Transbilayer Asymmetry of Phospholipids in the Plasma Membrane Regulates Exocytotic Release in Mast Cells

Naoto Kato, Mamoru Nakanishi, and Naohide Hirashima*

Graduate School of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

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ABSTRACT: To investigate the role of the asymmetric distribution of phospholipids of the plasma membrane in exocytosis, we examined the effects of disruption of this asymmetrical distribution of lipids on exocytotic release from mast cells (RBL-2H3). Lipid scramblase, which is activated by divalent cations and catalyzes the transbilayer movement of phospholipids, was overexpressed in mast cells. Exogenous lipid scramblase was expressed in the plasma membrane and the cytoplasm. Activation of scramblase by divalent cations disrupted the asymmetrical distribution of phospholipids in the plasma membrane. Exocytotic release induced by calcium ionophore and phorbol ester was significantly inhibited in the cells transfected with wild-type scramblase. This inhibition was observed with time lag of about 5 min. Furthermore, when the asymmetric distribution of lipids was disrupted before induction of exocytosis, the inhibition of exocytotic release was obvious from the beginning without time lag. These results suggest that the asymmetric distribution of phospholipids in the plasma membrane plays an essential role in fusion between secretory granules and the plasma membrane. This finding also demonstrates that the transbilayer asymmetry of phospholipids regulates exocytosis and gives a new insight into the significance of lipid asymmetry in the plasma membrane.

Over the past decade, the machinery of exocytosis has been studied intensively, and some key proteins such as SNARE (soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor) proteins (*1*−*3*) and some regulatory proteins such as munc-18 and complexin have been identified (*4*−*6*). However, the mechanism of exocytosis is not yet completely understood. Exocytosis ends with membrane fusion between secretory vesicles (granules) and the plasma membrane. Therefore, the distribution and the dynamic movement of membrane components should play a crucial role at the final stage of exocytosis. Although lipids are major components of biomembranes, their involvement in exocytosis has been poorly studied.

It is well-known that the plasma membrane exhibits an asymmetric distribution of phospholipids between the two leaflets of the bilayer. Aminophospholipids such as phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) reside in the inner leaflet of the plasma membrane, while phosphatidylcholine (PC) and sphingomyelin (SM) are distributed in the outer leaflet (*7*, *8*). The physiological significance of this asymmetric distribution is not well understood, but the translocation of PS from the inner to outer leaflet was observed during apoptosis and the activation of platelets (*9*, *10*). Some recent studies have suggested that the asymmetric distribution of phospholipids and the transbilayer movement of phospholipids are involved in exocytosis. For example, the asymmetric distribution of phospholipids is observed in both nerve terminal membrane and synaptic vesicle membrane, and PS resides in the leaflet that faces the cytoplasm in both membranes (*11*−*14*). In addition, Lee et al. reported the rapid scrambling of phospholipid in plasma membrane during the exocytotic release of acetylcholine from nerve terminals prepared from the electric organs of electric ray (*15*). In mast cells, it has been reported that PS was translocated to the outer leaflet during exocytosis, and this translocation was proportional to the extent of exocytosis (*16*). However, no direct evidence that the asymmetric distribution of phospholipids contributes to the exocytotic release.

In the present study, we investigated the role of the asymmetric distribution of lipids in exocytosis from RBL-2H3 cells. Lipid scramblase is a membrane protein that is activated by divalent cations and catalyzes the bidirectional migration of phospholipids, independent of the polar headgroup of phospholipid (*18*−*20*). The overexpression of lipid scramblase inhibited exocytosis induced by calcium ionophore and phorbol 12-myristate 13-acetate (PMA). Furthermore, disruption of the asymmetric distribution of phospholipids before stimulation inhibited exocytosis from the beginning of observation. These results suggest that the asymmetric distribution of phospholipids itself is essential for exocytosis in mast cells (RBL-2H3).

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmid of Scramblase and Mutated Scramblase. Poly(A)$^+$ RNA was obtained from 1 $\times$ 10^7 cells of NIH3T3 with a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) and served

* To whom correspondence should be addressed. Tel:+81-52-836-3412. Fax:+81-52-834-9309. E-mail:hirashim@phar.nagoya-cu.ac.jp.

[1] Abbreviations: RBL, rat basophilic leukemia cells; SNARE, soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; DIC, differential interference contrast.

