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A Role for Calmodulin in Organelle Membrane Tubulation

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Membrane tubules of uniform diameter (60–80 nm) and variable lengths have been seen to extend from the main bodies of the Golgi complex, *trans* Golgi network (TGN), and endosomes. In the case of endosomes, these tubules appear to mediate membrane and receptor recycling events. Brefeldin A (BFA) is a potent drug that completely blocks coated vesicle formation from the Golgi complex and TGN, but at the same time causes the enhanced formation of membrane tubules from these same organelles. Recently, experiments have shown that calmodulin antagonists inhibit the transport of receptors out of endosomes, perhaps by inhibiting the formation of recycling tubules. Using the potent calmodulin-specific antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13), and *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide (C-1), we found that the recycling of transferrin from endosomes to the cell surface was significantly inhibited, resulting in the formation of enlarged endosomal vacuoles. In addition, these same calmodulin antagonists also potently inhibited the formation of BFA-stimulated membrane tubules from the Golgi complex, TGN, and endosomes. In the case of the Golgi complex, failure to form tubules resulted in the inhibition of BFA-stimulated retrograde transport to the endoplasmic reticulum. These results suggest that calmodulin is a general regulator of membrane tubulation and is capable of influencing the morphology of several organelles.

INTRODUCTION

Since first proposed more than twenty-five years ago (Jamieson and Palade, 1967), the idea that membrane vesicles mediate protein transport between intracellular organelles in eukaryotic cells has gained wide acceptance. In fact, elegant experiments utilizing in vitro reconstitution and the generation of mutants in yeast have elucidated many molecular details associated with this important membrane trafficking process (for reviews see Pryer *et al.*, 1992; Anderson, 1993; Schmid, 1993; Rothman, 1994). Recent studies on the remarkable effects that the fungal metabolite Brefeldin A (BFA) exerts on organelle morphology and membrane trafficking suggest, however, that vesicles may not be the sole intermediaries of membrane transport. These studies have revitalized interest in the role that membrane tubules play in intracellular trafficking (for reviews see Klausner *et al.*, 1992; Mellman and Simons, 1992).

Intracellular membrane tubules of uniform diameter (60–80 nm), but variable lengths, have been previously shown to extend from the Golgi complex (Rambourg *et al.*, 1979; Rambourg and Clermont, 1990; Cooper *et al.*, 1990), TGN (formerly called the GERL; Novikoff *et al.*, 1971; Ladinsky *et al.*, 1994), and endosomes (Geuze *et al.*, 1983; Marsh *et al.*, 1986; Hopkins *et al.*, 1990; Tooze and Hollingshead, 1991). The function of Golgi and TGN tubules is not clear, however, several groups have proposed that membrane tubules may be involved in the recycling of material from the Golgi complex back to the endoplasmic reticulum (Hauri and Schweizer, 1992; Klausner *et al.*, 1992). In the case of endosomes, substantial evidence exists to conclude that tubules are involved in the recycling of membrane and receptors back to the plasma membrane or TGN (Geuze *et al.*, 1983, 1987, 1988; Dunn *et al.*, 1989; Hopkins *et al.*, 1994). These endosomal tu-

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bules may, through several rounds of formation, effectively separate membrane components destined for the plasma membrane or the TGN, from soluble components destined for lysosomes (Dunn *et al.*, 1989; Mayor *et al.*, 1993). Although these various membrane tubules may mediate different trafficking events, they appear to be morphologically identical, suggesting that a common underlying mechanism is utilized for their formation.

In spite of the well-documented existence of these various membrane tubules, virtually nothing is known about the molecular mechanisms that regulate their formation. This situation exists in part because of the relatively low abundance of tubules in vivo (compared with coated vesicles), and the lack of experimentally tractable assay systems. BFA is a potent drug that completely blocks anterograde membrane traffic by inhibiting the formation of several types of coated transport vesicles (for reviews see Klausner *et al.*, 1992; Mellman and Simons, 1992). In addition, and of more direct interest here, BFA causes the enhanced formation of membrane tubules from the Golgi complex, TGN, and endosomes (Lippincott-Schwartz *et al.*, 1990, 1991; Hunziker *et al.*, 1991; Wood *et al.*, 1991; Wood and Brown, 1992). By all criteria so far investigated, the BFA-induced tubules are identical to those normally seen extending from these organelles–they are just more numerous and longer. Controversy, however, still exists regarding the exact physiological relevance of the BFA-stimulated tubules (Klausner *et al.*, 1992): are they exaggerated manifestations of the normal tubules; are they directly linked to the failure to pinch off a coated bud; or, are they drug-induced artifacts of a cytotoxic compound? The answers to the first two questions remain to be determined. With regard to the last question, we would argue that BFA-stimulated tubules are very unlikely to be simply a drug artifact. First, as stated above, tubules normally found in cells appear to be identical to those enhanced during BFA treatment. Second, under the conditions used, BFA is not cytotoxic but fully reversible. Third, and perhaps most importantly, recent in vitro and in vivo studies have shown that both normal and BFA-enhanced membrane tubules require ATP (but not GTP), and cytosolic and membrane proteins for their formation (Donaldson *et al.*, 1990; Contradt *et al.*, 1993; Cluett *et al.*, 1993; Misteli and Warren, 1994; unpublished data).

The cytosolic factors required to induce membrane tubule formation have not yet been identified. Recently, Apodaca and colleagues have suggested that calmodulin may be one such membrane tubulation factor (Apodaca *et al.*, 1994). Using the potent calmodulin antagonists W-7 and W-13 (Hidaka *et al.*, 1981; Tanaka *et al.*, 1982), several groups have shown that calmodulin activity is required for the transcytosis of polymeric IgA receptors, and the recycling of endocytosed transferrin receptors to the basolateral plasma membrane in polarized MDCK cells (Apodaca *et al.*, 1994; Hunziker, 1994). In the presence of these compounds, large endosomal vacuoles accumulated that may have lacked the membrane tubules that their untreated counterparts display (Apodaca *et al.*, 1994). Based on these results, Apodaca *et al.* proposed that calmodulin antagonists may inhibit recycling by preventing the formation of recycling tubules.

In this paper, we directly test the above hypothesis that calmodulin is involved in the formation of membrane tubules. We reasoned that because tubulation of endosome membranes may be inhibited by calmodulin antagonists, and if BFA-induced tubulation of endosomes reflects a physiologically relevant extension of a normal process, then calmodulin antagonists should also inhibit BFA-stimulated tubulation. Using the calmodulin antagonists W-7, W-13, and C-1 (Hidaka *et al.*, 1981; Tanaka *et al.*, 1982), we provide evidence that calmodulin antagonists are potent inhibitors of endosome tubulation. Moreover, these same antagonists also inhibit the formation of tubules from the Golgi complex and TGN, indicating that calmodulin is a more general regulator of membrane tubulation.

MATERIALS AND METHODS

Materials and Cells

Normal rat kidney (NRK) cells, Clone 9 rat hepatocytes, and HeLa cells were grown in modified Eagle's medium (MEM) with 10% Calf Serum Supplement (CSS) and 1% penicillin/streptomycin. All cells were grown at 37°C in a humidified atmosphere of 95% air, 5% CO_2. BFA (Sigma Chemical, St. Louis, MO) was stored at −20°C in ethanol as a 10 mg/ml stock solution. *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), *N*-(4-aminobutyl)-5-chloro-2-naphthalene-sulfonamide (W-13), *N*-(4-aminobutyl)-5-chloro-1-napthalene-sulfonamide (C-1) (Sigma Chemical), and *N*-(4-aminobutyl)-2-naphthalenesulfonamide (W-12) (Seikagaku, St. Petersburg, FL), were stored at 4°C in H_2O as 5 mM stock solutions. CSS and penicillin/streptomycin were obtained from Life Technologies (Grand Island, NY). Horseradish peroxidase (HRP; Type II) was obtained from Sigma Chemical.

Mouse monoclonal antibodies directed against clathrin heavy chain (X22), β-coatamer complex (β-COP), and the cytoplasmic tail of the human transferrin receptor (HTR-H68), were generous gifts from F. Brodsky, University of California, San Francisco, T. Kreis, University of Geneva, Switzerland, and I. Trowbridge, Salk Institute, La Jolla, CA, respectively. Rabbit polyclonal antibodies were raised against purified bovine or rat liver M6P receptors and characterized as previously described (Brown and Farquhar, 1987). Rabbit polyclonal antibodies directed against α-mannosidase II were a generous gift from M. Farquhar (University of California San Diego, San Diego, CA). Mouse monoclonal anti-α-tubulin was purchased from Amersham (Arlington Heights, IL). All fluorescent secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, (West Grove, PA). Goat anti-rabbit Fab fragments conjugated with HRP were obtained from Biosys, S.A. (Compiegne, France).

