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Membrane Tubule-mediated Reassembly and Maintenance of the Golgi Complex Is Disrupted by Phospholipase A_2 Antagonists

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Although membrane tubules can be found extending from, and associated with, the Golgi complex of eukaryotic cells, their physiological function has remained unclear. To gain insight into the biological significance of membrane tubules, we have developed methods for selectively preventing their formation. We show here that a broad range of phospholipase A_2 (PLA_2) antagonists not only arrest membrane tubule–mediated events that occur late in the assembly of the Golgi complex but also perturb its normal steady-state tubulovesicular architecture by inducing a reversible fragmentation into separate "mini-stacks." In addition, we show that these same compounds prevent the formation of membrane tubules from Golgi stacks in an in vitro reconstitution system. This in vitro assay was further used to demonstrate that the relevant PLA_2 activity originates from the cytoplasm. Taken together, these results demonstrate that Golgi membrane tubules, sensitive to potent and selective PLA_2 antagonists, mediate both late events in the reassembly of the Golgi complex and the dynamic maintenance of its steady-state architecture. In addition, they implicate a role for cytoplasmic PLA_2 enzymes in mediating these membrane trafficking events.

INTRODUCTION

In many cultured cells, the interphase Golgi complex forms a large interconnected organelle (for reviews see Farquhar and Palade, 1998; Lippincott-Schwartz *et al.*, 1998; Warren and Malhotra, 1998). However, upon entry into mitosis, the Golgi complex undergoes a dramatic disassembly process involving its fragmentation, vesiculation, and dispersal throughout the cytoplasm (Lowe *et al.*, 1998; Warren, 1993). Currently, our understanding of the mechanism(s) by which Golgi complexes disassemble during mitosis are unclear; however, two not mutually exclusive models have been postulated. In the first model, intact, interconnected Golgi complexes undergo a fragmentation process to yield clusters of vesiculated Golgi membranes (VGMs) which disperse throughout the cytoplasm (Shima *et al.*, 1998). The second "recycling" model, patterned on suggested similarities to the dispersal of Golgi membranes by microtubule depolymerizing compounds (Cole *et al.*, 1996), postulates that mitotic Golgi disassembly occurs as a result of the disruption of a normal, dynamic cycle of membrane trafficking between the Golgi complex and endoplasmic reticulum (ER) (Lippincott-Schwartz and Smith, 1997). In this model, Golgi membranes and proteins recycle back to the ER as a prerequisite to the forma-

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Abbreviations used: $AACOCF_3$, arachidonyl trifluoromethyl ketone; ACA, *N*-(*p*-amylcinnamoyl) anthranilic acid; ARA, aristolochic acid; BEL, E-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; BFA, brefeldin A; COP, coatomer protein; EM, electron microscopy; ER, endoplasmic reticulum; GF, gel filtration; IQ, ilimaquinone; lyso-PC, lysophosphatidylcholine; ManII, α-mannosidase II; MEM, minimal essential media; NEM, *N*-ethyl-maleimide; ONO-RS-082, 2-(*p*-amylcinnamoyl) amino-4-chlorbenzoic acid; $PACOCF_3$, palmitoyl trifluoromethyl ketone; PLA_2, phospholipase A_2; RT, room temperature; TGN, *trans*-Golgi network; VGM, vesiculated Golgi membrane; VSV-G, vesicular stomatitis virus G.

tion of dispersed vesicle clusters. As a result, the intact Golgi complex eventually disassembles. Regardless of the disassembly mechanism, in postmitotic cells, the clusters of VGMs undergo a step-wise reassembly process, forming fully functional and morphologically intact Golgi stacks (Lucocq *et al.*, 1989; Rabouille *et al.*, 1995b).

In addition to mitosis, a number of pharmacological reagents have been shown to reversibly disrupt Golgi morphology, thus providing model systems for studying the disassembly and reassembly of the Golgi complex (for review, see Denesvre and Malhotra, 1996). For example, the sea sponge metabolite ilimaquinone (IQ) induces the formation of VGMs that share many features with Golgi vesicle clusters produced during mitosis (Takizawa *et al.*, 1993; Veit *et al.*, 1993). Golgi complex disassembly has also been extensively studied using brefeldin A (BFA), a fungal metabolite that stimulates membrane tubule-mediated retrograde movement of Golgi membranes to the ER, but which blocks forward trafficking out of the ER, thereby resulting in the loss of the Golgi complex (Lippincott-Schwartz *et al.*, 1990; Klausner *et al.*, 1992). Thus, BFA-induced Golgi disassembly shares important features with the recycling model of Golgi disassembly during mitosis.

One of the more fascinating aspects of the Golgi complex is its ability to rapidly reassemble in postmitotic cells or during recovery from treatment with IQ or BFA. Although early events in reassembly from IQ or BFA may differ, in that reassembly from BFA requires the exit of resident Golgi membrane proteins from the ER but IQ may not, and some uncertainty remains as to which compound most precisely mimics mitotic breakdown and postmitotic reassembly, all three model systems, postmitotic cells or recovery from BFA or IQ, have been fruitfully used to study the reassembly process. Each in its own way reveals the inherent capacity of cells to assemble an intact Golgi complex.

Most studies on the molecular mechanisms involved in the formation of a Golgi complex have focused on very early events that mediate the fusion of mitotic Golgi vesicles into cisternal elements, or the formation of cisternae into small mini-stacks. For example, the similarity between mitotic reassembly and recovery from IQ treatment is underscored by the finding that two distinct ATP-dependent enzymes, *N*-ethyl-maleimide (NEM)-sensitive factor and p97, an NEM-sensitive homologue of NEM-sensitive factor, directly participate in the early fusion and reassembly events of both processes (Acharya *et al.*, 1995a,b; Rabouille *et al.*, 1995a). Although each of these ATPases may function at different steps and in ways that are as yet unclear, both appear to work in concert to promote the fusion of VGMs and the reassembly of flattened cisternal elements, and each appears to require the presence of the t-SNARE, syntaxin 5 (Acharya *et al.*, 1995a; Rabouille *et al.*, 1998).

Although late events in the Golgi reassembly process have not been elucidated at the molecular level, there are morphological similarities between Golgi recovery from BFA, IQ, or mitosis. For example, in all cases, mini-stacks of spatially separate Golgi cisternae congregate in the juxtanuclear region of the cell and are kinetic and structural precursors to the fully reformed, interconnected Golgi ribbon (Lucocq *et al.*, 1989; Alcalde *et al.*, 1992; Veit *et al.*, 1993; Acharya *et al.*, 1995a). What mediates this final coalescence step? Various lines of evidence suggest that Golgi membrane tubules may play a key role in this process. First, electron micrographs of the Golgi complex have revealed that numerous membrane tubules (generally 60–80 nm in diameter and up to several micrometers long) are frequently found to form bridges between otherwise spatially distinct stacks of cisternal elements (Novikoff *et al.*, 1971; Rambourg *et al.*, 1979; Rambourg and Clermont, 1990). Second, results from in vitro experiments in which mitotic Golgi membrane vesicles were incubated under conditions that allow for their step-wise assembly into morphologically intact stacked cisternal elements have suggested that Golgi-derived membrane tubules may play important roles in multiple assembly events (Rabouille *et al.*, 1995b). Finally, real-time imaging of fluorescently labeled Golgi complexes revealed the continuous formation of thin membrane tubules that appear to dynamically connect spatially separate Golgi stacks (Cooper *et al.*, 1990).

It is important to note that the membrane tubules forming bridges between cisternal stacks are but one type of tubule that emanates from Golgi membranes. Three other classes of tubules can be seen: those that extend and detach from nearly all regions of the stack (Sciaky *et al.*, 1997; Presley *et al.*, 1998), those that are derived from the *trans*-Golgi network (TGN) (Hirschberg *et al.*, 1998; Ladinsky *et al.*, 1994; Toomre *et al.*, 1998), and those forming between cisternae of a single stack (Cunningham *et al.*, 1966; Morré *et al.*, 1970; Weidman *et al.*, 1993; Morré and Keenan, 1994). It seems likely that in all four cases tubule numbers increase in the presence of BFA (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991; Sciaky *et al.*, 1997; Presley *et al.*, 1998). Tubules exiting from the Golgi stack may function in retrograde trafficking to the ER, whereas those exiting from the TGN appear to be involved in delivery to the plasma membrane (Hirschberg *et al.*, 1998; Toomre *et al.*, 1998). Although these three classes of tubules have different fates, they may have similar mechanisms of formation. For example, membrane tubules that detach from the either the Golgi stack or TGN appear to be facilitated by microtubules and associated motor proteins (Lippincott-Schwartz *et al.*, 1995; Hirschberg *et al.*, 1998; Toomre *et al.*, 1998), although in the case of tubules emanating from the stack, microtubules are not absolutely required (Wood

et al., 1991; Klausner *et al.*, 1992). Whether bridging membrane tubules use microtubules is not known. Nevertheless, the similarities between these classes of tubules, their morphology, stimulation by BFA, and facilitation by microtubules, suggests a common mechanism of formation.

To better understand the physiological function of Golgi membrane tubules, and to identify molecules or enzyme activities that participate in their formation, we have developed a cell-free reconstitution system that uses isolated rat liver Golgi complexes and requires a small subset of proteins obtained from a preparation of bovine brain cytosol (Banta *et al.*, 1995). These tubules were morphologically similar to those seen emanating from Golgi complexes under normal conditions and after stimulation by BFA. Characterization of this tubulation factor in vitro suggested that it functioned as an enzyme, rather than as a dynamic coat protein–like complex, such as clathrin or coatomer protein I (COPI). To obtain clues as to the nature of an enzymatic activity that could dramatically remodel biological membranes, we have recently used BFA stimulation in vivo as a model system to screen for enzyme-specific compounds that inhibit Golgi tubulation. The results showed that a broad spectrum of phospholipase A_2 (PLA_2) antagonists potently inhibited Golgi and TGN membrane tubulation by BFA (de Figueiredo *et al.*, 1998). In the course of these studies, we observed that the PLA_2 antagonists by themselves caused a reversible fragmentation of intact Golgi ribbons into spatially separate mini-stacks. These mini-stacks of Golgi cisternae were quite different from those produced by microtubule-depolymerizing drugs, such as nocodazole, in that they were larger and remained located in the juxtanuclear region, and microtubule morphology was unaffected. These results suggested that the PLA_2 antagonists were perturbing the dynamic formation of Golgi membrane tubules that are required for the steady-state maintenance of a fully interconnected Golgi complex. In this paper, we directly test this idea by closely examining the behavior of Golgi membranes in vivo during recovery from PLA_2 antagonist-induced fragmentation. Moreover, hypothesizing that late events in Golgi reassembly may involve similar tubular intermediates, we provide evidence here that a wide variety of PLA_2 antagonists potently inhibited the formation of membrane tubule-mediated interconnections between spatially distinct Golgi mini-stacks during recovery from IQ and BFA. Finally, to more directly test the idea that tubulation of Golgi membranes requires a cytoplasmic PLA_2 activity, we examined the effects of PLA_2 inhibitors on the cytosol-dependent tubulation of Golgi membranes in a cell-free reconstitution system and found that tubule formation was significantly inhibited by these same compounds. These data suggest that membrane tubules play important roles for the assembly and maintenance of the Golgi complex, and moreover, that a cytoplasmic PLA_2 enzyme activity is a critical player in these tubule-mediated events.

