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Sphingosine Enhances Arachidonic Acid Liberation in Response to U46619 through an Increase in Phospholipase A_2 Activity in Rabbit Platelets

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Treatment of rabbit platelets for 1 min with 10-15 μM sphingosine enhanced arachidonic acid liberation after stimulation with U46619, although sphingosine or U46619 alone elicited little liberation of the lipid. Thrombin-induced arachidonic acid liberation was not influenced by sphingosine up to 10 μM, and was suppressed at concentrations higher than 20 μM. Sphingosine also promoted lysophosphatidylcholine formation in response to U46619, indicating that sphingosine caused an increase in hydrolytic action of phospholipase A_2 (PLA_2). The enhancing effect of sphingosine on arachidonic acid liberation decreased with increases in pretreatment time, accompanied by the conversion of sphingosine to sphingosine-1-phosphate. Of various sphingosine derivatives examined, sphingosine-1-phosphate, *N,N*-dimethylsphingosine, and *N*-acetylsphingosine (C_2-ceramide) showed little enhancing effect on arachidonic acid liberation. Sphingosine increased cytosolic PLA_2 activity in response to U46619 with enhancement of mitogen-activated protein kinase activity. These results indicate that sphingosine potentiates the transduction process of stimulus-PLA_2 activation, resulting in enhancement of arachidonic acid liberation.

Key words: arachidonic acid, mitogen-activated protein kinase, phospholipase A_2, platelet, sphingosine.

Membrane lipid metabolism is involved in receptor-operated signal transduction. Binding of exogenous agonists to the receptor leads to activation of phospholipase (PL) A_2, C, and D, the products of which act as second messengers. Recently, physiological metabolites of sphingolipids such as sphingosine, sphingosine-1-phosphate (Sph-1-P) and ceramide have been implicated in diverse cellular functions (*1-3*). The actions of these metabolites from glycerophospholipids and sphingolipids are considered to be closely related to operation of other lipid metabolic enzymes by positive or negative feedback mechanisms in the process of signal transduction.

Our previous studies showed that sphingosine activates phosphatidylinositol 4-kinase, which increases phosphatidylinositol 4-phosphate and phosphatidylinositol diphosphate levels (*4*), and enhances agonist-induced PLC activation, resulting in an increase in inositol 1,4,5-trisphosphate level (*5*). Other investigators have shown that sphingosine regulates the activation of enzymes involved in lipid metabolism, *i.e.*, PLD (*6*), phosphatidic acid phosphohydrolase (*7*), and diacylglycerol kinase (*8*). Thus, it is now clear that sphingosine influences several enzymes involved in phospholipid metabolism.

Upon stimulation, platelets release eicosanoids as mediators in signal amplification and blood clotting. Arachidonic acid, a precursor of the eicosanoids, is released from the *sn*-2 position of phospholipids by PLA_2 and metabolized by cyclooxygenase or lipoxygenase. In platelets, two types of PLA_2 have been identified: the 85-kDa PLA_2 which exists in the cytosolic fraction, and the 14-kDa PLA_2 which exists in cellular granules and is secreted into the extracellular medium upon stimulation (*9, 10*). Cytosolic PLA_2 requires submicromolar Ca^{2+} for activation (*11*), and phosphorylation of the enzyme by mitogen-activated protein (MAP) kinase or other kinases increases its activity (*12-14*).

Although sphingosine by itself does not elicit arachidonic acid liberation in platelets (*4*), little attention has been paid to the influence of sphingolipids on the liberation of arachidonic acid in response to agonists. We investigated the effects of sphingosine on arachidonic acid liberation by platelets.

MATERIALS AND METHODS

Materials—Sphingosine (from bovine brain sphingomyelin) and *N*-acetylsphingosine (C_2-ceramide) were purchased from Sigma Chemical (St. Louis, MO, USA), and sphingosine-1-phosphate (Sph-1-P) and *N,N*-dimethylsphingosine were from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Sphingolipids other than Sph-1-P were dissolved in ethanol at 300-fold higher than the final concentration (final ethanol concentration, 0.33%), and Sph-1-P was dissolved in methanol or BSA solution. [^{3}H]Arachidonic acid (76 Ci/mmol), [^{3}H]glycerol (19.4 Ci/mmol), and 1-stearoyl-2-[^{3}H]arachidonoyl-*sn*-glycero-

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Abbreviations: MAP kinase, mitogen-activated protein kinase; PL, phospholipase; Sph-1-P, sphingosine-1-phosphate.