Inhibition of BFA-stimulated Tubulation

In experiments in which Golgi or TGN morphology was examined, Clone 9 or NRK cells were grown in CSS-containing media to ~80%

confluency on glass coverslips. In some experiments (see figure legends for details), the coverslips were preincubated at 37°C with various concentrations of W-7, W-12, W-13, or C-1 for various lengths of time in CSS-containing media. BFA (10 μg/ml final concentration) was added (for various lengths of time) to this media. Cells were then fixed for 10 min in 3.7% formalin in phosphate-buffered saline, pH 7.4) at room temperature. Finally, cells were prepared for immunofluorescence microscopy as previously described (Wood *et al.*, 1991). For immunoperoxidase labeling at the electron microscopic level, cells were fixed, incubated successively with rabbit anti-M6PR antibodies followed by goat anti-rabbit-HRP conjugates, and prepared for diaminobenzidine (DAB) cytochemistry as described (Brown and Farquhar, 1989; Wood *et al.*, 1991).

Transferrin (Tfn) conjugated with fluorescein isothiocyanate (FITC) was prepared as a 4 mg/ml stock in phosphate-buffered saline (pH 7.4) and stored in the dark at 4°C as previously described (Maxfield *et al.*, 1978; Yamashiro *et al.*, 1984). Experiments using this reagent to visualize endosome morphology were performed on HeLa cells grown to ~80% confluency on glass coverslips in CSS-MEM. Coverslips were washed three times in CSS-free media, and then transferred to petri dishes containing Tfn-FITC (20 μg/ml) in CSS-free media. These petri dishes were incubated in the dark for 45 min at 37°C during which time Tfn-FITC was endocytosed into early, late, and recycling endosomes. Finally, Tfn-FITC–labeled cells were treated with calmodulin antagonists and BFA (in CSS-containing media) as described in the figure legends, before being fixed and prepared for immunofluorescence microscopy.

For labeling endosomes for electron microscopy, cells were incubated with HRP (8 mg/ml in MEM) for 45 min at 37°C, and then various drugs were added as described in RESULTS and figure legends. HRP-labeled cells were fixed in 1.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, for 1 h, and then processed for DAB cytochemistry as described (Apodaca *et al.*, 1994).

Inhibition of the Transferrin Receptor (TfnR) and Tfn-FITC Recycling

HeLa cells were grown in CSS-containing media to ~80% confluency on glass coverslips, washed three times in CSS-free media, and then incubated for 30 min in petri dishes containing a pulse solution of either Tfn-FITC (20 μg/ml) and 25 μM W-7 in CSS-free media, or Tfn-FITC alone (in same), during which time this fluorescent marker was endocytosed into endosomes. Cells were then washed three times in CSS-free media, and incubated in Tfn-FITC–free chase media for various lengths of time at 37°C, in the presence or absence of 25 μM W-7 (see figure legends for details). Finally, cells were fixed and prepared for immunofluorescence microscopy, using antibodies directed against the cytoplasmic tail of TfnR.

RESULTS

To investigate the role that calmodulin plays in BFA-stimulated tubulation, we treated Clone 9 rat hepatocytes, NRK, or HeLa cells with the potent calmodulin antagonists W-7, W-13, or C-1, before subsequent addition of BFA. These compounds inhibit calmodulin activity, to varying degrees, both in vivo and in vitro (Hidaka *et al.*, 1981; Tanaka *et al.*, 1982; Hidaka and Hartshorne, 1985). As a control, we also treated cells with *N*-4-aminobutyl-naphthalenesulfonamide (W-12), a compound that is significantly less potent at inhibiting calmodulin activity than its structural relative W-13. W-12 has been successfully employed to distinguish calmodulin-specific drug targets from those caused by nonspecific drug effects (Chafouleas *et al.*, 1982; Apodaca *et al.*, 1994).

Calmodulin Antagonists Block BFA-induced Tubulation of Golgi and TGN Membranes

To examine the morphology of the Golgi complex under various conditions, we prepared cells for immunofluorescence microscopy using a polyclonal antibody directed against a resident enzyme of the medial Golgi compartment, α-mannosidase II (αMII), and 10E6, a monoclonal antibody directed against an antigen located in *cis*-Golgi membranes (Wood *et al.*, 1991).

In untreated cells, αMII appeared as a discrete band of thick tubular elements in the juxtanuclear region of cells (Figure 1A). This juxtanuclear localization was also observed when cells were treated (10 min) with W-13 alone (25 μM) (Figure 1B), although in this case, the Golgi often appeared more compact than its untreated counterpart. When cells were treated for 5 min with BFA alone (10 μg/ml), Golgi membranes rapidly fused with the endoplasmic reticulum (ER), and a diffuse staining pattern, characteristic of the BFA-induced formation of a hybrid Golgi-ER network, was observed (Figure 1C). Remarkably, this BFA effect was completely inhibited, in a dose-dependent manner, by low concentrations of the calmodulin antagonist W-13. In contrast to the complete redistribution of Golgi membranes seen after 5 min of BFA treatment alone, inclusion of 1 μM W-13 slowed the process to the point that the tubular intermediates of the BFA-stimulated redistribution were often observed (Figure 1D). At 10 μM W-7, far fewer tubules were seen, and at 25 μM these tubules failed to form at all as the Golgi membranes remained in the juxtanuclear region of cells (Figure 1, E and F). Because membrane tubulation is the only means by which BFA-induced Golgi membrane redistribution to the ER is known to occur (Lippincott-Schwartz *et al.*, 1990, 1991), we interpret this inhibition of BFA-induced Golgi membrane redistribution to be a direct consequence of the failure of Golgi membrane tubules to form.

To quantitate the calmodulin antagonist–induced inhibition of BFA-stimulated Golgi-to-ER redistribution, we determined the percentage of cells with intact Golgi complexes versus those which had completely diffuse staining. The results revealed an IC_{50} = 38 μM for W13 (Figure 2A). Likewise, the calmodulin antagonists W-7 (IC_{50} = 32 μM) and C-1 (IC_{50} = 32 μM) also blocked BFA-stimulated Golgi tubulation and redistribution in a dose-dependent fashion (Figure 2A). W-12, however, failed to have a significant effect over the same concentration range (Figure 2A), suggesting, therefore, that the observed effects were calmodulin specific.

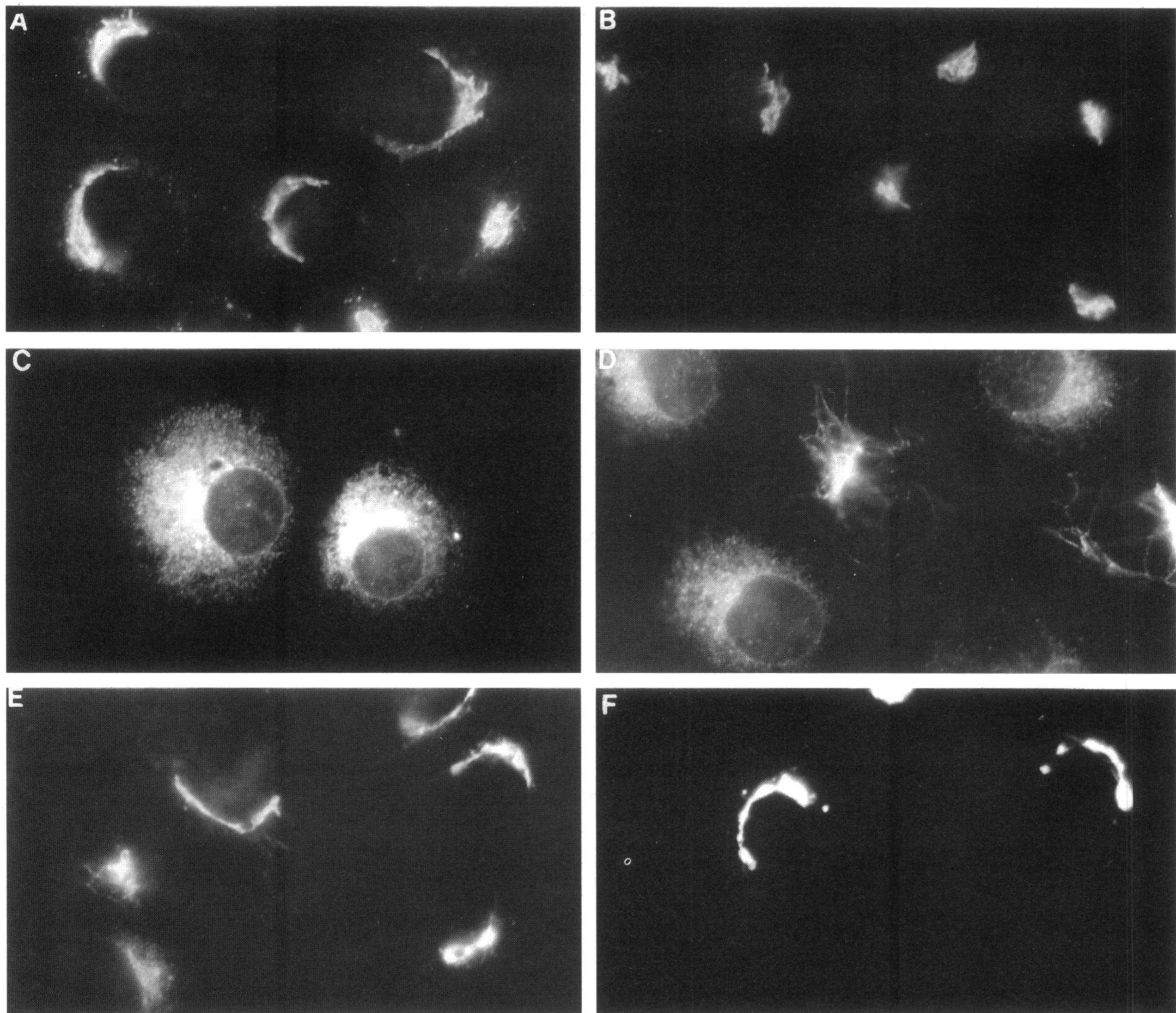

Figure 1. W-13 inhibits BFA-stimulated Golgi membrane tubulation and redistribution in a dose-dependent fashion. Clone 9 rat hepatocytes were treated with various concentrations of BFA and W-13 as follows: (A) untreated; (B) 25 μM W-13 alone for 10 min; (C) BFA (10 μg/ml) for 5 min; (D–F) pretreatment with 1 μM, 10 μM, and 25 μM W-13 (5 min), respectively, before subsequent addition of BFA (10 μg/ml) for 5 min. Cells were then fixed and prepared for immunofluorescence microscopy using polyclonal antibodies directed against α-mannosidase II, a medial Golgi marker, as described in MATERIAL AND METHODS.

