DECREASED PHOSPHOLIPASE D ACTIVITIES IN CERAMIDE-INDUCED APOPTOSIS OF RBL-2H3 MAST CELLS

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ABSTRACT

The treatment of 25 μ M *N*-acetyl sphingosine (C_2 -ceramide) resulted in marked morphological changes in rat basophilic leukemia (RBL-2H3) cells. Most of the cells became round within 6 h and were detached from culture dishes at 12 h. The Hoechst 33258 staining showed typical apoptotic changes; nuclear fragmentation and chromatin condensation. Apoptotic cell death was further assessed by intranucleosomal DNA fragmentation. An inactive C_2 -ceramide analog, dihydro C_2 -ceramide (Dh C_2 -ceramide) failed to induce apoptosis. The phospholipase D (PLD) activity was decreased in apoptotic cells. In membranes of RBL cells, three types of PLD activities are present, namely, 1) GTP γ S-dependent, 2) GTP γ S-independent and 3) oleate-dependent. These activities were markedly decreased in cells treated for 3 h with 25 μ M C_2 -ceramide, but not with Dh C_2 -ceramide. These results indicate that C_2 -ceramide induced marked apoptosis in RBL cells and inhibited these three types of PLD activities. In *in vitro* assay system, C_2 -ceramide had no effects on the oleate-dependent PLD activity in membranes from control cells, whereas it inhibited GTP γ S-dependent and -independent PLD activities. The mRNA levels of PLD genes, rPLD1a and rPLD1b, which encode small GTP-binding protein-dependent PLDs, time-dependently decreased after C_2 -ceramide treatment, suggesting that the decrease in GTP γ S-dependent PLD activity could be largely due to down-regulation of PLD1. In contrast, the mRNA level of rPLD2 was rather stable up to 12 h. Therefore, the mechanism(s) of decreased oleate-dependent and GTP γ S-independent PLD activities during apoptosis remains to be clarified but it could be due to down-regulation of PLD itself and/or by interference with their activation pathway directly or indirectly by unknown manner(s).

Mast cells play a pivotal role in acute allergic responses. Upon allergic stimuli, they release mediators stored in the granules such as histamine, serotonin, etc. and also generated lipidic mediators

such as leukotrienes, prostaglandins, etc.(21). Recent studies have shown that mast cell proliferation and maturation are controlled by IL-3 and stem cell factor (5, 26-28, 49). Deprivation of these

Abbreviations: C_2 -ceramide, *N*-acetyl sphingosine; Dh C_2 -ceramide, dihydro-*N*-acetyl sphingosine; PLA₂, phospholipase A₂; PLD, phospholipase D; RBL, rat basophilic leukemia; rARF, recombinant ADP-ribosylation factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); PBut, phosphatidylbutanol; PA, phosphatidic acid; 1,2-

DG, 1,2-diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP₂, phosphatidylinositol 4,5-bisphosphate; DPPC, L- α -dipalmitoylphosphatidylcholine; RT-PCR, reverse transcriptase-polymerase chain reaction.

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factors results in apoptosis of mast cells (20, 34). Mouse mast cells have been reported to undergo apoptosis following gamma irradiation (53) and cross-linking of Fas (18). However, intracellular signaling mechanism(s) leading to apoptosis in mast cells remains to be disclosed. Recent studies have demonstrated that cytokines, such as TNF- α (13), INF- γ (25), IL-1 β (2, 32) and withdrawal of nerve growth factor (12), induce activation of sphingomyelin cycle involving regulated conversion of sphingomyelin to ceramide during the apoptotic process in several types of cells. Sphingomyelin hydrolysis is also observed during apoptosis by removal of growth factors (22) and by physical stress such as heat shock (51) and radiation (46). Therefore, ceramide has been regarded as a potent inducer of apoptosis (41). Cell-permeable ceramides are often used for studying the mechanism of action of naturally occurring ceramides in signal transduction of apoptosis. Actually, C₂-ceramide, one of the most commonly used analogs, induces apoptosis in various cells, for example, promyelocytic leukemia HL-60 cells (44), primary cultured neurons (7), and C6 glioma cells (55). However, the precise mechanism(s) for ceramide-mediated apoptosis has not yet been delineated.

