Internalization of cholera toxin by different endocytic mechanisms

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SUMMARY

The mechanism of cholera toxin (CT) internalization has been investigated using Caco-2 cells transfected with caveolin to induce formation of caveolae, HeLa cells with inducible synthesis of mutant dynamin (K44A) and BHK cells in which antisense mRNA to clathrin heavy chain can be induced. Here we show that endocytosis and the ability of CT to increase the level of cAMP were unaltered in caveolin-transfected cells grown either in a non-polarized or polarized manner. Treatment of Caco-2 cells with filipin reduced CT-uptake by less than 20%, suggesting that caveolae do not play a major role in the uptake. Extraction of cholesterol by methyl-β-cyclodextrin, which removes caveolae and inhibits uptake from clathrin-coated pits, gave 30-40% reduction of CT-endocytosis. Also, CT-uptake in HeLa K44A cells was reduced by 50-70% after induction of mutant dynamin, which inhibits both caveolae- and clathrin-dependent endocytosis. These cells contain few caveolae, and nystatin and filipin had no effect on CT-uptake, indicating major involvement of clathrin-coated pits in CT-internalization. Similarly, in BHK cells, where clathrin-dependent endocytosis is blocked by induction of antisense clathrin heavy chain, the CT-uptake was reduced by 50% in induced cells. In conclusion, a large fraction of CT can be endocytosed by clathrin-dependent as well as by caveolae- and clathrin-independent endocytosis in different cell types.

Key words: Clathrin, Endocytosis, Dynamin, Caveolae, Cholera toxin

INTRODUCTION

Cholera toxin (CT), produced by the bacterium *Vibrio cholerae*, is an activator of adenylyl cyclase in eukaryotic cells. The toxin consists of five identical binding subunits (CTB) and a single A-chain, which after proteolytic cleavage gives rise to the enzymatically active A_1-peptide, which catalyses ADP-ribosylation of the α subunit of the heterotrimeric GTP-binding protein Gs (Ganguly and Kaur, 1996). This renders adenylyl cyclase constitutively active, thereby increasing the intracellular level of cAMP. The B-chains of the toxin pentamer bind specifically to the glycosphingolipid GM1 in the outer leaflet of the plasma membrane. The distribution pattern of GM1 in the plasma membrane is not clear. Visualized by CT, CTB or via biotin-labeling of GM1, GM1 has been reported to be evenly distributed in the plasma membrane (Kenworthy et al., 2000), but also to be concentrated in caveolae (Möbius et al., 1999; Parton, 1994; Montesano et al., 1982), specialized plasma membrane invaginations rich in the protein caveolin, cholesterol and glycolipids. GM1 has also been visualized in DIGs (detergent-insoluble glycosphingolipid rich domains) (Möbius et al., 1999), membrane patches with a similar cholesterol and glycolipid composition as caveolae, and in clathrin-coated pits (Möbius et al., 1999; Montesano et al., 1982; Parton, 1994), although to a much lesser extent than in caveolae.

It is generally accepted that intoxication of cells with CT requires endocytosis of the holotoxin before further intracellular transport and translocation to the cytosol. In certain cell types caveolae are reported to be involved in the internalization process (Schnitzer et al., 1996; Henley et al., 1998; Montesano et al., 1982; Tran et al., 1987). This has led to the notion that CT is a good marker for caveolae-mediated endocytosis. By using ^{125}I- or gold-labeled CT it has been reported that CT clusters in caveolae at low temperature and, after raising the incubation temperature, labeled CT is visualized in non-coated vesicles or multivesicular bodies (Schnitzer et al., 1996; Montesano et al., 1982; Tran et al., 1987). In addition, it has been reported that ^{125}I-CT labeled vesicles are released from isolated membranes in a cell-free system after raising the temperature (Gilbert et al., 1999). However, clustering of CT in caveolae is no direct evidence that the toxin is taken in via these structures. The molecules might be trapped in the invaginations, facilitating detection.

