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Sphingosine Enhances Phosphatidylinositol 4-Kinase Activity in Rabbit Platelets[1]

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The modulating effect of sphingosine on the metabolism of inositol phospholipids was investigated using rabbit platelets. When [^{3}H]arachidonic acid- or [^{3}H]inositol-labeled platelets were incubated at 37°C with sphingosine, the radioactivity of the phosphatidylinositol (PI) fraction obtained on TLC decreased time-dependently up to 5 min, and phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2) increased concomitantly, though neither arachidonic acid nor 1,2-diacylglycerol was formed. The effect of sphingosine was dose-dependent, the maximum effect being observed at 20 μM. Treatment with a sphingosine derivative, sphingosine-1-phosphate (Sph-1-P) or *N*-hexanoyl-sphingosine (C_6-ceramide), did not result in an increase in PIP. The increased radioactivity of PIP with sphingosine was attributable to an increase in phosphatidylinositol 4-phosphate, but not phosphatidylinositol 3-phosphate. Furthermore, wortmannin, an inhibitor of PI 3-kinase, did not affect the modulating effect of sphingosine at 100 nM, at which the enzyme is known to be completely inhibited. The activity of PI 4-kinase in the platelet lysate was increased by sphingosine but not by Sph-1-P. These results suggest that sphingosine enhances the activity of PI 4-kinase and thereby contributes to the regulation of inositol phospholipid metabolism.

Key words: phosphatidylinositol 4-kinase, phosphatidylinositol 4-phosphate, platelet, sphingolipid, sphingosine.

Sphingolipid metabolism has recently been shown to be involved in cell regulation processes; sphingosine, a sphingolipid metabolite, has emerged as a lipid modulator in diverse cellular processes (*1, 2*). Initially, sphingosine was discovered as a potent inhibitor of protein kinase C (*3, 4*). However, it is widely accepted that sphingosine affects not only protein kinase C, but also other enzymes playing important roles in signal transduction. Sphingosine has been shown to inhibit Ca^{2+}/calmodulin-dependent enzyme (*5*), Na^+/K^+-ATPase (*6*), and CTP:phosphocholine cytidylyltransferase (*7*), and to stimulate the tyrosine kinase activity of epidermal growth factor receptors (*8*). It also causes intracellular Ca^{2+} release or Ca^{2+} influx in various cells (*9, 10*), and focal contact assembly in Swiss 3T3 cells (*11*), and mediates the apoptotic signals of tumor necrosis factor-α in neutrophils (*12*).

In previous work, we obtained evidence that sphingosine promotes agonist-induced phospholipase C activation, thereby enhancing platelet aggregation (*13*). Other investigators have shown that sphingosine regulates the activation of enzymes involved in lipid metabolism, *i.e.*, phospholipase D (*14*), phosphatidic acid phosphohydrolase (*15*), and diacylglycerol kinase (*16*). Thus, it is now clear that sphingosine influences several enzymes involved in phospholipid metabolism.

In the stimulus-response coupling of many cells, including platelets, the binding of a wide range of agonists to their receptors on cells induces increased turnover in the phosphatidylinositol (PI)-cycle. Activation of phospholipase C specific for phosphatidylinositol bisphosphate (PIP_2) results in the formation of 1,2-diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate, which mobilizes Ca^{2+} from an internal store. PIP_2 is supplied *via* serial phosphorylation of PI through the actions of the corresponding kinases.

In the present work, we investigated the modulating effect of sphingosine on the metabolism of inositol phospholipids, and we propose that sphingosine modulates polyphosphoinositide metabolism through an increase in PI 4-kinase activity.

MATERIALS AND METHODS

Materials—Sphingosine (from bovine brain sphingomyelin), *N*-hexanoyl-sphingosine (C_6-ceramide), and PI, phosphatidylinositol monophosphate (PIP), PIP_2, and lyso-PI, as an inositol phospholipid standard, were purchased from Sigma Chemical (St. Louis, MO, USA). Sphingolipids were dissolved in ethanol at a concentration 300-fold higher than the final concentration (final ethanol concentration, 0.33%). Sphingosine-1-phosphate (Sph-1-P) was purchased from BIOMOL Research Laboratories (Plymouth

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Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol bisphosphate; Sph-1-P, sphingosine-1-phosphate.

