In AtT20 and HeLa Cells Brefeldin A Induces the Fusion of Tubular Endosomes and Changes Their Distribution and Some of Their Endocytic Properties

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Abstract. We have studied the effects of brefeldin A (BFA) on the tubular endosomes in AtT20 and HeLa cells (Tooze, J., and M. Hollinshead. 1991. J. Cell Biol. 115:635–653) by electron microscopy of cells labeled with three endocytic tracers, HRP, BSA-gold, and transferrin conjugated to HRP, and by immunofluorescence microscopy. For the latter we used antibodies specific for transferrin receptor, and, in the case of AtT20 cells, also antibodies specific for synaptophysin.

In HeLa cells BFA at concentrations ranging from 1 μg to 10 μg/ml causes the dispersed patches of network of preexisting tubular early endosomes to be incorporated within 5 min into tubules ~50 nm in diameter but up to 40–50 μm long. These long, straight tubular endosomes are aligned along microtubules; they branch relatively infrequently to form an open network or reticulum extending from the cell periphery to the microtubule organizing center (MTOC). As the incubation with BFA is prolonged beyond 5 min, a steady state is reached in which many tubules are located in a dense network enclosing the centrioles, with branches extending in a more open network to the periphery. This effect of BFA, which is fully reversed within 15–30 min of washing out, is inhibited by preincubating the cells with sodium azide and 2-deoxy-D-glucose.

In AtT20 cells BFA at 5 μg/ml or above causes the dispersed patches of network of preexisting tubular early endosomes to be incorporated within 5 min into tubules ~50 nm in diameter but several micrometers long. They branch and as a result often form discrete patches of network which, as far as can be judged from the examination of serial sections in the electron microscope, are not all joined together in a single reticulum. The previously reported properties of tubular endosomes (Tooze and Hollinshead, 1991), and the fact that they contain abundant transferrin receptor (see below), indicate that they are early endosomes. Tubular early endosomes are, however, morphologically distinct from the classical cisternal-vesicular early endosomes that have been characterized, by EM combined with other methods, in many cell lines and cells in tissues (reviewed by Gruenberg and Howell, 1989). In the cells we have studied, tubular early endosomes and classical cisternal-vesicular early endosomes coexist. It is, therefore, likely that the morphological differences between these two forms of early endosomes reflect functional differentiation. In the light of the work of Yamashiro et al. (1984) and Geuze et al. (1987), it would not be surprising if tubular early endosomes proved to be do-
remains, involved primarily in the sorting of molecules destined to be recycled to the plasma membrane rather than transported to late endosomes. Some of the data reported here are consistent with this notion.

Unfortunately, EM, which provides the resolution to distinguish tubular early endosomes from cisternal vesicular early endosomes, gives only a static image of what in vivo is a dynamic organelar system as, for example, Hopkins et al. (1990) have shown. In vivo there may well be a dynamic equilibrium between tubular early endosomes and cisternal-vesicular early endosomes. We find that tubular early endosomes are much more abundant in some cell types (e.g., PC12, AtT20, Hep2, and HeLa cells) than others (e.g., 3T3 and BHK cells) (Tooze and Hollinshead, 1991). These differences could simply reflect a shift in such an equilibrium in favor of one or the other morphological type of early endosomes.

During our initial characterization of tubular early endosomes we incubated AtT20 cells with brefeldin A (BFA)1 to cause the disassembly of the Golgi apparatus (Misumi et al., 1986; Doms et al., 1989; Lippincott-Schwartz et al., 1989, 1990), and found that the extent of accumulation of tubular endosomes around the microtubule organizing center (MTOC) was enhanced (Tooze and Hollinshead, 1991). Subsequently, Lippincott-Schwartz et al. (1991), as well as Wood et al. (1991) and Hunziker et al. (1991), reported that BFA not only causes disassembly of the Golgi apparatus but also induces extensive tubulation of the early endosomal system in several cell types. These groups did not, however, observe tubular early endosomes in the cells they studied before the addition of BFA. We therefore decided to investigate further the effects of BFA on cells containing abundant tubular early endosomes before adding the drug. For these experiments we chose neuroendocrine AtT20 cells and HeLa cells.

As the results presented here show, BFA has a more profound effect on the tubular early endosomes of AtT20 and the HeLa cells than we previously appreciated. It causes the dispersed discrete patches of tubular endosomal network present in control cells to join together to form a single tubular early endosomal reticulum made up of long tubules many tens of micrometers long and extending from the MTOC to the most distant parts of the cytoplasm. This reticulum, which requires microtubules to maintain its integrity, is very rapidly loaded with fluid phase endocytic tracer and moreover appears to be able to concentrate tracer. We discuss these findings in the context of the other recent reports on the effect of BFA on the early endosomal system (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991; Klausner et al., 1992).

**Materials and Methods**

**Cell Culture**

AtT20 cells were grown in DME with 3.5 g/liter of glucose and 10% FCS with 5% CO2 and HeLa cells were grown in 90% MEM and 10% FCS at 37°C with 5% CO2.

1. Abbreviations used in this paper: BFA, brefeldin A; MTOC, microtubule organizing center; TGN, trans-Golgi network.

**Immunofluorescence Microscopy**

AtT20 and HeLa cells grown on glass coverslips were fixed in either 3% paraformaldehyde in PBS at room temperature for 20 min or in methanol at −20°C for 5 min. The paraformaldehyde fixed cells were processed for immunofluorescence microscopy by the methods of Ash et al. (1977). The methanol-fixed cells were washed in PBS and, without quenching in NH4Cl solution, then processed by the method of Ash et al. (1977). We used a photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) with planapo × 40 and × 63 oil immersion objective lenses (E. Leitz, Inc., Rockleigh, NJ).

