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Sphingosine 1-Phosphate Breakdown in Platelets

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We examined the formation of sphingolipid mediators in platelets, which abundantly store, and release extracellularly, sphingosine 1-phosphate (Sph-1-P). Challenging [^{3}H]Sph-labeled platelet suspensions with thrombin or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) resulted in a decrease in Sph-1-P formation and an increase in sphingosine (Sph), ceramide (Cer), and sphingomyelin formation. Sph conversion into Cer, and Cer conversion into sphingomyelin were not affected upon activation, suggesting that Sph-1-P dephosphorylation may initiate the formation of sphingolipid signaling molecules. In fact, Sph-1-P phosphatase (but not lyase) activity was detected in platelets, but this activity was not enhanced by thrombin or TPA. When quantified with [^{3}H]acetic anhydride acetylation, followed by HPLC separation, the amounts of Sph-1-P and Sph decreased and increased, respectively, upon stimulation with thrombin or TPA, and these changes were attenuated by staurosporine. Under these TPA treatment conditions, over half of the [^{3}H]Sph-1-P (formed in platelets incubated with [^{3}H]Sph) was detected extracellularly, possibly due to its release from platelets, which was completely inhibited by staurosporine pretreatment. Furthermore, when TPA-induced Sph-1-P release was blocked by staurosporine after the stimulation, the extracellular [^{3}H]Sph-1-P radioactivity decreased, suggesting that the Sph-1-P released may undergo dephosphorylation extracellularly. To support this, [^{32}P]Sph-1-P, when added extracellularly to platelet suspensions, was rapidly degraded, possibly due to the ecto-phosphatase activity. Our results suggest the presence in anucleate platelets of a transmembrane cycling pathway starting with Sph-1-P dephosphorylation and leading to the formation of other sphingolipid mediators.

Key words: ceramide, lipid phosphate phosphatase, platelets, sphingomyelin, sphingosine, sphingosine 1-phosphate.

Abbreviations: Cer, ceramide; Sph, sphingosine; Sph-1-P, sphingosine 1-phosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; LPP, lipid phosphate phosphatase.

Recently, not only glycerophospholipids, but also sphingolipids have been shown to be involved in cellular signal transduction and to be sources of bioactive lipid mediators (*1–4*). Sphingomyelin is an important component of the membrane lipid bilayer, and its breakdown, through the mediation of signal-activated sphingomyelinase(s), leads to the formation of the important signaling molecule ceramide (Cer), although the *de novo* pathway for its synthesis may be involved in some systems (*1*, *2*, *4*). Deacylation of Cer by ceramidase results in the formation of sphingosine (Sph) (*1*, *2*, *4*). Sph is then phosphorylated by Sph kinase into Sph 1-phosphate (Sph-1-P) (*1*, *3*, *4*). Sph-1-P can be cleared by a lyase, a fatty aldehyde and ethanolamine phosphate being formed (*4*, *5*), although Sph can be regenerated from Sph-1-P through the action of Sph-1-P phosphatase (*6–8*). These sphingolipid signaling molecules, formed primarily through "sphingomyelin breakdown," have now emerged as lipid messengers exerting a variety of biological actions (*1–4*).

Sph-1-P, the sphingolipid mediator located most downstream from sphingomyelin degradation, has attracted a great deal of attention as a key cell signaling molecule, functioning as both an extracellular first messenger and an intracellular second messenger (*3*, *9*). As described above, the intracellular level of Sph-1-P is determined by the balance of Sph kinase-mediated synthesis and its degradation by Sph-1-P lyase or phosphatase. In general, the intracellular Sph-1-P level is kept low, possibly due to degradation by the lyase and phosphatase activities. Sph kinase is relatively inactive in the resting state (only a small fraction of exogenous Sph is converted to Sph-1-P intracellularly), but Sph kinase activity is stimulated and the Sph-1-P level is transiently increased by specific stimulants in cells in which Sph-1-P acts as an intracellular second messenger (*3*, *9–11*). It is unlikely that these characteristics are true of blood platelets (*9*); these anucleate cells possess a unique sphingolipid metabolic pathway, as follows. Sph-1-P lyase is a ubiquitous enzyme present in almost all tissues, but platelets are an impor-

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tant exception in that they are devoid of this lyase (*5*, *9*, *12*). In contrast, platelets possess a highly active Sph kinase (*9*, *12*). As a result, platelets accumulate abundant Sph-1-P (*9*, *13*). Furthermore, Sph-1-P, abundantly stored in platelets, can be released extracellularly by stimulants that are capable of activating protein kinase C (*9*, *12*, *14*).

In this study, using the highly unique cells platelets, we suggested the presence of a transmembrane cycling pathway starting with "Sph-1-P breakdown (dephosphorylation)," which is distinct from the well-known "sphingomyelin breakdown."

MATERIALS AND METHODS

Materials—[^{32}P]Sph-1-P and [3-^{3}H]Sph-1-P were prepared by [γ-^{32}P]ATP- and ATP-dependent phosphorylation of Sph and [3-^{3}H]Sph, respectively, which were both catalyzed by Sph kinase obtained from human platelets (*15*).