On the other hand, it has recently been accepted that phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA) plays an important role in signal transductions associated with various cell functions (14). PA can be further converted to DG by PA phosphohydrolase or lysoPA by phospholipase A₂ (PLA₂). PA and lysoPA are to be potent mitogens in fibroblasts (16). Long-lasting production of DG via PLD-mediated PC hydrolysis gives rise to prolonged protein kinase C (PKC) activation (39). Thus, PLD has been thought to be involved in cell proliferation, but its exact mechanisms remains unclear. Recently, existence of PLD isozymes (PLD1a, PLD1b, and PLD2) in mammalian cells has been shown (10, 11, 17, 29, 38), although the physiological roles of individual isozymes remain to be defined.

In this context, it is to be noted that C₂-ceramide inhibits the PLD activity in rat basophilic leukemia (RBL-2H3) cells (36), rat fibroblasts (16, 23), and in cell-free system using HL-60 cells (50). These observations have led us to ask whether PLD would be implicated in the apoptotic process. We have recently shown that mRNA expression of three PLD isoforms (PLD1a, PLD1b, and PLD2)

was changed during C₂-ceramide-induced apoptosis of C6 glioma cells (55). To our knowledge, however, no reports have been available regarding the ceramide-induced apoptosis in mast cells. In the present study, we have observed that C₂-ceramide induced marked apoptosis of RBL-2H3 cells, commonly used as a model of mucosal mast cells. Moreover, the three types of PLD activities were markedly decreased during C₂-ceramide-induced apoptosis. These results suggest that ceramide could function as a mediator to induce apoptosis in RBL-2H3 cells and that PLD might be involved in ceramide-induced apoptotic processes in RBL cells.

MATERIALS AND METHODS

Materials

RBL-2H3 cells were obtained from Health Science Research Resources (Osaka, Japan). Eagle's MEM, penicillin and streptomycin were from Life Technologies (Grand Island, NY). FBS was purchased from Irvine Scientific (Santa Ana, CA). PMA, PIP₂, egg PC, phosphatidylethanolamine (PE), and sodium oleate were from Sigma (St. Louis, MO). Guanosine 5'-O-(3-thiotriphosphate) (GTP γ S), proteinase K and DNase-free RNase were from Boehringer Mannheim GmbH (Mannheim, Germany). Silica gel LK6D plates were from Whatmann (Clifton, NJ). Phosphatidylbutanol (PBut) standard was obtained from Avanti (Albaster, AL). C₂-ceramide and dihydro C₂-ceramide (DhC₂-ceramide) were purchased from Matreya (Chalfont, PA). Hoechst 33258 (bisbenzimidazole) staining dye was from Wako, (Osaka, Japan). BCA protein assay reagents were from Pierce (Rockford, IL). Immobilon-P membrane filters were from Millipore (Bedford, MA). Isogen and SYBR Green were from Nippon gene (Tokyo, Japan). *Taq* DNA polymerase and random hexamer primers were purchased from Takara Shuzo Co. (Ohtsu, Japan). [2-Palmitoyl-9,10-³H] L- α -dipalmitoylphosphatidylcholine (DPPC; 42 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). *E. coli* bearing ADP-ribosylation factor (Arf1) plasmid was a kind gift from Dr. Joel Moss (NIH, MD). All other reagents used were of the highest analytical grade available.

Cell Culture

Monolayer cultures of RBL cells were maintained in MEM supplemented with 10% heat-inactivated

FBS, penicillin (100 units/mL) and streptomycin (100 μ g/mL) in a humidified atmosphere of 95% air/5%CO₂ at 37°C (36). For induction of apoptosis, 1.5×10^5 cells were grown in 100 mm culture plates for 24 h, rinsed twice with PBS, and cultured in MEM without FBS for another 12 h. Then, 10–50 μ M C₂-ceramide or 10–50 μ M DhC₂-ceramide were delivered into MEM without FBS. Control cells were incubated in the absence of FBS and C₂-ceramide.