Meeting, PA, USA) and dissolved in methanol or BSA solution. [^{3}H]Arachidonic acid (76 Ci/mmol) and [^{3}H-inositol]PI (11 Ci/mmol) were obtained from DuPont/NEN (Boston, MA, USA). myo-[^{3}H]Inositol (17 Ci/mmol) and [^{3}H-inositol]PIP (1 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., UK) and American Radiolabeled Chemicals (St. Louis, MO, USA), respectively. Staurosporine and wortmannin were from Kyowa Medex (Tokyo), and H-7 was from Seikagaku Kogyo (Tokyo). These reagents were 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 and Labeling—Fresh rabbit blood anti-coagulated with 0.1 volumes of 1% EDTA was centrifuged at $230\times g$ for 10 min at room temperature to obtain platelet-rich plasma. For labeling with [^{3}H]inositol, the platelets separated from platelet-rich plasma were washed once as described previously (*17*), and then incubated with [^{3}H]inositol (30 μCi/ml) at 37°C for 2.5 h. For labeling with [^{3}H]arachidonic acid, the platelet-rich plasma was incubated with [^{3}H]arachidonic acid (2 μCi/ml) at 37°C for 1.5 h. After incubation, the platelets were further washed twice. 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.

In the preparation of platelet lysate, unlabeled platelets were lysed by sonication in 0.5 volume of lysis buffer [145 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 50 μM leupeptin, 100 μM *p*-(amidinophenyl)methanesulfonyl fluoride, pH 7.0].

Lipid Analysis—[^{3}H]Inositol- or [^{3}H]arachidonic acid-labeled platelets were incubated at 37°C with sphingosine or other reagents in the presence of 1 mM $CaCl_2$ (final volume, 0.3 ml), and then the reaction was terminated by the addition of 2.1 ml of chloroform/methanol/HCl (100 : 200 : 2, by volume).

(1) For analysis of [^{3}H]inositol-labeled lipids, the suspension was separated into two phases by adding 0.7 ml of chloroform and 0.7 ml of 2.4 M HCl. The lower organic phase was removed, and the aqueous phase was re-extracted with 1 ml of chloroform. The combined organic phases were evaporated under N_2, and the residue was redissolved in chloroform/methanol. This solution was applied to a TLC plate that had been dipped in 1% potassium oxalate/2 mM EDTA in 50% ethanol and dried overnight (*18*), then the lipids were separated by developing the TLC plate in chloroform/methanol/water/NH_4OH (17 : 13.2 : 2.8 : 1, by volume) (*19*). To separate phosphatidylinositol 4-phosphate [PI(4)P] and phosphatidylinositol 3-phosphate [PI(3)P], the lipids were separated by TLC on a CDTA (1,2-cyclohexanediaminetetraacetic acid)-impregnated plate, developed with methanol/chloroform/pyridine/water/88% formic acid (50 : 40 : 30 : 5 : 2, by volume) containing 6.3% boric acid (*20*).

(2) For analysis of [^{3}H]arachidonic acid-labeled lipids, the suspension was separated into two phases by adding 0.7 ml of chloroform and 1 ml of 0.1 M KCl, and then the organic phase was subjected to TLC. Phospholipids were separated by two-dimensional TLC, developed with chloroform/methanol/NH_4OH (90 : 54 : 11, by volume) for the first dimension and chloroform/methanol/formic acid (13 : 5 : 2, by volume) for the second dimension. Diacylglycerol was separated by one-dimensional TLC as described previously (*21*).

Following chromatography, each lipid fraction was detected by comparison with authentic standards after staining with iodine vapor or Dittmer spray. The area corresponding to each lipid fraction was scraped off and the radioactivity was determined.

Measurement of PI 4-Kinase Activity in Platelet Lysate—The assay was performed in the incubation medium containing 50 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM EGTA, 0.4% Triton X-100, and 20 nM [^{3}H]PI. The reaction was started by addition of 500 μM ATP and the platelet lysate, and the mixture was incubated at 30°C for 15 min. After extraction of the lipids, the radioactivity of [^{3}H]PIP was measured as described above.

