Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner

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Abstract **Sphingosine 1-phosphate (S1P) is accumulated in platelets and released on stimulation by thrombin or Ca^{2+}. Thrombin-stimulated S1P release was inhibited by staurosporin, whereas Ca^{2+}-stimulated release was not. When the platelet plasma membrane was permeabilized with streptolysin O (SLO), S1P leaked out with cytosol markers, whereas granular markers remained in the platelets. The SLO-induced S1P leakage required BSA, probably for solubilization of S1P in the medium. These results indicate that S1P is localized in the inner leaflet of the plasma membrane and that its release is a carrier-mediated process. We also used alpha-toxin (ATX), which makes smaller pores in the plasma membrane than SLO and depletes cytosolic ATP without BSA-dependent S1P leakage. The addition of ATP drove S1P release from ATX platelets. The ATP-driven S1P release from ATX platelets was greatly enhanced by thrombin. An ATP binding cassette transporter inhibitor, glyburide, prevents ATP- and thrombin-induced S1P release from platelets. Ca^{2+} also stimulated S1P release from ATX platelets without ATP, whereas the Ca^{2+}-induced release was not inhibited by glyburide. Our results indicate that two independent S1P release systems might exist in the platelet plasma membrane, an ATP-dependent system stimulated by thrombin and an ATP-independent system stimulated by Ca^{2+}.**—Kobayashi, N., T. Nishi, T. Hirata, A. Kihara, T. Sano, Y. Igarashi, and A. Yamaguchi. **Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner.** ***J. Lipid Res.*** **2006.** 47: **614–621.**

Supplementary key words ATP binding cassette transporter • flip-flop • lipid mediator • streptolysin O • α-toxin

The secretion of signal transmitter molecules is a key step in the intercellular signal transduction process. It must be highly controlled. Although a lot of well-studied signal molecules are secreted through exocytosis (1), the secretion mechanisms for most amphiphilic signal molecules, such as steroid hormones (2), lipid mediators, and some peptide hormones (3), are unknown. There is no evidence for the secretion of these amphiphilic signal molecules via an exocytotic pathway, but most of their exporters have nevertheless been identified, the exception being ATP binding cassette (ABC) C1, which was reported to be an exporter of leukotriene C4 (LTC4) (4). Sphingosine 1-phosphate (S1P) is one such signal transmitter having an unknown secretion process (5).

S1P is a sphingolipid metabolite that functions as both extracellular and intracellular signaling mediators in the regulation of diverse biological processes, such as proliferation, differentiation, apoptosis, and cell motility (6, 7). The extracellular effects of S1P are mediated via members of the S1P receptor families, five subtypes of which ($S1P_1$–$S1P_5$) are now known (8). The first three receptors are widely expressed, whereas the distributions of $S1P_4$ and $S1P_5$ are more restricted in the immune and nervous systems (9). S1P is synthesized via the phosphorylation of sphingosine, which is catalyzed by sphingosine kinase (10). Once formed, S1P is rapidly cleaved into hexadecenal and phosphoethanolamine by S1P lyase or dephosphorylated by sphingosine phosphohydrolase (8, 11). Hence, the balance of the rates of its synthesis and degradation determines the intracellular concentration of S1P; as a result, many types of cells contain a small amount

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Abbreviations: ABC, ATP binding cassette; ACD, acid citrate-dextrose solution; ATX, α-toxin; LDH, lactate dehydrogenase; LTC4, leukotriene C4; PF4, platelet factor 4; PKC, protein kinase C; PLSCR, platelet phospholipid scramblase; PRP, platelet-rich plasma; SLO, streptolysin O; S1P, sphingosine 1-phosphate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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of S1P attributable to the high activities of S1P lyase and phosphatases (8, 11). However, there is one exception: the platelets. S1P is highly accumulated in platelets because of the high activity of sphingosine kinase and the lack of S1P lyase in the platelets (5, 12, 13). S1P is released from platelets induced by thrombin. Recent work demonstrated that >80% of potent chemoattractive activity generated in human serum during clotting results from platelet-derived S1P (14).

Platelets are extremely reactive cells that respond to various stimuli, such as thrombin, collagen, and ADP, and contribute to the filling in of any vascular rupture by secreting a number of modulators and physiologically active compounds (15). Platelets possess three kinds of intracellular granules: α-granules, dense granules, and lysosomes (15). These granules are known to secrete various signal molecules, such as platelet factor 4 (PF4), serotonin, and acid hydrolases, through exocytosis. Although the secretion pathway for platelet S1P is the key step in its intercellular signaling, little is known about this process (5). In this study, we investigated the process of S1P secretion from platelets.

