EFFECTS OF BREFELDIN A ON THE EXPRESSION AND TRANSPORT OF INFLUENZA A VIRUS HAEMAGGLUTININ, M1 AND M2 PROTEINS WITHIN THE CELL

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Summary. – Brefeldin A (BFA) decreased the expression of influenza A virus haemagglutinin (HA) and M2 protein on the plasma membrane of virus-infected MDCK cells. It caused a retention of M1 protein in the cell nucleus and a decrease of its expression on the plasma membrane. On the other hand, an increased labelling of the cytoplasmic domain of M2 protein on the plasma membrane in BFA-treated cells was observed in contrast to the labelling in BFA-untreated cells. The effects of BFA on the microtubules and cellular motors are discussed.

Key words: influenza virus; MDCK cells; haemagglutinin; M1 protein; M2 protein; Brefeldin A

Introduction

The influenza virus M2 protein is a type III integral membrane protein consisting of 97 amino acids, a 23-residue N-terminal extracellular domain and a 54-residue C-terminal intracellular cytoplasmic domain (Lamb *et al.*, 1985); it represents an ion channel with a homotetramer structure (Sugrue and Hay, 1991; Holsinger and Lamb, 1991; Shimbo *et al.*, 1996) that is expressed at the surface of virus-infected cells but is a relatively minor component of influenza virions (Zebedee and Lamb, 1988; Čiampor, 1993).

Amantadine and rimantadine are specific inhibitors of replication of influenza A viruses (Dolin *et al.*, 1982). An amantadine treatment of cells infected with influenza A viruses causes a M2 protein-mediated conversion of HA from its native to low-pH conformation. The structural alteration and hence drug action occur shortly after HA exits from the Golgi complex during its passage through the trans-Golgi region (Čiampor *et al.*, 1992a,b).

The influenza virus integral membrane proteins (HA, NA and M2) are synthesized on membrane-bound ribosomes and are translocated across the membrane of the endoplasmatic reticulum (ER) in an signal recognition particle (SRP)-dependent manner (Elder *et al.*, 1979; Hull *et al.*, 1988). The membrane proteins to be expressed at the plasma membrane are subsequently transported from the ER to the Golgi complex and beyond it via vesicular carriers that recycle between successive compartments along the pathway (Palade, 1975, 1982).

Such studies have been published in recent years as a result of the discovery of compounds that disrupt certain parts of the exocytotic pathway. One of them, BFA, is a hydrophobic fungal metabolite, an antiviral macrocyclic antibiotic isolated from *Penicilium brefeldinianum* Dodge

Abbreviations: BFA = Befeldin A; BSA = bovine serum albumin; ER = endoplasmatic reticulum; FITC = fluorescein isothiocyanate; GAR-FITC = goat anti-rabbit IgG-FITC conjugate; GAM-TRITC = goat anti-mouse IgG-TRITC conjugate; HA = haemagglutinin; MEM = Eagle's Minimal Essential Medium; MoAb = monoclonal antibody; PBS = phosphate buffered saline; RT = room temperature; SRP = signal recognition particle; TGN = trans-Golgi network; TRITC = tetramethylrhodamine isothiocyanate; VSV = vesicular stomatitis virus

by Härri *et al.* (1963). Originally it was reported that BFA blocks the transport of secretory proteins from ER to the Golgi complex with subsequent extensive disorganization of the latter (Kato *et al.*, 1989; Misumi *et al.*, 1986; Oda *et al.*, 1987; Takatsuki and Tamura, 1985).

In this paper, we describe the effects of BFA on the transport and expression of M2, HA and M1 proteins in the influenza A virus-infected MDCK cells.

Materials and Methods

Viruses and cells. Influenza virus A/chicken/Germany/34 (H7N1) Rostock strain was grown in 11-day-old fertile hens' eggs. MDCK cell monolayers were cultivated in Eagle's Minimal Essential Medium (MEM) supplemented with 5% foetal calf serum in a humidified atmosphere with 5% CO_2 at 37°C.

Antibodies. Anti-HA monoclonal antibody (MoAb) HC2, recognizing a peripheral domain of HA1 (site A), was produced against whole virus as described elswere (Sugrue *et al.*, 1990). Anti-M2 protein sera R53 and R54, raised in rabbits against C-terminal or N-terminal parts of M2 protein, were obtained from the National Institute for Medical Research, London, England (Grambas and Hay, 1992). The antisera R53 and R54 recognize the extracellular and cytoplasmic domains of M2 protein, respectively.

Anti-M1 monoclonal antibody (MoAb) 290 was prepared and characterized at the Institute of Virology, Bratislava (Varečková *et al.*, 1995).