**Antibodies**

A rabbit antibody against bovine 1,4β-galactosyltransferase was the generous gift of Dr. B. D. Shur (M. D. Anderson Cancer Center, Houston, TX). Antibodies against synaptophysin were generously donated by Dr. B. Wiedemann (Free University of Berlin, Berlin, Germany) and Dr. R. Jahn (Yale University, New Haven, CT). A human autoimmune serum that contains antibody against centrioles was kindly donated by our colleague Dr. E. Karsten (European Molecular Biology Laboratory, Heidelberg, Germany). The rat monoclonal antibodies LAMP 1 and II against the murine lysosomal membrane proteins (Chen et al., 1985, 1988) were kindly donated by Dr. T. August (Johns Hopkins University, Baltimore, MD). An affinity-purified rabbit antibody against rat liver CM-M6PR was the generous gift of our colleague Dr. T. Ludwig (European Molecular Biology Laboratory, Heidelberg, Germany). The monoclonal antibody B3/25 against human transferrin receptor was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. The monoclonal antibody RIT217 against murine transferrin receptor was the generous gift of Dr. J. Lesley and Dr. J. S. Trowbridge (The Salk Institute, San Diego, CA).

**Electron Microscopy**

Cells were washed with ice-cold PBS and fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate, pH 7.4, for 30 min, and then washed in buffer and postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 60 min at room temperature. After washing in water and then in 0.5% Mg uranyl acetate overnight at 4°C, the cells were washed in water again and then dehydrated in ethanol. Cells were either removed from the culture dish with 1,2-propylene oxide to make a pellet or left attached to the culture dish and then treated with BFA and 10 mg/ml HRP for varying times. Cells were washed, fixed as for HRP conjugates, and postfixed in 2% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. Immediately after fixation cells were washed with buffer and then reacted with DAB for 1 h and then with BFA and 20 mg/ml HRP for varying times. The reaction was stopped by washing with buffer and the cells were processed as for conventional EM. Cells were also incubated first in BFA (Epicentre Technologies, Madison, WI) at varying concentrations from 0.1 to 20 μg/ml for times varying from 1 to 90 min. Cells were washed with ice-cold PBS and fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate, pH 7.4, for 30 min. Immediately after fixation cells were washed with buffer and then reacted with DAB for 1 h, followed by DAB and H2O2 for 30 min. The reaction was stopped by washing with buffer and the cells were processed as for conventional EM. Cells were also incubated with HRP, with specific activities varying from 936 to 1,362 U/mg (Serva Fein Biochimica, Heidelberg, Germany) at varying concentrations from 0.1 to 20 mg/ml, for times varying from 1 to 90 min. Cells were washed with ice-cold PBS and fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate, pH 7.4, for 30 min. Immediately after fixation cells were washed with buffer and then reacted with DAB for 1 h and then with BSA-stabilized, 6-nm colloidal gold at a concentration of 0.1 μg/ml for 1 h and then with BFA and 10 or 20 mg/ml HRP for varying times. Cells were washed, fixed as for HRP conjugates, and processed as for conventional EM. The cells were left attached to the culture dish after reaction and were contrasted with lead citrate.

Cells were washed with buffered, serum-free medium at 37°C for 5 min and then washed with buffer and reacted with BFA and 20 mg/ml HRP for 30 min, washed in buffer, and immediately reacted with BFA and processed as for conventional EM. For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 40% formaldehyde in PBS for 40 min. The cells were then washed with ice-cold PBS, fixed in 0.5% glutaraldehyde for 30 min, and processed as for conventional EM. We used an E. M. 10 (Carl Zeiss, Inc.) operated at 60 and 80 kV.

For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 250 mM Hepes, pH 7.4, at 4°C for 10 min and then in 8% paraformaldehyde at room temperature for 50 min, then scraped off the rubber policeman and spun to a pellet. The cell pellet was infiltrated with 2.1 M sucrose and frozen.
in liquid nitrogen. Ultrathin sections were cut and immunogold labeled according to Tokuyasu (1980) and Griffiths et al. (1984).

**Results**

**Sequentially Added Tracers Colocalize in Tubular Early Endosomes**

We incubated AtT20 cells with medium containing 10 mg/ml HRP as a fluid phase endocytic tracer for 60 min at 37°C to saturate the tubular endosomes with HRP (Tooze and Hollinshead, 1991). The cells were quickly washed free of extracellular HRP, immediately incubated with medium containing BSA-gold (OD520 80) for 15 min, and then fixed and prepared for EM. The tubular endosomes at both the cell periphery and surrounding centrioles were seen to contain HRP reaction product and many also contained BSA-gold particles (Fig. 1). Since the two sequentially added endocytic traces colocalized, we can conclude that their endocytic pathways intersect in tubular early endosomes, as well as in cisternal-vesicular early endosomes (not shown).

**Tubular Endosomes Contain Transferrin Receptor**

When AtT20 or HeLa cells are incubated at 37°C for 60 min with HRP as a fluid phase endocytic tracer, it is necessary to use the peroxidase at concentrations of >5 mg/ml to optimally visualize the tubular endosomes after fixation and incubation with substrate. By contrast, when AtT20 or HeLa cells were incubated with 0.025 mg/ml of human transferrin conjugated to HRP for 60 min at 37°C, the tubular endosomes contained heavy deposits of reaction product (Fig. 2). Indeed, after only 10 min incubation with transferrin-HRP at this concentration many tubular endosomes were visualized (Fig. 2 C). Addition of an excess of transferrin to the medium containing 0.025 mg/ml transferrin-HRP inhibited uptake of the latter. We concluded that tubular endosomes contain transferrin receptor.