METHODS

Materials

Streptolysin O (SLO), BSA (fatty acid-free), alpha-toxin (ATX), glyburide, cyclosporin A, and sphingosine were obtained from Sigma. [^{3}H]sphingosine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]serotonin was from Perkin-Elmer (Boston, MA), and γ-[^{32}P]ATP (370 MBq/ml) was from Amersham Biosciences Corp. (Piscataway, NJ). Trypsin and proteinase K were purchased from Nacalai (Kyoto, Japan). Other chemicals were of reagent grade and from commercial sources.

Isolation of rat platelets

Wistar ST rats (9–13 weeks old, female) were anesthetized with diethylether. Then, whole blood was collected from their hearts using acid citrate-dextrose solution (ACD) as an anticoagulant, and platelets were collected in the platelet-rich plasma (PRP) obtained on centrifugation at 500 *g* for 15 min. For the S1P release assay, platelets were washed with a mixture of buffer A [20 mM HEPES-NaOH (pH 7.4), 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 138 mM NaCl, and 1 mg/ml glucose] and ACD, followed by immediate resuspension in fresh medium.

Measurement of [^{3}H]S1P release from platelets

S1P release from platelets was measured as reported previously by Yatomi et al. (13) with slight modifications. Platelet suspensions (0.5 ml, 2×10^7 platelets/ml) in buffer A containing 1% BSA were preincubated at 37°C for 10 min in the absence or presence of 1 μM staurosporin. Then, a mixture of 1 μl of 5 μM [^{3}H]sphingosine (0.1 μCi) and 24 μl of buffer A containing 1% BSA was added to each suspension (final concentration of sphingosine, 10 nmol/l) and incubated for 1 min at 37°C. Next, 25 μl of rat thrombin (final concentration, 5 U/ml), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (100 ng/ml), or A23187 (10 μM) and Ca^{2+} (2 mM) were added to the mixture, followed by incubation for 10 min except as indicated. After incubation, platelets and the medium were separated by centrifugation for 5 s at 12,000 *g*. Lipids were then extracted from the supernatant, and the platelets and analyzed by TLC with development with butanol-acetic acid-water (3:1:1). Radioactive bands were detected by autoradiography with a FLA-3000 Bioimaging Analyzer (Fuji Film Co., Tokyo, Japan).

Measurement of thrombin-induced PF4 and β-hexosaminidase secretion

After preincubation of platelets (2×10^7 platelets/ml) in buffer A containing 1% BSA at 37°C for 5 min, thrombin (final concentration, 5 U/ml) was added to induce platelet secretion. The platelets and the medium were separated by centrifugation for 5 s at 12,000 *g* after incubation for the indicated times. After precipitation with TCA, the resulting protein precipitate was subjected to SDS-PAGE, and PF4 was measured by Western blot analysis with anti-PF4 antibodies (AF595; R&D Systems). Band density was quantified using the computer program Image Gauge (Fuji Film Co.). The activity of β-hexosaminidase was measured as reported previously (16).

Measurement of serotonin secretion

[^{3}H]serotonin-preloaded platelets were prepared by addition of [^{3}H]serotonin to the PRP (final concentration, 1 μCi/ml PRP). The mixtures were then incubated for 1 h at 30°C. After incubation, the platelets were washed twice with a mixture of buffer A containing 1% BSA and ACD. The concentration of the platelets was adjusted to 2×10^7 platelets/ml. Then, the platelet suspension was incubated at 37°C for 5 min, followed by the addition of thrombin (final concentration, 5 U/ml) to induce platelet secretion. The platelets and the medium were separated by centrifugation for 5 s at 12,000 *g* after incubation for the indicated times. The [^{3}H]serotonin radioactivity was determined with a liquid scintillation counter, Ultima Gold™ XR (Perkin-Elmer).

SLO treatment of platelets

This experiment was performed based on a previous report (17). To measure the serotonin leakage by SLO treatment, the PRP was divided into two aliquots. [^{3}H]serotonin (specific activity, 30 Ci/mmol) was added to one of these aliquots (final concentration, 1 μCi/ml), and nonlabeled serotonin was added to the other aliquot as a control. The mixtures were then incubated for 1 h at 30°C. After incubation, the platelets were washed twice with a mixture of buffer A containing 1% BSA and ACD. The resulting serotonin-preloaded platelets (5×10^6 cells) were used for SLO treatment. As for the S1P leakage assay, [^{3}H]sphingosine (0.1 μCi; final concentration, 10 nmol/l) or nonlabeled sphingosine was added to the platelet suspension, followed by incubation for 5 min at 37°C. Sphingosine was taken up into the PRP and enzymatically converted into S1P. The [^{3}H]serotonin or [^{3}H]S1P-labeled platelets were placed on ice for 5 min, followed by incubation with 0.5 U/ml SLO at 4°C for 10 min. When the temperature of the SLO-treated platelets was shifted from 4°C to 30°C, SLO bound to the platelet plasma membrane. Then, the suspension was separated into platelets and a supernatant by centrifugation. The resulting platelets were resuspended in prewarmed buffer A containing the indicated concentrations of BSA, followed by incubation at 30°C for 5 min. At this step, the platelet plasma membrane was selectively permeabilized. The suspensions were then put on ice for 20 min to allow cytosol molecules to leak out from the platelets. Next, the platelets and supernatant were separated by centrifugation. The radioactivities of both the platelets and supernatants were measured as described above. Lactate dehydrogenase (LDH)

activity, as a cytosol marker, was measured with a LDH detection kit (Wako Co., Ltd., Osaka, Japan).