Infection and BFA-treatment of cells. MDCK cell monolayers on coverslips or microtiter plate wells were exposed to the virus at a multiplicity of infection of 10 – 100 PFU per cell for 1 hr at room temperature (RT) and then maintained in MEM with or without (control) 5 μg/ml BFA at 37°C until examined.

Immunofluorescence light microscopy and double-labelling. Cell cultures on glass coverslips were placed on ice, briefly washed with cold phosphate-buffered saline (PBS) pH 7.2, fixed and per-

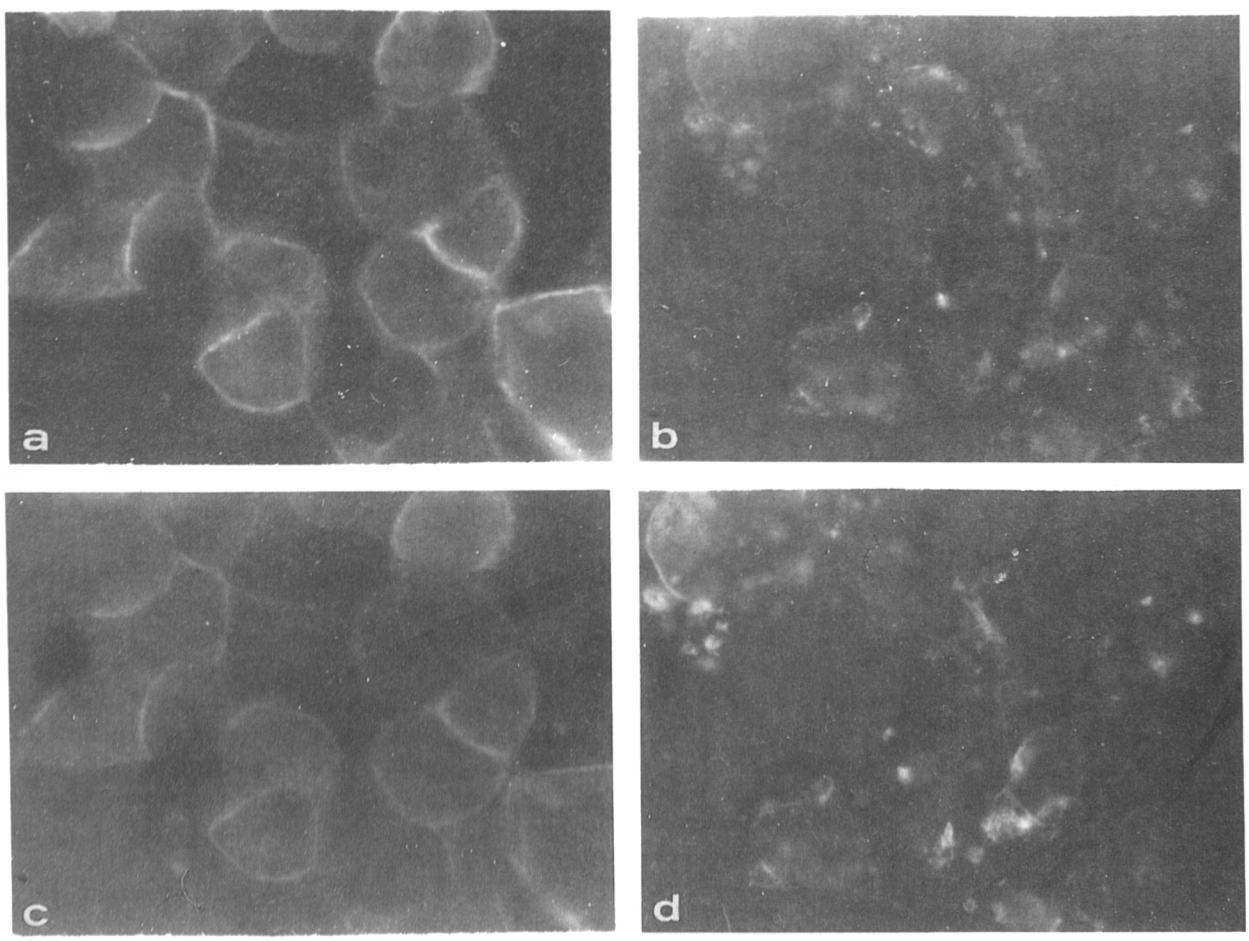

Fig. 1
Effect of BFA on the HA and M2 protein expression on the plasma membrane of virus-infected cells
16 hrs p.i. BFA-untreated (a,c) and BFA-treated (b,d) cells. Double labelling: HC2-GAM-TRITC (a,b); R53-GAR-FITC (c,d).