Because BFA-stimulated Golgi-to-ER redistribution was most sensitive to W7, we used this calmodulin antagonist to examine the time course of BFA-induced Golgi membrane redistribution (measured as the complete loss of distinct Golgi complex staining). The results showed that in the absence of calmodulin antagonists, Golgi membranes rapidly redistributed to the ER ($t_{1/2}$ = 3.5 min) (Figure 3). In the presence of 25 μM W-7, however, half-maximal Golgi membrane redistribution occurred after 7 min, and at 50 μM W-7, less than 20% of the cells contained redistributed Golgi membranes, even after 20 min in BFA (Figure 3).

In addition to promoting Golgi membrane tubulation and redistribution, BFA causes the TGN to tubulate and fuse with early endosomes (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991; Wood and Brown, 1992). To investigate whether calmodulin antagonists interfered with this particular BFA effect, we prepared cells for immunofluorescence microscopy using a polyclonal antibody directed against the cation-independent mannose 6-phosphate receptor (M6PR) (Brown and Farquhar, 1987). At steady state, M6PRs are primarily found in the TGN and vesicles of the prelysosomal/late endosome compartment in a

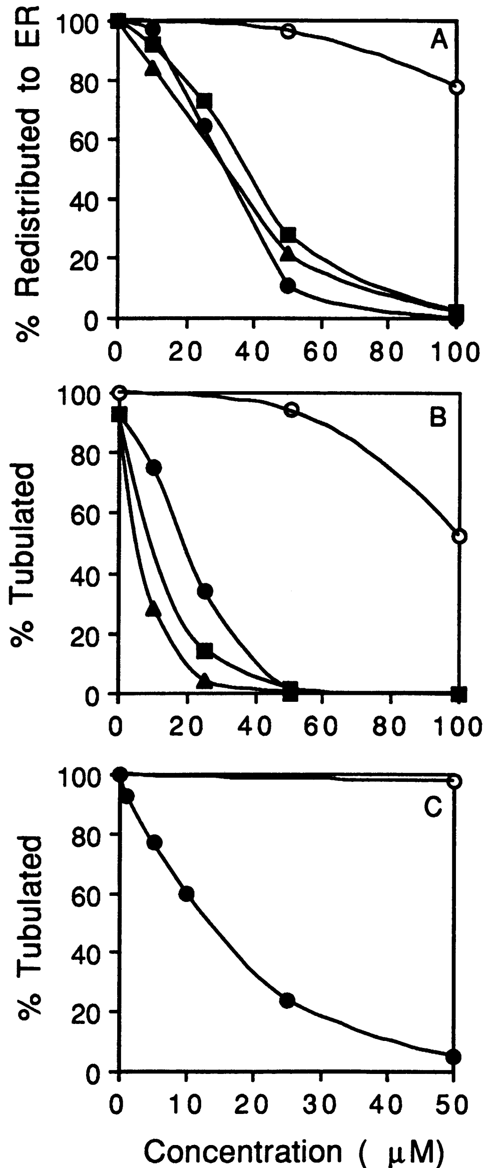

Figure 2. Calmodulin antagonists inhibit BFA-stimulated Golgi, TGN, and endosome membrane tubulation and redistribution. NRK cells were pretreated for 5 min with various concentrations of W-7 (solid circles), W-12 (open circles), W-13 (solid squares), and C-1 (solid triangles), before the subsequent addition of BFA (10 μg/ml for 5 min). Cells were then fixed and prepared for immunofluorescence microscopy using antibodies directed against the 10E6 *cis*-Golgi antigen (A), M6PRs (B), or the cytoplasmic tail of transferrin receptors (C). The percentage of cells with resident Golgi proteins that had completely redistributed to the ER (A), or greater than three M6PR-stained (B) or Tfn-FITC–stained (C) membrane tubules, was then determined and plotted. Each data point represents the average of two (C) or three (A and B) experiments.

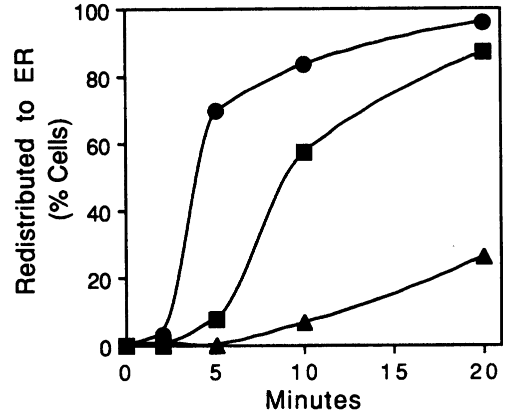

Figure 3. Time course of W-7 inhibition of Golgi membrane redistribution. Clone 9 rat hepatocytes were pretreated with solvent alone (circles), 25 μM (squares), or 50 μM (triangles) W-7 for 5 min before subsequent addition of BFA (10 μg/ml) for various lengths of time. Cells were then prepared for immunofluorescence microscopy using a polyclonal antibody directed against αMII. Finally, the percentage of cells with Golgi membranes that had fully redistributed to the ER was determined and plotted. Each data point represents the average of three experiments.

variety of cells (Geuze *et al.*, 1984; Brown *et al.*, 1986; Griffiths *et al.*, 1988). BFA, however, causes extensive tubulation of the TGN, but not the prelysosomal/late endosome compartment, which can be visualized by immunofluorescence with anti-M6PR antibodies (Wood *et al.*, 1991).

In untreated cells (Figure 4A), the M6PR is typically found in a cluster of vesicles and short tubules in the juxtanuclear region. Treating cells with W-7 (25 μM) alone for 5 min had no significant effect on the TGN (Figure 4C). When cells were treated for 5 min with BFA alone, the TGN formed thin tubules that extended throughout the cytoplasm (Figure 4E). Pretreating cells (5 min) with W-7 (25 μM) before subsequent addition of BFA, however, completely blocked this TGN tubulation (Figure 4G). The calmodulin antagonists W-13 (IC_{50} = 10 μM) and C-1 (IC_{50} = 6 μM) also blocked BFA-stimulated TGN tubulation (Figure 2B). W-12, on the other hand, showed no comparable inhibition over the concentration range tested (0–100 μM), suggesting therefore, that the observed effect was, once again, calmodulin specific (Figure 2B).

Calmodulin Antagonists Do Not Prevent the BFA-stimulated Dissociation of COPs and Clathrin

BFA prevents proteins of the coatomer- (COPs) and clathrin-complex from associating with Golgi and TGN membranes, respectively. This failure is reflected in the rapid loss of these coat proteins from membranes and their accumulation in the cytosol (Donaldson *et al.*, 1990; Robinson and Kreis, 1992; Wong and Brodsky, 1992). To investigate whether calmodulin antagonists interfere with these particular BFA effects, cells were prepared for immunofluorescence micros-

