Nordihydroguaiaretic acid (NDGA) caused disassembly of the Golgi apparatus of NRK cells in a dose-, time-, and energy-dependent manner but not in a microtubule-dependent manner. In contrast to brefeldin A, NDGA did not cause release of β-COP, a component of Golgi-derived vesicles. However, NDGA-induced disassembly was blocked by AlF$_4^-$, an activator of the heterotrimeric but not the small GTP-binding proteins. In digitonin-permeabilized cells, guanosine 5'-3-O-(thio)triphosphate (GTP$_7$S) as well as AlF$_4^-$ blocked the NDGA-promoted disassembly of the Golgi apparatus, and G$_{βγ}$ (by subunits of heterotrimeric G proteins) reversed this effect. Our present results suggest the possible involvement of heterotrimeric G proteins in the organization of the Golgi apparatus.

The Golgi apparatus consists of stacks of cisternae with dilated rims and is located in the perinuclear region via association with the microtubule organizing center. Although the principle of the organization of the Golgi apparatus is not fully understood, recent progress has revealed that a common fusion machinery works for vesicular traffic and the formation of cisternae from vesiculated Golgi membranes. Warren and coworkers (1, 2) developed a cell-free system that reconstitutes the vesicular transport machinery works for vesicular traffic and the formation of cisternae from vesiculated Golgi membranes. Warren and coworkers (1, 2) developed a cell-free system that reconstitutes the vesicular transport machinery works for vesicular traffic and the formation of cisternae from vesiculated Golgi membranes. Warren and coworkers (1, 2) developed a cell-free system that reconstitutes the vesicular transport machinery works for vesicular traffic and the formation of cisternae from vesiculated Golgi membranes. Warren and coworkers (1, 2) developed a cell-free system that reconstitutes the vesicular transport machinery works for vesicular traffic and the formation of cisternae from vesiculated Golgi membranes. Warren and coworkers (1, 2)

Possible Involvement of Heterotrimeric G Proteins in the Organization of the Golgi Apparatus*

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§ The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, NSF receptor; NDGA, nordihydroguaiaretic acid; ER, endoplasmic reticulum; G$_{βγ}$, G$_{βγ}$ subunits of heterotrimeric G proteins; Man II, mannosidase II; IQ, ilimaquinone; GTP$_7$S, guanosine 5'-3-O-(thio)triphosphate; ATP$_7$S, adenosine 5'-3-O-(thio)triphosphate; AMPFPN, adenosine 5'-β,γ-imino triphosphate; CHAPS, 3-[N-morpholino]propanesulfonic acid; COP, cost protein; C$_2$-NBD-ceramide, 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocarbonyl)phosphoglycerine; WGA, wheat germ agglutinin.

evisiae, which mediates the fusion of ER membranes during karyogamy (9, 10). Since the structures of assembled Golgi cisternae by NSF and p97 are different from each other, it was postulated that the two proteins have different roles in the assembly of the Golgi apparatus (2). Malhotra and co-workers (11, 12) also discovered the involvement of NSF and p97 in the assembly of vesiculated Golgi membranes. They found that IQ causes disassembly of the Golgi apparatus to form small vesicles with 60–90 nm diameter (13) and reconstituted the assembly of Golgi stacks from vesiculated Golgi membranes (11). In the present study, we screened transport inhibitors using an intra-Golgi transport assay reconstituted in a cell-free system (16) and found that NDGA, known as a dual inhibitor for lipoxygenases and cyclooxygenase, inhibits the transport assay (17). A recent study using BHK-21 cells showed that NDGA, without affecting microtubules, blocks protein transport from the ER to the Golgi apparatus and from the Golgi apparatus to the plasma membrane and retards the brefeldin A-mediated retrograde transport of mannosidase II (Man II) from the Golgi apparatus to the ER (18).

