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Brefeldin A and Exo1 Completely Releave the Block of Cholera Toxin Action by a Dipeptide Metalloendoprotease Substrate

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1. Introduction

Cholera toxin (CT), the enterotoxin secreted by *Vibrio cholerae* classical as well as *El Tor* biotypes, is the major causative agent of the acute diarrheal disease of humans. CT and the *Escherichia coli* heat labile enterotoxin (LT),are structurally and immunologically highly homologous,seeing that they belong to the same enterotoxin family (de Haan and Hirst, 2004; Spangler, 1992; Vanden Broeck et al., 2007). Both are oligomeric proteins of the A-B type. CT is composed of one A or activating subunit (CT-A Mr 27,400), which consists of two distinct polypeptide chains CT-A_1 (Mr 22,000) and CT-A_2 (Mr 5,400), linked by a single disulfide bridge, and 5 identical B subunits (Mr 11,600) arranged in a ring like configuration (CT-B).

The subunits are arranged in such a manner that CT-A occupies the central channel of the CT-B pentamer extending well above the plane of the pentameric ring (Sixma et al., 1991; Zhang et al., 1995). The CT-A_2peptide goes through the pore in the doughnut-like structure of the CT-B pentamer, and protrudes on the side, which binds cell surface receptors with its COOH-terminal KDEL sequence exposed. CT elicits a secretory response from intestinal epithelia by binding to the apical cell membrane through interaction between CT-B and the monosialoganglioside $GM_{1,}$ followed by entry of polypeptide A_1 into the cell, where it is able to stimulate the basolateral adenylatecyclase by catalyzing the ADP-ribosylation of Arg201 of the $G_{s\alpha}$ subunit of the stimulatory GTP-binding regulatory protein (de Haan and Hirst, 2004; Spangler, 1992; Vanden Broeck et al., 2007a; Sixma et al., 1991).

There is a distinct lag period between toxin binding and the activation of adenylatecyclase, during which the toxin must be internalized and processed.At the end of this lag period small amounts of CT-A_1 appear in the cells parallel to activation of the cyclase(Kassis et al., 1982).

Early morphologic studies showed that CT is preferentially clustered into non-coated membrane invaginations characteristic of caveolae and enters several cell types via smooth, non clathrin coated vesicles (Lencer et al., 1999).

Studies using cholesterol perturbing agents and chimeric toxins have shown that GM_1-mediated association with detergent-resistant membrane fractions (DRMS) or lipid rafts is required for toxic entry of CT (Orlandi and Fishman, 1998; Wolf et al., 1998, 2002).

Although CT is currently used as a marker for endocytosis without utilising clathrin-coated pits,it appears to be endocytosed simultaneously through both clathrin -dependent and-independent routes (Orlandi and Fishman, 1998; Nichols et al., 2001; Shogomori and Futerman, 2001; Torgersen et al., 2001; Vanden Broeck et al., 2007b).

In a recent study using fluorescence microscopy (Massol et al., 2004)it has been shown that apart from clathrin-, caveolin-endocytic pathways, CT also enters cells via a pathway that is regulated by the small GTPase Arf6 and possibly a fourth pathway that is dynamin and Arf6-independent. However, after blocking all three known endocytic payhways simultaneously by over expression of negative dominant mutants of dynamin and Arf6, fluorescent CT in the Golgi and ER became undetectable, although CT induced toxicity was hardly affected (Massol et al., 2004).These findings illustrate the difficulty in correlating morphologic data with the functional entry of a potent toxin such as CT.

Consistent with the multiple ports of entry into the cell, CT can be found in early and recycling endosomes (Tran et al., 1987; Nichols, 2002)and in caveolin-1 containing endocytic intermediates (Nichols, 2002),which have been proposed to be responsible for the functional transport of CT. For CT to be toxic it must be transported through the Golgi to the ER. Brefeldin A (BFA), a fungal metabolite that disrupts the structural and functional integrity of the Golgi apparatus (Klausner et al., 1992),renders cells resistant to CT cytotoxicity and blocks intracellular formation of CT-A_1(Orlandi et al., 1993; Nambiar et al., 1993; Lencer et al., 1993).

Movement into the Golgi can also be inhibited by blockage of COPI- and COPII- mediated vesicular transport, and this affects toxin function further implicating trafficking through the Golgi apparatus as a necessary step in toxin action (Richards et al., 2002; Majoul et al., 1998).

On reaching the ER, the reduced form of the lumenal chaperone protein disulfideisomerase binds to the A_1 chain, dissociates it from the B subunit and unfolds it(Tsai et al., 2001; Tsai and Rapoport, 2002; Fujinaga et al., 2003). Subsequent oxidation of PDI by the ER lumenal ERO1, the A_1 chain is released (Tsai and Rapoport, 2002)and is translocated to the cytosol probably via the Sec61 channel, identifying the rough ER as the compartment from which translocation occurs (Schmitz et al., 2000). It has been suggested that the rapid refolding (Tsai and Rapoport, 2002)may render the A_1 chain resistant to poly ubiquitination and provide the driving force for retro translocation to the cytosol (Rodighiero et al., 2002).

We previously reported that the metalloendoprotease substrate N-benzoyloxycarbonyl-Gly-Phe-NH_2(Cbz-Gly-Phe-NH_2) completely blocked the response of different celltypes in culture to CT. The effect was reversible, dose-and time-dependent. The dipeptide had no effect on the binding of CT to the cell surface and did not decrease its internalization but appeared to affect a later step in toxin action (De Wolf, 2000).

In this study we further investigated the mechanism by which Cbz-Gly-Phe-NH_2 blocks CT action.

2. Materials and methods

2.1.Materials

Highly purified CT was obtained from List Biological Laboratories (Campbell, Ca.). CT was radiolabeled with ^{125}I using the Iodo-gen method as described by Fraker and Speck (1978).

Unreacted ^{125}I-Na was removed using gel filtration on a Sephadex G-50 mini-column using the centrifugation procedure of Tuszynski et al. (1980).Cbz-Gly-Phe-NH_2, brefeldinA, 3-isobutylmethylxanthine, iodixanol (Optiprep™), 1,9-dideoxyforskolin, nocodazole and 2-deoxy-D-glucose were from Sigma. 2-(4-Fluorobenzoylamino)-benzoic acid methylester (Exo1) was from Calbiochem.1,3-Cyclohexane-bis(methylamine) (CBM) was purchased from Acros Organics. Na^{125}I was obtained from MP Biochemicals& Reagents (formerly ICN).

2.2.Cell culture

Vero cells originally obtained from Flow Laboratories were cultured in Medium 199 with Earle's salts supplemented with 5% fetal calf serum (FCS).Human intestinal epithelial T84 cells (obtained from ATCC) were propagated in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 2.5mM L-glutamine and 5% fetal bovine serum.Madin-Darby canine kidney (MDCK) cells (obtained from ATCC) were grown in Eagle's Minimum Essential Medium with 2mM L-glutamine and Earle's BSS adjusted to contain 1.5g/l sodium bicarbonate, 0.1mM non-essential amino acids, and 1.0mM sodium pyruvate with 10% fetal bovine serum. Growth medium was changed twice a week and cells were passed weekly (at confluency) using a 0.05% (w/v) trypsin solution (Gibco). Cells were counted in a hemocytometer.

2.3 Preparation of membranes from post nuclear supernatant of a Vero cell homogenate

Vero cells were grown to confluency in 175cm^2 culture flasks and maintained in culture for at least 14 days before harvesting. Cells were washed once with serum free medium to remove serum and harvested by short trypsinisation with a 0.05% trysin, EDTA solution in HBSS (Gibco). After 1 min the trypsinisation solution was removed by aspiration and the culture flasks were put at 37°C until the cells detached from the bottom. Cells needed for one gradient centrifugation experiment (5 culture flasks for each time interval) were collected in an Eppendorf tube, washed twice and suspended in 2ml of serum-free medium buffered with 25mM HEPES containing 0.1% BSA and chilled. Cells were then labeled with ^{125}I-CT (10^6cpm /2 ml≈ 10nM) in the same medium containing 0.01 % bovine serum albumin for 30 min at 4°C to allow binding without endocytosis. Subsequently, cells were washed and harvested immediately (t=0) or washed and incubated for 10,30,60 or 120 min in serum-free medium at 37°C and harvested.Cells were pelleted by low speed centrifugation. The pellets were resuspended in 50mM tris-HCl pH 7.4 containing 250mM sucrose (Tris-sucrose) buffer and homogenized on ice with 4 x 4 strokes of a Potter Elvejhem homogenizer (position 9). The homogenates were spun at 3,000 x g for 10 min to pellet unbroken cells, cell debris and nuclei to yield the PNS. Membranes from PNS were obtained by centrifugation of the PNS at 100,000 x g for 1 h at 4°C. The membrane pellet was resuspended in Tris-sucrose buffer.