Morphological Examination and DNA Fragmentation Assay

The cells detached from culture dishes were centrifuged, washed with PBS twice, and resuspended in 1% glutaraldehyde for 30 min for fixation. The cells were then rinsed with PBS and stained with 1 μ M Hoechst 33258 (55). For DNA fragmentation assay, cells were centrifuged at $3000 \times g$ for 10 min, washed twice in PBS, and resuspended in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate and 1% Triton X-100. The lysates were centrifuged at $20,000 \times g$ for 20 min and the resulting supernatants were treated with proteinase K (0.1 mg/mL) and DNase-free RNase (10 μ g/mL) for 2 h at 37°C. Genomic DNA was precipitated with ethanol and recovered by centrifugation at $20,000 \times g$ for 15 min. The resulting pellets were dissolved in Tris-EDTA buffer and electrophoresed on 2.0% agarose gel. The DNA was stained with 0.01% (v/v) SYBR Green and visualized under UV light.

Assay of PLD Activity

Collected RBL cells were washed twice with ice-cold PBS containing 1 mM EGTA. After centrifugation at $1,500 \times g$ for 5 min, cells were resuspended in ice-cold buffer A (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 0.5 mM PMSF, 5 mM DTT, 10 μ g/mL leupeptin and 1 mM EGTA) and were lysed by N₂ cavitation. The cytosol and membrane fractions were separated by centrifugation at $100,000 \times g$ for 30 min. PLD activities *in vitro* (GTP γ S-dependent, -independent and oleate-dependent) were measured according to the methods previously described (31, 43). For the assay of the GTP γ S-dependent and -independent PLD activities, mixed lipid vesicles (PE/PIP₂/egg PC, 160/14/10, μ M) containing [³H] DPPC (400,000 dpm/assay) were used as sub-

strate. The mixed lipid vesicles (20 μ L) were added to membranes (70 μ g protein) in a total of 120 μ L of 50 mM HEPES/NaOH buffer (pH 7.5) containing 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 1.2 mM CaCl₂ and 0.3% butanol. PLD activity assays were performed without or with 5 μ M rArf1, 30 μ M GTP γ S, 100 nM PMA and cytosol (70 μ g protein) at 37°C for 15–90 min. For the assay of oleate-dependent PLD activity, egg PC vesicles (20 μ L) containing egg PC (750 μ M) and [³H] DPPC (400,000 dpm) were prepared by sonication and then mixed with 5 μ L of 12 mM sodium oleate. The lipid vesicles thus prepared were added to membranes (70 μ g protein) in a total volume of 120 μ L of 50 mM HEPES/NaOH buffer (pH 7.0) containing 2 mM EGTA, 500 mM KCl, 1 mM MgCl₂, 2.1 mM CaCl₂ and 0.3% (v/v) butanol, and then incubated at 30°C for 15–90 min.

After these reactions were terminated by addition of 0.8 mL of methanol/chloroform (2 : 1, v/v), lipids were extracted by the method of Bligh and Dyer, (3). [³H] Phosphatidylbutanol (PBut) a potential parameter of PLD activity, was separated on silica gel LK6D plates by one dimensional thin-layer chromatography in a solvent system of the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13 : 2 : 3 : 10, v/v). The area corresponding to [³H] PBut, identified by co-migration with PBut standard, was scrapped off the plate and the radioactivity was measured in a liquid scintillation counter (Beckman LS 6500).

RNA Extraction and Reverse Transcription (RT)-PCR

Total RNA was isolated from RBL cells at various stages with Isogen according to the manufacturer's instruction. RNA (2 μ g) was reverse-transcribed using random hexamer-mixed primers. Primer sets for individual PLD isoforms were designed based on rat PLD cDNA (38, 54) by RT-PCR using degenerate primers. PCR primer sets for rPLD1a and rPLD1b were 5'-CGGTGGCGTTTGTGGGTGGGA-3' (sense) and 5'-GTCCTTGAAGA-CAAAGTTGCA-3' (antisense), and primer sets for rPLD2 were 5'-CTCCTGGTGGTAGACCA-AGCG-3' (sense) and 5'-GATGAAATCTTCA-AAAGGCCG-3' (antisense) (54). The PCR primer set for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was prepared as described previously (52). Temperatures used for PCR were as follows: denaturation 94°C for 30 s; annealing 58°C for 1 min; and

