**Introduction**

Ceramide causes a variety of cellular processes including differentiation and apoptosis (Kolesnick and Kronke, 1998). It is synthesized in the endoplasmic reticulum (ER) and transported to the Golgi apparatus via an ATP-dependent pathway distinct from that for secretory proteins (Fukasawa et al., 1999). In the Golgi apparatus, ceramide is converted to sphingomyelin and glucosylceramide, which are then predominantly delivered to the plasma membrane (Hoekstra and Kok, 1992; Rosenwald and Pagano, 1993a; van Meer, 1993). Upon stimulation of cells, ceramide is regenerated by the action of sphingomyelinases (Hannun, 1994).

Exogenously added short chain ceramide is rapidly incorporated into cells and accumulates in the Golgi apparatus (Lipsky and Pagano, 1985). The accumulation causes almost complete inhibition of protein transport at the level of the trans-Golgi region, whereas it slightly retards protein transport from the ER to the medial-Golgi region (Rosenwald and Pagano, 1993a; van Meer, 1993). Upon stimulation of cells, ceramide is regenerated by the action of sphingomyelinases (Hannun, 1994).

**Materials and Methods**

Materials

BFA, C2-ceramide, C6-ceramide and bovine brain ceramides were obtained from Wako Chemicals. Nocodazole, N-oleoyl ethanolamine (NOE) and sphingomyelinase from Staphylococcus aureus were used.

**Summary**

We examined the effects of short chain and long chain ceramides on the stability of the Golgi apparatus. Short chain ceramides, C2- and C6-ceramides, blocked brefeldin A-induced Golgi disassembly without affecting the rapid release of Golgi coat proteins, whereas they did not inhibit brefeldin A-induced tubulation of endosomes. Both short chain ceramides also retarded Golgi disassembly induced by nordihydroguaiaretic acid and nocodazole, suggesting that they stabilize the Golgi apparatus. In contrast to short chain ceramides, natural long chain ceramides, when incorporated into cells or formed within cells upon treatment with sphingomyelinase or metabolic inhibitors, enhanced brefeldin A-induced Golgi disassembly. These results suggest that sphingolipid metabolism is implicated in the stability of the Golgi apparatus.

Key words: Brefeldin A, Ceramide, Golgi apparatus, Nocodazole, Nordihydroguaiaretic acid, Sphingomyelinase

Takuya Fukunaga1, Masami Nagahama1, Kiyotaka Hatsuzawa1, Katsuko Tani1, Akitsugu Yamamoto2 and Mitsuo Tagaya1, *

1 School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan
2 Department of Physiology, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

*Author for correspondence (e-mail: tagaya@ls.toyaku.ac.jp)

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obtained from Sigma. Sphingosine, D, L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), D-erythro-C_{16}-ceramide and nordihydroguaiaretic acid (NDGA) were obtained from Biomol. Fluorescein-conjugated transferrin (Tf) was obtained from Molecular Probes.

Antibodies
Monoclonal antibodies against Man II and TGN38 were obtained from BAbCo and Affinity Bioreagents, respectively. A polyclonal anti-Tf antibody was obtained from Dako. A polyclonal anti-β-COP antibody was a generous gift from Dr Yamaguchi of this laboratory.

Microscopy
Immunofluorescence and electron microscopic analyses were performed as described by Tagaya et al. (1996) and Yamaguchi et al. (1997), respectively. A conventional fluorescence microscope was used to examine the localization of Man II because this apparatus was more suitable for obtaining image for diffused patterns of Man II than a confocal microscope. To investigate the localization of TGN38 and Tf, confocal immunofluorescence microscopy was performed with an Olympus Fluvew 300 laser scanning microscope.

Cell culture
NRK cells obtained from the Riken Cell Bank were grown on glass coverslips in α-minimum essential medium supplemented with 50 i.u./ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum in a humidified atmosphere containing 5% CO_{2} at 37°C.