**BFA Induced Redistribution of Early Endosomes Detected by Immunofluorescence Microscopy**

We examined the distribution of transferrin receptor in HeLa cells in the presence and absence of BFA using the monoclonal antibody B3/25, specific for human transferrin receptor. In control cells the transferrin receptor is distributed in fluorescent spots and some short tubes dispersed throughout the cytoplasm but with a significant accumulation in the perinuclear region (Fig. 3 A). After 1 min incubation with 5 μg/ml of BFA, however, some of the transferrin receptor was in tubules which became progressively longer, more numerous, and interconnected to form a network over a period of 5–15 min (Fig. 3 B and 7 A). In migrating cells these linear structures were aligned along the long axis of the cell. When cold methanol was used as a fixative instead of 3% paraformaldehyde in PBS, these tubules appeared as rows of fluorescent spots. The latter are a fixation artifact; cold methanol does not preserve fine structure. From ~5 min onward, in the presence of BFA, many of these long tubules were clustered close to the nucleus so that they could no longer be individually resolved (Fig. 3, B and C). After 60 min incubation in BFA, the longest time point examined, this pattern of labeling of transferrin receptor was maintained (Fig. 3 C). This effect of BFA was fully reversible; 5 min after washout the long tubular endosomes had begun to disappear and by 30 min the distribution of transferrin receptor was indistinguishable from that in control cells. Finally, when HeLa cells were incubated for 60 min in 0.02% sodium azide and 50 mM 2-deoxy-D-glucose, with 5 μg/ml BFA being added for the last 30 min, a network of long tubular endosomes was not observed after labeling with antibody to transferrin receptor, the distribution of which resembled that in controls.

We incubated HeLa cells at 37°C for 60 min with the monoclonal antibody B3/25 diluted 1:1,000 in medium. The cells were then either washed and fixed, or washed, incubated with medium containing 5 μg/ml BFA for 2.5, 5, or 15 min, and then fixed. The fixed cells were incubated with rhodamine-conjugated second antibody. The labeling of the
cells fixed without incubation in BFA was the same as that in control cells labeled after fixation first with the antitransferrin antibody and then with the second antibody (see above, and cf. Fig. 3, A and B). The labeling of the cells incubated in BFA before fixation and incubation with second antibody (Fig. 3, E and F) closely resembled that seen in HeLa cells incubated for 5 min with BFA and then processed normally for indirect immunofluorescence microscopy (see above and Fig. 3, B and C, and insert 7 A). We concluded first that the monoclonal antibody to transferrin receptor is concentrated in early endosomes by endocytosis of the receptor–antibody complex, and second that some of the endosomes containing antibody bound specifically to transferrin receptor give rise to the long tubules in the presence of BFA.

Single and double labeling with antibody against bovine galactosyltransferase and human transferrin receptor established that the tubular processes containing galactosyltransferase that emerge from the cisternae of the Golgi apparatus at early times after the addition of 5 μg/ml BFA (Fig. 3 G) are quite distinct from the tubules containing transferrin receptor (Fig. 3 H). At later times the galactosyltransferase fluorescence appeared in the ER, as expected from the results of Lippincott-Schwartz et al. (1990), while the transferrin receptor labeling was as described above.

Similar immunofluorescence microscopic studies were done with AtT20 cells and the results (not shown) can be summarized as follows. Within 5–10 min of the addition of 5 μg/ml BFA to AtT20 cells, fine tubules labeling with antibody to murine transferrin receptor were seen. After 60 min in BFA most of the transferrin receptor was in a large intensely fluorescent spot close to the nucleus and clearly different from the pattern of perinuclear labeling seen in controls. After 60 min incubation in BFA a similar image was obtained using several antibodies specific for synaptophysin, which occurs in early endosomes of neuroendocrine cells (Johnston et al., 1989; Linstedt and Kelly, 1991; Régnier-Vigoroux et al., 1991). Using a human serum containing antibodies that recognize centrioles, we saw that the centrioles colocalized with the perinuclear spots containing transferrin receptor and synaptophysin (see Fig. 11 A, insets). As expected from the results of Donaldson et al. (1990), immunofluorescence microscopy showed that BFA causes βCOP to dissociate from the Golgi apparatus of HeLa and AtT20 cells and become dispersed throughout the cytoplasm (not shown).

In short, BFA causes a rapid change in the distribution of transferrin receptor in both HeLa and AtT20 cells. At steady state in the presence of BFA much of the transferrin receptor is concentrated in the pericentriolar region, but some also occurs in tubules extending from the MTOC to the cell periphery, the latter being particularly prominent in HeLa cells.

**BFA Induced Redistribution of Tubular Endosomes Observed by EM**

HeLa cells were incubated for 30 min in 1 or 5 μg/ml BFA and then for 60 min in BFA and 10 mg/ml HRP. We observed two striking differences between the steady-state distribution of the tubular endosomes in the control and the BFA-treated cells (Fig. 4). First, in the latter but not the former there were many very long, single, unbranched tubular endosomes; profiles of up to 20–30 μm were measured in single sections. Some of these long tubules ran around the cell periphery, but most ran from the periphery toward the center of the cell. At intersections these tubules usually joined (Figs. 4 A and 7) to form a reticulum. On the other hand, the BFA-treated cells lacked the small, peripheral scattered patches of tubular endosome network and dispersed short tubular profiles, seen in the controls (Fig. 2 B). Second, in the BFA-treated cells the number of tubular endosomes around the MTOC was much higher than in the controls, and at this site the tubular endosomes were multibranched and appeared to be connected into a single dense network (Fig. 4, A and B). Golgi stacks were completely absent.

When AtT20 cells were incubated with 1 μg/ml BFA for 30 min and then with 1 μg/ml BFA and 10 mg/ml HRP for 60 min, the distribution of labeled tubular endosomes was essentially indistinguishable from that in controls. The Golgi cisternae were absent, but large clusters of vesicles and tubules were seen in the region of the MTOC. These clustered vesicles lacked HRP reaction product, or BSA-gold when it was used (Fig. 5), and therefore they are not endocytic compartments; their morphology is remarkably similar to that of the Golgi apparatus in mitotic pancreatic acinar cells (Melmed et al., 1973). Presumably derived from the Golgi apparatus, these vesicles were still present after 90 min incubation with 1 μg/ml BFA (Fig. 5).