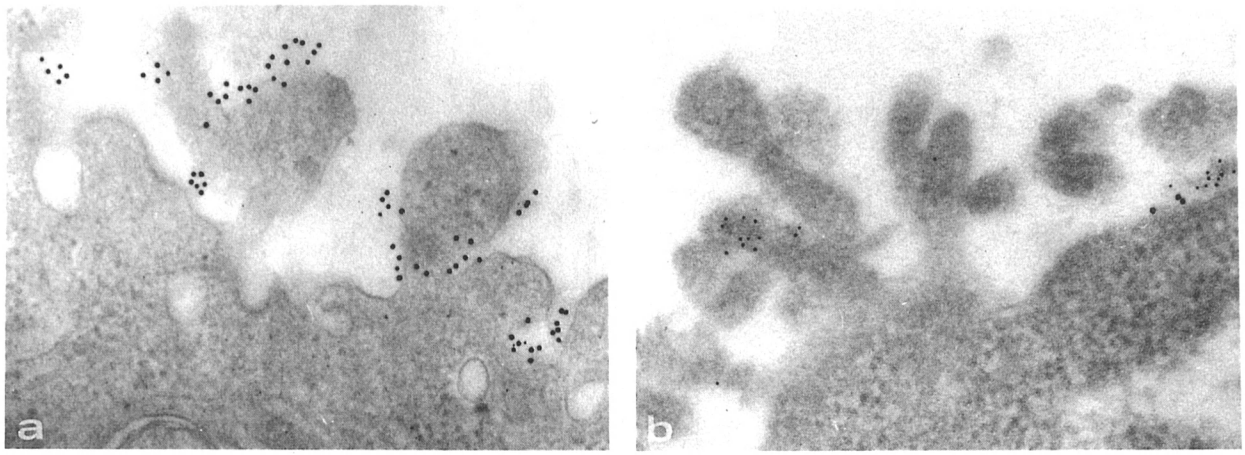

Fig. 2
Effect of BFA on the cell surface expression of M2 protein and HA in virus-infected cells
5.5 hrs p.i. BFA-untreated (a) and BFA-treated (b) cells. Double labelling: R53-GAR 5 nm gold; HC2-GAM 10 nm gold. Magnification 63,000 x (a) and 82,500 x (b).

meabilized with methanol at -20°C for 5 mins, or briefly washed with PBS at RT, fixed with 3% paraformaldehyde in PBS for 10 – 15 mins and washed with PBS at RT. To reduce a non-specific binding of antibodies, the cells were incubated with PBS containing 0.5% bovine serum albumin (BSA, IgG-free, Sigma) (PBS/BSA) for 2 x 15 mins before applying an antibody.

MoAb HC2, anti-M2 serum and MoAb 290 were diluted with PBS/BSA 1:100, 1:50 and 1:20, respectively, and applied to the cell monolayers for 30 – 45 mins at RT. The cells were repeatedly washed with PBS/BSA and labelled with a 1:125 dilution of goat anti-rabbit IgG-FITC conjugate (GAR-FITC, Sigma) or with 1:125 dilution of goat anti-mouse IgG-TRITC conjugate (GAM-TRITC, Sigma) for 30 – 45 mins at RT. In double-labelling experiments, GAM-TRITC was used as a first label and after several washings in PBS/BSA the cell monolayers were labelled with GAR-FITC. The labelled monolayers were washed several times with PBS and mounted in an anti-bleaching solution. The samples were observed under a Carl Zeiss Jena light microscope equiped for epifluorescence (NPL Fluotar objective x 100/1.32).

Gold-immunolabelling for electron microscopy. The immunolabelling was performed on LR Gold-embedded sections, or directly on fixed cells (Čiampor *et al.*, 1992a,b). Ultrathin sections were prepared on a Reichert OmU4 Ultracut FC ultramicrotome. The sections were stained with 2% aqueous uranyl acetate and lead citrate. The samples were examined under a Philips EM 300 electron microscope at 80 kV.

Enzyme-linked immunosorbent assay (ELISA) of influenza virus HA, M2 and M1 proteins in virus-infected cells was performed as follows. The cells on microtiter plate wells were fixed with 0.05% glutaraldehyde in PBS. ELISA was carried out on duplicate wells using ascitic fluids containing anti-HA MoAb HC2, anti-M2 polyclonal rabbit antibodies R53 or R54, anti-M1 MoAb 290, and anti-mouse and anti-rabbit horseradish peroxidase conjugates, respectively.

Results

Effect of BFA on the localization of HA and M2 protein on the cell surface of virus-infected cells

MoAb HC2, specific for the peripheral domain of HA_1 (site A), and antibodies R53 and R54, specific for extracellular and cytoplasmic domains of M2 protein, respectively, were used to localize the expression of the transmembrane viral proteins HA and M2 in influenza virus-infected cells (16 hrs p.i.) under the influence of BFA by immunofluorescence and immunogold electron microscopy, respectively.

