Ricin Transport in Brefeldin A-treated Cells: Correlation between Golgi Structure and Toxic Effect

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Abstract. Whereas brefeldin A (BFA) protected a number of cell lines against the protein toxin ricin, two of the cell lines tested were not protected but rather sensitized to ricin by BFA. EM studies revealed that upon addition of BFA the Golgi stacks in cells which were protected against the toxin rapidly transformed into a characteristic tubulo-vesicular reticulum connected to the endoplasmic reticulum, and subcellular fractionation experiments showed that galactosyl transferase disappeared from the Golgi fractions where it was normally located. EM and subcellular fractionation also indicated that in contrast to the Golgi stacks, the *trans*-Golgi network (TGN) remained intact and that internalized ricin was still localized in the TGN both when BFA was added before and after the toxin. Thus, BFA does not prevent fusion of ricin-containing vesicles with the TGN, and unlike resident proteins in Golgi stacks, ricin is not transported back to ER upon treatment of cells with BFA. Two kidney epithelial cell lines, MDCK and PtK2, were not protected against ricin by BFA, and EM studies of MDCK cells revealed that BFA did not alter the morphology of the Golgi complex in these cells. Also, subcellular fractionation revealed that, in contrast to the other cell types tested, the localization of galactosyl transferase in the gradients was not affected by BFA treatment. The data show that there is a correlation between BFA-induced disassembly of the Golgi stacks and protection against ricin, and they demonstrate that the structural organization of the Golgi apparatus is affected by BFA to different extents in various cell lines.

A number of toxic proteins such as ricin, abrin, modeccin, Shiga toxin, and diphtheria toxin inhibit protein synthesis after entry into the cytosol (for review see reference 17). The entry involves endocytosis of the toxins and translocation of the enzymatically active A moiety to the cytosol from an intracellular compartment (43). In the case of ricin it has been demonstrated both by EM and by subcellular fractionation that the toxin is transported to the Golgi apparatus (28, 32, 40), mainly to the *trans*-Golgi network (TGN)[1] (42). There is evidence that ricin A chain translocation takes place in the TGN or in a post-TGN compartment, and this seems to be the case for at least one other toxin, i.e., Shiga toxin (32). The exception is diphtheria toxin, where translocation of the enzymatically active chain seems to occur from endosomes (24, 27).

Drugs affecting the intracellular compartments involved in toxin entry are likely to affect the sensitivity of cells to toxins. The drug brefeldin A (BFA) has a marked effect on the Golgi complex in a number of cell types. Newly formed membrane proteins as well as virus proteins are retained in the ER (5, 13, 14). There is a rapid disorganization of Golgi stacks and a redistribution of Golgi membrane and proteins into the ER and nuclear envelope. BFA-induced changes in the Golgi complex has also been reported in a cell-free system (18). However, the structural pattern of this process varies somewhat from one report to another (6, 7, 13–15, 36, 39, 45).

We have in the present work studied the transport of ricin in various BFA-treated cell types and correlated the effects of BFA-treatment on Golgi structure with the ability of ricin to inhibit protein synthesis. We show that most cell lines are protected against ricin by BFA, and EM studies of four of these cell lines (T47D, A431, HEp-2, and Vero) reveal that the Golgi stacks but not the TGN disappear upon addition of BFA. Interestingly, ricin is still transported to the TGN. In contrast, MDCK and PtK2 cells are not protected against ricin by BFA, and there is no morphological effect of BFA on the Golgi apparatus of MDCK cells.

Materials and Methods

Materials

HRP type VI, pronase, diaminobenzidine, SPDP (3-[2 pyridyodithio]-propionic acid *N*-hydroxysuccinimideester), Hepes, and Tris were obtained from Sigma Chemical Co., St. Louis, MO. BFA was obtained from Epicentre Technologies, Madison, WI. Nycodenz was obtained from NYCOMED, Oslo, Norway; [^{3}H]leucine and Na^{125}I were from the Radio-

1. *Abbreviations used in this paper*: BFA, brefeldin A; TGN, *trans*-Golgi network.

chemical Centre, Amersham, UK. Shiga toxin was a generous gift from Dr. J. E. Brown. Ricin-HRP conjugates were prepared by the SPDP method as previously described (40).

Cells

The different cell lines were obtained from the following sources: Vero cells and HEp-2 cells were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland; A431, Caco-2, L929, PtK2, and MCF7 cells were from the American Type Culture Collection, Rockville, MD; MDCK and BHK cells were given to us by Dr. Kai Simons, EMBL, Heidelberg, Germany; NRK cells were obtained from Dr. T. Ege, NYCOMED; U-20S cells were from Dr. G. F. Vande Woude, National Cancer Institute, Frederick, MD; HeLa S3 cells were from Dr. Laland, University of Oslo, Norway; and T47D cells were obtained from the Fibiger Laboratory, Denmark (11). Cells were grown in Costar 3000 flasks (Costar, Badhoevedorp/The Netherlands) or T-25 flasks (NUNC, Roskilde, Denmark) and in the case of MDCK cells (strain I), also on polycarbonate filters (Costar Transwell, pore size 0.4 μm, diameter 24.5 mm). The cells were routinely seeded at a density of 10^6 per filter and used for experiments 2–5 days later (44). All filters used for experiments had a transepithelial resistance of at least 1,000 Ω cm^2 as measured with the Millicell-ERS equipment (Millipore Continental Water Systems, Bedford, MA) also at the end of the experiments. The medium used was DME (3.7 g/l sodium bicarbonate) (Flow Laboratories) containing 5% FCS (Gibco, Ltd., Paisley, Scotland) and 2 mM L-glutamine (Gibco, Ltd.). In the case of the PtK2 and CaCo-2 cells the medium contained 10% FCS and nonessential amino acids (Gibco, Ltd.).

Measurement of Cytotoxic Effect

After incubation of cells with toxin as described in legends to figures, the medium was removed, and the cells were incubated in the same medium (no unlabeled leucine) for 10 min at 37°C with 1 μCi of [^{3}H]leucine per ml. Then the solution was removed, and the cells were washed twice with 5% (wt/vol) TCA and solubilized in KOH (0.1 M). Finally, the acid-precipitable radioactivity was measured. The experiments were carried out in duplicate. The difference between duplicates was <10% of the average value.

Measurement of Receptor-mediated Endocytosis of ^{125}I-Transferrin and ^{125}I-Ricin

The amount of internalized transferrin was measured as described by Ciechanover et al. (4). Endocytosis of ^{125}I-labeled ricin was measured as the amount of toxin that could not be removed with lactose as previously described (23).

Subcellular Fractionation of Cells

The cells (10^7 cells growing in T-75 flasks) were scraped off the plastic with a rubber policeman after the addition of homogenization buffer (H-buffer: 0.3 M sucrose, 3 mM imidazole, pH 7.4). The pooled cells were pelleted by centrifugation for 10 min at 100 *g*. The pellet was resuspended in 1 ml H-buffer and homogenized by passing it 10 times up and down through a 1-ml blue tip on a Gilson pipette, followed by six times through a 1-ml syringe with a 22G 1 1/4 needle before homogenization by six strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 2,500 rpm for 10 min in 1.5 ml Eppendorf tubes in a centrifuge, (model 5415, Eppendorf) to obtain a nuclear pellet and a postnuclear supernatant. The postnuclear supernatant was subjected to discontinuous gradient centrifugation in a system similar to that reported by Sandberg et al. (22). In the bottom of SW 40 tubes, gradients were made of 4.5 ml light solution (1.15 M sucrose, 15 mM CsCl) and 1.5 ml heavy solution (1.15 M sucrose, 15 mM CsCl, 15% Nycodenz [wt/vol]). The gradients were made in a Biocomp Gradient Master, NYCOMED, Oslo (angle 74, speed 16, time 2 min, 45 s). Postnuclear supernatant (5.6 parts) was mixed with 2 M sucrose, 10 mM CsCl (4.4 parts), usually a total of 1.5 ml, and layered on top of the gradient. This was again overlayed with 3 ml 0.9 M sucrose, and finally 1–2 ml of 0.3 M sucrose. After 4 h at 33,000 rpm, the gradients were fractionated (25–30 fractions) and analyzed with respect to marker distribution.