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as a template for cDNA synthesis with SuperScript II RT (Gibco BRL). The full-length DNA sequence coding mouse scramblase (GenBank accession number: AF015790) was obtained by PCR with a primer pair, 5′-*GAATTC*ATGGAGGCTCCTCGCTC (sense; EcoRI site is italicized)/5′-*GTCGAC*CTACTCACAGCCTTCAAAAAACAT (anti-sense; SalI site is italicized), using a high-fidelity Taq polymerase EX-Taq (Takara, Tokyo, Japan). The cDNA obtained was ligated with a c-myc vector, pCMV-tag3 (Invitrogen), at EcoRI and SalI sites, and its sequence was verified by DNA sequencing. Mutated scramblase DNA in which GAT for Asp^{275} is substituted with GCT for Ala was prepared by the overlap extension method (*21*) using a primer pair containing a mutated region (5′-ACGGATTCG*GCT*AACTTTGG and 5′-CCAAAGTT*AGC*CGAATCCGT; mutated codon is italicized). The DNA coding mutated scramblase was also ligated with pCMV-tag3, and its sequence was confirmed by DNA sequencing.

Cell culture and Transfection. Rat basophilic leukemia 2H3 (RBL-2H3) cells were originally a gift from Dr. R. P. Siraganian (National Institutes of Health, Bethesda, MD). Cells were cultured in Eagle's minimal essential medium from Nissui (Tokyo, Japan) with 10% fetal calf serum (Boehringer Mannheim) at 37 °C in an atmosphere of 5% CO_2. RBL-2H3 cells were electroporated in cold phosphate-buffered saline with 40μg of plasmid DNA at 250 V and 950 μF using a Gene Pulser II (Bio-Rad). Stable transfectants were selected by G418.

Western blotting. RBL-2H3 cells (5×10^6) were lysed in lysis buffer (2% NP-40, 5 mM EDTA, 50 mM *N*-ethylmaleimide, 1 mM PMSF, and 1 mM leupeptin). After sonication for 10 s, lysate was mixed with an equal volume of Laemmli sample buffer and boiled for 5 min. Samples were electrophoresed by SDS−PAGE and transferred to PVDF membrane. After blocking with phosphate buffer containing 0.5% casein, blots were probed with primary antibody for c-myc (9E10, dilution 1:1000; Santa Cruz Biotechnology) or β-actin (AC-15, dilution 1:4000; Sigma) for 1 h. After washing with 0.1% Tween 20 in PBS, the membrane was treated with antimouse IgG conjugated with horseradish peroxidase. Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia) with a Lumi-Imager F1 (Roche Diagnostics).

Immunofluorescence Studies. Cells were fixed with 3% formaldehyde, 3 mM EGTA, and 2 mM $MgCl_2$ in phosphate buffer. After washing with phosphate buffer, cells were treated with 0.2% Triton-X100. Cells were labeled with primary antibody (5 μg/mL) for c-myc (9E10) for 15 min. After washing, a secondary antibody, FITC-conjugated antimouse IgG (Organon Teknika Corp., NC), was added (10 μg/mL), and the mixture was incubated for 15 min. After washing, immunofluorescence images were obtained with a confocal laser microscope (LSM510, Carl Zeiss, Oberkochen, Germany) with an argon ion laser. Samples were excited at 488 nm, and fluorescence was collected through a band filter (505−550 nm). The signal level of autofluorescence was obtained from nontransfected cells treated with the same staining procedure mentioned above. And this signal level was subtracted from original fluorescent images of samples using an image processing software of LSM510.

Detection of Externalized Phosphatidylserine. Scramblase was activated with A23187 in the presence of Ca^{2+} or Mn^{2+}. Externalized PS was detected with Cy3-conjugated annexin V (Medical and Biological Lab., Nagoya, Japan). Cells were incubated with Cy3-annexin V for 5 min in the presence of 0.5 mM Ca^{2+} without fixation, following the manufacturer's instruction. In the case of activation of scramblase with Mn^{2+}, cells were incubated with Cy3-annexin V for 5 min in the presence of Ca^{2+} and Mn^{2+}. Fluorescence images were obtained with a confocal laser microscope (LSM510). Samples were excited at 543 nm, and fluorescence longer than 560 nm was measured. To confirm the localization of Cy3-annexin V on the plasma membrane, we incubated cells with NBD-labeled phosphatidylethanolamine (1-palmitoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazo-l-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoethanolamine (NBD-PE); Avanti Polar Lipids) and Cy3-annexin V; NBD was excited at 488 nm, and fluorescence was detected using a band-pass filter (505−525 nm). The signal level of autofluorescence was obtained from nontransfected cells treated with the same staining procedure mentioned above. This signal level was subtracted from original fluorescent images of samples using an image processing software of LSM510.

Assay of Secreted β-Hexosaminidase. Degranulation of RBL cells was monitored by measuring the activity of the granule-stored enzyme, β-hexosaminidase, secreted into cell supernatant. Cells were stimulated with a calcium ionophore, A23187 (WAKO Pure Chemicals, Tokyo, Japan), and PMA (Sigma, St Louis, MO). Aliquots of supernatants were incubated with substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 100 mM citrate, pH4.5) for 45 min at 37 °C. After terminating the reaction with Na_2CO_3−$NaHCO_3$ buffer, we measured the absorbance at 405 nm. Data are reported as percentages of the total β-hexosaminidase content of the cells (determined from the value for control cells dissolved with 0.1% Triton X-100).

