Cholesterol Depletion Inhibits Store-Operated Calcium Currents and Exocytotic Membrane Fusion in RBL-2H3 Cells

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ABSTRACT: The effects of cholesterol depletion from the plasma membrane with methyl-β-cyclodextrin (MβCD) on exocytotic processes were investigated in rat basophil leukemia cells (RBL-2H3 cells). Pretreatment of the cells with MβCD inhibited antigen-evoked exocytotic release dose-dependently. To elucidate the mechanism of this inhibition, we performed experiments on the effects of MβCD on exocytotic membrane fusion and mobilization of Ca^{2+} and on the localization of the tyrosine kinase Lyn. Inhibition of degranulation by MβCD was observed even under stimulation with the phorbol ester and calcium ionophore. Therefore, MβCD affected a process downstream of Ca^{2+} influx, or membrane fusion between the granule and the plasma membrane. Intracellular calcium measurements revealed that MβCD inhibited the Ca^{2+} increase induced by antigen. Furthermore, we found that MβCD significantly inhibited Ca^{2+} influx from the extracellular medium through the store-operated calcium channel (SOC) but did not affect Ca^{2+} release from the intracellular Ca^{2+} store. Fluorescent image analysis of cells expressing Lyn-YFP showed that treatment with MβCD scarcely affected the localization and lateral mobility of Lyn in the plasma membrane. These results suggest that cholesterol depletion by MβCD decreases degranulation mainly by inhibiting the SOC and membrane fusion between the secretory granules and the plasma membrane in mast cells.

Exocytosis is the process of fusion between the secretory vesicles and the plasma membrane, resulting in the secretion of vesicular contents to the extracellular space. Various signaling molecules, such as neurotransmitters, hormones, and inflammatory mediators, are released through this process. The molecular machinery involved in this process has been studied intensively, and some key proteins, such as SNARE (soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor)[1] proteins (*1*−*5*), have been identified. On the other hand, the role of membrane components in exocytosis has been overlooked. Since exocytosis ends with membrane fusion between the secretory vesicles and the plasma membrane, components of the plasma membrane must affect this process. Several lines of evidence have suggested that the asymmetric distribution of phospholipids and the transbilayer movement of phospholipids in the plasma membrane are involved in exocytosis (*6*−*9*). Recently, we directly demonstrated that the asymmetric distribution of phospholipids was involved in exocytotic release in mast cells (*10*).

In addition to phospholipids, cholesterol is another major component of the plasma membrane. Recent studies have revealed that there are microdomains in the plasma membrane, called lipid rafts, where cholesterol is enriched. These domains are also abundant in various membrane-bound proteins such as Src family kinases and glycosylphosphatidylinositol (GPI)-anchored proteins, and they are thought to serve as platforms for the organization of the signaling cascades activated by extracellular stimulation (*11*−*13*). To examine the role of such rafts, methyl-β-cyclodextrin (MβCD) has been widely used to disrupt rafts by depleting cholesterol from the plasma membrane (*11*, *14*, *15*). Most studies that have used MβCD to investigate the role of rafts have focused on the very early events in the signaling cascade, such as the phosphorylation of receptors and the localization of the Src family tyrosine kinase (*11*, *12*, *16*), and the effects on subsequent responses have been poorly investigated. It is well-known that the depletion of cholesterol affects the physicochemical properties of membranes and membrane proteins. Therefore, MβCD might affect responses other than the early events after stimulation, especially those that are coupled to membranes or membrane proteins.

In mast cells, the role of rafts and the effects of MβCD have been studied by several groups (*17*−*20*), but there are some discrepancies in the effects and targets of MβCD. This is partly because investigations were restricted to very early events, as observed in other immune cells (*12*, *13*). In this study, therefore, we examined the effects of MβCD on the events downstream of receptor phosphorylation, or Ca^{2+} influx and exocytotic membrane fusion between the secretory granules and the plasma membrane. We found that MβCD inhibited not only exocytotic membrane fusion but also the

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[1] Abbreviations: RBL, rat basophilic leukemia cells; SNARE, soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; MβCD, methyl-β-cyclodextrin; FRAP, fluorescence recovery after photobleaching; SOC, store-operated calcium channel; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; DG, diacylglycerol; YFP, yellow fluorescent protein.

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store-operated calcium channel, while lateral diffusion of the tyrosine kinase Lyn and Ca^{2+} release from the intracellular Ca^{2+} store were slightly affected.