ATX treatment of rat platelets

[^{3}H]serotonin or [^{3}H]S1P-labeled platelets were prepared as described above except for suspension in buffer A containing 2 mM EGTA. Each platelet suspension was preincubated for 5 min in the absence or presence of 1 μM staurosporin, 200 μM glyburide, 10 μM cyclosporin A, or 50 μM MK571. Then, ATX (final concentration, 0.5 U/ml) was added to the suspension and the mixture was incubated at 37°C for 10 min. In some cases, MgATP (final concentration, 5 mM; pH 7.0) was added together with ATX. Next, either thrombin (final concentration, 5 U/ml) or $CaCl_2$ (final concentration, 2.5 mM) was added to the suspension, followed by incubation for 2 min in the case of serotonin and 10 min in the case of S1P at 37°C. After incubation, the platelets and medium were separated by centrifugation for 5 s at 12,000 *g*. The radioactivity of [^{3}H]serotonin or [^{3}H]S1P was measured as described above.

Determination of ATP release

This experiment was performed according to Holmsen and Dangelmaier (18). Platelets (95 μl, 2×10^7 platelets/ml) were preincubated at 37°C for 5 min. Five microliters of ATX (final concentration, 0.5 U/ml) or thrombin (final concentration, 5 U/ml) was added to the platelet suspension, followed by incubation for the indicated times. After incubation, the platelets and supernatant were separated by centrifugation. The platelets were then resuspended in 100 μl of buffer A containing 1% BSA. One hundred microliters of a prechilled mixture of 1 volume of 0.1 M EDTA (pH 7.4) and 9 volumes of 95% ethanol was added to the resulting platelet suspension and the separated medium, followed by immediate vortexing. After centrifugation, the amount of ATP in 5 μl of each supernatant was determined with the ATP Bioluminescence Assay kit CLSII (Roche Diagnostics, Penzberg, Germany).

RESULTS

Rat platelets release S1P

We measured S1P release from rat platelets (**Fig. 1**). [^{3}H]sphingosine was taken up into platelets and converted to [^{3}H]S1P. Without stimulation, [^{3}H]S1P almost completely remained in the platelets. When thrombin was added, [^{3}H]S1P was significantly released from the platelets. To detect S1P in the medium, BSA is necessary to extract S1P from the cell surface (13). The thrombin-stimulated [^{3}H]S1P release was greatly prevented by pretreatment of the platelets with staurosporin, which is known to be a protein kinase C (PKC) inhibitor. The PKC activator TPA also stimulated [^{3}H]S1P release, similar to thrombin. This TPA-stimulated release was also prevented by staurosporin pretreatment of the cells. Thus, the thrombin stimulation signal is transmitted via the PKC pathway. Ca^{2+} with the ionophore A23187 also stimulated [^{3}H]S1P release, whereas the Ca^{2+}-stimulated [^{3}H]S1P release was not inhibited by the staurosporin pretreatment, indicating a difference in the signal transduction pathway or the S1P release machinery. Because sphingosine kinase activity was not detected in the medium after thrombin stimulation (data not shown), S1P was not synthesized outside the platelets from sphingosine but released from the platelets as S1P. Sphingosine that was observed in the supernatant fraction after stimulation of platelets (Fig. 1) was derived from the dephosphorylation of released S1P, because the