The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA); Sph, Cer, C_6-Cer, lysophosphatidic acid, monoacylglycerol, phosphatidic acid, diacylglycerol, sphingomyelin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and bovine serum albumin (essentially fatty acid-free) (Sigma, St. Louis, MO); thrombin (Mochida Pharmaceutical Co., Tokyo, Japan); staurosporine (Kyowa Medex, Tokyo, Japan); and [γ-^{32}P]ATP (3,000 Ci/mmol), [3-^{3}H]Sph (22 Ci/mmol), [3-^{3}H]C_6-Cer (22.3 Ci/mmol), and [^{14}C]sphingomyelin (40–60 mCi/mmol) (NEN^{TM} Life Science Products, Inc., Boston, MA).

Cell Preparation—Platelets were isolated from the blood of healthy adult volunteers. The blood was anticoagulated with 3.8% sodium citrate (9 volumes of blood to 1 volumes of sodium citrate) and then centrifuged at 120 $\times$ *g* for 10 min to obtain platelet-rich plasma. Then, washed platelets suspended in a HEPES buffer [containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$, 1 mg/ml of glucose, 0.5% bovine serum albumin, and 20 mM HEPES (pH 7.4)] were prepared and handled as described previously (*16*). When indicated, 1 mM EDTA was added instead of $MgCl_2$. Unless otherwise stated, the washed platelets were used as platelet suspensions. All of the intact platelet reactions were performed at 37°C.

[^{3}H]Sphingolipid Formation in Platelet Suspensions Loaded with [^{3}H]Sph—Platelet-rich plasma was incubated with 100 nM [^{3}H]Sph (0.1 μCi/ml). The label was efficiently removed from the medium, and the uptake of [^{3}H]Sph was almost 100% at 10 min after the label addition, which was the same under the conditions where platelets were stimulated with thrombin or TPA. As described previously (*12*), [^{3}H]Sph incorporated into platelets was rapidly converted to [^{3}H]Sph-1-P; [^{3}H]Sph was also converted by *N*-acylation to [^{3}H]Cer, and then to [^{3}H]sphingomyelin, although slowly. After 2 h, about 60% of the [^{3}H]Sph originally added was converted to [^{3}H]Sph-1-P, and the ratio of [^{3}H]Sph-1-P to [^{3}H]Sph, which depends upon the relative conversion from Sph to Sph-1-P by Sph kinase and the reverse process by (possible) phosphatase, remained constant thereafter. Washed platelet suspensions were prepared from platelet-rich plasma incubated with [^{3}H]Sph for 2 h, adjusted to 3 $\times$ 10^8 cells/ml, and then used to examine the formation of sphingolipids in stimulated platelets. The [^{3}H]sphingolipid-labeled washed platelets (0.5 ml aliquots) were treated as indicated, the reactions were terminated by the addition of ice-cold chloroform/methanol/concentrated HCl (100:200:1), and then [^{3}H]sphingolipids were extracted and analyzed by TLC autoradiography, as previously described (*12*). The areas of silica gel containing radiolabeled sphingolipids, *i.e.*, Sph, Sph-1-P, Cer, and sphingomyelin, were scraped off and counted by liquid scintillation counting.

When [^{3}H]sphingolipid-labeled washed platelets (prepared as described above) were incubated at 37°C, the radioactivity of [^{3}H]Sph-1-P decreased, while that of [^{3}H]Sph, [^{3}H]Cer, and [^{3}H]sphingomyelin increased, even without stimulation; this may have been due to weak platelet activation occurring during the platelet preparation. Accordingly, the radioactivity distribution among [^{3}H]sphingolipids in the control platelets (incubated for the indicated duration without stimulation) was different from those in the labeled platelets immediately after preparation.

Metabolism of [^{3}H]Sph, [^{3}H]C6-Cer, [^{32}P]Sph-1-P, or [3-^{3}H]Sph-1-P—Washed platelets were incubated with 100 nM [^{3}H]Sph, [^{3}H]C_6-Cer, [^{32}P]Sph-1-P, or [3-^{3}H]Sph-1-P for the indicated times. The reactions were terminated by the addition of ice-cold chloroform/methanol/concentrated HCl (100:200:1), and lipids were extracted and analyzed by TLC autoradiography, as described previously (*12*).

The formation of [^{3}H]C_6-sphingomyelin from [^{3}H]C_6-Cer was confirmed by sphingomyelinase treatment of the extracted lipids, which was performed as described previously (*17*).

Sph-1-P Lyase and Phosphatase Activity Assays—Platelet homogenates were prepared by sonication, as reported (*15*). Sph-1-P lyase and phosphatase activities were measured essentially as described previously (*18*), except that [3-^{3}H]Sph-1-P instead of [4,5-^{3}H]dihydrosphingosine-1-phosphate was used as the substrate. The reaction time was 30 min, and the products were applied to silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). The plates were then developed in chloroform/methanol/acetic acid (50:50:1) or butanol/acetic acid/water (3:1:1). The areas of silica gel containing radioactive lyase metabolites and Sph were scraped off and counted by liquid scintillation counting to measure the Sph-1-P lyase and phosphatase activities, respectively. It was confirmed that [3-^{3}H]Sph formation increased linearly with time up to 60 min.