2.4 Gradient centrifugation

Subcellular fractionation was always performed on freshly prepared post nuclear membranes or PNS from cells labeled with ^{125}I-CT. Membrane pellets prepared from PNS (post nuclear membranes),resuspended in 3 ml of homogenization buffer (50mM Tris-HCl, pH 7.4 containing 0.25M sucrose) or equal volumes of PNS,werelayered on top of two layers of respectively 4 ml of 25% iodixanol and 4 ml of 50% iodixanol each in isoosmotic

homogenization buffer in a 11.5 ml centrifugation tube (Sorvall). The tubes were centrifuged in a (Sorvall TV-850) vertical rotor at 35,000 rpm for 18 h at 4°C using a Kontron centrifuge Centricon T2060. Fractions of 0.3 ml were collected from the bottom of the gradient using a device with a perforation needle and a density gradient fractionator. A 15 µl portion of each gradient fraction was used to determine its refractive index (η) as measured by an Abbe refractometer. From the refractive indices the densities of individual gradient fractions were calculated using the equation ς=3.4911η-3.6664 as reported by Graham et al. (1994). Fractions were further analyzed for protein by the method of Bradford (1976) and for the trans-Golgi marker UDP-galactosyltransferase(Verdon and Berger, 1983) as well as the classical subcellular organelle markers rotenone-insensitive NADPH cytochrome creductase, alkaline phosphatase, acid phosphatase and cytochromecoxidase as described before (De Wolf et al., 1985).

2.5 Assay of cellular cyclic AMP content

Monolayer cultures of cells were washed twice with Earle's balanced salt solution without Ca^{2+} and Mg^{2+} and treated with 0.05% (w/v) trypsin solution containing 0.02% EDTA in HBSS. After 1 min the trypsin solution was removed and 10-15 min later cells were suspended in culture medium. Cells were collected by centrifugation at 750 rpm for 5 min and resuspended in 1 ml serum-free medium containing 25mM Hepes, 0.01% (w/v) bovine serum albumin and 1mM 3-isobutyl-1-methylxanthine. 1 ml aliquots of cell suspension (10^5 cells/ml were divided over Eppendorf tubes and incubated with CT (1µg/ml) and the required effectors were added at the indicated times as described in the legend of figures. Afterwards, cell suspensions were put on ice, centrifuged for 6 min at 1000 rpm and resuspended in 0.1 ml 0.5M sodium acetate buffer (pH 6.2). The suspensions were boiled for 10 min and then sonicated for 30 s. After centrifugation for 10 min at 10000 rpm, 20 µl of cell extract was taken for cyclic AMP (cAMP) assay. cAMP was assayed using a cAMP assay kit (Pharmacia Amersham) based on a competitive protein-binding method. Results for cAMP represent the mean of values from duplicate samples each assayed in triplicate.

2.6 Generation of CT-A_1 and analysis by SDS-polyacrylamide gel electrophoresis

Vero cells were grown to confluency in small (∅=3cm) petri dishes (250.10^6 cells) and maintained in culture for at least one week before use. Cells were washed once with serum-free medium buffered with 25mM Hepes and incubated with and without Cbz-Gly-Phe-NH_2(3mM) for 30 min at 37°C.The medium was replaced with ice-cold serum-free medium/Hepes (total volume=3 ml) containing ^{125}I-CT (10^6cpm/ml;≈1nM CT) and 0.01% BSA and the cells were further incubated at 4°C for 30 min.The cells were then washed with ice-cold serum-free medium/Hepes and incubated at 37°C for 60 min by replacing the medium with warm serum-free medium-/Hepes. With each medium change, Cbz-Gly-Phe-NH_2(3mM) wasadded as required. The cell incubations were either stopped immediately or after the addition of BFA (1µg/ml), further incubated for 30 min at 37°C and then stopped. Incubations were stopped by adding 1 ml of ice-cold N-ethylmaleimide (NEM) (1mM) in phosphate-buffer saline to prevent any further reduction of CT (Kassis et al., 1982), scraped in PBS, and pelleted by low speed centrifugation. The cells were lysed and solubilised by addition of a small volume of 0.125M Tris/HCl (pH 8.0), 2mM phenyl-methyl sulfonylfluoride and 1 % SDS. After 10 min at 37°C the solubilised material was adjusted to 20% glycerol and 0.02% bromphenol blue and applied to the gels. The amount of CT-A_1 generated was determined by SDS-PAGE.

Each sample (≈10,000 cpm) was separated on 16% tris-glycine gel (Novex pre-cast gels). After the gels were run and stained with coomassie blue, protein bands corresponding to CT-A_1 were cut from the gel, which was subsequently dissolved in lumasolveand, after addition of lipoluma (Lumac, LSC), counted in a liquid scintillation counter. CT reduced with dithiothreitol and dialysed against 10mM tris/HCl pH 7.4 containing 10mM NEM, to remove the excess of reducing agent, was adjusted to 1% SDS, 20% glycerol and 0.02% bromphenol blue, warmed at 37°C for 10 min and run on each slab gel.

3. Results

3.1 Effect of Cbz-Gly-Phe-NH_2 on the intracellular retrograde transport of CT

In order to assess whether the metalloendoprotease substrate Cbz-Gly-Phe-NH_2 perturbs the uptake and intracellular trafficking of CT, we performed subcellular fractionation experiments on post-nuclear membranes of Vero cells with prebound^{125}I-CT.

The distribution profiles of marker enzymes after isopycnic gradient centrifugation of these membranes, using self-generating gradients of iodixanol, showed a reasonable separation of Golgi fractions (as represented by UDP-galactosyltransferase) from the ER fractions (rotenone-insensitive NADPH cytochrome c reductase) and plasma membranes (alkaline phosphatase) (Fig.1,C). Membrane proteins were distributed all over the gradient (profile not shown).

At the outset,^{125}I-CT was allowed to bind to Vero cells in suspension for 30 min at 4°C, minimizing endocytosis.Cells were washed and homogenized immediately (t=0) or they were washed and re-cultured for an additional 10 min (t=10); 30 min (t=30); 60 min (t=60) and 120 min (t=120) at 37°C prior to preparation of membranes from PNS. It was noticed that during re-culture, radioactivity was progressively released into the culture medium (after 10 h more than 60%, mostly (80%) as non-precipitable material).However, when the re-culture time was 120 min this amount was less than 10%.

As shown in Fig.1 panels A and C, the radioactivity profile at zero time corresponded to that of alkaline phosphatase, a cell-surface membrane marker. Upon increasing the re-culture time,ra-dioactivity shifted to higher densities. After 30 min most of the radioactivity equilibrated at densities (1.127g/ml) corresponding to Golgi-derived membranes, as evidenced by the distribution of the Golgi marker UDP-galactosyltransferase.At still later time points (60–120min),^{125}I-CT further moved to higher densities corresponding to those of membranes from the rough ER, which is in agreement with the generally accepted retrograde transport of the toxin to the ER. The distribution profiles of marker enzymes were not affected upon increasing the re-culture time (data not shown).

As shown in Fig.1,B, pre-treatment of Vero cells with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37° C, before binding and internalization of ^{125}I-CT, markedly affected the distribution profiles of radioactivity upon increasing re-culture times. Whereas the internalization,in agreement with our previous results (Schmitz et al., 2000), was not affected, further transport of the toxin appeared to be strongly perturbed. After re-culture the toxin still moved to densities corresponding to those of Golgi-derived membranes, but at an apparently lower rate, and further transport to the ER appeared to be blocked. Pre-exposure of cells to Cbz-Gly-Phe-NH_2 (3mM) did not significantly affect the distribution profiles of marker enzymes. Only the Golgi marker equilibrated at a somewhat higher density

(1.132g/ml versus 1.127g/ml) but clearly did not shift to densities corresponding to the ER, as was the case after pretreatment of cells with BFA (data not shown).