RESULTS

Expression of c-myc-Tagged Scramblase in RBL-2H3 Cells. To disrupt the asymmetric distribution of phospholipids, we transfected RBL-2H3 cells with c-myc-tagged scramblase, and four clones which stably expressed c-myc-scramblase were obtained. For the control experiments, we obtained three clones that expressed mutated scramblase which lacked scramblase activity by substituting Ala for Asp^{275} (*22*). All of these clones gave similar results in the following experiments; therefore, data derived from one of these clones are shown in this paper. Figure 1 shows the western blot analysis of exogenously introduced lipid scramblase detected with anti c-myc antibody. Both wild and mutated scramblase were detected at a relative molecular mass of about 36 kDa, which corresponds to the size deduced from transfected DNA. No detectable signal was observed from the cell lysate prepared from RBL-2H3 cells with mock transfection.

To examine the localization of c-myc-scramblase, we carried out an immunofluorescence study (Figure 2). Fluorescence signals were detected in both cells transfected with wild-type scramblase and mutated scramblase (Figure 2), while no fluorescence was observed in the cell with mock transfection. Fluorescence derived from c-myc-tagged scramblase was observed both in the plasma membrane and the cytoplasm with some granular staining. The mean fluorescent

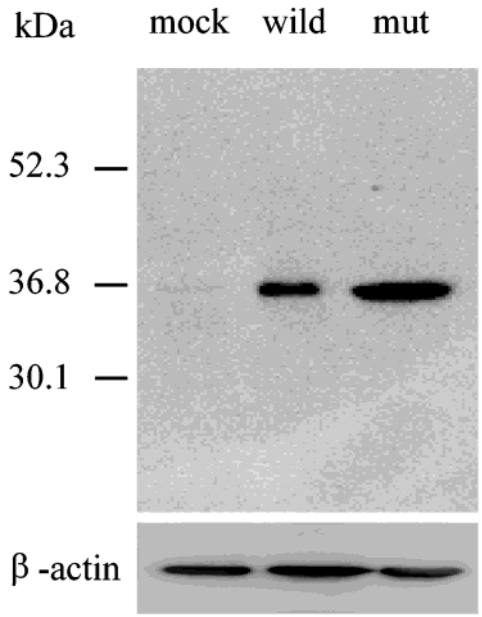

FIGURE 1: Western blot analysis of c-myc-tagged scramblase with or without mutation. Samples were prepared from cells transfected with wild-type (wt) and mutated scramblase (mut) and electrophoresed by SDS−PAGE. After transfer to PVDF membrane, blots were probed with primary antibody for c-myc (9E10) and visualized with antimouse IgG conjugated with horseradish peroxidase using chemiluninescence methods. A single band corresponding to c-myc-tagged scramblase was detected in transfected cells but not detected in cells with mock transfection (mock). The lower figure shows the expression of β-actin in the same lysate used to detect the expression of scramblase.