Enzyme Analysis

HRP was measured according to Steinman et al. (37), UDP-galactose/glycoprotein galactosyl transferase according to Brändli et al. (2), esterase and β-*N*-acetyl-glucosaminidase according to Beaufay et al. (1).

Processing for Electron Microscopy

Cells grown in monolayers in T-25 flasks were incubated with BFA (5 μg/ml) alone or with various combinations of BFA and ricin-HRP as described in the text. Thereafter the cells were fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature, washed with PBS, and incubated with diaminobenzidine-H_2O_2 as previously described (40). The cells were postfixed with OsO_4, treated with 1% uranyl acetate in distilled water, embedded in Epon, cut at 50 nm, and examined in an electron microscope (model 100 CX, JEOL) without further contrasting (40).

Results

Effects of BFA on the Structure of the Golgi Complex in Various Cell Lines

In this study the effect of BFA on the organization of the Golgi complex was examined ultrastructurally in five different cell lines: Vero, T47D, A431, HEp-2, and MDCK (see Table I). The key observations were identical for the four first-mentioned lines although Vero cells have small Golgi complexes without any marked stacked appearance or distinct TGN, and T47D cells are equipped with very extensive Golgi complexes often with distinct stacks and TGN.

Within 5 min of BFA treatment, most Golgi stacks had vesiculated and sometimes formed tubulo-vesicular appendices emerging from the lateral border of the stacks. After 15 min with BFA no structures resembling typical Golgi stacks could be identified in the cells, but a tubulo-vesicular Golgi reticulum (which was never observed in control cells) became very conspicuous (Fig. 1). After 30–60 min this Golgi reticulum had often "condensed" into some discrete, globular tubulo-vesicular lumps intimately associated with elements of the ER (Fig. 1). This was in particular evident in Vero cells where the small Golgi-derived reticula were well suited for serial section analysis (Fig. 2). Often vesicles and tubulo-vesicular processes appeared to connect these lumps with ER cisterns, in agreement with the concept of BFA-induced retrograde transport of Golgi membrane and content into the ER (13, 14, 18). The tubulo-vesicular Golgi reticulum which often occupied very large parts of the T47D cells after 15–60 min of BFA exposure (Fig. 3), gradually decreased in size with increasing time of BFA exposure. After 4–8 h of BFA exposure, the tubulo-vesicular Golgi reticulum had disappeared completely (data not shown). Under these conditions the ER was often highly dilated. Moreover, changes in the morphology of mitochondria became apparent. The BFA-induced transformation of Golgi stacks into tubulo-vesicular reticula was reversible. Thus, when cells were first incubated for 30 min in the presence of BFA and then washed and further incubated without BFA, typical Golgi complexes (stacks) were readily observed within 30 min (data not shown).

In contrast to our observations on Vero, T47D, A431, and HEp-2 cells, MDCK cells appeared morphologically unaffected by BFA treatment. Thus, even after 60 min at 37°C with 5 μg/ml BFA, normally appearing Golgi stacks with the same size and frequency as in controls could be identified (not shown), and no tubulo-vesicular reticula were observed.

The characteristic tubulo-vesicular reticulum reported here closely resembles the tubule network described recently in a cell-free system where the assembly of nonclathrin-coated Golgi transport vesicles was prevented by BFA treat-

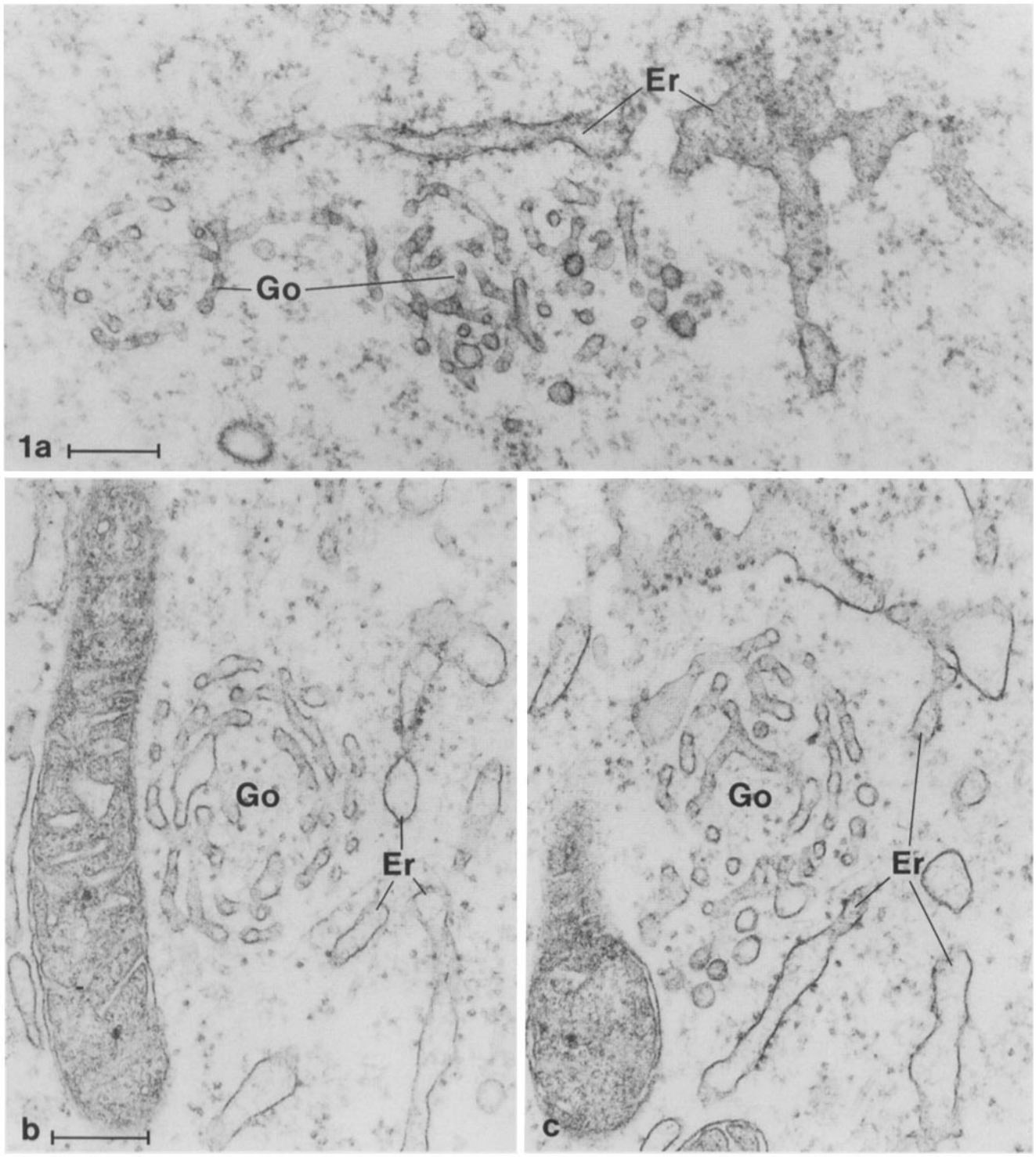

Figure 1. Effect of BFA treatment on the Golgi complex in Vero cells. (*a*) The Golgi-derived tubulo-vesicular reticulum (*Go*) after a 15-min exposure to 5 μg/ml BFA. (*b* and *c*) Sections no. 5 and 8 of a series of consecutive sections. The tubulo-vesicular Golgi reticulum is a characteristic, globular organelle after a 60-min exposure to BFA. Note the close relations between the Golgi reticulum and the endoplasmic reticulum (*Er*). Bars, 0.25 μm.

ment (18). In the cell-free Golgi system, however, structural relations to the ER could not be dealt with, and also no distinction between the Golgi stack and the TGN was made. It has been reported that resident Golgi enzymes of *cis*-, *medial*-, and *trans*-Golgi cisterns are transported back to the ER and nuclear envelope during BFA treatment whereas a marker enzyme of the TGN is not (3, 5, 13). In contrast, Fujiwara et al. (7) found in rat hepatoma cells that the rapid BFA-induced disassembly of the Golgi complex included the TGN, leaving no Golgi structures distinct from the ER. To

