Mepanipyrim, a Novel Inhibitor of Pharmacologically Induced Golgi Dispersion

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Received August 7, 2002; Accepted September 10, 2002

Mepanipyrim inhibited retrograde Golgi-to-ER trafficking induced by brefeldin A (BFA), nordihydroguaiaretic acid, clofibrate, and arachidonyl trifluoromethyl ketone in NRK and other types of cells, but did not inhibit anterograde trafficking of Golgi-resident proteins translocated to ER by BFA and newly synthesized VSV-G. However, mepanipyrim did not block the TGN38 dispersion induced by any of these compounds. Mepanipyrim acted on the Golgi, and swollen vesicular Golgi structures were formed and similar structures accumulated during rebuilding of the Golgi after BFA removal. These actions of mepanipyrim were readily reversed after its removal. Mepanipyrim did not stabilize microtubules, but prevented nocodazole-induced fragmentation and dispersion of the Golgi. These results suggest that the mepanipyrim-sensitive molecules participated in stabilizing the Golgi and its anchoring in the perinuclear region, and equally importantly, that the novel action of mepanipyrim may be used as a pharmacological tool for investigating membrane transport, Golgi membrane dynamics, and differentiation of the Golgi from TGN.

Key words: mepanipyrim; Golgi membrane dynamics; retrograde trafficking; microtubule; brefeldin A

The Golgi apparatus has a striking architecture in mammalian cells. Allied to this extraordinary architecture, the Golgi apparatus has a central role in the exocytic pathway.1 Many molecules are continually transported into and out of the Golgi, and thus the molecules that constitute this organelle are not in a static state.2 In addition, the Golgi breaks down at the onset of mitosis and, as cells exit from mitosis, the Golgi architecture seen in interphase cells is rebuilt.2-5 In marked contrast to the progress made in identifying molecules involved in traffic through the Golgi, only a very limited number of molecules have so far been identified that are involved in constructing this unique organelle. In addition, drugs are very limited that are useful in investigations of Golgi membrane dynamics.6

We have searched for inhibitors of intracellular trafficking. The Golgi apparatus plays pivotal functions in protein trafficking,1,2,6 and therefore it might be expected that drugs that act on the disassembly and/or rebuilding process of the Golgi should be concentrated among the inhibitors of protein trafficking. In the hope of finding drugs useful for studies on Golgi membrane dynamics, we have surveyed their action on the disassembly and rebuilding of the Golgi.

Mepanipyrim, a pyrimidinamine, has been developed as a fungicide and inhibits enzyme secretion in plant pathogens.7,8 Mepanipyrim inhibits the cell surface expression of vesicular stomatitis virus glycoprotein (VSV-G) and glucosylceramide in BHK cells.9 Until now, there has been little experimental evidence for its action mechanism. The principle of action of pyrimenthanil and CGA 219417, pyrimidinamine compounds structurally related to mepanipyrim, is assumed to be the inhibition of methionine biosynthesis.10,11 However, inhibition of virus multiplication by mepanipyrim could not be reversed in the presence of an excess amount of methionine (our unpublished results). In this report, we show that mepanipyrim blocked Golgi dispersion by inducers of retrograde trafficking such as BFA and microtubule-disrupting drugs such as nocodazole. However, mepanipyrim blocked neither the TGN dispersion induced by BFA or NDGA nor the anterograde trafficking of Golgi-resident proteins translocated to ER by BFA and de novo synthesized VSV-G.
This novel action of mepanipyrim may be useful for investigating membrane transport, Golgi membrane dynamics, and differentiation of the Golgi from TGN.

Materials and Methods

Antibodies and fluorescent probes. SlowFade and fluorescent secondary antibodies were purchased from Molecular Probes (Eugene, OR, U.S.A.). Fluorescent isothiocyanate-conjugated Helix pomatia lectin (FITC-HPL) was obtained from E-Y Laboratories (San Mateo, CA, U.S.A.). Monoclonal antibodies against ManII and α-tubulin were respective products of Berkeley Antibody Co. (Richmond, CA, U.S.A.) and Amersham International (Buckinghamshire, England). The monoclonal anti-TGN38 antibody was kindly presented by Dr. G. Banting (University of Bristol) or purchased from Oncogene Research Products (Boston, MA, U.S.A.). The polyclonal antibody against β-COP was kindly presented by Dr. M. Tagaya (Tokyo University of Pharmacy and Life Science). The polyclonal antibody against vesicular stomatitis glycoprotein (VSV-G) was raised in rabbits as previously described.13) Alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) was from Promega Co. (Madison, WI, U.S.A.).

Inhibitors. BFA was isolated from a culture of Penicillium sp.13) Mepanipyrim was obtained from Kumiai Chemical Industry Co. (Tokyo, Japan). PDMP (1,3-threo-1-phenyl-2-decanoylamino-3-morpholin-1-propanol) was purchased from Molecular Probes, and the other inhibitors were purchased from Sigma.