Increasing the concentration of BFA to 2.5 μg/ml or above resulted in the complete disappearance of these large clusters of Golgi vesicles; it also resulted in a striking redistribution of the tubular endosomes. The centrioles were enclosed in a massive accumulation of tubular endosomes, far in excess of the amount seen around the MTOC of controls. Examination of serial sections (Fig. 6, A–C) established that these pericentriolar tubular endosomes form a three-dimensional network in which the MTOC is embedded. In addition, there were tracts of endosomal network as well as some long straight tubules running from the MTOC into the neurites of the AtT20 cells which contained extensive networks of tubular endosomes as in controls (Fig. 6, D and E). Elsewhere in the cytoplasm of the treated AtT20 cells, for example, above the nucleus, there were few if any tubular endosomes. In short, in the BFA-treated cells most of the tubular endosomes appeared to have been recruited into a network extending from the MTOC via the shafts of the neurites to the growth cones.

**Figure 2.** Tubular endosomes in control cells labeled with transferrin conjugated to HRP. A shows pericentriolar tubular endosomes (arrows) in a HeLa cell incubated for 60 min with transferrin-HRP. The arrowhead indicates a centriole. B shows tubular endosomes at the periphery of a cell in the same culture. C shows a peripheral tubular endosome, ~3 μm long, in an AtT20 cell incubated for 10 min with transferrin HRP. Bars, 200 nm (A) and 500 nm (B and C).
Figure 3. Immunofluorescence microscopy of transferrin receptor and galactosyltransferase in HeLa cells. A shows a control HeLa cell labeled with antibody specific for human transferrin receptor. B and C show the labeling with this antibody in HeLa cells incubated for 15 and 60 min, respectively, with 5 μg/ml BFA. The inset to Fig. 3A shows the labeling seen after 5 min incubation with BFA. D–F show cells that were incubated for 60 min in a 1,000-fold dilution of the antibody to transferrin receptor, then fixed (D) or incubated with...
Figure 4. Tubular endosomes in HeLa cells incubated with BFA. A shows the massive accumulation of tubular endosomes around the MTOC in a HeLa cell incubated for 30 min in 1 μg/ml BFA followed by 60 min in BFA and 10 mg/ml HRP. The arrowhead indicates a centriole. B shows a similar region of a HeLa cell incubated for 30 min in 5 μg/ml BFA followed by only 1 min in BFA and 10 mg/ml HRP. Note in both micrographs the long profiles of some of the tubular endosomes. Bars, 1 μm.

BFA for 2.5 min (E) or 5 min (F) and then fixed and incubated with second antibody. G and H show HeLa cells incubated for 15 min with 5 μg/ml BFA and then double-labeled with antibodies against galactosyltransferase (G) and transferrin receptor (H). Where individual tubules can be resolved in the two micrographs they do not coincide.
**Figure 5.** Golgi-derived vesicle clusters in AtT20 cells treated with 1 μg/ml BFA. A shows clusters of Golgi-derived vesicles without HRP (arrows) and HRP-labeled pericentriolar tubular endosomes (arrowheads) in an AtT20 cell incubated for 30 min in 1 μg/ml BFA and 60 min in BFA and 10 mg/ml HRP. B shows Golgi vesicles (arrow) and BSA-gold-labeled tubular endosomes (arrowheads) around a centriole (open arrow) in a cell incubated as above but with BSA-gold instead of HRP as endocytic tracer. Bars, 200 nm.

**BFA Induces Redistribution of Preexisting Tubular Endosomes**

We allowed both HeLa and AtT20 cells to endocytose either 10 mg/ml HRP or 0.025 mg/ml transferrin-HRP for 60 min to saturate the tubular endosomes with fluid phase or receptor-mediated tracer. We then quickly washed the cells free of external tracer and incubated them with 5 μg/ml BFA for 5 and 10 min. (It requires at least 30 min to chase HRP out of tubular endosomes after they are loaded to saturation [Tooze and Hollinshead, 1991]). Within the first 5 min of exposure to BFA, the dispersed patches of tubular early endosomes, preloaded with HRP or transferrin-HRP, were largely replaced by very long, HRP-labeled tubules that rarely branched (Fig. 7). This result, confirming the immunofluorescence microscopy described above (Fig. 3), shows that at early times after the addition of BFA the network of long tubules extending from the cell periphery to the MTOC is derived at least in part from preexisting tubular endosomes. During the first 5 min of exposure to BFA the extent of accumulation of preloaded tubular endosomes around the MTOC also increased, but not to the extent seen after longer incubation with BFA; in other words, accumulation of the long tubular endosomes at the MTOC was progressive until a steady state was reached by 30 min.

**Figure 6.** HRP-labeled tubular endosomes in AtT20 cells incubated with 5 μg/ml BFA. A–C are micrographs of sections 1, 2, and 4 of a series. They show the massive accumulation of a tubular endosomal network enclosing the centrioles (arrowheads) in an AtT20 cell incubated for 30 min with 5 μg/ml BFA followed by 5 min with BFA and 20 mg/ml HRP. Note that with the concentration of BFA there are no Golgi vesicle clusters (compare A–C with Fig. 5). D shows part of a neurite of an AtT20 cell in the same culture with a network of tubular endosomes along its length. Note the length of individual profiles. E shows long profiles of tubular endosomes in part of another neurite. Bars, 500 nm, except D (1 μm).