EXPERIMENTAL PROCEDURES

Materials. Methyl-β-cyclodextrin, cholesterol-water soluble, PMA, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO). U73122 was obtained from Calbiochem (La Jolla, CA). A23187 and thapsigargin were from Wako Pure Chemicals (Tokyo, Japan). All other reagents were of the highest grade available commercially.

Cell Culture. Rat basophilic leukemia 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 (Boeringer Mannheim) at 37 °C in an atmosphere of 5% CO_2.

Plasmid Construction and Transfection. Poly(A)$^+$ RNA was obtained with a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) from 1×10^7 cells of RBL-2H3 and served as templates for cDNA synthesis with SuperScript II RT (Gibco BRL), as reported previously (*4*). The primer pair for rat Lyn was 5′-AGATCTTATGGGAT-GTATTAAATCAAAAAGGAA (sense; BglII site is underlined) and 5′-CCGCGGTCCTGGCTGCTGCTGATACTGC-3′ (anti-sense; SacII site is underlined). PCR products were extracted from agarose gel with Gene Clean (Bio 101) and subcloned into the TA cloning vector pCRII (Invitrogen). Cloned PCR products were sequenced with a DSQ1000 DNA sequencer (Shimadzu, Kyoto, Japan) using the FITC-labeled M13 universal primer, and verified cDNA was ligated with a p-EYFP (Clontech). RBL-2H3 cells were electroporated in cold phosphate-buffered saline with 20 μg of plasmid DNA at 250 V and 950 μF using Gene Pulser II (Bio-Rad) (*4*).

Assay of Secreted β-Hexosaminidase. Degranulation of RBL-2H3 cells was monitored by measuring the activity of a granule-stored enzyme, β-hexosaminidase, secreted in the cell supernatant (*10*). Cells were seeded in a 24-well plate (2×10^5 cells/well). After washing cells with HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.1% glucose, 0.1% BSA, and 10 mM HEPES, pH 7.4), cells were preincubated with MβCD in HEPES-buffered saline at 37 °C for 30 min before stimulation, to deplete cholesterol from the plasma membrane. Cells were sensitized by anti-DNP IgE (200 ng/mL) for 30 min and incubated with an average of six DNP groups conjugated with BSA (DNP_6-BSA) in 200 μL of HEPES-buffered saline for 30 min at 37 °C. Aliquots of supernatants were transferred to a 96-well plate (20 μL/well) and incubated with 20 μL of a substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 100 mM citrate, pH 4.5) for 1 h at 37 °C. After terminating the reaction with 160 μL of Na_2CO_3-$NaHCO_3$ buffer (1:1 mixture of 167 mM Na_2CO_3 and $NaHCO_3$), the absorbance at 405 nm was measured by a microplate reader (MPR-A4, TOSOH, Tokyo, Japan). The release activity relative to the total β-hexosaminidase content of the cells was calculated. The total β-hexosaminidase content was determined by dissolving the cells with 0.1% Triton-X100. Values were plotted as percentages of the release activity obtained from cells without the treatment of MβCD. For the repletion experiment with cholesterol, cells were treated with an MβCD−cholesterol complex (0.1 mM cholesterol) for 30 min after washing MβCD-treated cells (*18*).

Intracellular Ca^{2+} Measurement. RBL-2H3 cells (4.5×10^5 cells) were loaded with 2 μM Fura2/AM (Molecular Probes; Eugene, OR) for 30 min at 37 °C and washed twice with HEPES-buffered saline. Cells were sensitized with anti-DNP IgE (200 ng/mL) for 30 min and stimulated with DNP-BSA (100 ng/mL). The fluorescence intensity at 340 and 360 nm was measured at 37 °C, and the ratio (F340/F360) was calculated by a spectrofluorometer equipped with a personal computer (RF-5300PC; Shimadzu, Japan). For treatment with MβCD, cells were incubated with MβCD for 30 min with Fura2/AM. Ratio values were converted to Ca^{2+} concentration by the procedure reported previously (*21*).