Cell culture and virus multiplication. NRK, COS-1, Swiss 3T3, NIH 3T3, HeLa S3, and CCD 25-K cells were grown in Dulbecco-modified Eagle’s medium (DME; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% (vol/vol) fetal calf serum, and BHK-21 cells in Eagle’s minimal essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% (vol/vol) calf serum. They were incubated at 37°C in a water-saturated atmosphere of 5% CO2/95% air. The propagation and use of the VSV New Jersey serotype were as previously described.15)

VSV-G protein transport assay. The effect of mepanipyrim on the trafficking of newly synthesized protein from ER was monitored by following the acquisition of endo-N-acetylglucosaminidase H (EndoH) resistance of VSV-G that accumulated in ER in BFA-treated cells after BFA removal. BHK-21 cells were transferred into 6-well plates 3 d before use, and grown to confluence. The cells were infected with VSV at 37°C for 2 h, washed to remove the un-adsorbed virus, and incubated for a further 2 h in the presence of 10 μg/ml of BFA. Infected cells were then incubated for a further 90 min in MEM containing 40 μg/ml of mepanipyrim and 10 μg/ml of cycloheximide in addition to BFA. The cells were washed three times with MEM to remove BFA, and supplemented with MEM containing 10 μg/ml of cycloheximide with or without 40 μg/ml of mepanipyrim. The cells were then incubated at 37°C, and cell lysates were prepared at designated time intervals. The cell lysates were treated or false-treated at 37°C for 2 h with EndoH (1000 U/ml; BioLabs) and analyzed by western blotting.

Fluorescence microscopy. Cells were grown on glass coverslips contained in 6-well plates (Corning), experiments being carried out 2 d after passaging. Immunocytochemical studies followed various incubation schemes with mepanipyrim, BFA, nocodazole and/or other drugs, the cells being washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 3% paraformaldehyde/PBS for 10 min at room temperature. The cells were then washed three times with PBS, before being treated with –20°C methanol for 10 min. Thereafter, the cells were washed three times with PBS and then incubated for 30 min with 1% (wt/vol) BSA/PBS. The fixed and permeabilized cells were incubated for 30 min at room temperature with the primary antibody diluted in 1% (wt/vol) BSA/PBS (anti-ManII, diluted 1:100; anti-α-tubulin, diluted 1:100; anti-TGN38, diluted 1:500; anti-β-COP, diluted 1:100), this being followed by three washes with PBS. Thereafter, the fixed cells were incubated for 30 min with the secondary antibody, Alexa 488 goat anti-mouse IgG (H+L) conjugate (1:100 dilution) or Alexa 488 goat anti-rabbit IgG (H+L) conjugate (1:100 dilution), this being followed by three washes with PBS. For FITC-HPL staining, the fixed cells were treated with 10-μg/ml of this probe for 30 min. Finally, coverslips were mounted with Slow Fade and viewed under an epifluorescence microscope (AX-70; Olympus, Tokyo, Japan). The images were photographed with a cooled CCD video camera and prepared for visualization by using Canvas 7.0.

Western blot analysis. VSV-infected BHK cell lysates were resolved by SDS-PAGE on 7.5% separating gel under reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked with 10% skim milk in 10 mm Tris-HCl at pH 8, 150 mm NaCl, and 0.05% Tween 20 for 1 h at 25°C, and then incubated with a 1:2000 dilution of the polyclonal anti-VSV-G antibody for 1 h. After washing, the membrane was incubated at 25°C with alkaline phosphatase-conjugated goat anti-rabbit antibody. Following this incubation, the membrane was washed three times, and the protein bands were localized by incubating in a 10 mm Tris-HCl buffer at pH
Inhibition of Golgi Dispersion by Mepanipyrim

9.5 containing 10 mM NaCl, 0.5 mM MgCl₂, 0.33 mg/ml of nitroblue tetrazolium, and 0.16 mg/ml of 5-bromo-4-chloro-3-imidouracil.

Results

Mepanipyrim inhibited BFA-induced retrograde trafficking of cis-medial Golgi-resident proteins to ER in NRK cells in a time- and dose-dependent fashion