Quantitative Measurement of Sph and Sph-1-P by HPLC—Sph-1-P was first extracted into the upper aqueous phase under alkaline conditions by Folch's phase separation, and then reextracted into the lower chloroform phase under acidic conditions (*19*). The extracted Sph-1-P was *N*-acylated with [^{3}H]acetic anhydride into [^{3}H]C_2-Cer-1-P (*N*-[^{3}H]acetylated Sph-1-P), as described previously (*19*). Sph was extracted into the lower chloroform phase, and *N*-acylated with [^{3}H]acetic anhydride into [^{3}H]C_2-Cer (*N*-[^{3}H]acetylated Sph), as described previously (*20*). The acylated products were separated by reverse phase HPLC on a TSK-GEL ODS-80TM column

Table 1. **Radioactivity distribution among sphingolipids in platelet suspensions loaded with [^{3}H]Sph.**

	Sph-1-P	Sph	Cer	Sphingomyelin
Control	43.2 ± 4.3%	11.0 ± 2.4%	43.8 ± 4.9%	2.0 ± 0.2%
Thrombin	33.1 ± 4.1%	14.2 ± 2.9%	49.7 ± 4.9%	3.0 ± 0.3%
	(0.0049)	(0.0212)	(0.0086)	(0.0033)
TPA	35.1 ± 3.6%	15.1 ± 3.7%	46.7 ± 5.9%	2.7 ± 0.3%
	(0.0033)	(0.0356)	(0.0623)	(0.0067)

Washed platelet suspensions prepared after [^{3}H]Sph loading (see "MATERIALS AND METHODS") were stimulated without (control) or with 0.1 U/ml of thrombin or 1 μM TPA for 1 h. The radioactivities incorporated into Sph-1-P, Sph, Cer, and sphingomyelin were measured, and are expressed as percentages of the total sphingolipid radioactivity, which were 45,135 ± 5,614, 50,429 ± 4,280, and 46,268 ± 5,549 dpm for the control, thrombin-stimulated, and TPA-stimulated platelets, respectively. Data are expressed as means ± SD (n = 3). The decrease in Sph-1-P radioactivities, and increases in Sph, Cer, and sphingomyelin radioactivities (compared with the control) were statistically analyzed by the paired t-test, and p values are indicated in parentheses.

(4.6 mm × 150 mm) (TOSOH, Tokyo, Japan). The mobile phase consisted of 85% methanol plus 1% phosphoric acid, at the flow rate of 1 ml/min. Radioactivity in the eluate was monitored on-line with a 171 radioisotope detector (Beckman Coulter, Inc., Fullerton, CA).

When washed platelets were incubated at 37°C, the amounts of Sph-1-P and Sph decreased and increased, respectively, even without stimulation, probably due to weak platelet activation (occurring during the platelet preparation). Since there was variation between experiments in the undesired basal activation of platelets, the results of a representative experiment are shown.

Analysis of Extracellular [^{3}H]Sph-1-P in [^{3}H]Sph-Labeled Platelet Suspensions—Platelet suspensions (0.5 ml) were incubated with 100 nM [^{3}H]Sph (0.2 μCi) and then stimulated with the indicated agent. At the times indicated, the platelets were centrifuged for 15 s at 12,000 × g. Lipids were then extracted from the resultant supernatant and cell pellet, and analyzed for [^{3}H]Sph metabolism by TLC autoradiography, as described above. The extracellular Sph-1-P in the medium was calculated as 100 × ([^{3}H]Sph-1-P in medium)/(total [^{3}H]Sph-1-P in medium plus cell pellets), and extracellular Sph-1-P after stimulation as (percentage extracellular Sph-1-P in the medium upon stimulation) – (percentage extracellular Sph-1-P in the medium without stimulation).

Sph-1-P Ectophosphatase Activity Assay—Platelet suspensions were incubated with 50 nM [^{33}P]Sph-1-P in buffer containing 0.5% fatty acid-free BSA. The reactions (0.5 ml aliquots) were terminated by transfer to 0.5 ml of 1 M $HClO_4$. After centrifugation, Sph-1-P ectophosphatase activity was determined in the supernatants by measuring $^{33}P_i$ formation from the labeled Sph-1-P, as described previously (*21*, *22*).

RESULTS

Effect of Thrombin and TPA on [^{3}H]Sphingolipid Formation in Platelets Labeled with [^{3}H]Sph to a State of Equilibration—We first performed overall evaluation of sphingolipid metabolism during platelet activation. For this purpose, washed platelet suspensions incubated with [^{3}H]Sph to a state of equilibration (at least as far as Sph/Sph-1-P conversion was concerned) were prepared, and then the formation of [^{3}H]sphingolipids upon stimulation was examined. Thrombin acts on G protein-coupled seven-transmembrane receptors and strongly activates platelets, mainly through phosphoinositide turnover (*23*), while TPA stimulates platelets through direct protein kinase C activation (*24*). When [^{3}H]Sph-loaded platelets were stimulated with thrombin or TPA, a decrease in [^{3}H]Sph-1-P formation was observed (Table 1). It was considered that the decrease in Sph-1-P formation may have substantially affected the formation of other sphingolipids, since platelets abundantly store this phospholipid (*9*, *13*). In fact, increases in [^{3}H]Sph, [^{3}H]Cer, and [^{3}H]sphingomyelin formation were observed under the conditions employed (Table 1).