RESULTS AND DISCUSSION

Analysis of [^{3}H]Arachidonic Acid-Labeled Lipids—We first examined the effect of sphingosine on the amounts of the major molecular types of phospholipids. When [^{3}H]-arachidonic acid-labeled platelets were incubated at 37°C for 3 min with 20 μM sphingosine in the presence of 1 mM $CaCl_2$, the radioactivity in the PI fraction decreased to 79% of the control cell level, as shown in Table I. This incubation did not cause changes in the amounts of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine. The decrease in PI indicates its possible degradation by phospholipase A_2 and/or C, or phosphorylation. However, neither free arachidonic acid nor 1,2-diacylglycerol, the respective products of the enzymes, increased (Table I). In previous work we obtained a similar result, *i.e.*, that sphingosine (up to 30 μM) by itself had no effect on the formation of inositol 1,4,5-trisphosphate (*13*). On the other hand, Chao *et al.* reported that the hydrolysis of PI is induced by sphingosine alone in human foreskin fibroblasts (*22*). The reason for the conflicting results is unknown.

Although the radioactivity in phosphatidic acid slightly increased, the decrease in the amount of PI cannot all be

TABLE I. **Sphingosine-induced changes in the lipid contents of [^{3}H]arachidonic acid-labeled platelets.** [^{3}H]Arachidonic acid-labeled platelets were incubated at 37°C for 3 min with 20 μM sphingosine or the vehicle, ethanol (control), in the presence of 1 mM $CaCl_2$. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS"; then the radioactivity of each lipid was determined. The results are the means±SD of three separate experiments performed in duplicate. PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; AA, arachidonic acid; DAG, 1,2-diacylglycerol. Significant differences from control: $^*p<0.05$, $^{**}p<0.005$.

Addition	[^{3}H]Arachidonic acid distribution (dpm)						
	PI	PS	PC	PE	PA	AA	DAG
Control	15,346±1296	2,976±395	23,502±1921	19,933±1732	313±60	1,008±325	369±150
20 μM sphingosine	12,177±1350*	3,032±290	23,517±1978	20,814±1862	807±276**	841±314	336±35

accounted for by the conversion of PI to phosphatidic acid *via* 1,2-diacylglycerol. When the incubation of platelets with sphingosine was performed in the presence of 1.5% ethanol, phosphatidylethanol, which is a product resulting from phospholipase D activation, was not formed (data not shown). The significant increase in the phosphatidic acid level may be due to either the inhibition of phosphatidic acid phosphohydrolase or the activation of diacylglycerol kinase (*15, 16*). From these viewpoints, it seems probable that the decrease in PI on incubation with sphingosine is not due to hydrolysis but to stimulation of the phosphorylation of PI.

Analysis of [^{3}H]Inositol-Labeled Phospholipids—To test the above possibility, the alteration of polyphosphoinositides was examined. When platelet phospholipids were labeled with [^{3}H]inositol under the conditions mentioned in "MATERIALS AND METHODS," the ^{3}H-radioactivity was distributed in the inositol phospholipids as follows: 76.3% in PI, 10.2% in PIP, 8.4% in PIP_2, and 3.0% in lysoPI (means of four experiments). This is in agreement with the report of Hrbolich *et al.* (*23*). The results of incubation of [^{3}H]inositol-labeled platelets with sphingosine are shown in Figs. 1 and 2. There was a decrease in PI in the platelets treated with sphingosine, similar to the decrease in PI observed in [^{3}H]arachidonic acid-labeled platelets, whereas the radioactivities of PIP and PIP_2 increased time- and dose-dependently, in parallel with the decrease in PI. These increases became maximum with 20 μM sphingosine, and the magnitude of the increase in PIP was somewhat larger than that in PIP_2. These effects of sphingosine were observed even in the presence of 1 mM EGTA in the outer medium (data not shown), indicating that the action of sphingosine is independent of extracellular Ca^{2+}. Sphingosine did not exert any effect on the amount of lysoPI (second from the top in Table II), which also suggests no hydrolysis of PI by phospholipase A_2.