BFA fragments the Golgi, and proteins located in Golgi cisternae are transported to and merge with ER. This redistribution of Golgi-resident proteins to ER occurred rapidly in less than 5 min after the addition of BFA to cultured NRK cells when the localization of ManII, a cis-medial Golgi-resident protein, was followed by immunofluorescence microscopy (Fig. 1A, b). This relocation of ManII to ER was blocked when NRK cells had been treated with mepanipyrim before the addition of BFA (Fig. 1A, d). Mepanipyrim fragments the Golgi (compare c with a in Fig. 1A). Therefore, the Golgi fragmentation (Fig. 1A, d) seems not to be a reflection of a partial progression of retrograde trafficking, but rather to an effect of mepanipyrim itself on the Golgi. The blockade of Golgi-to-ER retrograde trafficking of ManII continued for 20 to 30 min. After a longer period of incubation time or at a subop-
timal concentration of mepanipyrim, extended necklace-like structures, which are assumed to have been intermediates in retrograde trafficking of the Golgi membrane proteins, were observed (data not shown), suggesting that mepanipyrim blocked the early stage of the fragmentation and retrograde trafficking induced by BFA. The blocking of retrograde trafficking to ER was not restricted to ManII. HPL is a lectin that recognizes proteins having an oligosaccharide modified by processing enzymes resident in the cis Golgi, and retrograde trafficking of HPL-reacting proteins was also blocked (Fig. 1A, h). Fragmentation of the Golgi by mepanipyrim was observed in this case (Fig. 1A, g), as in ManII staining (Fig. 1A, c). Inhibition of BFA-induced retrograde trafficking by mepanipyrim was not restricted to NRK cells, and was also apparent with BHK-21, COS-1, HeLa S3, and CCD 25-K cells (data not shown).

The blocking of retrograde trafficking of Golgi-resident proteins to ER by mepanipyrim depended both on its concentration and period of pretreatment time with it. Mepanipyrim concentration dependently blocked retrograde trafficking, and marked and nearly complete blocking was observed with 20 and 40 \( \mu \text{g/ml} \) of mepanipyrim, respectively (Fig. 1B). The degree of blocking of BFA-induced retrograde trafficking was also greatly influenced by the period of time of the mepanipyrim treatment before the BFA treatment (Fig. 1C). After 5 min of BFA treatment, a 30-min mepanipyrim treatment was enough to block retrograde trafficking in more than 70\% of the cells. However, a 90- to 120-min pretreatment was required to maintain the blocking for more than 15 min. Therefore, the cells were pretreated with 40 \( \mu \text{g/ml} \) of mepanipyrim for 120 min in the subsequent experiments, except being otherwise specified.

Inhibition of retrograde Golgi-to-ER protein trafficking by mepanipyrim was not restricted to BFA induction

BFA inhibits the GDP/GTP exchange reaction on the ADP-ribosylation factor, a small G-protein required for transport vesicle formation, and dissociates \( \beta \)-COP from the Golgi. This dissociation is assumed to be required for the induction of retrograde trafficking. Retrograde trafficking from the Golgi is induced by other pharmacological compounds unrelated to BFA in their structure and activity such as NDGA, clofibrate, and AACOCF3. Their induction mechanism has not yet been clarified, but they do not dissociate \( \beta \)-COP from the Golgi membrane and, therefore, they may be unrelated to BFA in their induction mechanism. We therefore investigated whether mepanipyrim would also block the retrograde trafficking induced by these compounds. Mepanipyrim inhibited the Golgi dispersion induced by any of these compounds (data not shown), suggesting that mepanipyrim could act on an unidentified common step beneath the apparently different induction mechanism for these compounds.

Mepanipyrim blocked neither BFA-induced \( \beta \)-COP dissociation from the Golgi membrane nor its reassociation after BFA removal

As predicted from the observation that mepanipyrim blocked the Golgi dispersion induced by pharmacological compounds irrespective of their effect on \( \beta \)-COP association with the Golgi membrane, mepanipyrim affected neither BFA-induced \( \beta \)-COP dissociation with the Golgi membrane nor its reassociation after BFA removal. \( \beta \)-COP localized in the ManII-stained structure (Fig. 2, compare A with E) was dispersed throughout the cytoplasm (Fig. 2B) accompanying the BFA-induced disappearance of...
Fig. 3. Blocking of Retrograde Trafficking and Golgi Fragmentation by Mepanipyrim was Reversible.

(A) The reversibility of the inhibition by mepanipyrim of BFA-induced retrograde Golgi-to-ER trafficking was followed after its removal. NRK cells, which had been grown on cover-slips, were incubated at 37°C for 120 min in DME containing 40 μg/ml of mepanipyrim, and then rinsed with DME to wash out mepanipyrim. BFA (10 μg/ml) was added to these cells at the time of mepanipyrim removal (a and b), and 5 min (c and d) and 10 min (e) after its removal, and cells were fixed after 5 min (a, c, and e) and 10 min (b and d) of the BFA treatment. The fixed cells were processed for ManII-staining. Bar, 10 μm. (B) Rebuilding of the Golgi stack from vesicles formed in the mepanipyrim-treated NRK cells was followed after its removal. NRK cells, which had been grown on coverslips, were incubated at 37°C for 120 min in DME containing 40 μg/ml of mepanipyrim. After washing out mepanipyrim, they were further incubated at 37°C. The cells were fixed at the time of BFA removal (a and b), and 15 min (b) and 30 min (c) after its removal. The fixed cells were processed for ManII-staining. Bar, 10 μm.