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3-phosphocholine (160 Ci/mmol) were obtained from DuPont/NEN (Boston, MA, USA). [^{3}H]Sphingosine (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$) was obtained from Cayman Chemical (Ann Arbor, MI, USA). RHC80267 was obtained from BIOMOL Research Laboratories and dissolved in dimethyl sulfoxide. The silica gel 60 plates for TLC were from Merck (Darmstadt, Germany). Other reagents were obtained from commercial sources.

Preparation of Platelets—Fresh rabbit blood anti-coagulated with 0.1 volume of 1% EDTA was centrifuged at 230×*g* for 10 min at room temperature to obtain platelet-rich plasma. The platelets separated from the platelet-rich plasma were washed twice as described previously (*15*). Finally, the platelet suspension was adjusted to 5×10^8 cells/ml in modified Tyrode-HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 2.9 mM NaH_2PO_4, 3.8 mM HEPES, pH 7.35) containing 0.35% BSA.

Lipid Analysis—The platelet-rich plasma was incubated at 37°C with [^{3}H]arachidonic acid (2 μCi/ml) for 1.5 h or with [^{3}H]glycerol (80 μCi/ml) for 3 h, and washed as described above. In measurement of arachidonic acid liberation, the platelets were pretreated with 100 μM BW755C (3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline, an inhibitor of both cyclooxygenase and lipoxygenase). After labeled platelets had been treated with various agents, the reaction was terminated by addition of chloroform/methanol/HCl (200 : 200 : 1, by volume), then the lipids were extracted. Arachidonic acid, 1,2-diacylglycerol and phosphatidic acid derived from [^{3}H]arachidonic acid-labeled platelets and lysophosphatidylcholine from [^{3}H]glycerol-labeled platelets were determined by TLC on silica gel 60 plates as described previously (*16, 17*).

Metabolism of Sphingosine—Washed platelets were incubated at 37°C for various times with 1 μM [^{3}H]sphingosine (0.1 μCi), then the reaction was terminated by addition of chloroform/methanol/HCl (200 : 200 : 1, by volume). The lipids were extracted and separated by TLC developed with butanol/acetic acid/water (3 : 1 : 1) (*18*). Sphingosine and Sph-1-P fractions, which were identified by comigration with authentic standards, were scraped off, then the radioactivities were determined.

Assay of MAP Kinase and PLA_2 Activities—Washed platelets were incubated at 37°C with 10 μM sphingosine and 2 μM U46619, then the mixture was centrifuged at 1,500×*g* for 10 min at 4°C in the presence of 3 mM EDTA. The pellets were washed once and resuspended in 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM EGTA, 100 μM leupeptin, 100 μM *p*-(amidinophenyl)methanesulfonyl fluoride. The cells were lysed by sonication and centrifuged at 100,000×*g* for 1 h at 4°C; then the resulting supernatant was subjected to the following assays. MAP kinase activity was determined with a kinase assay kit (Amersham International; Amersham, Bucks., UK). To measure cytosolic PLA_2 activity, the supernatant was treated with 5 mM dithiothreitol for 10 min to inhibit secretory PLA_2 activity, then incubated at 37°C for 5 min with 2 μM 1-stearoyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine as a substrate in the presence of 5 mM $CaCl_2$ and 100 mM Tris-HCl, pH 8.5. The released [^{3}H]arachidonic acid was determined as described by Sundaram *et al.* (*19*). The protein concentration in each sample was determined by the method of Lowry *et al.* (*20*).