Solubilization of long chain ceramides
D-erythro-C_{16}-ceramide and bovine brain ceramides were dissolved in ethanol/dodecane (98:2, v/v) at a concentration of 5 mg/ml, and then directly added to the medium. The final concentrations of ethanol and dodecane in the medium were 0.5% and 0.01%, respectively.

RESULTS
Effects of short chain ceramides on BFA-induced Golgi disassembly
We first examined the effects of short chain ceramides on BFA-induced Golgi disassembly. NRK cells were preincubated for 30 minutes in the absence or presence of 5 μM C_{6}- or C_{2}-ceramide, and then 10 μM BFA was added and the incubation was continued for 6 minutes. After fixation of cells with paraformaldehyde, both Man II, a medial-Golgi marker protein (Kornfeld and Kornfeld, 1985) and β-COP, a subunit of COPI (Waters et al., 1991), were visualized by fluorescence microscopy. In control cells Man II was localized at the perinuclear region with a ribbon-like structure (Fig. 1A). Upon BFA treatment, Man II was redistributed into the ER in almost all cells (Fig. 1C). In contrast, it was discernible at the perinuclear region when cells were incubated with C_{6}-ceramide (Fig. 1E) or C_{2}-ceramide (Fig. 1G). As shown in Table 1, the inhibitory effect of C_{2}-ceramide on BFA-induced Golgi disassembly was concentration-dependent. Essentially the same results were obtained when BHK-21 cells were used instead of NRK cells or when short chain ceramides obtained from different commercial sources were used (data not shown).

Table 1. Effect of C_{2}-ceramide on BFA-induced Golgi disassembly

<table>
<thead>
<tr>
<th>C_{2}-ceramide (μM)</th>
<th>Cells with disassembled Golgi apparatus (%)</th>
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<td>1</td>
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NRK cells were preincubated at 37°C for 30 minutes in the presence of C_{2}-ceramide at the indicated concentrations. BFA was added at a final concentration of 10 μM, and the cells were further incubated at 37°C for 6 minutes. They were immunostained with a monoclonal anti-Man II antibody (A,C,E,G) and a polyclonal anti-β-COP antibody (B,D,F,H). Control cells without BFA treatment (A and B). Bar, 20 μm.
present we cannot reconcile our results with those of Kok et al. (1998). However, the following results clearly showed that ceramides are closely related to the stability of the Golgi apparatus.

**Short chain ceramides do not block the BFA-induced rapid release of COPI from Golgi membranes**

Disassembly of the Golgi apparatus by BFA takes place following the rapid release of COPI from the Golgi apparatus (Klausner et al., 1992). This release is due to the inhibitory effect of BFA on the guanine nucleotide exchange factor for ARF1 (Donaldson et al., 1992; Helms and Rothman, 1992). Although C₆- and C₂-ceramides inhibited redistribution of Man II by BFA, they did not prevent the rapid release of β-COP, a component of COPI (Waters et al., 1991), from Golgi membranes (Fig. 1F,H). These results suggest that short chain ceramides inhibit BFA-induced Golgi disassembly without affecting the BFA-induced inhibition of the guanine nucleotide exchange factor for ARF1.

**Effects of short chain ceramides on BFA-induced tubulation of the trans-Golgi network (TGN) and endosomes**

BFA causes not only redistribution of Golgi components into the ER, but also tubulation of the TGN, endosomes, and lysosomes. The tubules from the Golgi and endosomes are fused, whereas the lysosomal tubules remain apart (Lippincott-Schwartz et al., 1991; Wood et al., 1991). We next examined whether or not short chain ceramides suppress the action of BFA on the TGN and endosomes. When cells were preincubated with 10 μM C₆-ceramide for 30 minutes, the morphology of the TGN represented by TGN38, a TGN marker protein (Luzio et al., 1990), changed from a ribbon-like structure to dot-like ones without changing the localization (Fig. 2C). Upon incubation with BFA for 6 minutes, no significant morphological change in the TGN38-containing dots was observed in the presence of C₆-ceramide (Fig. 2D). In the absence of C₆-ceramide, as described previously (Lippincott-Schwartz et al., 1991; Wood et al., 1991), TGN38-containing tubules appeared when cells were