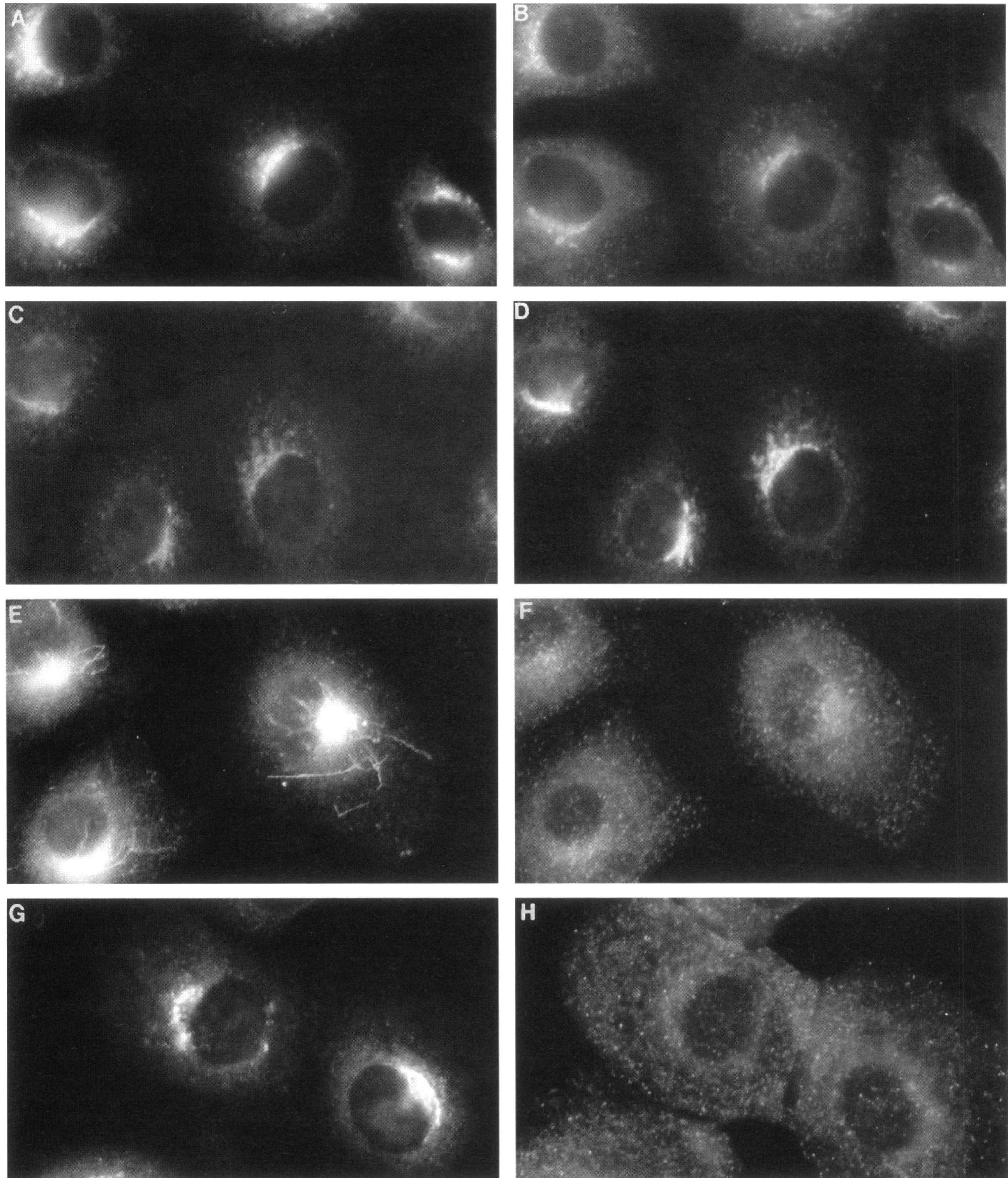

Figure 4. W-7 inhibits BFA-induced TGN membrane tubulation but not clathrin dissociation. Clone 9 rat hepatocytes were treated with various combinations of W-7 and BFA, and then prepared for double-label immunofluorescence microscopy using antibodies directed against M6PRs (left panels) and the clathrin heavy chain (right panels) as follows: (A and B) untreated; (C and D) 25 μM W-7 alone for 10 min; (E and F) BFA (10 μg/ml) for 5 min; (G and H) pretreatment with 25 μM W-7 (5 min), before subsequent addition of BFA (10 μg/ml) for 5 min.

copy using monoclonal antibodies directed against β-COP or the clathrin heavy chain.

In cells treated with W-7 alone (25 μM for 5 min), as in control cells, β-COP and αMII co-localized in the juxtanuclear region of cells, although some β-COP staining was also found to be associated with small vesicles (Figure 5, C and D). Similarly, clathrin, under these conditions, was found to be associated with the juxtanuclear TGN (Figure 4, C and D). Clathrin staining was also seen in coated pits and vesicles located at the cell surface, but these were not visible in these micrographs because the microscope was focused at the level of the TGN.

In cells treated with BFA alone, however, both clathrin and β-COP dissociated from membranes and were found dispersed throughout the cytoplasm, resulting in a low, diffuse signal (Figures 4F and 5F). The low signal was not due to degradation of clathrin or β-COP because removal of BFA from cells resulted in a recovery of intense Golgi and TGN staining for both proteins (our unpublished data), as has been previously shown (Donaldson *et al.*, 1990; Robinson and Kreis, 1992; Wong and Brodsky, 1992). When cells were pretreated with W-7 (25 μM) for 5 min, before subsequent addition of BFA, TGN and Golgi tubules failed to form as expected (Figures 4G and 5G). This block in BFA-stimulated tubulation did not arise because W-7 prevented proteins of the COP and clathrin complexes from dissociating from these membranes. As Figures 4H and 5H reveal, clathrin and β-COP failed to associate with their respective membranes under these conditions, and consequently, were found to be dispersed throughout the cytoplasm. Similar results were also obtained when the calmodulin antagonists W-13 and C-1 were used (our unpublished data).

The inhibition of BFA-stimulated tubulation of TGN membranes by W-7 was confirmed by localizing the M6PR at the EM level through immunoperoxidase staining. In cells treated with BFA alone for 5 min (by which time the Golgi complex has completely redistributed to the ER), the M6PR was found to be localized in numerous long tubules (Figure 6, A and B) as previously reported (Wood *et al.*, 1991). However, when cells were pretreated with W-7 (for 5 min) before subsequently adding BFA (5 min), M6PRs were found in the TGN adjacent to normal appearing Golgi stacks, and in late endosomes (prelysosomes) that appeared somewhat enlarged compared with their normal counterparts (Figure 6C). As predicted from the immunofluorescence results, few, if any, of the types of tubules produced by BFA were observed. Likewise, no tubules were observed extending from the Golgi stacks in these experiments. Other experiments designed to specifically investigate the inhibition of Golgi membrane tubulation by W-7 produced identical results (our unpublished data).

Calmodulin Antagonists Inhibit Tubulation of Endosome Membranes

To independently examine the effects that calmodulin antagonists have on the morphology of the tubular endosome network that forms in the presence of BFA, we incubated HeLa cells with Tfn-FITC (45 min) to allow for the receptor-mediated endocytic uptake of this fluorescent marker into early, late, and recycling endocytic compartments. After treating these cells with various combinations of calmodulin antagonists and BFA, we then prepared them for immunofluorescence microscopy using a monoclonal antibody directed against the cytoplasmic tail of the TfnR. Double labeling cells in this way allowed us to separately examine the effects that these drugs have on the fate of both recycling endocytic receptors (TfnR), and the ligand that those receptors transport (Tfn).

In untreated cells, or cells treated with W-7 alone for 10 min, both TfnR and Tfn colocalized in small endocytic vesicles dispersed throughout the cytoplasm (Figure 7, A and B). In the presence of BFA alone (5 min), however, endosomes formed an extensive tubular network (Figure 7, C and D). Pretreating cells with W-7 for 5 min completely blocked this BFA effect (IC_{50} = 13 μM), and Tfn and TfnR colocalized, under these conditions, in nontubular, vesicular organelles that appeared similar to those seen in cells treated with W-7 alone (Figure 7, E and F; Figure 2C). W-13 and C-1 also inhibited BFA-stimulated endosome tubulation in a dose-dependent fashion (our unpublished data). W-12, on the other hand, had no significant effect over the concentration range tested (0–100 μM) (Figure 2C).

The failure of BFA to tubulate organelle membranes in the presence of calmodulin antagonists did not result from a disruption of microtubule morphology as neither W-7, W-13, nor C-1 had any effect on tubulin staining (our unpublished data). In addition, when cells were treated with W-7 alone for 10 min (25 μM), and then washed in W-7–free media for 30 min, they recovered their untreated Golgi and TGN morphology and tubulated as did untreated controls in the presence of BFA alone (our unpublished data). Therefore, the W-7 drug effect was fully reversible within 30 min. Finally, calmodulin activity appeared to be necessary during the entire process of tubule growth and extension from organelle membranes. When Golgi membrane tubulation was initiated by briefly treating cells with BFA for 1 min before the subsequent addition of W-7 (50 μM) for various lengths of time, the rate of organelle redistribution to the ER–when compared with controls–was greatly diminished (Figure 8).

To confirm that W-7 inhibited the BFA-induced formation of endosomal tubules at the EM level, cells