RESULTS

Effect of Sphingosine on Arachidonic Acid Liberation in Response to U46619 or Thrombin—To determine the effect of sphingosine on arachidonic acid liberation induced by agonists, we used U46619, which induces little liberation, and thrombin, which evokes marked liberation through PLA_2 activation. As shown in Fig. 1A, when [^{3}H]arachidonic acid-labeled platelets were incubated at 37°C for 1 min with sphingosine and then exposed to 2 μM U46619, which induces full aggregation, sphingosine at 10-15 μM markedly enhanced the arachidonic acid liberation. This effect declined with increasing sphingosine concentration. In the case of thrombin stimulation, sphingosine at 10 μM failed to enhance the response and exerted a suppressive effect at higher concentrations of 20-30 μM (Fig. 1B). Sphingosine itself up to 30 μM had no effect on the amount of free [^{3}H]arachidonic acid released.

Activation of Cytosolic PLA_2 by Sphingosine and U46619—Arachidonic acid liberation is known to be induced by the action of PLA_2 or diacylglycerol lipase (*10, 21*). To ascertain which enzyme contributed to the enhancement of arachidonic acid liberation shown in Fig. 1A, formation of lysophosphatidylcholine, another product of PLA_2, was examined (Fig. 2). Neither sphingosine nor U46619 alone induced the formation of lysophosphatidylcholine, whereas a combination of 10 μM sphingosine and U46619 significantly evoked the formation of this product. This result is in agreement with the observations of arachidonic acid liberation.

Sphingosine increases 1,2-diacylglycerol level through enhancement of PLC activation induced by U46619 (*5*). In addition, sphingosine activates PLD (*6*), resulting in an

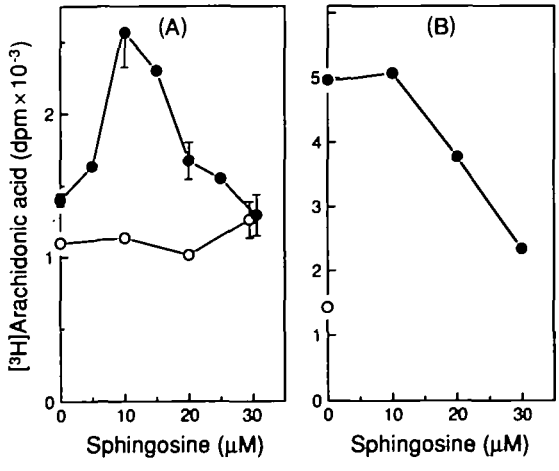

Fig. 1. **Effect of sphingosine on arachidonic acid liberation induced by U46619 or thrombin.** [^{3}H]Arachidonic acid-labeled platelets were incubated at 37°C for 1 min with various concentrations of sphingosine in the presence of 1 mM $CaCl_2$ and 100 μM BW755C, then stimulated for 2 min with 2 μM U46619 (A) or 0.05 U/ml thrombin (B). The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS," then the radioactivity of arachidonic acid was determined. ○, unstimulated.

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increase in the content of phosphatidic acid, which can be converted to 1,2-diacylglycerol by phosphatidic acid phosphohydrolase. As shown in Fig. 3A, phosphatidic acid generation was accelerated by sphingosine following stimulation with U46619. To examine the participation of diacylglycerol lipase in the enhancement of arachidonic acid liberation by sphingosine, platelets were pretreated with RHC80267, an inhibitor of the enzyme. The inhibitor at 25 or 75 μM had no influence on the arachidonic acid liberation induced by both sphingosine and U46619 (Fig. 3B).

On the other hand, since 14-kDa PLA_2 is known to be secreted from platelets upon stimulation (*9*), heparin was added to cell suspensions before addition of sphingosine and U46619 to prevent access of the enzyme to platelets. Even under these conditions, sphingosine enhanced the arachidonic acid liberation to a similar level as that without heparin (bottom of Table I).