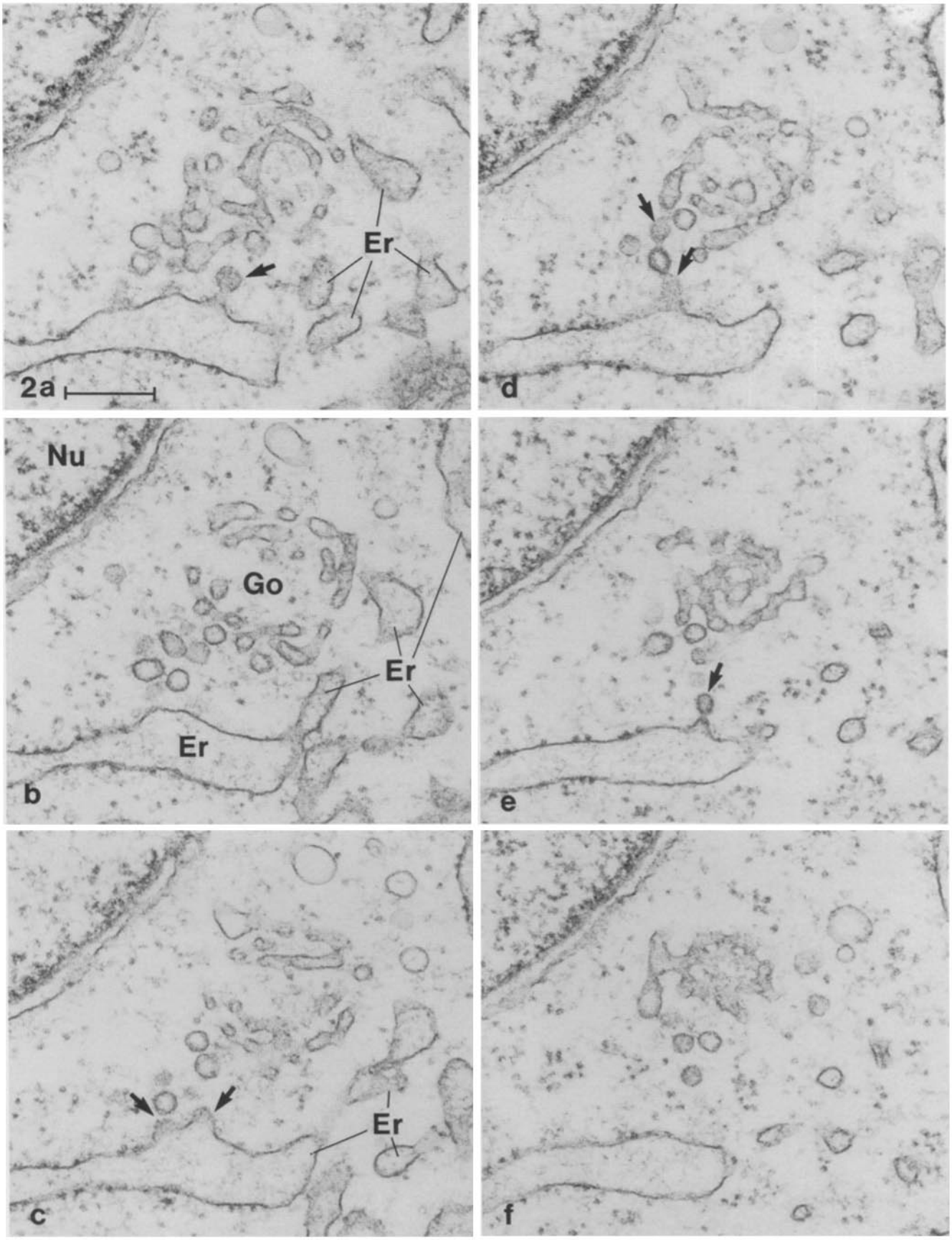

Figure 2. Six consecutive sections through a tubulo-vesicular Golgi reticulum (*Go*) of a Vero cell exposed to 5 μg/ml BFA for 60 min. The connections to the endoplasmic reticulum (*Er*) are shown (*arrows*). Bar, 0.25 μm.

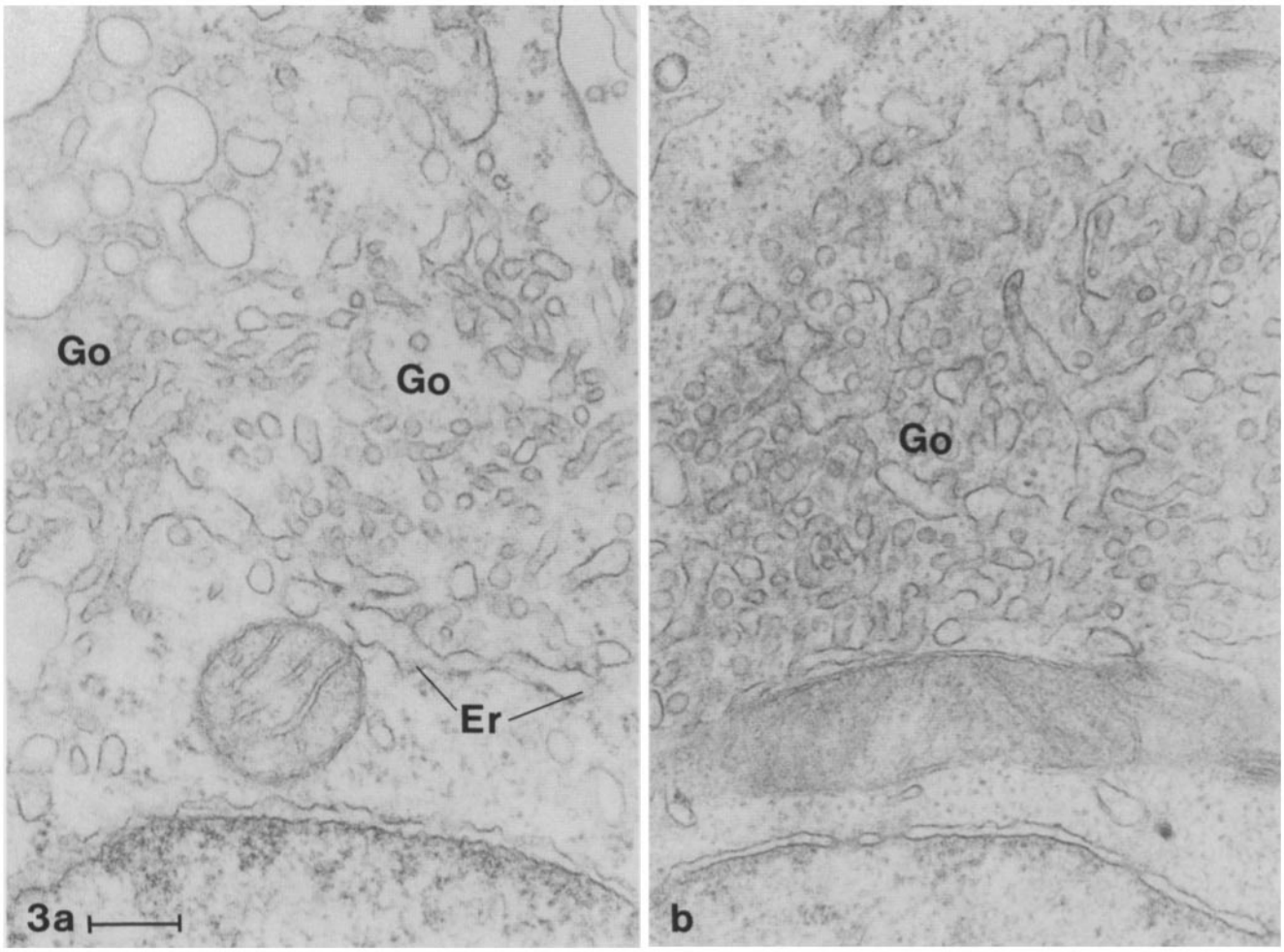

Figure 3. Tubulo-vesicular Golgi reticula (*Go*) in T47D cells exposed to 5 μg/ml BFA for (*a*) 15 or (*b*) 60 min. *Er*, endoplasmic reticulum. Bar, 0.25 μm.

