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# Stimulation by Ceramide of Phospholipase $A_2$ Activation through a Mechanism Related to the Phospholipase C-Initiated Signaling Pathway in Rabbit Platelets[1]

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**To study the involvement of sphingolipids in glycerophospholipid metabolism, the contribution of ceramide to the activation of group IV cytosolic phospholipase $A_2$ ($cPLA_2$) was investigated in platelets using cell-permeable $C_6$-ceramide (*N*-hexanoylsphingosine). The addition of ceramide led to potentiation of thrombin-induced activation of $cPLA_2$ and mitogen-activated protein kinase (MAPK) as well as arachidonic acid release and lysophosphatidylcholine formation. However, ceramide by itself did not induce any response. The arachidonic acid release due to the synergistic action of ceramide and thrombin was inhibited by PD98059, a MAPK kinase inhibitor. Ceramide also stimulated thrombin-induced protein kinase C (PKC) activation, but ceramide by itself failed to do so. Furthermore, ceramide synergistically enhanced diacylglycerol (DAG) formation and $Ca^{2+}$ mobilization with thrombin, and also DAG formation with $Ca^{2+}$-ionophore A23187. The DAG formation in response to ceramide with thrombin or A23187, as well as arachidonic acid release with thrombin were completely inhibited by U73122, a phospholipase C (PLC) inhibitor. These results suggest that ceramide triggers PLC activation through its synergistic action with thrombin, and subsequently potentiates the sequential PKC-MAPK cascade-$cPLA_2$ pathway, thus resulting in enhancement of arachidonic acid release.**

**Key words: ceramide, cytosolic phospholipase $A_2$, mitogen-activated protein kinase, phospholipase C, protein kinase C.**

Receptor-mediated stimulation of the sphingomyelin pathway *via* activation of sphingomyelinase generates biologically active sphingolipid metabolites, such as ceramide and sphingosine, which have been shown to act as cellular second messengers in a variety of signal transduction pathways (reviewed in Refs. *1* and *2*). Ceramide, a product of sphingomyelin hydrolysis, is known to be generated following stimulation with cytokines, growth factors, FAS ligand, and vitamin $D_3$, and is widely accepted to exhibit many biological activities, including cell proliferation, differentiation and apoptosis, in a variety of cells (reviewed in Refs. *3-5*).

To date, evidence has accumulated that receptor-stimulated activation of glycerophospholipid metabolism, which is initiated by the activation of a variety of phospholipases, *e.g.*, phospholipase (PL) $A_2$, C, and D, is linked to the activation of sphingomyelinase-initiated sphingomyelin metabolism, thus suggesting the occurrence of cross-talk between the glycerophospholipid and sphingomyelin signaling pathways. Indeed, cell-permeable $C_6$-ceramide (*N*-hexanoylsphingosine), sphingosine or sphingomyelinase treatment has been shown to stimulate PLD activation in Swiss 3T3 fibroblasts (*6*), neural-derived NG 108-15 cells (*7*), and human fibroblasts (*8*), whereas $C_2$-(*N*-acetylsphingosine) or $C_6$-ceramides prevent the activation in rat or human fibroblasts (*9-12*), rat basophilic leukemia cells (*13*), or HL-60 cells (*14*). Sphingosine has been shown to activate PLC and diacylglycerol kinase in Jurkat T cells (*15*) or airway smooth muscle (*16*). We have shown that sphingosine enhances U46619- and thrombin-induced PLC activation (*17*), and phosphatidylinositol 4-kinase activation (*18*) in rabbit platelets, suggesting the contribution of sphingosine to receptor-stimulated phosphatidylinositol turnover. Chao *et al.* also reported that sphingosine enhances inositol phosphate accumulation by stimulating PLC activity in human foreskin fibroblasts (*19*).

On the other hand, the involvement of sphingolipids in cellular responses associated with $PLA_2$ activation has been reported. $C_2$-Ceramide and sphingosine have been shown to stimulate interleukin-1$\beta$-induced prostaglandin $E_2$ generation concomitant with an increase in the induction of cyclooxygenase mRNA in human dermal fibroblasts (*20*). The $C_2$- and $C_6$-ceramides have also been reported to

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Abbreviations: $C_2$-ceramide, *N*-acetylsphingosine; $C_6$-ceramide, *N*-hexanoylsphingosine; $cPLA_2$, group IV cytosolic phospholipase $A_2$; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PL, phospholipase.

stimulate the expression of cyclooxygenase 2 and $cPLA_2$ mRNAs in murine fibrosarcoma L929 cells (*21*). On the contrary, it has been shown that arachidonic acid liberated upon stimulation with tumor necrosis factor $\alpha$ mediates ligand-induced sphingomyelinase activation followed by ceramide generation in HL-60 cells (*22*) and L929 murine fibroblast cell line (*23*), suggesting that $PLA_2$ plays an important role in the sphingolipid signaling pathway.