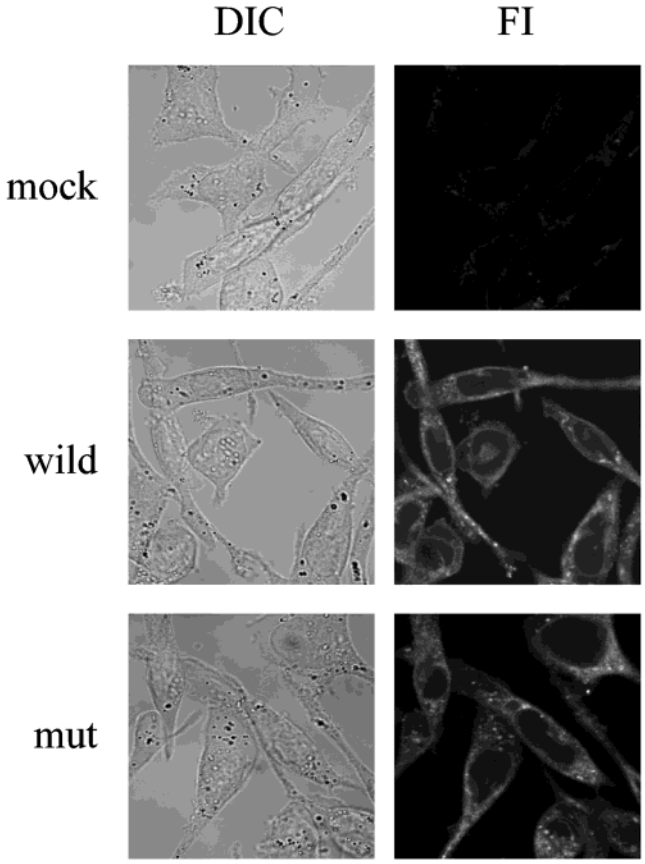

FIGURE 2: Localization of scramblase revealed by immunofluorescence analysis. Tagged-scramblase expressed in RBL-2H3 cells was stained with anti-c-myc antibody and FITC-labeled secondary antibody. Left and right images were DIC images and fluorescent images of the same fields, respectively. Top, middle, and bottom images were images of cells transfected with mock, wild-type, and mutated scramblase, respectively.

intensity of cells transfected with wild scramblase and mutated scramblase were 155 ± 31 and 148 ± 21 (mean ± S.D., $n > 50$), and no significant difference was observed.

Activation of Scramblase by Divalent Cation. Although lipid scramblase is activated by divalent cations such as calcium or manganese ion (*23*), calcium-induced changes in transbilayer distribution of phospholipids are not always observed in all types of cells (*24*). Therefore, we examined the scramblase activity in RBL-2H3 cells expressing exogenously introduced scramblase by measuring the externalization of PS to the outer leaflet. In measuring scramblase activity, however, there is difficulty specific to secretory cells such as RBL-2H3 cells. It has been reported that lipid scrambling occurs during exocytosis in nerve terminal and mast cells, resulting in the externalization of PS to the outer leaflet (*15*, *16*). Since the increase in intracellular calcium causes both exocytosis and the activation of scramblase, it is difficult to discriminate the externalization of PS by scramblase from that by exocytosis. To monitor the translocation of PS mediated by scramblase not by exocytosis, we stimulated RBL-2H3 cells with A23187 in the presence of manganese ion, which activates scramblase without inducing exocytosis (*25*). Cells were incubated with 0.5 mM Mn^{2+} and 1 μM A23187 for 30 min, and externalized PS was detected by Cy3-labeled annexin V (Figure 3). To examine whether exocytosis occurred in the presence of Mn^{2+}, we assayed exocytotic release by quantifying β-hexosaminidase secreted by cells. As shown in Figure 4, no exocytotic release was observed in the presence of Mn^{2+}. Therefore, the translocation of PS under this condition is thought to be a result of the activation of scramblase, not of exocytosis. As shown in Figure 5, translocation of PS to the outer leaflet was observed in cells transfected with wild-type scramblase, but not in cells with mutated scramblase nor in cells with mock transfection. To confirm that the fluorescence of Cy3 was on the plasma membrane, we also stained cells with NBD-labeled PE which localized on the plasma membrane. Double staining with Cy3-annexin V and NBD-PE revealed that the fluorescence of Cy3-annexinV localized on the plasma membrane (Figure 5). Therefore, these results indicate that c-myc-tagged scramblase is activated by Mn^{2+} in RBL-2H3 cells and can perturb the asymmetric distribution of phospholipids.

Exocytotic Release from Cells Transfected with Scramblase. To examine the role of lipid asymmetry in exocytosis, we stimulated RBL-2H3 cells which overexpress scramblase with A23187 and PMA in the presence of calcium ion. Under these experimental conditions, lipid scrambling and exocytosis were induced at the same time, since both scramblase and exocytosis were activated by the elevation of intracellular calcium concentration. Exocytotic release was monitored by quantifying secreted β-hexosaminidase and expressed as the percentge release in Figure 6. The total amount of β-hexosaminidase did not change by expressing scramblase. The percentage of β-hexosaminidase released was decreased in cells transfected with wild-type scramblase (Figure 6). As shown in Figure 7, substantial scrambling of lipid occurred in cells transfected with wild-type scramblase. Therefore, this