Relationship between Metabolic Conversion of Sphingosine to Sph-1-P and Arachidonic Acid Liberation—It has been reported that sphingosine added to human platelets is metabolically converted to Sph-1-P and ceramide; after 5 min, about 45% of the sphingosine added is converted to Sph-1-P (*18, 22*). We tried to confirm the time-dependent conversion of sphingosine to Sph-1-P using [^{3}H]sphingosine under the condition used here. As shown in Fig. 4A, [^{3}H]sphingosine added to platelet suspension decreased time-dependently with concomitant increase in the radioactivity of [^{3}H]Sph-1-P, and the residual sphingosine in the cells reduced to 56 and 23% of the initial level after 5 and 10 min of incubation, respectively. On the other hand, when the period of treatment with sphingosine of [^{3}H]-arachidonic acid-labeled platelets before addition of U46619 was prolonged, the enhancing effect of sphingosine on arachidonic acid liberation, which was observed within 1 min of treatment, decreased time-dependently (Fig. 4B). Incubation with sphingosine for 20 min did not further promote the arachidonic acid liberation in response to U46619.

Effects of Sphingosine Derivatives on Arachidonic Acid Liberation—Of the sphingosine derivatives which have been reported to exhibit biological activities in signal transduction, Sph-1-P, *N,N*-dimethylsphingosine, and *N*-acetylsphingosine (C_2-ceramide) were evaluated with re-

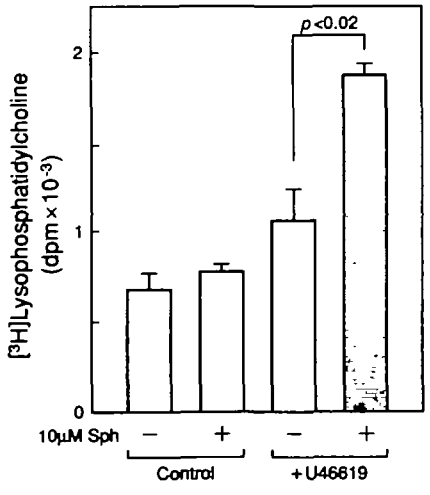

Fig. 2. **Effect of sphingosine on lysophosphatidylcholine formation induced by U46619.** [^{3}H]Glycerol-labeled platelets were incubated at 37°C for 1 min with 10 μM sphingosine (Sph) or the vehicle, ethanol, in the presence of 1 mM $CaCl_2$, then stimulated for 2 min with 5 μM U46619. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS," then the radioactivity of lysophosphatidylcholine was determined. The results are the means ± SE of triplicate determinations. Similar results were obtained in two other experiments.

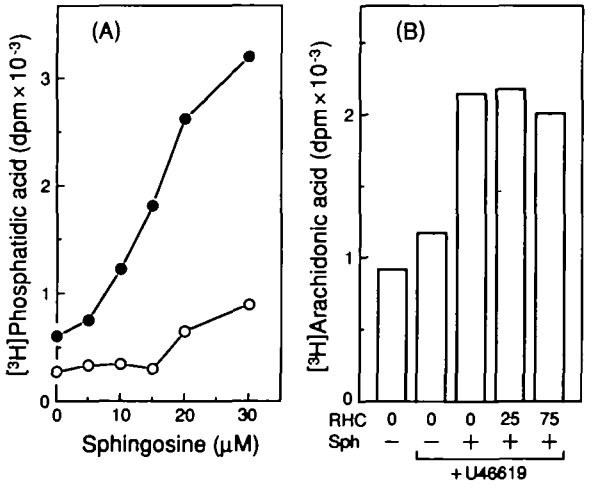

Fig. 3. **Effect of sphingosine on phosphatidic acid formation, and influence of RHC80267 on enhancement by sphingosine of arachidonic acid liberation induced by U46619.** [^{3}H]Arachidonic acid-labeled platelets were treated in the presence of 1 mM $CaCl_2$ and 100 μM BW755C as follows: (A) the platelets were incubated at 37°C for 1 min with various concentrations of sphingosine, then stimulated for 2 min with 2 μM U46619 (●) or the buffer vehicle (○); (B) after the platelets had been pretreated at 37°C for 15 min with 25 or 75 μM RHC80267 (RHC), the cells were incubated at 37°C for 1 min with 10 μM sphingosine (Sph), then stimulated for 2 min with 2 μM U46619. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS," then the radioactivities of phosphatidic acid (A) and arachidonic acid (B) were determined. The results are the means of duplicate determinations. Similar results were obtained in two other experiments.