further study whether the TGN of BFA-treated cells was included in the tubulo-vesicular reticulum or remained distinct, we allowed cells to endocytose monovalent ricin-HRP before adding BFA. Ricin (and monovalent ricin-HRP) is known to be transported to the TGN and can therefore be used as a marker for this compartment (28, 40, 42) (Fig. 4). The rationale was that if the TGN was incorporated in the tubulo-vesicular reticulum and thus moved backwards into the ER and the nuclear envelope, these compartments should be labeled with ricin-HRP. It turned out, however, that in BFA-treated T47D cells previously loaded with ricin-HRP, no labeling of the tubulo-vesicular reticulum was obtained (Fig. 4). Similarly, ricin-HRP labeling of the ER and the nuclear envelope was never seen (Fig. 4). In contrast, structures closely resembling TGN of control cells were often distinctly labeled (Fig. 4). Such structures were not labeled when the cells were incubated with HRP which detects all endocytic compartments but is not transported to the TGN (8, 41) (Fig. 5). Our results therefore suggest that whereas cisterns of the Golgi stack (*cis*-to-*trans*) are transformed into the tubulo-vesicular reticulum and transported in a retrograde way to the ER upon BFA treatment, the TGN becomes separated from the Golgi reticulum and remains intact. Similar TGN-like structures could also be labeled when T47D cells were BFA-treated before exposure to ricin-HRP. To confirm that ricin in BFA-treated cells enters the TGN and stays there, cell fractionation was used to study the distribution of galactosyl transferase as well as ^{125}I-labeled ricin in control and BFA-treated cells.

Subcellular Fractionation of Cells Treated with BFA

We have earlier shown that subcellular fractionation can be performed in such a way that the Golgi marker galactosyl transferase becomes separated from endosomes, lysosomes, and ER in MDCK cells (32). We have now found that the same method can be applied to T47D (Fig. 6) and A431 cells (data not shown), and we have therefore used this system to study changes in the localization of different markers after BFA treatment. To fractionate the cells we have used a linear gradient in the bottom of the tube, then a load zone, and on the top, two layers of sucrose. In control cells not treated with BFA, endosomes, lysosomes, and ER move down into the gradient (peak III in Fig. 6), whereas the Golgi markers move upwards (peak I in Fig. 6). Upon BFA treatment of T47D cells (Fig. 6) and A431 cells (data not shown) galac-

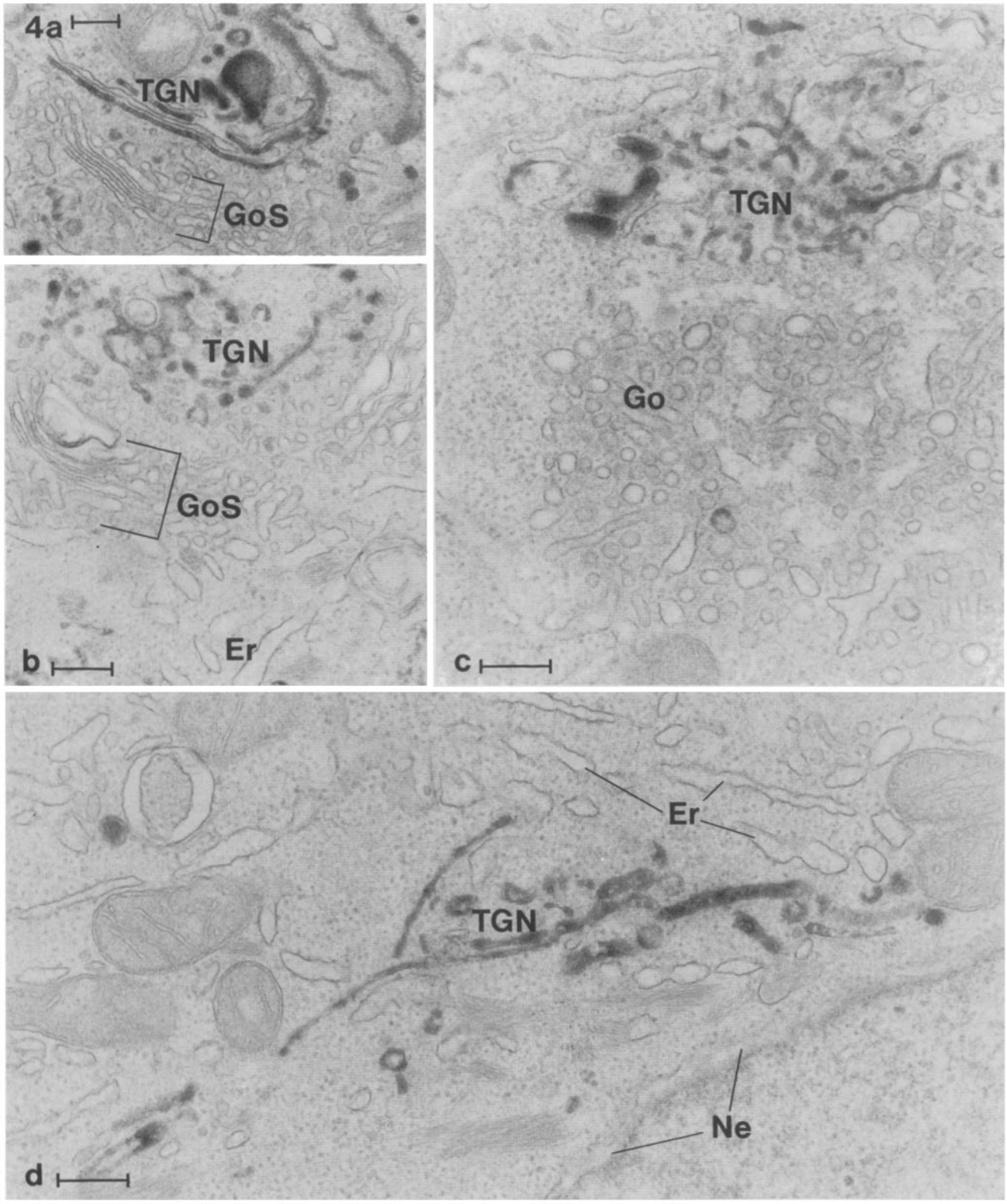

Figure 4. (*a* and *b*) Organization of the Golgi complex of control T47D cells incubated with ricin-HRP for 60 min at 37°C. The ricin conjugate clearly outlines the TGN, whether it is of cisternal (*a*) or tubular (*b*) appearance (cf. reference 9), while the Golgi stacks (*GoS*) are unlabeled. (*c* and *d*) From an experiment in which the TGN of T47D cells has been labeled by ricin-HRP for 60 min and thereafter exposed to BFA for 60 min. It is obvious that neither the tubulovesicular Golgi reticulum (*Go*), the endoplasmic reticulum (*Er*), nor the nuclear envelope (*Ne*) become labeled by the ricin conjugate. Bars, 0.25 μm.