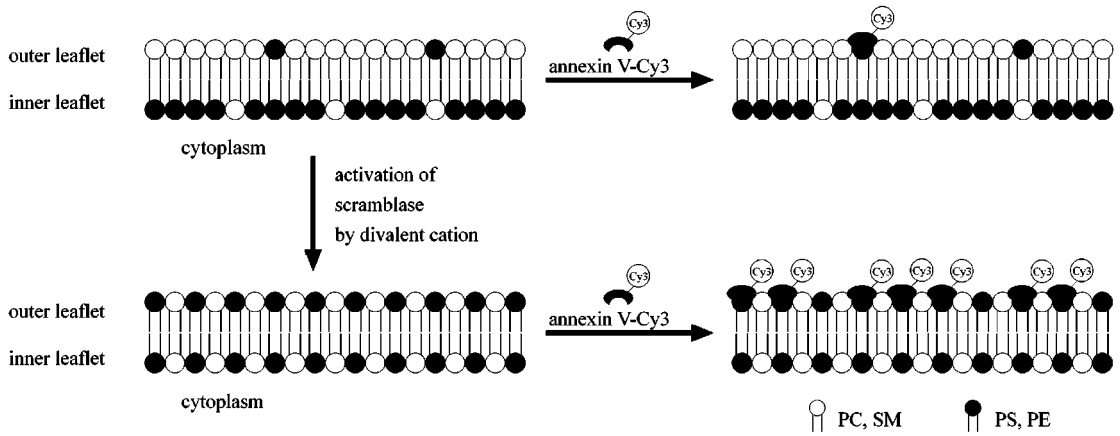

FIGURE 3: Schematic representation of the effects of the activation of lipid scramblase and its detection. The composition of phospholipids in the plasma membrane is different between the inner leaflet and the outer leaflet. Aminophospholipids such as PS and PE reside in the inner leaflet of the plasma membrane, while PC and SM are distributed in the outer leaflet. Activation of lipid scramblase disrupts this asymmetric distribution of phospholipids. Annexin V binds to PS specifically; therefore, lipid scrambling caused by the activation of scramblase can be monitored by labeling externalized PS with fluorescence-labeled annexin V (Cy3-labeled annexin V).

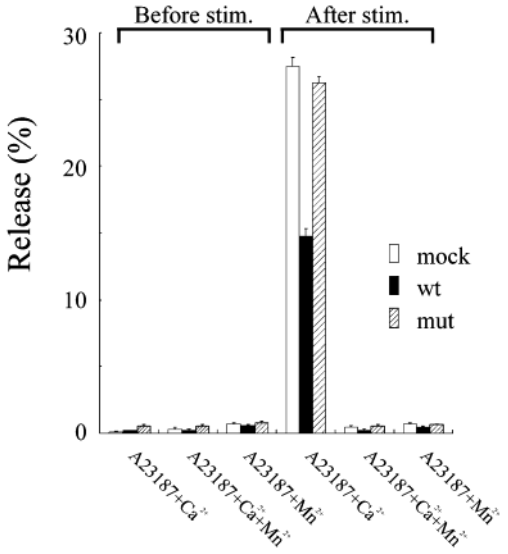

FIGURE 4: Effects of Mn^{2+} on the exocytotic release from RBL-2H3 cells. To confirm that Mn^{2+} does not cause exocytotic release, we quantified β-hexosaminidase in the presence or absence of Mn^{2+} and expressed it as percentages of the total β-hexosaminidase content of the cells (mean ± S.D.) Cells were stimulated with 1 μM A23187 and various combination of 1.0 mM Ca^{2+} and 0.5 Mn^{2+}, and released β-hexosaminidase was assayed at 30 min after stimulation. White, black, and hatched columns represent data from cells transfected with mock, wild-type, and mutated scramblase, respectively.

inhibition of exocytosis seemed to be due to the perturbation of asymmetry of lipids in the plasma membrane. It did not seem to be the result of nonspecific effects due to the overexpression of membrane protein because exocytosis was not attenuated in cells with mutated scramblase. Slight scrambling of lipids was also observed in the mock transfectant (Figure 7), probably due to the lipid scrambling caused by exocytosis as reported before (*16*). However, the lipid scrambling observed in transfected cells was greater than that of mock transfectant, even though the exocytosis itself was inhibited in transfected cells (Figure 7). This indicates that the lipid scrambling due to the activation of exogenous c-myc−scramblase was more intense than that caused by exocytosis. As shown in Figure 6, the inhibitory effects became significant at 5 min after stimulation. This time lag seemed to be due to the time necessary for the perturbation of asymmetrical distribution of phospholipids by scramblase. To confirm this notion, we disrupted the asymmetric distribution of phospholipids in the plasma membrane before stimulation of exocytosis. RBL-2H3 cells were pretreated with A23187 and Mn^{2+} for 30 min, which is sufficient to disrupt the asymmetric distribution of phospholipids as shown in Figure 5. After washing, cells were stimulated with A23187 and PMA in the presence of Ca^{2+} to induce exocytosis. As shown in Figure 8, disruption of the asymmetric distribution in advance inhibited exocytotic release. In contrast to Figure 6, this inhibition was obvious from the beginning of exocytotic release.

DISCUSSION

The mechanism of exocytosis has been studied intensively mainly in neuronal cells, and the SNARE hypothesis explains the roles of proteins involved in this process. In mast cells, which are often used to study exocytosis, we and other researchers have shown that SNARE proteins are expressed and involved in exocytosis, as in neuronal cells (*26*, *27*). In contrast to this abundance of studies on proteins, the role of membrane lipids in exocytosis has largely been overlooked. In the present study, we obtained RBL-2H3 cells which overexpressed lipid scramblase. In those cells, asymmetric distribution of phospholipids in the plasma membrane was supposed to be perturbed by activating scramblase, as shown in Figure 3.