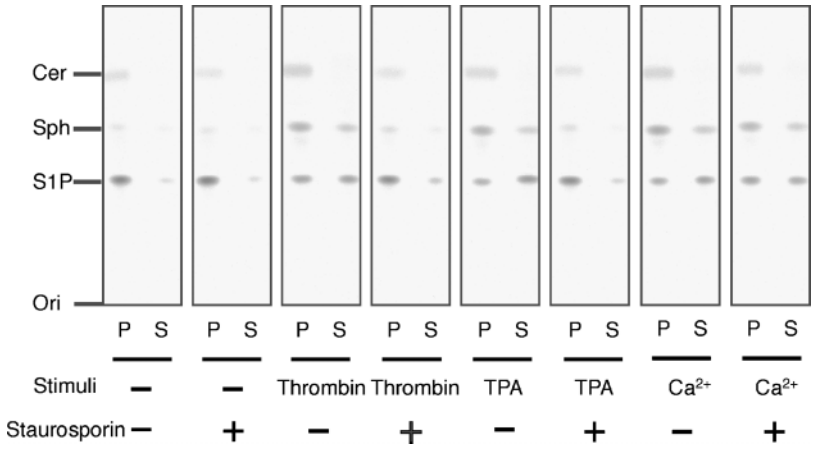

Fig. 1. Sphingosine 1-phosphate (S1P) release from rat platelets. Isolated platelets were preincubated with (+) or without (−) 1 μM staurosporin for 10 min at 37°C and then labeled with [^{3}H]sphingosine for 1 min at 37°C to obtain [^{3}H]S1P-preloaded platelets; S1P release was induced by thrombin (5 U/ml), 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 100 ng/ml), or Ca^{2+} (2 mM) and A23187 (10 μM). After incubation for 10 min at 37°C, platelet suspensions were centrifuged. Lipids extracted from the platelets (P) and supernatants (S) were analyzed by TLC. Spotting positions of samples, and the positions of the spots of ceramide, sphingosine, and S1P, are denoted by Ori, Cer, Sph, and S1P, respectively. The symbols − and + at bottom indicate the absence and presence of the protein kinase C inhibitor staurosporin during S1P preloading. The stimuli line shows the compounds used for induction. Experiments were performed more than three times, and similar results were obtained.

amount of sphingosine was decreased by the treatment of a phosphatase inhibitor, vanadate (data not shown) (19).

Time course of thrombin-induced platelet secretion

The contents of granules such as PF4 and serotonin are secreted from platelets upon thrombin stimulation through exocytosis (15). We compared the time course of [^{3}H]S1P release with those of PF4 and [^{3}H]serotonin. Approximately 80% of the [^{3}H]S1P was released from platelets within 6 min in a time-dependent manner after thrombin stimulation (**Fig. 2A**). On the other hand, 50% of an α granule marker, PF4, was secreted within 30 s, and ~80% of it was secreted only after 2 min (Fig. 2B). Similarly, a dense granule marker, serotonin, was secreted much faster than PF4 and S1P: 60% of [^{3}H]serotonin was secreted, and the maximum was reached within 30 s (Fig. 2C). In contrast, a lysosome marker, β-hexosaminidase, was hardly secreted at all (i.e., only up to ~20% after 30 min) (Fig. 2D). Thus, the characteristics of [^{3}H]S1P release are different from those of exocytosis-mediated secretion. In addition, granule markers were not secreted at all without stimulation, whereas a small but significant amount of [^{3}H]S1P (~10%) was released spontaneously without stimulation (Fig. 2A, at 5 min). Such spontaneous release of [^{3}H]S1P suggests that S1P is not occluded in the granules.

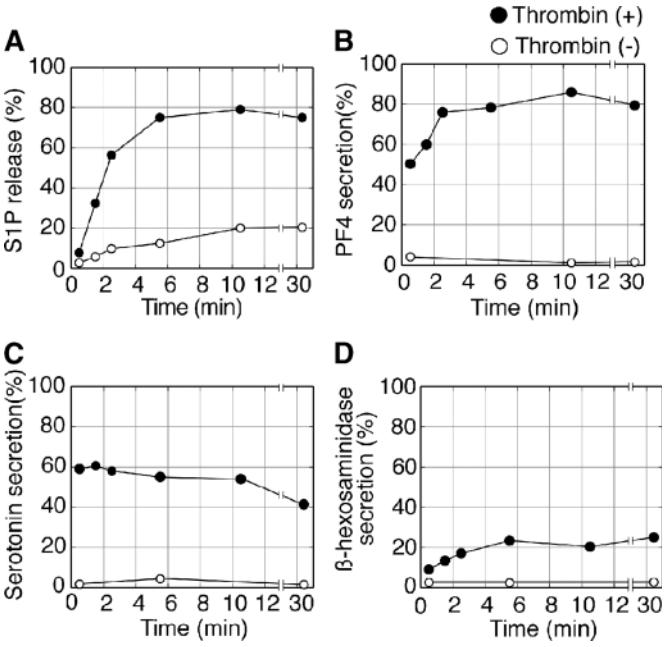

Fig. 2. Time courses of thrombin-induced secretion from platelets. [^{3}H]S1P-accumulated platelets (A), [^{3}H]serotonin-preloaded platelets (C), and nontreated platelets (B, D) were stimulated with thrombin for the indicated times, and then supernatants were separated from the platelets by centrifugation. The amounts of S1P (A), platelet factor 4 (PF4) (B), serotonin (C), and β-hexosaminidase (β-HEX) (D) in the supernatants and platelets were determined as described in Methods. Closed and open circles indicate the release from thrombin-treated and nontreated platelets, respectively. Percentage secretion was calculated as (amount of supernatant)/(total amount of supernatant + platelets). Experiments were performed more than two times, and similar results were obtained.

SLO permeabilization of rat platelets

To obtain direct evidence of the intracellular localization of S1P, plasma membranes were specifically permeabilized with SLO (20). SLO forms pores with cholesterol at 30°C but not at 4°C. The size of the pores ranges up to 30 nm, which is large enough for ions, nucleotides, and proteins of <100 kDa to leak out through the pores, whereas granular markers do not leak out because SLO does not make pores in granular membranes (20).