Fig. 1 Morphological changes of RBL-2H3 cells before and after C_2 -ceramide treatment. Cells were treated with C_2 -ceramide and DhC_2 -ceramide after incubation without FBS for 12 h. A, Untreated cells. B, Cells treated with $25 \mu M$ C_2 -ceramide for 3 h. C, Cells treated with $25 \mu M$ C_2 -ceramide for 6 h. D, Cells treated with $25 \mu M$ C_2 -ceramide for 12 h. E, Control cells incubated for 12 h without FBS and C_2 -ceramide. F, Cells treated with $25 \mu M$ DhC_2 -ceramide for 12 h. Cells were examined under a phase contrast microscope. Photographs shown are representative of three separate experiments.

extension $72^\circ C$ for 1 min. Numbers of amplification cycles were determined for individual primer sets to maintain the exponential rate of product amplification. The numbers of cycles determined for GAPDH, rPLD1a, rPLD1b and rPLD2 were 20, 30, 30 and 28, respectively. Amplified DNA fragments were subjected to electrophoresis on 1.5 % agarose gel and visualized by ethidium bromide staining. The intensity of bands was quantified by a densitometer (Atto Densitograph, Tokyo, Japan).

RESULTS

Morphological Changes in RBL Cells after C_2 -ceramide Treatment

To our knowledge, no reports have described the involvement of ceramide in apoptosis of RBL cells. Therefore, we first examined whether exogenously added ceramide induced apoptosis of RBL cells. Various concentrations of C_2 -ceramide, a short-chain cell-permeable analog, were administered to RBL cells after incubation for 12 h in the serum-

free medium. When examined under the phase-contrast microscope, drastic morphological changes were observed to occur with $25 \mu M$ C_2 -ceramide in a time-dependent manner. Cellular processes were retracted rapidly and cells became round 3 h after the C_2 -ceramide treatment and the population of floating cells increased time-dependently (Figs. 1A–D). Almost all the cells were detached from the culture dishes by 12 h (Fig. 1D). No significant morphological changes were observed in the control untreated cells after 24 h culture (12 h preincubation + 12 h incubation) in the serum-free medium (Fig. 1E). To be noted, an inactive C_2 -ceramide analog, DhC_2 -ceramide ($25 \mu M$), which lacks the 4–5 *trans* double bond in the sphingosine chain, had no effects on cellular shape (Fig. 1F).

Nuclear Staining and DNA Fragmentation in RBL Cells Treated with C_2 -ceramide

To examine whether the morphological changes induced by exogenous C_2 -ceramide were due to

Fig.2 Nuclear staining and DNA fragmentation of RBL-2H3 cells treated with C_2 -ceramide. RBL-2H3 cells were treated with (A) or without (B) $25 \mu M$ C_2 -ceramide for 6 h and then stained with Hoechst 33258 as described in *Materials and Methods*, and photographed under a fluorescent microscope. The photographs shown are representative of at least three independent cultures. For examination of intranucleosomal DNA fragmentation (C), genomic DNA was extracted from the control untreated cells (lane 1) and the cells treated with $25 \mu M$ C_2 -ceramide (lane 2) and $25 \mu M$ Dh C_2 -ceramide (lane 3) for 6 h as described in *Materials and Methods*. Intranucleosomal DNA fragments were visualized by staining gels with SYBR Green. Molecular size marker (123 bp ladder marker) was shown in lane M. The results shown are representative of at least three separate experiments.

apoptosis, cells were stained with Hoechst 33258. As shown in Fig. 2, chromatin condensation and nuclear fragmentation were observed, when cells were treated with $25 \mu M$ C_2 -ceramide for 12 h. Moreover, intranucleosomal DNA fragmentation was observed as DNA ladder by agarose gel electrophoresis (Fig.2C). In contrast, chromatin condensation, nuclear fragmentation and DNA ladder formation were not observed in either the cells treated with Dh C_2 -ceramide ($25 \mu M$) nor the control untreated cells. When the typical apoptotic cells stained with Hoechst 33258 were counted, the percentage of apoptotic RBL cells induced by C_2 -ceramide ($10 \mu M$ and $25 \mu M$) was increased time-dependently with being 52% at 12 h (Fig. 3). On the other hand, the percentages of apoptotic cells in the untreated control cells (FBS-free medium alone) and the cell treated with Dh C_2 -ceramide ($25 \mu M$) were approx. at most 10% at 12 h. These data indicate that C_2 -ceramide induced typical apoptosis of RBL cells.