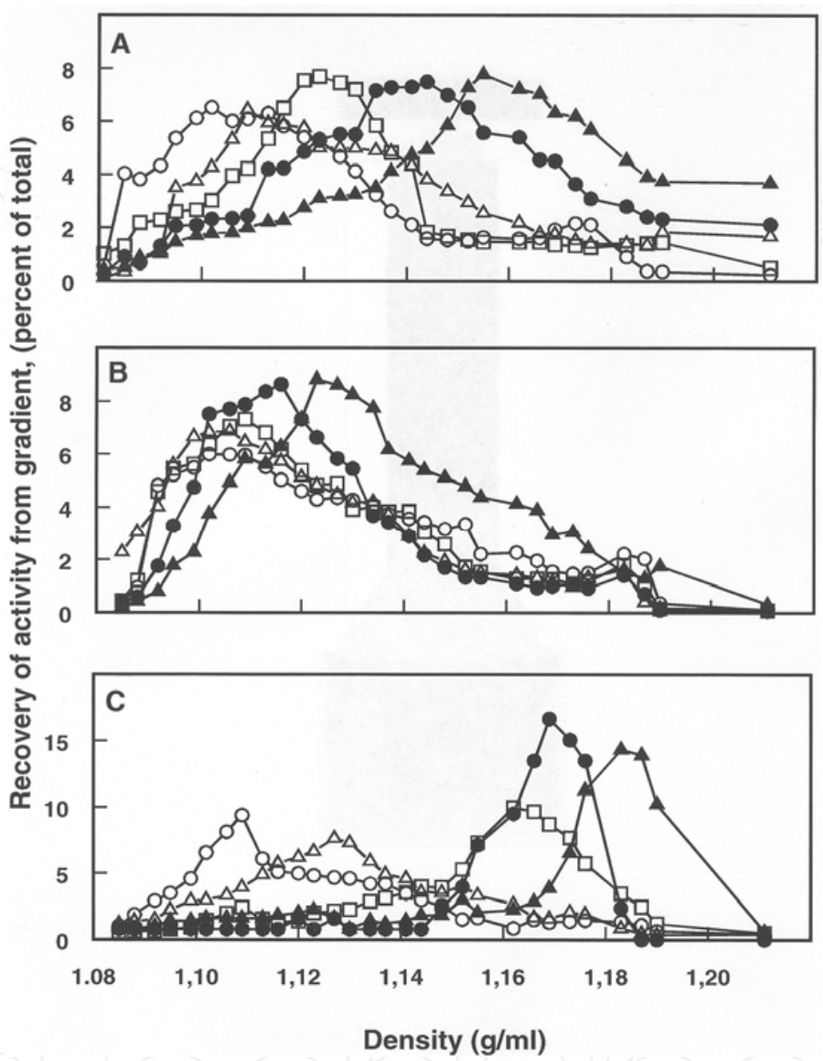

Fig. 1. Subcellular fractionation of post-nuclear membranes from Vero cells prelabeled with ^{125}I-CT.Vero cells prelabeled with ^{125}I-CT at low temperature to block endocytosis were, after washing, incubated at 37°C for 0 time (○), 15 min (△), 30 min (□), 60 min (●) or 120 min (▲) and post-nuclear membranes were prepared and centrifuged through a self-generating gradient of iodixanol as described under Materials and Methods. A. Subcellular distribution profiles of membrane-bound ^{125}I-CT after increasing re-culture periods.B. Subcellular distribution profiles of membrane-bound ^{125}I-CT after pretreatment of Vero cells with Cbz-Gly-Phe-NH_2 and different re-culture periods. Vero cells in suspension were pretreated with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37°C. The cells were chilled and after binding of ^{125}I-CT at 4°C, washed and re-cultured for different periods of time in the presence of Cbz-Gly-Phe-NH_2 (3mM) at 37°C.C. Distribution profiles of subcellular marker enzymes at zero time (no-reculture),(○) Alkaline phosphatase, (△) UDP- galactosyltransferase, (●) NADPH - cytochrome creductase, (□) Acid phosphatase, (▲) cytcoxidase. The distribution profiles are representative for three similar experiments.

3.2 Effect of drugs perturbing the Golgi structure on theCbz-Gly-Phe-NH_2-mediated inhibition of CT action

From the density gradient centrifugation experiments it is clear that the metalloendoprotease substrate Cbz-Gly-Phe-NH_2 affects the intracellular transport of CT and that in its presence the toxin appears to be trapped in an intracellular compartment, which cofractionated with a marker of the Golgi apparatus.In order to find out whether in the presence of Cbz-Gly-Phe-NH_2 the toxin travels beyond the trans-Golgi network (TGN) and reaches the cisternae of the Golgi complex, we explored whether drugs that are able to redistibute Golgi membranes and its content into the ER also caused a reversal of Cbz-Gly-Phe-NH_2-mediated inhibition of CT action.

As shown in Fig.2,A,C and in agreement with previous results (De Wolf, 2000),prior incubation of Vero cells and T84 cells with Cbz-Gly-Phe-NH_2 or with BFA for 30 min at 37°C resulted in respectively a complete or strong inhibition of CT action in a dose-dependent way.

However, when these cells were preincubated with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37 °C and then incubated in the presence of CT (1µg/ml) for an additional 60 min time period, a time at which CT - as evidenced by the gradient centrifugation experiments - becomes trapped in a compartment where it is unable to reach the cytosol and raise intracellular cAMP levels, subsequent addition of BFA completely reversed the Cbz-Gly-Phe-NH_2-mediated inhibition of CT action in a dose-dependent way(EC_{50}≈0.5µg/ml) (Fig.2,B,D).

The concentrations at which reversal of the inhibition occurred, corresponded to the concentrations needed for inhibition of CT action and induction of the redistribution of Golgi membranes into the ER (Fig.2,A,C) (Doms et al., 1989; Lippincott-Schwartz et al., 1989).

We next determined the effect of 2-(4-fluoro-benzoylamino)-benzoic acid methylester (Exo1), a novel chemical inhibitor of the exocytotic pathway (Feng et al., 2003).Like BFA, Exo1 induces the release of ADP-ribosylation factor (ARF)1 from Golgi membranes, inducing/generating/stimulating a rapid collapse of the Golgi into the endoplasmic reticulum in different cell types. However, unlike BFA this drug has less effect on the organization of the trans-Golgi network (Feng et al., 2003). As shown in Fig.2,A,C,E, prior exposure to Exo1 blocked the CT induced cAMP accumulation in all cell types tested. The effect was dose-dependent with an IC_{50} value of ≈0.35µM. This value is almost two orders of magnitude lower than that reported for its inhibitory effect on the anterograde movement of the viral glycoprotein VSVG from the ER to the Golgi and its stimulation of the release or ARF1 from Golgi membranes in BSC1 fibroblasts (Feng et al., 2003).

As expected, Exo1 also completely reversed the Cbz-Gly-Phe-NH_2-mediated inhibition of CT ac-tion in a similar dose-dependent way (Fig.2,B,-D,F).

We previously showed (De Wolf, 2000)that there are some similarities in the inhibitory effects of Cbz-Gly-Phe-NH_2 and BFA on CT action. However, Madin-Darby canine kidney epithelial (MDCK) cells, of which the Golgi structure is BFA resistant (Hunziker et al., 1991)and as a consequence are insensitive to the inhibitory effect of BFA on CT action, were sensitive to the inhibitory effect of Cbz-Gly-Phe-NH_2.In agreement with our previous results (De Wolf, 2000),prior exposure of these cells toCbz-Gly-Phe-NH_2(Fig.2,E) completely suppressed (IC_{50}=0.5mM) CT action. Therefore, it was of interest to determine whether BFA is also able to reverse the inhibitory effect of Cbz-Gly-Phe-NH_2 on CT action in MDCK cells.