MATERIALS AND METHODS

Materials

Phospholipase and other inhibitors were obtained from the following sources: *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA), aristolochic acid (ARA), 2-(*p*-amylcinnamoyl) amino-4-chlorbenzoic acid (ONO-RS-082), arachidonyl trifluoromethyl ketone ($AACOCF_3$), palmitoyl trifluoromethyl ketone ($PACOCF_3$), 7,7-dimethyleicosadienoic acid, and E-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (or bromoenol lactone [BEL]) were from Biomol Research Laboratories (Plymouth Meeting, PA); IQ, originally a gift from V. Malhotra (University of California, San Diego, CA), was later obtained from Biomol Research Laboratories; quinacrine, *p*-bromophenacyl bromide (BPB), and all other common reagents were from Sigma (St. Louis, MO).

Cell Culture and Treatments

Clone 9 rat hepatocytes were maintained in minimal essential media (MEM) with 10% FBS and 1% penicillin/streptomycin at 37°C in an atmosphere of 95% air and 5% CO_2. The 8G5-H7 anti-vesicular stomatitis virus G (VSV-G)–producing hybridoma cells were grown in HT media with 10% NU-Serum (Life Technologies, Grand Island, NY). In a typical experiment, cells were plated onto glass coverslips (for immunofluorescence) or directly onto 35-mm dishes (for immunoperoxidase) and allowed to grow for 2 d before treatment with IQ, BFA, and/or PLA_2 inhibitors as described. Specific details of each experiment will be given in the figure legends. Each inhibitor was freshly prepared as a 1000-fold stock solution in organic solvent (either 100% ethanol or dimethylsulfoxide as appropriate) and then diluted into MEM just before use. To ensure that microtubule morphology was not disrupted under the various conditions tested, some experiments were performed in the presence of the microtubule stabilizing reagent paclitaxel (see below).

The ts045 temperature-sensitive mutant of VSV was propagated for 2 d in BHK21 cells grown as monolayers at 32°C, and the virus-containing culture supernatant was collected and stored at −80°C for later use. Trypan blue dye exclusion experiments were performed to examine the effects of various drugs on cell viability; we found no changes in viability when compared with untreated controls at any of the reported conditions.

Membrane Trafficking Assay

We used immunofluorescence to examine the effects of assorted PLA_2 inhibitors on the trafficking of the temperature-sensitive variant (ts045) of VSV-G membrane glycoprotein (Knipe *et al.*, 1977). To study ER-to-Golgi transport during various stages of Golgi reassembly, clone 9 hepatocytes were infected with ts045 VSV, incubated in normal growth media for 2 h at the restrictive temperature of 40°C to accumulate VSV-G in the ER, washed three times in serum-free MEM, and incubated in the presence of BFA (10 μg/ml) for 15 min at 40°C. Cells were then rapidly washed with MEM (40°C) and incubated in prewarmed (40°C) BFA-free media for various lengths of time in the presence or absence of assorted PLA_2 inhibitors to allow the step-wise reassembly of Golgi complex mini-stacks to occur but not the exit of VSV-G from the ER. Cells were then shifted to the permissive temperature (32°C) for various lengths of time to induce the synchronous anterograde movement of VSV-G protein from the ER to the reassembled Golgi mini-stacks. Finally, cells were processed for double-label immunofluorescence microscopy using

monoclonal anti-VSV-G antibodies and polyclonal anti-α-mannosidase II (ManII) antibodies.

Immunofluorescence and Immunoperoxidase Labeling for Electron Microscopy

Cells were processed for single or double immunofluorescence labeling (de Figueiredo *et al.*, 1998) or for immunoperoxidase labeling and electron microscopy (EM) (Brown and Farquhar, 1989) as described. Primary antibodies used in these studies were as follows: polyclonal ManII (from M.G. Farquhar, University of California, San Diego, CA; or K. Moreman, University of Georgia, Athens, GA); monoclonal anti-β-COP (from Affinity Bioreagents, Golden, CO); the anti-VSV producing hybridoma cell line (8G5-H7; from W. Balch, Scripps Research Institute, La Jolla, CA); and polyclonal antibodies directed against VSV-G membrane glycoprotein (from C. Machamer, Johns Hopkins University, Baltimore, MD). Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-rabbit immunoglobulin G Fab fragments coupled to horseradish peroxidase were from Biosys (Compiegne, France).

Preparation of Bovine Brain Cytosol and In Vitro Golgi Membrane Tubulation

Bovine brain cytosol and a Golgi-enriched fraction were prepared as previously described by, respectively, Banta *et al.* (1995) and Cluett and Brown (1992). In vitro Golgi membrane tubulation assays using a whole-mount EM-negative stain assay (Cluett *et al.*, 1993) were performed, and the extent of tubulation was quantified as previously described (Banta *et al.*, 1995). Briefly, a tubule was defined as a membranous extension that was 60–80 nm in diameter and at least three times as long. The percent tubulation was defined as the number of Golgi profiles that exhibited at least one tubule divided by the total number of Golgi profiles multiplied by 100. Generally, tubule-inducing conditions with control cytosol resulted in a maximum of ~50–70% of all whole-mount Golgi profiles exhibiting at least one membrane tubule. To compare results from different experiments and with different PLA_2 inhibitors (which have different solvent controls), results of the in vitro tubulation assays will be expressed as percent maximal tubulation (which is the percentage of experimentally treated whole-mount Golgi stacks exhibiting at least one membrane tubule divided by the percentage of Golgi stacks exhibiting tubules when incubated with active cytosol alone multiplied by 100). In all experiments, >50% of Golgi stacks contained tubules after incubation with control, active cytosol.

Immunogold Labeling of Negatively Stained Golgi Membranes

Golgi complexes were immunolabeled with polyclonal anti-ManII antibodies using a whole-mount, negative stain EM-immunogold protocol. Golgi-enriched fractions from rat liver were incubated in the presence of various concentrations of assorted combinations of buffer, cytosol, and PLA_2 inhibitors to induce or suppress membrane tubulation, as described above. Drops (10 μl) of these reaction mixtures were then placed on carbon- and Formvar-coated nickel grids for 15 min to allow attachment of Golgi complexes. Grids were then incubated in 0.5 ml of periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974) for 5 min at room temperature (RT) in a closed microfuge tube. Grids were then washed three times (10 min each) in buffer A (0.1% ovalbumin in PBS, pH 7.4) at RT and permeabilized (2 min) in 0.05% Triton X-100 in buffer A. The grids were washed again as above before being incubated overnight at 4°C in microfuge tubes containing 50 μl of anti-ManII antibody diluted 1:100 in buffer A. The next day, grids were washed and fixed (as above), washed again, and then incubated in goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles (diluted in buffer A) for 3 h at RT. The labeled whole-mount Golgi complexes on these grids were washed, fixed, washed again (as above), and then negatively stained for various lengths of time with 2% phosphotungstic acid, pH 7.2 (Cluett *et al.*, 1993), and analyzed by transmission EM.

Preparation of Fractions Enriched in Radiolabeled Golgi Complexes

Clone 9 cells, grown to confluence in two large tissue culture trays (225 mm^2), were incubated for 3 d in the presence of [^{3}H]choline (1 μCi/ml final) in MEM containing 10% Calf Supreme Supplemented (Sigma) to allow for the metabolic incorporation of the radiolabel into cellular phosphatidylcholine. The labeling media were aspirated, and the cells were washed (three times) with ice-cold serum-free media. The cells were then harvested by scraping with a rubber policeman (on ice) and centrifugation in a clinical centrifuge (5 min at 4°C). The pelleted cells were resuspended in 4 ml of ice-cold homogenization buffer (0.25 M sucrose, 10 mM Tris, and 1 mM EDTA, pH 7.4), and homogenized by serial passages through 21- and 23-gauge needles until a uniform suspension, as assessed by light microscopic visualization, was obtained. A postnuclear supernatant was prepared by centrifuging the homogenized material in a clinical centrifuge as above, and then Golgi complexes were enriched by subjecting the postnuclear supernatant to ultracentrifugation on a sucrose step gradient exactly as previously described (Cluett and Brown, 1992). The harvested, Golgi-enriched fraction was used in subsequent phospholipase assays.

Phospholipase Activity Assays

As a measure of the efficacy of PLA_2 inhibitor treatments, two separate assays were performed. In the first, the level of PLA_2 activity in cells was measured indirectly by determining the amount of [^{3}H]lysophosphatidylcholine (lyso-PC) present in total lipid extracts from control and treated cells as previously described (de Figueiredo *et al.*, 1998). In the second, the ability of bovine brain cytosol to hydrolyze [^{3}H]PC from Golgi-enriched fractions (prepared as above) was measured. These cytosol-dependent phospholipase assays were performed under conditions similar to those used in our whole-mount Golgi membrane tubulation assays. Briefly, in a typical experiment various combinations of svPLA2 (1 mg/ml final), bovine brain cytosol (1.5 mg/ml final), BEL (25 μM final), or control buffer were incubated (15 min at 37°C) in the presence of an equal volume (50 μl) of radiolabeled Golgi-enriched fractions, during which time esterified fatty acids were hydrolyzed from membrane phospholipids. The reaction was stopped, the components were extracted, and the products were resolved by TLC as previously described (de Figueiredo *et al.*, 1998). Finally, spots corresponding to PC and lyso-PC were excised and quantified by liquid scintillation counting as previously described (de Figueiredo *et al.*, 1998).

RESULTS

PLA_2 Antagonists Inhibit Golgi Reassembly after IQ and BFA Treatment

When rat clone 9 hepatocytes were incubated in the presence of IQ or BFA and then examined by immunofluorescence microscopy using anti-ManII antibodies, membranes of the juxtanuclear Golgi complex disassembled, resulting in both cases as a diffuse fluorescence pattern. (Figure 1, A–D). In the case of BFA, this disassembly resulted in the relocation of Golgi membranes to the ER (Lippincott-Schwartz *et al.*, 1990), and in the case of IQ, the formation of VGMs (Takizawa *et al.*, 1993) (Figure 2). However, we also