Fig.3 Time-dependent increase in apoptotic RBL-2H3 cells induced by C_2 -ceramide. The cells were treated with vehicle (□), $10 \mu M$ (◇), $25 \mu M$ (●) of C_2 -ceramide or $25 \mu M$ Dh C_2 -ceramide (△) for the indicated period of time. Typical apoptotic cells stained with Hoechst 33258 were counted among more than 1200 cells. Results are given as means $\pm$ SD from at least three separate experiments.

Fig. 4 PLD activities in membranes of RBL-2H3 cells. (A) GTP γ S-dependent and -independent PLD activities were measured using $[^3\text{H}]\text{DPPC}$ in PE/PIP $_2$ /egg PC vesicles (160/14/10 μM) as substrates. Membranes (70 μg protein) were incubated for the indicated period in the presence of cytosol (70 μg protein) with or without 5 μM rArf, 30 μM GTP γ S, 100 nM PMA as described in *Materials and Methods*. (B) Oleate-dependent PLD activity in membranes (70 μg protein) was measured using egg PC vesicles containing egg PC (750 μM) and 400,000 dpm of $[^3\text{H}]\text{DPPC}$ in the presence of 0.5 mM oleate as described in *Materials and Methods*. PLD activity was determined by measuring formation of $[^3\text{H}]\text{PBut}$. The data shown are represented as the means $\pm$ SD from three separate experiments, each performed in duplicate.

Fig. 6 Effects of cytosols from cells pretreated with C₂-ceramide or DhC₂-ceramide on RBL-2H3 membrane PLD activity. Membranes (70 μg protein) isolated from RBL cells were incubated with cytosol (70 μg protein) from cells pretreated with 25 μM C₂ Ceramide (Cyt-C₂) ($\diamond$) or 25 μM DhC₂-ceramide (Cyt-DhC₂) ($\circ$) for 3 h. GTP γ S-dependent PLD activity was measured using $[^3\text{H}]\text{DPPC}$ in PE/PIP $_2$ /egg PC vesicles (160/14/10 μM) in the presence of 30 μM GTP γ S and 100 nM PMA. PLD activity was determined by measuring formation of $[^3\text{H}]\text{PBut}$ as described in *Materials and Methods*. The data shown are represented as the means $\pm$ SD from two separate experiments, each performed in duplicate.

Fig. 5 Effects of C₂-ceramide or DhC₂-ceramide on GTP γ S-dependent (A), -independent (B) and oleate-dependent (C) PLD activities in membranes. Membranes (70 μg protein) isolated from RBL cells pretreated with 25 μM C₂-ceramide ($\circ$), 25 μM DhC₂-ceramide ($\diamond$) for 3 h or untreated cells ($\square$) were incubated with (A) and without (B) 30 μM GTP γ S and 100 nM PMA in the presence of cytosol (70 μg protein), or with (C) 0.5 mM oleate. GTP γ S-dependent (A) and -independent (B) PLD activities were measured using $[^3\text{H}]\text{DPPC}$ in PE/PIP $_2$ /egg PC vesicles (160/14/10 μM) and oleate-dependent PLD (C) activity was measured $[^3\text{H}]\text{DPPC}$ in egg PC vesicles as substrates. PLD activity was determined by measuring the formation of $[^3\text{H}]\text{PBut}$ as described in *Materials and Methods*. The data shown are represented as the means $\pm$ SD from two separate experiments, each performed in duplicate.

Fig. 7 Effects of C₂-ceramide on the membrane PLD activities. PLD activities (A: GTPγS-dependent, B: oleate-dependent) in membranes (70 μg protein) isolated from the control RBL cells were determined by measuring formation of [³H]PBut in the presence of the indicated concentrations of C₂-ceramide (0–50 μM). The data shown are represented as the means ± SD from three separate experiments, each performed in duplicate.

Changes in PLD Activity During C₂-ceramide-induced Apoptosis in RBL Cells

We have previously shown that C₂-ceramide inhibited IgE-mediated PLD activation in RBL-2H3 cells (36). More recently, we have demonstrated a decrease in the GTPγS-dependent PLD activity during C₂-ceramide-induced apoptosis in C6 glial cells (55). In the present study, changes in PLD activity were examined during the apoptotic process of RBL cells induced by C₂-ceramide.