TABLE I. **Effects of sphingosine derivatives on arachidonic acid liberation induced by U46619.** [^{3}H]Arachidonic acid-labeled platelets were incubated at 37°C for 1 min with 10 μM sphingosine or its derivatives in the presence of 1 mM $CaCl_2$ and 100 μM BW755C, then stimulated for 2 min with 2 μM U46619. Pretreatment with heparin was conducted at 10 U/ml for 2 min before addition of sphingosine. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS," then the radioactivity of arachidonic acid was determined. The results are the means ± SE of quadruplicate determinations. Sph-1-P, sphingosine-1-phosphate; dimethyl-Sph, *N,N*-dimethylsphingosine; C_2-ceramide, *N*-acetylsphingosine. Significantly different from vehicle in U46619-stimulated cells: $^*p<0.001$.

Treatment	[^{3}H]Arachidonic acid (dpm)
Unstimulated	
Vehicle	1,046±74
U46619-stimulated	
Vehicle	1,486±116
Sph-1-P	1,625±74
Dimethyl-Sph	1,418±148
C_2-ceramide	1,326±138
Sphingosine	2,864±171*
Heparin+sphingosine	3,197±166*

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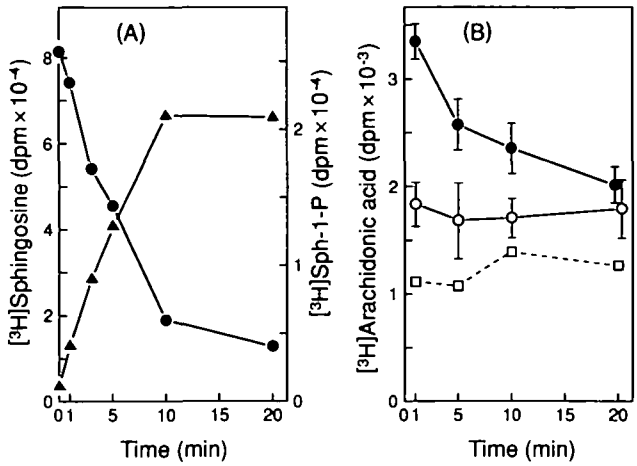

Fig. 4. **Time courses of sphingosine metabolism and U46619-induced arachidonic acid liberation by sphingosine treatment.** (A) Washed platelets were incubated at 37°C for various times with [³H]sphingosine. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS," then the radioactivities of sphingosine (●) and Sph-1-P (▲) were determined. The results are the means of duplicate determinations. (B) [³H]Arachidonic acid-labeled platelets were incubated at 37°C for various times with 10 μM sphingosine (●) or the vehicle, ethanol (○, □), in the presence of 1 mM $CaCl_2$ and 100 μM BW755C, then stimulated for 2 min with 2 μM U46619 (●, ○) or the vehicle alone (□). The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS," then the radioactivity of arachidonic acid was determined. The results are the means ± SE of triplicate determinations. Similar results were obtained in two other experiments.

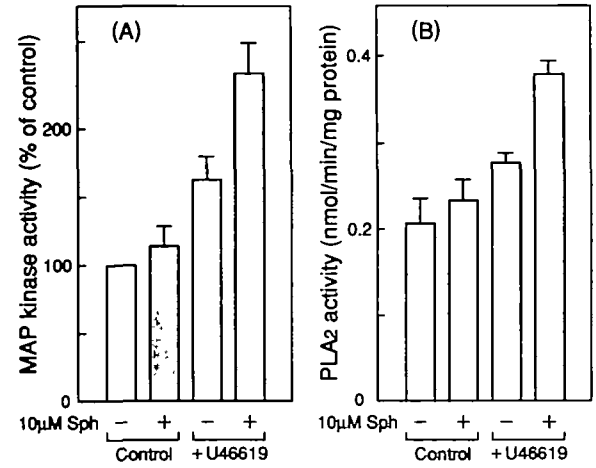

Fig. 5. **Effect of sphingosine on activation of MAP kinase and PLA_2 induced by U46619.** Washed platelets were incubated at 37°C for 1 min with 10 μM sphingosine (Sph) or the vehicle, ethanol, in the presence of 1 mM $CaCl_2$, then stimulated for 1 min with 2 μM U46619. The MAP kinase (A) and cytosolic PLA_2 (B) activities in the supernatant of the cell lysate were determined as described in "MATERIALS AND METHODS." The results are the means ± SE of triplicate determinations. Similar results were obtained in two other experiments.

gard to an effect on arachidonic acid liberation in response to U46619. As shown in Table I, these derivatives at 10 μM did not exert any effect on the radioactivity of [³H]arachidonic acid in U46619-stimulated platelets.