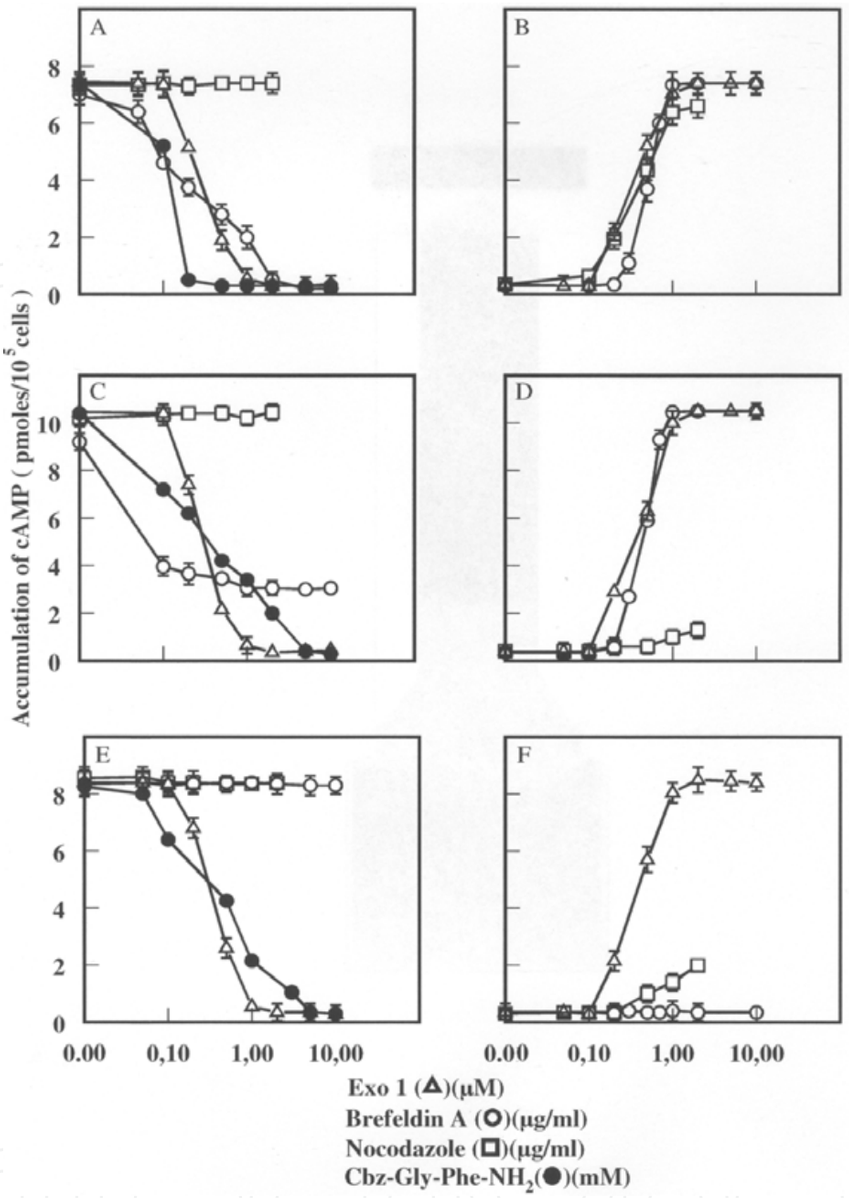

Fig. 2. Inhibition of CT action by different concentrations of BFA, Exo1 and nocodazole and their effect on the Cbz-Gly-Phe-NH_2 - mediated inhibition of CT action in several cell types. A, Vero cells; C, T84 human intestinal epithelial cells and E, MDCK cells in suspension were preincubated at 37°C for 30 min in the presence of the indicated amounts of BFA (○), Exo1 (△), nocodazole (□) or Cbz-Gly-Phe-NH_2 (●). After the addition of CT (1μg/ml) cells were further incubated for 90 min at 37°C and the cAMP accumulation measured. B, Vero cells; D, T84 human, intestinal epithelial cells and F, MDCK cells were preincubated with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37°C. Following the addition of CT (1μg/ml) cells were further incubated for 60 min at 37°C. Finally, the indicated amounts of BFA (○),Exo1 (△) and nocodazole (□)were added and the cells incubated for an additional 30 min at 37°C and cAMP measured. Values are the means ±SD of triplicate assays from one of three similar experiments. Error bars are indicated when they were more than 5% of the mean.

As shown in Fig.2,E,F, BFA did neither prevent CT induced cAMP accumulation nor reverse the inhibitory effect of Cbz-Gly-Phe-NH_2 on CT action in MDCK cells. In contrast, Exo1, which is able to prevent CT action in these cells, was also able to reverse the inhibition by Cbz-Gly-Phe-NH_2 in a similar dose-dependent way (Fig.2,-E,F).

This is in line with the proposal (Feng et al., 2003)that although Exo1 and BFA are exerting similar effects they probably have different protein targets. Whereas BFA blocks GDP to GTP exchange on ARF1 and therefore reduces the concentration of ARF1-GTP on Golgi membranes, it is believed that Exo1 also reduces the concentration of ARF1-GTP on Golgi membranes by accelerating the hydrolysis of GTP bound to ARF1 by an activation of ARF1-GAP activity(Feng et al., 2003).

By causing the release of ARF1 and COPI from membranes, BFA and Exo1 directly interfere with the Golgi-ER retrograde trafficking machinery and this likely perturbs normal recycling from the Golgi to the ER. Therefore it was of interest to see whether a treatment of cells, which affects the Golgi structure without directly interfering with the retrograde transport, also caused a reversal of the inhibition of the CT action by Cbz-Gly-Phe-NH_2.

A constant influx of membrane from the ER is required to maintain the Golgi structure. Microtubule disruption prevents this influx by blocking the peripherial pre-Golgi intermediates from tracking into the Golgi region (Cole et al., 1996; Storrie et al., 1998). The microtubule depolymerizing agent nocodazole, which blocks the forward traffic into the Golgi complex without a corresponding effect on recycling, leads to the fragmentation of the Golgi complex and redistribution of its material to the site of perturbation (Cole et al., 1996).

Whereas prior exposure of cells to nocodazole did not affect CT action (Fig.2,A,C,E), it was able to reverse the Cbz-Gly-Phe-NH_2-mediated inhibition of CT action in a dose-dependent way. Maximal reversal was observed at a concentration of 0.5µg/ml. The effect was most pronounced in Vero cells (Fig.2,B), whereas in T84 and MDCK cells the effect was minimal. Increasing the concentration of nocodazole above 2µg/ml impairedthe effect because of strongly reduced cell viability.

3.3 Time dependence of the BFA-, Exo1-and nocodazole-induced reversal of the inhibitory effect of Cbz-Gly-Phe-NH_2on CTaction in Vero cells

As depicted in Fig.3 BFA (2µg/ml), Exo1 (2µM) and nocodazole (1µg/ml) caused a rapid increase in the cAMP concentration after CT had accumulated in an intracellular compartment in the presence of Cbz-Gly-Phe-NH_2 (3mM). Already within 1 min after the addition of each drug a significant increase in cAMP accumulation could be observed and after a 5 to 10 min time period the cAMP concentration reached its maximal value. This time course is similar to that observed for the redistribution of Golgi membranes into the ER (Lippincott-Schwartz et al., 1989; Feng et al., 2003; Sciaky et al., 1997)and indicates that once CT has reached the Golgi, the BFA-, Exo1-or nocodazole-induced redistribution of Golgi membranes and content into the ER is followed by a very fast activation of the adenylyl cyclase.Thisimplies that within approximately one minute a fraction of the toxin reaches the ER, becomes activated by reduction, translocates into the cytosol and gains access to its substrate the $G_{S\alpha}$subunit of G_S, which finally activates the cyclase after mono-ADP ribosylation.

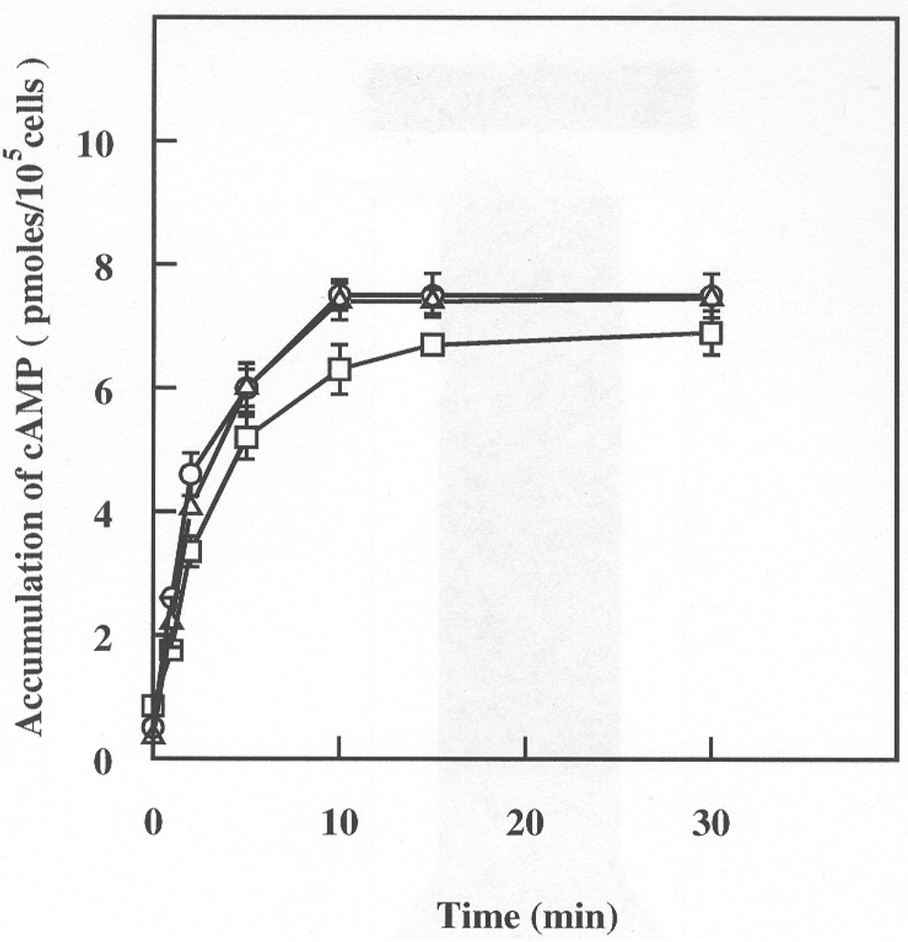

Fig. 3. Time dependence of the BFA-, Exo1- and nocodazole-induced reversal of the inhibitory effect of Cbz-Gly-Phe-NH_2 on the CT-induced accumulation of cAMP in Vero cells. Vero cells in suspension were preincubated with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37°C. After the addition of CT (1µg/ml) cells were further incubated for 60 min at 37°C. Subsequently, BFA (2µg/ml) (○), Exo1 (2µM) (△) and nocodazole (1µg/ml) (□) were added and the cells incubated for the indicated times followed by a determination of cAMP. Data points are the means of triplicate assays from one of at least three similar experiments.