Actions of mepanipyrim on the retrograde trafficking and Golgi structure were readily reversed after its removal

Reversibility of action is another important characteristic of a pharmacological probe to be useful in biochemical studies. We first addressed whether the blocking by mepanipyrim of BFA-induced retrograde trafficking was reversible or not. When BFA was added at 0 or 5 min after mepanipyrim removal, retrograde trafficking was completed in less than 10 min (Fig. 3A, b and d), even though it had been delayed and Golgi vesicles had been observed in the perinuclear region after 5 min of BFA treatment (Fig. 3A, a and c) when retrograde trafficking had been completed in the control cells not treated with mepanipyrim. ManII was translocated to ER in less than 5 min when BFA was added 10 min after the removal (Fig. 3A, e), as in the case of the control cells not treated with mepanipyrim. These results indicate that the sensitivity to BFA-induced retrograde trafficking recovered in less than 10 min after mepanipyrim removal.

Mepanipyrim disassembled the Golgi stacks into vesicles (Fig. 1A, c). This action of mepanipyrim was reversible, and the Golgi vesicles (Fig. 3B, a) progressed to extended, partly stacked tubulovesicles and then to stacks at 15 and 30 min after mepanipyrim removal, respectively (Fig. 3B, b and c). The stacked Golgi structure was rebuilt from the vesicles formed after BFA removal in the presence of mepanipyrim and, in addition, these vesicles were sensitive to BFA-induction of retrograde trafficking when mepanipyrim was removed, as described in the following section. These observations indicate that the mepanipyrim action was readily reversed after its removal.

Mepanipyrim inhibited neither BFA- nor NDGA-induced TGN38 dispersion

BFA induces merging of TGN with endosome/lysosome in addition to merging of cis and medial and possibly trans Golgi-resident proteins with ER.\(^{26}\) TGN38, compared to ManII, is relatively resistant to clofibrate-induced dispersion.\(^{24}\) Thus, while the Golgi proper and TGN are in close proximity, they can be pharmacologically dissected into distinct compartments, as pointed out by Chege and Pfeffer.\(^{26}\) To address whether mepanipyrim could dissect the Golgi and TGN, its effect on the pharmacological dispersion of TGN was monitored by TGN38 staining. Extensive necklace- or thread-like structures were observed after 10 min (Fig. 4A, b). A subset of TGN38 is resistant to BFA and NDGA and is associated with the centrosome throughout the cell cycle,\(^{27}\) and a cloud of TGN38 staining was observed in the perinuclear region even after 30 min of treatment (Fig. 4A, d). When the NRK cells had been treated for 120 min with mepanipyrim, TGN was swollen into vesicles in the perinuclear region (Fig. 4A, e) as well as the Golgi stained for ManII (Fig. 1A, c). The time-course characteristics for the morphological change of TGN38-stained organelles after the BFA treatment were similar between the control (Fig. 4A, b–d) and mepanipyrim-treated cells (Fig. 4A, f–h).
Mepanipyrim did not inhibit BFA- and NDGA-Induced Dispersal of TGN in NRK Cells.

(A) NRK cells, which had been grown on coverslips, were incubated for 120 min in DME without (a–d) or with (e–h) 40 μg/ml of mepanipyrim. For the PDMP treatment (i–l), the cells were incubated for 60 min in the presence of 100 μM PDMP. The cells were then incubated in the presence of 10 μg/ml of BFA, and the effect on TGN was followed at 10-min intervals by staining TGN38. Bar, 10 μm. (B) NRK cells, which had been grown on coverslips, were treated with the vehicle, DMSO (a), mepanipyrim (b) or PDMP (c), as in (A) and then with 200 μM NDGA. Images after 10 min of the NDGA treatment are shown. Bar, 10 μm.

NDGA induces movement of both Golgi and TGN proteins to ER.22) As in the case of BFA, mepanipyrim did not inhibit the NDGA action on TGN38 (Fig. 4B, b), even though a cloud of TGN38 staining remained in the perinuclear region, as it did in the control. Similar results were obtained with clofibrate and AACOF3 (data not shown).

PDMP blocks the pharmacologically induced dispersion of TGN38 as well as ManII.28) To certify the relative insensitivity to mepanipyrim of TGN38 dispersion by inducers of retrograde Golgi-to-ER trafficking, the effect of PDMP was examined at the same time. TGN38-stained vesicles were observed in the perinuclear region of PDMP-treated cells after 30 min of BFA (Fig. 4A, l) and 10 min of NDGA treatment (Fig. 4B, c). The PDMP effects examined at the same time confirmed that mepanipyrim did not protect TGN from dispersion by inducers of retrograde Golgi-to-ER trafficking and indicate that mepanipyrim dissected the Golgi and TGN with respect to their sensitivity to these pharmacological compounds. Although inducers of dispersion such as BFA and clofibrate dissect the Golgi and TGN, to our knowledge, mepanipyrim is the first to be found to dissect the two organelles among the inhibitors of Golgi dispersion.