**BFA Increases Rate of Entry of HRP into Tubular Endosomes**

In control cells some tubular endosomes receive HRP within
5 min but about 30 min of continuous endocytosis are needed to accumulate threshold levels of HRP in the entire tubular endosome system of AtT20 and HeLa cells (Tooze and Hollinshead, 1991). To determine whether or not BFA-treated cells behaved in this regard like control cells, we incubated HeLa and AtT20 cells for 30 min in BFA at 5 μg/ml to achieve the steady-state distribution of tubular endosomes in the presence of the drug and then incubated them with both BFA and 10 or 20 mg/ml HRP for periods ranging between 1 and 10 min. With both cell types the tubular endosomes in many cells were heavily labeled with peroxidase reaction product after only 1 min incubation with medium containing HRP (Figs. 4 B and 8 A and B), and after 5 min uptake the entire network of long tubules extending from the cell periphery to the MTOC was filled with reaction product in essentially all the cells. As expected, incubation of control cells for 1-10 min with the same concentration of HRP resulted in only partial labeling of the tubular endosomes. At steady state in the presence of concentrations of BFA sufficient to completely disassemble the Golgi apparatus, the tubular endosomes are filled with HRP to the threshold of detection at least fivefold faster than in controls.

After 60 min receptor-mediated endocytosis of 0.025 mg/ml transferin-HRP, the tubular endosomes in the BFA-treated AtT20 and HeLa cells appeared identical in amount and distribution to those seen after 60 min endocytosis of HRP, as expected. (Likewise, with control cells the tubular endosomes appeared the same after 60 min endocytosis of HRP or transferrin-HRP). However, using AtT20 cells we found that after short incubations with transferrin HRP the picture was very different from that after short incubations with HRP. After 30 min incubation in BFA to reach steady state followed by 5 min in BFA and 0.025 mg/ml transferrin-HRP, very few tubular endosome profiles contained HRP reaction product; most of the latter was confined to conventional early endosomes (Fig. 10, D and E). In further independent experiments we compared the extent of the labeling of tubular endosomes in control and BFA-treated AtT20 cells after 5 and 10 min incubation with 0.025 mg/ml transferrin-HRP. At least as many, if not more, tubular endosomes were labeled in the control cells as in the BFA-treated cells, after these short incubations with a tracer of receptor-mediated endocytosis, precisely the opposite of the result obtained using HRP, a tracer of fluid phase endocytosis (see above).

**BFA Increases the Concentration of HRP in AtT20 Tubular Endosomes**

To investigate whether or not BFA affected the amount of HRP accumulated in AtT20 tubular endosomes, we preincubated cells with 5 μg/ml BFA for 30 min followed by 60 min incubation with BFA and 0.1, 0.25, or 0.5 mg/ml HRP. Control cells were incubated in parallel for 60 min with these concentrations of HRP. The cells were then processed in parallel for electron microscopy and Fig. 8, C and D, shows the results. In control cells tubular endosomes lacked reaction product and were not visualized, but multivesicular bodies and late endosomes contained reaction product (Fig. 8 C). By contrast, in the BFA-treated cells the tubular endosomes (Fig. 8 D) as well as late endosomes (not shown) contained reaction product. When the BFA-treated cells were incubated in 0.5 mg/ml HRP for only 10 min the tubular endosomes contained small patches of reaction product, rather than being filled along their entire lengths. Clearly, in the presence of BFA the amount of fluid phase tracer accumulated in tubular endosomes increased with time, and at steady state after 60 min it exceeded the amount accumulated in tubular endosomes in control cells. In both control and BFA-treated cells HRP accumulated to the threshold of detection in late endosomes during 60 min.

**BFA Steady-State Distribution of Tubular Endosomes Depends on Microtubules**

We incubated AtT20 cells with 10 μg/ml of BFA for 30 min and then with BFA and 10 mg/ml HRP. After 10 min we added 20 μM nocodazole and continued the incubation for 50 min. These cells had very few long individual tubular endosomes and the dense network of tubular endosomes around the MTOC, characteristic of BFA-treated cells (Fig. 6, A-C), had dispersed. Instead we observed dispersed patches of network of variable size, some very large indeed, scattered throughout the cytoplasm (Fig. 9). The characteristic BFA steady-state distribution of tubular endosomes had clearly been disrupted by the depolymerization of microtubules with nocodazole.

We then incubated HeLa cells for 60 min in 5 μg/ml BFA to which 20 μM nocodazole was added for the last 30 min. The cells were fixed, labeled with antibody to transferrin receptor, and examined by immunofluorescence microscopy. As the inset to Fig. 9 shows, the long tubules (see Fig. 3) had disappeared and the transferrin receptor was present in clumps of various sizes.

**Conventional EM of AtT20 Tubular Endosomes in the Presence of BFA**

In thin sections of conventionally fixed and embedded control HeLa and AtT20 cells in the absence of an electron-dense endocytic tracer, it is virtually impossible to identify tubular endosomes. There is no way of distinguishing one smooth tubular or circular profile from another. However,