Therefore we assume that, at least in the presence of Cbz-Gly-Phe-NH_2,CT also becomes activated at the level of the Golgi apparatus, and that in the ER the A_1 fragment is rapidly translocated into the cytosol. A generation of CT-A_1 at the level of the Golgi complex is in line with our previous observation (De Wolf, 2000)that in the presence of Cbz-Gly-Phe-NH_2 (3mM), CT-A_1 can still be formed. Furthermore, in this study we found that upon direct quantitation of the amount of CT-A_1 generated, the BFA-induced redistribution of Golgi membranes into the ER did not increase the fraction of CT-A that became reduced in cells pretreated with Cbz-Gly-Phe-NH_2 (3mM) (data not shown).

Likewise, the BFA-induced reversal of the inhibitory effect ofCbz-Gly-Phe-NH_2on the CT action was hardly affected by the addition of agents that are able to change the redox potential of the ER and affect the unfolding of CT and the generation of CT-A_1 fragment

(Tsai and Rapoport, 2002). As shown in Table I, incubation of cells with 5mM dithiothreitol (DTT), a reducing agent known to permeate into the ER of intact cells (Braakman et al., 1992)or the oxidant diamide (0.5mM),did almost not influence the BFA-induced reversal of the inhibitory effect ofCbz-Gly-Phe-NH_2. Also, the membrane-permeant sulfhydryl blocker NEM, which at a concentration of 10μM has been shown to completely inhibit the reduction of CT to CT-A_1 by intact CaCo-2 cells (Orlandi, 1997),had only a minor effect on the BFA-induced restoration of CT toxicity in the presence of Cbz-Gly-Phe-NH_2 (Table I).

Treatment	Accumulation of cAMP
	pmoles/10^5 cells
Control	7.5 ± 0.3
DTT (5mM)	6.7 ± 0.3
Diamide (0.5mM)	7.5 ± 0.3
NEM (0.01mM)	7.4 ± 0.2
NEM (0.1mM)	5.8 ± 0.2
2-deoxy-D-glucose (50mM) + 0.02 % sodium azide	2.0 ± 0.2
Nocodazole (2μM)	7.4 ± 0.3
1,3-cyclohexanebis(methylamine) (2mM)	7.5 ± 0.2
All treatments no BFA added	0.4 ± 0.2

Table 1. Effect of several treatments on the BFA-induced reversal of the inhibitory effect of Cbz-Gly-Phe-NH_2 on CT cytotoxicity. Vero cells in suspension were preincubated for 30 min at 37°C with Cbz-Gly-Phe-NH_2 (3mM). After addition of CT (1μg/ml) cells were further incubated for 60 min at 37°C. The indicated amounts of agents were added and the cells incubated for 15 min at 37°C. Finally, BFA (1μg/ml) wad added and the incubation continued for 30 min at 37°C.

3.4 Kinetics of CT transport in the presence of Cbz-Gly-Phe-NH_2 to a compartment that redistributes into the ER upon addition of BFA

The rapidBFA-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2 allowed us to estimate the time it takes for CT to reach, in the presence of Cbz-Gly-Phe-NH_2,a compartment that redistributes into the ER following the addition of BFA.

In these experiments, cells (Vero,T84 and MDCK) were preincubated with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37°C. CT (1μg/ml) was added and the cells further incubated for the indicated times. Finally, BFA (5μg/ml) was added and the incubation continued for 90 min and cAMP accumulation measured.

As shown in Fig.4, in the presence of Cbz-Gly-Phe-NH_2 substantial amounts of CTreached, within approximately 5 to10 min, a compartment that redistributes into the ER following the addition of BFA.

This time period corresponds to the time needed for CT to reach the Golgi complex, as evidenced by our subcellular fractionation experiments (Fig.1) and previous immunofluorescence studies (Majoul et al., 1996) on Vero cells.

In these experiments theBFA concentration was kept high to induce rapid (within less than 5 min) redistribution of the Golgi lipids and proteins into the ER.

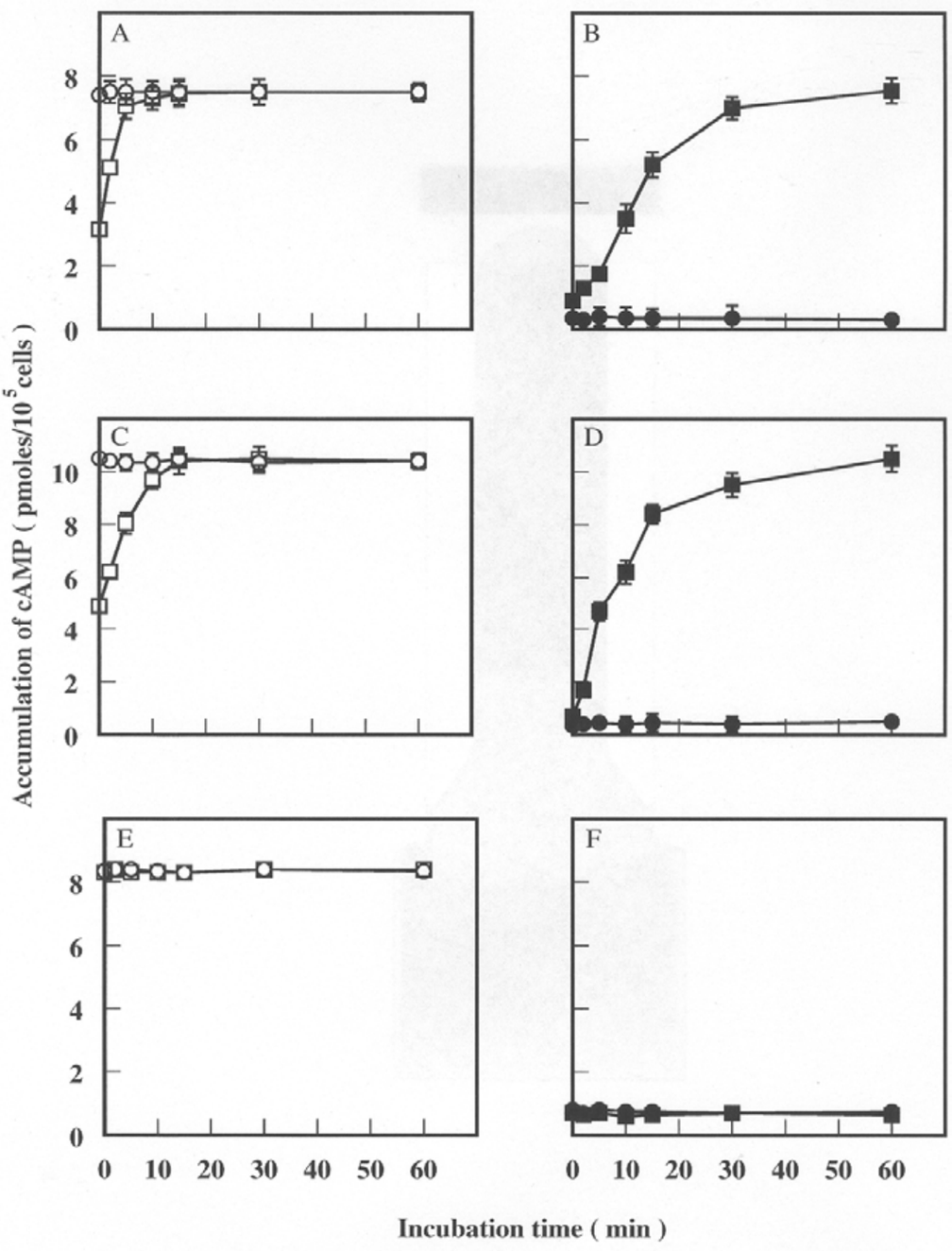

Fig. 4. Kinetics of CT transport in the presence of Cbz-Gly-Phe-NH2 to a compartment which is redistributed to the ER following the addition of BFA. Vero cells (A,B), T84 (C,D) and MDCK cells (E,F) were preincubated with (●,■) and without (○,□) Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37°C. Subsequently, CT (1µg/ml) was added and the cells incubated at 37°C for the indicated times. Finally, cells were incubated in the presence (□,■) and absence (○,●) of BFA (2µg/ml) for 90 min at 37°C and the cAMP accumulation determined. Values are the means ±SD of triplicate assays from one of three similar experiments. Error bars are indicated when they were more than 5% of the mean.

3.5 Characteristics of the BFA-and Exo1- induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2

It has been shown previously (Lippincott-Schwartz, 1990)that within minutes of adding BFA to most cells, the Golgi apparatus disassembles, giving rise to long, uncoated tubules that extend along microtubules fusing uniquely with the ER. Several treatments have been shown to inhibit this BFA-induced tubule formation. These included AlF_4^-, nocodazole, forskolin and some of its derivatives, reduced temperature and energy depletion (Orlandi, 1997; Majoul et al., 1996; Lippincott-Schwartz, 1990).