Fluorescent Microscopy and FRAP Assay. Cells (2×10^5 cells) were plated in a ZOG-3 glass-bottom chamber (Elekon; Chiba, Japan). Fluorescent images were taken with a confocal laser scanning microscope (Zeiss, LSM-510) with a 63× objective lens (Plan-Apochromat 63×/1.4 oil). Samples were excited at 488 nm with an Ar laser, and fluorescence longer than 515 nm was collected. The lateral diffusion of Lyn was observed by fluorescence recovery after photobleaching (FRAP). A rectangular region (1×2.5 μm along the plasma membrane) was bleached by a 488 nm laser, and the time course of the recovery of the fluorescence intensity of the bleached region was measured. Photobleaching during the measurement was corrected with the total fluorescence in the whole-cell images obtained at the beginning and end of the measurement. When a FRAP assay was carried out while changing the bleaching area, the times required to attain half of the recoverable fluorescence intensity were almost proportional to the bleached area. This indicates that the major contribution to the fluorescence recovery is the lateral diffusion of Lyn, not the exchange of Lyn between the plasma membrane and the cytoplasmic pool, since the exchange of protein between the plasma membrane and the cytoplasmic pool is independent of the bleaching area (*22*).

RESULTS

Effects of MβCD on Degranulation. The cross-linking of IgE receptors by a multivalent antigen causes the activation of protein tyrosine kinases, such as Lyn and Syk. The consequent activation of phospholipase C (PLC) leads to the production of IP_3 and diacylglycerol (DG). IP_3 mobilizes Ca^{2+} from calcium stores, and DG and elevated Ca^{2+} levels activate PKC. Depletion of the Ca^{2+} stores causes Ca^{2+} influx through the calcium channel in the plasma membrane (store-operated calcium channel), and this Ca^{2+} influx triggers degranulation. We first investigated the effects of MβCD on the degranulation in mast cells (RBL-2H3). For the treatment of cells with MβCD, the cells were incubated with the indicated concentration of MβCD at 37 °C for 30 min. Degranulation induced by a multivalent antigen was inhibited by MβCD dose-dependently (Figure 1). This result was similar to that obtained by Yamashita et al. (*18*). To examine the effects of MβCD on the process after the elevation of intracellular Ca^{2+}, we stimulated cells with the phorbol ester and calcium ionophore. Since stimulation with the phorbol

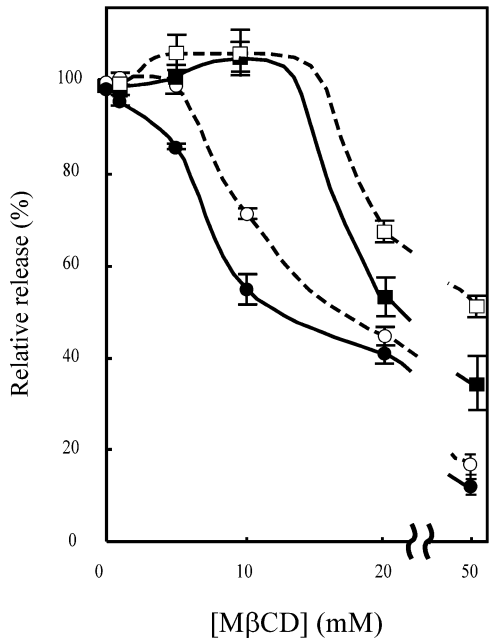

FIGURE 1: Effects of MβCD on the exocytotic release from RBL-2H3 cells. Cells were preincubated with the indicated concentrations of MβCD and stimulated with antigen (closed circle) or PMA/A23187 (open circle). For repletion experiments, cells were stimulated with antigen (closed square) or PMA/A23187 (open square) after treatment with cholesterol. Values are plotted as percentages of the release activity obtained from cells treated with 0 mM MβCD (mean ± SD).

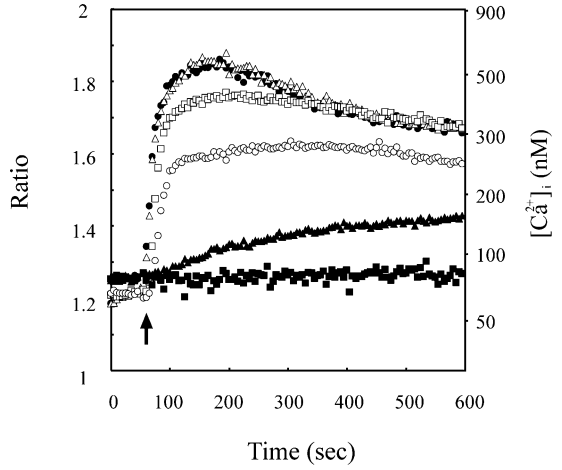

FIGURE 2: Effects of MβCD on the dynamics of the intracellular Ca^{2+} concentration. Cells were preincubated with the indicated concentrations of MβCD for 30 min. Each symbol represents the concentration of MβCD (mM) as follows: ●: 0; △: 1; □: 5; ○: 10; ▲: 20; and ■: 50. Antigen (100 ng/mL) was added at the time indicated by an arrow. Ratio values (F340/F360) were converted to the Ca^{2+} concentration and are shown as a second ordinate.