Mepanipyrim did not inhibit anterograde trafficking of Golgi-resident proteins retrogradely translocated to ER and de novo synthesized proteins

The action of BFA was readily reversed upon its removal by washing, and Golgi resident proteins translocated to ER by the BFA treatment were reassembled into Golgi stacks after its removal in less than 1 h (Fig. 5A, c). The effect of mepanipyrim on the egress of retrogradely translocated Golgi-resident proteins from ER was analyzed by using the advantage of this reversible action of BFA. To differentiate anterograde trafficking of proteins retrogradely translocated to ER from that synthesized de novo in ER, cycloheximide was added to the cell culture before the induction of retrograde trafficking by BFA and was present throughout the experiment. An accumulation of ManII-stained vesicles in the cytoplasm and perinuclear region was detected 15 min after BFA removal (Fig. 5A, a). Golgi stacks began to be formed by 30 min (Fig. 5A, b), and were apparently completed at 45 min (Fig. 5A, c). Compared with these kinetics for Golgi stack rebuilding in the control, there was no apparent delay with the kinetics for vesicle formation in the perinuclear region of mepanipyrim-treated NRK cells (Fig. 5A, d and e). However, Golgi stacks had not formed after 45 min of incubation in the presence of mepanipyrim (Fig. 5A, f). The vesicular structures formed in the presence of mepanipyrim at this time of BFA removal were similar to those in the mepanipyrim-treated cells (Fig. 1A, c), suggesting that incomplete rebuilding of Golgi in the mepanipyrim-treated NRK cells after BFA removal was caused by the Golgi-disrupting action of mepanipyrim. ManII-stained vesicles were also stained with FITC-HPL upon double staining (data not shown), indicating that the similar kinetics for anterograde trafficking of Golgi proteins translocated to ER by BFA are general for Golgi proteins.

The next question we addressed was whether mepanipyrim would affect the anterograde trafficking of newly synthesized proteins. For this, the effect of mepanipyrim was analyzed with anterograde trafficking of VSV-G that had been synthesized de novo and accumulated in ER in the presence of BFA. The New Jersey serotype of VSV does not effectively multiply in NRK cells, and BHK-21 cells were used in this experiment. Mepanipyrim blocked BFA-induced retrograde trafficking of Golgi-resident proteins in BHK-21 cells as well as in NRK cells (data not shown).
Mepanipyrim did not inhibit anterograde trafficking of Golgi proteins that had translocated to ER by BFA and de novo synthesized VSV-G. (A) NRK cells, which had been grown on coverslips, were incubated in DME containing 10 μg/ml of BFA for 30 min. The cells were incubated at 37°C in the presence of BFA (a–c) or BFA and 40 μg/ml of mepanipyrim (d–f) for a further 90 min. The cells were then rinsed with prewarmed DME to wash out BFA, and incubated for a further 15, 30, or 45 min in DME containing the vehicle, DMSO (a–c) or mepanipyrim (d–f). The fixed cells were stained for ManII. Bar, 10 μm. (B) BHK-21 cells, which had been grown on coverslips, were infected with VSV, and incubated at 37°C for 120 min in MEM. The cells were then rinsed to remove unadsorbed VSV, and incubated at 37°C for a further 120 min in MEM containing 10 μg/ml of BFA. To the VSV-infected, BFA-treated BHK-21 cells was added the vehicle, DMSO (a) or 40 μg/ml of mepanipyrim (b), in addition to 10 μg/ml of cycloheximide. After a 90-min incubation at 37°C, the cells were rinsed with prewarmed MEM to wash out BFA, and refed with MEM containing 0.2% DMSO and cycloheximide (a) or mepanipyrim and cycloheximide (b). The cells were then fixed and processed for immunocytochemical staining for VSV-G at 15-min intervals after BFA removal for 45 min. Images 30 min after BFA removal are shown. Bar, 10 μm.

Golgi vesicles formed in the presence of mepanipyrim after BFA-removal were functional in oligosaccharide processing, BFA sensitivity, and Golgi stack rebuilding.