**Fig. 2.** C₆-ceramide but not C₂-ceramide suppresses BFA-induced tubulation of the TGN. NRK cells were preincubated at 37°C for 30 minutes in the presence of 0.1% DMSO (A and B), 10 μM C₆-ceramide (C and D) or 10 μM C₂-ceramide (E and F). BFA was added at a final concentration of 10 μM, and the cells were further incubated at 37°C for 6 minutes. After fixation, they were immunostained with a monoclonal anti-TGN38 antibody. Cells before (A,C,E) and after (B,D,F) BFA treatment. Bar, 20 μm.

**Fig. 3.** Short chain ceramides do not inhibit BFA-induced tubulation of recycling endosomes. NRK cells were preincubated in the absence of fetal calf serum at 37°C for 1 hour. To the medium was added 25 μg/ml fluorescein-conjugated Tf, and the incubation was continued for another 1 hour in the presence of 0.1% DMSO (A and B), 10 μM C₆-ceramide (C and D) or 10 μM C₂-ceramide (E and F). BFA was added at a final concentration of 10 μM, and the cells were further incubated at 37°C for 6 minutes. After fixation, they were immunostained with a polyclonal anti-Tf antibody. Cells before (A,C,E) and after (B,D,F) BFA treatment. Bar, 20 μm.
incubated with BFA (Fig. 2B). In the case of C2-ceramide, fragmentation of the TGN occurred (Fig. 2E), but BFA-induced tubulation was not significantly suppressed (Fig. 2F).

To examine the effects of short chain ceramides on BFA-induced tubulation of recycling endosomes, we investigated the morphology of recycling endosomes containing Tf. For this purpose, cells were preincubated with 25 μg/ml fluorescein-conjugated Tf in the presence or absence of 10 μM C6- or C2-ceramide for 1 hour, and then treated with BFA for 6 minutes. Since the fluorescence intensity of fluorescein-conjugated Tf was weak, cells were further immunostained with an anti-Tf antibody followed by a fluorescein isothiocyanate-labeled secondary antibody. Despite a partial inhibition of Tf recycling by C6-ceramide (Chen et al., 1995), Tf was uptaken by cells into endosomes, and the patterns of Tf staining were not significantly different between control cells (Fig. 3A) and short chain ceramide-treated cells (Fig. 3C, E). Neither C6-ceramide (Fig. 3D) nor C2-ceramide (Fig. 3F) prevented BFA-induced tubulation of recycling endosomes.

**Electron microscopic analysis of C6-ceramide-treated cells**

To confirm the immunofluorescence observations, we performed electron microscopic analysis of C6-ceramide-treated NRK cells. In agreement with a previous observation (Linardic et al., 1996), the distal Golgi region was apparently dilated (Fig. 4B), but the nuclear envelope and ER remained intact. This structural change may be relevant to the morphological change in the TGN38-containing compartment (Fig. 2). As well as this effect, C6-ceramide caused the formation of large distended saccules, often containing other membrane-bound structures within them, as reported previously (Rosenwald and Pagano, 1993b). In addition, it produced membrane aggregates located close to the Golgi region. Consistent with the immunofluorescence observations (Fig. 1), BFA-induced Golgi disassembly did not occur when cells were incubated with C6-ceramide (Fig. 4D). Interestingly, the distended saccules disappeared during BFA treatment, but we did not further examine this phenomenon.

**Short chain ceramides retard Golgi disassembly induced by NDGA and nocodazole**

To examine whether or not short chain ceramides generally stabilize the Golgi apparatus, we used other compounds that are known to cause Golgi disassembly in mechanisms different from that of BFA. We and others previously showed that NDGA causes Golgi disassembly without affecting the attachment of COPI to Golgi membranes (Yamaguchi et al., 1997; Drecktrah et al., 1998; Fujiwara et al., 1998). When cells were preincubated with 10 μM C2-ceramide for 30 minutes, NDGA-induced Golgi disassembly was markedly prevented (Fig. 5C). C6-ceramide at the same concentration had a similar but lesser effect. Golgi membranes became fragmented but not completely vesiculated (Fig. 5B).