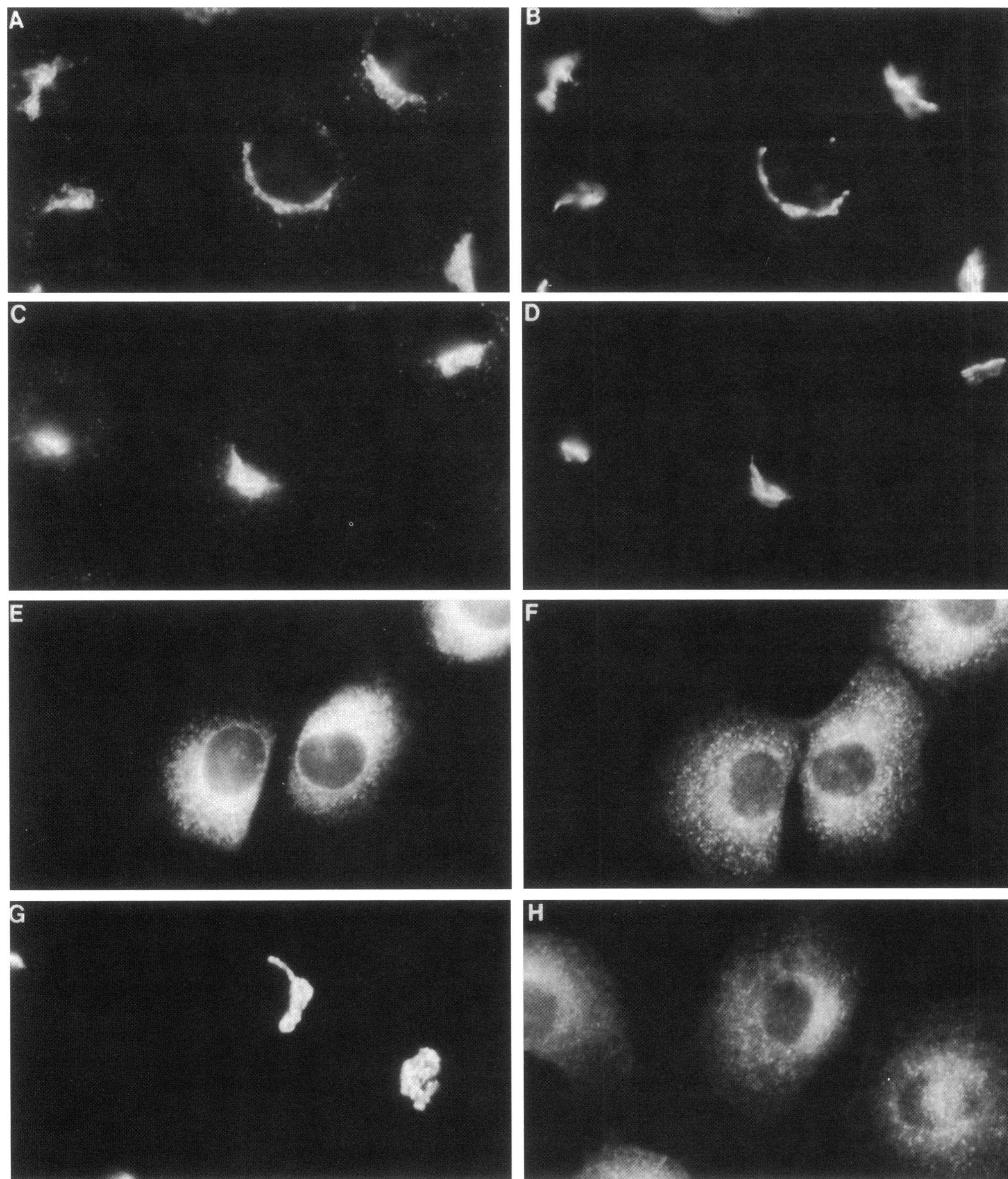

Figure 5. W-7 inhibits Golgi membrane tubulation and redistribution but not β-COP dissociation. Clone 9 rat hepatocytes were treated with various combinations of W-7 and BFA and then prepared for double-label immunofluorescence microscopy using antibodies directed against αMII (left panels) and β-COP (right panels) as follows: (A and B) untreated; (C and D) 25 μM W-7 alone for 10 min; (E and F) BFA (10 μg/ml) for 5 min; (G and H) pretreatment with 25 μM W-7 (5 min), before subsequent addition of BFA (10 μg/ml) for 5 min.

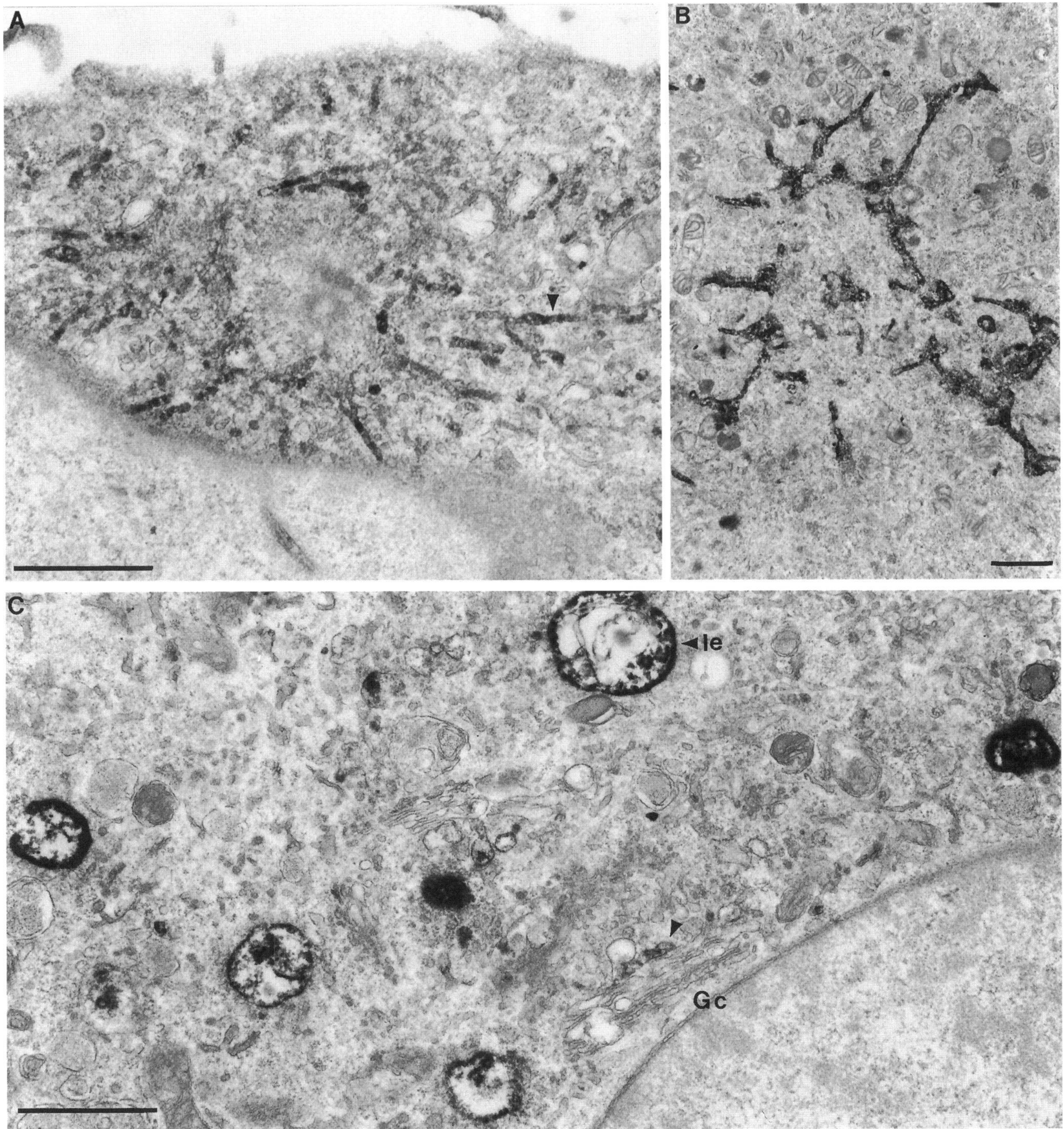

Figure 6. Immunoelectron microscopy of W-7 inhibition of BFA-stimulated TGN membrane tubulation. (A and B) Cells treated with BFA (10 μM) for 5 min display extensive tubulation of TGN membranes (arrowhead) as revealed by immunoperoxidase staining for the M6PR. (C) Cells pre-treated with W-7 (25 μM) for 5 min before BFA treatment appear as do untreated cells, with typical stacked Golgi cisternae (Gc) adjacent to M6PR-stained TGN membranes (arrowhead). Few tubules were observed to extend from either TGN or stacked Golgi membranes. In these W-7–treated cells, the M6PR-stained late endosome/prelysosome vesicles (le) appear somewhat enlarged. Bars, 1 μm.

were incubated with soluble HRP for 45 min to label early and late endosomes, followed by DAB cytochemistry. In control, untreated cells, internalized HRP was delivered to endosomes that were primarily