Recently, we showed that sphingosine and $C_6$-ceramide enhance thrombin- and U46619-induced arachidonic acid liberation in rabbit platelets (*24, 25*). In the present study, we extended our recent studies by defining the mechanism by which ceramide enhances thrombin-stimulated arachidonic acid liberation using cell-permeable $C_6$-ceramide in rabbit platelets. Although the activation mechanism is well characterized in platelets, the step of receptor-stimulated arachidonic acid liberation followed by thromboxane formation is a critical one for platelet activation. Several studies have demonstrated that the thrombin-stimulated arachidonic acid liberation is caused by the catalytic action of group IV cytosolic $PLA_2$ ($cPLA_2$) (*26-28*). Furthermore, it has been reported that phorbol 12,13-dibutyrate, a protein kinase C (PKC) activator, phosphorylates mitogen-activated protein kinase (MAPK), which in turn results in the phosphorylation of $cPLA_2$ in platelets (*29*), suggesting that $cPLA_2$ activation is regulated, at least in part, by PKC through MAPK.

Therefore, in the present work we focused on the involvement of ceramide in thrombin-induced $cPLA_2$ activation *via* the PLC-initiated sequential pathway including PKC and MAPK. Although platelets generate little ceramide upon stimulation because of the low activity of sphingomyelinase, they seem to be more suitable for investigating the effect of ceramide, since in platelets the effect of ceramide-induced signals to the nucleus on the enzyme activation can be excluded.

## MATERIALS AND METHODS

*Materials*—$C_6$-Ceramide (*N*-hexanoylsphingosine) and phorbol 12-myristate 13-acetate were obtained from Sigma. A23187 and PD98059 (2′-amino-3′-methoxyflavone) were from Calbiochem (La Jolla, CA, USA). Thrombin (bovine plasma) was from Mochida Pharmaceuticals (Tokyo), fura 2-pentaacetoxymethyl ester from Dohjin Chemicals (Kumamoto), U73122 ((1-[6-[[17$\beta$-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]-hexyl]-1*H*-pyrrole-2) from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA), and methyl arachidonyl fluorophosphonate from Cayman Chemical (Ann Arbor, MI, USA). [$^3$H]-Arachidonic acid (100 Ci/mmol), [$^3$H]glycerol (18.2 Ci/mmol), and 1-stearoyl-2-[$^3$H]arachidonoyl-glycerophosphocholine (160 Ci/mmol) were from New England Nuclear (Boston, MA, USA). Other reagents were from Nacalai Tesque (Kyoto) or Wako Pure Chemical Industries (Osaka).

*Preparation of Washed Rabbit Platelets*—Platelet-rich plasma was obtained from rabbit blood anticoagulated with a one-tenth volume of 1% EDTA by centrifugation at 230 × *g* for 10 min. Platelets were sedimented by centrifugation of the platelet-rich plasma at 800 × *g* for 15 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 2.9 mM $NaH_2PO_4$, 5.6 mM glucose, 0.35% BSA, and 3.8 mM HEPES, pH 6.5) containing 0.4 mM EGTA. The washed platelets were resuspended in HEPES buffer (pH 7.4).

*Lipid Metabolism*—Platelet-rich plasma was incubated with [$^3$H]arachidonic acid (1 $\mu$Ci/ml) or [$^3$H]glycerol (60 $\mu$Ci/ml) at 37°C for 1.5 h, and then washed as described above. The labeled platelets ($5 \times 10^8$ cells/ml) were pretreated with 100 $\mu$M BW755C, a cyclooxygenase and lipoxygenase inhibitor (*30*), at 37°C for 2 min in the presence of 1 mM $CaCl_2$. The platelets were further treated with $C_6$-ceramide and then stimulated with various agonists. When platelets were treated with PD98059 or U73122, the inhibitor was added before the treatment with $C_6$-ceramide. The reaction was terminated by adding chloroform/methanol/HCl (200:200:1, v/v/v). Lipids were extracted and separated by TLC on silica gel G plates with the following development systems: for the analysis of arachidonic acid and diacylglycerol, petroleum ether/diethyl ether/acetic acid (40:40:1, v/v/v); and for the analysis of lysophosphatidylcholine, chloroform/methanol/$H_2O$ (65:35:6, v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