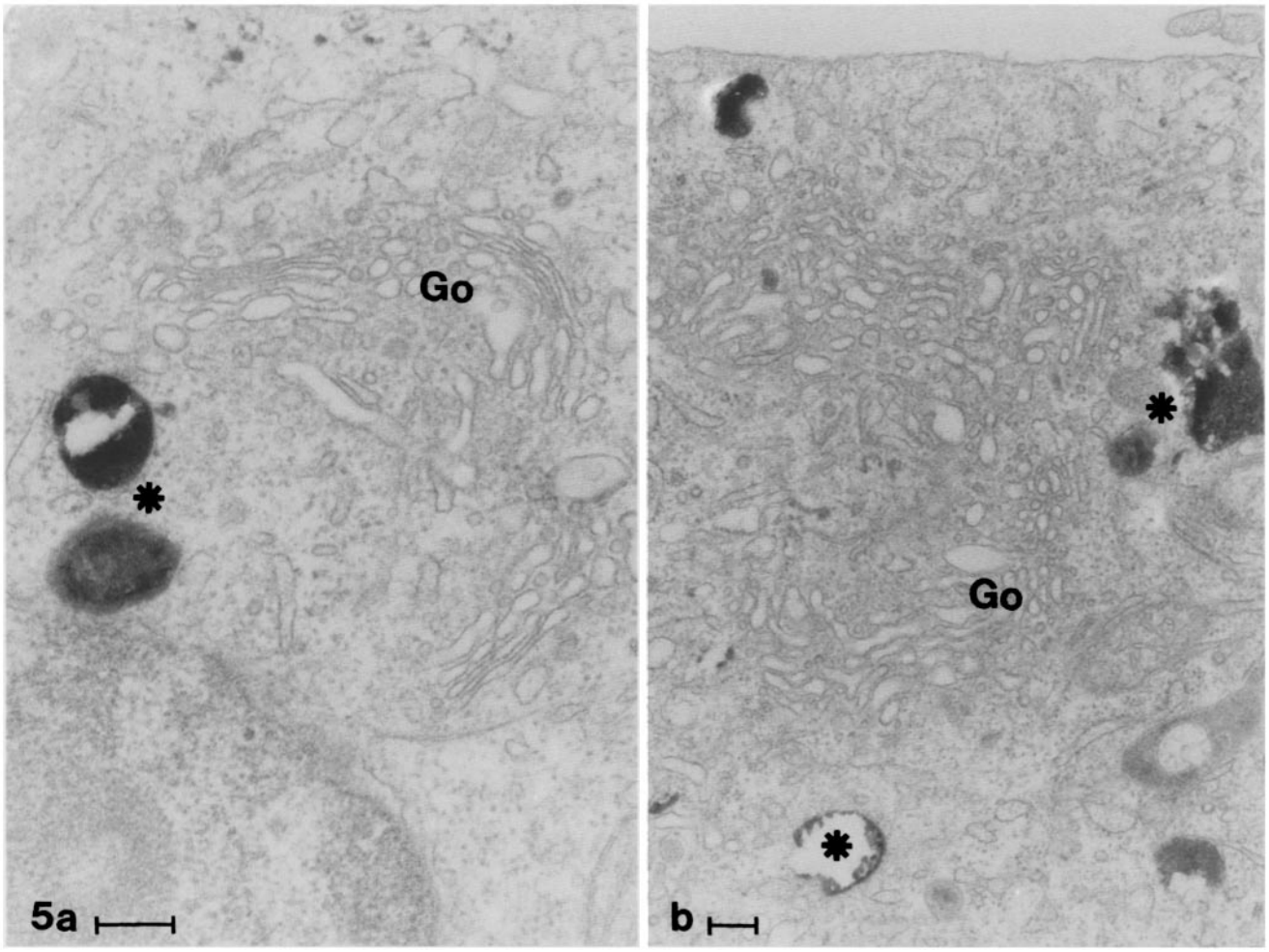

Figure 5. Control T47D cells incubated with HRP for 60 min at 37°C. Labeling of endosomes/lysosomes (*) is distinct, whereas the Golgi complexes (*Go*) are unlabeled. Bars, 0.25 μm.

tosyl transferase was shifted to a different position in the gradient. Instead of moving upwards from the load zone, the enzyme now moved downwards. On the other hand, there was no change in the localization of ^{125}I-labeled ricin (Fig. 6). As in control cells, a fraction of the toxin moved upwards in the gradients also when the cells were incubated with BFA, demonstrating that incoming vesicles containing ricin can still fuse with the TGN. Ricin transport to the TGN was measured in a number of different experiments, and the data are summarized in Fig. 7. As shown, BFA treatment seems to give only a slight reduction in the amount of ricin transported to the TGN. Transport of ricin to the TGN was measured both when BFA was present during the incubation with ricin and after a preincubation with BFA which was then removed before the addition of ricin. Also, when ricin was first allowed to accumulate in the TGN and BFA was then added, ricin stayed in the Golgi fractions. As shown in Fig. 7, we found that ~5% of internalized ricin was present in the Golgi apparatus, a value identical to that previously obtained by quantitative immunogold labeling (42).

In contrast to the results obtained with T47D and A431 cells, there was no change in the localization of any marker in the MDCK cells (Fig. 8).

The Effect of BFA on the Sensitivity of Cells to Ricin

To study the effect of BFA on the sensitivity of different cell types to ricin, we preincubated cells for 30 min at 37°C with the indicated concentrations of BFA, added increasing concentrations of toxin, and measured the protein synthesis 3 h later. As shown in Fig. 9 and Table I, Vero, T47D, A431, and a number of other cell types were protected against ricin upon addition of BFA. Vero cells were also protected by BFA against modeccin, Shiga toxin, abrin, volkensin, and viscumin (data not shown). All these toxins may depend on transport to the Golgi apparatus for entry into the cytosol. In contrast, there was no effect of BFA on the intoxication with diphtheria toxin, which enters the cytosol from endosomes (27).

The protective effect of BFA was not due to inhibition of transport of newly synthesized toxin receptors to the cell surface since the drug protected even after prebinding of the toxins (data not shown). BFA protected well even when added several minutes after ricin, an observation that is in agreement with the rapidly observed changes in the Golgi apparatus upon addition of this drug. Also, the effect of BFA on the Golgi apparatus seems to be rapidly reversible (13). The same was the case with the effect on toxicity, the cells

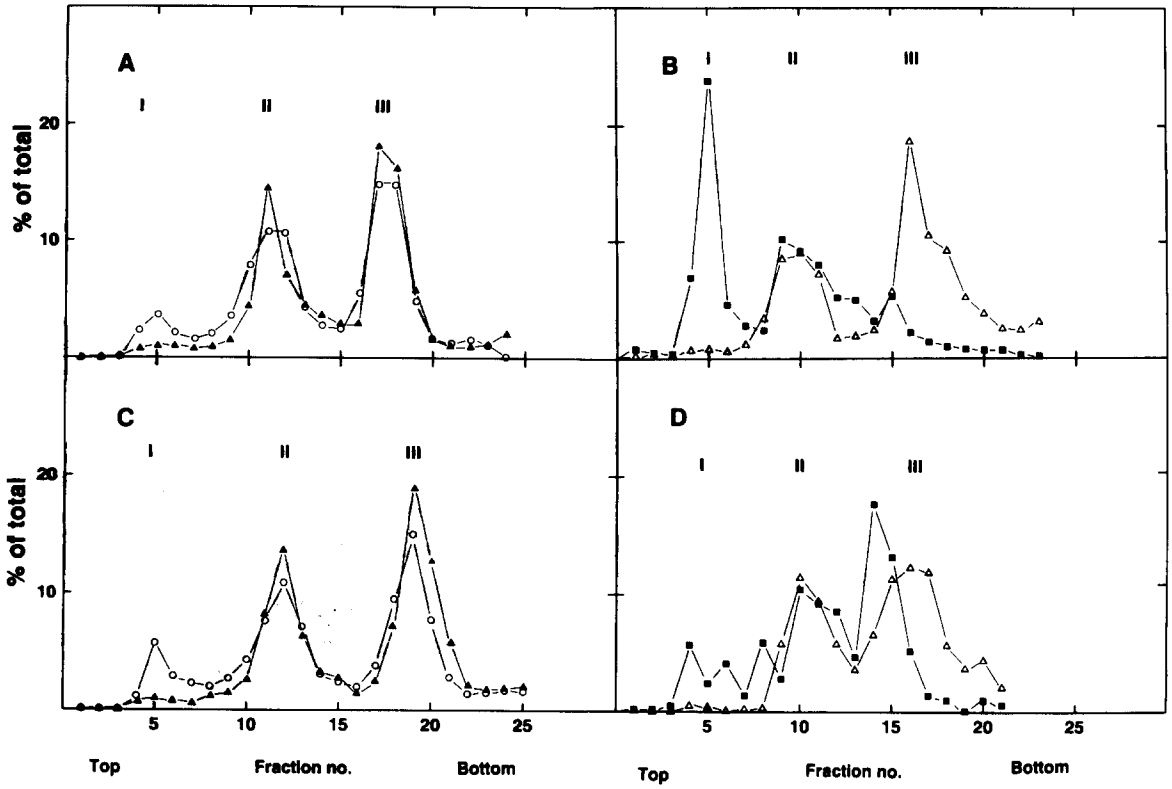

Figure 6. Effect of BFA on the subcellular distribution of different markers in T47D cells. The cells were incubated in the absence and presence of BFA (1 μg/ml) and ^{125}I-labeled ricin (100 ng/ml; 40,000 cpm/ng) for 60 min at 37°C. In experiments in which ricin had been added, cell surface-bound toxin was then removed with lactose, and the cells were homogenized and fractionated as described in Materials and Methods. The amounts of ^{125}I-labeled ricin and marker enzymes in the different fractions were measured as described in Materials and Methods. (*A* and *B*) No BFA added; (*C* and *D*) BFA added. (○) ^{125}I-labeled ricin; (▲) HRP; (■) galactosyl transferase; (△) β-hexosaminidase. (I) Golgi fractions; (II) load zone; (III) endosomes and lysosomes.

were as sensitive as before when BFA was removed after a 1-h incubation in the presence of the drug (data not shown).