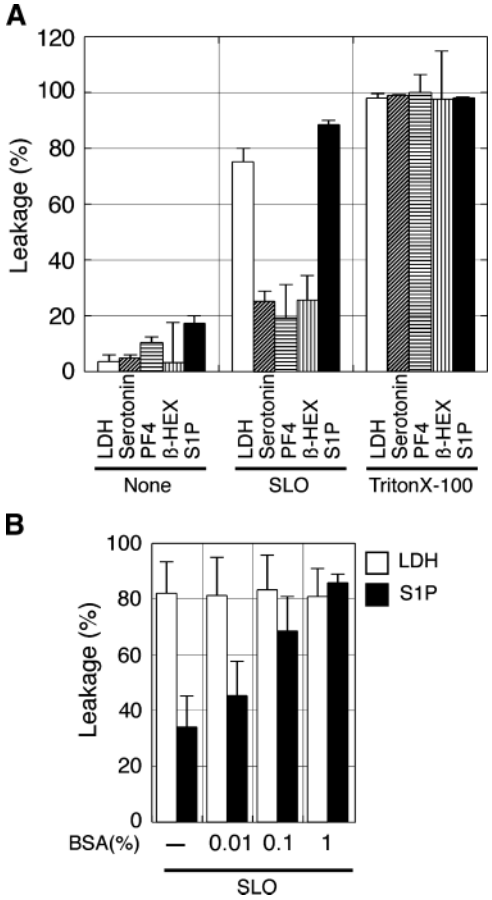

Fig. 3. Leakage of S1P and other cytosolic and granular markers from streptolysin O (SLO)-permeabilized platelets. A: [^{3}H]S1P or [^{3}H]serotonin-preloaded platelets were treated with SLO (0.5 U/ml) or 0.2% Triton X-100 as described in Methods. Then, the platelets and supernatant were separated by centrifugation. The percentage leakage of lactate dehydrogenase (LDH) [cytosol marker (open bars)], [^{3}H]serotonin [dense granule marker (bars with diagonal lines)], PF4 α-granule marker (bars with horizontal lines)], β-hexosaminidase (β-HEX) [lysosome marker (bars with vertical lines)], and [^{3}H]S1P (closed bars) from the platelets was determined as described in Methods. Error bars indicate SD of more than three independent experiments. B: Experiments were performed as described for A except for the indicated BSA concentration after SLO permeabilization. The percentage leakage of LDH and [^{3}H]S1P are indicated as open and closed bars, respectively. Experiments were performed more than three times, and error bars indicate SD.

Actually, although the activity of a cytosol marker, LDH, did not leak out from platelets at 4°C in the presence of SLO (data not shown), after an increase in the temperature to 30°C, LDH began to leak out from the platelets, up to ~80% in 20 min (**Fig. 3A**). On the other hand, an α granule marker, PF4, a dense granule marker, serotonin, and a lysosome marker, β-hexosaminidase, remained in the platelets after SLO treatment (Fig. 3A). Because these marker molecules are small enough to penetrate through the SLO pores, these results indicate that SLO actually permeabilized only the platelet plasma membrane. Under these conditions, [^{3}H]S1P leakage was observed up to 90%, similar to that of LDH (Fig. 3A). These data also indicate that S1P is not occluded in the secretory granules.

BSA is essential for the [^{3}H]S1P leakage from SLO-permeabilized platelets

S1P is barely soluble in water. In the experiments on S1P release (Figs. 1, 2A) and leakage (Fig. 3A), BSA was used for the solubilization of S1P in the medium. The leakage of [^{3}H]S1P from SLO-permeabilized platelets was actually dependent on BSA (Fig. 3B). Under SLO-treated conditions, BSA could go inside the cells through the SLO pore and bind to the S1P inside the cells; then, BSA-S1P complexes leaked out through the SLO pore into the medium. The degree of [^{3}H]S1P leakage was dose-dependent with the concentration of BSA, whereas LDH leakage was independent of BSA (Fig. 3B). Without BSA addition, however, ~30% of the [^{3}H]S1P leaked out into the medium