There is a possibility that the decrease in PI and the increase in PIP may result from suppression by sphingosine of PIP phosphatase activity. To evaluate the effect of sphingosine on the enzyme, the activity was determined in terms of the conversion of PIP into PI using a mixture of [^{3}H-inositol]PIP as the substrate and platelet lysate as the enzyme source. When [^{3}H]PIP (23,860 dpm) was incubated at 37°C for 3 min with platelet lysate in the absence of sphingosine, the radioactivity of [^{3}H]PI increased from 192±13 to 2,505±145 dpm. However, there was no significant change in the increase in [^{3}H]PI in the presence or absence of 20 μM sphingosine, although the increase was somewhat lower in the presence of sphingosine (2,127± 174 dpm). These results indicate that sphingosine causes the phosphorylation of PI to PIP and PIP_2. In addition, even

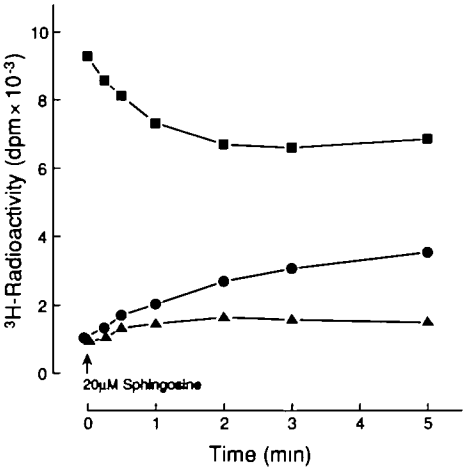

Fig. 1. **Time-dependent change in the inositol phospholipid content caused by sphingosine.** [^{3}H]Inositol-labeled platelets were incubated at 37°C with 20 μM sphingosine for various times in the presence of 1 mM $CaCl_2$. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS." The radioactivities of PI (■), PIP (●), and PIP_2 (▲) were determined. The results are the means of duplicate determinations. Similar results were obtained in two other experiments.

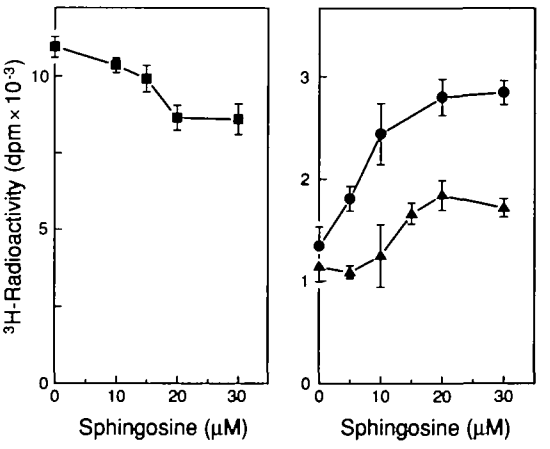

Fig. 2. **Concentration-dependent changes in inositol phospholipid contents caused by sphingosine.** [^{3}H]Inositol-labeled platelets were incubated at 37°C for 3 min with various concentrations of sphingosine in the presence of 1 mM $CaCl_2$. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS." The radioactivities of PI (■), PIP (●), and PIP_2 (▲) were determined. The results are the means±SE of triplicate determinations. Similar results were obtained in two other experiments.

TABLE II. **Effects of sphingosine and its derivatives on the inositol phospholipid contents of [^{3}H]inositol-labeled platelets.** [^{3}H]Inositol-labeled platelets were incubated at 37°C for 3 min with sphingosine or its derivatives in the presence of 1 mM $CaCl_2$. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS"; then the radioactivity of each lipid was determined. The results are the means±SE of triplicate determinations. Similar results were obtained in two other experiments. Sph-1-P, sphingosine-1-phosphate. Significant differences from control: $^*p<0.005$, $^{**}p<0.001$.