As shown in Fig. 1a, MoAb HC2 localized the HA expression on the surface of virus-infected cells not treated with BFA. In contrast, as a consequence of the BFA effect on the Golgi complex, the labelling of the cell surface with MoAbs HC2 was reduced to small patches in BFA-treated cells (Fig. 1b).

The immunogold electron microscopy of cells labelled for HA showed a decoration of the cell surface with gold particles in BFA-untreated cells (Fig. 2a), but a reduced number of gold particles in BFA-treated cells (Fig. 2b).

Double labelling experiments with R53 antibody and MoAB HC2 recognizing the N-terminal domain of M2 protein and HA, respectively, localized both proteins in ER and Golgi complex compartments and on the cell plasma membrane.

As shown in Fig. 1c, R53 antibody localized the M2 expression on the surface of virus-infected BFA-untreated cells. In contrast, in BFA-treated cells, the intensity of labelling of the cell surface was reduced and the fluorescence on the surface was not regular, but concentrated in small patches (Fig. 1d).

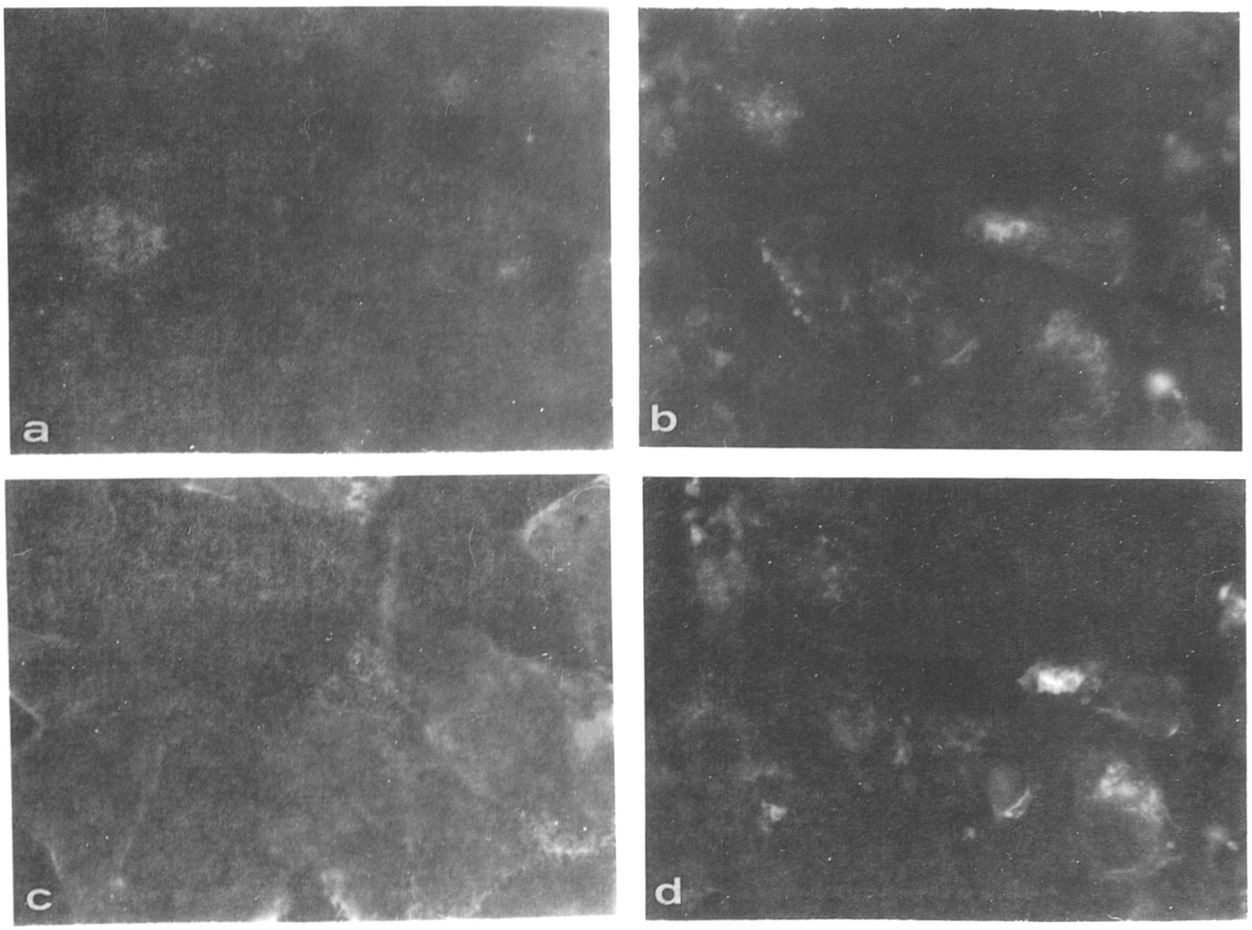

Fig. 3
Effect of BFA on the distribution and labelling of HA and the cytoplasmic domain of M2 protein on the plasma membrane of virus-infected cells
16 hrs p.i., double labelling. BFA-untreated cells: R54-GAR-FITC (a); HC2-GAM-TRITC (c). BFA-treated cells: R54-GAR-FITC (b); HC2-GAM-TRITC (d).