In order to further examine whether the BFA- and Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2 is related to tubulation and disassembly of the Golgi apparatus, similar treatments affecting Golgi tubule formation were applied.

To this end, Vero cells in suspension were preincubated for 30 min at 37°C with Cbz-Gly-Phe-NH_2 (3mM). Subsequently, CT (1µg/ml) was added and the cells further incubated for 60 min at 37°C. The cells were then treated with the different reagents and finally BFA (1µg/ml) or Exo1 (2µM) wasadded and the incubation continued for 30 min at 37°C and thecAMP content determined.

3.5.1 Effect of AlF_4^- on the BFA- and Exo1- induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2

*A*lF_4^- stabilizes coatomer binding to Golgi membranes in vitro and in vivo and renders COPI highly resistant to removal by BFA, apparently by inhibition of COPI dissociation(Donaldson et al., 1991a; Finazzi et al., 1994).

The mechanism by which AlF_4^- stabilizes COPI binding is not well understood but probably in-volvestrimeric G-proteins(Donaldson et al., 1991b; Helms et al., 1998). Another possibility is that AlF_4^-locksARF1 in a GDP.AlF_4^-binding transition state together with a limiting cofactor GAP, as shown for other small GTPases,and in this way reduces the overall rate of GTP hydrolysis (Rittinger et al., 1997; Scheffzek et al., 1997).

As shown in Fig.5,A, treatment of Vero cells with 30mM NaF plus 50µM $AlCl_3$ elicted, however, only a minor inhibitory effect on the BFA induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2. This slight reduction of the reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2 appearedto be cell specific, since it was not observed with T84 cells (data not shown).

It has been reported that AlF_4^-blocks the ability of Exo1 to induce dissociation of membrane-bound ARF^{wt}-GTP from Golgi membranes (Feng et al., 2003). Therefore, we also determined the effect of AlF_4^-on the Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2. From Fig.5,A it is clear that AlF_4^-almostcompletely blocked the ability of Exo1 to reverse the effect of Cbz-Gly-Phe-NH_2.

AlF_4^- is able to directly interact with heterotrimeric G-proteins and, as described above, with small GTP binding proteins complexed with specific GTPase activating proteins.

However, the effect of AlF_4^-on the BFA-and Exo1-induced reversal of the inhibitory action of Cbz-Gly-Phe-NH_2 wasmost likely not influenced by an increase of the cAMP level by a direct activation of G_S,since addition of $AlF_4^-$to intact cells hadonly a minor effect on the cAMP level

(Fig.5,B). This is in contrast to its effect on the adenylyl cyclase activity of membrane preparations or crude cell lysates. In addition, CT- induced mono ADP ribosylation of $G_{S\alpha}$ depends on active ARF1 as a cofactor, therefore, an effect of AlF_4^- at the level of ARF1 should also be considered. A previous study (Kahn, 1991), however , has demonstrated that AlF_4^- does not activate ARF1 and does not affect the CT induced mono ADP ribosylation of G_S.

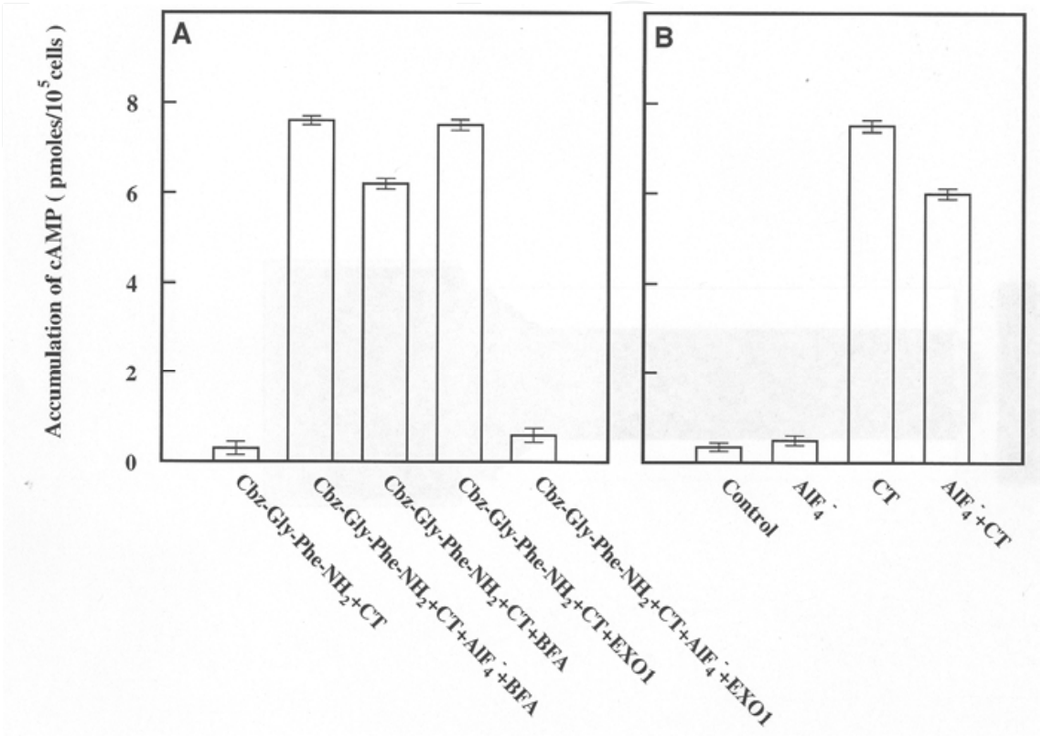

Fig. 5. Effect of AlF4 on the BFA- and Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH2.A. Vero cells in suspension were incubated with Cbz-Gly-Phe-NH2 (3mM) for 30 min at 37°C. CT (1μg/ml) was added and the cells were incubated for an additional 60 min at 37°C. Then cells were further incubated for 15 min at 37°C with and without NaF (30mM) and AlCl3 (50μM). Finally cells were treated with BFA (1 μg/ml) or Exo1 (2μM) for 30 min at 37°C and the intracellular concentration of cAMP measured. B. In parallel experiments Vero cells were preincubated with and without NaF (30mM) and AlCl3 (50μM) for 15 min at 37°C and further incubated for 60 min at 37°C with or without CT (1mg/ml) and the intracellular concentration of cAMP determined. Values are the means ±SD of triplicate assays from one of three similar experiments. Error bars are indicated when they were more than 5% of the mean.

Furthermore, as shown in Fig.5,B, prior exposure of Vero cells to AlF_4^- did not enhance but rather slightly reduced the CT-induced cAMP accumulation. A similar reduction was observed in the BFA-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2. Wealso looked whether1,3-cyclohexanebis-(methylamine)(CBM), a drug that interacts with COPI coatomer and inhibits coatomer binding to Golgi membranes (Hu et al., 1999), interferes with the BFA- or Exo1-induced reversal of the inhibition of CT action byCbz-Gly-Phe-NH_2. As shown in Table I, CBM (2mM) had no effect on the BFA- or Exo1-induced

reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2.This dibasic compound itself did also not reverse the inhibitory effect of Cbz-Gly-Phe-NH_2 (data not shown), which is consistent with its inability to cause a redistribution of Golgi membranes into the ER (Hu et al., 1999).

In agreement with previous results (Chen et al., 2002),pre-treatment of Vero cells with CBM (2mM) did not affect the CT-induced elevation of the cAMP level (data not shown).

These experiments indicate that there is no straightforward relationship between COPI dissociation and redistribution of Golgi membranes into the ER and also argue against COPI-and KDEL-dependent functional retrograde transport of CT.