*Measurement of the Activities of $cPLA_2$, MAPK, and PKC*—Washed platelets ($5 \times 10^8$ cells/ml) were treated with $C_6$-ceramide in the presence of 1 mM $CaCl_2$, and then stimulated with thrombin. After centrifugation in the presence of 3 mM EGTA, the platelets were washed and lysed by sonication in lysis buffer consisting of 100 mM NaCl, 20 mM $\beta$-glycerophosphate, 1 mM $Na_3VO_4$, 2 mM EGTA, 100 $\mu$M leupeptin, 100 $\mu$M *p*-(amidinophenyl)-methanesulfonyl fluoride, and 10 mM Tris-HCl (pH 7.4). The lysate was centrifuged at 100,000 × *g* for 1 h at 4°C, and the pellet was resuspended in the lysis buffer. The supernatant was subjected to assaying for the activity of $cPLA_2$ or MAPK. The pellet was subjected to PKC assaying. The protein concentration of each sample was determined with a commercial assay kit (Pierce, Rockford, IL). For the $cPLA_2$ assay, the supernatant was treated with 5 mM dithiothreitol to inhibit secretory $PLA_2$ activity (*31*). $cPLA_2$ activity in the supernatant was determined by incubation of the supernatant with a mixture of 1-stearoyl-2-[$^3$H]arachidonoylglycerophosphocholine and the unlabeled compound (25 Ci/mol, 10 $\mu$M) as a substrate at 37°C for 5 min in the presence of 5 mM $CaCl_2$ and 100 mM Tris-HCl (pH 8.5). [$^3$H]Arachidonic acid liberated was determined as described by Sundaram *et al.* (*32*). The MAPK activity in the supernatant and the PKC activity in the pellet were measured using commercial assay kits (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

*Measurement of $Ca^{2+}$ Mobilization*—Washed platelets were incubated with fura 2-pentaacetoxymethyl ester (2 $\mu$M), a fluorescent dye, at 37°C for 30 min. The fura 2-loaded platelets were washed twice, and then resuspended in HEPES buffer (pH 7.4). The platelets ($1.25 \times 10^8$ cells/ml) were treated with $C_6$-ceramide in the presence of 1 mM $CaCl_2$, and then stimulated with thrombin. The fluorescence of the reaction mixture was continuously monitored with a spectrofluorometer (F-2000, Hitachi), with excitation at 340 and 380 nm, and emission at 500 nm. The intracellular $Ca^{2+}$ concentration was calculated according to the method of Grynkiewicz *et al.* (*33*).

## RESULTS

*Potentiation of Receptor-Mediated cPLA$_2$ Activation*—To investigate the cross-talk between glycerophospholipid and sphingolipid metabolism, the effect of ceramide on receptor-mediated signal transduction for cPLA$_2$ activation was investigated in platelets. Cell-permeable C$_6$-ceramide, when added to platelets, did not induce any arachidonic acid release, but potentiated thrombin-stimulated release dose-dependently, as shown in Fig. 1A. We confirmed that the arachidonic acid liberation induced by thrombin or by thrombin and ceramide was completely inhibited by methyl arachidonyl fluorophosphonate, an inhibitor of cPLA$_2$ (*34*) (data not shown). Under the conditions used thrombin-induced lysophosphatidylcholine production was also stimulated significantly (Fig. 1B). These results suggest that C$_6$-ceramide enhances cPLA$_2$ activation through its synergistic action with thrombin. Actually, as shown in Fig. 2A, thrombin-induced cPLA$_2$ activation was significantly increased by C$_6$-ceramide, although C$_6$-ceramide by itself did not induce such an effect. Since the activation of cPLA$_2$ is known to be regulated by MAPK (p42/44 MAPK)-catalyzed phosphorylation (*35*), we examined the effect of ceramide on MAPK activity. The results in Fig. 2B show that C$_6$-ceramide apparently enhanced thrombin-induced MAPK activation.