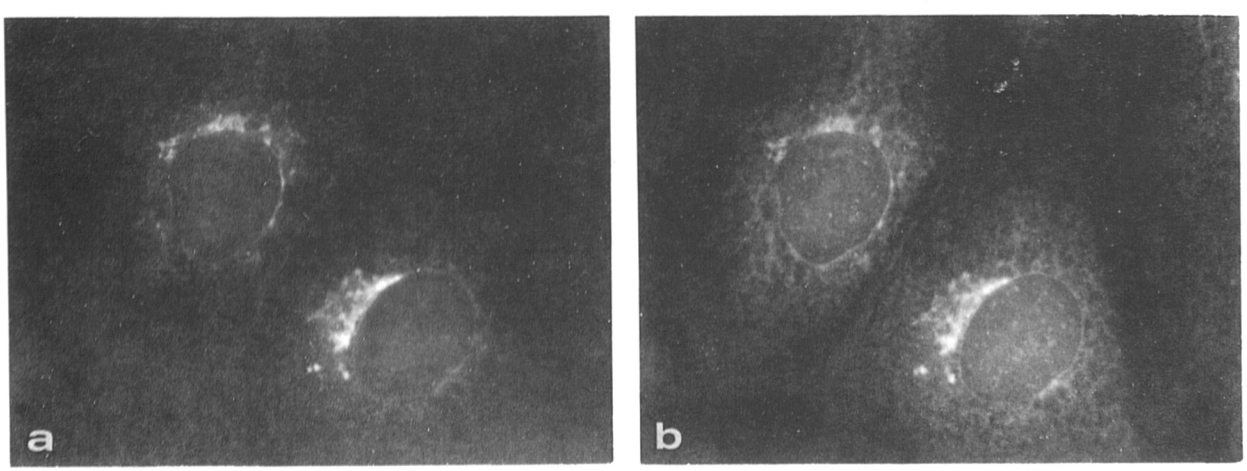

Fig. 4
Localization of HA and M2 protein in BFA-untreated virus-infected cells
5.5 hrs p.i., permeabilized cells. Labelling: HC2-GAM-FITC (a); R53-GAR-TRITC (b).

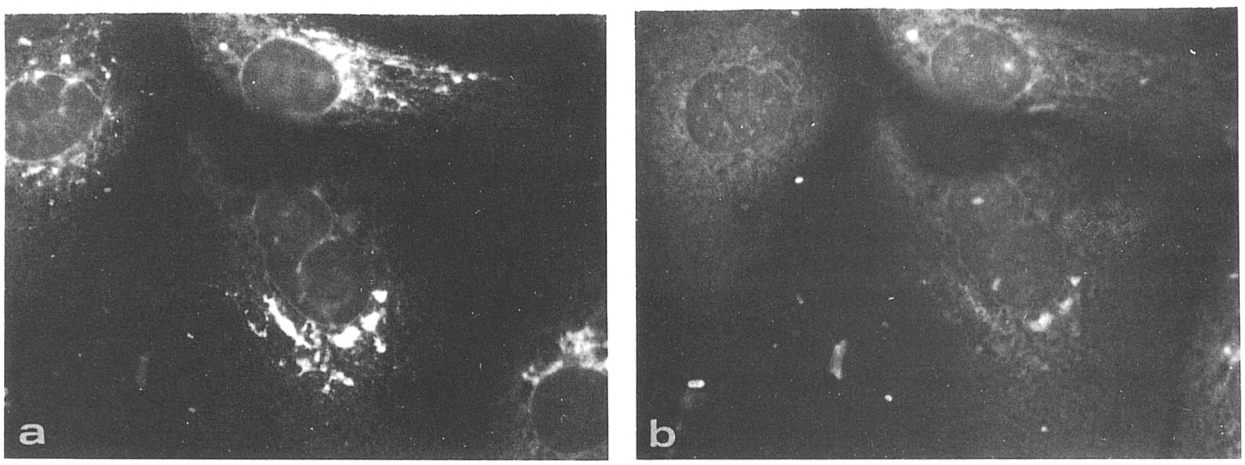

Fig. 5
Formation of HA- and M2 protein-enriched TGN-derived membrane tubules induced by BFA in virus-infected cells
5.5 hrs, permeabilized cells. Labelling: HC2-GAM-FITC (a); R53-GAR-TRITC (b).