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*Figure 7. Very long tubular endosomes in BFA-treated HeLa cells. A shows a network of very long tubular endosomes at the periphery of a HeLa cell incubated for 60 min with 10 mg/ml HRP and then washed and incubated for 5 min in 5 μg/ml BFA. Note that the small patches of peripheral tubular endosomes seen in control cells incubated with tracer (see Fig. 2 B) are absent. The inset shows a HeLa cell incubated for 5 min with 5 μg/ml BFA and then labeled for indirect immunofluorescence microscopy with antibody against human transferrin receptor. B and C are, respectively, low magnification and higher magnification micrographs of a tubular endosome 23 μm long (measured between the arrowheads in B) in a HeLa cell incubated with 0.025 mg/ml transferrin-HRP for 60 min and then washed and incubated in 5 μg/ml BFA for 5 min. Note in C the tubular endosome passing above (or below) a mitochondrion (arrow). The arrow in B indicates the same region. Compare the constant diameter of the tubular endosomes in A and B with cisternae of the RER (open arrows) which have variable diameters and low contrast in these thick sections which were not contrasted with lead citrate. Bars, 500 nm (A and C) and 1 μm (B).*
after incubation in 5 or 10 mg/ml BFA for 30 or 60 min the Golgi stacks are absent and, particularly in AT20 cells, tubular endosomes are massively accumulated around the MTOC (see Fig. 6). This makes it possible to identify in Epon thin sections of AT20 cells treated with BFA tubular endosomes surrounding the centrioles in the absence of HRP reaction product. As Fig. 10, A–C, shows, they are smooth-membrane tubules, 50 nm in diameter, that branch repeatedly and form a network. Examination of single and serial sections showed that the pericentriolar network of tubular endosomes was not connected to the rough ER. Some branches of the tubular endosomes had terminal swellings that were coated (Fig. 10, A and B); identical, coated bulbous ends were seen on tubular endosomes of control cells as reported previously (Tooze and Hollinshead, 1991). After endocytosis of BSA-gold (OD_{520} 40) the tracer was seen in the lumens of these pericentriolar tubular endosomes (Fig. 10 C), including those with coats (Fig. 10 C, inset).

**AT20 Tubular Endosomes Contain Synaptophysin**

Antibodies specific for synaptophysin give an intense pericentriolar immunofluorescent labeling in AT20 cells treated with BFA (Fig. 11 A, inset). We therefore incubated AT20 cells with 10 µg/ml of BFA for 30 min followed by BFA and 10 mg/ml of HRP for 60 min and fixed and embedded them for cryoelectron microscopy. Double and single labeling experiments with antibodies specific for synaptophysin and HRP showed that synaptophysin was present in the membrane of the tubular endosomal networks around the MTOC (Fig. 11 A). The pericentriolar tubular endosomes could not be labeled by either antibody against rat CI-M6PR or the rat monoclonal antibodies LAMP I and II against two different lysosomal membrane glycoproteins, but these three antibodies labeled late endosomes in the same sections, providing a positive control (see below).

**BFA Has No Detectable Effect on AT20 Late Endosomes**

In AT20 cells incubated with 10 µg/ml BFA for 60 min before fixation and Epon embedding, the late endosomes had the same morphology and intracellular distribution as that seen in control cells (Tooze and Hollinshead, 1991). In sections of AT20 cells incubated for 30 min with 10 µg/ml BFA and then 60 min with BFA and 10 mg/ml HRP the proportion of late endosomes that contained HRP reaction product was similar to that in controls. BFA treatment does not therefore totally block delivery of HRP to late endosomes. In cryosections of AT20 cells incubated as above with BFA and then BFA and HRP, the endocytic tracer could be detected with antibody to HRP in the late endosomes (Fig. 11 B), which could also be labeled with affinity-purified rabbit antibody specific for rat CI-M6PR (11, D–F). We quantitated the immunogold labeling of CI-M6PR over late endosomes in control and BFA-treated AT20 cells, which were fixed, embedded, and labeled in parallel. As Table I shows, prolonged BFA treatment did not cause a significant decrease in the extent of CI-M6PR labeling observed.

Finally, indirect immunofluorescence microscopy of AT20 cells incubated 5–180 min in 5, 10, or 20 µg/ml BFA showed that the two murine lysosomal membrane glycoproteins recognized by rat monoclonal antibodies LAMP I and II (Chen et al., 1985, 1988) remain associated with granules in the cytoplasm, as in control cells (Fig. 11 C). These immunofluorescent granules must be the AT20 late endosomes since the...
latter are the only structures immunogold-labeled above background by these antibodies in thin cryosections of BFA-treated and control cells (Fig. 11 B). In short, using the LAMP I and II antibodies for both immunogold and immunofluorescence labeling we never observed in the AtT20 cells treated with BFA for periods of up to 180 min any LAMP-positive tubular structures emanating from the late endosomes. Furthermore, neither the LAMP I nor the LAMP II antibody gave immunofluorescence labeling of the pericentriolar spot which, in BFA-treated AtT20 cells, was heavily labeled by antibodies specific for murine transferrin receptor and synaptophysin.

Discussion

Based on the kinetics of the loading and unloading of tubular endosomes with fluid phase tracer, and some other properties, we concluded that they are part of the early endosomal system but morphologically distinct from cisternal-vesicular early endosomes with which they coexist (Tooze and Hollinshead, 1991). By showing here that these tubular endosomes in HeLa and AtT20 cells are enriched in transferrin receptor, and that in AtT20 cells they contain synaptophysin, we have confirmed that tubular endosomes are indeed early endosomes. The fact that tubular early endosomes contain these two membrane proteins, one of which is recycled to the plasma membrane while the other, synaptophysin, is destined for small synaptic-like vesicles in neuroendocrine cells (Johnston et al., 1989; Linstedt and Kelly, 1991; Régnier-Vigoroux et al., 1991), suggests that they may well be involved in sorting (and sequestering) membrane proteins out of the pathway from early to late endosomes.

Recently Lippincott-Schwartz et al. (1991), Hunziker et al. (1991) and Wood et al. (1991) have reported detailed studies of the effects of BFA on early endosomes in several cell types. These three groups discovered that BFA causes early endosomes to tubulate and become part of a tubular endosomal reticulum. The micrographs of Lippincott-Schwartz (1991), Wood et al. (1991), and Klausner et al. (1992) show that this tubular endosomal reticulum extends from the perinuclear region to the cell periphery. These groups did not, however, demonstrate the presence of the tubular early endosomes we have described (Tooze and Hollinshead, 1991) in any of the cell types they studied before the addition of BFA. Since they did not use the optimal conditions for detecting this compartment, which include the use of high specific activity HRP and the examination of relatively thick sections of flat embedded cells, it would be premature to conclude that preexisting tubular early endosomes were absent from all of the cell types involved.