Furthermore, we provided evidence that PD98059, a MAPK kinase inhibitor (*36*), dose-dependently abolished the synergistic effect of C$_6$-ceramide on thrombin-induced arachidonic acid liberation (Fig. 3). Under these conditions, it was confirmed that the MAPK activity enhanced by ceramide and thrombin was completely returned to the control level by 200 $\mu$M PD98059 (data not shown). These findings suggest that MAPK activation is actually involved in the ceramide-induced cPLA$_2$ activation. However, the

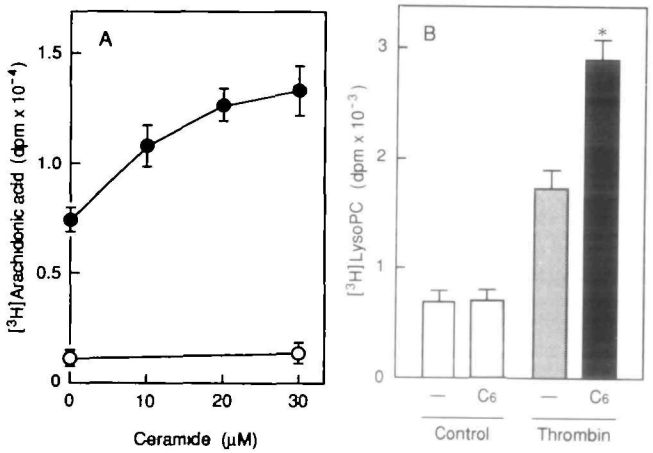


Fig. 1. **Stimulation of thrombin-induced arachidonic acid liberation (A) and lysophosphatidylcholine production (B).** (A) [$^3$H]Arachidonic acid-labeled platelets ($5\times10^8$ cells/ml) were pretreated with 100 $\mu$M BW755C at 37°C for 2 min in the presence of 1 mM $CaCl_2$, and then incubated with various concentrations of C$_6$-ceramide for 1 min before stimulation with (●) or without (○) 0.1 U/ml thrombin for a further 2 min. (B) [$^3$H]Glycerol-labeled platelets ($5\times10^8$ cells/ml) were treated with BW755C as described above, and then incubated with ethanol (−) or 30 $\mu$M C$_6$-ceramide (C$_6$) for 1 min before stimulation with (Thrombin) or without (Control) 0.1 U/ml thrombin for 2 min. The [$^3$H]arachidonic acid released (A) or [$^3$H]-lysophosphatidylcholine produced (B) was determined as described under "MATERIALS AND METHODS." The results are the means±SE for three experiments. *$p<0.01$, relative to the cells exposed to thrombin without ceramide.

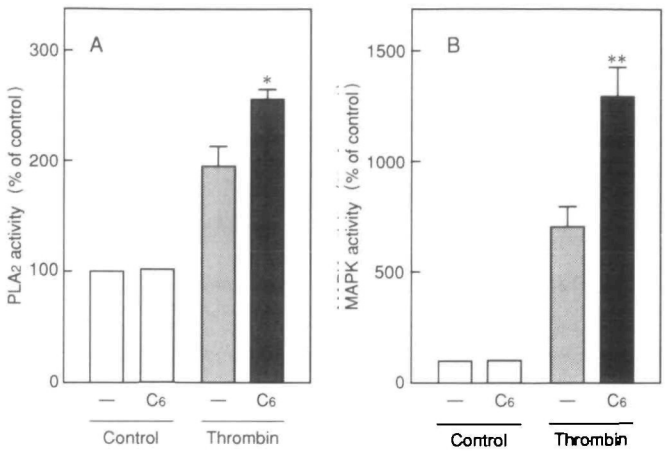


Fig. 2. **Stimulation of thrombin-induced cPLA$_2$ (A) and MAPK (B) activation.** Washed platelets ($5\times10^8$ cells/ml) were treated with ethanol (−) or 30 $\mu$M C$_6$-ceramide (C$_6$) at 37°C for 1 min, and then stimulated with (Thrombin) or without (Control) 0.1 U/ml thrombin for 2 min. The activities of cPLA$_2$ (A) and MAPK (B) in the supernatant, which was obtained by centrifugation of the cell lysate at 100,000×$g$ for 30 min at 4°C, were determined as described under "MATERIALS AND METHODS." The results are expressed relative to the activities in intact cells (0.39±0.01 nmol/min/mg protein for cPLA$_2$, and 4.36±0.03 pmol/min/mg protein for MAPK, $n=3$), and are the means±SE for three experiments. *$p<0.01$ and **$p<0.05$, relative to the cells exposed to thrombin without ceramide.