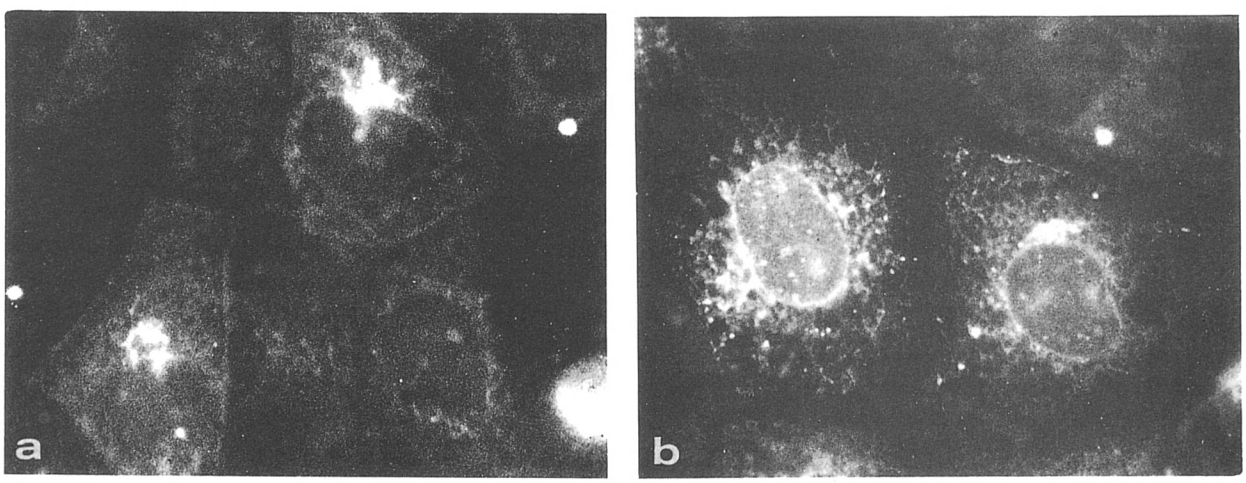

Fig. 6
Effect of BFA on the localization of the cytoplasmic domain of M2 protein in virus-infected cells
5.5 hrs p.i. BFA-untreated (a) and BFA-treated (b) cells. Labelling: R54-GAR-FITC.

On the other hand, the labelling of the virus-infected cell surface with R54 antibody was poor (Fig. 3a). In the virus-infected BFA-treated cells, labelled for M2 protein with R54 antibody, the labelling was observed just in small patches (Figs. 3b,c and 4d).

Effect of BFA on the localization of HA and M2 proteins in permeabilized virus-infected cells

Double labelling experiments localized both HA nad M2 protein in virus-infected cells 5.5 hrs p.i. juxtanuclearly, in rough ER and Golgi complex (Figs. 4a and 4b). BFA induced a rapid formation of HA- and M2 protein-enriched, TGN-derived membrane tubules, but did not affect the medial part of Golgi complex (Figs. 5a and 5b).

Antibody R54 recognized M2 protein on the virus-infected cell surface very poorly (Fig. 3a), but in virus-infected permeabilized cells quite well. The labelling was localized in the juxtanuclear region and Golgi complex (Fig. 6a). In BFA-treated cells, antibody R54 similarly to antibody R53 labelled M2 protein-enriched, TGN-derived membrane tubules (Fig. 6b).

Effect of BFA on the localization of M1 protein on the surface and inside the permeabilized virus-infected cells

At 5.5 hrs p.i., we observed a cell surface labelling of M1 protein with MoAb 290 in BFA-untreated cells