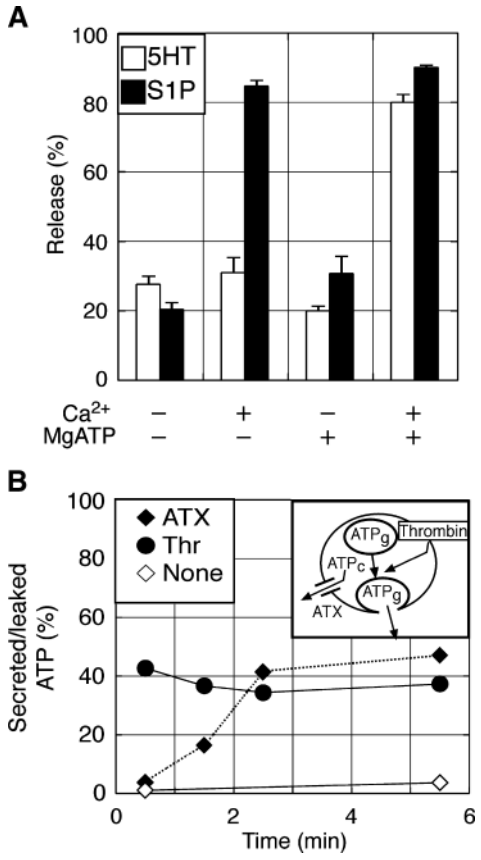

Fig. 4. Serotonin and S1P release from permeabilized platelets with α-toxin (ATX) treatment. A: [^{3}H]serotonin and [^{3}H]S1P-preloaded platelets were permeabilized with ATX for 10 min at 37°C in the presence or absence of 5 mM MgATP, and then the serotonin and S1P release was induced by the addition of Ca^{2+} for 2 min (serotonin) or 10 min (S1P). The addition and no addition of 2 mM Ca^{2+}, as an inducer, and 5 mM MgATP, as an energy source, are denoted by + and −, respectively. The percentage release of [^{3}H]serotonin and [^{3}H]S1P are indicated by open bars and closed bars, respectively, and error bars indicate SD. B: Time course of ATP release from platelets when treated with ATX or stimulated with thrombin. The levels of ATX-induced ATP leakage from the cytosol and thrombin-stimulated secretion from the granules through exocytosis were determined as described in Methods. The percentage of leaked/secreted ATP from platelets with thrombin stimulation, ATX treatment, and no treatment is indicated by closed circles, closed diamonds, and open diamonds, respectively. The inset shows a model of ATP release from cytosolic (ATPc) and dense (ATPg) granules. 5HT, serotonin.

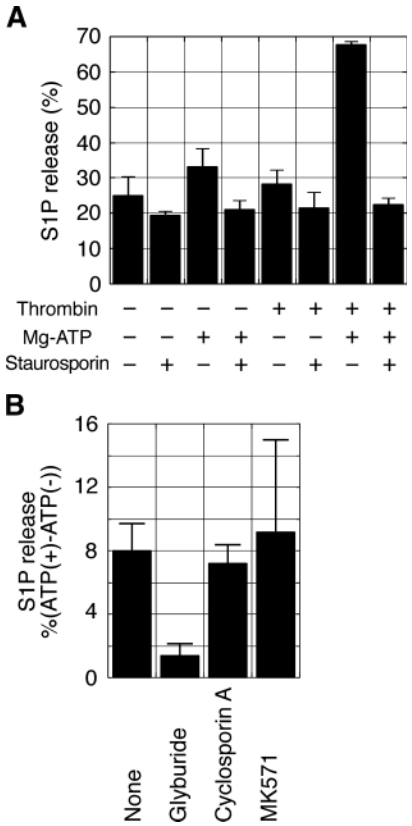

Fig. 5. Effects of inhibitors on ATX-induced S1P release. A: Isolated platelets were preincubated with (+) or without (−) 1 μM staurosporin for 10 min at 37°C and then labeled with [^{3}H]sphingosine to obtain [^{3}H]S1P-preloaded platelets, followed by permeabilization with ATX in the presence or absence of 5 mM MgATP for 10 min at 37°C. S1P release from ATX-permeabilized platelets was induced by the addition of thrombin. Error bars indicate the SD of three independent experiments. B: Effects of ATP binding cassette (ABC) transporter inhibitors on ATP-driven S1P release from ATX-treated platelets. Experiments were performed as described for A except that the platelets were preincubated with various inhibitors (200 μM glyburide, 10 μM cyclosporin A, and 50 μM MK571) in place of staurosporin. ATP-induced S1P release is shown as a percentage: (% release in the presence of ATP) − (% release in the absence of ATP). Error bars indicate SD.

(Fig. 3B). This leakage was attributable to the carryover BSA from the SLO binding step. When [^{3}H]S1P-labeled platelets were sonicated and ultracentrifuged without BSA, [^{3}H]S1P remained in the membrane fraction (data not shown). Proteinase K and trypsin treatment hardly liberated [^{3}H]S1P from the membranes (data not shown), suggesting that S1P on the cytoplasmic membranes may not be captured by a membrane-bound protein. The addition of 1% BSA to the sonicated membranes liberated ~75% of the [^{3}H]S1P into the soluble fraction. Thus, S1P is thought to be located on the inner leaflet on the cytoplasmic side of the membranes.

