

# Forskolin Inhibits and Reverses the Effects of Brefeldin A on Golgi Morphology by a cAMP-independent Mechanism

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**Abstract.** Brefeldin A (BFA) causes rapid redistribution of Golgi proteins into the ER, leaving no definable Golgi apparatus, and blocks transport of proteins into post-Golgi compartments in the cell. In this study we follow the disassembly of the Golgi apparatus in BFA-treated, living cells labeled with NBD-ceramide and demonstrate that forskolin can both inhibit and reverse this process. Long, tubular processes labeled with NBD-ceramide were observed emerging from Golgi elements and extending out to the cell periphery in cells treated with BFA for 5 min. With longer incubations in BFA, the NBD label was dispersed in a fine reticular pattern characteristic of the ER. Treatment with forskolin inhibited these effects of BFA as well as BFA's earliest morphologic effect on the Golgi apparatus: the redistribution to the cytosol of a 110-kD Golgi peripheral membrane protein. In addition, forskolin could reverse BFA's block in protein secretion. Forskolin inhibition of BFA's effects was dose dependent and reversible. High concentrations of BFA could overcome forskolin's inhibitory effect, suggesting for-

skolin and BFA interact in a competitive fashion. Remarkably, in cells already exposed to BFA, forskolin could reverse BFA's effects causing the 110-kD Golgi peripheral membrane protein to reassociate with Golgi membrane and juxtannuclear Golgi complexes to reassemble. Neither membrane permeant cAMP analogues nor cAMP phosphodiesterase inhibitors could replicate or enhance forskolin's inhibition of BFA. 1,9-Dideoxyforskolin, which does not activate adenylyl cyclase, was equally as effective as forskolin in antagonizing BFA. A derivative of forskolin, 7-HPP-forskolin, that is less potent than forskolin at binding to adenylyl cyclase, was also equally effective as forskolin in antagonizing BFA. In contrast a similar derivative, 6-HPP-forskolin, that is equipotent with forskolin at binding to adenylyl cyclase, did not inhibit BFA's effects. These results suggest that forskolin acts as a competitive antagonist to BFA, using a cAMP-independent mechanism to prevent and reverse the morphologic effects induced by BFA.

Two fundamental questions in cell biology are how membrane organelles of the secretory system maintain their identity and how selective transport between these organelles is achieved. *In vitro* reconstitution of interorganelle transport (Balch et al., 1984; Beckers et al., 1987), and the application of yeast genetics for the identification of specific genes required for movement through the secretory pathway (Schekman, 1985) are two lines of research that are beginning to illuminate the biochemical basis for selective interorganelle transport. These approaches have revealed integral components of the secretory transport machinery but have yet to define the molecular basis of organelle identity. The finding that the drug brefeldin A (BFA)<sup>1</sup> has pronounced effects on both transport out of and

the existence of the Golgi apparatus has provided an additional approach for addressing the questions of organelle identity and selective membrane transport.

Three phenotypic changes are seen in cells exposed to BFA: (a) protein transport within the secretory pathway beyond the Golgi apparatus is blocked (Misumi et al., 1986; Oda et al., 1987); (b) the Golgi apparatus rapidly disassembles and no longer exists as a distinct cellular compartment (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989); and (c) resident and itinerant Golgi proteins, as well as Golgi lipid, are redistributed into the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Young et al., 1990). Whether all these phenotypic changes are the result of the perturbation of a single molecular target remains to be established. BFA's effects, however, fulfill a variety of criteria for the type of pharmacologic tool that might yield insight into the mechanisms underlying organelle identity and interorganelle

1. *Abbreviations used in this paper:* BFA, brefeldin A; IBMX, isobutyl methylxanthine; Man II, mannosidase II.

trafficking. BFA's effects appear to be specific for the Golgi apparatus and are rapidly and completely reversed by removing the drug (Misumi et al., 1986; Lippincott-Schwartz et al., 1989; Fujiwara et al., 1989). In addition, disruption of the Golgi apparatus by BFA occurs at relatively low concentrations (50–100 nM). Finally, minor chemical modifications of BFA completely abrogate its cellular effects (Donaldson, J. G., J. Lippincott-Schwartz, and R. Klausner, unpublished observations).

Previous studies with BFA have detailed the phenotypic changes within cells as a consequence of BFA treatment using morphological and biochemical techniques. Resident membrane proteins of the *cis*-, medial, and *trans*-Golgi system have been observed to redistribute rapidly into the ER after treatment of cells with BFA in contrast to components of the *trans*-Golgi network (Fujiwara et al., 1989; Lippincott-Schwartz et al., 1989, 1990; Chege and Pfeffer, 1990). As a consequence of this, carbohydrate side chains of resident or retained proteins in the ER are processed as if they have been transported to the Golgi complex (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Ulmer and Palade, 1989). Although no definable Golgi structures remain in cells exposed to BFA (Fujiwara et al., 1988), once BFA is removed Golgi proteins rapidly sort out of the ER and a recognizable Golgi apparatus is reformed (Lippincott-Schwartz et al., 1989).

Recent studies with BFA have identified specific biochemical changes of the Golgi apparatus in cells treated with this compound. Donaldson et al. (1990) observed rapid alteration in the intracellular localization of a 110-kD cytoplasmically oriented, peripheral membrane protein of the Golgi apparatus (Allan and Kreis, 1986) upon addition of BFA to cells. Within 30 s of BFA treatment, the intracellular location of the 110-kD protein shifted from a Golgi distribution to a diffuse cytoplasmic pattern. This shift preceded all other observable morphologic changes in the Golgi apparatus and was reversible. Upon removal of BFA, the 110-kD protein rapidly reassociated with newly forming Golgi structures.

These observations point to the presence of critical molecules (i.e., the molecular targets of BFA) that must be regulating and maintaining both the structure of the Golgi apparatus and its ability to allow anterograde membrane transport. Deciphering how BFA is working in cells ultimately will require the identification of BFA's binding site(s). An important step toward this goal is the understanding of the structure and function of BFA through the identification of drugs that either mimic, antagonize, or alter the effects of BFA. We report here on one such reagent, the naturally occurring diterpene, forskolin, which initially was observed to interfere with BFA's effects by J. Glickman and M. Sheetz.

Forskolin is known to have multiple effects on cells, including stimulation of the enzyme adenylyl cyclase, leading to a dramatic rise in cAMP levels in cells (Seamon and Daly, 1986), and the cAMP independent inhibition of both membrane transport and channel proteins (Laurenza et al., 1989). In this paper we demonstrate that forskolin can inhibit and reverse BFA's morphologic effects on the Golgi apparatus using the vital stain *N*-(7-(4-nitrobenzo-2-oxa-1,3-diazole))-6-aminocaproylsphingosine (NBD-ceramide) and antibodies to the *cis*/medial Golgi enzyme mannosidase II (Man II), as markers for the Golgi complex. Forskolin inhibition and reversal of BFA action occurred in a competitive manner by a mechanism that was cAMP independent. These results

suggest forskolin acts as a competitive antagonist to BFA, making it a useful probe for the dissection of the mechanism by which BFA disrupts Golgi structure and membrane trafficking.

## Materials and Methods

### Materials and Cells

NRK (normal rat kidney) cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin, and streptomycin. TCR $\alpha$ GPI cells were a kind gift of Mark Davis (Stanford University, CA) and were developed as described in Lin et al. (1990). Cells were grown on 12-mm round glass coverslips for 1–2 days before use.

Brefeldin A was either purchased from Epicentre Technologies (Madison, WI) or obtained from Sandoz Ltd. (Basel, Switzerland). It was stored at –20°C as a stock solution of 1 mg/ml in methanol. NBD ceramide was obtained from Molecular Probes Inc. (Eugene, OR) and was stored as a solid at –20°C or as 200 nmol/ml stock in 2:1 chloroform/methanol at 4°C. Forskolin, dibutyl cAMP, and IBMX were of highest available grade and were purchased from Sigma Chemical Co. (St. Louis, MO). 6-HPP-forskolin, and 7-HPP-forskolin were prepared according to Robbins, J., A. Laurenza, G. O. O'Malley, B. Spahl, R. W. Kosley, and K. B. Seamon (manuscript submitted for publication). 1,9-Dideoxyforskolin was purchased from Calbiochem-Behring Corp., San Diego, CA.