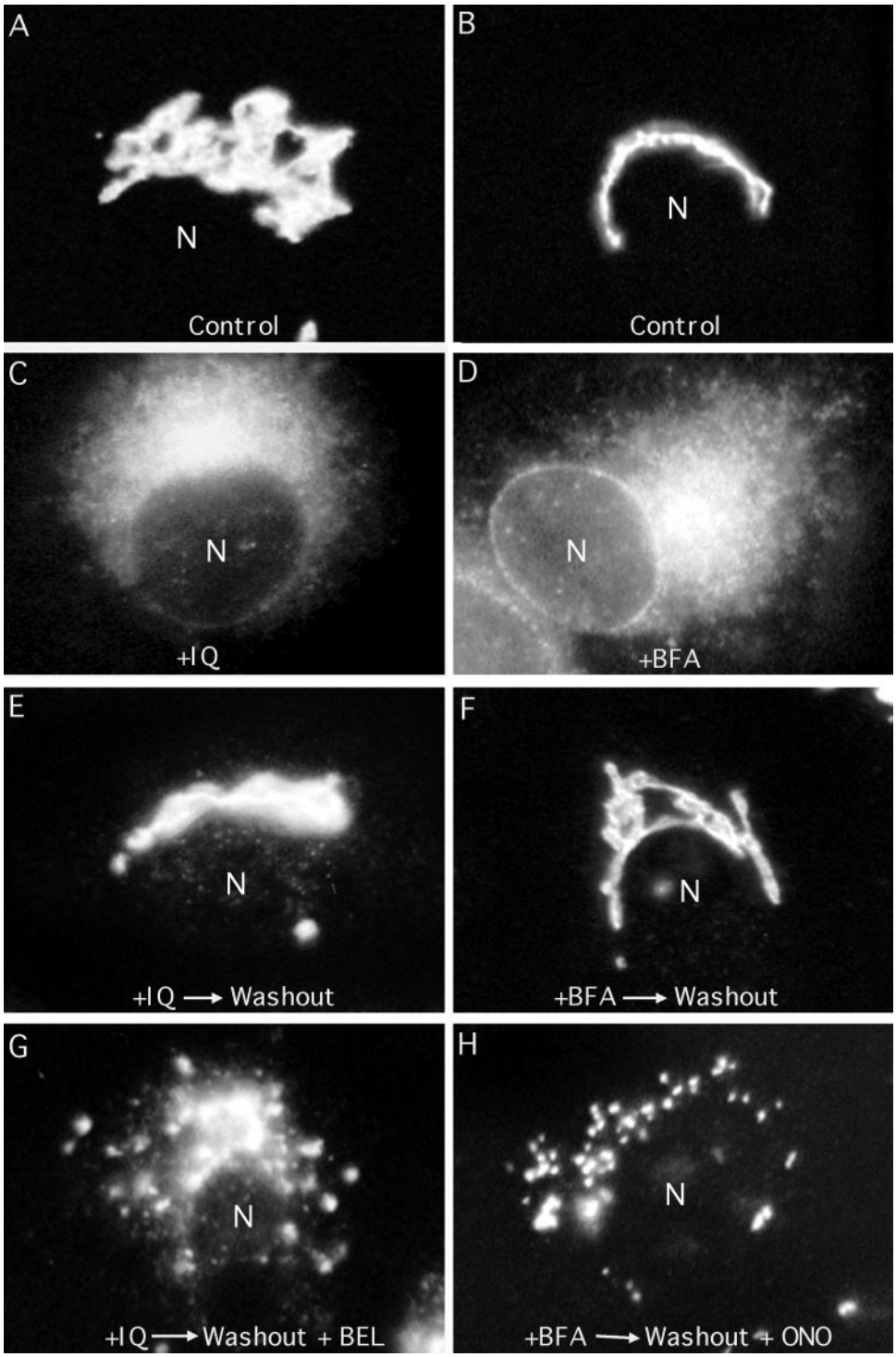


Figure 1. PLA_2 antagonists arrest the reassembly of the Golgi complex during recovery from IQ or BFA treatment as seen by immunofluorescence staining with the medial Golgi enzyme ManII. Control cells display a compact, interconnected Golgi complex in the juxtanuclear region of the cell (A and B). Treatment with either IQ (10 μM for 60 min) or BFA (5 μg/ml for 30 min) resulted in a diffuse Man II staining pattern (D). When IQ- or BFA-treated cells were then washed free of these drugs and incubated in normal media, Golgi complexes were fully reassembled within 1 h (E and F). However, when recovery from IQ or BFA for the same length of time was done in the presence of the PLA_2 inhibitors BEL (10 μM; G), or ONO-RS-082 (ONO, 1 μM; H), Golgi complexes reassembled only to the point of forming large, disconnected punctate structures in the juxtanuclear region of the cell. N, nucleus.

note that in our experience, IQ also induces some retrograde movement of ManII to the ER, as evidenced by faint nuclear envelope staining (Figure 1C). When similarly IQ- or BFA-treated cells were subsequently washed extensively and then incubated in drug-free media for various lengths of time, Golgi complexes reassembled in a step-wise manner, which included, at later stages, the formation of separate mini-stacks, seen as punctate staining located in the juxtanuclear region (Alcalde *et al.*, 1992). Within 60

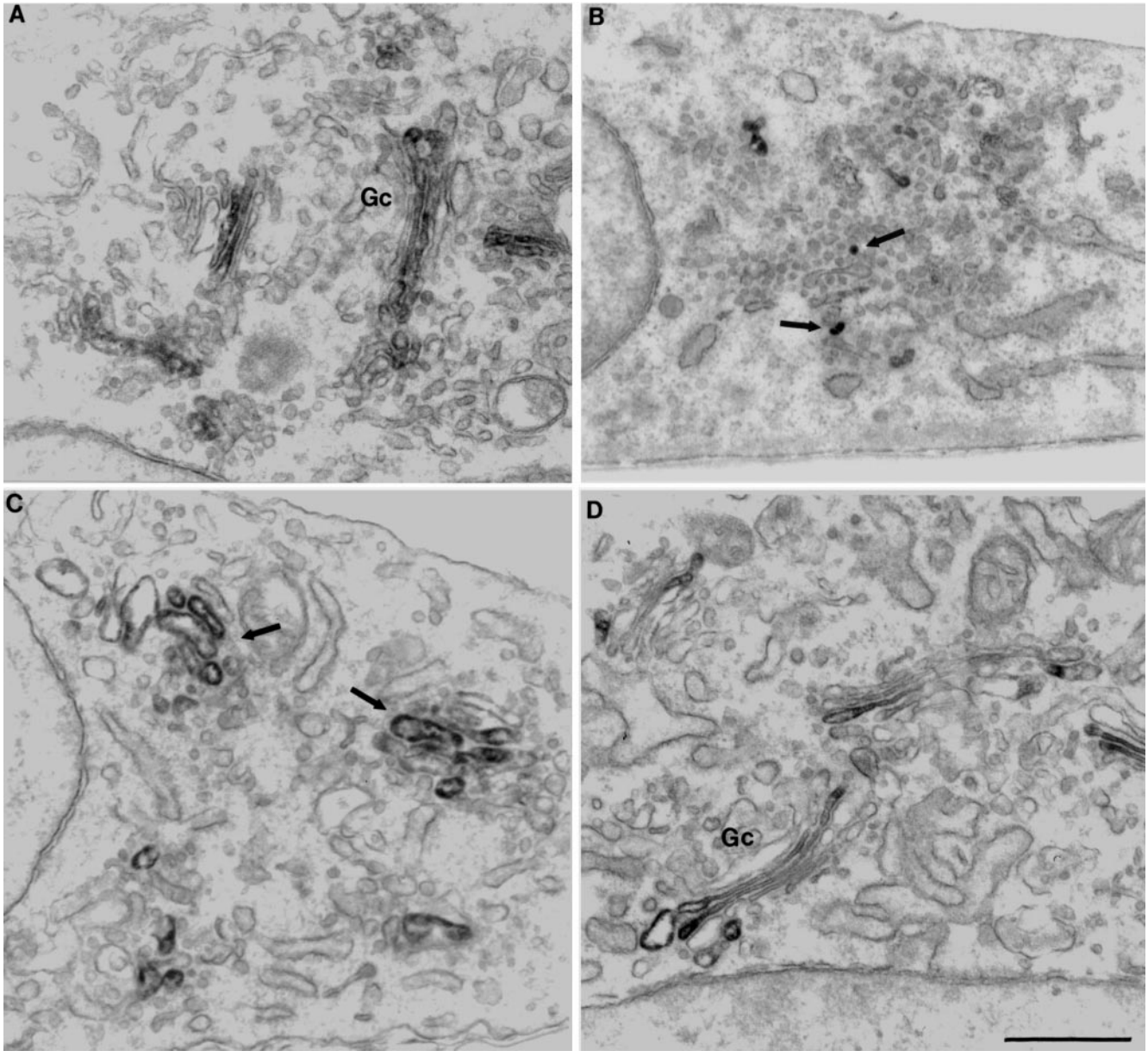


Figure 2. Immunoperoxidase localization of ManII during recovery from IQ treatment. (A) Control cells showing the typical stacked Golgi cisternae (Gc) with ManII labeling generally restricted to one or two medial cisternae. (B) Cells treated with IQ (10 μM for 60 min) reveal the formation of clusters of VGMs throughout the cytoplasm; within these clusters a few of the vesicles stain for ManII (arrows). (C) Cells treated with IQ as above and then washed and incubated without IQ but in the presence of the reversible PLA_2 inhibitor ONO-RS-082 (1 μM) for 1 h are capable of reassembling stacked Golgi cisternae that are labeled with ManII antibodies (arrows). (D) Cells treated as in C, but then washed free of ONO-RS-082 and incubated for 1 h, demonstrate that the Golgi complex further reassembles into a larger interconnected organelle with ManII staining more restricted to one or two cisternae. All micrographs were printed to the same final magnification. Bar, 0.5 μm.

min after removal of the drugs, these mini-stacks had coalesced into a fully recovered, interconnected ribbon of Golgi stacks (Figure 1, E and F).

In contrast, when IQ- or BFA-treated cells were washed and incubated for various lengths of time in the presence of BEL (at 10 μM) or ONO-RS-082 (1 μM), potent and selective irreversible (BEL) and reversible (ONO-RS-082) antagonists of intracellular PLA_2 activities (Banga *et al.*, 1989; Hazen *et al.*, 1991; Ackermann *et al.*, 1995), this reassembly process was dramatically arrested before complete coalescence of separate Golgi stacks into a fully formed organelle. A

time course of reassembly in the presence of these PLA_2 antagonists revealed that 60 min after IQ or BFA removal, ManII was found in collections of small (relative to intact Golgi ribbons) punctate structures that were clustered in the juxtanuclear region of the cytoplasm (Figure 1, G and H). Immuno-EM of ManII on cells after 60 min of recovery from IQ treatment in the presence of ONO-RS-082 revealed that the punctate structures seen by immunofluorescence were, in fact, stacks of three to five closely apposed Golgi cisternal elements (Figure 2C). The cisternae of these stacks appeared to be somewhat less flattened and elongated when compared with control cells (Figure 2A) or cells that were allowed to subsequently recover from the ONO-RS-082 treatment (Figure 2D). Nevertheless, based on the immunofluorescence and electron microscopic observations, we conclude that these mini-stacks represent a late stage in the reassembly of the intact Golgi complex, and moreover, that they are functionally capable of conducting membrane trafficking events (see below). Similar ultrastructural observations were made on cells that had recovered from BFA treatment and that had recovered in the presence of the irreversible inhibitor BEL (our unpublished data).

Using the immunofluorescence assay to follow recovery, we were able to quantify the effects of PLA_2 inhibitors on reassembly (Figure 3). A time course of Golgi reassembly from IQ in the absence of PLA_2 inhibitors revealed that after a lag phase of ~15 min, ≥90% of the cells recovered to exhibit fully intact Golgi complexes by 60 min (Figure 3A). However, in the presence of 5 μM ONO-RS-082, recovery was significantly slowed and in 25 μM recovery was almost completely inhibited. Using a fixed time point of recovery (60 min), dose–response curves for recovery from either BFA or IQ revealed that both BEL and ONO-RS-082 inhibited reassembly in the low micromolar range, and recovery from BFA was more sensitive to both inhibitors than was recovery from IQ (Figure 3B).