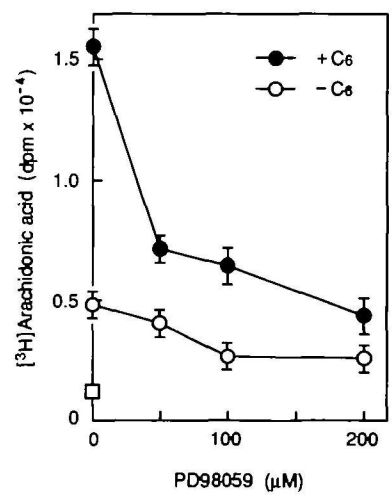


Fig. 3. **Inhibitory effect of PD98059 on arachidonic acid liberation due to the synergistic action of ceramide and thrombin.** [$^3$H]Arachidonic acid-labeled platelets ($5\times10^8$ cells/ml) were pretreated at 37°C with various concentrations of PD98059 for 15 min and then with 100 $\mu$M BW755C for 2 min in the presence of 1 mM $CaCl_2$. Then, the cells were treated with ethanol (○) or 30 $\mu$M C$_6$-ceramide (●) for 1 min, and then stimulated with 0.1 U/ml thrombin for a further 2 min. The [$^3$H]arachidonic acid released was determined as described under "MATERIALS AND METHODS." The results are the means±SE for three experiments. □: intact cells.

results in Fig. 2, in which we show that ceramide by itself failed to activate $cPLA_2$ and MAPK, suggests that ceramide affects a factor upstream of the MAPK cascade.

*Stimulation of PKC Activation*—It has been shown that ceramide can activate PKC (PKCζ), which results in activation of the MAPK cascade (*37-39*). We tried to determine, therefore, the effect of ceramide on PKC activity, and obtained the results shown in Fig. 4, *i.e.*, $C_6$-ceramide accelerated thrombin-induced PKC activation, suggesting the possibility that ceramide has a potentiating effect on PKC activation. However, $C_6$-ceramide by itself did not activate PKC (Fig. 4). Furthermore, since phorbol ester is known to cause MAPK-stimulated $cPLA_2$ phosphorylation (*29*), we investigated the effect of ceramide on phorbol 12-myristate 13-acetate-stimulated $cPLA_2$ activation. The results showed that ceramide did not exert such an effect (data not shown). Collectively, these results indicate that ceramide might act as an upstream activator of PKC in the ceramide-stimulated $cPLA_2$ pathway.

*Potentiation of Thrombin-Stimulated PLC Activation*—To examine the mechanism by which thrombin-induced PKC activation is enhanced by ceramide, we investigated the effect of ceramide on the activation of PLC, which in turn causes the accumulation of diacylglycerol (DAG) to induce PKC activation. As shown in Fig. 5, $C_6$-ceramide by itself did not generate any DAG but it potentiated the

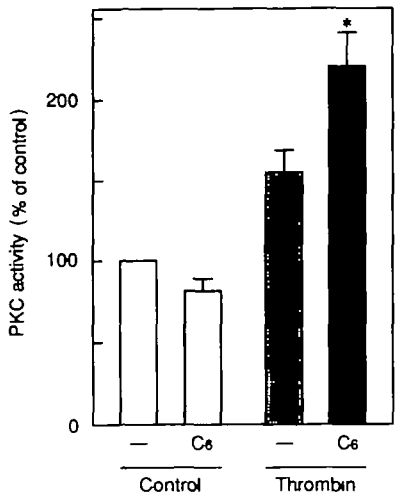


Fig. 4. **Stimulation of thrombin-induced PKC activation.** Washed platelets ($5\times10^8$ cells/ml) were treated with ethanol (−) or 30 μM $C_6$-ceramide ($C_6$) at 37°C for 1 min, and then stimulated with (Thrombin) or without (Control) 0.1 U/ml thrombin for 2 min. The activity of PKC in the pellet, which was obtained by centrifugation of the cell lysate at $100,000\times g$ for 30 min at 4°C, was determined as described under "MATERIALS AND METHODS." The results are expressed relative to the activity in intact cells (20.0 pmol/min/mg protein, the means for three experiments), and are the means ± SE for three experiments. $^*p<0.05$, relative to the cells exposed to thrombin without ceramide.