### Antibodies

Rabbit IgG directed against Golgi Man II (Moremen and Tbuster, 1985) was the kind gift of Dr. K. Moremen (Massachusetts Institute of Technology, Cambridge, MA). The mouse monoclonal antibody M3A5 recognizes the 110-kD Golgi peripheral membrane protein (Allan and Kreis, 1986). The mouse monoclonal antibody, A2B4-2, binds to the TCR $\alpha$  chain (Samelson et al., 1983). Rhodamine- and fluorescein-labeled goat anti-mouse and goat anti-rabbit IgG were purchased from Organon-Teknika (Westchester, PA) and used at 1:500 dilution of manufacturer's stock solution.

### NBD-Ceramide Labeling of Cells

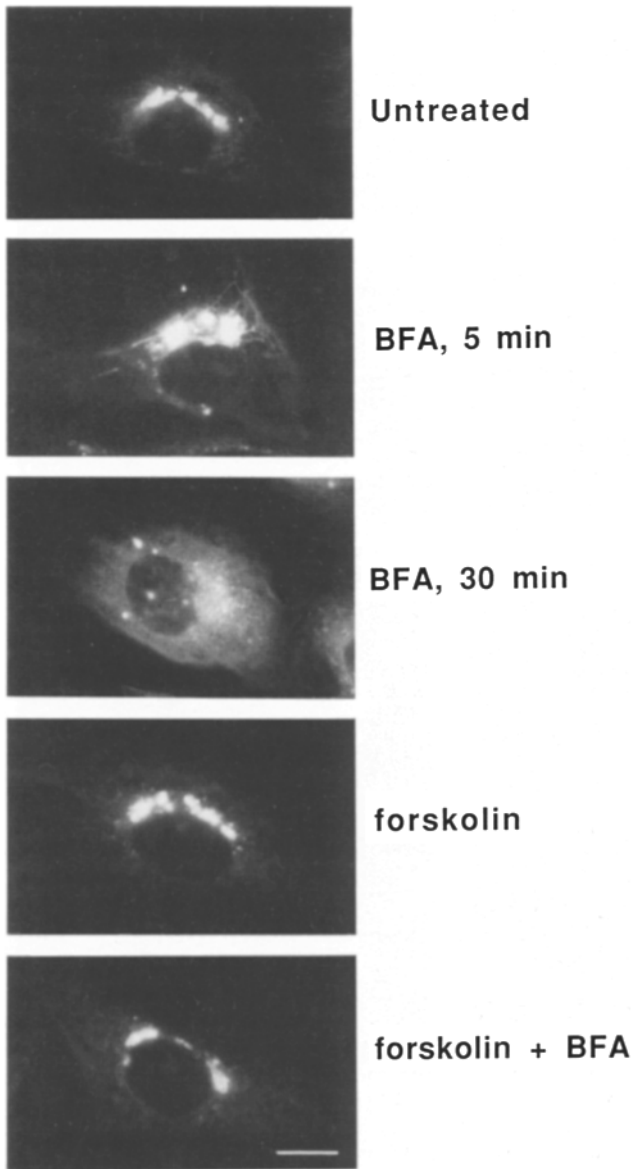
NBD-ceramide-BSA conjugates were prepared in the appropriate serum-free medium according to Lipsky and Pagano (1985). Cells plated on glass coverslips were washed once with serum-free medium, incubated for 10 min at 37°C with NBD-ceramide-BSA, and washed three times with complete media (5 min at 37°C for each wash). Labeled cells were maintained at 37°C until use and were used within 30–45 min of labeling. Images of labeled cells were obtained using the fluorescein channel on a Zeiss microscope.

### Immunofluorescence Microscopy

NRK cells were grown on No. 1 glass coverslips to 60–70% confluency. After appropriate incubation conditions, the cells were fixed for 10 min at room temperature in 2% formaldehyde in PBS and washed in PBS containing 10% fetal calf serum. For permeabilization, cells were incubated in methanol at 0°C for 1 min and then rinsed in PBS/10% serum. Cells were incubated in primary antibody in PBS/serum and 0.2% saponin for 1 h, washed free of antibody in PBS/serum, and then incubated in fluorescently labeled secondary antibody in PBS/serum/0.2% saponin for 1 h. After a final washing in PBS/serum and then PBS alone the cells were mounted onto glass slides in fluoromount G (Southern Biotechnology Associates), and viewed with a 63 $\times$  oil planapo lens on a Zeiss microscope equipped with barrier filters to prevent crossover of fluorescein and rhodamine fluorescence.

### Metabolic Labeling and Immunoprecipitation

For metabolic labeling, cells were preincubated for 5 min at 37°C in methionine-free medium containing 10% fetal bovine serum. The cells were then labeled for 30 min with 0.25 mCi/ml [<sup>35</sup>S]methionine (Trans-<sup>35</sup>S-label; ICN Biomedicals, Inc., Irvine, CA) in methionine-free medium containing 10% fetal bovine serum. At the end of the pulse, radioactive medium was removed and the cells were chased in complete medium for 2 h. The chase was terminated by placing the cells on ice. Solubilization



**Figure 1.** Effects of BFA and forskolin on the morphology of the Golgi apparatus in living NRK cells stained with the fluorescent lipid NBD-ceramide. NRK cells were stained with NBD-ceramide and treated with nothing (*Untreated*), with 1  $\mu\text{g/ml}$  BFA for 5 min (*BFA, 5 min*), with 0.1  $\mu\text{g/ml}$  BFA for 30 min (*BFA, 30 min*), with 100  $\mu\text{M}$  forskolin for 1 h (*forskolin*), or with 100  $\mu\text{M}$  forskolin for 30 min and then forskolin (100  $\mu\text{M}$ ) plus BFA (0.1  $\mu\text{g/ml}$ ) for 30 min (*forskolin + BFA*). Living cells were viewed by fluorescence microscopy using fluorescein optics. In untreated cells, NBD-ceramide-labeled Golgi structures appeared as juxtannuclear, tightly packed cisternae. Treatment of cells with BFA for 5 min caused the cisternae to enlarge and to extend thin, tubular processes out to the cell periphery (*BFA, 5 min*). After 30 min of BFA treatment, the NBD-ceramide label now appeared in a diffuse pattern with some accumulation in juxtannuclear vesicles (*BFA, 30 min*). Pretreatment of cells with forskolin inhibited BFA-induced dispersal of NBD-ceramide (*forskolin + BFA*), while treatment with forskolin alone had no effect on the Golgi distribution of NBD-ceramide. Bar, 10  $\mu\text{m}$ .

and immunoprecipitation using antibody adsorbed to protein A-agarose (Bethesda Research Laboratories, Gaithersburg, MD) were performed as previously described (Lippincott-Schwartz et al., 1988). Immunoprecipitated proteins were analyzed by SDS-PAGE on polyacrylamide gels (13%)

using the buffer systems of Laemmli as described in Samelson et al. (1985). Digestion with the enzyme endo H (Genzyme, Boston, MA) was performed by incubating the immunoprecipitates for 16 h at 37°C with 5 mU of endo H in 50  $\mu\text{l}$  of 0.1 M sodium phosphate buffer (pH 6.1) containing 0.1% Triton X-100, 0.03 M SDS, and 20 mM EDTA.