Although the only reported effects of BFA until now are on the Golgi apparatus, we tested the effect of the drug on endocytosis after a 3-h incubation, since a reduced uptake from the cell surface would affect translocation of toxin to the cytosol. However, BFA treatment had no effect on the uptake of transferrin which is taken up by clathrin-coated vesi-

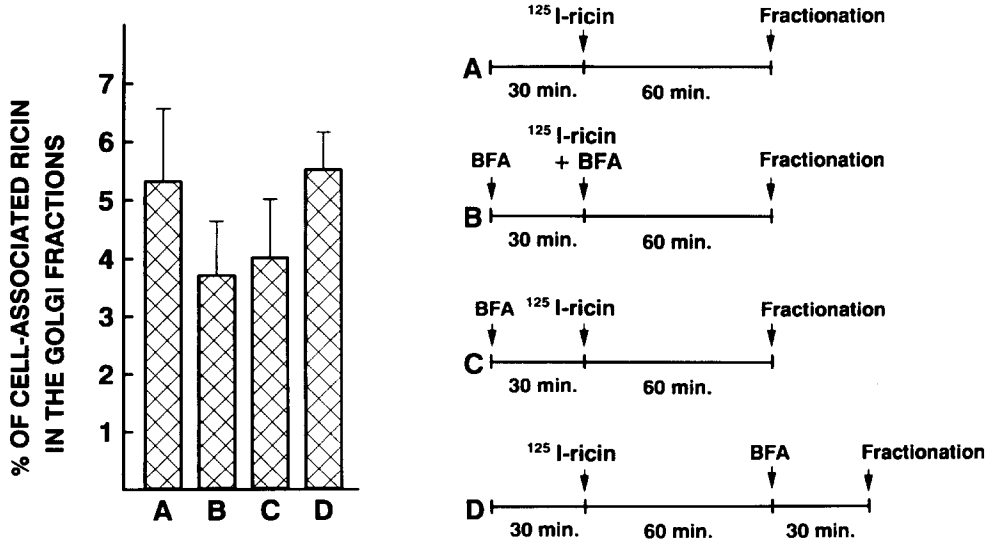

Figure 7. Effect of BFA on the localization of ^{125}I-ricin in the Golgi fractions of T47D cells. T47D cells (10^7 cells growing in T-75 flasks) were incubated in a Hepes-containing medium at 37°C with and without BFA (1 μg/ml) as indicated in the figure. Then ^{125}I-ricin (40,000 cpm/ng; 100 ng/ml) was added, and the incubation was continued for 60 min more before cell surface-bound toxin was removed with lactose and the cells were homogenized as described in Materials and Methods. In *D*, the incubation was continued for an additional 30 min in the presence of BFA before the cells were homogenized. The results shown are the mean values + SD. (n = 5 in *A* and *B*; n = 3 in *C* and *D*).

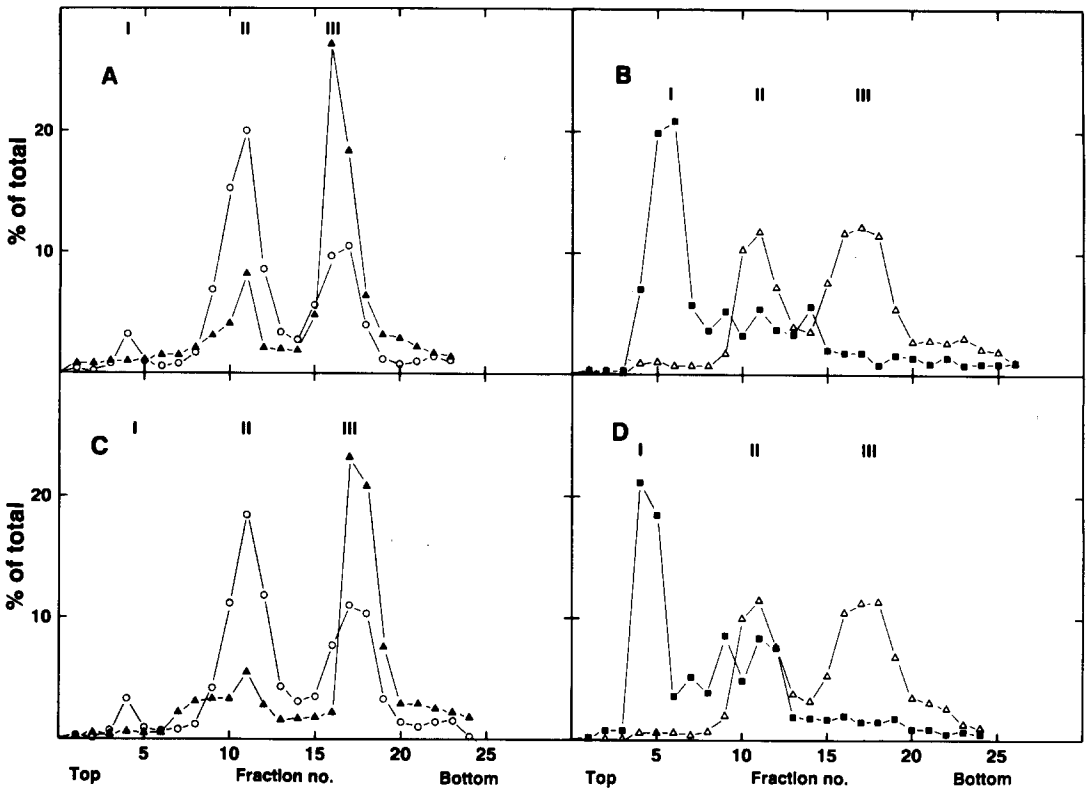

Figure 8. Effect of BFA on the subcellular distribution of different markers in MDCK cells. The experiment was performed in the same way as described in the legend to Fig. 6. (*A* and *B*) No BFA added; (*C* and *D*) BFA added. (○) ^{125}I-labeled ricin; (▲) HRP; (■) galactosyl transferase; (△) β-hexosaminidase. (I) Golgi fractions; (II) load zone; (III) endosomes and lysosomes.

cles (10) and it had no effect on the uptake of ricin which is internalized both by clathrin-coated vesicles and from uncoated areas of the cell membrane (26, 29–31). Also, normal appearing endosomes and lysosomes containing ricin-HRP were observed by EM in BFA-treated cells (data not shown). Thus, it seems likely that the protection against ricin by BFA is due to its effect on the Golgi apparatus, and that the change in this compartment somehow interferes with the translocation of the toxin A chains to the cytosol.

Addition of BFA to MDCK and PtK2 cells gave no protection against ricin (Fig. 9 and Table I), the cells were rather sensitized to ricin. In different experiments the sensitization

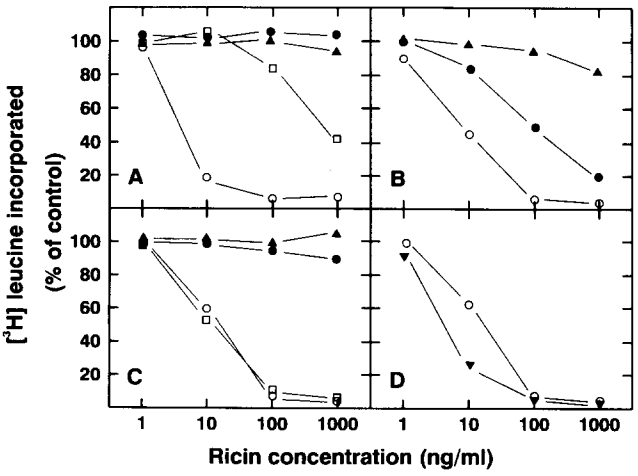

Figure 9. Effect of Brefeldin A on the sensitivity of (*A*) Vero, (*B*) T47D, (*C*) A431, and (*D*) MDCK cells to ricin. Cells growing in 24-well disposable trays were incubated in a Hepes-containing medium with and without the indicated concentrations of BFA for 30 min at 37°C. Then increasing concentrations of toxin were added and the cells were incubated for 3 h at 37°C before protein synthesis was measured as described in Materials and Methods. (○) No BFA; (▼) 5 μg/ml BFA; (▲) 1 μg/ml BFA; (●) 0.1 μg/ml BFA; (□) 0.01 μg/ml BFA.