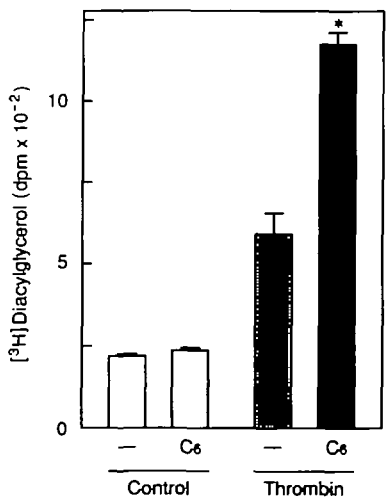


Fig. 5. **Stimulation of thrombin-induced DAG generation.** [$^3$H]Arachidonic acid-labeled platelets ($5\times10^8$ cells/ml) were pretreated with 100 μM BW755C at 37°C for 2 min in the presence of 1 mM $CaCl_2$, and then incubated with 30 μM $C_6$-ceramide ($C_6$) for 1 min before stimulation with (Thrombin) or without (Control) 0.1 U/ml thrombin for a further 2 min. The [$^3$H]DAG generated was determined as described under "MATERIALS AND METHODS." The results are the means ± SE for three experiments. $^*p<0.01$, relative to the cells exposed to thrombin without ceramide.

TABLE I. **Stimulation of thrombin-induced $Ca^{2+}$ mobilization.** Fura 2-loaded platelets ($5\times10^8$ cells/ml) were pretreated with ethanol (Thrombin) or 30 μM $C_6$-ceramide (Thrombin + $C_6$) at 37°C for 1 min, and then stimulated with 0.1 U/ml thrombin. The intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) were determined as described under "MATERIALS AND METHODS." The results represent the concentrations at the peak and 2 min after stimulation, and are the means ± SE for three experiments. $^*p<0.05$ and $^{**}p<0.01$, relative to the cells exposed to thrombin without ceramide.

| Addition | $[Ca^{2+}]_i$ | |
|---|---|---|
| | Peak | 2 min after |
| Thrombin | 804 ± 15 | 692 ± 44 |
| Thrombin + $C_6$ | 925 ± 28* | 851 ± 44** |

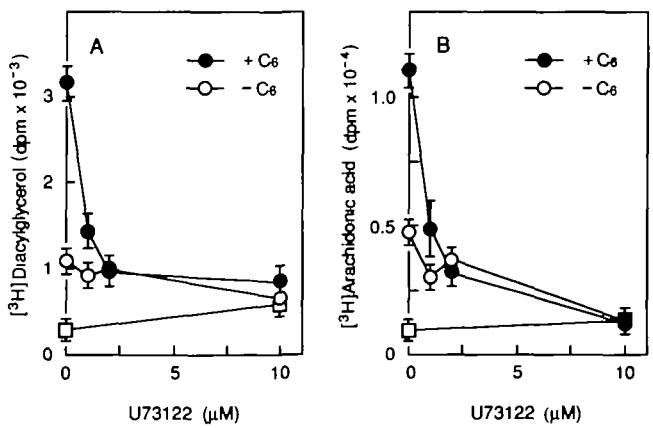


Fig. 6. **Inhibitory effects of U73122 on DAG generation (A) and arachidonic acid liberation (B) due to the synergistic action of ceramide and thrombin.** [$^3$H]Arachidonic acid-labeled platelets ($5\times10^8$ cells/ml) were pretreated with various concentrations of U73122 at 37°C for 15 min and then with 100 μM BW755C for 2 min in the presence of 1 mM $CaCl_2$. Then, the cells were treated with ethanol (○) or 30 μM $C_6$-ceramide (●) for 1 min, and then stimulated with 0.1 U/ml thrombin for a further 2 min. The [$^3$H]DAG generated (A) and [$^3$H]arachidonic acid released (B) were determined as described under "MATERIALS AND METHODS." The results are the means ± SE for three experiments. □: intact cells.