Nocodazole causes depolymerization of microtubules (Zieve et al., 1980), leading to Golgi disassembly (Rogalski and Singer, 1984). Recent studies suggest that this compound promotes redistribution of Golgi components into the ER, and the redistributed components may coalesce at or about ER exit sites to regenerate Golgi mini-stacks (Cole et al., 1996; Storrie et al., 1998; Drecktrah and Brown, 1999). When cells were preincubated with short chain ceramides for 30 minutes followed by incubation with 15 μg/ml nocodazole for 90 minutes Golgi dispersion was considerably blocked. In this case, 5 μM C6-ceramide had a more significant effect (Fig. 6C) than 5 μM C2-ceramide (Fig. 6D). Neither ceramide significantly affected microtubule disassembly by nocodazole (data not shown).

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**Fig. 4.** Analysis of C6-ceramide-treated cells by electron microscopy. NRK cells were preincubated at 37°C for 30 minutes in the presence of 0.1% DMSO (A and C) or 10 μM C6-ceramide (B and D). BFA was added at a final concentration of 10 μM, and the cells were further incubated at 37°C for 6 minutes (C and D). Representative Golgi apparatus (G), vesicles (V), saccules (S), nucleus (N) and aggregates (A) are marked. Bar, 1 μm.
Effects of ceramide on the Golgi stability

Sphingomyelinase treatment enhances the action of BFA

The results described above raise the possibility that ceramide stabilization of the Golgi apparatus. We next examined whether ceramide generation inside cells also suppresses BFA-induced Golgi disassembly. For this purpose, cells were preincubated for 1 hour with 500 mU/ml sphingomyelinase. Previous studies showed that treatment of cells with 1 U/ml sphingomyelinase for 1 hour increases ceramide levels 2- to 4-fold over control levels (Kolesnick, 1989; Olivera et al., 1992; Oda et al., 1995). The incubation of cells with sphingomyelinase caused a slight fragmentation of the Golgi apparatus, represented by Man II staining (Fig. 7C). When the resultant cells were incubated with 10 μM BFA for 3 minutes, surprisingly, Man II staining became dispersed in almost all sphingomyelinase-treated cells (Fig. 7D). On the other hand, Golgi dispersion occurred in about 70% of control cells. These results raised the possibility that endogenously formed ceramides stimulate BFA-induced Golgi disassembly. It should be noted, however, that BFA-induced Golgi disassembly was still inhibited by C_6-ceramide (Fig. 7E) or C_2-ceramide (Fig. 7F) even after cells had been treated with sphingomyelinase. We carried out a similar experiment except that the incubation time with BFA was 2 minutes because no significant Golgi dispersion occurred in control cells during this short incubation. Under this condition, Golgi disassembly occurred in about 60% of sphingomyelinase-treated cells. These experiments were repeated several times and essentially the same results were obtained each time.

Inhibitors of sphingolipid biosynthesis affect the action of BFA

To confirm that the accumulation of endogenous ceramides enhances the action of BFA, we used two metabolic inhibitors of sphingolipid biosynthesis, NOE and PDMP. NOE inhibits ceramidase, the enzyme that mediates the conversion of ceramide to sphingosine (Sugita et al., 1975). PDMP is an inhibitor for the synthesis of glucosylceramide and sphingomyelin from ceramide (Radin et al., 1993). Ceramide levels are known to increase 2- to 2.5-fold by treatment of cells with NOE (Wiesner and Dawson, 1996; Spinedi et al., 1999) or PDMP (Maceyka and Machamer, 1997).

When cells were preincubated without or with 20 μM NOE for 2 hours, and then treated with 10 μM BFA for 2 minutes, dispersed staining patterns for Man II were observed in many NOE-treated cells (Fig. 8D), whereas no significant Man II dispersion occurred in control cells (Fig. 8B).

