The cells were treated with 10 mM glutamate for the indicated periods of time. Determination of PEt level was performed as described in Materials and Methods. Data are means ± SEM from triplicate experiments. *$P < 0.05$, significantly different compared to control conditions in the absence of glutamate.

Effect of glutamate on PLD activity in PC12 cells

PC12 cells were labeled with [^{3}H]-palmitic acid then exposed to glutamate to detect a change of PLD activity measured by transphosphatidylation reaction, that is, the generation of PEt. To determine whether glutamate changes the PLD activity, cells were incubated with 10 mM glutamate and the PLD activities after various times of exposure were monitored (Figure 3). A significant decrease in PLD activity was seen as early as 30 min after exposure to glutamate, reaching a minimal level at 1 h incubation. In addition to these results, a dose-dependent curve for glutamate revealed maximal decrease in PLD activity at 10 mM glutamate compared to control values (data not shown).

Effect of glutamate on sphingolipid metabolism in PC12 cells

PC12 cells were labeled with [^{3}H]-L-serine to detect sphingomylin degradation induced by treatment of glutamate. When the cells were exposed to 10 mM glutamate, increase in ceramide level was observed in a time-dependent manner, reaching a maximal level at 6 h. On the other hand, sphingomyelin level was decreased after 30 min, reaching minimal level

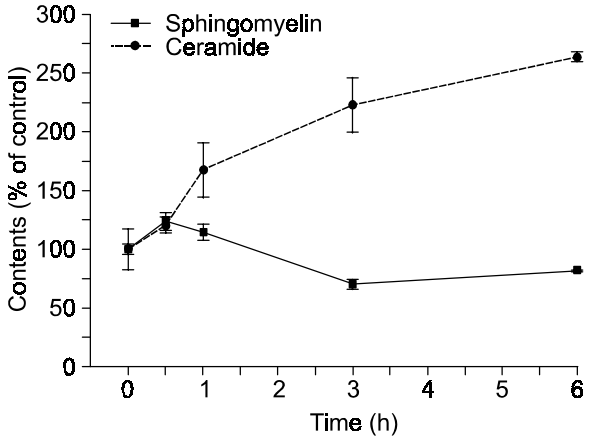

Figure 4. Effect of glutamate on sphingolipid metabolism in PC12 cells. Serum-starved cells on 6-well plates (1×10^6 cells/ml) were incubated in serum-free medium containing 5 μCi/ml of [^{3}H]-serine for 18 h. After stimulation with 10 mM glutamate for the indicated periods of time, the cells were harvested and intracellular levels of sphingomyelin and ceramide were determined according to the methods described in Materials and Methods.

at 3 h (Figure 4).

Effect of C6-ceramide on PLD activity in PC12 cells

It has been reported that treatment of exogenous ceramide substantially suppressed the PLD activity (Park *et al.*, 1999). To determine the effect of ceramide on PLD activity, PC12 cells were incubated with exogenous 25 μM C6-ceramide and PLD activities after various periods of time exposure were monitored. Incubation of PC12 cells with exogenous 25 μM C6-ceramide showed a time-dependent decrease in PLD activity, reaching minimal level at 12 h (Figure 5). 1, 3, 6, and 12 h after addition of 25 μM C6-ceramide, PLD activity was decreased approximately 12%, 25%, 40% and 45%, respectively compared to control.

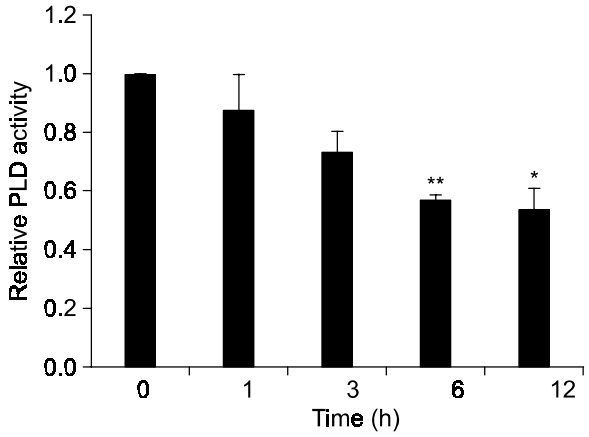

Figure 5. Effect of C6-ceramide on PLD activity in PC12 cells. Cells were treated with 25 μM C6-ceramide for the indicated periods of times. The PLD activity was measured using the transphosphatidylation reaction as described in Materials and Methods. After treatment, lipids were extracted and separated by TLC. PEt was quantitated by liquid scintillation spectrometry. Data are means ± SEM from triplicate experiments. $*P < 0.05$; $**P < 0.01$, significantly different compared to control conditions in the absence of C6-ceramide.