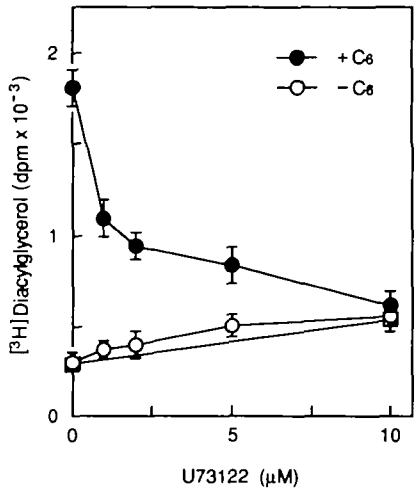


Fig. 7. **Inhibitory effect of U73122 on ceramide-induced DAG generation upon stimulation with A23187.** [$^3$H]Arachidonic acid-labeled platelets ($5\times10^8$ cells/ml) were pretreated with various concentrations of U73122 at 37°C for 15 min and then with 100 $\mu$M BW755C for 2 min in the presence of 1 mM $CaCl_2$. Then, the cells were treated with ethanol (○) or 30 $\mu$M $C_6$-ceramide (●) for 1 min, and then stimulated with 200 nM A23187 for a further 2 min. The [$^3$H]DAG generated was determined as described under "MATERIALS AND METHODS." The results are the means±SE for three experiments. □: intact cells.

generation on stimulation with thrombin synergistically. In addition, $C_6$-ceramide increased the intracellular $Ca^{2+}$ concentration, that peaked after exposure to thrombin, and suppressed the subsequent decrease by 2 min (Table I). This observation is consistent with the results reported by Wong and Li (*40*). These data suggest that ceramide potentiated thrombin-induced PLC activation. This was confirmed by the results in Fig. 6A, *i.e.*, U73122, a PLC inhibitor (*41*), completely inhibited the DAG generation in response to $C_6$-ceramide, and thrombin. We examined further whether or not PLC activation enhanced by ceramide is a trigger stimulating the downstream signal pathway to $cPLA_2$ activation. For this purpose, the effect of U73122 on arachidonic acid release due to the synergistic action of $C_6$-ceramide with thrombin was examined. As shown in Fig. 6B, U73122 dose-dependently suppressed the release similar to the inhibitory pattern of DAG generation (Fig. 6A).

*Synergistic Action with A23187 as to PLC Activation*—There is a possibility that the enhancement of PLC activation due to the synergistic action of ceramide and thrombin results from augmentation of thrombin binding to the receptor. Therefore, we used $Ca^{2+}$-ionophore A23187, which stimulates platelets through a non-receptor mechanism. When A23187 was added to platelets, little generation of DAG was observed, but stimulation with A23187 in the presence of $C_6$-ceramide led to a remarkable increase in DAG generation (0 $\mu$M U73122 in Fig. 7), which was dose-dependently inhibited by U73122 (Fig. 7), suggesting that ceramide could elicit PLC activation through a mechanism independent of receptor-stimulation.

## DISCUSSION

Although a number of studies have demonstrated a close relationship between the glycerophospholipid and sphingolipid signaling pathways, the role of sphingolipids in receptor-mediated signal transduction for $PLA_2$ activation, which triggers arachidonic acid metabolism, remains poorly understood. It has been reported, however, that arachidonic acid released subsequent to the initial activation of $cPLA_2$ upon stimulation with tumor necrosis factor $\alpha$ mediates the generation of ceramide, which leads to cell death (*22, 23*). While this finding suggests that $cPLA_2$ plays a central role in the triggering of sphingolipid signaling, we demonstrated that the addition of cell-permeable ceramide enhances thrombin- or U46619-stimulated arachidonic acid release, suggesting the contribution of sphingolipid to the $PLA_2$-initiated arachidonic acid metabolism in platelets (*25*). However, the precise mechanism underlying ceramide-induced $PLA_2$ activation remains unexplored.

In the present work, we showed that cell-permeable $C_6$-ceramide remarkably potentiated thrombin-stimulated arachidonic acid liberation through stimulation of $cPLA_2$ activation. Although the precise mechanism underlying ceramide-induced $cPLA_2$ activation was not fully elucidated in the present work, our data suggest that ceramide synergistically potentiates thrombin-induced PLC activation, and subsequently causes the accumulation of DAG and inositol phosphate, which in turn stimulate PKC activation and intracellular $Ca^{2+}$ mobilization, respectively. The activation of PKC leads to stimulation of the downstream MAPK cascade, which subsequently increases $cPLA_2$ activity through phosphorylation of the enzyme, while an increase in the intracellular $Ca^{2+}$ concentration stimulates the translocation of $cPLA_2$ to membranes. Consequently, $cPLA_2$ can hydrolyze membrane phospholipids to release arachidonic acid.