In the present study, we examined the effect of NDGA on the Golgi apparatus in NRK cells. We found that NDGA causes reversible disassembly of the Golgi apparatus in a microtubule-independent manner. Interestingly, the effect of NDGA was markedly protected by AlF$_4^-$, an activator of the heterotrimeric but not the small molecular weight GTP-binding proteins (19, 20). Using digitonin-permeabilized cells, we showed that GTP$_7$S also blocks the NDGA-mediated disassembly of the Golgi apparatus. This effect of GTP$_7$S was reversed by the addition of G$_{βγ}$. Our present results raise the possibility that heterotrimeric G proteins are involved in the organization of the Golgi structure in cells.

EXPERIMENTAL PROCEDURES

Materials—NDGA was obtained from Biomol Research Laboratories Inc. and freshly dissolved in dimethyl sulfoxide before use. Cycloheximide, ACl$_3$, and NaF were obtained from Wako Chemicals. Digitonin was obtained from Merck. C$_2$-NBD-ceramide was obtained from Molecular Probes. Cholera toxin was purchased from List Biological Laboratories Inc. Pertussis toxin was purchased from Nakon Pharma. Plastic coverslips (Celldesk FL1) were purchased from Sumitomo Bakelite. A polyclonal antibody against the amino-terminal fragment (residues 1–100) of bovine G$_β$ was obtained from Upstate Biotechnology, Inc. Mono-
clonal antibodies against α-tubulin (clone DM1A), Man II (clone 53F3C3), and protein disulfide isomerase were purchased from BioMa-
kor, BAbCo, and Fuji Chemical Industry, respectively. Polyclonal anti-
53FC3), and protein disulfide isomerase were purchased from BioMa-

RESULTS
NDGA Causes Disassembly of the Golgi Apparatus in a Mi-
crotubule-independent Manner—NRK cells were incubated with NDGA at 37 °C for 30 min, and the distribution of the Golgi apparatus was analyzed with immunofluorescence micro-
copy using an antibody against Man II, a medial Golgi marker protein. As shown in Fig. 1, NDGA caused dispersion of Man II in a concentration-dependent manner. At 125–150 μM NDGA, the dispersed pattern of Man II was observed in more than 85% of cells. Microtubules play an important role in the perinuclear localization of the Golgi apparatus (25). However, the morphology of microtubules was not affected by NDGA up to 150 μM (Fig. 1). When the Golgi apparatus was stained with C6-NBD-ceramide (26) or rhodamine-conjugated WGA, the lat-
doubly stained with uranyl acetate and lead citrate and were observed
ethanol and were embedded in epoxy resin. Ultrathin sections were

BHK-21 cells (18). To ensure the blockade of protein synthesis, we added 20 μg/ml cycloheximide with NDGA throughout the experiments using intact cells. When cycloheximide was not added, the Golgi apparatus was disassembled in the same kinetics as that in the presence of cycloheximide (data not shown).

Electron microscopic analysis confirmed the results of the immunofluorescence study (Fig. 4). At 15 min after addition of NDGA, Golgi membranes were converted into aggregates of small vesicles and tubulovesicular structures. At 50 min, no aggregates of vesicles were observed in the perinuclear region in control cells; instead, small vesicles appeared to be dispersed
throughout the cytoplasm. Electron microscopic analysis also showed that no significant morphological changes occurred in mitochondria, the nucleus or the plasma membrane. A slight
dilation of the ER was observed, but this phenomenon was
generally observed when vesicular transport was inhibited.
These results suggest that NDGA specifically affects the Golgi apparatus and the vesicular transport pathways.

β-COP Is not Released by NDGA—Brefeldin A causes disassembly of the Golgi apparatus and promotes the retrograde transport of Golgi components to the ER (for a review, see Ref. 28). The effect by brefeldin A detectable within 30 s is the release of β-COP (29), a component of Golgi-derived transport vesicles (30–32). We therefore examined whether or not β-COP is rapidly released from the Golgi apparatus by NDGA. When disassembly of the Golgi apparatus started at 5 min after addition of NDGA, β-COP was still associated with it (data not shown). In addition, β-COP was co-localized with Man II-containing vesicles even after complete disassembly occurred (Fig. 5). These results suggest that the disassembly of the Golgi apparatus is not due to the release of coat proteins from the Golgi apparatus.