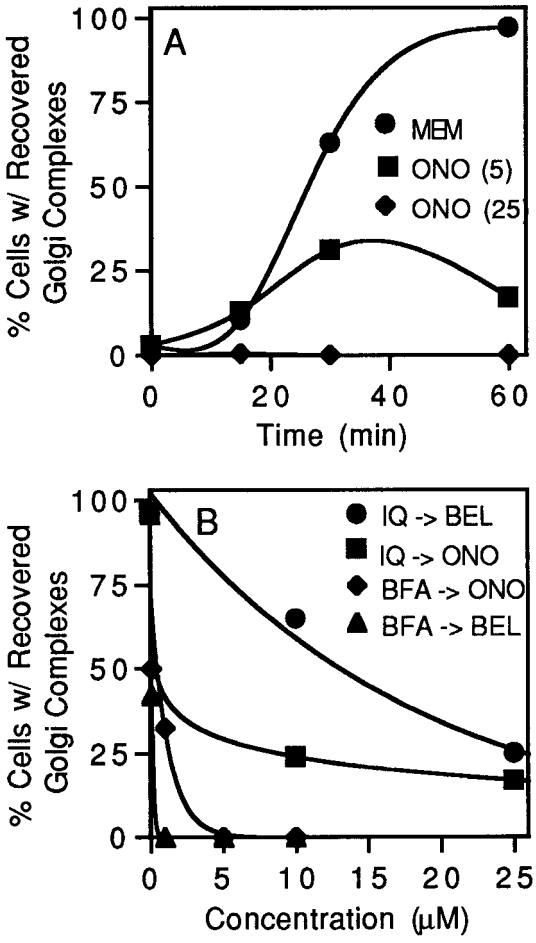


Figure 3. (A) Time course of Golgi reassembly during recovery from IQ treatment. Cells were first treated with IQ (10 μM for 60 min), washed to remove IQ, and then incubated in the absence or presence of the PLA_2 inhibitor ONO-RS-082 (ONO, 5 or 25 μM as indicated). Cells were fixed, processed for ManII immunofluorescence, and then counted to determine the percentage that had fully reassembled Golgi complexes (as illustrated by Figure 1, E and F). (B) Dose response of PLA_2 inhibitors on the reassembly of Golgi complexes during recovery from IQ or BFA treatment. Cells were treated with IQ or BFA as in Figure 1, washed free of the drugs, and incubated for 1 h with various concentrations of BEL or ONO-RS-082 as indicated.

PLA_2 Antagonists Do Not Perturb COPI Coatomer Localization

The COPI coatomer complexes play a key role in forming the coated vesicles that mediate trafficking within the Golgi complex and, in addition, are important for maintaining the structure of the Golgi complex (Dascher and Balch, 1994; Schekman and Orci, 1996). To investigate whether BEL and ONO-RS-082 were exerting their effects on Golgi reassembly by perturbing the localization of COPI coatomer proteins, we analyzed the location of β-COP, a protein constituent of the COPI coatomer complex. In BFA-treated cells, β-COP was found, as expected, diffusely throughout the cytoplasm, and ManII was in the ER (Figure 4, A and B). After washout of BFA, the Golgi rapidly reformed and colocalized to a large extent with β-COP (Figure 4, C and D). During recovery from BFA treatment in the presence of ONO-RS-082, β-COP was found associated with Golgi mini-stacks (Figure 4, E and F). These observations are consistent with previous work demonstrating that β-COP binding to isolated Golgi membranes in vitro was not affected by PLA_2 antagonists (de Figueiredo *et al.*, 1998) and suggest, moreover, that PLA_2 antagonist inhibition of Golgi reassembly does not arise because of failure of β-COP binding to membranes.

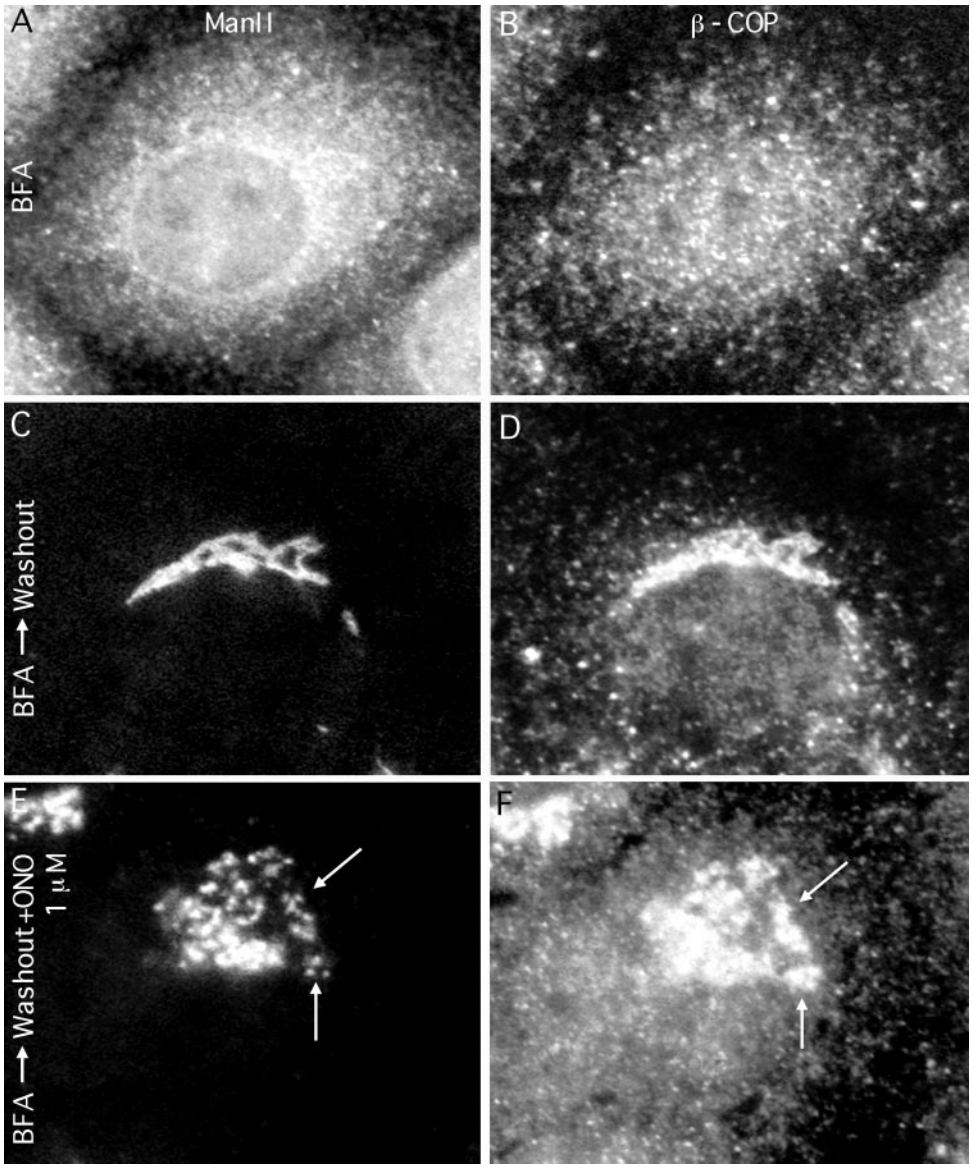


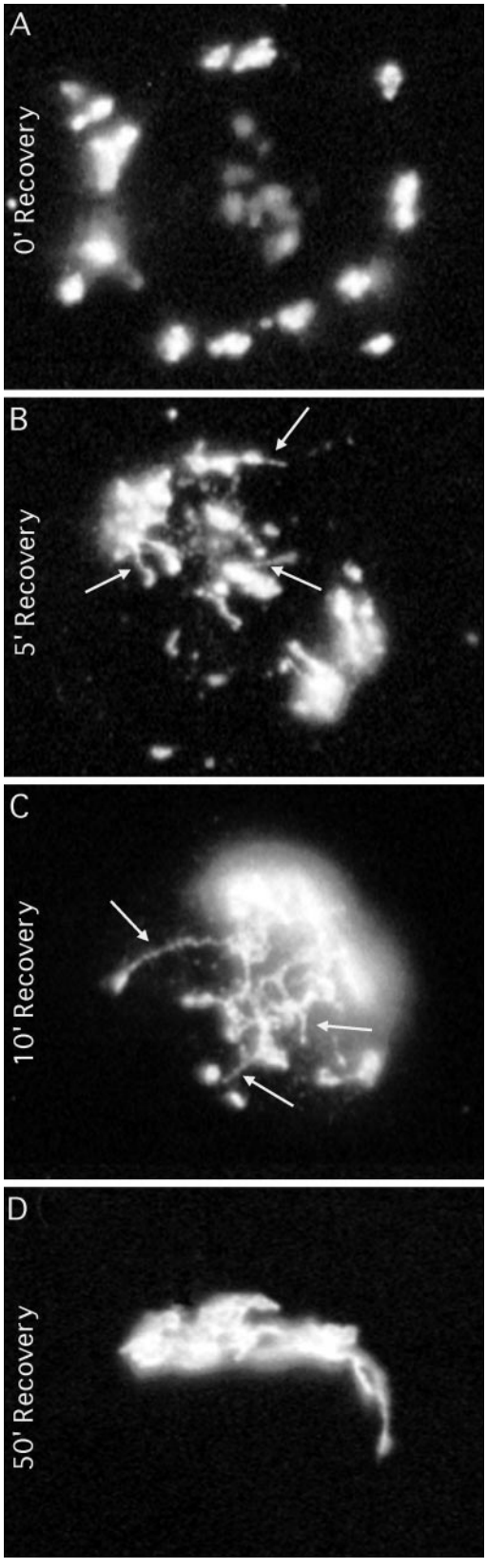
Figure 4. ONO-RS-082 inhibits Golgi reassembly but does not prevent the association of β-COP with Golgi membranes during recovery from BFA treatment. Each set of side-by-side panels shows double-immunofluorescent labeling of ManII (left panels) and β-COP (right panels). Cells treated with BFA (5 μg/ml for 15 min) show the typical diffuse immunofluorescence staining pattern of both ManII and β-COP (A and B). Treatment with BFA followed by a washout period of 1 h in drug-free media resulted in the reassembly of the Golgi complex and reassociation of β-COP with these membranes (C and D). Recovery from BFA in the presence of 1 μM ONO-RS-082 (ONO) resulted in the reformation of tightly clustered but unconnected mini-stacks in the juxtanuclear region to which β-COP was localized (E and F).

Golgi Membrane Tubules Mediate Mini-Stack Assembly into an Intact, Juxtanuclear Golgi Ribbon

To gain insight into the mechanisms by which mini-stacks reassembled into an intact Golgi complex, we observed the recovery process at early times after the removal of the reversible PLA_2 inhibitor ONO-RS-082. In these experiments, the Golgi complex was allowed to form mini-stacks by first washing BFA out of treated cells in the presence of ONO-RS-082 and then allowing cells to recover from ONO-RS-082 for various periods before being processed for immunofluorescence using anti-ManII antibodies (Figure 5). The results showed that within 5 min of recovery from ONO-RS-082, thin membrane tubules sprouted from many of the spatially separate mini-stacks (Figure 5B). By 10 min, many, if not most, of the mini-stacks were connected by numerous, thin membrane tubules (Figure 5C). Finally, by 50 min all of the separate mini-stacks had coalesced into an interconnected, intact Golgi complex (Figure 5D).