The next question we addressed was whether or not the vesicular Golgi structure formed after BFA removal in mepanipyrim-treated cells could recover its normal functions. Newly synthesized glycoproteins become EndoH-resistant when they are transported to the Golgi, a consequence of the processing of their oligosaccharide moiety by enzymes located in the Golgi. In many cells, glycoproteins that have been synthesized and retained in ER in BFA-treated cells become EndoH-resistant, because Golgi-resident oligosaccharide-processing enzymes merge with ER in BFA-treated cells and convert them to EndoH-resistant ones. However, the membrane glycoprotein of the VSV New Jersey serotype accumulated in ER of BHK-21 cells was not modified to an EndoH-resistant form in the presence of BFA. VSV-G retained in ER migrated faster than the control did (Fig. 6A, compare lane 3 with lane 1) by SDS-PAGE, and kept its EndoH-sensitivity for up to 7 h in the presence of BFA (Fig. 6A, lane 4). This property for VSV-G of the New Jersey serotype enabled us to follow the rebuilding of functional Golgi structures by monitoring the acquisition of EndoH-resistance. A portion of the control VSV-G retained its EndoH sensitivity even after prolonged incubation in the presence of cycloheximide (Fig. 6A, lane 2), because one of the two oligosaccharide chains of the VSV New Jersey serotype is heterogeneous in its EndoH-sensitivity. VSV-G oligosaccharide processing was monitored every 30 min from 0 to 90 min after BFA removal. No apparent change in the migration by SDS-PAGE was apparent during this period of time, irrespective of the mepanipyrim treatment (Fig. 6A, lanes 7–10). VSV-G migrated similarly to that synthesized during 7 h in the presence of BFA (Fig. 6A, lane 6) and faster than the mature type (Fig. 6A, lane 5), indicating that a 90-min incubation after BFA removal was not enough for VSV-G to mature. The EndoH-treatment made it easy to differentiate the mature control from immature VSV-G that had been synthesized in the presence of BFA, because the migration distance was substantially shown), but did not affect the kinetics of anterograde ER-to-perinuclear region trafficking of VSV-G when it was followed at 15-min time intervals up to 45 min after BFA removal. Vesicles stained for VSV-G were detected in the perinuclear region irrespective of the mepanipyrim treatment (Fig. 5B), as in the case of ManII-stained vesicles (Fig. 5A, a and d), indicating that mepanipyrim did not inhibit anterograde trafficking to the perinuclear region of both retrogradely translocated and de novo synthesized proteins.
Fig. 6. Golgi Vesicles Formed after BFA Removal in the Presence of Mepanipyrim were Functional.

(A) VSV-G oligosaccharide acquired EndoH-resistance with similar kinetics in the absence or presence of mepanipyrim. VSV infection and treatment with BFA, mepanipyrim, and/or cycloheximide were carried out as described in Fig. 5B unless otherwise specified. One set of infected cells was incubated for a long time to confirm that VSV-G did not acquire EndoH-resistance in the presence of BFA. For this purpose, VSV-infected BHK-21 cells were incubated for a long time in MEM containing the vehicle, DMSO or 10 μg/ml of BFA, throughout the experiment. To the cells was added 10 μg/ml of cycloheximide 5 h after the infection, and the cells were incubated for a further 120 min in the presence of cycloheximide (lanes 1 and 2) or both cycloheximide and BFA (lanes 3 and 4). VSV-G protein in the cell was analyzed by western blotting, using a polyclonal antibody against VSV-G after treatment (lanes 2 and 4) or false treatment (lanes 1 and 3) with EndoH. Cell-associated VSV-G in the control was stained weakly compared with that in the BFA-treated cells (compare lane 1 with lane 3), because a large part of VSV-G was incorporated into virus particles and released from the cell during the period of the cycloheximide treatment. Another set of VSV-infected cells was processed 0, 30, 60, and 90 min after BFA removal to follow the kinetics of EndoH-resistance acquisition. The results at 0 and 90 min in the presence (lanes 9, 10, 13, and 14) and absence (lanes 7, 8, 11, and 12) of mepanipyrim are shown. As controls, samples containing mature and ER-retained immature VSV-G used for lanes 1 and 3 were loaded into lanes 1 and 2, respectively. Arrowheads indicate a minor EndoH-sensitive VSV-G.

(B) Golgi vesicles formed after BFA removal were sensitive to the second BFA attack. NRK cells were treated as described in Fig. 5A to accumulate Golgi vesicles, and BFA was then added again 25 min after BFA removal. Mepanipyrim was removed just before the second BFA addition. Cells incubated in the absence of mepanipyrim were fixed at 3-min intervals from 0 to 15 min, and the mepanipyrim-treated cells at 10-min intervals from 0 to 50 min. The fixed cells were stained for ManII. Images at the indicated times are shown. Bar, 10 μm.