Addition	[^{3}H]Inositol phospholipids (dpm)			
	PI	LysoPI	PIP	PIP_2
Control	9,365±195	511±63	1,252±84	1,314±88
20 μM sphingosine	7,719±578*	405±13	2,881±239**	1,975±149*
20 μM Sph-1-P	11,092±573*	452±58	1,520±88	1,276±113
20 μM C_8-ceramide	9,723±74	578±11	1,353±72	1,323±21

at 15-30 s after the addition of sphingosine to [^{3}H]inositol-labeled platelets, the radioactivity of PIP_2 increased (Fig. 1), indicating that the increase in PIP and PIP_2 was not due to rapid conversion from PI in compensation for initial degradation of PIP_2 by phospholipase C activation.

Effects of Protein Kinase C Inhibitors and Sphingosine Derivatives—Sphingosine is known to inhibit protein kinase C, which regulates PI turnover (*3, 4*). We examined the effects of other inhibitors of protein kinase C in order to determine whether or not the effect of sphingosine is dependent on the action on protein kinase C. Staurosporine and H-7 are potent inhibitors of protein kinase C; nevertheless, neither of them caused changes in inositol phospholipids (data not shown). The results, therefore, suggest that phosphorylation of PI by sphingosine is not related to inhibition of protein kinase C activity.

Several metabolites of sphingolipids, other than sphingosine, have also been reported to exhibit biological activities in signal transduction. For example, sphingosylphosphorylcholine and sphingosine 1-phosphate (Sph-1-P) induce Ca^{2+} mobilization from the inositol 1,4,5-trisphosphate-sensitive calcium pool (*9, 24*), and stimulate the proliferation of Swiss 3T3 fibroblasts (*25*). Ceramide has been reported to be a mediator of tumor necrosis factor-α signaling in HL-60 cells (*26*), and a stimulator of cytosolic protein phosphatase in T9 glioma cells (*27*). Lysosphingolipids such as psychosine and lyso-G_{M3} inhibit protein kinase C and induce tyrosine phosphorylation of the epidermal growth factor receptor (*4, 28*). Of these sphingolipids, Sph-1-P and C_6-ceramide (membrane-permeable short-chain ceramide) were evaluated with regard to quantitative changes in polyphosphoinositides, and the results obtained are shown in Table II. When [^{3}H]inositol-labeled platelets were incubated at 37°C with 20 μM Sph-1-P or C_6-ceramide under the same conditions as used for sphingosine, the amount of PIP did not change. Although the radioactivity of PI significantly increased in the case of treatment of Sph-1-P, the mechanism remains to be elucidated.

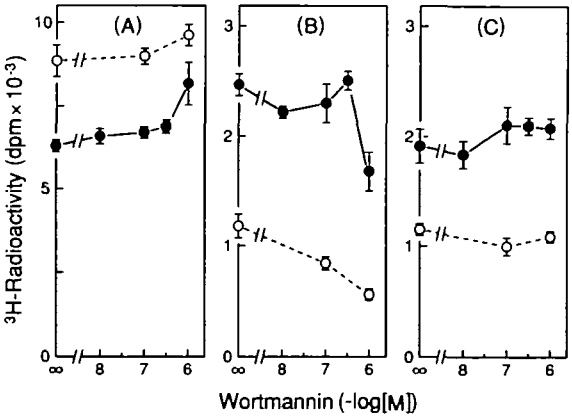

Fig. 3. **Effect of wortmannin on the sphingosine-induced changes in inositol phospholipid contents.** After [^{3}H]inositol-labeled platelets had been treated at 37°C for 10 min with various concentrations of wortmannin, they were incubated at 37°C for 3 min with 20 μM sphingosine (●) or the vehicle, ethanol (○), in the presence of 1 mM $CaCl_2$. The lipids were extracted and separated by TLC as described in "MATERIALS AND METHODS." The radioactivities of PI (A), PIP (B), and PIP_2 (C) were determined. The results are the means±SE of triplicate determinations. Similar results were obtained in two other experiments.

Analysis of the PIP Increase—Recent studies have demonstrated that PI 3-kinase catalyzes the phosphorylation of inositol phospholipids at the D-3 position of the inositol ring to form PI(3)P or phosphatidylinositol 3,4,5-trisphosphate in platelets (*29*). The question now arises: which increases on treatment with sphingosine, PI(3)P or PI(4)P?