PLA_2 Antagonists Reveal Dynamic Tubule-mediated Connections between Golgi Stacks

Previous three-dimensional reconstruction of Golgi complex ultrastructure revealed the presence of thin membrane tubules that connected spatially separate cisternal stacks (Rambourg *et al.*, 1979; Rambourg and Clermont, 1990). Based on our results above, we wondered whether the tubules that formed during reas-

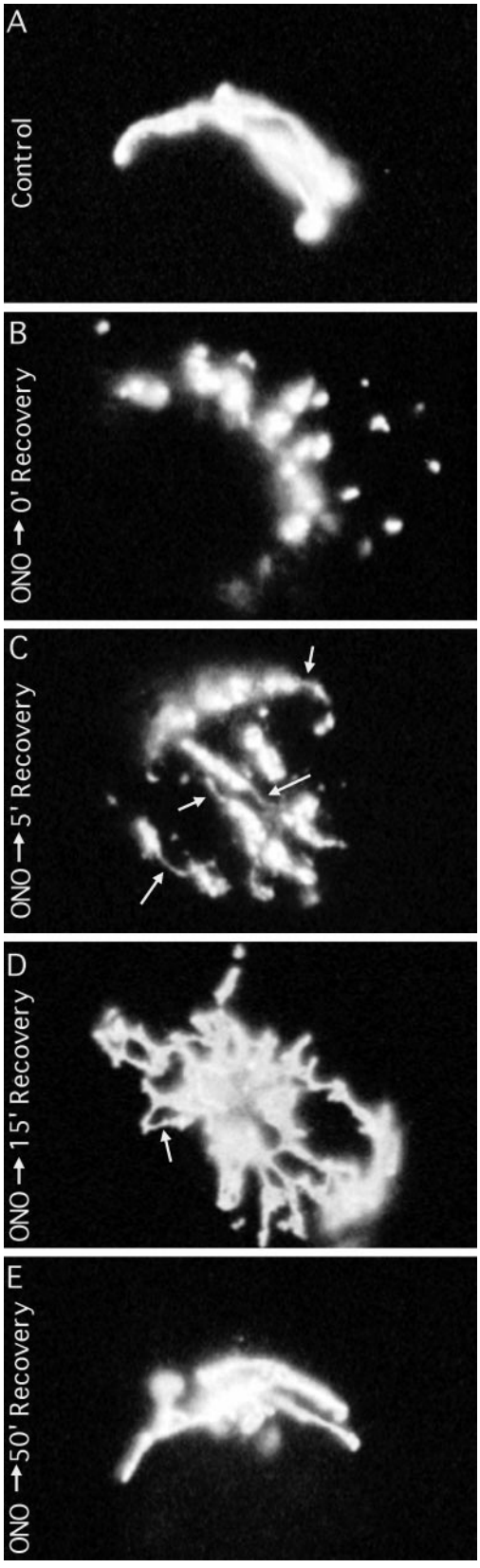


sembly might be similar to those that connect stacks at steady state. This question was stimulated by our recent finding that certain PLA_2 inhibitors, such as BEL, alone cause the interconnected Golgi complex to become fragmented into mini-stacks (de Figueiredo *et al.*, 1998). Indeed, treatment of cells with ONO-RS-082 (5 μM for 1 h) caused the intact Golgi to disassemble into smaller, disconnected fragments that nevertheless remained in the juxtanuclear region (Figure 6B). Immuno-EM revealed that these fragments were in fact composed of stacks of Golgi cisternae that were similar to those mini-stacks formed during recovery from IQ or BFA in the presence of PLA_2 inhibitors (Figure 2C). Recovery from ONO-RS-082 treatment alone was virtually identical to that which occurred after removal of PLA_2 inhibitors from BFA- or IQ-recovering cells (as in Figure 5). That is, thin membrane tubules emanated from spatially separate mini-stacks by 5 min of recovery (Figure 6C) and increased in number until virtually all mini-stacks were interconnected by tubules after 10 min of recovery (Figure 6D), and by 50 min an intact Golgi had reformed (Figure 6E). Additional observations during early (5–15 min) recovery from ONO-RS-082 treatment alone, or recovery from ONO-RS-082 arrest of reassembly after BFA washout (as in Figure 5), revealed thin, ManII-stained membrane tubules that formed bridges between the reforming central Golgi region and outlying mini-stacks (Figure 7). Also, it was not uncommon to find ManII-stained tubules extending from the central Golgi region that did not appear to connect with any outlying mini-stacks (Figure 7B).

Golgi Mini-Stacks Support ts045 VSV-G Trafficking from the ER

The observation that PLA_2 antagonists prevent tubule-mediated reassembly of the Golgi complex during recovery from BFA or IQ treatment might indicate that the separate mini-stacks are functionally defective in other membrane trafficking events. To address this

Figure 5. Membrane tubules form from Golgi mini-stacks after recovery from PLA_2 inhibitor arrest of Golgi reassembly. In these experiments, cells were treated with BFA (5 μg/ml for 30 min), washed free of BFA, and incubated with the reversible PLA_2 inhibitor ONO-RS-082 (5 μM) for 60 min to allow reassembly up to the point of mini-stack formation (A). Cells were then washed free of the inhibitor and allowed to recover in normal media for 5 min (B), 10 min (C), or 50 min (D). After each experiment, cells were processed for immunofluorescence with anti-ManII antibodies. Cells incubated for 5 min in inhibitor-free media exhibit ManII-stained, thin membrane tubules emanating from many of the mini-stacks (arrows) (B). By 10 min of recovery, nearly all mini-stacks were interconnected by thin membrane tubules (arrows), and by 50 min the Golgi complex had completely reformed (D). Some of the tubules indicated by the arrows appear to be of somewhat different diameters; however, these differences are actually due to out-of-focus light from tubules in different focal planes.



issue, we investigated the transport of ts045 VSV-G protein from the ER to the newly reformed mini-stacks. The results showed that Golgi mini-stacks that formed during recovery from BFA treatment in the presence of ONO-RS-082 (1 μM) were capable of receiving ts045 VSV-G from the ER after shift from the restrictive to the permissive temperature (Figure 8). Similarly, when IQ-treated cells were washed and transferred to IQ-free media containing BEL or ONO-RS-082, conditions under which VGMs were allowed to reassemble into mini-stacks, ER-to-Golgi transport of ts045 VSV-G was not prevented (our unpublished data). These observations suggest that under the conditions used here, BEL and ONO-RS-082 did not interfere with COPI- or COPII-coated vesicle transport machinery but instead were specifically disrupting other membrane trafficking events associated with the Golgi reassembly process.

PLA_2 Antagonists Inhibit Golgi Membrane Tubulation In Vitro

The analysis of Golgi reassembly by immunofluorescence strongly suggests that BEL- and ONO-RS-082-sensitive assembly steps are mediated by Golgi membrane tubules. To more directly test the hypothesis that Golgi membrane tubulation requires a cytoplasmic PLA_2 activity, we used a system that reconstituted the cytosol-dependent formation of membrane tubules from isolated Golgi stacks (Banta *et al.*, 1995). As we have previously shown by EM of thin sections, the organelles contained within a typical preparation of the subcellular fraction used in this assay consist primarily of individual Golgi stacks, each with an average of 3.7 closely apposed cisternae (Cluett and Brown, 1992), and which appear by negative staining of whole-mount preparations as a clump of unresolved membranous elements (Cluett *et al.*, 1993). Tubulation in this in vitro assay was analyzed by preparing whole-mount preparations of control or tubulated Golgi complexes for negative stain, electron microscopic visualization. Using this in vitro assay, an organelle-free extract of bovine brain cytosol was shown to induce the time- and dose-dependent for-

Figure 6. Normal Golgi architecture is reversibly altered by PLA_2 antagonists, as shown by immunofluorescence localization of ManII. (A) Control cell. (B) Treatment of cells with ONO-RS-082 (ONO) alone (5 μM for 60 min) produced large fragments that remained in the juxtanuclear region, which were, in fact, also mini-stacks (de Figueiredo *et al.*, 1998). (C–E) Reassembly of intact Golgi complexes during recovery from ONO-RS-082 treatment. Cells were treated as in B and then washed and incubated in normal media for 5 min (C), 15 min (D), and 50 min (E). By 5 min of recovery, thin, ManII-stained membrane tubules can be observed to emanate from, and link together, spatially separate mini-stacks; by 15 min virtually all ManII-stained elements are completely interconnected by thin tubules, and by 50 min the entire Golgi complex has reformed.

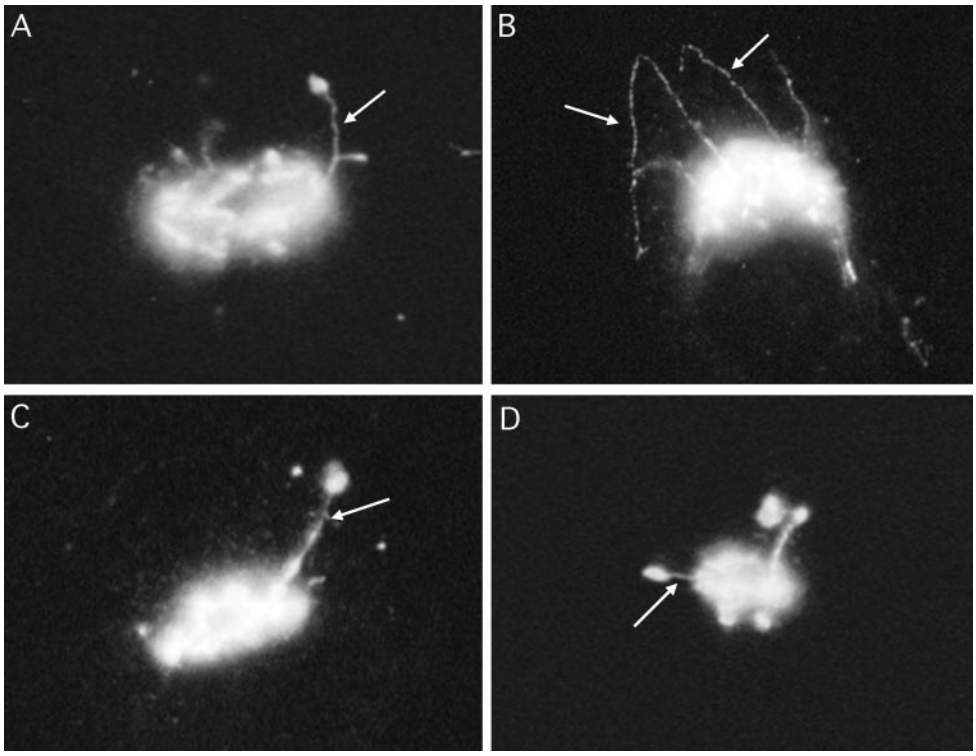


Figure 7. Cells recovering from ONO-RS-082 treatment display ManII-stained tubular connections between large centrally located Golgi elements and more peripheral Golgi mini-stacks. (A) Cells were treated with BFA for 30 min, washed free of BFA, incubated with ONO-RS-082 (5 μM) for 60 min to allow reassembly up to the point of mini-stack formation, and then allowed to recover from ONO-RS-082 for 10 min before fixing for immunofluorescence. (B–D) Cells were treated with ONO-RS-082 alone (5 μM for 60 min) to generate mini-stacks and then allowed to recover in drug-free growth medium for 10 min (B and C) or 15 min (D).

mation of numerous Golgi membrane tubules (60–80 nm in diameter and up to several micrometers in length), which were similar to those seen emanating from cisternal membranes in vivo (Cluett *et al.*, 1993; Banta *et al.*, 1995).

To better characterize the cytosol-induced formation of Golgi membrane tubules in vitro, and to determine whether the tubules grown in vitro bore relevance to those that form during reassembly in vivo, we subjected the whole-mount Golgi preparations to an immunogold labeling procedure using anti-ManII antibodies. Under control conditions in the absence of cytosol, the whole-mount Golgi preparations were roughly spherical, with a small number of associated buds, vesicles, and short tubules (Figure 9A). Immunogold labeling revealed that ManII was present across the entire whole-mount preparation (Figure 9D). In contrast, when incubated with bovine brain cytosol, Golgi complexes were induced to form numerous tubules (60–80 nm in diameter) that extended from the stack (Figure 9B), and moreover, these tubules were heavily immunolabeled by anti-ManII antibodies along their entire length (Figure 9E). In some cases, as in illustrated in Figure 9E, all of the induced tubules were labeled with anti-ManII antibodies. However, in many other cases, only about half of the Golgi tubules were labeled with ManII antibodies, and in double-labeling experiments that localized ManII and mannose 6-phosphate receptors (located in *trans* elements), separate tubules were stained. These results showed that tubules can arise independently from both medial- and *trans*-Golgi elements. To determine whether Golgi tubules generated in vitro were formed by a PLA_2-dependent mechanism, cytosol was incubated with the irreversible PLA_2 inhibitor BEL before mixing with isolated Golgi complexes. Qualitatively, we found that BEL significantly inhibited tubule formation without any other obvious morphological effects on the Golgi complexes (Figure 9C).