ester and calcium ionophore bypasses events before the increase in Ca^{2+}, we can see whether MβCD affects the membrane fusion between the secretory granules and the plasma membrane. When stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL), and A23187 (1 μM) in the presence of 1 mM Ca^{2+}, exocytosis was also inhibited by MβCD dose-dependently (Figure 1). This suggests that MβCD inhibited the exocytotic membrane fusion itself. The dose−inhibition curve when cells were subjected to antigen stimulation was shifted toward a lower concentration of

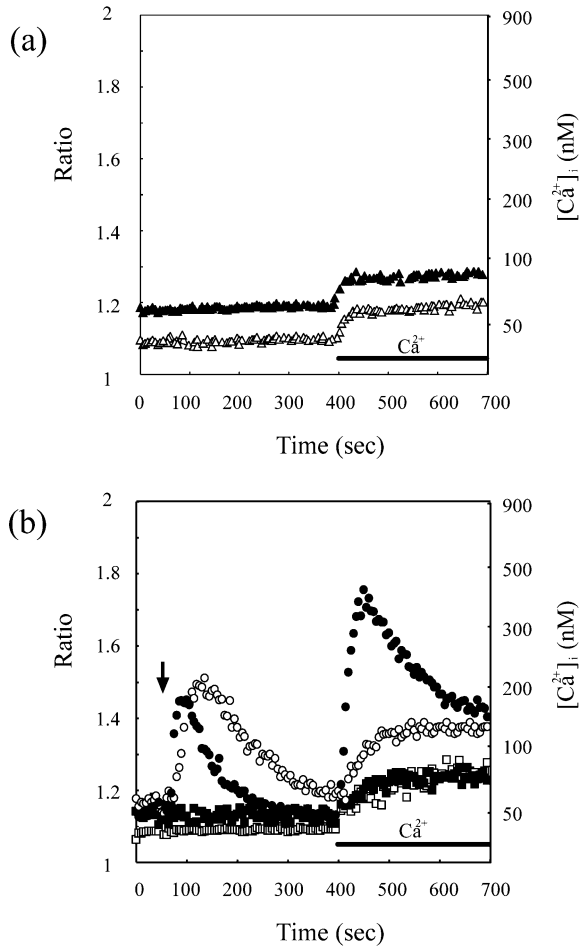

FIGURE 3: Effects of MβCD on the SOC activated by antigen stimulation. Cells were stimulated in the absence of extracellular Ca^{2+}. The concentration of calcium ion in the extracellular solution was elevated to 1 mM in the period indicated by a solid line. (a) Effects of MβCD on the basal level of the intracellular calcium concentration. With an increase in the extracellular Ca^{2+} concentration alone, a small increase in the intracellular Ca^{2+} concentration was observed (▲: +MβCD; △: −MβCD). (b) Cells were stimulated with antigen (Ag) in the absence of extracellular Ca^{2+}. After recovery to the basal level, the extracellular concentration was elevated (●: Ag; ○: Ag + MβCD; □: Ag + U73122; and ■: Ag + U73122 + MβCD).

MβCD as compared to that when cells were stimulated by PMA and A23187. Since this implies that MβCD inhibited the processes before exocytotic membrane fusion, we examined the effects of MβCD on Ca^{2+} mobilization.

To examine whether the inhibitory effects of MβCD are due to the depletion of cholesterol, MβCD-treated cells were subsequently incubated with water-soluble cholesterol. Cholesterol repletion completely reversed the attenuated exocytosis activity for up to 10 mM MβCD, suggesting that the effects of MβCD are based on cholesterol depletion at a lower concentration of MβCD.