First, we checked the expression and activity of c-myc-tagged scramblase. The tagged scramblase expressed in the plasma membrane and the cytoplasm (Figure 2). Although the structure or organelle in which c-myc-tagged scramblase expressed in the cytoplasm remains to be elucidated, it is likely that scramblase expressed in ER and intracellular granules. As shown in Figure 5, c-myc-tagged scramblase was functional in the plasma membrane of RBL-2H3 cells, since the translocation of PS from the inner to the outer leaflet of the plasma membrane was occurred by the stimulation of Mn^{2+}. Similar increased exposure of PS was observed when GFP-tagged scramblase was expressed in Raji cells (*28*). Next, we investigated the effects of the activation of scramblase on the exocytosis. To see the effects of lipid

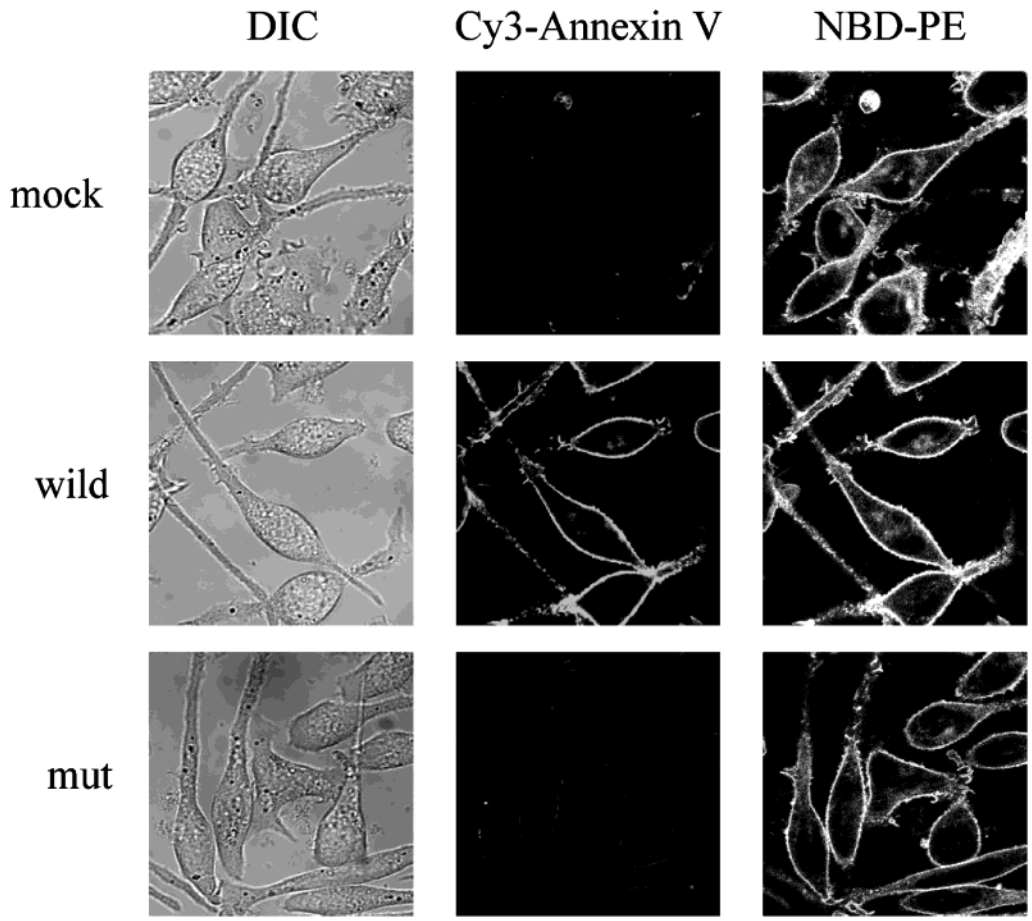

FIGURE 5: Externalization of PS by the activation of scramblase. Externalized PS was visualized with Cy3-labeled annexin V. Scramblase was activated by the incubation with 0.5 mM Mn^{2+} and 1 μM A23187 for 30 min. Cells were incubated with Cy3-labeled annexinV in the presence 0.5 mM Mn^{2+}, 1 μM A23187, and 0.5 mM Ca^{2+} for binding of PS and annexinV. To clarify the location of annexinV, we also stained cells with NBD-PE which localized on the plasma membrane. Left, middle, and right images are DIC images and fluorescence images of Cy3-annexin and NBD-PE, respectively. Top, middle, and bottom images are cells transfected with mock, wild-type, and mutated scramblase, respectively.