Using this in vitro reconstitution assay, we quantified the effects of PLA_2 inhibitors on cytosol-dependent Golgi membrane tubulation and found that membrane tubulation was potently inhibited by a broad spectrum of PLA_2 antagonists (Figure 10A). In these experiments, however, we could not distinguish whether the PLA_2 antagonists were inhibiting an activity in cytosol or on Golgi membranes. To address this issue, we took advantage of the fact that BEL is a site-specific, irreversible inhibitor that covalently binds to enzyme active sites (Daniels *et al.*, 1983; Hazen *et al.*, 1991; Ackermann *et al.*, 1995; Balboa *et al.*, 1997). We found that when cytosol was first treated with BEL, dialyzed extensively to remove unbound inhibitor, and then applied to Golgi-enriched fractions, the level of tubulation was reduced nearly to that of nondialyzed BEL-treated cytosol (Figure 10A). Previously, we have shown that extensive biochemical fractionation of our standard bovine brain cytosol

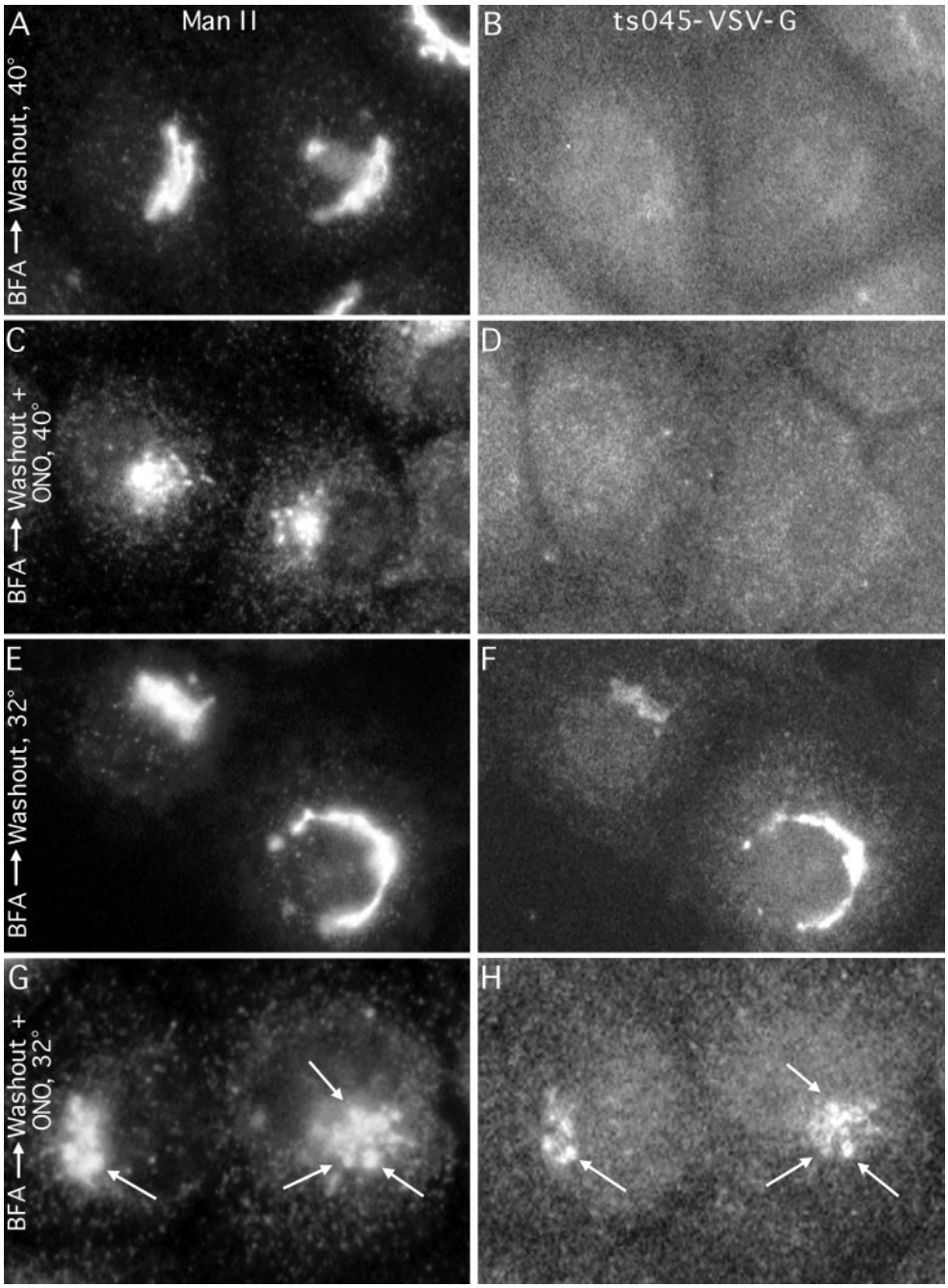


Figure 8. Golgi mini-stacks formed in the presence of PLA_2 inhibitors during reassembly after BFA washout receive membrane traffic from the ER. Cells were infected with ts045 VSV at 40°C to entrap VSV-G in the ER, treated with BFA (5 μg/ml for 15 min) to recycle the Golgi back to the ER, washed free of BFA, and then incubated under various conditions to allow Golgi reassembly and/or transport of VSV-G out of the ER. Cells were then fixed and processed for double-label immunofluorescence to localize ManII (left panels) and VSV-G (right panels). Conditions during the washout from BFA were as follows. (A and B) Cells incubated with drug-free media at 40°C allow the Golgi complex to completely reform, but VSV-G remains diffusely in the ER. (C and D) Cells incubated in media with ONO-RS-082 (ONO) at 40°C reassemble the Golgi into punctate mini-stacks, but VSV-G still remains in the ER. (E and F) Cells incubated with drug-free media but shifted to the permissive temperature of 32°C reassemble an intact Golgi complex to which VSV-G is now transported, as evidenced by its colocalization with ManII. (G and H) Cells incubated with ONO-RS-082 at 32°C reassemble the Golgi only to the point of forming punctate mini-stacks to which VSV-G is transported (arrows point to double-labeled mini-stacks).

preparation, culminating in a gel filtration (GF) column, yielded a GF peak that was highly enriched in tubulation activity and a subset of cofractionating proteins (Banta *et al.*, 1995). Adding back increasing amounts of this GF fraction to BEL-treated cytosol restored tubulation activity (Figure 10B). These data indicate that the relevant BEL-sensitive targets originated in the cytosolic fraction.

To determine whether the PLA_2 inhibitors were working within the same concentration ranges in vitro as in vivo, numerous dose–response experiments were performed, for example, with ONO-RS-082, as shown in Figure 10C. Based on these experiments, we were able to estimate the IC_{50} for many of the PLA_2 inhibitors (Table 1). Concentrations at which BEL and ONO-RS-082 inhibited late Golgi assembly events in vivo were similar to those that inhibited in vitro tubulation, generally in the low micromolar range. Moreover, these concentrations are similar to those reported for the inhibition of intracellular PLA_2 activ-

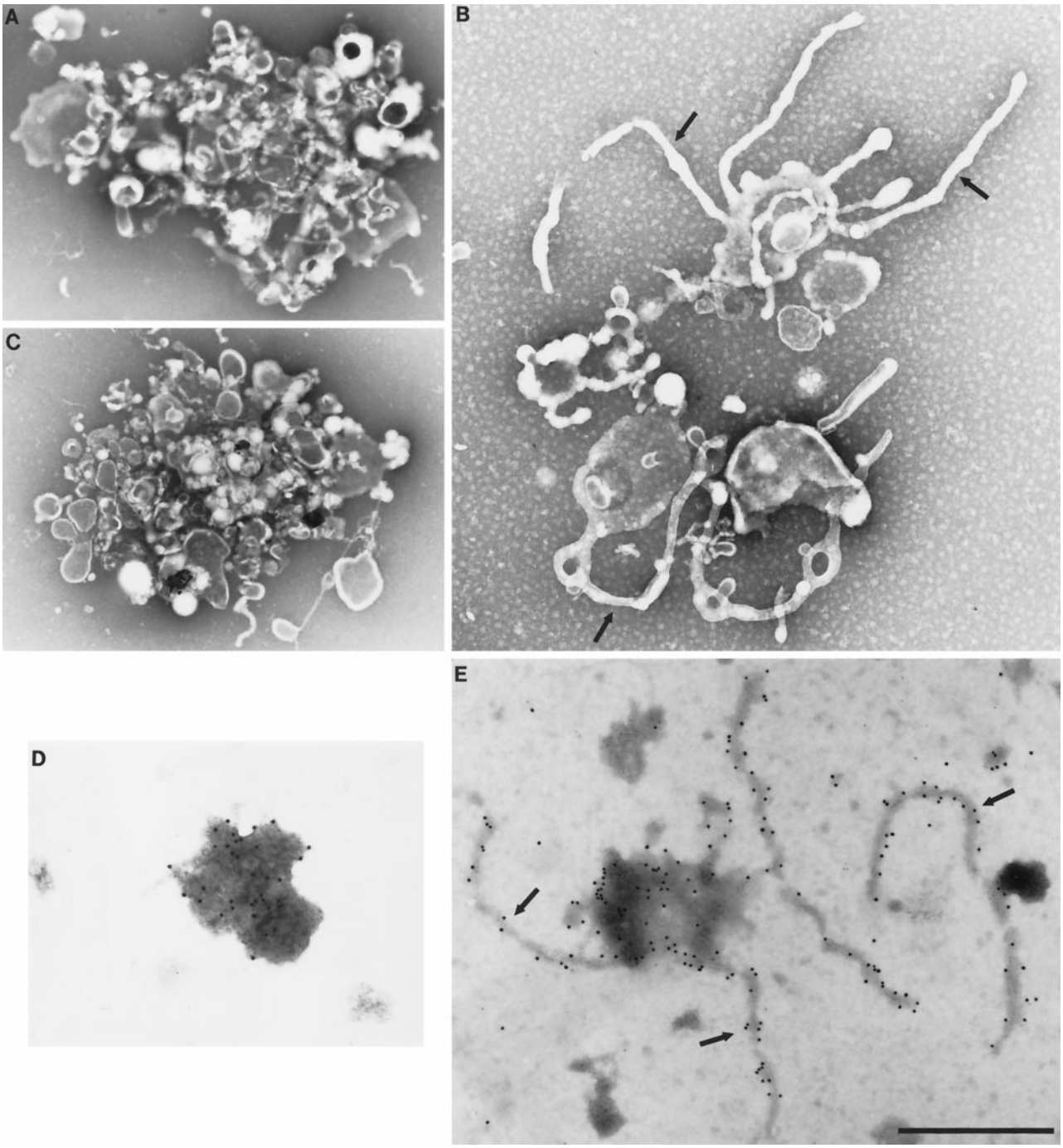


Figure 9. Electron microscopic observations of Golgi membrane tubulation in a cytosol-dependent, in vitro reconstitution system and immunogold localization of ManII on Golgi membrane tubules. Golgi-enriched fractions were incubated in vitro under various conditions and applied as whole-mount preparations onto EM grids for standard negative staining (A–C) or for a modified protocol involving a combination of immunogold labeling of ManII followed by negative staining (D and E). (A) Golgi complex incubated with buffer control. (B) Golgi complex incubated with bovine brain cytosol under conditions that induce membrane tubule formation. Arrows indicate a few of the numerous 60- to 80-nm-diameter membrane tubules that formed. (C) Golgi complex incubated with cytosol that had first been treated with the PLA_2 inhibitor BEL (25 μM). (D) Control Golgi complex immunogold labeled to show distribution of ManII. (E) Golgi complexes incubated with cytosol under tubulation conditions and then immunolabeled to show ManII distribution. Arrows indicate several tubules with a nearly uniform distribution of gold particles along the length of each tubule. As shown in E, all of the induced tubules were labeled with anti-ManII antibodies; however, in many other cases, only about half of the Golgi tubules were labeled with ManII antibodies, and in double-labeling experiments that localized ManII and mannose 6-phosphate receptors (located in *trans* elements), separate tubules were stained. Bar, 0.5 μm.