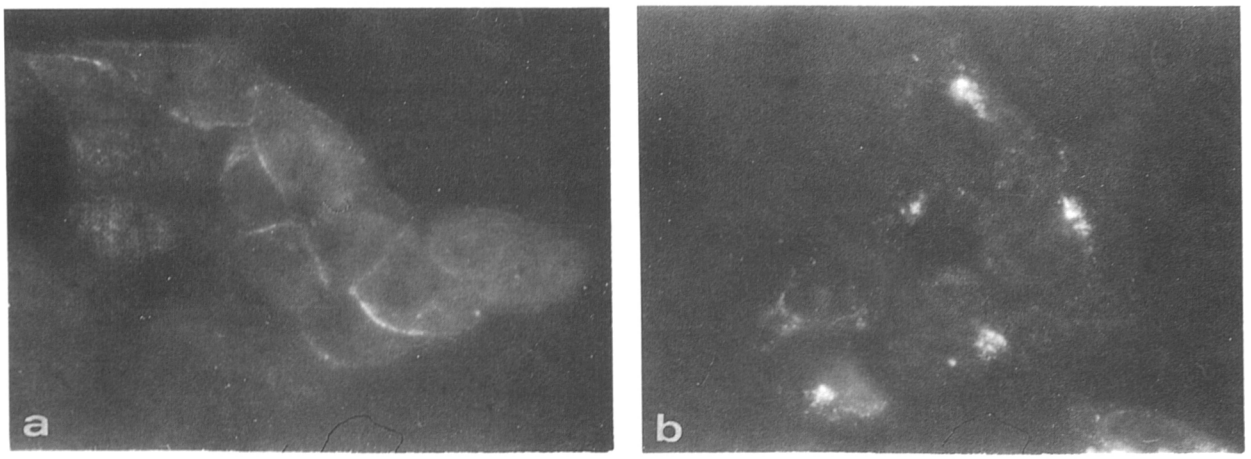

Fig. 7
Effect of BFA on the localization of M1 protein on the plasma membrane of virus-infected cells
5.5 hrs (a) and 16 hrs (b) p.i. BFA-untreated (a) and BFA-treated (b) cells. Labelling: 290-GAM-FITC.

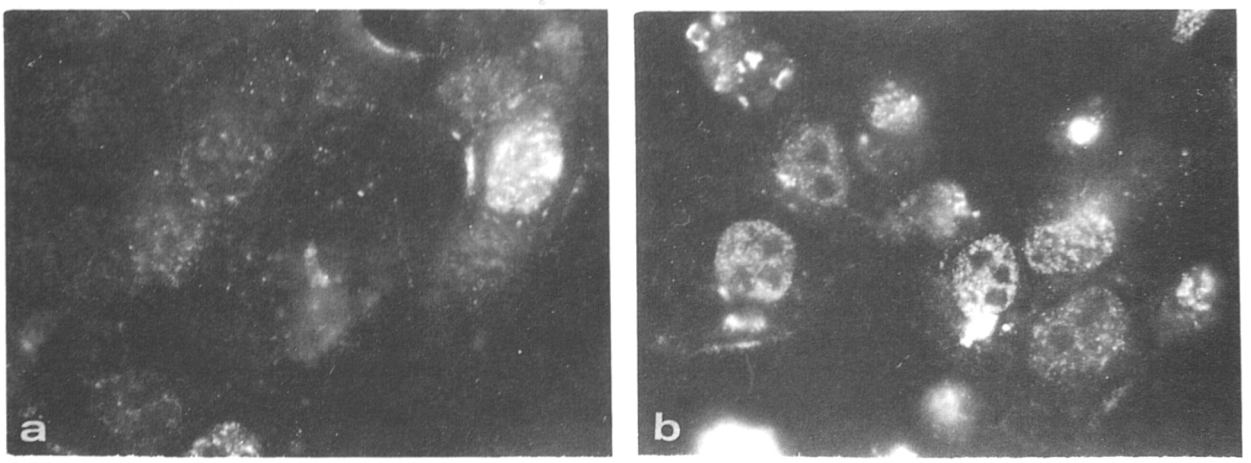

Fig. 8
Effect of BFA on the localization of M1 protein in virus-infected cells
16 hrs p.i., permeabilized cells. BFA-untreated (a) and BFA-treated (b) cells. Labelling: 290-GAM-FITC.

(Fig. 7a). At 16 hrs p.i. in BFA-treated cells, we observed no cell surface labelling of M1 protein, but just small patches of a fluorescent material (Fig. 7b).

In permeabilized virus-infected BFA-untreated cells, we detected a nuclear localization of M1 protein, but only small patches of a fluorescent material in the cytoplasm and on the cell surface (Fig. 8a). In BFA-treated cells, an increased nuclear labelling and a weak cell surface labelling of M1 protein was observed (Fig. 8b).

Effects of BFA on HA, M2 and M1 proteins in virus-infected cells as examined by ELISA

The ELISA examination of the localization of HA, M2 and M1 proteins on the surface of and in whole virus-infected cells under influence of BFA confirmed the results of the immunomicroscopic experiments. The influence of BFA on the distribution of the tested viral proteins in virus-infected MDCK cells is summarized in Table 1. BFA decreased the labelling of HA, M2 (as de-

tected by antibody R53) and M1 proteins on the cell surface, but increased that of M2 protein (as detected by antibody R54) on the cell surface and that of intracellular M1 protein.

Discussion

The trans-Golgi network (TGN) is an organelle engaged in the exocytosis and the site of the amantadine-induced, M2-mediated conversion of influenza A virus HA from its native to the low-pH conformation (Čiampor *et al.*, 1992a,b).