## Results

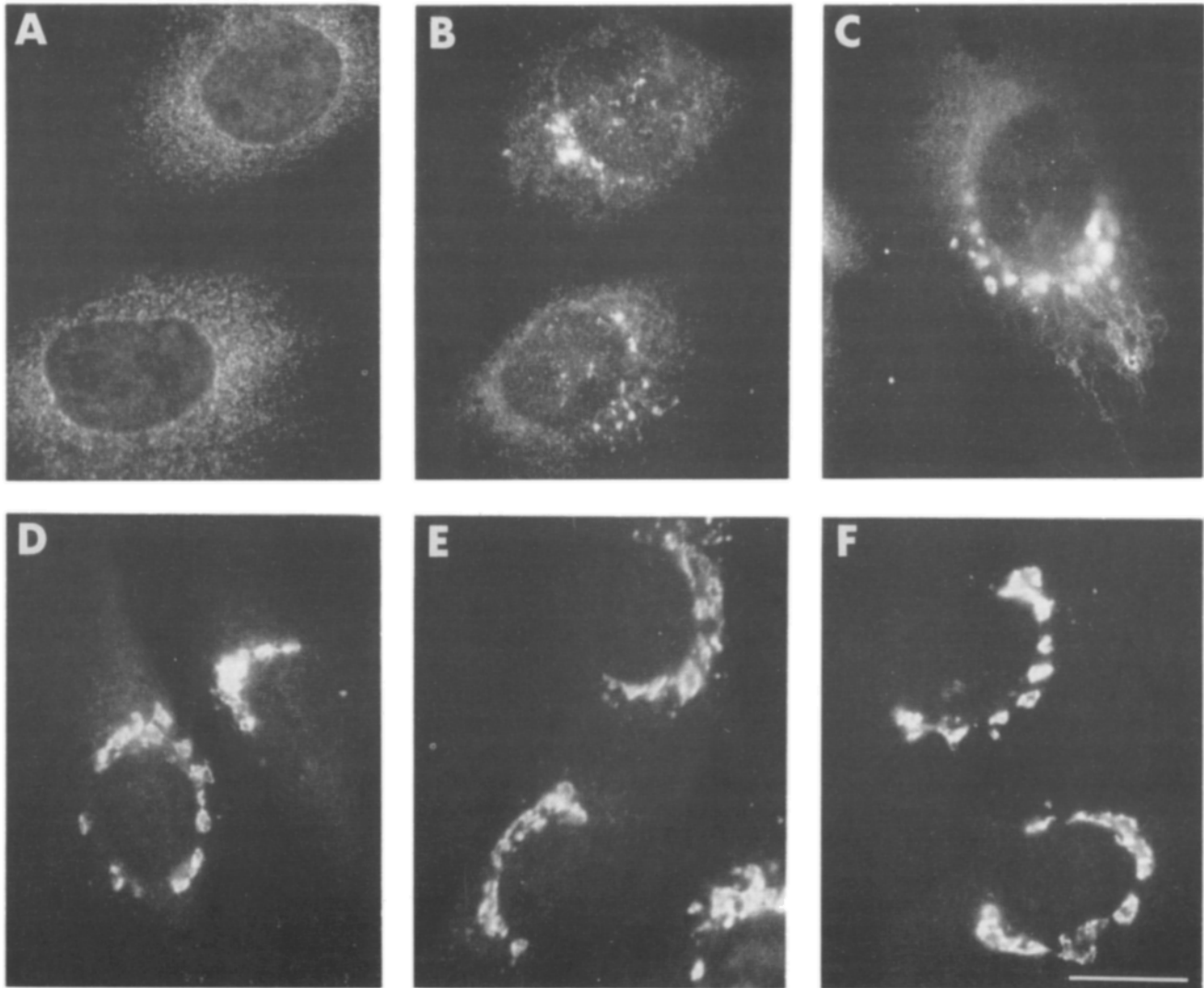
### *Effects of BFA on the Membranes of the Golgi Apparatus in Living Cells*

Previous work has demonstrated that BFA causes the rapid and reversible redistribution of Golgi resident and itinerant proteins into the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Fujiwara et al., 1989). To determine the fate of Golgi membranes in BFA-treated cells we examined the effect of this drug on the distribution of the fluorescent lipid NBD-ceramide, which specifically associates with Golgi membranes of living cells (Lipsky and Pagano, 1985). Since the NBD-ceramide label remains associated with the Golgi apparatus for 2–3 h and can be easily visualized with fluorescence optics, this system is excellent for monitoring movement of Golgi membranes in living cells (Ho et al., 1989; Cooper et al., 1990).

In untreated NRK cells, NBD-ceramide strongly and specifically labeled a perinuclear membrane compartment (Fig. 1, *Untreated*) which previously has been shown to be the Golgi apparatus by double labeling with markers for Golgi-specific proteins (Lipsky and Pagano, 1985). The NBD-ceramide-labeled Golgi complex in NRK cells ranged in appearance from elongated, interconnected networks to a tightly massed ring or half ring surrounding the nucleus. Addition of BFA to the cells had a rapid and striking effect on the morphology of the NBD-ceramide-labeled Golgi complex. Within 5 min of BFA treatment at 37°C, numerous long, thin tubular processes extending out of brightly-stained and enlarged Golgi structures were evident (Fig. 1, *BFA, 5 min*). These processes appeared as continuous tubules that extended up to 10  $\mu\text{m}$  from Golgi elements. By 30 min, the Golgi label was dispersed in a fine reticular pattern throughout the cell (Fig. 1, *BFA, 30 min*). In some cells, in addition to this dispersed pattern, numerous small vesicles in a juxtannuclear location were labeled with the fluorescent dye. However, no fluorescence in large Golgi-like structures remained. These results with NBD-ceramide in living cells closely parallel the morphological changes observed for Golgi resident proteins during BFA treatment (Lippincott-Schwartz et al., 1990). Thus, Golgi lipid appears to share the fate of resident Golgi proteins upon BFA treatment.

### *Effects of Forskolin on the Distribution of NBD-Ceramide in BFA-treated Cells*

Since many cellular functions are under the regulatory influence of cAMP, and cAMP-dependent protein kinase II has been localized to the Golgi apparatus (Nigg et al., 1985), we wondered whether BFA's effects on Golgi structure and dynamics is either due to or regulated by changes in cellular cAMP levels. To explore this possibility we examined the effect of forskolin, an activator of adenylyl cyclase, on BFA's action. As shown in Fig. 1 (*forskolin + BFA*), pretreatment of cells with 100  $\mu\text{M}$  forskolin for 30 min before addition of 0.1  $\mu\text{g/ml}$  BFA for 30 min resulted in no change in morphol-