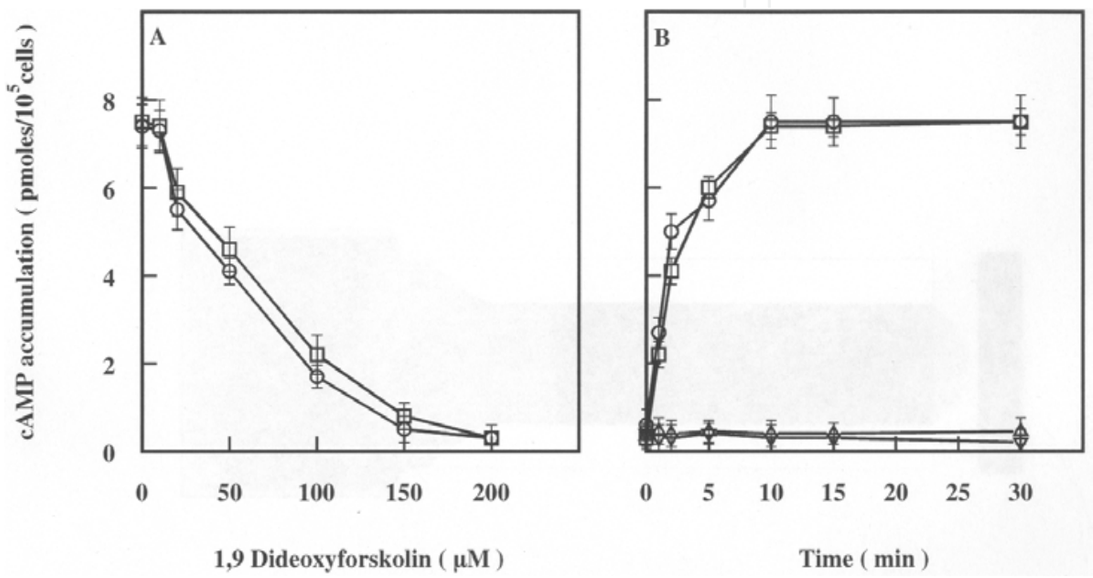

Fig. 6. Effect of 1,9-dideoxyforskolin on the BFA- or Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH2. A. Effect of 1,9-dideoxyforskolin concentration.Vero cells in suspension were preincubated for 30 min at 37°C with Cbz-Gly-Phe-NH2 (3mM). CT (1µg/ml) was added and the cells further incubated for 60 min at 37°C. The indicated amounts of 1,9-dideoxyforskolin were added and after 15 min BFA (1 µg/ml) (○) or Exo1 (2µM) (□) were added and the cells finally incubated for an additional 30 min at 37°C and cAMP accumulation measured. B. Effect of 1,9-dideoxyforskolin on the time courses of the BFA or Exo1 induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH2.Cells were preincubated as described under A. After CT treatment cells were incubated in the presence and absence of 1,9 dideoxyforskolin (150µM) for 15 min and BFA (1µg/ml) (○,△) or Exo1 (2µM) (□,▽) were added and the cells further incubated at 37°C for the indicated times. Values are the means ±SD of triplicate assays from one of three similar experiments. Error bars are indicated when they were more than 5% of the mean.

3.5.2 Effect of 1,9-dideoxyforskolin on the BFA- and Exo1-induced reversal of the inhibition of CT action byCbz-Gly-Phe-NH_2

It has previously been shown that forskolin inhibits and reverses the effects of BFA on Golgi morphology(Lippincott-Schwartz, 1991a).Also, 1,9-dideoxyforskolin, a naturally occurring

analogue of forskolin that does not activate adenylyl cyclase and reproduces many of the cAMP independent effects of forskolin(Laurenza et al., 1989),exerted a similar effect (Lippincott-Schwartz, 1991a). Therefore, it was of interest to look at the effect of 1,9-dideoxyforskolin on the BFA- and Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2.

As illustrated in Fig.6,A, the forskolin analogue completely antagonized the effect of BFA or Exo1 on the Cbz-Gly-Phe-NH_2-induced inhibition of CT action in a dose-dependent (IC_{50} ≈60μM) way. The effect of 1,9-dideoxyforskolin was very fast,since after its additionno raise in cAMP level could be observed one minute after the further addition of BFA or Exo1 (Fig.6,B).

3.5.3 Effect of reduced temperature and energy depletion on the BFA-induced reversal of the inhibition of CT action byCbz-Gly-Phe-NH_2

As shownin Table I, reduction in cellular ATP levels using the metabolic inhibitors 2-deoxy-D-glucose (50mM) and sodium azide (0.02%) strongly suppressed the BFA-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2. Also, lowering the temperature to below 15°C abolished (Fig.7) the BFA-induced restoration of toxicity. The inhibitory effectsof these treatments are in line with their influence on the BFA-induced disassembly of the Golgi apparatus (Lippincott-Schwartz et al., 1990).

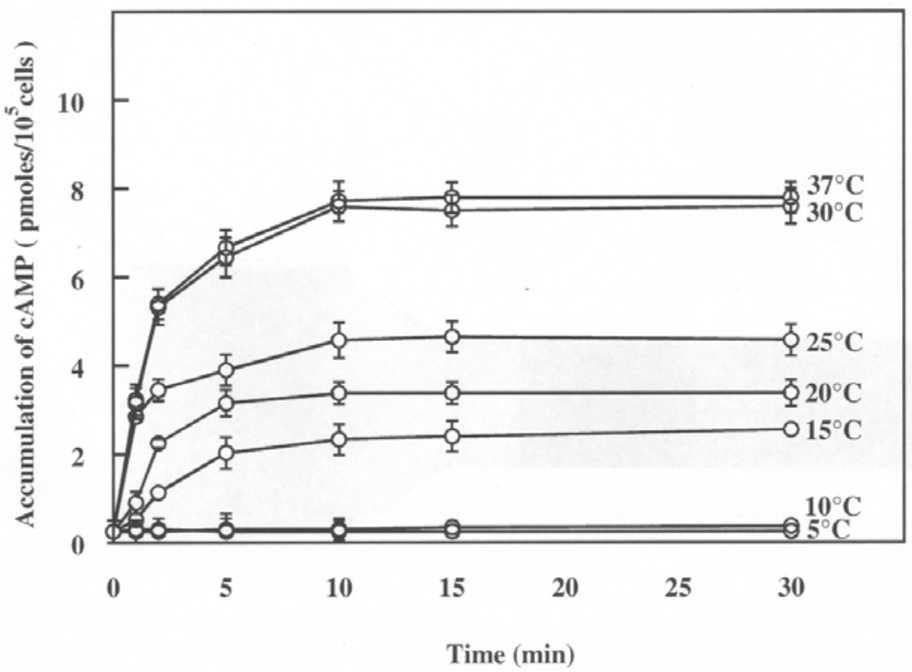

Fig. 7. Temperature dependence of the kinetics of the BFA-induced reversal of the Cbz-Gly-Phe-NH_2-mediated inhibition of CT action on Vero cells. Vero cells in suspension were preincubated with Cbz-Gly-Phe-NH_2 (3mM) for 30 min at 37°C. CT (1μg/ml) was added and the cells further incubated for 60 min at 37°C. Cells were subsequently cooled to the indicated temperatures and after the addition of BFA (5μg/ml) incubated for the indicated times and cAMP measured.Values are the means ±SD of triplicate assays from one of three similar experiments. Error bars are indicated when they were more than 5% of the mean.

4. Discussion

The metalloendoprotease substrate Cbz-Gly-Phe-NH_2, but not its inactive analogue Cbz-Gly-Gly-NH_2, renders cells completely resistant to the action of CT without apparently affecting the binding and internalization of the toxin (De Wolf, 2000).

In this study we further examined the Cbz-Gly-Phe-NH_2-induced inhibition of CT action by looking at the effect of thisdipeptide on the intracellular trafficking of the toxin by using gradient centrifugation experiments and treatment of cells with agents affecting intracellular vesicular transport.

Density gradient centrifugation experiments of post nuclear membranes or supernatants of Vero cells prelabeled with ^{125}I-CT revealed that Cbz-Gly-Phe-NH_2 does not affect the internalization of the toxin but blocks its transport to the ER. Following pretreatment of cells with Cbz-Gly-Phe-NH_2 (3mM) the toxin appears to be trapped in an intracellular compartment, which cofractionates with UDP-galactosyltransferase a marker of the Golgi apparatus.

To further explore whether in the presence of Cbz-Gly-Phe-NH_2 the toxin travels beyond the trans Golgi-network (TGN) and actually reaches cisternae of the Golgi complex, we looked whether drugs known to redistribute Golgi membranes into the ER are also able to cause a reversal of Cbz-Gly-Phe-NH_2-induced inhibition of CT action.

The idea for such an approach came from our previous experiments on the time dependence of the BFA effect on the CT-induced cAMP accumulation in Vero cells (De Wolf, 2000).In these experiments we noticed that addition of BFA 15 min after the addition of CT did not inhibit but rather enhanced the CT-induced increase in the cAMP level. As an explanation we proposed that once the toxin has reached the Golgi complex, the BFA-mediatedredistribution of Golgi membranes into the ER increases the rate of delivery of the toxin to the ER.

In this study we showed that the fungal metabolite brefeldin A (BFA) and a novel chemical inhibitor of anterograde vesicular transport Exo1 are able to completely reverse the Cbz-Gly-Phe-NH_2-induced inhibition of CT action. Both drugs have been shown to cause a rapid release of ADP-ribosylation factor (ARF)1 and COPI from Golgi membranes into the cytosol, followed by massive tubulation and collapse of the Golgi apparatus into the endoplasmic reticulum (Feng et al., 2003; Sciaky et al., 1997; Lippincott-Schwartz et al., 1991a). BFA and to a much lesser extent Exo1 also cause tubulation of the trans-Golgi network (TGN), however, no redistribution of TGN membranes into the ER occurs, instead theyfuse with early and recycling endosomes (Feng et al., 2003; Lippincott-Schwartz et al., 1991b).Therefore, our results indicated that, in the presence of Cbz-Gly-Phe-NH_2, CT is able to travel to the Golgi, however, further retrograde transport to the ER is blocked.