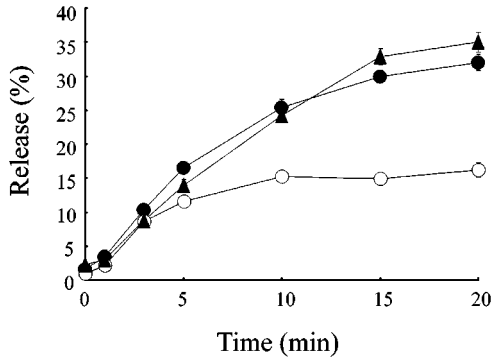

FIGURE 6: Effects of the expression of scramblase on exocytotic release. Exocytotic release from mast cells was evaluated by quantification of β-hexosaminidase. Cells were stimulated with PMA (50 ng/mL) and A23187(1 μM) in the presence of 1 mM Ca^{2+} to induce exocytosis. Values were represented as percentages of the total β-hexosaminidase content of the cells (mean $\pm$ S. D.) and plotted against time (●; wild scramblase, ▲; mock transfection, ○; mutated scramblase).

scrambling on fusion between secretory vesicle and the plasma membrane, we stimulated cells with a calcium ionophore and PMA, which mimic the activation of mast cells, by bypassing events before calcium increase. We also examined whether the expression of scramblase itself affected exocytosis, since the overexpression of membrane protein itself might affect membrane fusion. Since the expression of mutated scramblase which lacks scramblase activity did not affect exocytosis, the effects of the expression of scramblase observed in this study could be attributed to lipid scrambling. The inhibitory effect of scramblase on the exocytosis (Figure 6) suggests that scramblase is not involved in exocytosis, although lipid scrambling is observed during exocytosis in mast cells and nerve terminals (*15*, *16*). Devaux suggested the involvement scramblase in this lipid scrambling during exocytosis (*17*). However, Lee et al. concluded that the lipid scrambling during neurotransmitter release is too fast to explain by the activation of scramblase, and our present data also suggest that scramblase is not involved in exocytotic release, although RBL-2H3 cells express endogenous lipid scramblase (*29*).

Cy3-annexin fluorescence in cells stimulated with Ca^{2+} shown in Figure 7 seems to be punctated and less in intensity compared to that in cells stimulated with Mn^{2+} shown in Figure 5. One possible reason for this is that length of the stimulation of scramblase with Mn^{2+} is longer (30 min) in Figure 5 than that with Ca^{2+} (20 min) in Figure 7. Another and more likely reason is that the stimulation with Ca^{2+} caused exocytosis, and this caused an increase in the surface area of the plasma membrane in addition to disorder and ruffling of the plasma membrane, while Mn^{2+} caused no exocytotic release (Figure 4).

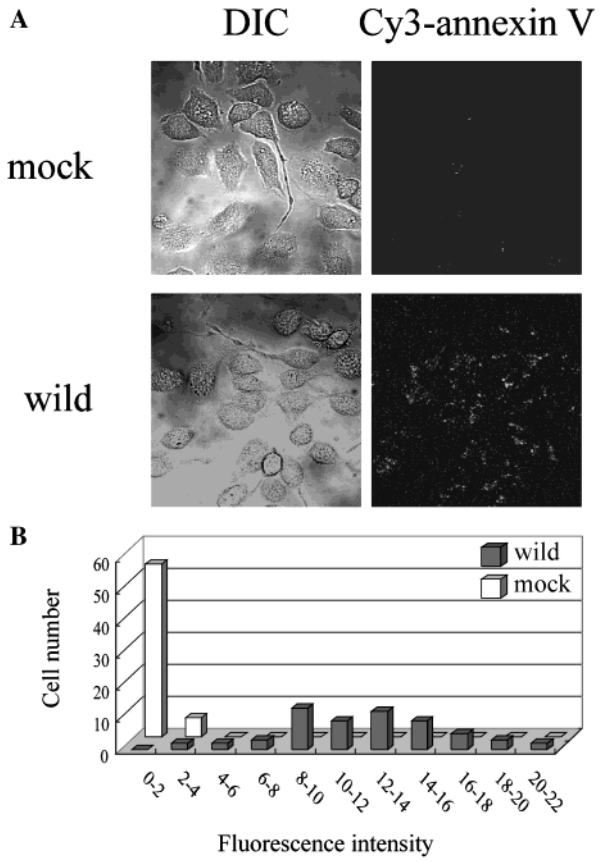

FIGURE 7: Externalization of PS caused by the stimulation of exocytotic release. (A) Exocytosis was induced by the same stimulation as that in Figure 6, PMA (50 ng/mL) and A23187 (1 μM) and Ca^{2+} (1 mM). Externalization of PS caused by the stimulation was observed at 20 min after stimulation by staining with Cy3-labeled annexinV. Upper and lower images are obtained from cells transfected with mock and wild scramblase, respectively. Left and right panels are DIC and fluorescent images, respectively. (B) Histogram of fluorescence intensity of Cy3-labeled annexinV. Total fluorescent intensity of individual cell was measured. Sixty cells were examined for each cells transfected with mock (white) and wild scramblase (black).