Effects of Thrombin and TPA on Sph Conversion into Cer, and Cer Conversion into Sphingomyelin—Since it is known that platelets lack Sph-1-P lyase activity (*5*, *12*), the above findings indicate that Sph-1-P phosphatase activity is present in these anucleate cells and plays an important role in the formation of Sph. Although it was possible that the increases in the Cer and sphingomyelin radioactivity levels were also due to enhanced Sph-1-P hydrolysis, the involvement of Cer synthase and sphingomyelin synthase, respectively, could not be ruled out. Accordingly, we examined the effects of thrombin and TPA on Sph conversion into Cer (reflecting Cer synthase activity), and Cer conversion into sphingomyelin (reflecting sphingomyelin synthase activity). As shown in Table 2, both thrombin and TPA did not affect these activities.

Detection of Sph-1-P Phosphatase (but Not Lyase) Activity in Platelets—The above metabolic studies indicate that in platelets, Sph-1-P phosphatase activity plays an important role in the formation of Sph, Cer, and

Table 2. **Effects of thrombin and TPA on Sph conversion into Cer, and Cer conversion into sphingomyelin.**

	Sph conversion into Cer (%)	Cer conversion into sphingomyelin (%)
Control	7.5 ± 0.6	2.0 ± 0.2
Thrombin	7.2 ± 1.0	2.0 ± 0.3
TPA	7.3 ± 0.8	1.9 ± 0.3

To analyze Sph conversion into Cer, platelets were treated with [^{3}H]Sph alone (control), [^{3}H]Sph plus 0.1 U/ml of thrombin, or [^{3}H]Sph plus 1 μM TPA for 1 h. Lipids were extracted, and [^{3}H]Cer formation from [^{3}H]Sph was analyzed by TLC autoradiography. Cer formation was calculated as 100 × [^{3}H]Cer formed/[^{3}H]Sph originally added. To analyze Cer conversion into sphingomyelin, platelets were treated with [^{3}H]C_6-Cer alone (control), [^{3}H]C_6-Cer plus 0.1 U/ml of thrombin, or [^{3}H]C_6-Cer plus 1 μM TPA for 2 h. Lipids were extracted, and [^{3}H]C_6-sphingomyelin formation from [^{3}H]C_6-Cer was analyzed by TLC autoradiography. Sphingomyelin formation was calculated as 100 × [^{3}H]C_6-sphingomyelin formed/[^{3}H]C_6-Cer originally added. Data are expressed as means ± SD (n = 3).

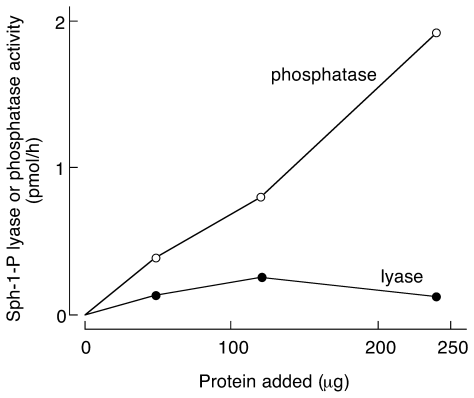

Fig. 1. **Presence of phosphatase (but not lyase) activity in platelets.** Various protein amounts of platelet homogenates were assayed for Sph-1-P phosphatase (open circles) and lyase (solid circles) activities by measuring the conversion of [3-^{3}H]Sph-1-P into [^{3}H]palmitaldehyde, [^{3}H]palmitic acid, and [^{3}H]palmitol (lyase activity), and [^{3}H]Sph (phosphatase activity), respectively. Similar results were obtained in three other experiments.

sphingomyelin. When [^{3}H]Sph-1-P was added to platelet homogenates under established assay conditions for Sph-1-P lyase (*18*), no lyase products (palmitaldehyde, palmitic acid, and palmitol) were formed (Fig. 1), as reported previously (*12*). In contrast to the absence of lyase activity, the conversion of [^{3}H]Sph-1-P into [^{3}H]Sph, reflecting Sph-1-P phosphatase activity, was detected in platelets (Fig. 1), as expected.

When homogenates of 2×10^8 platelets (treated without or with thrombin or TPA under the conditions employed for Table 1) were analyzed, thrombin or TPA failed to enhance Sph-1-P phosphatase activity (Table 3).

Quantification of Sph-1-P and Sph in Platelets—Next, we quantitatively measured Sph and Sph-1-P by their *N*-acylation with [^{3}H]acetic anhydride into [^{3}H]C_2-Cer (*N*-[^{3}H]acetylated Sph) and [^{3}H]C_2-Cer-1-P (*N*-[^{3}H]acetylated Sph-1-P), respectively. In this study, we detected the final acetylated products with an HPLC system, instead of scraping off the spots on TLC silica gels [as we did previously (*19*)], for accurate measurement; [^{3}H]C_2-Cer and [^{3}H]C_2-Cer-1-P, which were derived from Sph and Sph-1-P, respectively, were clearly detected with the newly-developed reverse phase HPLC system (Fig. 2).

Table 3. **Effect of thrombin and TPA on Sph-1-P phosphatase activity.**

	Sph-1-P phosphatase activity (%)
thrombin	91 ± 5
TPA	94 ± 6

Platelets were treated without or with 0.1 U/ml of thrombin or 1 μM TPA for 1 h. Platelet homogenates were prepared and assayed for Sph-1-P phosphatase activity by measuring the conversion of [3-^{3}H]Sph-1-P into [^{3}H]Sph. Data are expressed as percentages of the control activity without treatment (mean ± SD, n = 3).