By contrast, we know that tubular early endosomes are abundant in HeLa and AtT20 cells, which is why we chose them for this study. The data presented here establish first that in these cells BFA induces the same sort of endosomal reticulum as in the cells studied by the other three groups, and second that the preexisting tubular early endosomes are incorporated into this reticulum as it forms. Indeed, we have observed a correlation between the extent of tubular early endosomes in control cells and the extent of the BFA-induced endosomal reticulum; as we have shown here, BFA induces a very extensive reticulum in HeLa and AtT20 cells but in 3T3 cells which have very few if any tubular early endosomes (Tooze and Hollinshead, 1991) the BFA-induced endosomal reticulum is seen, under the immunofluorescence microscope, to be small and sparse, while in NRK cells the situation is intermediate between these two extremes (unpublished data). In HeLa and AtT20 cells the tubular early endosomes appear to be close to quantitatively incorporated into the BFA-induced endosomal reticulum, the tubules of which have the same diameter (~50 nm) as tubular early endosomes. We therefore believe that tubular early endosomes are the source of a large proportion of the membrane in the BFA-induced endosomal reticulum, which is not to say that these endosomes are the sole precursors of the latter.

In control cells many of the tubular early endosomes occur as discrete patches of highly branched network and long, straight profiles are rare. By contrast, in HeLa and AtT20 cells as well as the cells studied by Lippincott-Schwartz et al. (1991) and Klausner et al. (1992), the BFA-induced endosomal reticulum, is characterized especially at the periphery by remarkably straight infrequently branching tubules tens of micrometers long, and aligned along microtubules. BFA does not, therefore, simply induce the fusion together of patches of preexisting tubular endosomes. Rather, BFA appears to promote the growth of long tubules, enhance alignment along microtubules, and suppress tubule branching. In BFA-treated HeLa and AtT20 cells the density of tubular endosomal reticulum is greatest around the MTOC and from there the tubules radiate out along microtubules to the cell’s extremities. This suggests to us that the endosomal reticulum can move in both retrograde and anterograde directions along microtubules, like the endoplasmic reticulum, which uses microtubules to achieve a steady-state distribution extending from the MTOC to the cell periphery (Terasaki et al., 1986).

Fig. 10 shows that some of the branches of the BFA-induced endosomal reticulum in AtT20 cells are coated. Since BFA causes β coat protein (βCOP), nonclathrin Golgi coats (Donaldson et al., 1990) and trans-Golgi network (TGN), γ-adaptin clathrin coats (M. Robinson, cited in Klausner et al., 1992) to disassemble, the coats shown in Fig. 10 are presumably neither of these two classes. It remains to be seen whether they are clathrin coats like those on the plasma membrane, which are not affected by BFA (K.

Figure 10. Tubular endosomes and early endosomes in BFA-treated AtT20 cells. A and B show pericentriolar tubular endosomes (arrows) in thin sections of conventionally fixed and Epon-embedded AtT20 cells incubated for 60 min in 10 μg/ml BFA. These sections were contrasted with lead citrate. The arrowhead in B indicates a centriole. Note the coats (open arrows) on some buds at the ends of tubular endosomal profiles. C shows tubular endosomes in the pericentriolar region of an AtT20 cell incubated with 5 μg/ml BFA for 30 min and BFA and BSAGold for 60 min. Note the accumulation of the BSAGold (arrowheads) in the tubular endosomal lumen. The inset to C shows that BSAGold (small arrows) occurs in tubular endosomes with coated buds (arrowheads) close to centrioles (arrow). D and E show classic early endosomal profiles in AtT20 cells incubated in BFA for 30 min and BFA and 0.025 mg/ml transferrin-HRP for 5 min. Prolonged incubation with BFA does not eliminate classic early endosomes. Bars, 200 nm (A–D) and 100 nm (E).
Figure 11. Cryo-immunoelectron microscopy of tubular and late endosomes in BFA-treated cells. A shows tubular endosomes in the pericentriolar region in a cryosection of an AtT20 cell incubated with BFA for 30 min and BFA and 10 mg/ml HRP for 60 min. The section was labeled with antibody against HRP (6 nm gold, arrowheads) and monoclonal antibody against synaptophysin (9 nm gold, arrows). This establishes that synaptophysin is in the tubular endosomal membrane. The insets to A show immunofluorescence micrographs of three AtT20 cells incubated with BFA and double-labeled for synaptophysin (left) and centriolar material (right). B shows a late endosome in a cell in the same preparation labeled with antibody to HRP (6 nm gold, arrowheads) and the rat monoclonal antibody LAMP I (9 nm...
Table I. Quantitation of CI-M6PR Labeling of AtT20 Late Endosomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gold/μm²</th>
<th>Profiles counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtT20 control</td>
<td>171 ± 16</td>
<td>n = 32</td>
</tr>
<tr>
<td>AtT20 5 μg/ml BFA 60'</td>
<td>196 ± 21</td>
<td>n = 34</td>
</tr>
<tr>
<td>AtT20 5 μg/ml BFA 180'</td>
<td>162 ± 12</td>
<td>n = 23</td>
</tr>
<tr>
<td>AtT20 1 μg/ml BFA 60'</td>
<td>172 ± 22</td>
<td>n = 27</td>
</tr>
</tbody>
</table>

Values are means and corrected error of means.

Beck, cited in Klausner et al. (1992), or a novel coat as predicted by Klausner et al. (1992).