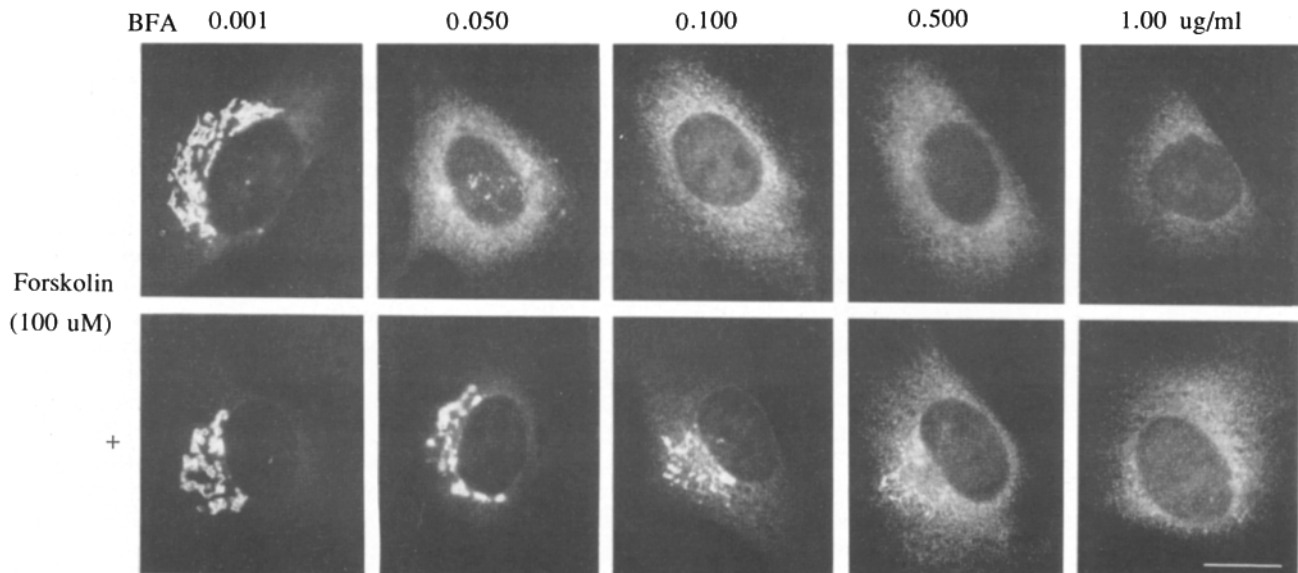


**Figure 2.** Dose response to forskolin in BFA-treated cells. NRK cells were treated with 0 (A), 10 (B), 25 (C), 50 (D), 100 (E), or 200  $\mu\text{M}$  (F) forskolin for 30 min at 37°C. BFA (0.1  $\mu\text{g}/\text{ml}$ ) was then added to the cells for an additional 1-h incubation in the continued presence of forskolin. The cells were then fixed, permeabilized, and stained for immunofluorescence using antibodies to Man II. At 0.1  $\mu\text{g}/\text{ml}$  BFA (A), Man II labeling appeared only in the nuclear envelope and in a diffuse reticular pattern due to disassembly of the Golgi apparatus induced by BFA. Preincubation of cells with forskolin at concentrations at and above 50  $\mu\text{M}$  (D, E, and F) before BFA treatment inhibited Golgi disassembly. At these concentrations of forskolin, Man II appeared in normal looking juxtannuclear Golgi structures. Man II staining in cells treated with 10 (B) or 25  $\mu\text{M}$  (C) forskolin before BFA treatment showed diffuse ER staining in addition to staining of distinct vacuoles and tubular processes. Bar, 10  $\mu\text{m}$ .

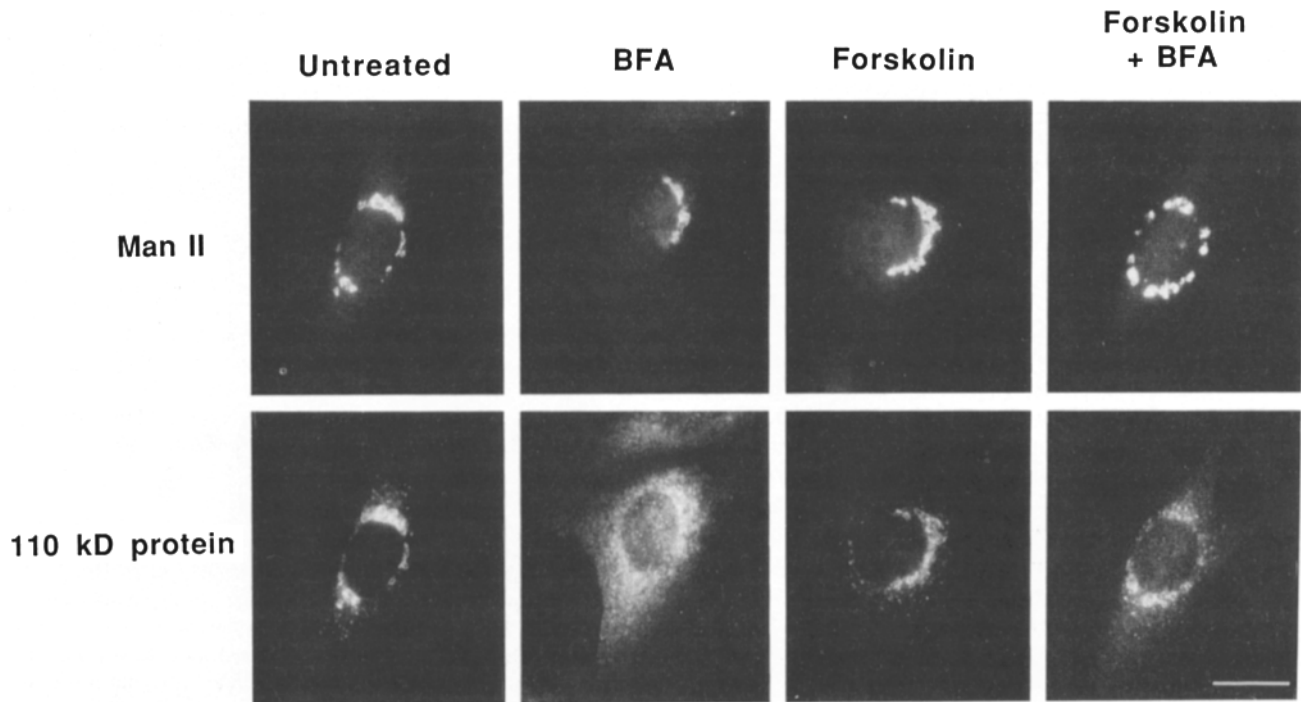
ogy of the Golgi apparatus. Since cells treated for 30 min with 0.1  $\mu\text{g}/\text{ml}$  BFA alone showed dispersal of NBD-ceramide from perinuclear Golgi structures into a diffuse ER-like network (Fig. 1, *BFA, 30 min*), these results suggested that forskolin could inhibit BFA's effects on Golgi morphology. Removal of forskolin in the continued presence of BFA resulted in rapid dispersal of NBD-labeled Golgi elements and the redistribution of the NBD label into a fine punctate ER-like pattern indicating forskolin's inhibitory effects on BFA were reversible (data not shown). Forskolin treatment alone had very little effect on the distribution of NBD-ceramide within cells (Fig. 1, *forskolin*). If anything, the Golgi apparatus was more brightly stained by NBD-ceramide in forskolin-treated cells and appeared more compact than in control cells.

#### ***Forskolin Acts as a Competitive Antagonist to BFA's Effect on the Golgi Apparatus***

We sought to determine whether forskolin acted at the same site as BFA or at an independent target distal to the BFA target. To distinguish between these possibilities, we examined the concentration dependence of forskolin's inhibition of BFA using antibodies to the cis/medial Golgi enzyme, Man II, as marker for the Golgi apparatus. As shown in Fig. 2 A, cells treated with 0.1  $\mu\text{g}/\text{ml}$  BFA alone for 1 h showed no staining of Golgi structures. Instead, Man II was localized in the nuclear envelope and in a diffuse reticular pattern that previously has been shown by immunoelectron microscopy and biochemical techniques to include their distribution in the ER (Lippincott-Schwartz et al., 1989). Cells pretreated



**Figure 3.** Dose response to BFA in forskolin-treated cells. NRK cells were treated with or without 100  $\mu$ M forskolin for 30 min at 37°C. BFA at the noted concentrations was then added for an additional 1 h. The cells were then fixed, permeabilized, and stained with Man II antibodies for immunofluorescence. At low concentrations of BFA (0.001  $\mu$ g/ml), Man II labeling appeared in Golgi cisternae surrounding the nucleus. Addition of BFA at concentrations of 0.05  $\mu$ g/ml or higher caused Man II to redistribute into a fine punctate/reticular pattern characteristic of ER labeling. Pretreatment of cells with forskolin shifted the concentration of BFA required to observe Man II redistribution into the ER to >0.5  $\mu$ g/ml. Bar, 10  $\mu$ m.