In contrast to the Golgi apparatus of MDCK cells, TGN is sensitive to BFA (Wagner *et al.*, 1994). At a concentration of 1μg/ml, BFA promoted extensive tubulation of TGN, while the medial Golgi marker alfa-mannosidase II was not affected. Extensive structural alterations of TGN were accompanied by functional disruptions, such as an extensive missorting of influenza HA, and by a release of the TGN-marker gamma-adaptin. These results suggest the involvement of BFA-sensitive adaptor proteins in the TGN-surface transport.

The data of Wood *et al.* (1991) showed that BFA causes a microtubule-mediated fusion of TGN with the early endosomes, and revealed a membrane transport cycle between TGN and the early endosomes, perhaps used for the secretion or delivery of molecules to the cell surface.

Pelham (1991) reviewed the recent data on multiple targets of BFA and on species-specific effects of BFA on the vesicular transport that suggest several distinct sites of action of BFA on the endomembrane system.

BFA inhibits the export of proteins from distal Golgi compartments to the cell surface of BHK-21 cells (Miller *et al.*, 1992). The results of these authors suggested that BFA blocked the export via both the constitutive and regulated pathways. In contrast, the endocytosis and recycling of the vesicular stomatitis virus (VSV) G protein were not blocked by BFA, but it did block the constitutive secretion of glycosaminoglycan chains that had been synthesized and sulfatated in the trans-Golgi cisternae.

On the other hand, Low *et al.* (1992) showed a selective inhibition of protein targeting to the apical domain of BFA-treated MDCK cells.

The presented experiments confirm and extend further the data obtained in our earlier studies on the transport of HA and M2 protein in the cytoplasm of infected cells including the role of TGN in conformational changes of HA dependent on M2 protein (Čiampor *et al.*, 1992a,b; Čiampor, 1993), and on the effect of BFA on influenza virus-infected MDCK cells (Závodská *et al.*, 1995; Čiampor *et al.*, 1996). We also show additional results concerning the effect of BFA on the M1 protein transport from the nucleus to plasma membrane.

Table 1. Influence of BFA (5 μg/ml) on the distribution of virus proteins in influenza virus-infected MDCK cells as determined by ELISA

Virus proteins	Antibodies	Cell surface*	Whole cell*
HA	HC2	61.5	85.8
M1	290	83.7	125.1
M2	R53	73.5	91.0
M2	R54	116.0	98.3

*The ELISA values of the BFA-untreated controls were taken for 100%.

BFA inhibited the expression of HA as well as of M2 protein on the plasma membrane. HA at the plasma membrane was reduced to 61.5% and total HA to 85.8% of the control, respectively. A similar reduction was observed for the extracellular domain of M2 protein at plasma membrane (to 73.5%) and for the total one (to 91.0%).

Surprising results were obtained with the labelling of the cytoplasmic domain of M2 protein. In infected BFA-untreated cells, the labelling of the plasma membrane was regularly poor but strongly positive in BFA-treated cells. The corresponding intracellular labelling was not significantly affected by BFA (reduction to 98.3% only).

Similarly, M1 protein, which is normally transported directly from the cell nucleus to the plasma membrane and its transport does not depend on the Golgi complex, accumulated in BFA-treated cells in the cell nucleus and its expression on the plasma membrane was reduced.

Studies of Lippincott-Schwarz *et al.* (1989, 1990) showed that BFA caused a microtubule dependent redistribution of the Golgi complex in relation to ER and thus provided a morphological evidence for a retrograde, incompartmental transport pathway between the Golgi cisternae and the ER. In this article, we show that BFA first of all affects cytoskeletal proteins, microtubules and that all the changes in the transport pathway are the result of this primary effect. The accumulation of M1 protein in the cell nucleus observed by us corresponds to the results of Lippincott-Schwarz *et al.* (1989, 1990).

The increased labelling of the extracellular domain of M2 protein on the cell surface provokes a speculation that the position of transmembrane proteins in the plasma membrane of vesicles transporting via TGN depends not only on intramembraneous interactions but also on an interaction with cytoskeletal proteins, e.g. microtubules. The apical or basolateral transport of vesicles from TGN is affected by BFA (Low *et al.*, 1992).

BFA has multiple targets (Pelham, 1991), but our results comparing the transmembrane protein transported via the

Golgi stack or directly from the nucleus to the cell surface indicate one common target – cytoskeletal proteins, mainly the microtubules and cell motors. BFA by affecting the microtubules causes microtubule-mediated changes in the endocytosis and exocytosis pathways.

Our results represent only a partial support to this hypothesis an direct our attention to a more detailed investigation of the role of the microtubules and cell motors in these processes.

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