PLD activity in membranes was measured using the exogenous [³H]DPPC as a substrate. Formation of [³H]PBut, a specific product of transphosphatidylase activity of PLD in the presence of 0.3% butanol, was measured to monitor PLD activity. As previously reported (36, 45, 42), a nonhydrolyzable GTP analog, GTPγS (30 μM) stimulated membrane PLD activity, only when cytosol was added in the reaction mixture. The cytosolic factors were thought to be small G proteins (Arf and Rho family) and PKC (4, 6, 9, 42, 47, 48). Even in the absence of GTPγS, time-dependent [³H]PBut formation was detectable (Fig. 4A, GTPγS(–)), although the magnitude was much less than in the presence of GTPγS. This GTPγS-independent PLD activity did not require cytosol (data not shown) but did PIP₂ in

the substrate mixture. In the absence of PIP₂, [³H]PBut accumulation was not observed (Fig. 4B, control). GTPγS-dependent PLD activity was enhanced by addition of PMA (100 nM), with being highest in the co-existence of PMA (100 nM) and rArf (5 μM) (Fig. 4A, PMA/GTPγS/Arf). This could be PLD1-type activity, which is synergistically activated by small G proteins (Rho family, Arf) and PKC (17). As shown in Fig. 4A, time-dependent [³H]PBut formation was observed in the presence of oleic acid (0.5 mM), suggesting the oleate-dependent PLD activity which has been reported in other cell types (31, 43). This activity was assayed using [³H]DPPC substrate without PIP₂ and cytosol. These results indicated that at least three types of PLD activities are present in RBL membranes; 1) GTPγS-dependent, 2) GTPγS-independent, and 3) oleate-dependent. The former two enzymes require PIP₂ but not the last one.

In membranes isolated from apoptotic cells treated with C₂-ceramide (25 μM) for 3 h, both GTPγS-dependent and -independent [³H]PBut formations were decreased to 4% and 18% of those from control cells, respectively (Figs. 5A, B). Addition of cytosol from control cells failed to restore GTPγS-dependent PLD activity in membranes from apoptotic cells. In contrast, cytosol from either apoptotic or control cells was able to stimulate PLD activity in membranes from control cells to a nearly equal extent (Fig. 6). These results suggest that inhibition of GTPγS-dependent PLD activity in apoptotic cells was caused by change(s) of membrane component(s) but not by cytosolic factor(s). Oleate-dependent PLD activity in membranes from apoptotic cells was also decreased to 25% of that from control cells (Fig. 5C). DhC₂-ceramide, which does not induce apoptosis, did not affect these three types of PLD activities.

Effects of C₂-ceramide on Membrane PLD Activities

The above results indicate that pretreatment of cells with C₂-ceramide diminished three types of PLD activities (GTPγS-dependent, -independent and oleate-dependent). In order to better understand the mechanism underlying decreased PLD activities, the effects of C₂-ceramide on PLD activities *in vitro* were examined in membranes isolated from control cells. C₂-ceramide (0–50 μM) was added to the PLD assay mixture and incubated for 0.5 h. GTPγS-dependent [³H]PBut formation in the presence of PMA was decreased by C₂-ceramide in a concentration-dependent manner (Fig. 7A).

Fig. 8 Changes in mRNA levels of rPLD1a and rPLD1b (A), and rPLD2 (B) during C_2 -ceramide treatment. Levels of mRNAs were analyzed by RT-PCR. Total RNA was isolated from RBL cells pretreated with $25 \mu\text{M}$ C_2 -ceramide at indicated times. RNA ($2 \mu\text{g}$) was reverse-transcribed using random hexamer-mixed primers. cDNA fragments were amplified as described in *Materials and Methods*. Amplified DNA fragments were subjected to electrophoresis on 1.5 % agarose gel and visualized by ethidium bromide staining. The intensity of bands was quantified by a densitometer (Atto Densitograph, Tokyo, Japan). The data shown are represented as means $\pm$ SD from three experiments, each performed in duplicate.