S1P release and serotonin secretion from ATX-treated platelets

ATX is a bacterial toxin that forms pores smaller than SLO in the cytoplasmic membrane (21). The diameter of the ATX pores is ~4.5 nm, which permits molecules of <0.5 kDa to diffuse through the pores (21). Because BSA does not penetrate through ATX pores, [^{3}H]S1P did not leak out from ATX-treated platelets even in the presence of BSA (**Fig. 4A**). On the other hand, almost all of the cytoplasmic ATP leaked out from ATX platelets within 5 min, whereas ATP occluded in the granules that remained (Fig. 4B, closed diamonds). The amount of ATP present in the granules was determined as the ATP secreted through exocytosis upon thrombin stimulation (Fig. 4B, closed circles). Figure 4B indicates that approximately equal amounts of ATP are present in the cytoplasm and inside the granules. When Ca^{2+} was added to ATX-treated platelets, serotonin, which is a dense granule marker, was not secreted without ATP, because exocytosis requires cytoplasmic ATP (22) (Fig. 4A). However, [^{3}H]S1P was released upon addition of Ca^{2+} without cytosolic ATP (Fig. 4A). Upon addition of ATP without Ca^{2+}, ~10% of [^{3}H]S1P was released from the platelets in addition to its background release (Fig. 4A). These results indicate that S1P release stimulated by Ca^{2+} ions and by ATP are independent of each other.

In ATX-treated platelets, thrombin caused a slight stimulation of [^{3}H]S1P release without ATP, and the addition of ATP greatly enhanced the thrombin-induced [^{3}H]S1P release (**Fig. 5A**), probably as a result of the requirement of cytosolic ATP for thrombin signal transmission. This ATP-induced [^{3}H]S1P release was inhibited by a PKC inhibitor, staurosporin, suggesting phosphorylation of the transporter proteins. It is possible that ATP-induced S1P release is mediated by ABC transporters. Thus, we examined the several common ABC transporter inhibitors. An ABCA protein inhibitor, glyburide (23), can block ATP-induced [^{3}H]S1P release (Fig. 5A, B). However, neither the ABCB1 inhibitor cyclosporin A (24–26) nor the ABCC1 inhibitor MK571 (27) showed any effect on [^{3}H]S1P release (Fig. 5B). Glyburide also inhibited the thrombin-induced [^{3}H]S1P release from intact platelets in a dose-dependent manner (**Fig. 6**). These results indicate that an ABCA-like ATP-dependent exporter might be responsible for the thrombin-stimulated S1P release from platelets.

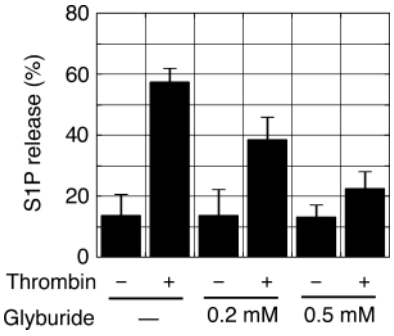

Fig. 6. Effect of glyburide on thrombin-induced S1P release from intact platelets. Experiments were performed as described for Fig. 1 except that the platelets were preincubated with the indicated concentrations of glyburide for 10 min. Experiments were performed more than two times, and error bars indicate SD.

DISCUSSION

S1P release from platelets is caused by thrombin stimulation in the presence of BSA. However, it has not been determined whether the S1P is released through exocytosis or not, because thrombin also induces exocytosis (15). In this study, we revealed that S1P release is not mediated by exocytosis, because the characteristics of the [^{3}H]S1P release process are not the same as those of any other

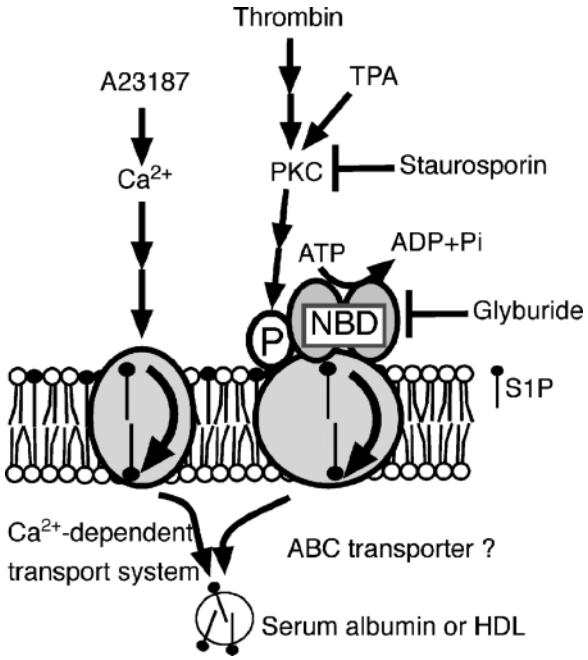

Fig. 7. Schematic model of S1P secretion from platelets. S1P release appeared to be mediated by two independent transporters: one is ATP-dependent and the other is Ca^{2+}-dependent. S1P molecules transported to the outer surface of the plasma membrane by the transporters are then extracted from the membrane by HDL or serum albumin (28, 29). NBD, nucleotide binding domain; Pi, inorganic phosphate; PKC, protein kinase C.