The possible involvement of the MAPK cascade in the mechanism is supported by the present finding that PD98059, a MAPK kinase inhibitor, completely inhibited the arachidonic acid liberation induced by ceramide and thrombin. A number of studies have revealed that cell-permeable ceramide could induce MAPK activation in HL-60 cells (*42*), Swiss 3T3 fibroblasts (*43*), endothelial cells (*44*), and vascular smooth muscle cells (*45*). Furthermore, ceramide has been shown to induce the phosphorylation of Raf-1, which is known to act as an upstream activator of the MAPK cascade, through ceramide-activated protein kinase, thus evoking MAPK activation (*46, 47*). However, our data showed that ceramide by itself could not induce MAPK activation or $cPLA_2$ activation. These results indicate that in platelets the ceramide-induced signaling pathway *via* ceramide-activated protein kinase is not involved in ceramide-induced MAPK activation, and suggest that ceramide might act on a factor upstream of the MAPK cascade.

It has been shown that ceramide could phosphorylate and activate PKC$\zeta$, which in turn induces MAPK activation (*37-39*). Actually, the present results indicated that ceramide potentiated thrombin-induced PKC activation. However, ceramide by itself did not have any effect on PKC activity, and, furthermore, ceramide did not enhance

phorbol 12-myristate 13-acetate-induced $cPLA_2$ activation (data not shown). Therefore, we suggest that ceramide is not a potent activator for PKC in platelets, and that the activation of PKC is not the step which triggers the sequential ceramide-$cPLA_2$ pathway.

However, we showed that ceramide potentiated DAG generation through its synergistic action with thrombin, although it was not induced by ceramide by itself. Similarly, ceramide apparently increased DAG generation on stimulation with a $Ca^{2+}$ ionophore, A23187. These results obviously indicate that ceramide could potentiate thrombin-stimulated PLC activation without a receptor-mediated mechanism. We also demonstrated that U73122, a potent PLC inhibitor, completely abolished the synergistic effect of ceramide on thrombin-induced arachidonic acid liberation in parallel with the inhibition of DAG formation. U73122 was also shown to suppress the DAG formation on stimulation with ceramide and A23187. These data imply that ceramide potentiates thrombin-induced PLC activation, and subsequently the sequential activation of PKC-MAPK cascade-$cPLA_2$ leads to arachidonic acid liberation. We showed in the present work that ceramide facilitated and sustained the thrombin-induced increase in the intracellular $Ca^{2+}$ concentration, it being apparent that this effect of ceramide contributes to the induction of arachidonic acid liberation.

Although the mechanism by which PKC stimulates the downstream MAPK cascade has not been fully elucidated, it was recently reported that MAPK is activated through a PKC$\alpha$-Raf-1-dependent pathway in monocytic leukemia cells (*48*). Since PKC$\alpha$ activation is dependent on DAG as well as $Ca^{2+}$, the pathway including PKC$\alpha$ and Raf-1 may be involved in the mechanism underlying the PKC-MAPK pathway examined in the present work.

Several studies have shown that sphingosine-1-phosphate could activate MAPK in airway smooth muscle (*49*) and U937 cells (*50*). Therefore, the possibility exists in the present study that ceramide added to platelets is metabolized to sphingosine-1-phosphate, which in turn stimulates MAPK, followed by $cPLA_2$ activation. However, since in platelets ceramide has been shown not to be converted to other sphingolipid metabolites in 2-3 h (*51*), such a mechanism should be ruled out under our experimental conditions, in which ceramide was added 1 min prior to stimulation with thrombin, followed by incubation for 2 min.

The mechanism underlying the synergistic effect of ceramide on thrombin-stimulated PLC activation remains obscure. It seems possible to consider that the addition of ceramide leads to perturbation of membrane phospholipids, which in turn increases the accessibility of PLC as well as $PLA_2$ to the phospholipid bilayer and the resultant hydrolyzing activity towards phospholipids (*52*). However, such a mechanism seems unlikely, because in our previous study we found that other ceramide analogues, such as dihydro $C_6$-ceramide or $C_2$-ceramide, exhibited such an effect on thrombin-induced arachidonic acid liberation (*25*). Further studies are needed to determine the precise mechanism.

Overall, we conclude that ceramide triggers PLC activation through its synergistic action with thrombin, and subsequently activates PKC and the downstream MAPK cascade, which induces $cPLA_2$ activation, with concomitant acceleration of intracellular $Ca^{2+}$ mobilization, thus resulting in stimulation of arachidonic acid release. These observations may reflect a physiological role of ceramide through activation of such sequential pathways in receptor-mediated signal transduction.

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