The amounts of Sph-1-P and Sph decreased and increased, respectively, upon stimulation with thrombin or the direct protein kinase C activator TPA (*24*) (Fig. 3), which was consistent with the data obtained using [^{3}H]Sph-loaded platelets (Table 1). Furthermore, these stimulation-dependent changes were attenuated by staurosporine (Fig. 3), a potent inhibitor of protein kinases, including protein kinase C (*25*). Accordingly, it was considered that the increase in the Sph amount and the decrease in the Sph-1-P amount, probably reflecting Sph-1-P dephosphorylation, may be related to protein kinase C activation; under similar conditions, we confirmed that thrombin and TPA strongly activated protein kinase C, which was abolished by staurosporine (*26*). However, further studies may be needed to confirm this, due to the use of a non-selective inhibitor.

Analysis of Extracellular [^{3}H]Sph-1-P in [^{3}H]Sph-labeled Platelet Suspensions—We previously reported that Sph-1-P, abundantly stored in platelets, can be released by protein kinase C activation; thrombin or TPA strongly induced platelet Sph-1-P release, which was abolished by staurosporine (*12*, *14*). Accordingly, we checked for the extracellular presence of Sph-1-P, under the conditions employed for examining the levels of sphingolipids in platelet suspensions, to accurately evaluate Sph-1-P dephosphorylation to Sph; extracellular Sph-1-P could be determined mainly by Sph-1-P release from platelets and Sph-1-P degradation on platelets.

Upon TPA stimulation, which induced Sph-1-P conversion to Sph, over 50% of the [^{3}H]Sph-1-P (formed in platelets incubated with [^{3}H]Sph) was detected extracellularly

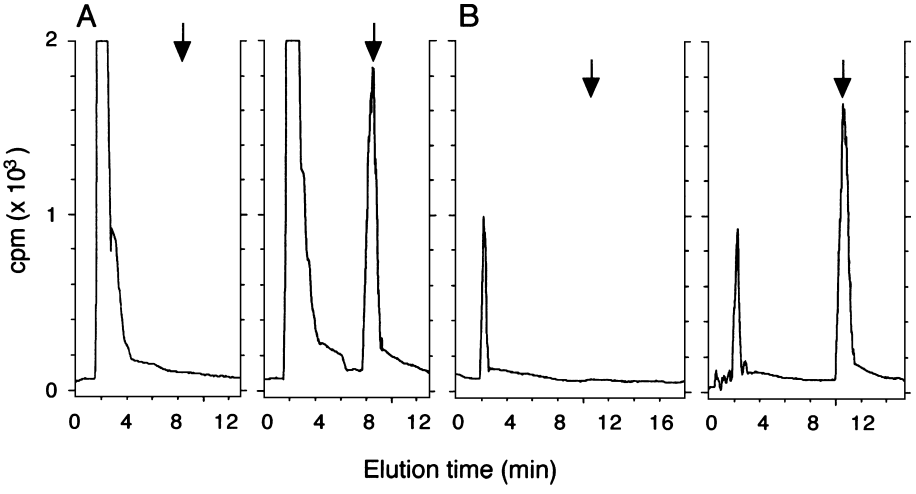

Fig. 2. **Elution profiles of *N*-[^{3}H]acetylated products of Sph-1-P and Sph.** In A, 0 (Left) and 3 (Right) nmol of Sph-1-P were *N*-[^{3}H]acetylated with [^{3}H]acetic anhydride to [^{3}H]C_2-Cer-1-P. In B, 0 (Left) and 3 (Right) nmol of Sph were *N*-[^{3}H]acetylated with [^{3}H]acetic anhydride to [^{3}H]C_2-Cer. The acetylated products were separated by reverse phase HPLC (see "MATERIALS AND METHODS"). The elution locations corresponding to C_2-Cer-1-P (A) and C_2-Cer (B) are indicated by arrows in the chromatographs.

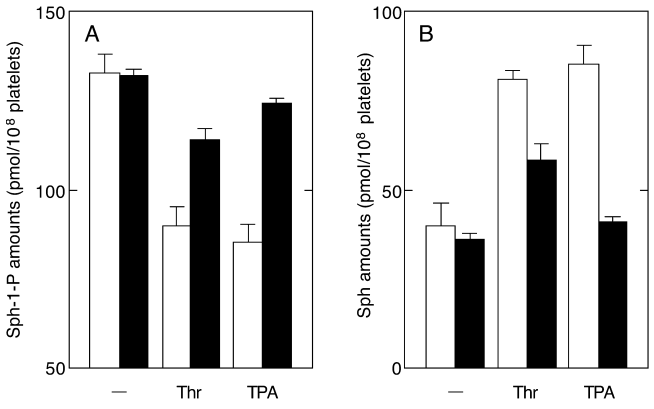

Fig. 3. **Effects of thrombin and TPA on the Sph-1-P and Sph amounts in platelet suspensions.** Platelet suspensions preincubated without (open columns) or with (solid columns) 1 μM staurosporine for 15 s were stimulated with 0.1 U/ml of thrombin (Thr) or 1 μM TPA for 1 h. Sph-1-P and Sph were extracted and quantified by *N*-[^{3}H]acetylation with [^{3}H]acetic anhydride to [^{3}H]C_2-Cer-1-P and [^{3}H]C_2-Cer, respectively. The columns and error bars represent the means ± range for a representative experiment. Similar results were obtained in three other experiments.