AlF₄⁻ Markedly Prevents the Disassembly of the Golgi Apparatus by NDGA—Recent studies revealed the involvement of heterotrimeric G proteins in the membrane traffic processes (for a review, see Ref. 33). We examined whether or not heterotrimeric G proteins are involved in the disassembly of the Golgi apparatus. For this purpose, NRK cells were preincubated with or without AlF₄⁻ (50 μM AlCl₃ and 30 mM NaF) at 37 °C for 10 min, and then NDGA was added at a final concentration of 125 μM, and incubation was continued for another 15 min. AlF₄⁻ is an activator of the heterotrimeric but not the small molecular weight G proteins (19, 20). It is believed that AlF₄⁻ acts as a mnemonic for the γ-phosphate and activates the GDP-bound form of Ga. As shown in Fig. 6, the preincubation of cells with AlF₄⁻ markedly blocked the disassembly of the Golgi apparatus by NDGA, whereas 50 μM AlCl₃ alone did not exhibit such an effect. When AlF₄⁻ and NDGA were simultaneously added, some protective effect was detected but not as remarkable compared with the cells preincubated with AlF₄⁻ (data not shown). These results raise the possibility that heterotrimeric G proteins are involved in the disassembly of the Golgi apparatus by NDGA. When 150 μM NDGA was added, significant disassembly of the Golgi apparatus by NDGA, whereas 50 μM AlCl₃ alone did not exhibit such an effect. When AlF₄⁻ and NDGA were simultaneously added, some protective effect was detected but not as remarkable compared with the cells preincubated with AlF₄⁻ (data not shown). These results raise the possibility that heterotrimeric G proteins are involved in the disassembly of the Golgi apparatus by NDGA. When 150 μM NDGA was added, significant disassembly of the Golgi apparatus was observed even when cells were preincubated with AlF₄⁻ (data not shown). This may suggest that NDGA and AlF₄⁻ interact in a competitive manner. Electron microscopic analyses confirmed that the Golgi apparatus was not disassembled by NDGA when cells were preincubated with AlF₄⁻ (Fig. 7).

GTPγS Blocks the NDGA-promoted Disassembly of the Golgi Apparatus in Digitonin-permeabilized Cells—To provide a more direct line of evidence for the involvement of heterotrimeric G proteins in the disassembly of the Golgi apparatus by
NDGA, we reconstituted the disassembly event in permeabilized NRK cells and examined the effect of membrane-nonpermeable effectors for heterotrimeric G proteins. Digitonin was used for permeabilization because it selectively permeabilizes the cholesterol-rich plasma membrane but not other organelle membranes. This selectivity has allowed the reconstitution of a variety of membrane traffic events (34–36).

We permeabilized NRK cells with 40 mg/ml digitonin at 0 °C for 10 min because under this condition the transport of vesicular stomatitis virus-encoded protein from the ER to the Golgi apparatus could be reconstituted by the addition of an ATP-regenerating system and cytosol (36). When permeabilized NRK cells were incubated with 60 μM NDGA in the absence of an ATP-regenerating system and cytosol at 37 °C for 10 min, and then incubated without (A) or with 125 μM NDGA (B-D) in the presence of AlCl₃ (C) or AlF₄⁻ (D) for 15 min. Man II was immunostained. Bar represents 20 μm.