At $50 \mu\text{M}$ C_2 -ceramide, [^3H]PBut formation was decreased by 80 %. GTP γ S-independent PLD activity was also reduced to 15% at $50 \mu\text{M}$ C_2 -ceramide (data not shown). In contrast, the oleate-induced [^3H]PBut formation was unaffected with the same treatment (Fig. 7B). These results suggest that C_2 -ceramide directly affects GTP γ S-dependent and -independent PLD activities but not oleate-dependent PLD activity.

Changes in mRNA Expression of PLD Isozymes in C_2 -ceramide-induced Apoptotic RBL Cells

The changes in the mRNA levels of rat PLD isoforms rPLD1a, rPLD1b and rPLD2 were examined in RBL cells during C_2 -ceramide-induced apoptosis by RT-PCR (Fig. 8). No significant changes were observed in the levels of GAPDH mRNA (reference control) throughout the time course up to 12h. It was observed that the mRNA levels of rPLD1a and rPLD1b time-dependently decreased by C_2 -ceramide treatment. Significant

decrease was detectable as early as 1 h following C_2 -ceramide addition. In contrast, the mRNA level of rPLD2 did not decrease but was rather stable throughout the time course examined. These findings are compatible with our previous results in C6 glial cells (55).

DISCUSSION

It has been reported that mast cell numbers in tissues under normal conditions are relatively constant from individual to individual (15). However, they increase in number in response to a variety of pathogenic conditions (8, 19, 30, 33, 40). The mechanisms of mast cell proliferation and differentiation (maturation) have recently been disclosed. Since cytokines such as IL-3 and stem cell factor regulate these cell responses (5, 26-28, 49), removal of these factors results in apoptosis of tissue mast cells (20, 34). Gamma irradiation, TGF- β and Fas have also been reported to induce apoptosis of mast cells (18, 35, 53). Recently, the involvement of Bruton's tyrosine kinase has been reported in activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) leading to apoptosis of murine mast cells (24). However, little is known about the apoptotic signaling mechanisms in mast cells. The studies of apoptotic pathways in mast cells will provide insight into further understanding of the mechanism of mast cell survival and the control of pathologic increases of mast cells.

In the present study, we have shown that a cell-permeable C_2 -ceramide induced a typical marked apoptosis of RBL cells. The treatment with $25 \mu\text{M}$ C_2 -ceramide resulted in morphological changes; cellular processes were retracted gradually and cells became round. Most of the cells were detached from the culture dishes at 12 h after the C_2 -ceramide treatment. The Hoechst 33258 staining showed typical apoptotic changes such as nuclear fragmentation and chromatin condensation. Intracellular DNA fragmentation was observed as a characteristic ladder pattern when examined by agarose gel electrophoresis. In contrast, an inactive C_2 -ceramide analog, Dh C_2 -ceramide failed to induce apoptosis. Therefore, it is reasonable to consider that ceramide, a product of sphingomyelin hydrolysis by sphingomyelinase, acts as a second messenger of apoptosis in RBL cells as demonstrated in many types of cells (41).

PLD has been believed to play an important role in cellular signaling (14). The receptor-mediated PLD activation is implicated in a variety of cellu-

lar responses; rapid responses (*e.g.* secretion and superoxide generation) as well as long-term responses (*e.g.* proliferation, differentiation and apoptosis). The implication of PLD in proliferation is attributed to the observations that several growth factors stimulate PLD activity and that second messengers (PA, DG and lysoPA) produced by the PLD pathway have the ability to stimulate cell growth. Interestingly, several studies including ours have recently shown that ceramide exerts an inhibitory effect on PLD activity (16, 23, 36, 50). We have previously reported that C_2 -ceramide inhibits the rapid IgE-mediated activation of PLD in RBL cells by blocking Ca^{2+} -influx and PKC translocation to membranes (36). Similar inhibitory action of ceramide in agonist-induced PLD activation has also been observed in fibroblasts stimulated by PA and lysoPA (16) or by bradykinin (50) and neutrophils stimulated by a chemotactic peptide, FMLP (37). C_2 -ceramide was observed to inhibit PLD activity in *in vitro* assay system (50). Ceramide prevented translocation of activators for PLD such as PKC, ARF and RhoA (36, 1). Recently, we have demonstrated that PLD activity was markedly repressed during C_2 -ceramide-induced apoptosis in rat C6 glial cells (55).