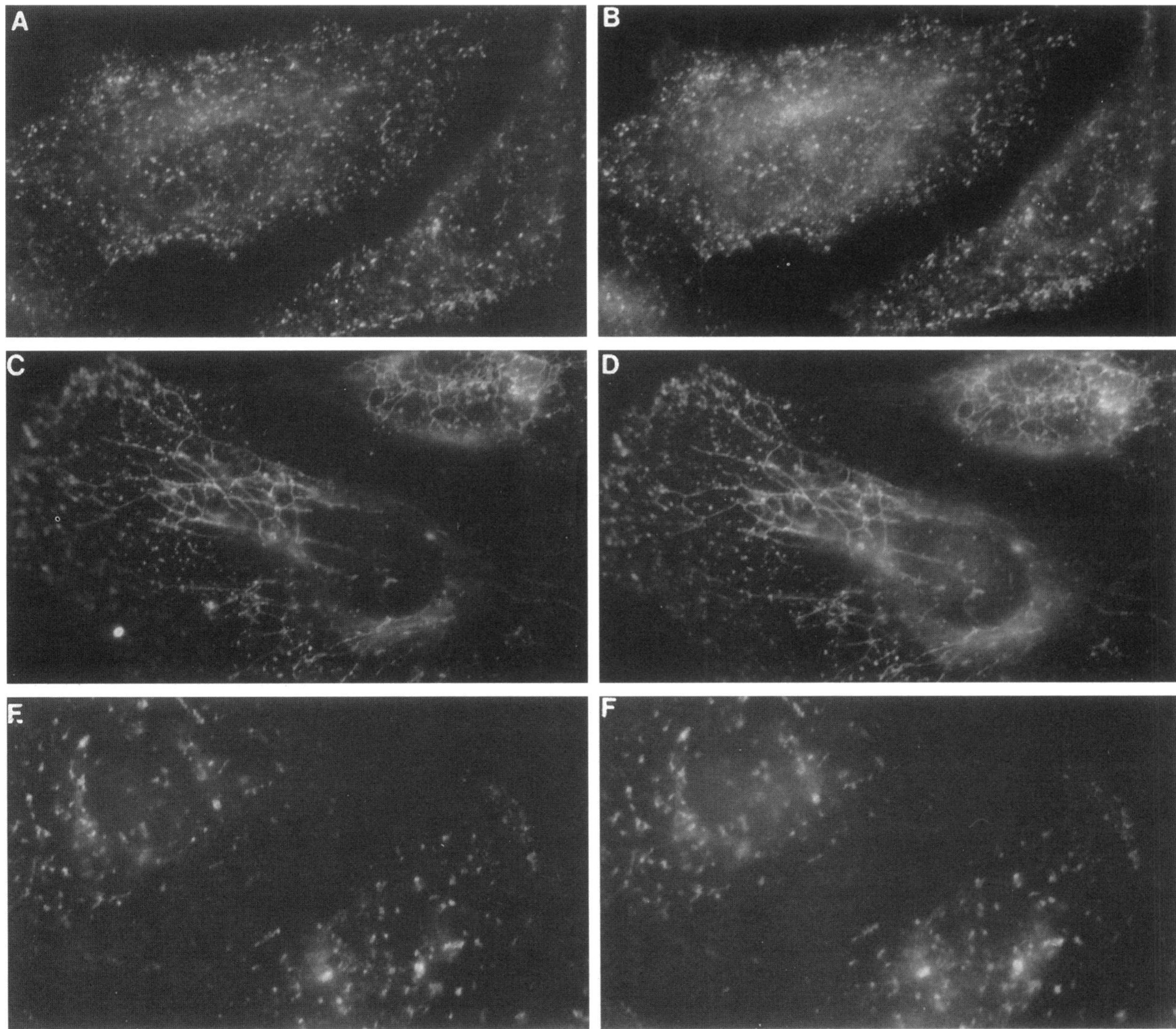

Figure 7. W-7 inhibits BFA-stimulated endosome tubulation. Early, late, and recycling endosomes in HeLa cells were labeled by endocytic uptake of Tfn-FITC (left panels) for 45 min. Labeled cells were then incubated with various combinations of W-7 and BFA before being prepared for immunofluorescence microscopy using a monoclonal antibody directed against the cytoplasmic tail of the TfnR (right panels). Cells were treated as follows: (A and B) untreated; (C and D) BFA (10 μg/ml) for 5 min; (E and F) pretreatment with 25 μM W-7 (5 min), before subsequent addition of BFA (10 μg/ml) for 5 min.

spherical and 0.1–0.4 μm in diameter (Figure 9A). However, when cells were treated with BFA for just 5 min after uptake of HRP, extensive tubular endosomes formed (Figure 9B). Significantly, treatment of cells with W-7 for 10 min before addition of BFA prevented the formation of tubular endosomes (Figure 9C). As seen by immunofluorescence, the endosomes of W-7 pre-treated cells, although spherical, were somewhat larger in diameter (0.1–0.8 μm) than those of control cells. Again, cells treated in this manner display normal appearing Golgi stacks.

Calmodulin Antagonists Inhibit Recycling of the TfnR and Tfn-FITC

To examine the effects that calmodulin antagonists have on endosome morphology in the absence of BFA, we incubated HeLa cells for 30 min in a pulse solution containing either Tfn-FITC (20 μg/ml) and W-7 (25 μM), or Tfn-FITC alone (control), to allow for the receptor-mediated endocytic uptake of this fluorescent marker into recycling endocytic compartments. To examine the fate of previously endocytosed Tfn-FITC as it trafficked through the endocytic system,

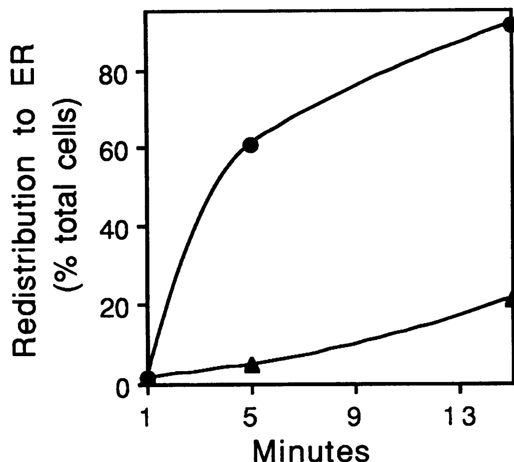

Figure 8. Calmodulin activity is required for BFA-stimulated Golgi membrane tubule elongation. Clone 9 rat hepatocytes were pretreated for 1 min with BFA (10 μg/ml) to induce some tubule formation (but not enough to complete Golgi membrane redistribution to the ER), before the subsequent addition of solvent alone (circles) or W-7 (50 μM) (triangles), for various lengths of time. Cells were then fixed and prepared for immunofluorescence microscopy using a polyclonal antibody directed against αMII. Finally, the percentage of cells with Golgi membranes that had fully redistributed to the ER was determined and plotted. Each data point represents the average of three experiments.

these pulse-labeled cells were then incubated for various lengths of time in a chase solution lacking Tfn-FITC, but containing, when appropriate, W-7 (25 μM). Finally, cells were fixed and prepared for immunofluorescence microscopy using a monoclonal antibody directed against TfnRs.

In control cells incubated with Tfn-FITC alone for 30 min, Tfn-FITC and TfnRs colocalized in small endocytic vesicles that were dispersed throughout the cytoplasm (Figure 10, A and B). In W-7–treated cells, however, Tfn-FITC and TfnRs colocalized in vacuoles that were significantly larger than those found in control cells (Figure 10, C and D).

When cells were pulsed with Tfn-FITC for 30 min and then chased in Tfn-FITC-free media in the absence of W-7, Tfn-FITC was recycled to the plasma membrane and released into the media, resulting in a significant loss of fluorescent signal as expected from previous studies (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). For example, after 15 min of chase, a small decrease in the amount of Tfn-FITC staining associated with endocytic vesicles was observed (Figure 10E), but by 1 h in chase, almost all the Tfn-FITC had recycled, and very little remained in cells (Figure 10I). The amount of Tfn-FITC staining after a 60-min chase was, therefore, markedly decreased, when compared with a 30-min pulse alone. The steady-state distribution of TfnRs, however, remained unchanged during the entire chase period (Figure 10, F and J).

However, when cells were pulsed with Tfn-FITC for 30 min and then chased in Tfn-FITC–free media containing W-7 (25 μM) for even up to 1 h, Tfn-FITC was not significantly recycled and lost from intracellular compartments. Instead, Tfn-FITC remained in large endocytic vacuoles (Figure 10, G and K), and colocalized, under these conditions, with TfnRs (Figure 10, H and L). W-13 similarly inhibited Tfn-FITC and TfnR recycling, whereas W-12 did not (our unpublished data), suggesting, therefore, that the observed effects were calmodulin specific.

Finally, we determined that the effect of W-7 on Tfn transport was reversible. As expected from the above experiments, Tfn-FITC internalized for 45 min in the presence of W-7 colocalized with the TfnR, and both markers accumulated in enlarged endosomal vacuoles (Figure 11A). This block in Tfn recycling was relieved when cells pre-loaded with Tfn-FITC in the presence of W-7 were washed free of the antagonist for 90 min. Under these conditions, Tfn-FITC was lost from cells and the TfnR was found to stain small vesicles throughout the cytoplasm (Figure 11B). Likewise, when cells were first treated with W-7 for 45 min, and then washed free of the antagonist for 90 min, Tfn-FITC added subsequently was internalized and delivered to small endosomal vesicles that colocalized with the TfnR, as in untreated control cells (Figure 11C).

DISCUSSION

The idea that intracellular membrane tubules may be important intermediaries in membrane trafficking is not new. In fact, intracellular membrane tubules of relatively uniform diameter (50–80 nm), but variable lengths, have been found extending from the endoplasmic reticulum (Lee and Chen, 1988), Golgi complex (Novikoff *et al.*, 1971; Rambourg *et al.*, 1979), TGN (Cooper *et al.*, 1990; Rambourg and Clermont, 1990; Ladinsky *et al.* 1994), and endosomes (Geuze *et al.*, 1983; Marsh *et al.*, 1986). In addition, endosome tubules are thought to be the means by which membrane and endocytosed transmembrane receptors are efficiently recycled back to the plasma membrane (Geuze *et al.*, 1983; Dunn *et al.*, 1989; Mayor *et al.*, 1993).