**Figure 4.** Forskolin inhibits BFA-induced dissociation of the 110-kD protein from the Golgi apparatus. NRK cells were treated with nothing (*Untreated*), with BFA (0.1  $\mu$ g/ml) for 20 min (*BFA*), with forskolin (100  $\mu$ M) for 1 h (*Forskolin*), or treated with forskolin (100  $\mu$ M) for min and then BFA (0.1  $\mu$ g/ml) plus forskolin (100  $\mu$ M) for an additional 20 min (*Forskolin + BFA*), all at 37°C. The cells were fixed, permeabilized, and stained by double-label immunofluorescence with antibodies to Man II and the 110-kD protein. In untreated cells, Man II was localized to the Golgi apparatus which appeared as a compact, perinuclear structure in these cells. The distribution of the 110-kD protein colocalized with Man II labeling of the Golgi apparatus but also occurred in peripheral vesicles. After 20 min of BFA (0.1  $\mu$ g/ml) treatment, the 110-kD protein was widely scattered throughout the cytoplasm, while Man II remained largely associated with the Golgi apparatus due to the low concentrations of BFA used. At these concentrations of BFA both the dissociation of the 110-kD protein and subsequent redistribution of Golgi membrane into the ER are not as rapid as occurs with higher concentrations of BFA (i.e., 5  $\mu$ g/ml and greater) (see Donaldson et al., 1991). Pretreatment of cells with forskolin (100  $\mu$ M) before the addition of BFA prevented BFA-induced dissociation of the 110-kD protein from the Golgi apparatus. Forskolin treatment alone had no noticeable effect on the distributions of Man II or the 110-kD protein compared to untreated cells. Bar, 10  $\mu$ m.

with different concentrations of forskolin for 30 min before the addition of 0.1  $\mu\text{g/ml}$  BFA, however, showed a dose-dependent inhibition of Golgi apparatus dispersal. Low concentrations of forskolin (10  $\mu\text{M}$ ) had only a slight inhibitory effect on BFA-induced Golgi redistribution into the ER (Fig. 2 B). At this concentration of forskolin, Man II appeared in distinct vesicles surrounding the nucleus in addition to a diffuse reticular pattern. At 25  $\mu\text{M}$  forskolin, more distinct Golgi-like structures were apparent in the BFA-treated cells and tubular elements were frequently seen extending out of these structures (Fig. 2 C). At concentrations of forskolin  $\geq 50$   $\mu\text{M}$  (Fig. 2, D, E, and F) pronounced inhibition of BFA's effects was observed. At these concentrations of forskolin, essentially normal Golgi structures were observed. Longer incubation times at these higher concentrations of forskolin (100 and 200  $\mu\text{M}$ ) showed no delayed effect of BFA on Golgi morphology (not shown).

If forskolin acted at a site distinct from the site of BFA action then raising the concentration of BFA at an effective dose of forskolin would not be expected to overcome forskolin's effects. If, however, forskolin acted at the same site as BFA, raising the concentration of BFA might be expected to overcome forskolin's inhibitory effects. A dose-response curve of BFA in the presence of 100  $\mu\text{M}$  forskolin was performed to distinguish between these possibilities. As shown in Fig. 3, rather than inhibit BFA's effects at all concentrations of BFA, 100  $\mu\text{M}$  forskolin appeared only to shift the dose dependence of BFA action. Whereas 50 ng/ml BFA was normally sufficient to cause Golgi apparatus dispersal in NRK cells, >500 ng/ml BFA was required to achieve the same result in the presence of 100  $\mu\text{M}$  forskolin. These results suggested that forskolin and BFA act in a competitive fashion.

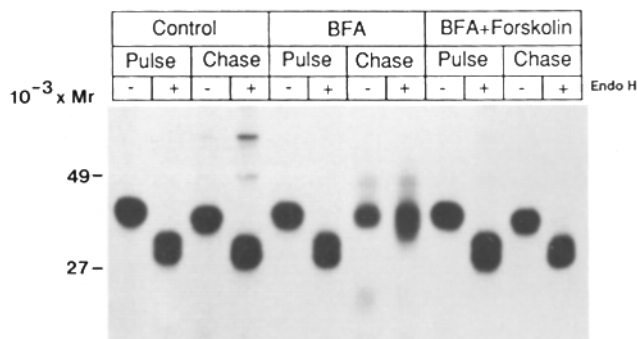
#### Forskolin Inhibits BFA's Earliest Effects on the Golgi Apparatus

We have previously shown that within minutes of BFA treatment Golgi morphology is affected (Lippincott-Schwartz et al., 1990). In addition to spreading numerous, long, thin membrane processes, the Golgi complex itself is altered in appearance: the normal compact and flattened cisternal elements become replaced by numerous vacuoles. Recently, Donaldson et al. (1990) have reported that a 110-kD cytoplasmically oriented peripheral membrane protein redistributes to the cytosol within 30 s of adding 5  $\mu\text{g/ml}$  BFA to cells. This precedes all other morphologic changes observable at the light microscope level in BFA-treated cells. The kinetics of dissociation of the 110-kD protein and movement of Man II into the ER in BFA-treated cells was found to be dose dependent. With lower concentrations of BFA, the 110-kD protein took longer to dissociate from the Golgi apparatus and subsequent transport of Golgi proteins into the ER was slower (Donaldson et al., 1990). To determine whether forskolin's inhibition of BFA's effects extended to the 110-kD protein, we examined the effect of forskolin on the dissociation of the 110-kD protein using antibodies to the 110-kD protein. In control cells, the distribution of the 110-kD protein significantly overlapping with Man II staining of the Golgi apparatus in the same cell (Fig. 4, *Untreated*). The 110-kD protein, however, appeared less tightly associated with cisternal Golgi elements compared to Man II, and also appeared to be associated with peripheral vesicles. Forskolin treatment alone had very little effect on the distribution of

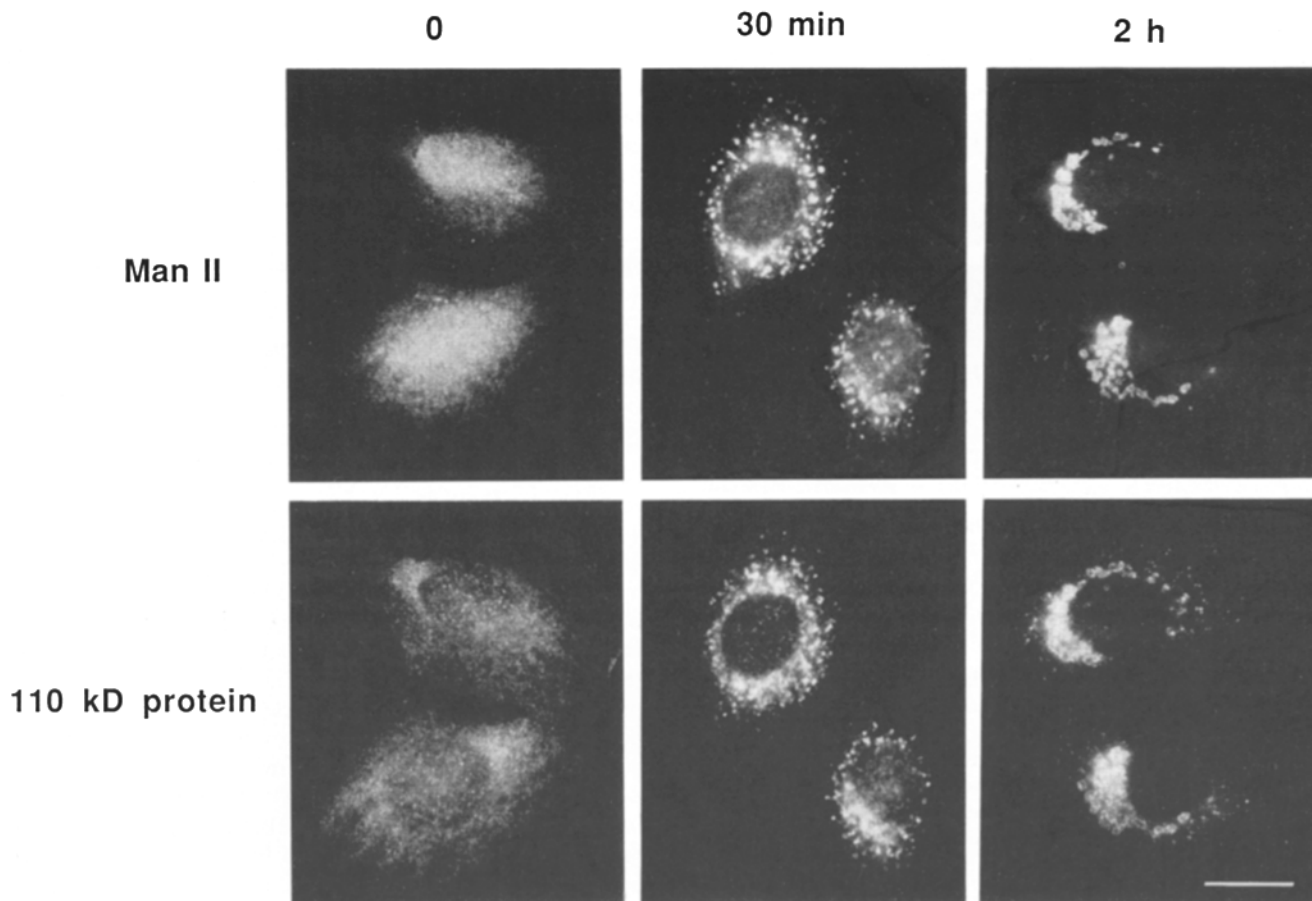
both the 110-kD protein and Man II (Fig. 4, *Forskolin*). Treatment with 0.1  $\mu\text{g/ml}$  BFA for 20 min, however, caused the 110-kD protein to dissociate from the Golgi apparatus and to distribute into a diffuse punctate pattern throughout the cytoplasm (Fig. 4, *BFA*). Due to the low concentration of BFA used in this experiment, Man II remained predominantly in perinuclear Golgi structures at this time of BFA treatment. Upon longer incubations with BFA (i.e., 30–60 min), Man II no longer appeared in Golgi structures and instead had redistributed into an ER-like pattern (see Fig. 6). The 110-kD protein retained its diffuse punctate pattern during longer BFA treatments and never colocalized with the Golgi marker. When cells were first treated with forskolin for 30 min and then BFA was added, remarkably no dissociation of the 110-kD protein from perinuclear Golgi structures was observed (Fig. 4, *Forskolin + BFA*). The 110-kD protein remained associated with perinuclear Golgi structures at all times in cells treated with forskolin plus BFA including prolonged periods (data not shown). These results indicate forskolin inhibits BFA's earliest observable effect on the Golgi apparatus; the dissociation of the 110-kD peripheral Golgi membrane protein.