Conversely, the fact that little CT is detected in clathrin-coated pits (Parton, 1994; Montesano et al., 1982) does not exclude the possibility that the toxin is rapidly endocytosed via these structures, and is therefore more difficult to visualize. It should be noted that clathrin-coated pits have a half-life of only 1 minute at the cell surface (Marsh and McMahon, 1999). In fact, two recent studies have indicated partial involvement of clathrin-dependent endocytosis in the uptake of CT in COS-7 cells (Nichols et al., 2001) and in hippocampal neurons (Shogomori and Futerman, 2001). In addition, CT is internalized in cells without caveolae. For example, lymphocytes lack detectable levels of caveolin-1 and do not

express morphologically distinct caveolae (Fra et al., 1994), yet they bind and respond to CT (Kassis et al., 1982; Orlandi and Fishman, 1998). DIGs have been proposed to act as the vehicle for CT entry in Jurkat T lymphoma cells (Orlandi and Fishman, 1998), but there are no data indicating how DIGs might be internalized. Whether the uptake resembles what happens with caveolae is not at all obvious, since caveolin is not present. In addition to clathrin-dependent endocytosis and possible uptake by caveolae, there are also clathrin- and caveolae-independent forms of endocytosis (Lamaze and Schmid, 1995; Sandvig and van Deurs, 1994; van Deurs et al., 1989; Lamaze, 2001), and both dynamin-dependent and -independent forms have been described. These mechanisms might contribute to the internalization of CT. Further quantitative studies on the endocytic mechanisms of CT are clearly needed to fully understand how this protein toxin is internalized.

In the present study we have investigated the importance of different endocytic mechanisms for CT uptake in a CaCo-2 cell line transfected with caveolin-1 (Vogel et al., 1998), in which caveolae are expressed basolaterally after transfection. Both the endocytosis and the effect of the toxin were investigated after transfection, and since caveolin has been shown to interact directly with GM1 (Fra et al., 1995a), also the polarized distribution of GM1 in the cells was studied. The role of dynamin in CT-uptake was investigated using the HeLa K44A cell line which, in an inducible manner, produce mutant dynamin (Damke et al., 1994). Finally, the involvement of clathrin-coated pits in CT-internalization was specifically investigated using a BHK cell line that can be induced to produce antisense clathrin heavy chain (CHC), with a subsequent block in clathrin-dependent endocytosis (Llorente et al., 2001; Iversen et al., 2001). Interestingly, the uptake of CT by different endocytic mechanisms are not affected to the same extent by actin depolymerization and by tyrosine kinase inhibition. Together, the experiments rule out that CT is exclusively internalized via caveolae or glycosphingolipid-rich domains. Clathrin-dependent and caveolae- and clathrin-independent endocytosis are major pathways involved in the uptake of CT in the cell types studied.

MATERIALS AND METHODS

Materials

Cholera toxin was purchased from Calbiochem, La Jolla, CA. Hepes, BSA, MESNa (2-mercaptoethanesulfonic acid, sodium salt), tetracycline, methyl-β-cyclodextrin, IBMX, cytochalasin D, genistein, nystatin and filipin III were purchased from Sigma Chemical Co., St Louis, MO. Geniticin was obtained from Life Technologies Inc., Gaithersburg, MD. [^{3}H]-labeled arachidonic acid (AA) and Na^{125}I were obtained from NEN Life Science Products, Boston, MA. Transferrin was labeled with ^{125}I as described (Fraker and Speck, 1978).