Table I. Effect of Brefeldin A on the Sensitivity of Different Cell Lines to Ricin

Cell line	Tissue of origin	Protection
MDCK I	Madin–Darby canine kidney	–
MDCK II	Madin–Darby canine kidney	–
PtK2	Potoroo kidney	–
T47D	Human mamma carcinoma	+
HEp-2	Human carcinoma laryngis	+
A431	Human epidermoid carcinoma	+
Vero	African green monkey kidney	+
HeLa S3	Human cervical carcinoma	+
L929	Murine fibroblast	+
NRK	Normal rat kidney	+
Caco-2	Human intestine	+
BHK	Baby hamster kidney	+
MCF7	Human adenocarcinoma	+
U20S	Human osteogenic sarcoma	+

Cells growing in 24-well disposable trays were incubated with 1–5 μg/ml BFA in a Hepes-containing medium for 30 min. Then increasing concentrations of ricin were added, and the protein synthesis was measured 3 h later as described in Materials and Methods.

varied somewhat, but in all cases 4–10 times less ricin was required to give a 50% reduction of the protein synthesis in BFA-treated cells compared to nontreated cells. Similarly, MDCK cells were not protected by BFA against abrin and modeccin (data not shown). Even when the cells were preincubated for 4 h in the presence of BFA before ricin was added, there was no protection against the toxin. The lack of protection was not due to inactivation of BFA by the cells. The cells were still sensitized when the drug was added several times during the experiment and, furthermore, medium from MDCK cells incubated with BFA was, when transferred to Vero cells, still able to protect these cells against ricin. BFA was unable to protect MDCK cells against ricin under all culture conditions studied. The drug did not protect newly trypsinized cells against ricin, nor did it protect polarized MDCK cells grown on polycarbonate filters. As mentioned above, BFA actually sensitized MDCK cells to ricin. We have previously shown that cycloheximide sensitizes Vero and Hela cells to ricin (28). Cycloheximide also sensitized MDCK cells to the toxin. However, the combination of BFA and cycloheximide did not sensitize more than addition of either drug alone (data not shown), suggesting that the reason for the sensitization may be the same in the two cases. BFA in fact also inhibited the growth of MDCK cells (data not shown). However, a long-term incubation with BFA could induce changes of a different type than the short-term effects here described.

Discussion

The main finding in the present paper is that the effect of BFA on Golgi structure correlates with the toxic effect of ricin, and that cell lines respond quite differently to BFA treatment. This latter observation may reflect differences in the organization or dynamics of the Golgi apparatus.

Protection against ricin by BFA correlates with the formation of a tubulo-vesicular reticulum in several, quite different cell lines. This reticulum closely resembles the tubule network generated from Golgi stacks by BFA treatment in a cell-free system made from CHO cells (18). In this system Golgi stack elements apparently fuse and allow exchange of Golgi molecules by lateral diffusion. Our present observations establish this tubulo-vesicular Golgi reticulum as a distinct intermediate station during the BFA-induced retrograde movement of Golgi elements into the ER.

Several lines of evidence have suggested that the TGN is structurally and functionally different from the *cis-trans* stacks (3, 9), and Chege and Pfeffer (3) found that mannose 6-phosphate receptors were able to reach the TGN after endocytosis in BFA-treated cells. Similarly, our present studies with both EM and cell fractionation indicate that even when the stacked cisterns have become transformed into the tubulo-vesicular reticulum and moved in a retrograde way to the ER, the TGN remains apparently intact, and can still be reached by endocytosed molecules, in this case the protein toxin ricin. Ricin-HRP has thus allowed us to visualize the TGN after BFA treatment.

BFA has been reported to inhibit sialylation of vesicular stomatitis virus G-protein (5), T cell antigen receptor-α chain (13), class I-restricted antigen (16), and the EGF receptor (35). These results are in agreement with the notion that sialylation occurs in the TGN, and that BFA treatment prevents the contact between substrate and enzyme because TGN stays intact. However, it was recently shown that BFA has different effects on sialylation depending on the linkage type of the oligosaccharides. N-linked saccharides were not sialylated whereas O-linked saccharides were sialylated in the presence of BFA (35). This suggests that the different enzymes responsible for sialylation may have different localization in the Golgi complex and could be the explanation for the sialylation of O-linked carbohydrates of glycophorins occurring in the presence of BFA (39). Roth and co-workers (20, 21) in fact reported that β-galactoside α2,6-sialyltransferase is localized significantly in the *trans*-Golgi cisternae in addition to the TGN in various cells examined, and Takami et al. (38) reported that sialyltransferase was localized both in the *trans*-Golgi stack and in TGN in choriocarcinoma cells. They also reported sialylation of N-linked carbohydrates in the same cells after BFA treatment. Thus, sialyltransferase is not necessarily a good marker to test the integrity of TGN after for instance BFA treatment.

The fate of the TGN during BFA exposure is in particular interesting in relation to the ability of ricin to inhibit protein synthesis. Hence ricin A chain has to be translocated from an intracellular compartment (25), and different lines of evidence suggest that ricin is translocated to the cytosol from the TGN or from a post-TGN location (28, 33, 43). BFA was in fact recently found to protect Vero cells and BHK cells against ricin (33, 46), and it was suggested by Yoshida et al. (47) that BFA inhibits transport of ricin to the TGN (8) and that this could be the explanation for the protection. However, as shown in the present study, ricin seems to enter the TGN also after BFA treatment. One can therefore only speculate about the reasons for the protection. The TGN in BFA-treated cells seems to end up as a separate organelle which can no longer participate in retrograde transport of molecules to Golgi stacks. Also, it seems reasonable to believe that the formation of exocytic vesicles is rapidly abolished. Therefore, if ricin normally enters the cytosol at a post-TGN stage, BFA would protect. Another possibility is that ricin has to be modified by Golgi enzymes before it enters the cytosol, and that BFA treatment leads to removal of these enzymes, which may be transferred back to the ER. Although TGN seems to be unaffected morphologically, we can not exclude the possibility that BFA changes the composition of this organelle. It is known from other studies that proteolytic processing can occur in the Golgi apparatus (12, 19, 34), and ricin may have to undergo proteolytic processing before being translocated.

The finding that BFA actually sensitized the MDCK cells to ricin shows that the drug affects also these cells, although in a different manner than it affects most other cell types tested. In spite of the lack of structural changes of the Golgi stacks, recent experiments reported by Dr. Ira Mellman and co-workers clearly show that BFA can affect intracellular transport in MDCK cells (personal communication from Dr. Ira Mellman, Yale University, New Haven, CT). Their results show that transcytosis of dimeric IgA is strongly inhibited in MDCK cells upon addition of BFA. Furthermore, the inhibition of cell growth caused by BFA, although being a long-term effect, also shows that the drug affects these cells.