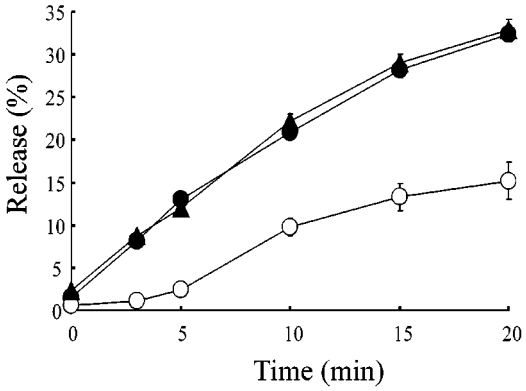

FIGURE 8: Effects of disruption of lipid asymmetry before stimulation on the exocytotic release. Cells were preincubated with 0.5 mM Mn^{2+} and 1 μM A23187 for 30 min to disrupt the asymmetric distribution of phospholipids. After washing with buffer without divalent cation, cells were stimulated with PMA (50 ng/mL) and A23187 (1 μM) in the presence of 1 mM Ca^{2+} to induce exocytosis. Exocytotic release from cells was evaluated as mentioned in Figure 6. Time courses of β-hexosaminidase released by exocytosis are plotted (●, wild scramblase; ▲, mock transfection; ○, mutated scramblase).

As shown in Figure 6, the inhibition of exocytosis began at about 5 min after stimulation. In this case, scramblase was activated by Ca^{2+}, and lipid scrambling starts at the same time as the activation of exocytosis. The time lag is thought to reflect the amount of time needed to disrupt the asymmetric distribution of phospholipids. To confirm this notion, we perturbed asymmetric distribution before stimulation of exocytosis. In this condition, exocytosis was inhibited without lag time (Figure 8). This suggests that a predisturbed distribution of phospholipids attenuated the membrane fusion of granules and the plasma membrane. There are several possible explanations for the reason the exocytotic release proceeded even after the asymmetry distribution had been perturbed by pretreatment with Mn^{2+} (Figure 8). One is the difference in the ability of the activation of scramblase between Ca^{2+} and Mn^{2+}. Another explanation is based on the mechanism of exocytotic release in mast cells. It is known that contents in secretory granules were released through two pathways in mast cells (*30*). One is the fusion of granules with the plasma membrane. The other one is the fusion of granules with granules which have fused with the plasma membrane. Through the latter pathway, granules that reside near the granules fused with the plasma membrane can release their contents without fusing with the plasma membrane. Due to the loss of asymmetry of lipids, only a small number of granules fused with the plasma membrane at the beginning in the case of Figure 8. Subsequently, fusion of granules with the granules which had fused with the plasma membrane occurred, resulting in decreased but significant release of β-hexosaminidase with time lag.

We cannot exclude the possibility that the asymmetric distribution of phospholipids in secretory granule membrane also affects exocytosis, since c-myc-tagged scramblase expressed in granular structures in the cytoplasm and some of these might be secretory granules.

Since scramblase transports phospholipids independent of polar headgroups, we cannot conclude which phospholipid is important in terms of the asymmetric distribution of phospholipids. Several lines of evidence suggest that the asymmetrical distribution of PS is one of the most influential factors in exocytosis. PS is localized in the cytoplasmic leaflet of the plasma membrane and granule membrane that face each other at membrane fusion (*13*, *14*). The relative amount of PS translocated during exocytosis is greater than that of PC and PE (*15*). It is well-studied that calcium ion which triggers exocytosis induces membrane fusion between liposomes containing PS (*31*, *32*). Another intriguing mechanism is that the disruption of asymmetrical distribution of phospholipids may affect the structure of a membrane domain, such as lipid raft. Recently, several groups reported that SNARE proteins are localized in raft in PC12 cells (*33*, *34*). Therefore, it is possible that disruption of the asymmetrical distribution changed the structure of raft, resulting in an inhibition of exocytosis.

Our results demonstrate the importance of the lipid composition in exocytosis and give new insight into the physiological significance of the asymmetric distribution of phospholipids in the plasma membrane. RBL-2H3 cells that overexpress lipid scramblase might also be useful for investigating the involvement of membrane structure in the mechanism of signal transduction as well as exocytosis.

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