We permeabilized NRK cells with 40 μg/ml digitonin at 0 °C for 10 min because under this condition the transport of vesicular stomatitis virus-encoded protein from the ER to the Golgi apparatus could be reconstituted by the addition of an ATP-regenerating system and cytosol (36). When permeabilized NRK cells were incubated with 60 μM NDGA in the absence of an ATP-regenerating system or cytosol at 32 °C for 40 min, the Golgi apparatus was considerably dispersed (Fig. 8D). It is not certain whether or not this dispersion is relevant to the disassembly of the Golgi apparatus in intact cells because ATP is required in the latter case. When an ATP-regenerating system was included in the assay, the Golgi apparatus was not significantly disassembled by NDGA (Fig. 8C). When permeabilized cells were incubated with 60 μM NDGA in the presence of an ATP-regenerating system and bovine brain cytosol, the Golgi apparatus became dispersed throughout the cells (Fig. 8D). The dispersion was remarkably suppressed when permeabilized cells were incubated in the presence of an ATP-depleting system and cytosol (data not shown), suggesting the requirement of ATP hydrolysis for this process. The dispersion pattern of the Golgi apparatus in the presence of an ATP-regenerating system and cytosol was somewhat different from that in intact cells and rather similar to the ER pattern. Double staining revealed that the distribution of Man II significantly, but not completely, coincides with that of protein disulfide isomerase, an ER marker protein, suggesting that a part of Man II is redistributed to the ER (data not shown). This redistribution of...
The disassembly of the Golgi apparatus was significantly suppressed when the assay mixture contained 0.014% CHAPS, which was derived from a solution containing GTPγS (data not shown). In this case, the assay mixture contained 0.014% CHAPS, which was derived from a solution containing GTPγS. Bar represents 20 μm.

β-COP might be related to the retrograde transport of Man II to the ER. At present we cannot reconstitute the disassembly of the Golgi apparatus without the redistribution of Man II to the ER. However, the dispersion of the Golgi apparatus in permeabilized cells was also blocked by AlF4− (data not shown), suggesting that the initial stage of the disassembly is relevant to that in intact cells. The requirement of a lower concentration of NDGA for the disassembly of the Golgi apparatus may reflect the fact that NDGA was partly adsorbed to serum components in the case of intact cells. Indeed, 60 μM NDGA was enough for complete disassembly of the Golgi apparatus when intact cells were incubated without fetal calf serum.

When 20 μM GTPγS was added in the complete mixture containing an ATP-regenerating system, cytosol, and 60 μM NDGA, the disassembly of the Golgi apparatus was blocked (Fig. 8E). A similar result was observed when the concentration of GTPγS was 2 μM (data not shown). In contrast, addition of 20 μM AMP-PNP (Fig. 8F) or 2 μM ATPγS (data not shown) showed little effect. It should be noted that the concentration of ATP in the assay mixture is 50 μM. When 20 μM ATPγS was added, the disassembly of the Golgi apparatus was significantly suppressed. AMP-PNP might be less effective than ATPγS with respect to the inhibition of ATP hydrolysis. These results suggest that the activation of GTP-binding proteins blocks the NDGA-induced disassembly of the Golgi apparatus.

Effect of βγ Subunits of Heterotrimeric G Proteins—To exclude the possibility that small GTP-binding proteins are involved in the NDGA-promoted Golgi disassembly in permeabilized cells, we next examined the effect of βγ subunits of heterotrimeric G proteins on the disassembly of the Golgi apparatus. Gβγ bind to Go but not to ras-related small GTP-binding proteins (37, 38). In the resting state, the GDP-bound form of Go is associated with Gβγ. When the bound GDP is replaced with GTP, Go is activated and released from Gβγ. When excess Gβγ are present, Go is associated with them, which results in the inactivation of Go. If this scheme is also applicable to the case for the disassembly of the Golgi apparatus, it is expected that the addition of Gβγ would prevent the effect of GTPγS and cause disassembly of the Golgi apparatus. As shown in Fig. 9D, addition of 1 μM detergent-free Gβγ to the assay mixture containing GTPγS resulted in disassembly of the Golgi apparatus. In contrast, addition of buffer alone had no effect (data not shown). Furthermore, Gβγ alone did not significantly cause disassembly of the Golgi apparatus in the absence of NDGA (Fig. 9E). Similar results were obtained when 180 nM Gβ1γ2 were used instead of Gβγ (data not shown). In this case, the assay mixture contained 0.014% CHAPS, which was derived from a solution containing Gβ1γ2. This concentration of CHAPS without Gβ1γ2 had no significant effect on the Golgi morphology (data not shown).