Activities of MAP Kinase and PLA_2 in Response to U46619 or Thrombin—The mechanism by which sphingosine enhanced the arachidonic acid liberation by PLA_2 on stimulation with U46619 was examined. Cytosolic PLA_2 activity has been reported to increase when it is phosphorylated by MAP kinase or other kinases (*12-14*). To measure MAP kinase and PLA_2 activities, we prepared the supernatant of lysate obtained from platelets which had been treated with sphingosine and U46619. Both enzyme activities in the supernatant increased with U46619 stimulation, and further treatment of the platelets with 10 μM sphingosine significantly potentiated the activities (Fig. 5). However, the PLA_2 activity in the supernatant of the lysate from platelets treated with U46619 was suppressed when 10 μM sphingosine was added to the supernatant (PLA_2 activity: 0.32 and 0.14 nmol/min/mg protein in the absence and presence of sphingosine, respectively, average of duplicate determination).

On the other hand, sphingosine even at 10 μM did not potentiate the arachidonic acid liberation upon stimulation with thrombin, as shown in Fig. 1B. This result suggests the possibility that PLA_2 as well as MAP kinase may be fully activated by thrombin alone to liberate arachidonic acid. Therefore, we examined the activities of PLA_2 and MAP kinase in response to thrombin under the conditions used in Fig. 1B. The activities of PLA_2 and MAP kinase increased to 220 and 520%, respectively, relative to unstimulated platelet responses. These increased activities markedly exceeded the sphingosine-potentiated activities of PLA_2 and MAP kinase in response to U46619 (the activities of PLA_2 and MAP kinase upon stimulation with sphingosine plus U46619 were 180 and 230%, respectively, relative to unstimulated responses).

DISCUSSION

Several agonists evoke the rapid liberation of arachidonic acid from membrane phospholipids. Since arachidonic acid is a precursor of prostaglandin endoperoxides, thromboxane A_2 and other eicosanoids, which also mediate platelet activation and actions on other cells, arachidonic acid liberation is a key step in signal transduction. We showed here that treatment for 1 min with sphingosine enhanced the liberation of arachidonic acid by U46619-stimulated platelets (Fig. 1). This enhancing effect increased with a peak at 10 μM sphingosine, and decreased at higher concentrations. Following thrombin stimulation, a suppressive effect on arachidonic acid liberation was observed at sphingosine concentrations higher than 20 μM. The release of arachidonic acid from thrombin-stimulated platelets is attributable largely to the action of PLA_2 (*23*), while protein kinase C potentiates the arachidonic acid liberation by PLA_2 (*24*). Since sphingosine at 25-100 μM inhibits protein kinase C activity in platelets (*25, 26*), the suppressive effect of high concentrations of sphingosine on thrombin-induced arachidonic acid liberation may be due to negative feedback on protein kinase C. Sphingosine is a long-chain base and is structurally analogous to fatty acids which inhibit platelet responses (*16*). The effect of sphingosine at high concentrations may be based on a mechanism similar to that of fatty acids. On the other hand, we suggest that the failure of 10 μM sphingosine to induce arachidonic acid liberation on thrombin stimulation is due to sufficient

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activation of PLA_2 by thrombin for maximal liberation of arachidonic acid. In fact, the activity of PLA_2 in response to thrombin markedly exceeded the activity upon stimulation with sphingosine plus U46619, as shown in "RESULT."