#### Effect of Forskolin on the Processing of N-linked Oligosaccharide Chains in BFA-treated Cells

One consequence of the redistribution of Golgi proteins into the ER in BFA-treated cells is the modification of carbohydrate chains of ER resident and retained proteins by Golgi enzymes. This effect on carbohydrate processing has been observed for a variety of proteins (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Ulmer and Palade, 1989). If forskolin inhibits BFA-induced redistribution of Golgi pro-



**Figure 5.** Forskolin abrogates BFA's effect on the carbohydrate processing of ER retained proteins. NRK cells were pulse labeled with [ $^{35}\text{S}$ ]methionine for 30 min and chased for 2 h with normal medium (*Control*), with 0.1  $\mu\text{g/ml}$  BFA (*BFA*), or with forskolin (100  $\mu\text{M}$ ) alone for 15 min and then forskolin plus BFA (0.1  $\mu\text{g/ml}$ ) for 2 h (*BFA + forskolin*). The cells were detergent solubilized, immunoprecipitated with anti-TCR  $\alpha$  chain antibodies, and treated with or without endo H before analysis by SDS-PAGE under reducing conditions. In control cells, TCR $\alpha$ GPI migrated on the gel at  $\sim 40$  kD at the end of either the pulse or chase. Endo H digestion cleaved TCR $\alpha$ GPI to a faster migrating species. In the presence of BFA, TCR $\alpha$ GPI matured to a slightly higher molecular mass species that was largely resistant to cleavage by endo H. Treatment with forskolin plus BFA abrogated this effect of BFA and produced an endo H-sensitive form of TCR $\alpha$ GPI in the chase that was indistinguishable from control cells.

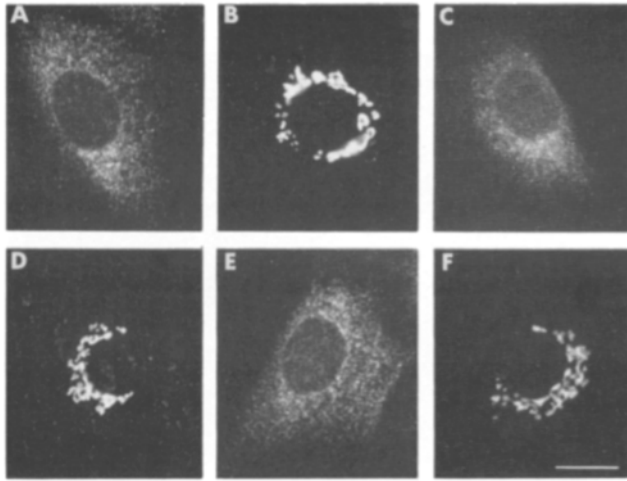


**Figure 6.** Addition of forskolin reverses the effects of BFA on the Golgi apparatus. NRK cells were treated with BFA (0.1  $\mu\text{g/ml}$ ) for 1 h at 37°C. Forskolin (100  $\mu\text{M}$ ) was then added to the cells for 0, 30 min, or 2 h at 37°C in the continued presence of BFA. After fixation and permeabilization, the cells were stained and prepared for double-label immunofluorescence using antibodies to Man II and the 110-kD protein. During BFA treatment alone (0), the 110-kD protein and Man II were not localized to Golgi structures but instead were diffusely distributed in the cytosol or associated with ER membrane, respectively. Upon addition of forskolin, both proteins co-localized in distinct vesicles scattered throughout the cytoplasm (30 min) which later coalesced into juxtannuclear Golgi-like structures (2 h). Bar, 10  $\mu\text{m}$ .

teins into the ER, as suggested by our morphologic observations, than one would predict that Golgi processing of ER resident proteins would not occur in cells treated with BFA plus forskolin. To test this possibility we studied a truncated form of the T cell antigen receptor alpha chain that lacks transmembrane and cytoplasmic peptide domains but contains a phosphatidyl inositol glycan linkage (TCR $\alpha$ GPI) (Lin et al., 1990). When expressed in CHO cells, TCR $\alpha$ GPI is retained within the membranes of the ER throughout its lifetime (Suzuki, C., manuscript submitted for publication). In these cells, because TCR $\alpha$ GPI never reaches the Golgi apparatus its oligosaccharide chains remain sensitive to digestion by endoglycosidase H (endo H), an enzyme which cleaves "high mannose" but not "complex" type oligosaccharides (Tarentino and Maley, 1974; Kornfeld and Kornfeld, 1985). This is demonstrated in the metabolic labeling experiment of Fig. 5, where TCR $\alpha$ GPI appeared endo H sensitive after either a 30-min pulse or 2-h chase. In cells exposed to BFA during the 2-h chase period, TCR $\alpha$ GPI became endo H resistant due to redistribution of Golgi enzymes into the ER. In cells treated with BFA plus forskolin during the 2-h chase period, however, only endo H sensitive forms of TCR $\alpha$ GPI appeared, consistent with forskolin inhibiting BFA-induced redistribution of Golgi proteins into the ER.

#### ***Rapid Reformation of the Golgi Apparatus in BFA-treated Cells Exposed to Forskolin***

We have shown in the above experiments that cells preincubated with forskolin can be protected from the effects of BFA. To determine what effect, if any, forskolin has on the distribution of Golgi proteins in cells already exposed to BFA, we treated cells with BFA for 1 h at 37°C to disassemble the Golgi apparatus and then added forskolin for various times (Fig. 6). In the presence of BFA alone Man II staining was diffusely distributed in punctate structures characteristic of its distribution in the ER while the 110-kD protein was dispersed throughout the cytoplasm. No colocalization between the two markers was observed. Addition of forskolin, however, changed this. Within 30 min, Man II and the 110-kD protein appeared colocalized in discrete vesicles throughout the cytoplasm of most cells. These vesicles became larger with time, collecting at a perinuclear location. By 2 h in the presence of forskolin plus BFA, both Man II and the 110-kD protein colocalized in structures resembling an untreated Golgi apparatus. In control cells treated with BFA only, Man II immunofluorescence retained its ER pattern distinct from the diffuse distribution of the 110-kD for the duration of the study (not shown).