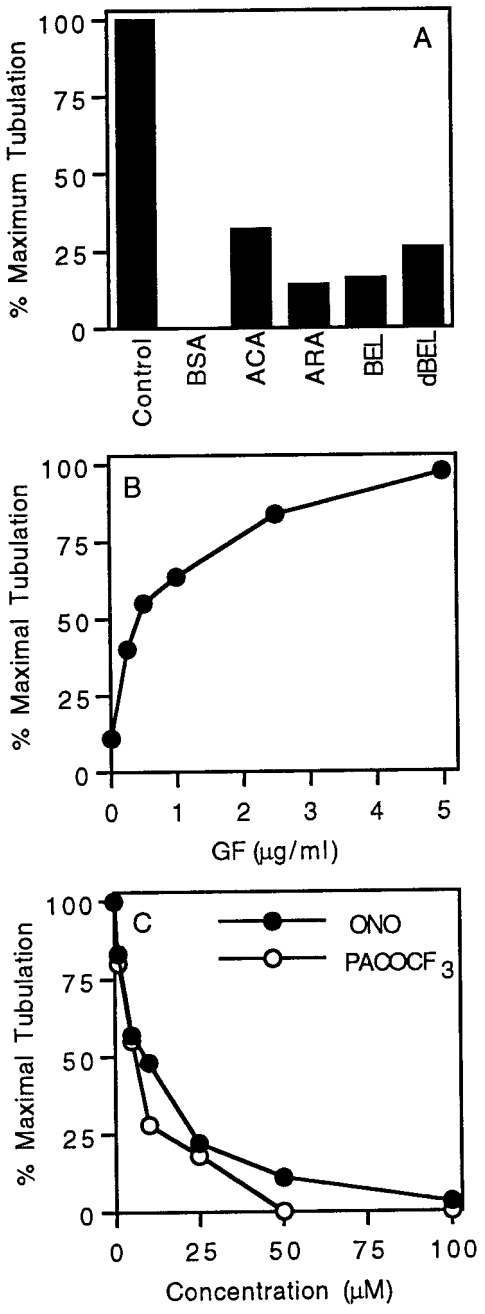


Figure 10. Quantitation of Golgi membrane tubulation inhibition by PLA_2 antagonists. (A) Aliquots of an active fraction of bovine brain cytosol, containing tubulation activity, were incubated in the absence or presence of various PLA_2 inhibitors, and then each was mixed with isolated rat liver Golgi complexes in our standard in vitro tubulation assay. Concentrations were BBC (bovine brain cytosol), 1.5 mg/ml; BSA, 1.5 mg/ml; ACA, ARA, and BEL, 5 μM. The bar labeled dBEL (for dialyzed BEL) shows the level of tubulation that resulted when BBC was first incubated with BEL (20 μM for 15 min at 37°C) and then dialyzed to remove excess, unbound BEL. The extent of tubulation was measured as described in MATERIALS AND METHODS, and the data are expressed as the percent maximal tubulation to compare results from different experiments. (B) Addition of the enriched GF fraction restored tubulation activity to

Table 1. IC_{50} for various PLA_2 inhibitors on in vitro Golgi tubulation

Compound[a]	IC_{50} (μM) Golgi tubulation
$AACOCF_3$	10
ACA	8
ARA	8
BEL	5
BPB	26
DEDA	25
ONO-RS-082	6
$PACOCF_3$	8

[a] Abbreviations for each compound are given in MATERIALS AND METHODS.

ities in various in vitro and in vivo systems (Hazen *et al.*, 1991; Ackermann *et al.*, 1995). In addition to BEL and ONO-RS-082, a variety of other compounds known to differentially antagonize intracellular PLA_2 activities, including ACA, BPB, and ARA, inhibited in vitro Golgi tubulation (Table 1) and late events in Golgi reassembly after removal of BFA or IQ from treated cells. Finally, we found that two active site-directed inhibitors that are selective for cytoplasmic versus secretory PLA_2s, $AACOCF_3$ and $PACOCF_3$ (Street *et al.*, 1993; Gelb *et al.*, 1994), also potently inhibited in vitro Golgi membrane tubulation (Table 1). These results are consistent with the idea that these compounds may be exerting their effects by disrupting intracellular PLA_2 activities.

To verify that the tested PLA_2 inhibitors were having their expected effects on intracellular PLA_2 activities, the relative amount of one PLA_2 hydrolysis product, lyso-PC, was measured in total lipid extracts from cells that had been metabolically labeled with [^{3}H]choline and treated with different PLA_2 inhibitors (Table 2). We found that BEL and ONO-RS-082 reduced levels of [^{3}H]lyso-PC by ~20–25%, consistent with the inhibition of a small subset of total cellular PLA_2s (Dennis, 1997). We previously reported a similar reduction in cellular lyso-PC levels by BEL in BFA-treated cells (de Figueiredo *et al.*, 1998).

If a cytoplasmic PLA_2 is, in fact, acting on Golgi membranes to induce tubulation, then we would expect to find the liberation of inhibitor-sensitive PLA_2

Figure 10 (cont). BEL-inactivated cytosol. BBC (1.5 mg/ml) was incubated with BEL (as above), dialyzed extensively, incubated with increasing amounts of enriched GF fraction, and then used in the standard in vitro tubulation assay. The data points are averages of duplicate experiments. (C) Typical dose–response experiment showing the loss of cytosolic tubulation activity with increasing amounts of the PLA_2 inhibitors ONO-RS-082 and $PACOCF_3$. All data points are the averages of duplicate experiments.

Table 2. PLA_2 antagonists inhibit intracellular and cytosol-stimulated, Golgi membrane-derived [^{3}H]lyso-PC formation

Condition	% control[a]
Changes in cellular [^{3}H]lysoPC content[b]	
Control	100
IQ	95
BFA	86
ONO-RS-082	75
BEL	71
IQ + BEL	72
BFA + BEL	69
BFA + ONO-RS-082	59
Changes in formation of [^{3}H]lyso-PC from Golgi membranes by bovine brain cytosol[c]	
Control	100
BBC	159
BBC + BEL	87
$svPLA_2$	192

[a] Results are expressed as percent control which was determined by dividing the counts per minute in [^{3}H]lyso-PC of experimental samples by those of control samples times 100. In all cases, measurements were determined from at least triplicate samples.

[b] Concentrations of compounds were IQ, 10 μM; BFA, 10 μg/ml; ONO-RS-082 and BEL, 25 μM.

[c] Concentrations were BBC (bovine brain cytosol), 1.5 mg/ml; BEL, 25 μM; $svPLA_2$ (snake venom PLA_2), 1 mg/ml.

products from these membranes. To examine this issue, enriched Golgi fractions prepared from clone 9 cells that had been metabolically labeled to incorporate [^{3}H]choline into cellular phospholipids were incubated with various concentrations and combinations of buffer, cytosol, and PLA_2 antagonists (Table 2). These data show that bovine brain cytosol stimulates an increase in the amount of [^{3}H]lyso-PC in this Golgi-enriched fraction, and that this increase was significantly inhibited by BEL, at concentrations similar to those that inhibited Golgi membrane tubulation in vivo and in vitro.

Recently, we have found that fractions of bovine brain cytosol that are enriched in tubulation activity (Banta *et al.*, 1995) also contain PLA_2 activity (our unpublished data); however, we have not yet ascribed this activity to a specific protein or known cytoplasmic PLA_2. The ability of Golgi complexes to form tubules in response to PLA_2 hydrolysis appears, however, to be specific for a particular type of enzyme, because incubation of the enriched Golgi fraction with either purified snake venom PLA_2 or the 85-kDa Ca^{2+}-independent PLA_2 (Tang *et al.*, 1997) did not induce Golgi membrane tubulation in our in vitro system.

DISCUSSION

We have found that a broad spectrum of PLA_2 antagonists arrested the reassembly of the Golgi complex in vivo during recovery from BFA or IQ. The step in reassembly that was inhibited occurred after the formation of Golgi mini-stacks and their congregation in the juxtanuclear region but before their coalescence into a single, interconnected organelle. That this step involved the formation of membrane tubules was clearly suggested by studies in which reversible PLA_2 antagonists were used; upon washout of the antagonist, tubules could be seen sprouting from mini-stacks, which were eventually linked together by these tubules. We therefore envision that these tubules facilitate the coalescence of spatially separate Golgi stacks into a single, interconnected organelle.

The observation that mini-stacks of reassembling Golgi complexes appeared to become linked together by PLA_2 antagonist-sensitive membrane tubulation suggested that, perhaps, a similar mechanism operates during interphase to dynamically link spatially separate cisternal stacks. Evidence for this idea came from the observation that ONO-RS-082, a reversible antagonist of cytoplasmic PLA_2s, caused interphase Golgi complexes to fragment into mini-stacks (de Figueiredo *et al.*, 1998), which, after removal of the drug, rapidly (within 5 min) began to form membrane tubules. Importantly, the coalescence of separate stacks after washout from ONO-RS-082 treatment alone was morphologically identical to that which occurred after the release from the PLA_2 antagonist-induced arrest of Golgi reassembly after BFA or IQ washout. These results strongly suggest that the maintenance of normal steady-state architecture of the Golgi complex requires the dynamic and continuous formation of the interconnecting PLA_2-mediated tubules.

Of course, our immunofluorescence observations only establish a correlation between tubular connections and the coalescence of mini-stacks into an intact Golgi ribbon. It is difficult to definitively demonstrate tubular connections by immunofluorescence microscopy and, because of the small number of tubules that do form, even more challenging to directly show these connections by EM. However, the fact that mini-stacks remained spatially separate, and that few if any tubules were observed, in the presence of PLA_2 antagonists, clearly suggests that tubules play some role in the coalescence of mini-stacks. We theorize that these tubules "probe" the cytoplasm for their appropriate targets, so that at early times after formation, they would most likely have free ends (as in Figure 5B) but later might sometimes hit their target and form bridges (as in Figures 5C, 6C, and 7, A, C, and D) or, alternatively, miss and remain as tubules with free ends (as in Figure 7B). Also, the establishment of tubular bridges may only be one aspect of the final coalescence into an intact Golgi ribbon. For example, three-dimensional image reconstructions of various cell types show extensive expanses of cisternal stacks (Novikoff *et al.*, 1971; Rambourg *et al.*, 1979; Rambourg

and Clermont, 1990; Ladinsky *et al.*, 1994), which would suggest that after connection of mini-stacks via tubules, either the tubules themselves expand into cisternal elements or additional cisternal connections are made.