A renewed interest in the role that membrane tubules play in intracellular membrane trafficking has emerged from studies with the fungal metabolite BFA, a compound that has been shown not only to prevent COPs and clathrin from associating with Golgi and TGN membranes, respectively (Donaldson *et al.*, 1990; Robinson and Kreis, 1992; Wong and Brodsky, 1992), but also to cause those same membranes to dramatically increase the number and length of their associated membrane tubules (Lippincott-Schwartz *et al.*, 1990, 1991; Wood *et al.*, 1991; Wood and Brown, 1992). Although the molecular mechanism by which these tubules form have yet to be identified, some studies

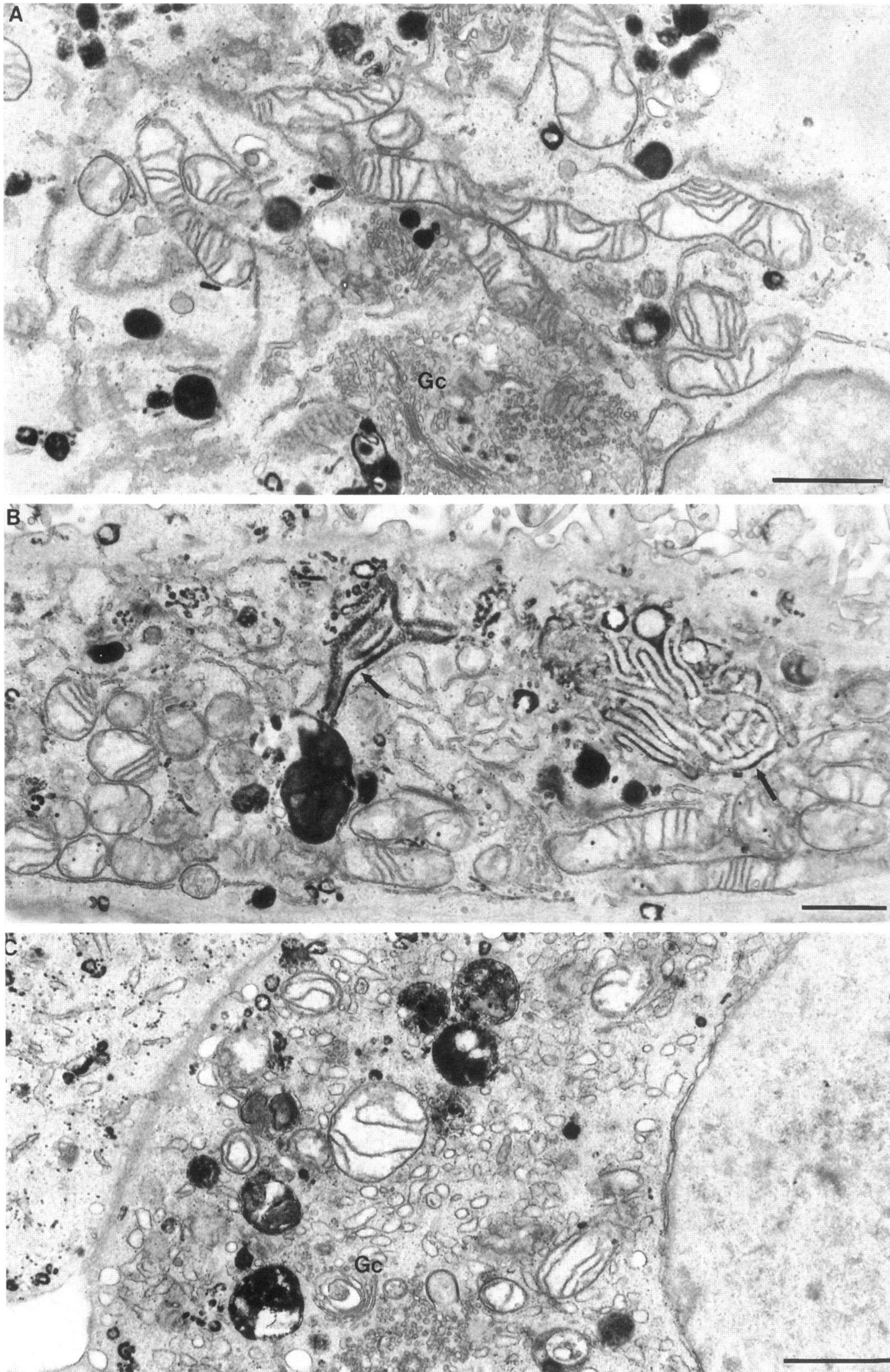

Figure 9. Electron microscopic observations of W-7 inhibition of BFA-stimulated endosome tubulation. (A) In control, untreated cells, HRP internalized continuously for 45 min is delivered to a spectrum of early, late, and recycling endosomes, which appear predominately as spherical 0.1–0.4 μm (diameter) vesicles. (B) After uptake of HRP for 45 min, a brief 5-min treatment with BFA produces extensive tubulation of endosomal compartments (arrows). The tubules can reach many microns in length. (C) Pretreatment of cells with W-7 (25 μM for 5 min) completely abolishes the tubulation of endosomes by subsequently added BFA. The endosomes in this case are generally spherical and larger than their untreated counterparts. Bars, 1 μm.

suggest that BFA may render organelle membranes especially susceptible to the activity of cytosolic tubulation factors (Conradt *et al.*, 1993; Cluett *et al.*, 1993; de Figueiredo *et al.*, unpublished data). In this regard, Apodaca and colleagues have recently provided evidence suggesting that calmodulin may be important

for forming endosome membrane tubules in polarized epithelial cells (Apodaca *et al.*, 1994). We have extended this work in a number of ways to implicate a role for calmodulin activity in membrane tubulation and trafficking in general. First, the potent calmodulin antagonists W-7, W-13, and C-1 inhibited, in a dose-dependent manner, the formation of BFA-stimulated tubules from Golgi, TGN, and endosome membranes. Importantly, W-12 did not have a significant inhibitory effect over a similar concentration range, suggesting, therefore, that this inhibition was calmodulin specific. Second, the calmodulin antagonist–induced inhibition of BFA-stimulated membrane tubulation and redistribution occurred, even though immunoflu-

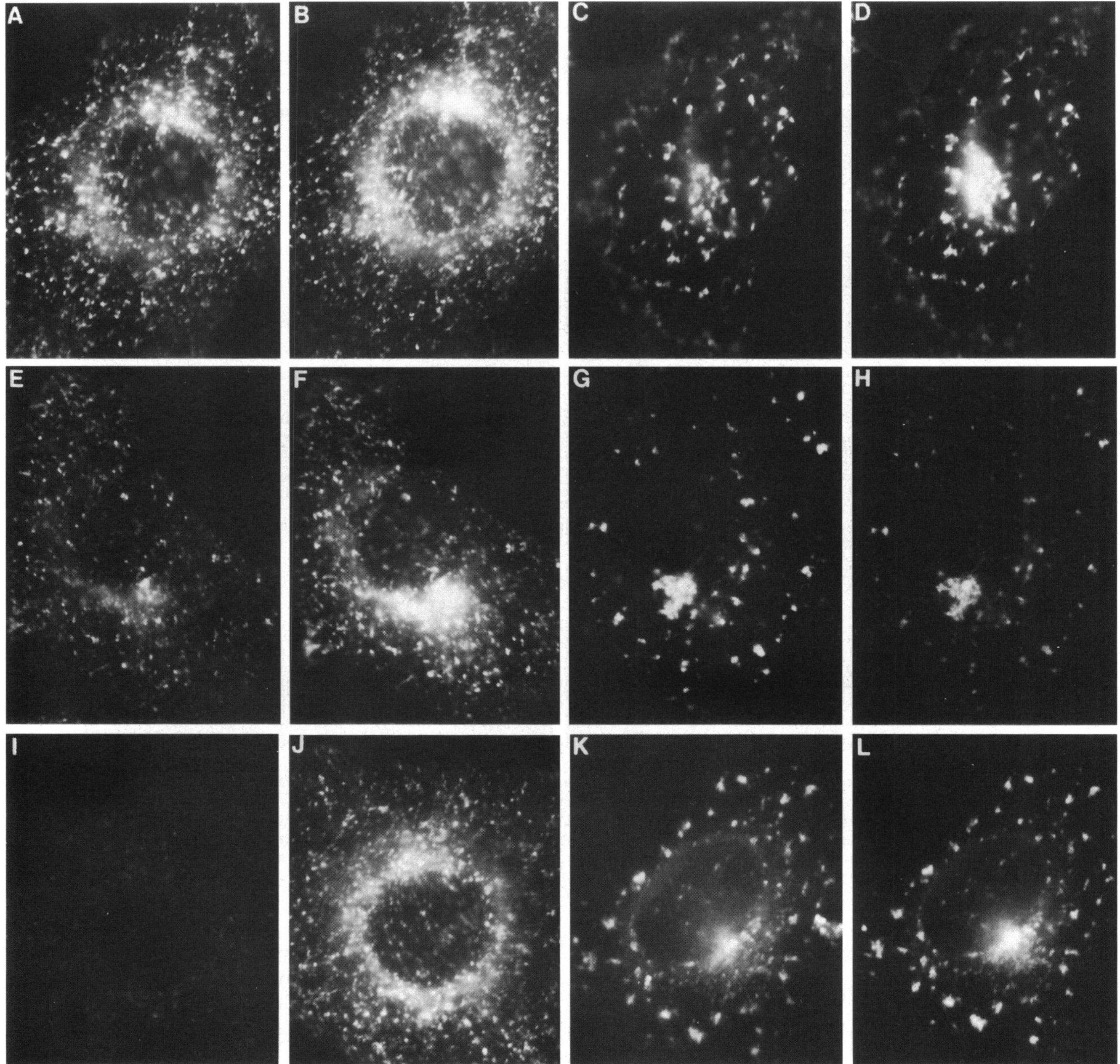

Figure 10. W-7 inhibits the recycling of Tfn-FITC and the TfnR. HeLa cells were incubated with media containing either Tfn-FITC (20 μg/ml) and 25 μM W-7 (C, G, and K), or Tfn-FITC alone (A, E, and I), during which time this fluorescent marker was endocytosed into endosomes (A–D). Cells were then incubated in Tfn-FITC-free chase media for 15 (E–H) or 60 (I–L) min, in the presence or absence of W-7 (25 μM). Finally, cells were fixed and prepared for immunofluorescence microscopy using antibodies directed against the cytoplasmic tail of the TfnR (B, D, F, H, J, and L).