Suppression of glutamate-induced cell death by PLD overexpression

To investigate the role of PLD activity in glutamate-induced cell death, PC12 cell lines expressing human PLD1 and PLD2 were constructed using retroviral infection, respectively (PLD1-PC12 and PLD2-PC12). The morphology and growth rate of PLD1- PC12 and PLD2-PC12 cells were almost the same as those of mock cells (data not shown). Also, we could not detect any changes of expression levels of endogenous PLD2 in PLD1-PC12 cells, and endogenous PLD1 in PLD2-PC12 cells, respectively (Figure 6A). As shown in Figure 6A, overexpressions of 120-kDa PLD1 in PLD1-PC12 cells and 105-kDa PLD2 in PLD2-PC12 cells were confirmed by immunoprecipitation and Western blotting with anti-PLD antibody, respectively.

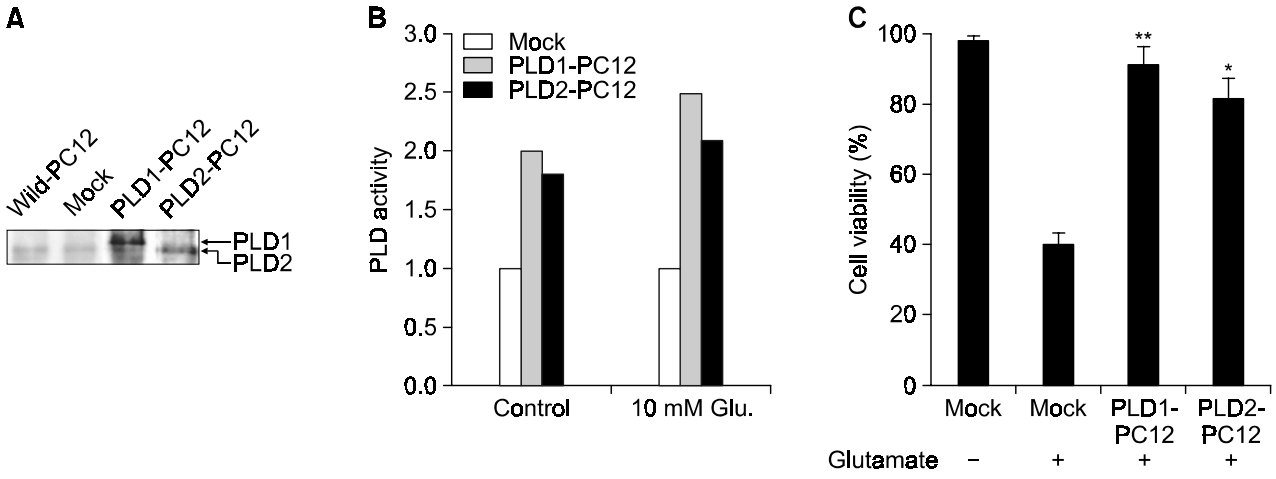

Figure 6. Suppression of glutamate-induced cell death by PLD overexpression. PC12 cells were infected with recombinant retrovirus encoding human PLD1 and PLD2, respectively as described in Materials and Methods. A) Immunoprecipitation and Western blot analysis of PLD1 and PLD2 in mock, PLD1-PC12 and PLD2-PC12 cell. PC12 cell lysates were immunoprecipitated with anti-PLD antibody and analyzed by SDS-PAGE, followed by transfer of proteins to nitrocellulose membrane and Western blotting with same antibody. B) Changes in PLD activity of PLD1-PC12 and PLD2-PC12 cells. Cells were infected with recombinant retrovirus encoding human PLD1 or PLD2. Cells were treated with 10 mM glutamate for 6 h. Cells were radiolabeled with 5 μCi/ml [^{3}H]-palmitic acid and preincubated with 1 % ethanol for 15 min prior to stimulation with glutamate. C) Effect of PLD overexpression (PLD1 and PLD2) on glutamate-induced cell death. PLD1-PC12 and PLD2-PC12 cells were cultured for 6 h in the absence or presence of 10 mM glutamate. Data are means ± SEM from triplicate experiments. $*P < 0.05$; $**P < 0.01$, significantly different compared to mock cells in the presence of glutamate.

Both of PLD1-PC12 cells and PLD2-PC12 cells showed higher basal activities than control mock cells. When treated with 10 mM glutamate, basal PLD activities in both of PLD1-PC12 and PLD2-PC12 cells were also higher than that of control mock cells (Figure 6B). To determine the role of PLD activity in glutamate-induced cell death, we used PLD1-PC12 and PLD2-PC12 cells. In the PC12 cells overexpressing PLD1 or PLD2, glutamate-induced cell death was suppressed indicating that both of PLD1 and PLD2 activities are involved in suppressing glutamate-induced cell death. As shown in Figure 6C, survival rate in PC12 cells overexpressing PLD1 or PLD2 compared with vector-transfected mock cells was increased. After treatment of 10 mM glutamate for 6 h, nearly 52% of mock cells underwent cell death, but 9% of PLD1-PC12 cells and 18% of PLD2-PC12 cells became apoptotic.