(Fig. 4A), possibly due to its release from platelets by protein kinase C activation (*12*, *14*). As expected, this TPA-induced Sph-1-P release from platelets was completely inhibited by staurosporine pretreatment (Fig. 4B). Furthermore, when TPA-induced Sph-1-P release was blocked by staurosporine after the stimulation, the extracellular [^{3}H]Sph-1-P radioactivity was found to be reduced (Fig. 4B). This indicates that, in TPA-stimulated platelet suspensions, the presence of extracellular Sph-1-P is balanced by Sph-1-P release from the inside and extracellular Sph-1-P degradation; thus, blockage of Sph-1-P release by staurosporine resulted in a reduction in extracellular [^{3}H]Sph-1-P. It was suggested that at least part of the Sph-1-P released extracellularly undergoes dephosphorylation by an ectoenzyme.

Presence of Sph-1-P Ectophosphatase Activity on Platelets—To check the Sph-1-P ectophosphatase activity, we examined the metabolism of radiolabeled Sph-1-P added to platelet suspensions. As expected, [^{32}P]Sph-1-P was degraded very rapidly when added extracellularly to platelet suspensions (Fig. 5A). Similar results were obtained when [^{33}P]Sph-1-P was employed (data not shown); [^{33}P]P_i was recovered in the extracellular medium, indicating ectophosphatase activity (see Fig. 6). In contrast, the radioactivity of [3-^{3}H]Sph-1-P, added similarly, decreased only very slowly, with [^{3}H]Sph and [^{3}H]Cer appearing (Fig. 5B). These results can be best explained by the idea that non-polar [3-^{3}H]Sph, formed from polar [3-^{3}H]Sph-1-P by means of ectophosphatase activity, is incorporated into platelets and then phosphorylated to regenerate [3-^{3}H]Sph-1-P intracellularly by means of Sph kinase or converted to [^{3}H]Cer (and then to

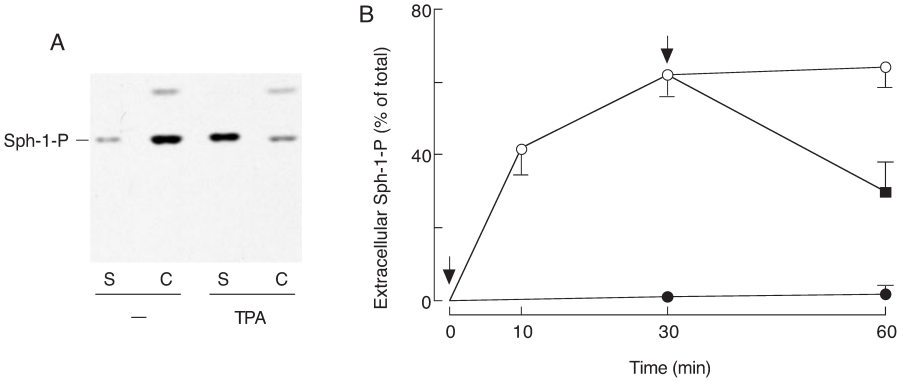

Fig. 4. **Analysis of extracellular [^{3}H]Sph-1-P in [^{3}H]Sph-labeled platelet suspensions.** (A) Platelet suspensions incubated with [^{3}H]Sph were stimulated without (–) or with 1 μM TPA. After 60 min incubation, samples were centrifuged, and lipids were extracted from the resultant supernatant (S) and cell pellet (C), and analyzed by TLC autoradiography. (B) Platelets were preincubated without (open circles) or with (solid circles) 1 μM staurosporine for 1 min, and then stimulated with 1 μM TPA. When indicated (a solid square), platelets were first treated with 1 μM TPA and then 1 μM staurosporine (30 min after TPA addition). Extracellular Sph-1-P (in the medium supernatant) was measured as described under "MATERIALS AND METHODS." Data are expressed as means ± SD (n = 3).