Effect of Pertussis and Cholera Toxins—Pertussis and cholera toxins are useful tools for determining which type of heterotrimeric G proteins are involved in signal transduction (39, 40). Pertussis toxin catalyzes the ADP-ribosylation of α-subunit of Gi, which results in the uncoupling of the Gi from activation. Cholera toxin, on the other hand, catalyzes the ADP-ribosylation of α-subunit of Gs, which results in constitutive activation of the Gs. We next examined the effect of pertussis and cholera toxins on the NDGA-promoted disassembly of the Golgi apparatus. When pertussis toxin- or cholera toxin-treated cells were incubated with 60 μM NDGA, the Golgi apparatus was disassembled by NDGA as in the case of nontreated cells (data not shown), suggesting that heterotrimeric G proteins involved in the process of the disassembly are different from pertussis toxin- or cholera toxin-sensitive ones.
DISCUSSION

In the first half of this study, we showed that NDGA causes disassembly of the Golgi apparatus of NRK cells in a dose-, time-, and energy-dependent manner. Ikehara et al. at Fukuoka University also obtained similar results. A kinetic analysis using electron microscopy revealed that the Golgi apparatus is first converted to aggregates of small vesicles and then dispersed. Although microtubules were not disassembled by NDGA, disassembled Golgi vesicles seemed to be dispersed throughout the cytoplasm. In contrast to brefeldin A (29), NDGA did not promote the rapid release of β-COP, a component of COP-coated vesicles (30–32). Since no significant morphological changes were observed in mitochondria, the nucleus, or the plasma membrane, it is obvious that NDGA interacts with a limited number of components involved in the organization of the Golgi apparatus.

Malhotra and co-workers (13, 41) reported that IQ causes the Golgi apparatus to break down. There are several differences in the effect of the two compounds. First, NDGA strongly inhibits protein synthesis (18), whereas IQ does not (13). Second, NDGA does not disassemble microtubules, whereas IQ does (41). However, the disassembly of the Golgi apparatus by IQ is not due to this effect because the disassembly occurs even when microtubules are stabilized by Taxol (41). Third, NDGA causes disassembly of the Golgi apparatus in a limited number of cell lines. No remarkable disassembly of the Golgi apparatus was observed when BHK-21, PC12, COS7, HeLa, Chinese hamster ovary or L-cells were incubated with 100–125 μM NDGA (Ref. 18 and data not shown). The target molecules for NDGA may have different sensitivities to the compound in different cell lines. On the other hand, IQ causes the Golgi apparatus to break down in a wide variety of cell lines (13).

Despite these differences, the fundamental mechanism of the disassembly of the Golgi apparatus appears to be quite similar between NDGA and IQ. IQ causes disassembly of the Golgi apparatus, but not the nuclear envelope, in a time- and energy-dependent manner. The Golgi apparatus is converted to aggregates of small vesicles and tubulovesicular structures and then dispersed throughout the cytoplasm. β-COP is not rapidly released by IQ. The similarities in the effects of IQ and NDGA strongly suggest that their target molecules are the same or, as described below, one of the components in the heterotrimeric G protein-signal transduction pathway.

In the latter half of this study, using intact and digitonin-permeabilized NRK cells, we obtained several lines of evidence that heterotrimeric G proteins are involved in the NDGA-promoted disassembly of the Golgi apparatus. First, ALF₄⁻ markedly prevented this disassembly in intact cells and digitonin-permeabilized cells. Second, GTPγS had the same effect in digitonin-permeabilized cells. Third and most convincing, Gβγ suppressed the effect of GTPγS. These results strongly suggest that NDGA causes the disassembly of the Golgi apparatus via a pathway involving heterotrimeric G proteins.