granular markers, such as serotonin, PF4, and β-hexosaminidase (Fig. 2). Moreover, we determined that [^{3}H]S1P is located on the inner surface of the plasma membrane and released from platelets upon stimulation with thrombin and Ca^{2+} (Figs. 1, 3). For the S1P release process from platelets in vitro, BSA is necessary for the extraction of S1P molecules from the outer surface of the platelet plasma membrane (**Fig. 7**). When BSA is absent, exported S1P might remain on the outer surface of the plasma membrane. HDL and/or serum albumin plays a BSA-like role in S1P release in vivo (28, 29). These results strongly suggest that S1P is released through a carrier-mediated process on the plasma membrane and that there are two independent S1P exporters, thrombin-induced and Ca^{2+}-induced, in platelets (Fig. 7). The former is stimulated by the PKC signaling pathway, requires ATP, and is inhibited by glyburide, which is an ABC transporter inhibitor. The latter is not mediated by the PKC pathway, is independent of ATP, and is not inhibited by glyburide (data not shown). It should be noted that we observed the release of the newly synthesized radiolabeled S1P in this experiment. The possibility cannot be ruled out that the newly synthesized S1P might not fully reflect the behavior of the preformed S1P molecules in the platelet.

It seems that thrombin stimulation did not cause the activation of the Ca^{2+}-dependent S1P transport system in rat platelets, because thrombin-induced S1P release is almost completely inhibited by staurosporin. Although thrombin causes a transient increase in the intracellular Ca^{2+} concentration (30), it is known that a platelet phospholipid scramblase (PLSCR) is not induced by thrombin until the vesicular Ca^{2+}-ATPase is inhibited by an inhibitor such as thapsigargin (31). The thrombin-stimulated temporary increase in the intracellular Ca^{2+} concentration might be too short and low as a result of the Ca^{2+}-reuptake pump to induce the activation of PLSCR (31) and the Ca^{2+}-dependent S1P exporter. In addition, S1P release from platelets induced by Ca^{2+} plus A23187 was inhibited by the PLSCR inhibitor R5421 (32) (data not shown). However, because the inhibitory action of R5421 is not always specific to PLSCR, it remains uncertain whether or not the Ca^{2+}-dependent S1P release is mediated by PLSCR. Although a Ca^{2+}-dependent transport system exists in rat platelets, the physiologically important pathway for S1P release on stimulation might be an ATP-dependent one, because a Ca^{2+}-dependent pathway is not induced by intercellular signal molecules such as thrombin.

Some ABC family transporters are known to export lipid derivatives, including lipid mediators (33). Among them, ABCB1 and ABCC4 were reported to export signal molecules such as platelet-activating factor (34, 35) and prostaglandins E1 and E2 (36) when they were cloned and expressed in a cell line or in primary cultured cells. However, there is no direct evidence regarding whether or not such proteins act as exporters of these signal molecules. Regarding the ABC transporter family, only ABCC1-mediated LTC4 transport showed its physiological importance in ABCC1 knockout mice, which showed an impaired response to inflammatory stimuli associated with decreased LTC4 secretion (37). Because S1P is also a lipid mediator, there is a possibility that ATP-dependent S1P release from platelets might be mediated by ABC family transporters. The fact that the ABCA1 inhibitor glyburide (23) prevents ATP-dependent [^{3}H]S1P release from platelets supports the idea that an ABCA family transporter might mediate the S1P release. Previously, we revealed that ABCA7 is preferentially expressed in rat platelets (38). In addition, ABCA3 is expressed in platelets as well as liver and kidney (38). It has also been revealed that ABCA1 plays an important role in platelet function (39, 40). Recently, Aoki et al. (29) reported that S1P release from platelets was also supported by HDL in addition to serum albumin. However, apolipoprotein A-I, which is a component of HDL and plays an important role in cholesterol transport by ABCA1, did not support the S1P release from the platelet (data not shown). Thus, it is not clear what kind of ABC family transporter mediates the S1P release.

As for the regulation of signal molecule secretion, the rate-limiting steps for the secretion of LTC4, prostaglandin, and platelet-activating factor are controlled by their intracellular concentrations, because they are synthesized in a stimulation-dependent manner (41, 42). In contrast, S1P is accumulated in platelets without stimulation and released from cells upon stimulation (Fig. 1). Thus, the release should be regulated at the export step. The stimulation-dependent S1P exporters we revealed in this study should play important physiological roles in S1P signaling.

Our findings suggest that membrane transporters may mediate the secretion of a lot of amphiphilic signal molecules, such as lipid mediators. The identification of such signal molecule exporters is an interesting target for future work, which should be important not only for understanding intercellular signal transduction but also for developing exporter-oriented drugs.

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