RBL cells have at least three types of PLD activities; GTP γ S-dependent and -independent and oleate-dependent. Cytosolic factors such as small G-proteins and PKC are required for the assay of the GTP γ S-dependent PLD activity, whereas the latter two types of do not require cytosol. At present, two mammalian PLDs, (PLD1a, PLD1b and PLD2) have been cloned (10, 11, 17, 29, 38). GTP γ S-dependent PLD activity given here, may be due to PLD1 which is dependent on small G-proteins and PKC (17), and GTP γ S-independent PLD activity could be PLD2 type which is independent of these factors but requires PIP_2 in *in vitro* assay (11, 29). Oleate-dependent activity is distinct from either PLD1 or PLD2, since they are inhibited by oleate (11, 29). Partial purification of oleate-dependent PLD from pig lung has been reported (43), but its molecular nature has not yet been disclosed. In membranes obtained from apoptotic cells treated with C_2 -ceramide (25 μ M for 3h), these three types of PLD activities were observed to be decreased. In contrast, Dh C_2 -ceramide, which does not induce apoptosis, had no effect on these PLD activities.

The mechanism(s) underlying decreased PLD activities during C_2 -ceramide-induced apoptosis was further examined. In *in vitro* assay system,

C_2 -ceramide inhibited GTP γ S-dependent and -independent PLD activities of membranes from control cells. It has been suggested that ceramide may compete with PIP_2 (1) which is an essential cofactor for the assay of GTP γ S-dependent and -independent PLD activities in *in vitro*. Thus, such competition with PIP_2 is one of the possible mechanisms for the decreases in GTP γ S-dependent and -independent PLD activities in C_2 -ceramide-induced apoptosis.

Alternatively, it has been reported that ceramide inhibits translocation of Arf and Rho from cytosol to plasma membrane, leading to a decrease in GTP γ S-dependent PLD activity (1). In the present study, the cytosolic fraction from apoptotic cells was equally potent to that from control cells in the ability to stimulate GTP γ S-dependent membrane PLD activity. Moreover, cytosol from control cells failed to stimulate GTP γ S-dependent membrane PLD activity in apoptotic cells. Thus, the decrease in GTP γ S-dependent PLD activity was thought to be due to alterations in membrane component(s) in apoptotic RBL cells. As an approach to prove this possibility, expression of mRNAs was examined by RT-PCR. We have recently cloned genes encoding PLD isozymes (rPLD1a, rPLD1b, and rPLD2) and determined their chromosomal mapping (38). The mRNA levels of rPLD1a and rPLD1b showed the time-dependent decreases during the C_2 -ceramide treatment (Fig.8). Therefore, it can be assumed that the decrease of GTP γ S-dependent PLD activity during apoptosis might be attributable to down-regulation of rPLD1. We have previously observed similar findings in C6 glial cells treated with C_2 -ceramide (55). Thus, it is tempting to speculate that decrease in PLD1 would be involved in apoptotic process. However, to prove this, the protein levels of the PLD enzymes need to be determined by Western blot analysis, when adequate immunoprecipitable antibodies are available. We are currently preparing them.

GTP γ S-independent PLD activity decreased in spite of no appreciable changes in the mRNA level of rPLD2 during apoptotic process. The decrease in GTP γ S-independent PLD activity could be due to reduction of the protein and/or inhibition by C_2 -ceramide probably through competition with a cofactor, PIP_2 . Unlike GTP γ S-dependent and -independent PLD activities, in *in vitro* assay system, the oleate-dependent PLD activity was not directly affected by C_2 -ceramide. Therefore, decrease in oleate-dependent PLD activity during apoptosis could be caused by down-regulation of PLD pro-

tein itself or by interfering with its activation by some unknown mechanism(s). Oleate-type PLD gene has not yet been cloned and the antibody is not available. In order to clarify the mechanisms of decreased PLD activities, protein levels of PLD isozymes need to be examined, by Western blot analysis using specific antibodies.

In conclusion, the results obtained here have indicated that C₂-ceramide induced apoptosis in RBL cells and nearly abolished three types of PLD activities during apoptosis. Therefore, C₂-ceramide could be implicated in apoptosis of mast cell line RBL cells at least by suppressing PLD activities. However, to prove this possibility, further work including identification of PLD isozymes involved in apoptosis should be required and is under current progress.

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