Effects of MβCD on the Ca^{2+} Elevation Induced by Antigen Stimulation. We examined the effects of MβCD on intracellular changes in the Ca^{2+} concentration. As shown in Figure 2, the basal Ca^{2+} concentration in MβCD-treated cells was slightly higher than that in cells that had not been pretreated with MβCD. The antigen-induced Ca^{2+} increase was inhibited by MβCD in a dose-dependent manner (Figure 2). To identify the Ca^{2+} sources that are affected by MβCD, we measured the intracellular Ca^{2+} concentration first in the

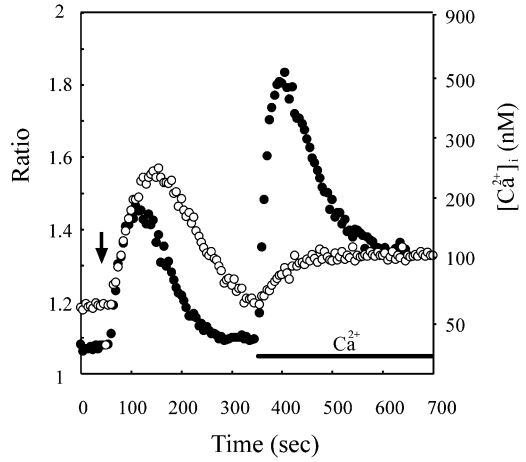

FIGURE 4: Effects of MβCD on the SOC activated by thapsigargin. The intracellular Ca^{2+} concentration was observed using the same protocol as in Figure 3. Cells treated with (open circle) or without (closed circle) MβCD were stimulated with thapsigargin instead of antigen at the time indicated by an arrow.

absence of extracellular Ca^{2+} and then in the presence of extracellular Ca^{2+}. As shown in Figure 3a, pretreatment with MβCD increased the basal level slightly but did not inhibit Ca^{2+} elevation due to Ca^{2+} release from the Ca^{2+} store in the absence of extracellular Ca^{2+}. With the addition of Ca^{2+} to the extracellular solution (final 1 mM Ca^{2+}), a small increase in the Ca^{2+} concentration was observed (Figure 3a). When cells were stimulated with antigen in the absence of extracellular Ca^{2+}, the antigen induced a transient increase in the Ca^{2+} concentration due to Ca^{2+} release from the Ca^{2+} store (Figure 3b). After the elevation of Ca^{2+} in the extracellular solution, the intracellular Ca^{2+} concentration increased again due to Ca^{2+} influx through the store-operated calcium channel (Figure 3b). Upon pretreatment with 10 mM MβCD, the onset of the initial Ca^{2+} increase was significantly delayed, but its amplitude was augmented as compared to that without pretreatment. The Ca^{2+} increase after elevation of the extracellular Ca^{2+} concentration was greatly decreased by MβCD. To estimate the Ca^{2+} increase due to pathways other than store-operated calcium currents, the production of IP_3 was inhibited by U73122, a specific inhibitor of phospholipase C. The initial Ca^{2+} increase was completely inhibited by U73122 (10 μM). Under this condition, store-operated calcium channels are thought to be inactive, and the Ca^{2+} increase with an increase in extracellular Ca^{2+} gives an estimate of the Ca^{2+} influx other than via SOC. As shown in Figure 3b, a major component of the Ca^{2+} increase after elevation was the SOC, which was remarkably inhibited by MβCD.

Effects of MβCD on the Calcium Elevation Induced by Thapsigargin. To further investigate the effects of MβCD on SOC, we stimulated cells with thapsigargin, which causes Ca^{2+} release from the Ca^{2+} store by inhibiting sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPases (SERCAs). Cells were stimulated with thapsigargin in the absence of extracellular Ca^{2+}. The intracellular Ca^{2+} concentration increased transiently with the addition of thapsigargin (Figure 4). Regarding the onset of the increase in the Ca^{2+} concentration, the delay observed with antigen stimulation was not observed, even following pretreatment with MβCD. However, augmentation of the amplitude of the transient increase in Ca^{2+}