One concern with the use of PLA_2 antagonists is that their effects might be indirect; however, a number of lines of evidence suggest otherwise. First, all early events during recovery from BFA or IQ occurred in a similar manner in both the presence and absence of PLA_2 antagonists. This observation demonstrates that all budding and fusion steps needed to form mini-stacks (Lucocq *et al.*, 1989; Acharya *et al.*, 1995a,b; Rabouille *et al.*, 1995a,b) were not perturbed by the PLA_2 antagonists. Second, ER-to-Golgi transport of newly synthesized VSV-G protein was also not qualitatively affected by PLA_2 antagonists. Third, the antagonist's effects on Golgi membrane tubulation and reassembly were fully reversible. And, fourth, although the tested PLA_2 antagonists have different structures, and in some cases different mechanisms of action and secondary targets (Gelb *et al.*, 1994; Mukherjee *et al.*, 1994), each one nevertheless inhibited the same late assembly event, that is, the step at which an interconnected Golgi complex forms from otherwise spatially distinct cisternal stacks. Finally, complementary support for these studies has come from the observation that stimulators of PLA_2 activity, including melittin and PLA_2-activating protein peptide (Clark *et al.*, 1991), enhance cytosol-dependent tubulation (Polizotto and Brown, unpublished data). These observations therefore suggest that the PLA_2 antagonists were all acting on the same molecular target(s). Our results also showed that PLA_2 antagonists had no adverse effects on the structure or function of microtubules, which are known to facilitate membrane tubule formation in response to BFA in vivo (Lippincott-Schwartz *et al.*, 1990, 1991; Wood *et al.*, 1991). First, immunofluorescence revealed no obvious changes in microtubule number or distribution in the presence of BEL or ONO-RS-082. Second, movement of VSV-G from the ER to the Golgi complex, which appears to be facilitated by microtubules (Presley *et al.*, 1997), was not affected by the antagonists. And third, microtubule-facilitated recovery from BFA, leading to the centripetal movement of Golgi proteins to form mini-stacks in the juxtanuclear region (Alcalde *et al.*, 1992; Lippincott-Schwartz *et al.*, 1998), was also not inhibited by the antagonists.

The conclusion that a PLA_2 activity is required for Golgi membrane tubulation in vivo was supported by results from experiments performed with the in vitro reconstitution system. Under the particular conditions of this assay, intact Golgi complexes, when incubated with a fraction of bovine brain cytosol, formed membrane tubules that reached up to several micrometers in length. These tubules formed in the absence of microtubules, and the tubulation activity present in cytosol displayed characteristics of an enzyme (Banta *et al.*, 1995). Thus, the cytosolic tubulation activity most likely acted directly on the Golgi membranes. The fact that the IC_{50} values for a variety of PLA_2 antagonists were similar for both in vitro and in vivo tubulation suggests that a similar mechanism is used under both conditions. Our studies do not address or rule out the possibility that, in vivo, the formation of interstack membrane tubules might be facilitated by microtubules or other cytosketelal elements. In fact, we previously suggested that Golgi-to-ER retrograde tubules induced by BFA would appear to require a sequential two-step mechanism of formation (de Figueiredo *et al.*, 1998). Initially, the activity of a cytoplasmic PLA_2 would induce the formation of a short tubule that subsequently would be pulled out along microtubules. A similar two-step mechanism could function to produce stack-to-stack bridging tubules.

The use of different PLA_2 antagonists, especially the irreversible suicide substrate BEL (Daniels *et al.*, 1983; Hazen *et al.*, 1991; Ackermann *et al.*, 1995), in the in vitro and in vivo assays allows us to make certain conclusions and predictions about the nature of the PLA_2 activity that appears to be required for membrane tubulation. For example, the finding that BEL-treated and dialyzed cytosol still inhibited in vitro tubulation argues strongly that the relevant PLA_2 originates from the cytoplasm. The inhibition of tubulation by BEL-inactivated, dialyzed cytosol also argues against the possibility that the PLA_2 antagonists, which are to varying degrees hydrophobic, might simply be perturbing the phospholipid bilayer. Also, the possibility that a secretory-type PLA_2, released from membrane-bound organelles during homogenization, is responsible for the tubulation activity in the in vitro assay seems very unlikely, because many of the most potent inhibitors of tubulation, including BEL, ONO-RS-082, $PACOCF_3$, and $AACOCF_3$, would be active against only cytoplasmic-type, and not secretory-type, PLA_2s at the concentrations used in our studies (Hazen *et al.*, 1991; Street *et al.*, 1993; Gelb *et al.*, 1994).

The identity of the relevant PLA_2 is not yet known; however, our results here and elsewhere (de Figueiredo *et al.*, 1998) strongly suggest that a cytoplasmic Ca^{2+}-independent PLA_2 is responsible, because Ca^{2+}-independent PLA_2s are 1000-fold more sensitive than Ca^{2+}-dependent enzymes to BEL and would be primarily affected at the concentrations used here (Hazen *et al.*, 1991; Ackermann *et al.*, 1995; Tang *et al.*, 1997). Also, in vitro tubulation of Golgi complex membranes is Ca^{2+}-independent (Banta *et al.*, 1995). Although phospholipases often have broad activity against phospholipids found in many biological membranes, it is important to note that tubulation appears to require a specific PLA_2, because snake venom PLA_2 (a secretory-type enzyme) and a BEL-sensitive, 85-kDa

cytoplasmic Ca^{2+}-independent PLA_2 (Balboa *et al.*, 1997; Tang *et al.*, 1997) did not induce tubulation in the in vitro assay. Thus, tubulation cannot be induced by the general hydrolysis of membrane lipids.

Our in vitro studies here and other in vivo experiments (de Figueiredo *et al.*, 1998) suggest that a cytoplasmic PLA_2 could cause Golgi complexes to form tubules by directly acting on the membranes. Although a PLA_2 could stimulate a lipid-based signal transduction pathway, for example, via arachidonic acid, that indirectly activates the tubulation machinery, we have previously found that inhibition of arachidonic acid conversion to prostaglandins or leukotrienes did not prevent BFA stimulation of Golgi membrane tubule formation (de Figueiredo *et al.*, 1998). Alternatively, a cytoplasmic PLA_2 could specifically produce a localized accumulation of the immediate products of PLA_2 hydrolysis, principally lysophospholipids, that directly induce a planar lipid bilayer to form membrane tubules by selectively increasing the surface area on the cytoplasmic side of organelle membranes. Such a mechanism of membrane shape change was originally described by the bilayer-couple hypothesis (Sheetz and Singer, 1974) and has been experimentally shown to occur by PLA_2 hydrolysis of both red blood cell plasma membranes and artificial liposomes (Fujii and Tamura, 1979; Israelachvili *et al.*, 1982; Christiansson *et al.*, 1985; Mui *et al.*, 1995). Therefore, we envision that a cytoplasmic PLA_2, whose activity is tightly regulated, could similarly induce tubules to form directly from Golgi membranes.

A variety of cytoplasmic Ca^{2+}-independent PLA_2s have been characterized to the level of cDNA cloning and sequencing (for review, see Dennis, 1997; Tang *et al.*, 1997; Underwood *et al.*, 1998; Watanabe *et al.*, 1998) or as enzyme activities (Hirashima *et al.*, 1992; Miyake and Gross, 1992), so any one of these, or an enzyme yet to be discovered, could be the relevant one associated with tubulation activity. Although the antagonists used here strongly point to a cytosolic Ca^{2+}-independent PLA_2 activity, most of them have activity against other enzymes such as PLA_1, platelet-activating factor acetylhydrolase, and lysophospholipase (Mukherjee *et al.*, 1994). We should also emphasize that the in vitro assay may only reveal the minimal requirements for tubulation and may not therefore reconstitute any regulatory aspects of tubulation, such as the possible regulation of tubulation by calmodulin, antagonists of which have been shown to inhibit BFA-stimulated tubulation (de Figueiredo and Brown, 1995). In this regard, we note the interesting finding that modulation of intracellular calcium homeostasis, likely involving transport from intracellular stores, also appears important in the response of Golgi complexes to BFA (Ivessa *et al.*, 1995; Kok *et al.*, 1998). In addition, Mironov *et al.* (1997) and Weigert *et al.* (1997) have shown that BFA-stimulated tubulation is inhibited by certain coumarin and quinone compounds that antagonize a membrane-associated mono-ADP-ribosylation activity. Thus, Golgi membrane tubulation could possibly be regulated in a variety of ways.

In this paper, we have focused on those membrane tubules that appear to help link cisternal stacks into a single, interconnected Golgi ribbon and have provided evidence that this normal steady-state architecture and the reassembly of the Golgi after recovery from BFA or IQ require the dynamic formation of PLA_2-dependent membrane tubules. Irrespective of the role that tubules appear to play, it is clear that many types of mammalian cells invest significant resources to ensure that the architecture of an intact, interconnected Golgi complex is reproducibly rebuilt during recovery from drug-induced disassembly and during each round of the cell cycle. But, to what end? Many eukaryotic cells such as plant and algal cells do not have interconnected stacks (Dupree and Sherrier, 1998); some yeasts do not have stacked cisternae under normal conditions (Rambourg *et al.*, 1995); and in some cell types transport through the secretory pathway is not significantly inhibited even when Golgi complexes are fragmented after microtubule depolymerization (for discussion, see Bloom and Goldstein, 1998). Thus, the purpose of forming an interconnected ribbon of Golgi stacks remains a mystery.

In addition to the membrane tubules that form bridges between spatially separate stacks, three other types of tubules, with similar morphologies, can be seen emanating from Golgi complex: those apparently destined for ER retrograde transport, especially as revealed by BFA (Lippincott-Schwartz *et al.*, 1989, 1990; Sciaky *et al.*, 1997; Presley *et al.*, 1998); those emanating from the TGN and traveling to the plasma membrane (Ladinsky *et al.*, 1994; Hirschberg *et al.*, 1998; Toomre *et al.*, 1998); and those forming between cisternae of a single stack (Weidman *et al.*, 1993; Morré and Keenan, 1994; Cunningham *et al.*, 1966). The fact that BFA stimulation of Golgi tubule formation was also inhibited by PLA_2 antagonists (de Figueiredo *et al.*, 1998) suggests that at least two classes of Golgi tubules form by similar mechanisms and therefore would be expected to have been inhibited in our studies. Although membrane tubules that mediate retrograde trafficking to the ER have only been definitively shown to form after BFA treatment (Lippincott-Schwartz *et al.*, 1989, 1990; Sciaky *et al.*, 1997), it seems very likely that similar retrograde tubules form under normal conditions, as seen by fluorescence time-lapse imaging studies of GFP-labeled Golgi resident membrane proteins (Sciaky *et al.*, 1997; Presley *et al.*, 1998). Thus, a prediction from our studies here and elsewhere (de Figueiredo *et al.*, 1998) is that some portion of retrograde trafficking from the Golgi complex to the ER would be inhibited by PLA_2 antagonists. Indeed,

preliminary studies suggest that this is in fact the case (our unpublished data).

The accumulating evidence now indicates that the formation of at least two kinds of Golgi-associated membrane tubules, those that connect spatially separate cisternal stacks both at steady state and during reassembly and those induced by BFA, which move retrogradely to the ER, appear to require a cytoplasmic PLA_2 activity, strongly suggesting that a common basic mechanism is involved in membrane tubulation. It is important to note, however, that these two classes of Golgi-associated tubules are not likely to be molecularly identical, because they have different fusion targets. The availability of specific reagents that selectively perturb the formation of these tubules should continue to aid in establishing the function(s) of these tubules and to identify the cytoplasmic PLA_2 enzyme(s) involved.

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