Immunofluorescence microscopy indicates that the long endosomal tubules induced by BFA are networked to form a single reticulum. Our finding that the entire BFA-induced reticulum in HeLa and AtT20 cells is loaded with HRP to the threshold of detection within 1-5 min also implies that the long tubules are all interconnected, allowing tracer to diffuse rapidly throughout the lumen of the entire system. In living cells Wood et al. (1991) observed essentially the same effect: Lucifer yellow was rapidly delivered to the endosomal reticulum of cells in BFA. During incubation of 10 min or less with transferrin-HRP at a low concentration the endosomal reticulum in BFA-treated cells was not labeled faster than some patches of tubular early endosome in control cells. This result is also the expected one if the endosomal reticulum is a single compartment because endocytosed transferrin receptor, with the HRP-tagged ligand bound, is free to migrate by lateral diffusion throughout essentially all the early endosomal membrane in the cell and therefore to be initially diluted.

Lippincott-Schwartz et al. (1991) and Wood et al. (1991) have interpreted their observations as showing that BFA not only causes tubulation of early endosomes but also induces fusion of early endosomal tubules with tubules derived from the TGN; Lippincott-Schwartz et al. (1991) envisage early endosomes and the TGN as a "homotypic" membrane system. Data concerning the effects of BFA on the trans side of the Golgi apparatus are, however, discrepant (see Reaves and Banting, 1992, and Alcante et al., 1992, and references therein), in large part because of the scarcity of unambiguous markers of the TGN. Lacking such markers, we have not been able to investigate directly the effect of BFA on the TGN in AtT20 and HeLa cells. We have, however, made an observation that may be related to this issue; namely, that in AtT20 cells the amount of HRP accumulated at steady state in the lumen of the BFA-induced endosomal reticulum is greater than the amount accumulated in the lumen of tubular early endosomes in control cells.

This effect of BFA can be interpreted as a consequence of (1) the BFA-induced fusion of early endosomes with the TGN, and (2) the significant (65-75 %) inhibition by BFA of delivery of endocytic tracers to late endosomes and lysosomes (Lippincott-Schwartz et al., 1991). We have not directly measured the extent of the inhibition of delivery of fluid phase tracers to late endosomes and lysosomes in AtT20 cells treated with BFA, but we assume that it is in the same range as that reported by Lippincott-Schwartz et al. (1991). At steady state the concentration of fluid phase tracer in the early endosomal system will depend upon the rate of uptake, the rate of recycling out of the cell, and the rate of delivery from early to late endosomes. If BFA inhibits the latter without changing the other two rates, HRP will accumulate in the early endosomal compartment. The TGN in AtT20 cells is a compartment in which secretory proteins are concentrated and brought out of solution to form the cores of secretory granules. Therefore, the TGN membrane may well be enriched in pumps able to eliminate water. Fusion of the TGN would deliver these pumps to the endosomal reticulum membrane where, by eliminating water, they could play a role in concentration fluid phase tracers such as HRP.

But regardless of the mechanism involved, we believe that this effect of BFA on the amount of HRP in the endosomal reticulum resolves an apparent discrepancy between our work and that of Lippincott-Schwartz et al. (1991) and Hunziker et al. (1991). These two groups achieved labeling on the endosomal reticulum in BFA-treated cells using low specific activity HRP, which, in our hands, has repeatedly failed to label tubular early endosomes in control AtT20, HeLa, and other cells; as a result, we no longer use preparations of HRP with a lower specific activity. We interpret the results of Lippincott-Schwartz et al. (1991) and Hunziker et al. (1991) as showing that BFA treatment leads to an increase in the concentration of HRP accumulated in the endosomal reticulum such that even with low specific activity tracer the threshold of detection by the peroxidase-DAB method is reached.

The response of cells to BFA is known to vary. For example, the Golgi apparatus in PtK cells is resistant to BFA (Kistakis et al., 1991) at concentrations that cause tubulation of early endosomes in these cells (Lippincott-Schwartz et al., 1991), and at low concentrations of BFA the same is true of MDCK cells (Hunziker et al., 1991; Low et al., 1991). Our data indicate that in AtT20 cells the situation is the opposite: the Golgi apparatus is more sensitive to a low concentration (1 μg/ml) of BFA than the early endosomes. In HeLa cells this concentration induces the full BFA phenotype.

The response of late endosomes/lysosomes also varies between cell types. In NRK and CEF cells BFA caused tubulation of lysosomes (Lippincott-Schwartz et al., 1991); on the other hand, tubules were not induced from the prelysosomes of MDBK cells (Wood et al., 1991) or from the late endosomes/prelysosomes of AtT20 cells, as shown here. We could detect no morphological or immunocytochemical changes to the prelysosomes of AtT20 cells even after incubation for up to 3 h with concentrations of up to 20 μg/ml.

gold, arrows) against a murine lysosomal membrane protein. C shows an AtT20 cell incubated with 5 μg/ml BFA for 60 min and then labeled for indirect immunofluorescence microscopy with a 50:50 mixture of the rat monoclonal antibodies LAMP I and II. The labeling is restricted to discrete cytoplasmatic granules; the same pattern of labeling was seen in control cells and cells incubated with BFA for 5-180 min. D-F show, respectively, late endosomes in a control AtT20 cell and cells incubated for 60 and 120 min with BFA. The sections were labeled in parallel with affinity-purified antibody against rat liver CI-M6PR. Quantitation of the experiment is given in Table I. Bars, 100 nm.
BFA. Moreover, as Table I shows, incubation with BFA did not alter the amount of immunogold labeling of CI-M6PR in Art20 prelysosomes, whereas Wood et al. (1991) concluded from immunofluorescence microscopy that BFA induced the loss of CI-M6PR from MDBK cell prelysosomes. As far as we can judge, Art20 prelysosomes are unaffected by BFA; in these cells, therefore, the hierarchy of decreasing sensitivity is Golgi stacks, early endosomes, and late endosomes/prelysosomes.

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