(C) Stacked Golgi structure was rebuilt from vesicles accumulated in the presence of mepanipyrim after BFA removal. NRK cells were treated as described in Fig. 5A. One set of cell cultures was then removed from mepanipyrim 25 min after the BFA removal, fixed at 15-min intervals after a further incubation at 37°C, and processed for ManII staining. Images at the indicated times are shown. Another set of cell cultures was incubated in the presence of mepanipyrim throughout the experiment after BFA removal to confirm that stacked Golgi was not formed in its presence (data not shown). Bar, 10 μm.
different in each case (Fig. 6A, compare lane 2 with lane 4). On the contrary, the EndoH-treatment of VSV-G synthesized in the presence of BFA did not result in any marked change in its behavior by SDS-PAGE (Fig. 6A, compare lane 3 with lane 4), and this fact made it difficult, in some cases, to differentiate the EndoH-sensitivity of immature VSV-G. However, VSV-G synthesized during 2 h in the presence of BFA was EndoH-sensitive and migrated faster than that not treated with EndoH (Fig. 6A, compare lane 7 or 10 with lane 11). We have no explanation for the observation that EndoH-treated VSV-G at 0 min showed another smear band indicated by the arrowhead (Fig. 6A, lanes 11 and 13). This band was observed with VSV-G at 60 min (data not shown) but not at 90 min after BFA removal, irrespective of the mepanipyrim treatment (Fig. 6A, lanes 12 and 14). Further characterization of the oligosaccharides of this VSV-G await the determination of their structure. VSV-G both in the presence and absence of mepanipyrim was EndoH-sensitive at 60 min after BFA removal (data not shown), but acquired EndoH-resistance at 90 min (Fig. 6A, compare lanes 11 and 13 with lanes 12 and 14, respectively). This acquisition of EndoH-resistance between 60 and 90 min is in relatively good accordance with the formation of apparently mature Golgi stacks 45 min after BFA removal in the absence of mepanipyrim (Fig. 5A, c). This similarity in the kinetics of EndoH-resistance acquisition in the presence and absence of mepanipyrim indicates that the aberrant Golgi formed in the presence of mepanipyrim is fully functional with respect to VSV-G oligosaccharide processing.

Golgi vesicles formed after BFA removal in the presence or absence of mepanipyrim were sensitive to BFA, and ManII was relocated to ER when BFA was added again (Fig. 6B). However, the intermediate vesicles seemed to be less sensitive to BFA-induced retrograde trafficking than intact Golgi, as indicated by the time required for BFA to induce retrograde trafficking to ER. Vesicular structures remained after 3 min of BFA treatment (Fig. 6B, b), by which time retrograde trafficking from the Golgi stack had been completed. Necklace-like structures were observed even at 6 min (Fig. 6B, c), and ManII merging with ER seems to have been incomplete even at 12 min, a bright, punctate stain being observed (Fig. 6B, d). Vesicles formed in the presence of mepanipyrim after BFA removal were resistant to the second attack of BFA when mepanipyrim was present throughout the experiment (data not shown). However, this blocking was reversed when mepanipyrim was removed (Fig. 6B, e–h), and ManII was substantially and completely translocated to ER at 20 min and 30 min, respectively (Fig. 6B, g and h). These observations indicate that the vesicles formed after BFA removal were sensitive to BFA, although less sensitive than intact Golgi.

Stacked Golgi structures were rebuilt after BFA removal (Fig. 5A, c), but vesicular structures accumulated in the presence of mepanipyrim (Fig. 5A, f). This may have been caused by mepanipyrim action to reversibly disintegrate the Golgi (Fig. 3B). We addressed whether the Golgi stacks were also rebuilt from the vesicles accumulated in the presence of mepanipyrim after BFA removal. The Golgi stacks were rebuilt in less than 60 min after mepanipyrim removal (Fig. 6C), and this time for Golgi stack rebuilding was not markedly different from that in NRK cells not treated with mepanipyrim after BFA removal (Fig. 5A, a–c). These observations indicate that the vesicles accumulated in the presence of mepanipyrim were functional in that stacked Golgi structures formed after mepanipyrim removal.

Mepanipyrim blocked Golgi dispersion by microtubule-depolymerizing drugs without preventing microtubule depolymerization

Microtubules have a crucial role in the organization of the Golgi. In addition, they participate in retrograde Golgi-to-ER trafficking, as indicated by the observations that nocodazole, a microtubule depolymerizing drug, inhibited BFA- and clofibrate-induced retrograde trafficking. Mepanipyrim disorganizes the Golgi and inhibits BFA-induced retrograde Golgi-to-ER trafficking, so the effect of mepanipyrim on microtubules and nocodazole action was evaluated.

Nocodazole disrupted microtubules (Fig. 7A, c). Mepanipyrim neither disrupted microtubules nor protected them from the action of nocodazole (Fig. 7A, b and d). Nocodazole fragmented the Golgi into small vesicles and dispersed them throughout the cytoplasm, but it did not induce their merging with ER (ref. 34 and Fig. 7A, c). Unexpectedly, after its action on nocodazole-induced depolymerization of microtubules, mepanipyrim protected the Golgi from further fragmentation and dispersion by nocodazole, and vesicles were retained in the perinuclear region (Fig. 7B, d). Such vesicles in the perinuclear region were not apparent when NRK cells had been treated with nocodazole and then with mepanipyrim (data not shown), ruling out the possibility that mepanipyrim tethered nocodazole-induced Golgi vesicles in the perinuclear region.

Mepanipyrim did not have a profound effect on BFA- or NDGA-induced relocation of TGN38 (Fig. 4). We investigated whether or not mepanipyrim could protect TGN from the action of nocodazole. Nocodazole fragmented TGN into vesicles and dispersed them throughout the cytoplasm (Fig. 7C, c) as well as the Golgi (Fig. 7B, c). Mepanipyrim induced swelling of TGN (Fig. 7C, b), but protected it from the action of nocodazole, and it
Fig. 7. Mepanipyrim Blocked Nocodazole-induced Fragmentation and Dispersion of the Golgi and TGN without Preventing Microtubule Depolymerization.