**Figure 7.** Effect of dibutyryl cAMP and forskolin analogues on BFA-induced dispersal of the Golgi apparatus. NRK cells were treated with BFA (0.1  $\mu\text{g/ml}$ ) for 1 h (A); with forskolin (100  $\mu\text{M}$ ) for 30 min and then forskolin plus BFA (0.2  $\mu\text{g/ml}$ ) for 1 h (B); with dibutyryl cAMP (10  $\mu\text{M}$ ) for 10 min and then dibutyryl cAMP plus BFA (0.1  $\mu\text{g/ml}$ ) for 1 h (C); with 1,9 dideoxyforskolin (100  $\mu\text{M}$ ) for 30 min and then 1,9 dideoxyforskolin plus BFA (0.1  $\mu\text{g/ml}$ ) for 1 h (D); with 6-HPP-forskolin (100  $\mu\text{M}$ ) for 30 min and the 6-HPP-forskolin plus BFA (0.1  $\mu\text{g/ml}$ ) for 1 h (E); and with 7-HPP-forskolin (100  $\mu\text{M}$ ) for 30 min and then 7-HPP-forskolin plus BFA (0.1  $\mu\text{g/ml}$ ) for 1 h (F). The cells were then fixed, permeabilized and stained for immunofluorescence using antibodies to Man II. After treatment of cells with BFA alone for 1 h (A), Man II was distributed in a punctate pattern that is characteristic of its distribution in the ER and associated vesicles. In cells treated with forskolin before the addition of BFA (B), Man II appeared only in juxtannuclear Golgi structures essentially indistinguishable from its distribution in control cells. Raising intracellular cAMP levels with dibutyryl cAMP before the addition of BFA (C) had no inhibitory effect on BFA-induced redistribution of Man II into the ER. Treatment with either 1,9 dideoxyforskolin (D) or 7-HPP-forskolin (F), however, prevented BFA-induced disassembly of the Golgi apparatus while treatment with 6-HPP-forskolin did not (E). These results suggest forskolin's ability to inhibit BFA's effects on the Golgi apparatus exhibits specificity for forskolin structure and occurs by a cAMP independent mechanism. Bar, 10  $\mu\text{m}$ .

The process of Golgi recovery after addition of forskolin to BFA-treated cells was nearly indistinguishable from the sequence of events observed previously for the reassembly of the Golgi apparatus upon removal of BFA (see Lippincott-Schwartz et al., 1990, and Donaldson et al., 1990). In both recovery processes, the 110-kD protein rapidly reassociated with Man II-containing vesicles that were scattered throughout the cytoplasm. These vesicles aggregated into larger structures with time and then migrated into a juxtannuclear location to form elongated interconnected Golgi networks. These results suggest that forskolin can act to reverse BFA's effects on Golgi morphology.

As demonstrated in the above experiment protein export from the ER occurs in BFA-treated cells exposed to forskolin. Whether protein export to more distal sites in the cell than the Golgi apparatus occurs under these conditions was tested by examining the rate of secretion of fibronectin in cells exposed to BFA or BFA plus forskolin (data not shown). In cells treated with BFA alone, secretion of newly synthe-

sized fibronectin was essentially undetectable at 100 ng/ml BFA over 3 h. In cells exposed to forskolin (100  $\mu\text{M}$ ) plus BFA (100 ng/ml), however, secretion was clearly detected over this same period. The rate of fibronectin secretion in cells treated with BFA plus forskolin was somewhat slower than untreated cells, however. This may be explained, in part, by the fact that forskolin alone demonstrated slower rates of fibronectin secretion. These results indicate that forskolin can reverse, at least partially, the block in protein secretion induced by BFA.

#### ***Forskolin Acts by a cAMP Independent Mechanism to Inhibit BFA-induced Redistribution of Golgi Membrane into the ER***

Forskolin has been studied most extensively for its effects on adenylyl cyclase, but has other effects on cells as well (Laurenza et al., 1989; Seamon and Daly, 1986). To determine whether forskolin inhibition of BFA is related to increased cAMP levels that accompany forskolin stimulation of adenylyl cyclase we attempted to reproduce the effects of forskolin by incubation with dibutyryl cAMP, a membrane permeant analogue of cAMP that has been used previously to raise intracellular cAMP levels (Nigg et al., 1985). Preincubation with 10 mM dibutyryl cAMP, however, had essentially no effect on the redistribution of Golgi membrane induced by BFA (Fig. 7 C). Similar results were obtained with 8-bromo-cAMP (data not shown). Pretreatment of cells with 0.5 mM isobutyl methylxanthine (IBMX), a potent inhibitor of cAMP phosphodiesterase also had no inhibitory effect upon BFA-induced movements and did not potentiate the inhibitory effects of forskolin (data not shown). The latter phenomenon, a result of forskolin's and IBMX's synergistic effects on the accumulation of intracellular cAMP, has been observed for a number of cAMP-dependent phenomena (Forscher et al., 1987).

Forskolin analogues with differing potencies towards adenylyl cyclase (see Fig. 8) were tested for their abilities to inhibit BFA's effects. 1,9-Dideoxyforskolin is a naturally occurring analogue of forskolin that does not activate adenylyl cyclase and reproduces many of the cAMP independent effects of forskolin (Laurenza et al., 1989). 1,9-Dideoxyforskolin inhibited BFA-induced redistribution of Golgi proteins into the ER with a similar potency as forskolin (Fig. 7 D). Another analogue, 7-HPP-forskolin, which is much less potent than forskolin at binding to adenylyl cyclase, also inhibited BFA-induced redistribution of Golgi proteins (Fig. 7 F). In contrast, a similar analogue, 6-HPP-forskolin, which is equipotent with forskolin in binding to adenylyl cyclase, did not inhibit BFA-induced Golgi dispersal (Fig. 7 E). These results are consistent with forskolin inhibiting BFA action by a cAMP independent mechanism that demonstrates specificity for different forskolin analogues.

#### ***Discussion***

Perhaps the most striking characteristic of the secretory pathway is the dynamic nature of membrane traffic. Membrane and secretory proteins are known to move rapidly and selectively through different subcompartments of this pathway yet the identity and function of these compartments are maintained. The development of reversible (temperature



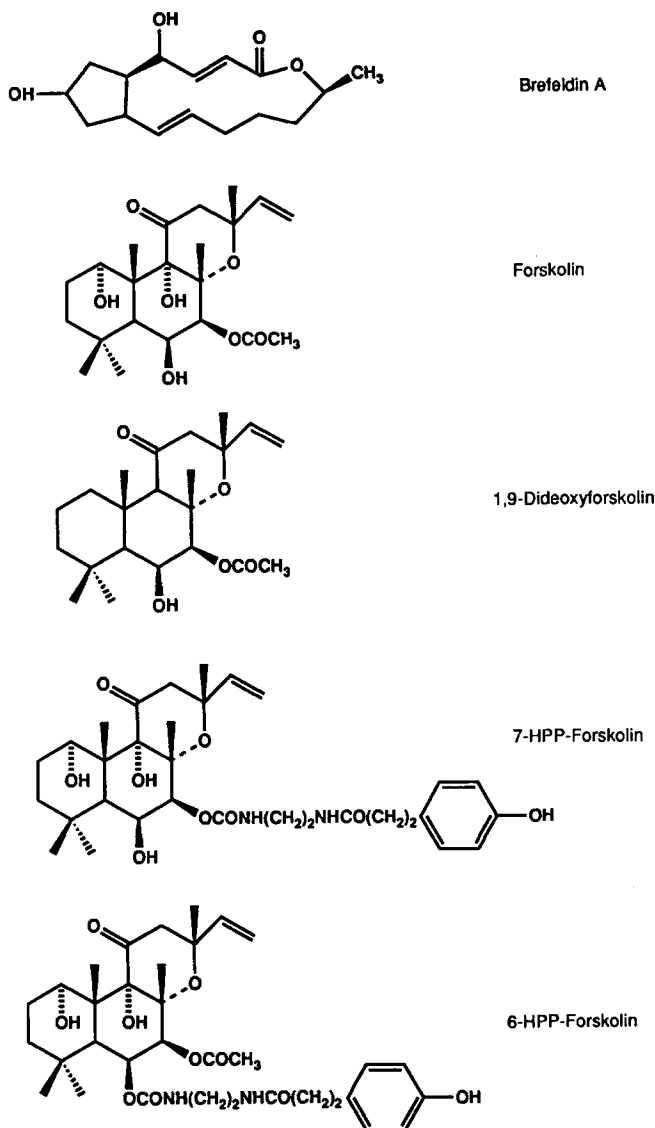


Figure 8. Structure of BFA, forskolin and forskolin analogues.