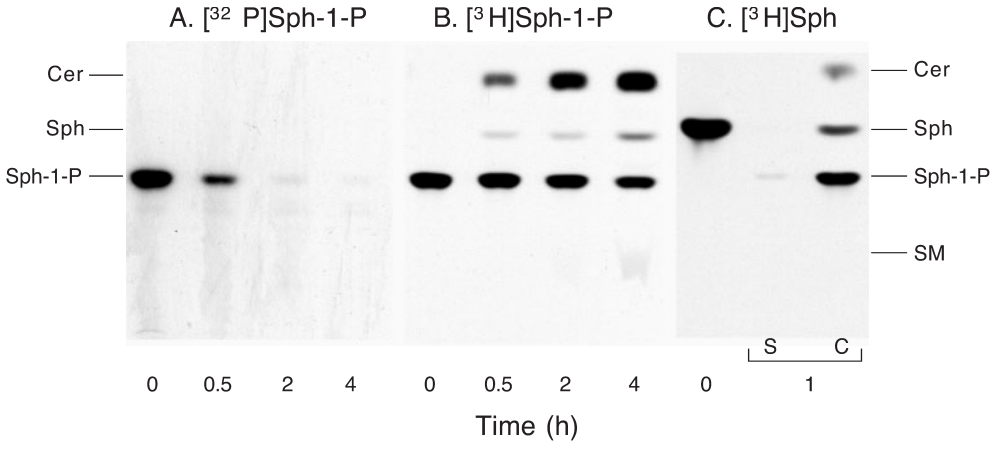

Fig. 5. **Metabolic fates of [^{32}P]Sph-1-P, [3-^{3}H]Sph-1-P, and [^{3}H]Sph exogenously added to platelet suspensions.** Platelet suspensions were incubated with [^{32}P]Sph-1-P (A), [3-^{3}H]Sph-1-P (B), or [^{3}H]Sph (C) for the indicated times. After the reactions, lipids were extracted from the whole cell suspensions in A and B, and from (after separation by centrifugation) the cell pellet (C) and supernatant (S) in C. Metabolic changes in each radiolabeled sphingolipid were analyzed. The locations of standard lipids are indicated on the left.

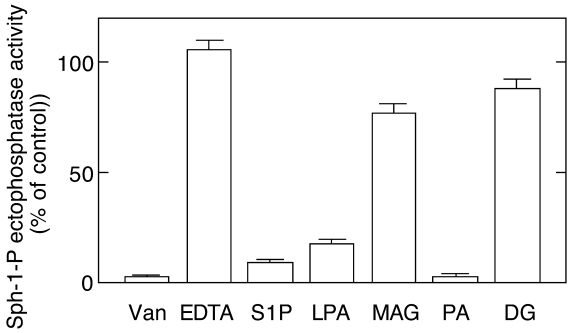

Fig. 6. **Platelet Sph-1-P ectophosphatase activity.** Platelet suspensions were incubated with [^{33}P]Sph-1-P in the absence or presence of 1 mM vanadate (Van), 1 mM EDTA (instead of $MgCl_2$), 5 μM (non-radioactive) Sph-1-P (S1P), 5 μM lysophosphatidic acid (LPA), 5 μM monoacylglycerol (MAG), 5 μM phosphatidic acid (PA), or 5 μM diacylglycerol (DG) for 10 min. [^{33}P]Sph-1-P phosphatase activity was determined by counting water-soluble ^{33}Pi radioactivity and is expressed as a percentage of the control value. The columns and error bars represent means ± SD (n = 3).

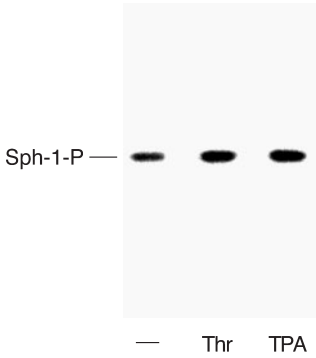

Fig. 7. **Effect of thrombin and TPA on the metabolism of [^{32}P]Sph-1-P exogenously added to platelet suspensions.** Platelet suspensions were incubated with [^{32}P]Sph-1-P for 30 min under simultaneous stimulation with 0.1 U/ml of thrombin (Thr), 1 μM TPA, or nothing (–). After the reactions, lipids were extracted from the whole cell suspensions, and then metabolic changes of [^{32}P]Sph-1-P were analyzed.

sphingomyelin); Sph (but not Sph-1-P) is hydrophobic and easily passes through the lipid bilayer. In fact, [3-^{3}H]Sph, added exogenously, was rapidly incorporated into platelets, and converted to [3-^{3}H]Sph-1-P and [3-^{3}H]Cer (Fig. 5C).

We next characterized the ectophosphatase activity expressed on platelets. [^{33}P]Sph-1-P dephosphorylation was strongly inhibited in the presence of the phosphatase inhibitor vanadate, but was not affected on omission of Mg^{2+} (Fig. 6). Furthermore, the [^{33}P]Sph-1-P dephosphorylation was inhibited not only by non-labeled Sph-1-P, but also by lysophosphatidic acid and phosphatidic acid (Fig. 6). In contrast, monoacylglycerol and diacylglycerol failed to mimic the effects of their phosphorylated counterparts, *i.e.*, lysophosphatidic acid and phosphatidic acid (Fig. 6). These characteristics of platelet phosphatase activity are compatible with those of lipid phosphate phosphatase (LPP), also known as type 2 phosphatidic acid phosphatase (*7*, *21*, *22*, *27–31*).

Given that Sph-1-P released from the inside of platelets undergoes dephosphorylation, that of exogenously-added, radio-labeled Sph-1-P should be hampered by (non-labeled) Sph-1-P released from the inside of platelets. Accordingly, we checked the effects of thrombin and TPA (which induce platelet Sph-1-P release) on [^{32}P]Sph-1-P ectophosphatase activity. Dephosphorylation of [^{32}P]Sph-1-P (see Fig. 5A for the time course) was decreased by treatment with each of these stimulants (Fig. 7); TPA (1 μM) enhanced the radioactivity of [^{32}P]Sph-1-P (remaining outside the platelets) to 204 ± 27% (n = 3) of the control level. It seems likely that extracellular Sph-1-P release, but not Sph-1-P ectophosphatase activity, regulates the rate of Sph-1-P dephosphorylation in stimulated platelets since the effect of thrombin or TPA on the Sph-1-P phosphatase activity itself was marginal, as described above.