NRK cells, which had been grown on coverslips, were incubated at 37°C for 90 min in DME containing the vehicle, DMSO (a and c) or 40 μg/ml of mepanipyrim (b and d). To one set of cells (c and d) was added 10 μg/ml of nocodazole, and both sets were incubated for a further 60 min. They were fixed, and finally processed for immunocytochemical staining by using antibodies against α-tubulin (A), ManII (B), and TGN38 (C). Bar, 10 μm.

was retained in the perinuclear region (Fig. 7C, d) as well as the Golgi (Fig. 7B, d). Mepanipyrim protected both the Golgi and TGN from the action of colchicine and vinblastin, microtubule-depolymerizing drugs each having an action mechanism different from that of nocodazole (data not shown). Cumulatively, these results indicate that mepanipyrim protected both the Golgi and TGN from the action of microtubule-depolymerizing agents without preventing microtubule depolymerization.

Discussion

BFA, NDGA, clofibrate, and AACOCF3 induce retrograde protein trafficking from the Golgi to ER.14,21–25 They are unrelated to each other in their structure and reported action. Therefore, they might induce retrograde trafficking through different mechanisms, even though it remains a possibility that they induce it through the same mechanism while having apparently different actions on others. Mepanipyrim inhibited retrograde Golgi-to-ER trafficking induced by any of these compounds, suggesting that a mepanipyrim-sensitive common step or target is involved in their action or that mepanipyrim changes sensitivity to these compounds by modifying a cellular function.

Mepanipyrim did not markedly affect anterograde trafficking from ER of retrogradely translocated Golgi proteins and de novo synthesized VSV-G protein (Fig. 5), and the Golgi vesicles that accumulated in its presence were functional with respect to oligosaccharide processing, BFA sensitivity, and rebuilding Golgi stacks (Fig. 6). However, mepanipyrim disintegrated the Golgi and TGN scaffolds (Figs. 1A and 6A) and blocked pharmacologically induced retrograde Golgi-to-ER trafficking (data not shown). Mepanipyrim inhibits the egress of VSV-G and glucosylceramide from the Golgi and/or TGN.9 Therefore, the action of mepanipyrim seems to have been restricted to the Golgi and TGN anchored in the perinuclear region, even though TGN was less sensitive to mepanipyrim action.

Experimental evidence for the action mechanism of mepanipyrim is very limited. Mepanipyrim, CGA 219417, and pyrimenthanil are pyrimidinamine compounds, and the principle of their fungicidal action is assumed to be the inhibition of methionine biosynthesis.10,11 However, inhibition by mepanipyrim of both the multiplication of VSV and Newcastle disease virus in BHK cells and BFA-induced retrograde trafficking in NRK cells was not prevented in the presence of methionine up to 10 mM (our unpublished results). Our present work does not propose any clear model for the mechanism by which mepanipyrim stabilized the Golgi against pharmacologically induced fragmentation and dispersion. The blocking of nocodazole-induced Golgi dispersion seems to shed some insight into its mechanism of action.

The organization and dynamics of the Golgi architecture depend on microtubules, and nocodazole induced the depolymerization of microtubules and Golgi dispersion (ref. 30 and Fig. 7). However, mepanipyrim blocked nocodazole-induced fragmentation and dispersion of the Golgi without preventing microtubule depolymerization (Fig. 7). This observation indicates that polymerized microtubules per se would not be required to organize the Golgi in the perinuclear region and suggests that some cellular
components other than polymerized microtubules are crucial for this. One possible candidate is the centrosome. Compounds that block the Golgi dispersion induced by either BFA and nocodazole act on the centrosome and accelerate its microtubule-nucleating activity, while those that block only the before do not. Golph membranes are coordinated with cell-cycle progression and the Golgi is fragmented and dispersed at the onset of mitosis, but sub-populations of Golgi proteins such as golgin-97 associate with the centrosome, even in the mitotic phase. These centrosome-associated Golgi proteins might nucleate Golgi proteins into rebuilding the Golgi architecture after mitosis. Mepanipyrim accelerated the microtubule-nucleating activity of the centrosome and induced splitting of duplicated centrosomes (our unpublished results), suggesting that mepanipyrim acted on the centrosome. The next goal of our investigation is to clarify the centrosome function in the organization of the Golgi by using the novel action of mepanipyrim.

Acknowledgment

We thank Dr. Makoto Muroi for help throughout the present work, Dr. George Banuing for the monoclonal anti-TGN38 antibody, Dr. Mitsuo Tagaya for the polyclonal anti-β-COP antibody, Dr. Kaoru Sugawara for the cultured cell lines, and Dr. Gareth Griffiths for critically reading this manuscript. This work was financially supported in part by grants under the Bioarchitect Research Program and Multi-bioprobe Research Program from RIKEN.

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