sensitive) mutants of the secretory pathway (sec mutants) of *Saccharomyces cerevisiae* (Novick et al., 1980; Schekman, 1985) has provided an important tool for the identification of specific biochemical components involved in the regulation of secretion. In these mutants, the ability to reversibly inactivate individual genes and/or gene products has revealed the crucial function of particular proteins in the secretory system. More complex eukaryotic cells are, at present much less amenable to these sorts of genetic manipulations. However, we can consider a pharmacologic analogue to these temperature-sensitive mutants that does function in higher organisms. BFA is the first drug that can reversibly alter the normal membrane dynamics within the early secretory pathway. Not only has it been a useful tool for experimentally manipulating protein and membrane movement through the secretory pathway but the striking changes in cells induced by BFA promise to reveal a variety of processes that normally take place in the organelles of the early secretory pathway but are difficult to observe. Understanding the mechanism of action of BFA, therefore, will likely lead to

the identification of critical molecules that control the structure and function of one or more of the organelles of this pathway.

The identification of drugs that can either mimic, enhance or antagonize BFA's effects on cells could be of great use for identifying either BFA's site of action or potentially to separate different effects of BFA. When two of us (J. Glickman and M. Sheetz) attempted to pharmacologically manipulate the effects of BFA on cells in culture, addition of forskolin to cells was observed to block the morphologic changes in the Golgi apparatus normally seen after the addition of BFA. Forskolin was tested because of its ability to activate adenylyl cyclase and to thereby raise intracellular cAMP levels which was thought might alter BFA's action. In the studies reported here with forskolin and forskolin derivatives, we have followed the fate of Golgi components using a lipid marker of the Golgi apparatus, NBD-ceramide, antibodies to a *cis*/medial Golgi marker, Man II, and antibodies to a Golgi peripheral membrane protein of 110 kD.

Observable effects of BFA on the Golgi apparatus follow a temporal pattern that is first detected within 30 s after addition of BFA when the 110-kD Golgi peripheral membrane protein dissociates from the Golgi apparatus into a diffuse cytosolic distribution (Donaldson et al., 1990). This is followed by the movement of Golgi membrane proteins into long tubular processes which over time fuse with the ER resulting in the mixing of Golgi proteins with the ER (Lippincott-Schwartz et al., 1990). The fluorescent lipid marker of the Golgi apparatus NBD-ceramide, examined in this study in living cells and in a previous study in fixed cells (Young et al., 1990), followed this temporal pattern of movement in the presence of BFA further suggesting there is a nonselective, likely complete breakdown of the Golgi apparatus in response to BFA.

All of the above mentioned effects of BFA could be abrogated by pretreating cells with forskolin. This is in contrast to other treatments that inhibit the redistribution of Golgi markers into the ER (low temperatures or nocodazole) but do not block the ability of BFA to induce the rapid redistribution into the cytosol of the 110-kD protein (see Donaldson et al., 1990). Thus, we cannot distinguish the morphology of a cell that has been exposed to forskolin plus BFA from that of an untreated cell.

In addition to forskolin's ability to inhibit BFA's morphological effects on the Golgi apparatus, forskolin also appeared to be able to reverse BFA's functional effects on protein secretion. In cells exposed to BFA plus forskolin, protein secretion occurred. This was in contrast to cells treated with BFA alone, where protein secretion was undetectable over 3 h of drug treatment.

Forskolin's ability to inhibit BFA's effects extended to other phases of BFA treatment. Thus, if forskolin was added to cells after BFA had been added and at any point after BFA's morphologic changes in the cell had been established, the result was a complete reversal in the morphology of the cell to an untreated state. These events closely resembled those after removal of BFA. Although the effects observed upon removing BFA could be the result of the rapid metabolic inactivation of BFA, we think that it more likely reflects the reversible binding of BFA to its cellular target(s). If this is true, then forskolin's ability to reverse BFA's effects is consistent with its binding to the same cellular target(s) as BFA.

Forskolin was originally tested in our system because of its ability to activate adenylyl cyclase; subsequent examination however, clearly dissociated its activity from alterations in cellular cAMP levels. Two membrane permeable analogues of cAMP were unable to mimic the effects of forskolin. Furthermore, the addition of a phosphodiesterase inhibitor, IBMX, did not potentiate the action of forskolin or reveal effects due to the cAMP analogues.

It is known that forskolin has effects other than the activation of adenylyl cyclase (Laurenza et al., 1989). Thus forskolin has been reported to inhibit the glucose transporter, the multidrug resistance gene product and a variety of gated ion channels (Kashiwagi et al., 1983; Coombs and Thompson, 1987). The synthesis of a variety of forskolin derivatives (Robbins, J., A. Laurenza, G. O. O'Malley, B. Spahl, R. W. Kosley, and K. B. Seamon, manuscript submitted for publication) has proven useful in delineating the different cellular effects of forskolin. Many of these derivatives demonstrate only selective cellular actions. To further define the mechanism by which forskolin inhibits BFA's cellular effects, therefore, we used several of these in our studies (Fig. 8). 1,9-Dideoxyforskolin does not activate adenylyl cyclase and 7-HPP-forskolin is not potent at binding to adenylyl cyclase. However, both analogues were equally effective as forskolin at antagonizing BFA. In contrast, 6-HPP-forskolin, which is equipotent with forskolin at binding to adenylyl cyclase, showed essentially no ability to antagonize BFA. Taken together, these data rule out almost any role of cAMP in antagonizing the effects of BFA in cells. Furthermore, the ability of the 7-HPP-forskolin but not the 6-HPP-forskolin to antagonize BFA implicates specific structural determinants in forskolin activity.

All of these observations are consistent with forskolin acting as a competitive antagonist of BFA. This was further supported by dose-response competitions between the two drugs. Either of the drugs can overcome the effects of the other simply by raising the dose of drug added. Thus, the dose-response curve for BFA is shifted progressively by increasing the concentration of added forskolin. Despite these observations, we do not know whether forskolin is indeed a competitive antagonist of BFA at the molecular level. Establishing this will require the demonstration that it binds to the same cellular target(s) as BFA. Although we currently favor this interpretation, other explanations are possible. Forskolin could be acting to "inactivate" BFA, either by inducing its metabolism or altering its access to the cell. However we would not expect this mechanism to generate simple symmetric shifts in dose response curves. Alternatively, forskolin could be "activating" a molecular target whose action is to antagonize the effects of BFA. According to this interpretation, forskolin would be activating an antagonistic pathway but would not be interacting with the same molecular target as BFA.

An interesting question raised by forskolin's interaction with BFA, regardless of the molecular mechanism, is whether forskolin is a pure antagonist or whether it exerts independent effects of the structure and function on the Golgi apparatus. Although no distinct morphologic effects on the Golgi apparatus by forskolin alone have been observed, other possible effects on the Golgi system by this drug are being investigated. The availability of functional derivatives of forskolin which can be used for covalent affinity labeling promises to

provide new tools for establishing the molecular basis for how BFA and forskolin together can regulate the identity and function of an intracellular organelle.

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