To examine the effect of PDMP, cells were preincubated with 70 μM PDMP for 1 minute or 2 hours, and then treated with 10 μM BFA for 2 minutes. In this experiment, PDMP was omitted during incubation with BFA because Kok et al. (1998) previously reported that PDMP inhibits BFA-induced Golgi disassembly by perturbing calcium homeostasis. This inhibitory effect was reversible, and was not observed when PDMP was omitted during incubation with BFA (T. Fukunaga and M. Tagaya, unpublished results). As shown in Fig. 9, preincubation of cells with PDMP for 2 hours significantly enhanced BFA-induced Golgi disassembly, whereas preincubation for 1 minute had essentially no effect. The requirement of a long time incubation with PDMP for the stimulation of the action of BFA may reflect a gradual accumulation of endogenous ceramides in the presence of PDMP.

Effects of D-erythro-C_16-ceramide on the action of BFA

To test more directly that natural long chain ceramides enhance the action of BFA on the Golgi apparatus, D-erythro-C_16-ceramide, an abundant species of endogenous ceramides, was dissolved in ethanol/dodecane, and then added to cells. Under these conditions, long chain ceramides can be incorporated into cells (Ji et al., 1995).

When cells were preincubated with 25 μg/ml D-erythro-C_16-ceramide for 1 hour, the Golgi apparatus became slightly

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Fig. 5. Short chain ceramides inhibit NDGA-induced Golgi disassembly. NRK cells were preincubated at 37°C for 30 minutes in the presence of 0.1% DMSO (A), 10 μM C_6-ceramide (B) or 10 μM C_2-ceramide (C). NDGA was added at a final concentration of 20 μM, and the cells were further incubated at 37°C for 15 minutes. After fixation, they were immunostained with a monoclonal anti-Man II antibody. Bar, 20 μm.
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Fig. 6. Short chain ceramides retard nocodazole-induced Golgi disassembly. NRK cells were preincubated at 37°C for 30 minutes in the presence of 0.1% DMSO (B), 10 μM C₆-ceramide (C) or 10 μM C₂-ceramide (D). Nocodazole was added at a final concentration of 15 μg/ml, and the cells were further incubated at 37°C for 90 minutes. After fixation, they were immunostained with a monoclonal anti-Man II antibody. Control cells (A). Bar, 20 μm.

Fig. 7. Sphingomyelinase treatment enhances BFA-induced Golgi disassembly. Cells were preincubated at 37°C for 1 hour without (A and B) or with 500 mU/ml sphingomyelinase (C and D), 500 mU/ml sphingomyelinase plus 10 μM C₆-ceramide (E) or 500 mU/ml sphingomyelinase plus 10 μM C₂-ceramide (F). BFA was added at a final concentration of 10 μM, and the cells were further incubated at 37°C for 3 minutes. After fixation, they were immunostained with a monoclonal anti-Man II antibody. Cells before (A and C) and after (B,D,E,F) BFA treatment. Bar, 20 μm.

fragmented (Fig. 10C), as observed for sphingomyelinase-treated cells (Fig. 7). Incubation of the cells with 10 μM BFA for 2 minutes caused a significant Golgi dispersion (Fig. 10D), whereas no significant Golgi dispersion occurred in cells preincubated with ethanol/dodecane alone (Fig. 10B). Similar results were obtained when natural ceramides from bovine brain were used (data not shown). It should be noted that C₆-ceramide (Fig. 10E) and C₂-ceramide (Fig. 10F) inhibited BFA-induced Golgi disassembly even in the presence of D-erythro-C₁₆-ceramide.

DISCUSSION

In the present study we examined the effects of short chain and long chain ceramides on the stability of the Golgi apparatus. We found that exogenously added short chain ceramides markedly inhibit BFA-induced Golgi disassembly without affecting the rapid release of COPI from Golgi membranes. Short chain ceramides also retarded Golgi disassembly induced by NDGA and nocodazole, suggesting that they stabilize the Golgi apparatus. On the other hand, natural long chain ceramides enhanced BFA-induced Golgi disassembly. These results, in conjunction with the previous finding that short chain ceramide inhibits protein traffic through the Golgi apparatus (Rosenwald and Pagano, 1993b), suggest that the effect of ceramides on the properties of Golgi membranes is a general one.