Although the mechanism by which sphingosine increases PIP and PIP_2, with a decrease in PI, is unknown, we suggest the involvement of the activation of PI 4-kinase in the mechanism for the following reasons. First, a large portion of PIP was PI(4)P, as analyzed by the TLC method of Walsh *et al.* (*20*): the radioactivities were 889±39 dpm and 2,002±96 dpm ($p<0.001$) in the absence and presence of 20 μM sphingosine, respectively (the means±SE of quadruplicate determinations). Radioactivity of the PI(3)P fraction was hardly detected under the experimental conditions used, with ^{3}H-labeled inositol (the radioactivities were 78±12 and 69±10 dpm in the absence and presence of sphingosine, respectively). The PI(4)P production increased with increase in the sphingosine concentration (data not shown). Secondly, we determined the influence of wortmannin, a potent inhibitor of PI 3-kinase, on the changes in inositol phospholipids caused by sphingosine. However, wortmannin, even at 300 nM, failed to inhibit the increase in PIP or the decrease in PI (Fig. 3). This reagent is known to inhibit completely the activity of PI 3-kinase at 100 nM, although it also inhibits other enzymes at higher concentrations (*30*). Thirdly, the total radioactivity of PI, PIP, and PIP_2 did not vary on sphingosine treatment, indicating only interconversion among these inositol phospholipids. Fourthly, the activity of PI 4-kinase in the platelet lysate was increased by 20 μM sphingosine but not by Sph-1-P (Table III). These findings indicate that sphingosine induces PI 4-kinase activation, thereby causing the increase in PI(4)P. The increase in PIP_2 appears to be due to the activation of PIP kinase or to be secondary to the increase in PIP, a substrate for the enzyme.

We found that sphingosine enhanced the activity of PI 4-kinase, but not that of PI 3-kinase, thus causing an increase in PI(4)P. Nevertheless, we cannot rule out the possibility that sphingosine might also result in the formation of another product, *e.g.*, phosphatidylinositol 3,4,5-trisphosphate, through the activation of PI 3-kinase. This would be consistent with the facts that PI 3-kinase activity is regulated by tyrosine kinase (*31*), and that sphingosine activates some types of tyrosine kinase (*8, 11*). Thus, there

TABLE III. **Effect of sphingosine on PI 4-kinase activity in the platelet lysate.** [^{3}H-Inositol]PI was incubated at 30°C for 15 min with or without the platelet lysate in the presence of 500 μM ATP and sphingosine or Sph-1-P. The lipids were extracted, and the radioactivity of [^{3}H]PIP was determined as described in "MATERIALS AND METHODS." The results are the means±SE of triplicate determinations. Similar results were obtained in another experiment. Sph-1-P, sphingosine-1-phosphate. Significant difference from control: *$p<0.01$.

Addition	[^{3}H]PIP (dpm)	
	None	Lysate
Control	460±89	3,342±122
20 μM sphingosine	—	5,817±432*
20 μM Sph-1-P	—	3,682±82

is a need for further investigation.

Recent studies suggested that some agonists and changes in medium conditions induce sphingomyelin hydrolysis to produce sphingosine through the sequential actions of sphingomyelinase and ceramidase (*2, 32*). In addition, sphingosine is reported to be converted into Sph-1-P by the corresponding kinase in platelets (*33*). However, the mechanism underlying agonist-induced production of sphingolipids, including sphingosine, is still not completely elucidated, and the physiological role of the individual sphingolipid molecules is uncertain. We propose that sphingosine may behave as a modulator of PI turnover in the signal transduction system. One possibility is that sphingosine may contribute to the supply of PIP_2, an important precursor of bioactive messengers such as 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. Such an effect may also explain our previous finding that sphingosine enhanced agonist-induced inositol 1,4,5-trisphosphate generation and aggregation (*13*).

In conclusion, the treatment of platelets with sphingosine leads to the activation of PI 4-kinase, followed by increases in PI(4)P and PIP_2. This regulation by sphingosine of inositol phospholipid turnover may be one part of its action as a signal modulator.

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