Recent data indicate that heterotrimeric G proteins are present on the Golgi apparatus and regulate vesicular transport. The first suggestion came from the finding that ALF₄⁻ inhibits intra-Golgi protein transport in a cell-free system (14). A later study revealed that Goₛ₃ is present on the Golgi apparatus and regulates the secretion of heparan sulfate proteoglycan in LLC-PK₁ cells (42). In MDCK cells, Gₛ and Gₐ regulate the apical and basolateral transport pathways, respectively (43). In PC12 cells, the formation of large dense core vesicles is inhibited by Gₛ and stimulated by Gₐ (44). Denker et al. (45) recently demonstrated that Goₛ₁, Gₛ, and Goₛ₃₁₁ are present on the Golgi apparatus. Our present finding points to a new role of heterotrimeric G proteins: regulation of the organization of the Golgi apparatus.

We previously suggested that the target molecules for NDGA may be phospholipase A₂ (17). This conclusion was based on the observation that phospholipase A₂ inhibitors, such as unsaturated fatty acids and analogues of arachidonic acid including NDGA, inhibit intra-Golgi protein transport (17). If this were true, the present results could be explained as follows. The structure of the Golgi apparatus may be maintained by phospholipase A₂, which is downstream in heterotrimeric G protein-signaling. Therefore, inhibition of this activity by NDGA would cause disassembly of the Golgi apparatus. Activators for heterotrimeric G proteins through transducing signals may somehow enhance the activity of the inhibited phospholipase A₂ and block the disassembly of the Golgi apparatus. We examined whether the addition of arachidonic acid or hog pancreas phospholipase A₂ reverses the effects of NDGA in digitonin-permeabilized cells. Either molecule failed to prevent the disassembly of the Golgi apparatus by NDGA (data not shown). Therefore, we do not prefer this possibility. Another possibility in our mind is that NDGA may directly interact with heterotrimeric G proteins, which are insensitive to pertussis and cholera toxins. This is suggested by the finding that NDGA and ALF₄⁻ interact in a competitive manner. The heterotrimeric G proteins involved in the organization of the Golgi apparatus are most likely different from the one that regulates the association of β-COP with the Golgi apparatus (46) because the latter is known to be sensitive to pertussis toxin (47). The lack of the rapid release of β-COP from the Golgi apparatus by NDGA in intact cells is consistent with this idea. Recently, Gₛ, formally a member of the Gₛ family, was found to be inactivated by arachidonate and related unsaturated fatty acids (48). Interestingly, this protein is insensitive to pertussis toxin (49). Although Gₛ is predominately expressed in platelets and neurons, this does not necessarily mean that this protein is not expressed in other types of cells. NSF, which consists of general fusion machinery for vesicular transport in cells, is also predominantly expressed in neurons (50, 51).

How do heterotrimeric G proteins regulate the disassembly of the Golgi apparatus? One possible explanation is that heterotrimeric G proteins on the Golgi apparatus transduce signals in a way similar to those on the plasma membrane. In this scenario, signal molecules bind to receptors upstream of heterotrimeric G proteins. Heterotrimeric G proteins modulate enzyme activities in the signaling pathway, and finally the Golgi apparatus is disassembled. Another possibility is that heterotrimeric G proteins on the Golgi apparatus may transduce signals in a different way from that on the plasma membrane. This possibility is raised by the finding that Gβγ may not be on the Golgi apparatus (45). Since no signals were required for constitutive vesicular transport, heterotrimeric G proteins may be regulated by a mechanism other than by signals formed by the interaction between signal molecules and their receptors.

At the onset of mitosis, the Golgi apparatus is converted into small vesicles in both COP-I-dependent and -independent pathways (3, 4), and the vesiculated membranes are equally dispersed in the mitotic cytoplasm (52–54). This disassembly may be due to the cessation of budding of vesicles and the inhibition of their fusion (55). We do not know whether or not the mechanism of the NDGA-induced disassembly of the Golgi apparatus is exactly the same as that of the break down of the Golgi apparatus at the onset of mitosis. Further study is required to address this question.

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