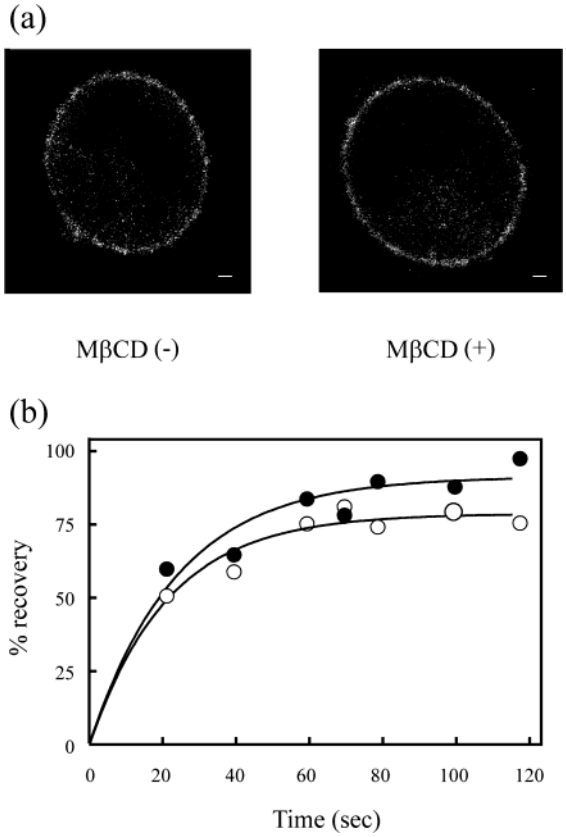

FIGURE 5: Effects of MβCD on the lateral mobility of Lyn. (a) Fluorescent images of RBL-2H3 cells expressing Lyn-YFP. Images were obtained after incubation with (right) or without 10 mM MβCD (left) for 30 min. Scale bars represent 1 μm. (b) Time course of fluorescence recovery after photobleaching. A rectangular region along the plasma membrane was bleached after incubation with (closed circle) or without (open circle) 10 mM MβCD for 30 min. Regression curves were obtained by fitting with a single-exponential equation.

was observed. After elevation of the extracellular Ca^{2+} concentration, remarkable inhibition of the Ca^{2+} increase was observed (Figure 4), as with antigen stimulation (Figure 3b). These results suggest that the depletion of cholesterol by MβCD inhibits the SOC.

Effects of MβCD on the Lateral Mobility of Lyn. The tyrosine kinase Lyn is localized in rafts, and its localization is thought to be crucial for signal transduction in mast cells. We investigated the effects of MβCD on the lateral mobility of Lyn on the plasma membrane by FRAP (fluorescence recovery after photobleach) assay. We expressed YFP-tagged Lyn in RBL-2H3 cells and observed FRAP by confocal laser scanning microscopy. YFP-tagged Lyn was distributed on the plasma membrane, and this distribution was not affected by treatment with MβCD (Figure 5a). Figure 5b shows a typical time course of the recovery of fluorescent Lyn-YFP. No significant difference was observed in the rate of recovery of fluorescence intensity, while the mobile fraction in an MβCD-treated cell (94.7% $\pm$ 6.8, $n = 5$; mean $\pm$ SD) was higher than that in a nontreated cell (75.2% $\pm$ 5.6, $n = 5$: mean $\pm$ SD). These results suggest that MβCD had little effect on the lateral mobility of Lyn, but some fraction of Lyn was immobilized by a cholesterol-dependent structure.