DISCUSSION

We previously reported that the formation of Sph was induced in platelets stimulated with thrombin or TPA (*32*). In that study, however, we could not identify the source of the enhanced Sph formation. In the present study, we modified the experimental conditions; the incubation time for platelet stimulation was prolonged, and more sensitive methods for Sph and Sph-1-P quantitation were employed. As a result, we found that Sph-1-P dephosphorylation led to Sph formation in platelets stimulated with thrombin or TPA. Furthermore, it seems that Sph-1-P dephosphorylation is the first step of the formation of not only Sph but also Cer and sphingomyelin. This is because the radioactivity of Cer and sphingomyelin (in addition to Sph) increased, in parallel with a decrease in that of Sph-1-P, upon stimulation with thrombin or TPA, while both Cer and sphingomyelin synthase activities were not affected by such treatment. However, as we have already reported (*32*), a change in the chemical amount of Cer was not actually detected under the conditions that the Sph level increased. Presumably, it may be difficult to measure accurately small changes in the

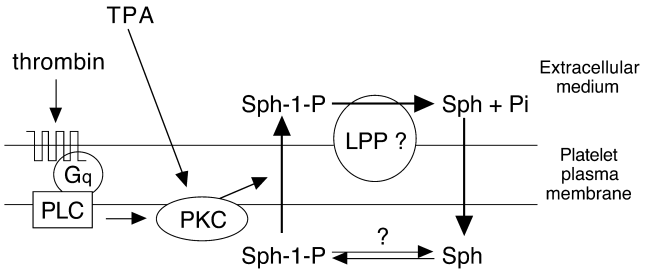

Fig. 8. **Proposed scheme for a transmembrane cycling pathway of sphingolipid metabolism in platelets.** Platelets, which store Sph-1-P abundantly, release this lysophospholipid extracellularly upon stimulation, possibly through mediation by protein kinase C (PKC). The released Sph-1-P is dephosphorylated by an ectophosphatase, possibly LPP, to non-polar Sph, which is then incorporated into platelets. Intracellularly, Sph may be phosphorylated to regenerate Sph-1-P by means of Sph kinase or converted to Cer (and then to sphingomyelin). Thus, the levels of Sph-1-P and Sph decreased and increased, respectively, in stimulated platelets. There is also the possibility that part of Sph-1-P that remains inside platelets (even after stimulation) undergoes dephosphorylation intracellularly.

amount of Cer (formed from Sph) in platelets, because the amount of Cer is much greater than that of Sph.

It is noteworthy that Sph-1-P conversion (dephosphorylation) into Sph may take place extracellularly through mediation by an ectophosphatase. Several isoenzymes of mammalian LPP have been cloned, and they are believed to act at the outer leaflet of the cell surface bilayer, accounting for the ecto-phosphatase activities toward Sph-1-P, lysophosphatidic acid, phosphatidic acid, and Cer 1-phosphate (*21*, *22*, *27–31*). Although this was recently confirmed in platelets (*31*), the involvement of LPP in Sph formation from Sph-1-P remains to be examined. Considering that thrombin and TPA strongly induce extracellular Sph-1-P release (possibly through protein kinase C activation) but seemingly fail to enhance Sph-1-P ectophosphatase activity, Sph-1-P release, but not Sph-1-P ectophosphatase activity, may regulate the rate of Sph-1-P dephosphorylation in stimulated platelets. Our present findings, summarized in Fig. 8, may be surprising, considering the well-established sphingomyelin breakdown model for the formation of sphingolipid signaling molecules (*1*, *2*, *4*).

There are several unique aspects in platelet sphingolipid metabolism. Platelets exceptionally lack Sph-1-P lyase, which degrades Sph-1-P into phosphoethanolamine and a fatty aldehyde (*5*, *9*, *12*). Accordingly, Sph-1-P dephosphorylation is the only possible pathway for the degradation of Sph-1-P in platelets. On the other hand, platelets possess a highly active Sph kinase, which, together with a lack of Sph-1-P lyase, is responsible for abundant Sph-1-P accumulation in these anucleate cells (*9*, *12*, *13*). Furthermore, Sph-1-P, abundantly stored in platelets, can be released extracellularly upon protein kinase C activation (*9*, *12*, *14*). Finally, platelets express the ectophosphatase LPP (*31*), for which Sph-1-P is a good substrate (*21*, *22*). Considering these points, the presence in anucleate platelets of a transmembrane cycling pathway starting with Sph-1-P dephosphorylation and leading to the formation of other sphingolipid mediators appears to be reasonable.

Recently, evidence has accumulated showing that sphingolipids have an important function in signaling; these lipids serve as intracellular second messengers and extracellular mediators (*1–4*). Basically, the sphingolipid metabolism for the formation of these signaling molecules is conserved from yeast to mammals. Platelets are anucleate cells, and have nothing to do with cell growth, differentiation, programmed cell death, or stress responses, the biological processes in which sphingolipids play an important role. Presumably, sphingolipid metabolism in anucleate platelets is specialized for specific purpose(s), which remain to be clarified in further studies.

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