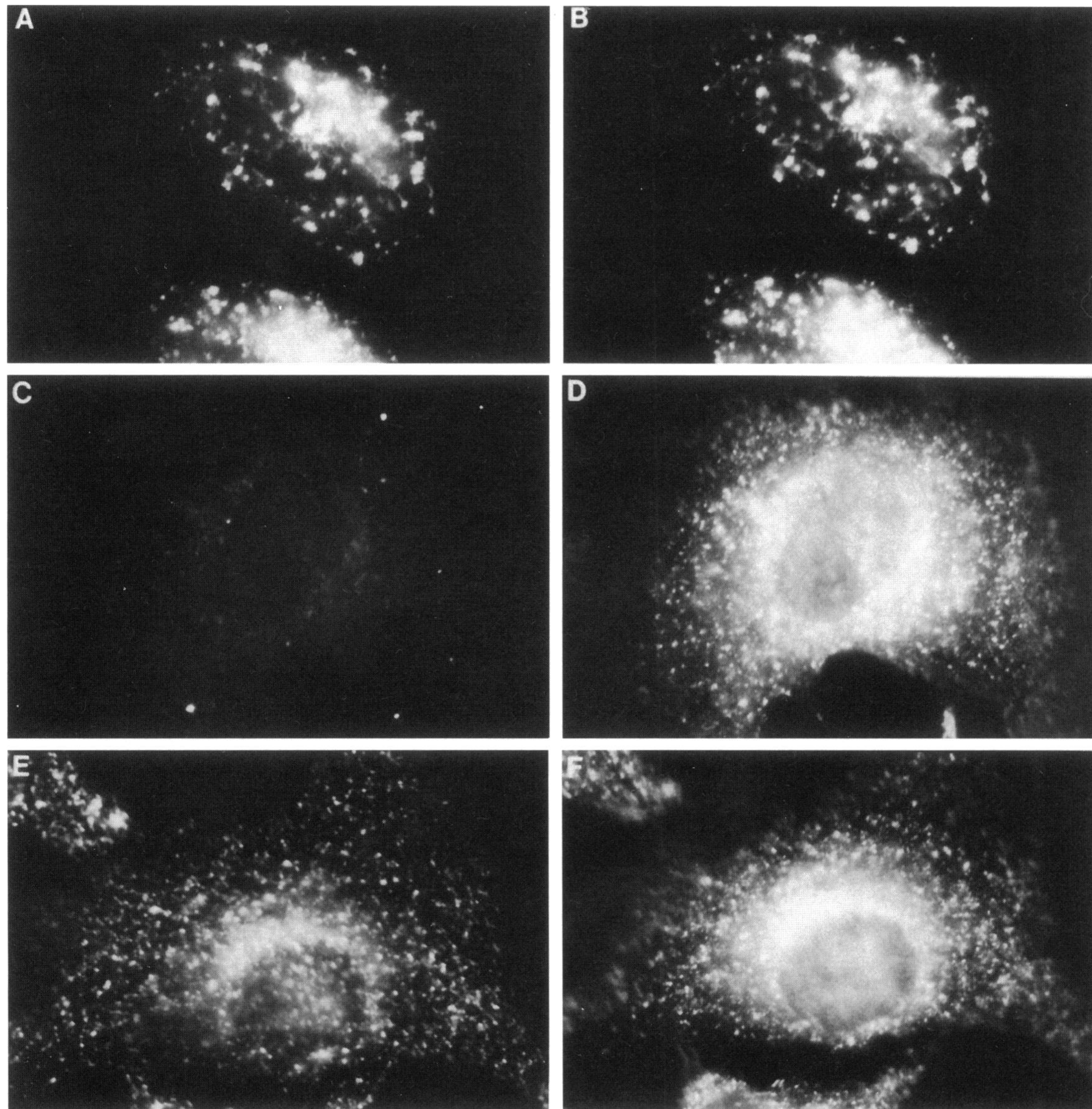

Figure 11. Reversible inhibition of Tfn-FITC recycling by W-7. HeLa cells were incubated with media containing Tfn-FITC (20 mg/ml) and W-7 (25 μM) for 45 min, during which time this fluorescent marker accumulated in enlarged endocytic vacuoles (A), that colocalized with TfnRs (B). Identically treated cells were subsequently washed free of W-7 for 90 min, during which time the Tfn-FITC was lost from cells (C), and the TfnR was found to stain small vesicles throughout the cytoplasm (D). Finally, cells were first treated with W-7 (25 μM) for 45 min, washed free of the antagonist for 90 min, and then incubated with Tfn-FITC (20 mg/ml) for 45 min (E), before subsequent fixation and staining for the TfnR (F).

orescence microscopy clearly revealed that proteins of the coatamer and clathrin complexes were no longer associated with Golgi and TGN membranes, respectively. Clearly, the inability to form membrane tubules in the presence of calmodulin antagonists was not due to an inhibition of the BFA effect on COPs or clathrin. It seems likely, therefore, that calmodulin is more directly linked to the membrane tubulation machinery itself, than to either the BFA-sensitive COP or clathrin coat proteins.

Third, when calmodulin antagonists were added to cells after Golgi tubules had already formed (by pretreating those cells with BFA for a short time), the rate of Golgi membrane tubule growth and redistribution, when compared with controls, dramatically decreased. These observations suggest that a calmodulin-dependent activity is required throughout the process of membrane tubule initiation and elongation. Finally, the inhibition of BFA-stimulated tubulation was neither cell-type- nor species-specific, as W-7,

W-13, and C-1, potently inhibited membrane tubulation in rat liver (Clone 9) and kidney (NRK) cells, and human fibroblasts (HeLa). Taken together, our data support the idea that calmodulin is an ubiquitous tubulation factor required in a variety of cells for the elongation of BFA-stimulated tubules from Golgi, TGN, and endosome membranes.

Many questions remain concerning the role calmodulin plays in forming the tubules that are known to extend from Golgi, TGN, and endosome membranes in the absence of BFA (Novikoff *et al.*, 1971; Lee and Chen, 1988; Cooper *et al.*, 1990; Rambourg and Clermont, 1990; Ladinsky *et al.*, 1994). Although we have not tackled this question directly, some of our data are very revealing. First, as Apodaca and colleagues have shown (Apodaca *et al.*, 1994), and we have now verified, the recycling of Tfn from endosomes to the plasma membrane is greatly inhibited for relatively long periods of time (1 h) in cells treated with low concentrations of calmodulin antagonists. In addition, under these conditions, novel relatively nontubular endocytic structures accumulate in cells. These effects are entirely consistent with the idea that calmodulin activity is involved in the formation of the recycling endosome tubules that have been implicated in returning membrane and receptors to the cell surface (Geuze *et al.*, 1983; Dunn *et al.*, 1989; Mayor *et al.*, 1993). Second, in the presence of calmodulin antagonists, the Golgi complex becomes much more compact (as viewed by immunofluorescence) than its untreated counterpart, even though Golgi stacks are still observed by electron microscopy. A possible explanation for this change in Golgi morphology is that calmodulin antagonists may decrease the number and length of the membrane tubules and tubular fenestrations that have previously been shown to link adjacent stacks of Golgi cisternae (Rambourg *et al.* 1979; Rambourg and Clermont, 1990). It seems, therefore, that our data suggest that calmodulin is a molecular link between two morphologically similar processes occurring on diverse organelle membranes: BFA-stimulated and BFA-independent membrane tubulation.

That calmodulin activity is required for membrane trafficking events is also not a new idea. In fact, calmodulin activity has previously been implicated in regulated exocytosis (Nishikawa *et al.*, 1980; Henquin *et al.*, 1981; Steinhardt and Alderton, 1982), phagocytosis (Horwitz *et al.*, 1981), and endocytosed receptor recycling to the plasma membrane (Grasso *et al.*, 1990; Apodaca *et al.*, 1994). Given that calmodulin plays the role of a second messenger molecule in diverse intracellular signaling systems, it seems likely that calmodulin operates somewhere upstream of the actual tubulation machinery. Calmodulin may, for example, activate cellular kinases, phosphatases, or cytoskeletal-associated proteins (for reviews see Walsh and Hartshorne, 1982; Klee, 1988), which in turn activate or are a component of, the membrane tubulation machinery itself.

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