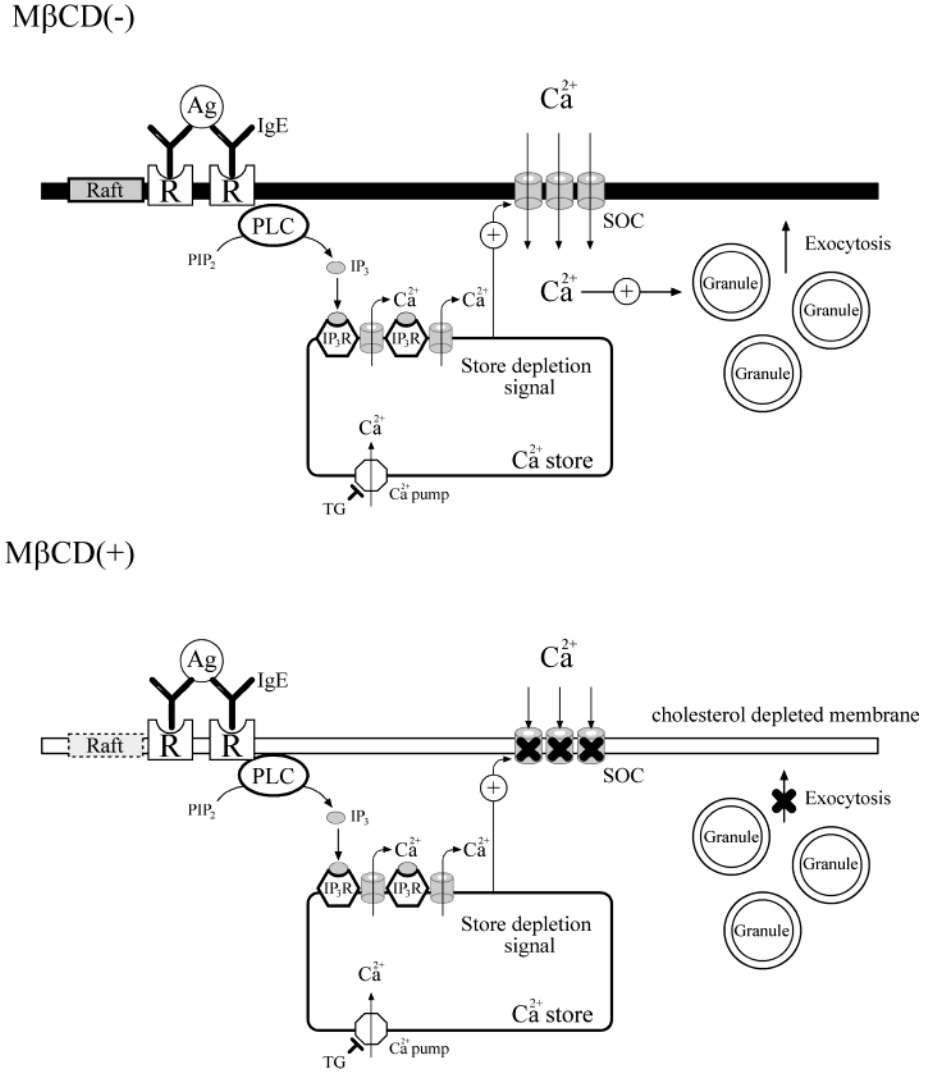

FIGURE 6: Schematic representation of the effects of MβCD on a degranulation process in mast cells. Signal transduction from receptor activation to exocytotic release in a control cell in the absence of MβCD (upper panel). Antigen (Ag) cross-links IgE receptor (R) and activates phospholipase C (PLC), which produces IP_3 from PIP_2. IP_3 causes Ca^{2+} release from the Ca^{2+} store by activating the IP_3 receptor (IP_3R). Depletion of the Ca^{2+} store activates store-operated Ca^{2+} channel (SOC), and this induces membrane fusion between the secretory granule and the plasma membrane (exocytosis). Cholesterol depletion by the treatment with MβCD inhibited SOC and the exocytotic membrane fusion, while Ca^{2+} release from the Ca^{2+} store induced by antigen stimulation or thapsigargin (TG) was not impaired. Although tyrosine kinase Lyn is thought to be localized in lipid rafts, MβCD did not affect its lateral mobility very much.

DISCUSSION

We first investigated the effects of the depletion of cholesterol from the plasma membrane on exocytosis in mast cells. We found that MβCD inhibited the exocytosis induced by antigen stimulation, and the results were essentially the same as those reported by Yamashita et al. (*18*). However, Sheets et al. reported that 10 mM MβCD did not significantly inhibit antigen-induced exocytosis (*17*). To confirm our results and clarify the mechanism of this inhibition, we examined the effects of MβCD on the exocytosis induced by PMA and A23187, which mimic antigen stimulation bypassing the early events before Ca^{2+} influx. Exocytosis induced by PMA and A23187 was inhibited by MβCD, and this suggests that the action of MβCD was on the membrane fusion itself. Yamashita et al. reported that MβCD did not inhibit the degranulation induced by PMA and A23187 (*18*). The reason for this discrepancy is not clear, but the elevation of the intracellular Ca^{2+} concentration by A23187 was not inhibited by MβCD treatment (data not shown). We also showed that the repletion of cholesterol recovered the degranulation induced by PMA and A23187 (Figure 1; open square), suggesting that the effect of MβCD is restricted to the depletion of cholesterol. Cholesterol depletion changes the properties of the membrane and membrane proteins (*23*). Therefore, it is reasonable to suppose that exocytotic membrane fusion is inhibited by MβCD. Membrane fusion is thought to be affected by various physicochemical properties of the biomembrane, such as membrane stability, membrane fluidity, lipid packing, bilayer curvature, and hydration elasticity. The lipid composition, including cholesterol, changes those properties. Many lines of evidence suggest that cholesterol is needed for the membrane fusion activity of viruses (*24*−*26*). Cholesterol destabilizes the bilayer structure and induces the formation of hexagonal H_{II} (*27*), which is thought to be involved in membrane fusion. Recently, lipid rafts have been reported to play a role in various cellular functions. Interestingly, it has been reported that SNARE proteins are localized in rafts in MDCK cells

and PC12 cells (*28*−*30*). Although the localization of SNARE proteins in rafts has not been reported in mast cells, it is probable that the disruption of rafts by MβCD inhibited the efficient assembly of SNARE proteins, resulting in the inhibition of exocytosis. In addition to this raft-related mechanism, the direct effects of cholesterol depletion on the activity of SNARE proteins cannot be excluded.