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References

1. Beaufay, H., A. Amar-Costesec, E. Feytmans, A. Thinès-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* 61:188–200.
2. Brändli, A. W., G. C. Hansson, E. Rodriguez-Boulan, and K. Simons. 1988. A polarized epithelial cell mutant deficient in translocation of UDP-galactose into the Golgi complex. *J. Biol. Chem.* 263:16283–16290.
3. Chege, N. W., and S. R. Pfeffer. 1990. Compartmentalization of the Golgi complex: brefeldin A distinguishes *trans*-Golgi cisternae from the *trans*-Golgi network. *J. Cell Biol.* 111:893–899.
4. Ciechanover, A., A. L. Schwartz, A. Dautry-Varsat, and H. F. Lodish. 1983. Kinetics of internalization and recycling of transferrin and transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. *J. Biol. Chem.* 258:9681–9689.
5. Doms., R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* 109:61–72.
6. Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* 263:18545–18552.
7. Fujiwara, T., K. Oda, and Y. Ikehara. 1989. Dynamic distribution of the Golgi marker thiamine pyrophosphatase is modulated by brefeldin A in rat hepatoma cells. *Cell Struct. Funct.* 14:605–616.
8. Griffiths, G., S. Pfeiffer, K. Simons, and K. Matlin. 1985. Exit of newly synthesized membrane proteins from the *trans* cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol.* 101:949–964.
9. Griffiths, G., S. D. Fuller, R. Back, M. Hollinshead, S. Pfeiffer, and K. Simons. 1989. The dynamic nature of the Golgi complex. *J. Cell Biol.* 108:277–297.
10. Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell.* 35:321–330.
11. Keydar, I., L. Chen, S. Karby, F. R. Weiss, J. Delarea, M. Radu, S. Chaitcik, and H. J. Brenner. 1979. Establishment and characterization of a cell line of human breast carcinoma origin. *Eur. J. Cancer.* 15:659–670.
12. Lepage-Lezin, A., P. Joseph-Bravo, G. Devilliers, L. Benedetti, J.-M. Launay, S. Gomez, and P. Cohen. 1991. Prosomatostatin is processed in the Golgi apparatus of rat neural cells. *J. Biol. Chem.* 266:1679–1688.
13. Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with Brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell.* 56:801–813.
14. Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of Brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.
15. Misumi, Y., K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. 1986. Novel blockade by Brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* 261:11398–11403.
16. Nuchtern, J. G., J. S. Bonifacio, W. E. Biddison, and R. D. Klausner. 1989. Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen. *Nature (Lond.).* 339:223–226.
17. Olsnes, S., and K. Sandvig. 1988. How protein toxins enter and kill cells. *In* Immunotoxins. A. E. Frankel, editor. Kluwer Academic Publishers, Boston. 39–73.
18. Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183–1195.
19. Redding, K., C. Holcomb, and R. S. Fuller. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in Yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 113:527–538.
20. Roth, J. 1987. Subcellular organization of glycosylation in mammalian cells. *Biochim. Biophys. Acta.* 906:405–436.
21. Roth, J., D. J. Taatjes, J. Weinstein, J. C. Paulson, P. Greenwell, and W. M. Watkins. 1986. Differential subcompartmentation of terminal glycosylation in the Golgi apparatus of intestinal and goblet cells. *J. Biol. Chem.* 261:14307–14312.
22. Sandberg, P.-O., L. Marzella, and H. Glaumann. 1980. A method for rapid isolation of rough and smooth microsomes and Golgi apparatus from rat liver in the same sucrose gradient. *Exp. Cell Res.* 130:393–400.
23. Sandvig, K., and S. Olsnes. 1979. Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. *Exp. Cell Res.* 121:15–25.
24. Sandvig, K., and S. Olsnes. 1981. Rapid entry of nicked diphtheria toxin into cells at low pH characterization of the entry process and effects of low pH on the toxin molecule. *J. Biol. Chem.* 256:9068–9076.
25. Sandvig, K., and S. Olsnes. 1982. Entry of the toxic proteins abrin, modeccin, ricin, and diphtheria toxin into cells. II. Effect of pH, metabolic inhibitors, and ionophores and evidence for toxin penetration from endocytotic vesicles. *J. Biol. Chem.* 257:7504–7513.
26. Sandvig, K., and B. van Deurs. 1990. Selective modulation of the endocytic uptake of ricin and fluid phase markers without alteration in transferrin endocytosis. *J. Biol. Chem.* 265:6382–6388.
27. Sandvig, K., A. Sundan, and S. Olsnes. 1984. Evidence that diphtheria toxin and modeccin enter the cytosol from different vesicular compartments. *J. Cell Biol.* 98:963–970.
28. Sandvig, K., T. I. Tønnessen, and S. Olsnes. 1986. Ability of inhibitors of glycosylation and protein synthesis to sensitize cells to abrin, ricin, Shigella toxin, and Pseudomonas toxin. *Cancer Res.* 46:6418–6422.
29. Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105:679–689.
30. Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. 1988. Inhibition of endocytosis from coated pits by acidification of the cytosol. *J. Cell. Biochem.* 36:73–81.
31. Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. 1989. Control of coated-pit function by cytoplasmic pH. *Methods Cell Biol.* 32:365–382.
32. Sandvig, K., K. Prydz, M. Ryd, and B van Deurs. 1991. Endocytosis and intracellular transport of the glycolipid-binding ligand Shiga toxin in polarized MDCK cells. *J. Cell Biol.* 113:553–562.
33. Sandvig, K., K. Prydz, and B. van Deurs. 1991. Endocytic Uptake of Ricin and Shiga Toxin. Springer Verlag New York Inc., New York. In press.
34. Schnabel, E., R. E. Mains, and M. Gist Farquhar. 1989. Proteolytic processing of pro-ACTH/endorphin begins in the Golgi complex of pituitary corticotropes and AtT-20 cells. *Mol. Endocrinol.* 3:1223–1235.
35. Shite, S., T. Seguchi, H. Mizoguchi, M. Ono, and M. Kuwano. 1990. Differential effects of brefeldin A on sialylation of N- and O- linked oligosaccharides in low density lipoprotein receptor and epidermal growth factor receptor. *J. Biol. Chem.* 265:17385–17388.
36. Shite, S., T. Seguchi, T. Shimada, M. Ono, and M. Kuwano. 1990. Rapid turnover of low-density lipoprotein receptor by a non-lysosomal pathway in mouse macrophage J774 cells and inhibitory effect of brefeldin A. *Eur. J. Biochem.* 191:491–497.
37. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereologic analysis. *J. Cell Biol.* 68:665–687.
38. Takami, N., K. Oda, T. Fujiwara, and Y. Ikehara. 1990. Intracellular accumulation and oligosaccharide processing of alkaline phosphatase under disassembly of the Golgi complex caused by brefeldin A. *Eur. J. Biochem.* 194:805–810.
39. Ulmer, J. B., and G. E. Palade. 1989. Targeting and processing of glycophorins in murine erythroleukemia cells: use of Brefeldin A as a perturbant of intracellular traffic. *Proc. Natl. Acad. Sci. USA.* 86:6992–6996.
40. van Deurs, B., T. I. Tønnessen, O. W. Petersen, K. Sandvig, and S. Olsnes. 1986. Routing of internalized ricin and ricin conjugates to the Golgi complex. *J. Cell Biol.* 102:37–47.
41. van Deurs, B., O. W. Petersen, S. Olsnes, and K. Sandvig. 1987. Delivery of internalized ricin from endosomes to cisternal Golgi elements is a discontinuous, temperature-sensitive process. *Exp. Cell Res.* 171:137–152.
42. van Deurs, B., K. Sandvig, O. W. Petersen, S. Olsnes, K. Simons, and G. Griffiths. 1988. Estimation of the amount of internalized ricin that reaches the *trans*-Golgi network. *J. Cell Biol.* 106:253–267.
43. van Deurs, B., O. W. Petersen, S. Olsnes, and K. Sandvig. 1989. The ways of endocytosis. *Int. Rev. Cytol.* 117:131–177.
44. van Deurs, B., S. H. Hansen, O. W Petersen, E. L. Melby, and K. Sandvig. 1990. Endocytosis, intracellular transport and transcytosis of the toxic protein ricin by a polarized epithelium. *Eur. J. Cell Biol.* 51:96–109.
45. Yamashina, S., O. Katsumata, H. Tamaki, and A. Takatsuki. 1990. Morphological effects of brefeldin A on the intracellular transport of secretory materials in parotid acinar cells. *Cell Struct. Funct.* 15:31–37.
46. Yoshida, T., C. Chen, M. Zhang, and H. C. Wu. 1990. Increased cytotoxicity of ricin in a putative Golgi-defective mutant of Chinese hamster ovary cell. *Exp. Cell Res.* 190:11–16.
47. Yoshida, T., C. Chen, M. Zhang, and H. C. Wu. 1991. Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and Pseudomonas toxin. *Exp. Cell Res.* 192:389–395.