Our results showed that 10 μM sphingosine promoted the formation of lysophosphatidylcholine, another product of PLA_2, on stimulation with U46619 (Fig. 2), and that RHC80267, an inhibitor of diacylglycerol lipase, failed to suppress the arachidonic acid liberation under these conditions (Fig. 3). Furthermore, addition of heparin, which prevents the action of secretory PLA_2 on membranes, exerted no effect on the liberation (Table I). These results imply that the arachidonic acid liberated by both sphingosine and U46619 is derived from the action of cytosolic PLA_2.

Yatomi *et al.* reported that sphingosine added to intact platelets is converted to Sph-1-P, and subsequently to ceramide and sphingomyelin (*18, 22*), and we also confirmed the conversion (Fig. 4A). This phenomenon can be explained by the fact that platelets possess an active sphingosine kinase and no lyase activity for degradation of Sph-1-P. When [^{3}H]arachidonic acid-labeled platelets were incubated with sphingosine for periods longer than 1 min, the sphingosine-enhanced arachidonic acid liberation in response to U46619 decreased time-dependently to reach the level without sphingosine after 20 min (Fig. 4B). The most likely explanation for this is that sphingosine may be converted to other metabolites such as Sph-1-P and ceramide. In fact, sphingosine was converted to Sph-1-P time-dependently when it was added to platelet suspension (Fig. 4A), and the time-dependent decrease in sphingosine was parallel with the decrease in the potential effect of sphingosine on U46619-stimulated arachidonic acid liberation. Furthermore, Sph-1-P, *N*-acetylsphingosine (membrane-permeable ceramide), and *N,N*-dimethylsphingosine did not enhance arachidonic acid liberation in U46619-stimulated platelets (Table I). Therefore, the development of the hydrolytic action of cytosolic PLA_2 appears to be due to the action of sphingosine alone.

Cytosolic PLA_2 is regulated by intracellular Ca^{2+}, which induces translocation of the enzyme to membranes (*27*), and by phosphorylation, which leads to an increase in the activity (*12-14*). The observation in the present study that sphingosine enhanced the U46619-stimulated increase in PLA_2 activity (Fig. 5B) may represent one of the mechanisms by which sphingosine promotes arachidonic acid liberation upon U46619 stimulation. However, sphingosine may not directly act on PLA_2, because the PLA_2 activity in lysates was suppressed by addition of sphingosine. This is in agreement with the report that sphingosine inhibited PLA_2-catalyzed hydrolysis of phosphatidylcholine liposomes (*28*). It is likely that sphingosine functions in a process of stimulus-PLA_2 activation, and therefore we suggest that sphingosine may accelerate phosphorylation of PLA_2 through MAP kinase activation. Indeed, MAP kinase was reported to phosphorylate and to increase the activity of PLA_2 (*13*), and we also observed in the present study that sphingosine significantly enhanced MAP kinase activity in U46619-stimulated platelets (Fig. 5A). MAP kinase activity is regulated by MAP kinase kinase (*29, 30*), which is activated, in part, *via* a protein kinase C-dependent mechanism (*31*). Although it is not yet known which process in the MAP kinase cascade is affected by sphingosine, there are two likely hypotheses. Firstly, sphingosine may increase 1,2-diacylglycerol level through enhancement of U46619-stimulated PLC activation (*5*), followed by an increase in protein kinase C activity, resulting in acceleration of the MAP kinase cascade. Alternatively, sphingosine may cause protein-tyrosine phosphorylation, resulting in activation of MAP kinase (*32-34*).

As an alternative mechanism, sphingosine may have enhanced arachidonic acid liberation in response to U46619, due to an increase in intracellular Ca^{2+} concentration (*5*). Ca^{2+} is required for PLA_2 activation, and induces translocation of the enzyme to membranes, as mentioned above. Furthermore, arachidonic acid liberation by cytosolic PLA_2 is also regulated by GTP-binding protein (*35, 36*), and sphingosine derivatives such as Sph-1-P have been reported to stimulate the protein (*37*). Whether the action of sphingosine is mediated through GTP-binding protein, however, remains to be determined.

In this study, we showed that sphingosine enhanced arachidonic acid liberation by platelets in response to U46619 through an increase in cytosolic PLA_2 activity due to MAP kinase activation. This finding corroborates the view that sphingosine acts as a modulator of lipid metabolism.

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