As shown in Figure 1, the dose−inhibition curve was shifted toward a lower concentration of MβCD in the case of antigen stimulation as compared to that with PMA/A23187 stimulation. This led us to examine the effects of MβCD on the process before exocytotic membrane fusion (i.e., calcium mobilization). As shown in Figure 2, MβCD inhibited the elevation of the intracellular Ca^{2+} concentration induced by antigen stimulation. Using a protocol that allowed us to discriminate between Ca^{2+} sources, we found that MβCD inhibited Ca^{2+} influx from the extracellular solution or SOC channel (Figure 3). This was confirmed by the result that MβCD inhibited the SOC channel activated by the Ca^{2+} store depletion induced by thapsigargin (Figure 4). There is considerable evidence regarding the effects of cholesterol depletion on the Ca^{2+} channel (*31*). One possible mechanism of these effects is the direct effect of cholesterol depletion on the Ca^{2+} channel. It has been reported that an increase in cholesterol content in the plasma membrane increased Ca^{2+} influx through the Ca^{2+} channel (*32*, *33*), while a decrease in cholesterol caused a reduction in the Ca^{2+} influx (*34*, *35*), although the precise mechanisms remain unknown. Second, the localization of the Ca^{2+} channel in a cholesterol-rich microdomain might explain the effects of cholesterol depletion. Lockwich et al. reported that Trp1, an SOC, is localized in a lipid raft, and this localization is crucial for the activity of SOC (*36*). Another possibility is that cholesterol depletion blocks the store-depletion signal from the Ca^{2+} store, while the Ca^{2+} channel itself remains intact.

Although the initial Ca^{2+} increase due to the Ca^{2+} release from the Ca^{2+} store was little affected by 10 mM MβCD, both the onset and the decay of the Ca^{2+} increase were delayed. Since a delayed onset was not observed with thapsigargin stimulation, MβCD inhibited the processes from receptor activation to IP_3 production. The inhibitory action of MβCD on these early processes was clearly observed at a higher dose (Figure 2). At an MβCD concentration of 50 mM, the initial Ca^{2+} increase was completely inhibited. As for the delayed decline in the Ca^{2+} transient observed by both antigen and thapsigargin stimulation, impaired activity of the system in the plasma membrane that removes Ca^{2+} from the cytoplasm, such as the Ca^{2+} pump or Na/Ca exchanger, might explain this finding (*31*).

Finally, we compared the intracellular distribution of Lyn in an MβCD-treated cell and a control cell. Since Lyn is localized in lipid rafts and its localization is closely related to signal transduction in mast cells, MβCD could affect early events in the activation of mast cells. As shown in Figure 3, however, Ca^{2+} release from the Ca^{2+} store was not affected by MβCD, suggesting that early events leading to Ca^{2+} were released hardly inhibited by MβCD. The intracellular localization of Lyn in an MβCD-treated cell before and after stimulation did not seem to be different from that in a control cell. This is consistent with the result that MβCD had no significant effects on Ca^{2+} release from the Ca^{2+} store.

The signaling process of antigen-induced degranulation and the effects of cholesterol depletion on this process are summarized in Figure 6. Cross-linking of the high affinity IgE receptor (FcϵR I) induces the activation of protein tyrosine kinases such as Lyn and Syk followed by the activation of phospholipase C, which hydrolyzes phosphatidylinositol and produces inositol 1,4,5-trisphosphate (IP_3). Binding of IP_3 to the IP_3 receptor on the intracellular Ca^{2+} store causes Ca^{2+} release to the cytoplasm. The depletion of Ca^{2+} ion in the store activates the store-operated Ca^{2+} channel (SOC) in the plasma membrane, and the sustained elevation of the intracellular Ca^{2+} concentration induces degranulation. As shown in Figure 3, however, Ca^{2+} release from the Ca^{2+} store was not affected by MβCD, suggesting that early events leading to Ca^{2+} release were hardly inhibited by MβCD. Consistent with these observations, Yamashita et al. reported that MβCD did not affect either the phosphorylation level of the β-chain of the IgE receptor or the IP_3 production (*18*). Interestingly, it has been shown that palmitoylation-defective Lyn, which has a decreased ability to localize into lipid rafts, was able to initiate early events including the phosphorylation of the β and γ chain of the IgE receptor and the elevation of the intracellular Ca^{2+} concentration (*26*). This observation also supports our finding that MβCD had no significant effects on Ca^{2+} release from the Ca^{2+} store.

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