The observation that short chain and natural long chain ceramides have different effects on membrane dynamics is not peculiar to the present case. Accumulation of long chain ceramides by PDMP causes early Golgi resident proteins such as ERGIC-53 to be redistributed into the ER, whereas exogenously added short chain ceramide does not show the same effect (Maceyka and Machamer, 1997). In addition, short chain ceramide partially inhibits the endocytic pathways (Chen
et al., 1995; Li et al., 1999), whereas the formation of long chain ceramides by sphingomyelinase treatment rather promotes the ATP-independent formation of endocytic vesicles (Zha et al., 1998). It appears that short chain ceramide inhibits the formation of vesicles and/or tubules from membranes, whereas long chain ceramide enhances it.

How does ceramide affect the Golgi structure? One possibility is that ceramide modulates the activities of proteins involved in the organization of the Golgi apparatus by phosphorylation and dephosphorylation. It is well known that ceramides activate several kinases (Westwick et al., 1995; Zhang et al., 1997) and a phosphatase (Dobrowsky and Hannun, 1992) in a species-dependent manner (Wolff et al., 1994). Previous studies showed that the process of vesicular transport is regulated by phosphorylation and dephosphorylation (Davidson et al., 1992; Jamora et al., 1999), and accumulating bodies of evidence suggest that the mechanism of the organization of the Golgi apparatus is quite similar to that of vesicular transport (Warren and Malhotra, 1998).

An alternative possibility is that ceramide affects the physical properties of the Golgi apparatus. Natural long chain ceramides, like diacylglycerol, are known to mix poorly with phospholipids in bilayers and facilitate the formation of inverted hexagonal phases, thereby causing destabilization of phospholipid membranes (Veiga et al., 1999). At preset we favor the latter possibility because in a similar fashion short chain ceramides affect Golgi disassembly that occurs through different mechanisms. Perhaps the different effects of C_6- and C_2- ceramides on different Golgi-disrupting reagents are due to the different physicochemical properties of these ceramides.

Recent studies clearly demonstrated the importance of the lipid structure for the formation of transport vesicles (Zha et al., 1998; Schmidt et al., 1999; Weigert et al., 1999). Conversion of lysophosphatidic acid, an inverted cone-shaped lipid, to phosphatidic acid, a cone-shaped lipid, induces fission of membranes from the Golgi (Weigert et al., 1999) and plasma membrane (Schmidt et al., 1999). Since natural long chain ceramide does not have a bulky polar head group, it should be a cone-shaped lipid as phosphatidic acid. On the contrary, short chain ceramide is perhaps a cylindrical or inverted cone-shaped lipid because the length of the acyl group at sn-2 position is short. The presence of the cylindrical or inverted cone-shaped lipid at a high amount in Golgi membranes may inhibit the formation of tubules and/or vesicles, thereby inhibiting Golgi disassembly as well as protein traffic through the Golgi apparatus (Rosenwald and Pagano, 1993b). In contrast,
accumulation of natural long chain ceramides, cone-shaped lipids, may facilitate the formation of tubules and/or vesicles and cause the destabilization of the Golgi apparatus. In this context, it should be noted that short chain ceramides still inhibit BFA-induced Golgi disassembly even when the levels of long chain ceramides are increased. The quantitative balance between long chain and short chain ceramides may be crucial for the stability of the Golgi apparatus.

Although our results clearly demonstrated that short chain ceramides stabilize the Golgi apparatus, it should be noted that they are not normally present in cells. Therefore, the effect of long chain ceramides may be crucial for the stability of the Golgi apparatus.

Although our results clearly demonstrated that short chain ceramides stabilize the Golgi apparatus, it should be noted that they are not normally present in cells. Therefore, the effect of long chain ceramides may be crucial for the stability of the Golgi apparatus.

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