The modes of action of BFA and Exo1, however, appear to be different, as illustrated by the effect of AlF_4^-. Whereas AlF_4^- has only a minor or no effect (depending on the cell type) on the BFA-induced reversal of inhibition of CT action by Cbz-Gly-Phe-NH_2, it completely preventsthe reversal of inhibition by Exo1. This is consistent with earlier observations showing that, whereas AlF_4^- slows but does not prevent the BFA-induced dissociation of ARF1 from Golgi membranes (Feng et al., 2003),it completely blocks the Exo1-induced dissociation of ARF1 from these membranes (Feng et al., 2003).

This agrees with the proposal (Feng et al., 2003)that BFA and Exo1 have different targets. In contrast with BFA, Exo1 appears not to interfere with the activity of guanine nucleotide exchange factors specific for Golgi associated ARF's, but probably acts at a step downstream from the ARF1-GTP loading step, most likely by increasing the rate of GTP hydrolysis through the activation of an ARF-GAP-dependent step (Feng et al., 2003).

The difference in the mechanisms of action of BFA and Exo1 is also apparent from their divergent effects on the action of CT in MDCK cells and reversal of the Cbz-Gly-Phe-NH_2-induced inhibition of CT action in these cells. MDCK cells have a Golgi complex which is resistant to the action of BFA (Hunziker et al., 1991),but apparently,as shown in this study,not to that of Exo1. Kinetic analysis of the BFA- or Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2 revealed that the redistribution of Golgi membranes into the ER is a fast process. Furthermore, sincethe toxin-induced increase in cAMP content reached 50%of its maximal levelonly two minutes after the addition of BFA or Exo1, the subsequent steps in toxin action must also be taken very rapidly. Golgi tubules are known to mediate retrograde traffic in cells treated with BFA. In BFA treated cells, Golgi tubules resembling those of untreated cells are observed, but they are formed at a more rapid rate. Moreover, the tubules fail to detach from Golgi structures. This leads to the formation of a dynamic Golgi tubule network within 5-8 min of adding the drug. When one or more of the Golgi tubules fuses with the ER, Golgi membranes redistribute rapidly (within 30 sec) in the ER, leaving no Golgi structure behind (Sciaky et al., 1997).

Since BFA and Exo1, by perturbing ARF1 function and COPI binding, directly interfere with the Golgi to ER retrograde trafficking machinery, we also investigated the effect of the microtubule depolimerizing drug nocodazole. Microtubule disruption by nocodazole is known to block the inward translocation of pre-Golgi intermediates along microtubules without significant effects on the Golgi to ER traffic (Cole et al., 1996),causing a more naturalrecyclingof Golgi components to the ER. As shown in this study,nocodazole also partially reverses the Cbz-Gly-Phe-NH_2 induced inhibition of CT action. The extent of reversal, however, appears to be cell type dependent.

We previously reported (De Wolf, 2000)that whereas Vero cells pretreated with BFA areunable to reduce CT to the CT-A_1 peptide and arecompletely resistant to CT action, treatment of the same cells with Cbz-Gly-Phe-NH_2 only partially reduced their ability to generate the CT-A_1 peptide, although they also become completely insensitive to toxin action. To account for this observation we argued that the total amount of reduced toxin may not reflect toxin that can be translocated to the cytosol, for instance, in the presence of Cbz-Gly-Phe-NH_2, reduced toxin may be trapped in a compartment where translocation to the cytosol is impossible or much less efficient. Several lines of evidence have indicated that a protein-disulfideisomerase (PDI; EC 5.34.1) mediates the reduction of CT-A (Tsai et al., 2001; Orlandi, 1997). This enzyme is found predominantly as a resident soluble protein within the lumen of the ER (Freedman et al., 1989),but has also been ascribed to the Golgi apparatus (Taylor and Varandani, 1985), the trans-Golgi network and the plasma membrane of mammalian cells (Varandani et al., 1978). Our results indicated that at least in the presence of Cbz-Gly-Phe-NH_2, CT can be reduced and thus activated at the level of the Golgi complex. Modifications of CT structure at the level of the Golgi complex have been reported previously (Bastiaens et al., 1996). In this study evidence waspresented indicating that CT-A dissociates from CT-B in the Golgi, after which CT-A is transported in oxidized

form to the ER via a KDEL-dependent mechanism. Reduction of CT in an intermediate compartment before reaching the Golgi is unlikely, since in the presence of BFA, which as Cbz-Gly-Phe-NH_2 does not impair the binding and internalization of CT, no reduction of CT-A occurs. Conditions prevailing in the Golgi (lower pH, less oxidizing) may also be favourable for the reduction of CT-A. For instance, it has been shown that in vitro the PDI mediated reduction of CT-A is maximalat a pH 5.5-6.0 (Tsai et al., 2001).

Previous studies (Lippincott-Schwartz et al., 1991a)have reported that forskolin inhibits and even reverses the effects of BFA on Golgi structure. These effects arealso reproduced by 1,9-dideoxyforskolin, a naturally occurring analogue of forskolin that does not activate adenylyl cyclase(Laurenza et al., 1989). In this study we demonstrated that 1,9-dideoxyforskolin also antagonizes the effect of BFA on the Cbz-Gly-Phe-NH_2-induced inhibition of CT action in a dose-dependent way. It has been speculated that forskolin interferes with the action of BFA by (competition)/competing for the binding of BFA to its target protein, the Golgi-localized nucleotide exchange factor specific for ARF1(Lippincott-Schwartz et al., 1991a). A subsequent study (Nickel et al., 1996), however, showed that in vitro forskolin does not prevent inhibition of Golgi-catalyzed nucleotide exchange by BFA. Therefore, it was concluded that it is unlikely that forskolin and BFA bind to the same target protein. Forskolin treatment of CHO cells, however, resultsin increased levels of Cys-BFA, the major BFA conjugate secreted by CHO cells, in the medium, which led to the suggestion that the effect of forskolin on BFA-induced disassembly of the Golgi apparatus might be due to an enhanced detoxification of the drug. The present results do not favor this hypothesis, since 1,9-dideoxyforskolin also blocks the Exo1-induced reversal of the inhibition of CT action by Cbz-Gly-Phe-NH_2. In addition,the fast action of 1,9-dideoxyforskolin is difficult to reconcile with the proposed enhanced detoxification and removal of BFA. In this light it is also interesting to note that forskolin also prevents the redistribution of Golgi membranesinto the ER, induced by the Epidermal-cell differentiation inhibitor (EDIN), an exoenzyme (ADP-ribosyltransferase) produced by Staphylococcus aureus with a substrate specificity of the rho protein (Sugai et al., 1992). Therefore, it is clear that forskolin inhibits the action of structurally totally unrelated compounds, which all cause disassembly of the Golgi complex. The exact target and mechanism of action of forskolin and its derivatives in this phenomenon therefore remains to be defined.

Taken together, the results of this study are in agreement with the view that intoxication by CT requires retrograde transport of CT from the plasma membrane to the ER, involving passage through the TGN and Golgi apparatus. A direct transport from the TGN to the ER as recently proposed (Feng et al., 2004)is unlikely since Cbz-Gly-Phe-NH_2 and Exo1 completely abolish toxicity,whereastransport of the toxin to the TGN still occurs. We also demonstrated that the metalloendoprotease substrate blocks the retrograde transport of CT from the Golgi complex to the ER. Therefore,metalloendoproteases may not only play a role in vesicular transport and secretion of newly synthesized proteins as previously proposed (Lennarz and Strittmatter, 1991),but may also be involved in retrograde transport between the Golgi and the ER. Finally, we also showed that successive addition of two strong inhibitors of CT action at the appropriate time points can annihilate their inhibitory effects.

5. Acknowledgment

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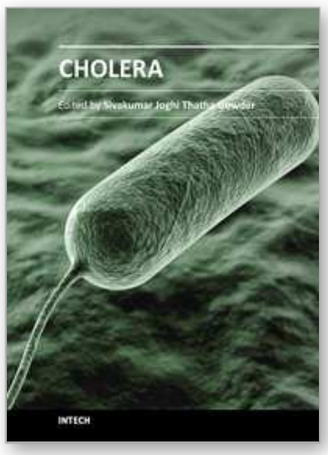

Cholera

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Cholera, a problem in Third World countries, is a complicated diarrheal disease caused by the bacterium Vibrio cholerae. The latest outbreak in Haiti and surrounding areas in 2010 illustrated that cholera remains a serious threat to public health and safety. With advancements in research, cholera can be prevented and effectively treated. Irrespective of "Military" or "Monetary" power, with one's "Own Power", we can defeat this disease. The book "Cholera" is a valuable resource of power (knowledge) not only for cholera researchers but for anyone interested in promoting the health of people. Experts from different parts of the world have contributed to this important work thereby generating this power. Key features include the history of cholera, geographical distribution of the disease, mode of transmission, Vibrio cholerae activities, characterization of cholera toxin, cholera antagonists and preventive measures.

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