Discussion

In this work, we investigated the effect of glutamate on cellular viability and anti-apoptotic role of PLD in glutamate-induced cell death of PC12 cells. The results presented show that exposure of PC12 cells to glutamate induces a reduction on cell viability, which becomes statistically significant for glutamate concentrations of 10 mM and after 3 h incubation (Figures 1 and 2). Sphingomyelin pathway has been claimed to be involved in the apoptotic process and ceramide, the product of sphingomyelin hydrolysis by sphingomyelinase, appears to be a functional mediator of this pathway (Hannun and Obeid, 1995; Testi, 1996). In this present study, we demonstrated that glutamate increases sphingomyelin hydrolysis, therefore, increases intracellular ceramide level in PC12 cells (Figure 4). Ceramide has been known to induce apoptosis in FRTL-5 thyroid cells (Park *et al.*, 1999), fibroblast (Obeid *et al.*, 1993), neurons (Brugg *et al.*, 1996) and reproductive cells (Kaipia *et al.*, 1996). Considering several findings indicating that sphingomyelin pathway is involved in apoptotic signaling in various type of cells (Boesen-de Cock *et al.*, 1998), our results also suggested that ceramide may play a key role in glutamate-induced apoptosis in PC12 cells.

Recently, many investigators have demonstrated that ceramide inhibits PLD activity in a number of cells including FRTL-5 thyroid cells (Park *et al.*, 1999), rat basophilic leukemia cells (Nakamura *et al.*, 1996), rat fibroblast (Gomez-Munoz *et al.*, 1995; Jones and Murray, 1995) and C6 glial cells (Yoshimura *et al.*, 1997). Because PLD activation is involved in cell proliferation and differentiation (Liscovitch and Cantley, 1995), it is tempting to examine that inhibition of PLD activity would be implicated in induction of apoptotic cell death by ceramide. Our results showed that glutamate or ceramide induced decrease of PLD activity (Figures 3 and 5). These results indicated that the PLD pathway could be an important target for the modulation of signal transduction by ceramide. Although the exact mechanism of this effect is unclear at present, considering that PLD activity is regulated by Arf, Rho (Cockcroft *et al.*, 1994; Malcolm *et al.*, 1994) and PKC (Balboa *et al.*, 1994), it is possible that ceramide may inhibits translocation of these PLD activator to membrane, leading to a decrease in PLD activity (Nakamura *et al.*, 1996; Park *et al.*, 1999). In addition, it is well known PA which is produced by PLD from PC may act as a potent mitogen in cells (Spiegel and Milstien, 1996). This is followed by DAG generation via a PA phosphohydrolase and then DAG activates PKC. Therefore, it is possible that ceramide-induced inhibition of PLD activity attenuates PA production and subsequent DAG accumulation, and results in insufficient PKC activation for cell growth. Accordingly, we propose that ceramide may play an important role in inhibiting mitogenic pathway in an early phase during apoptotic process.

Up to now relatively little information exists concerning the mechanisms by which glutamate leads to cell death on PC12 cells, even though many reports suggested the possibility that PLD activity may be implicated in the apoptotic process (Yoshimura *et al.*, 1997; Nakashima and Nozawa, 1999; Park *et al.*, 1999). However, role of the PLD activity in these apoptotic processes is still unclear. Recently, two groups reported that PLD2 activity acts as survival factor against apoptosis. Lee *et al.* (2000) reported that hydrogen peroxide-induced apoptosis was blocked by overexpression of PLD2 in PC12 cells. This suppressive effect of PLD2 on hydrogen-peroxide-induced cell death was also observed in a different cell line, 3Y1 fibroblast. Yamakawa *et al.* (2000) also reported that exogenous *S. chromofuscus* PLD prevented hypoxic cell death in PC12 cells and when PLD2 was transiently overexpressed in PC12 cells, hypoxia-induced apoptosis was significantly inhibited suggesting that PLD2 activation may play an anti-apoptotic role. To investigate the role of PLD activity in glutamate-induced cell death in PC12 cells, we overexpressed PLD1 and PLD2 in PC12 cells using retroviral transfer, respectively. When PLD1 or PLD2 was expressed in PC12 cells, glutamate-induced apoptosis was suppressed in both of transfected cells (Figure 6C). Furthermore, inhibitory effect of glutamate on PLD activity was overcome in both of PLD1-PC12 and PLD2-PC12 cells (Figure 6B). Taken together, these results suggest that not only PLD2 activity but also PLD1 activity has an anti-apoptotic role in glutamate-induced cell death in PC12 cells and provide strong evidence to support the existence of a close relationship between

the activation of PLD and cell survival in the PC12 cells.

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