Cells

CaCo-2 cells transfected with caveolin-1 (clone 33-2) and control vector (clone 12-2) (Vogel et al., 1998) were grown in DMEM (Dulbecco's modified Eagle's MEM) supplemented with 10% fetal calf serum (FCS), nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.65 mg/ml geneticin. The cells were seeded out on Transwell filters (Costar; pore size 0.4 μm, diameter 12 mm) 4 days prior to experiments at a density of 1.6×10^5 cells/filter. The transepithelial resistance of the monolayer was measured with a Millicell-ERS equipment (Millipore Corporation, Bedford, MA) at the beginning of the experiments. Filters with a transepithelial resistance higher than 300 Ωcm^2 were used. In the experiments, 0.25 ml of Hepes buffered MEM medium was added to the apical side and 0.5 ml was added to the basolateral side. HeLa K44A mutant dynamin cells (Damke et al., 1994) were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 μg/ml puromycin, 0.4 mg/ml geneticin and 1 μg/ml tetracycline. Two days prior to experiments the cells were seeded with and without tetracycline in 24-well plates at a density of 2×10^4 cells/well. BHK21-tTA cells transfected with antisense CHC (Llorente et al., 2001; Iversen et al., 2001) were grown in DMEM supplemented with 7.5% FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml puromycin, 0.2 mg/ml geneticin and 2 μg/ml tetracycline. Two days prior to experiments the cells were seeded with and without tetracycline in 24-well plates at a density of 2×10^4 cells/well.

Measurement of endocytosis of TAG-labeled CT and ^{125}I-labeled transferrin

Endocytosis of CT was performed essentially as previously described (Skretting et al., 1999). Briefly, CT was labeled with tris (bipyridine)-chelated ruthenium(II) (TAG-label) (IGEN Inc., Rockville, MD) and biotinylated with the reducible ImmunoPure NHS-SS-Biotin (Pierce). Biotinylation of CT has previously been used and does not influence the toxin molecule (Lencer et al., 1995). The cells were washed once with Hepes medium and incubated with TAG- and biotin-labeled CT (9-12.5 ng/ml) in Hepes buffered MEM medium in the presence of BSA (2 mg/ml) for 2-20 minutes at 37°C. Half of the wells were then treated with 0.1 M MESNa at 0°C for 1 hour to reduce the SS-linked biotin in cell surface-bound toxin (Smythe et al., 1992). Only toxin that is TAG-labeled and still biotinylated is detected in the cell lysate using streptavidin beads (Dynal, Oslo, Norway) and ORIGEN Analyzer (IGEN Inc.). Cells treated with MESNa give the amount of endocytosed toxin, while untreated cells give the total amount of toxin associated with the cells. In this assay there is a background of 2.5% after MESNA-treatment, which is subtracted from all endocytosis values given in this paper. The background is constant from 2 to 20 minutes of endocytosis and is similar in all the cells studied.

The cells were preincubated with the various drugs for 30 minutes at the concentration stated in the figure legend, and then the endocytosis of CT was determined in the presence of the drug. Endocytosis of ^{125}I-transferrin was performed as previously described (Rodal et al., 1999).

Measurement of cAMP, arachidonic acid metabolite release and proliferation

For the determination of cAMP, polarized CaCo-2 cells were washed in Hepes medium, Hepes medium with IBMX (0.5 mM) was added, and the cells were incubated at 37°C for 1 hour with CT (10 μg/ml) added apically or basolaterally. The content of cAMP in the cells was measured by a cyclic AMP-[^{3}H] assay system from Amersham Corp., as previously described (Sandvig et al., 1994). The generation of cAMP in response to cholera toxin was extremely low in both the HeLa K44A and the BHK antisense CHC cells used here.

The release of arachidonic acid (AA) metabolites after incubation with CT was measured by preincubating CaCo-2 cells grown in 24-well plates with 0.25 μCi/ml of [^{3}H]-labeled AA for 24 hours, and then incubating the cells with 10 μg/ml CT for 1 hour after two washes with Hepes. Free cells were removed from the incubation medium by centrifugation, and the amount of [^{3}H]-labeled material released into the medium was measured.

For measurement of proliferation, CaCo-2 cells were seeded out in 24-well plates, and 1 and 10 μg/ml CT were added after 8 hours, 1 day or 2 days. After 3 days of incubation, the cells were washed in

PBS and lysed with 0.1 M KOH. The amount of protein was measured using BCA Protein Assay Reagent (Pierce).

Electron microscopy

BHK antisense CHC cells and HeLa K44A cells were incubated with a conjugate of CTB-chain coupled covalently to HRP (Sigma type IV) at 0°C to visualize binding of CT and at 37°C for endocytosis experiments. After 30 minutes of incubation with the conjugate (10 μg/ml), the cells were washed with PBS and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 hour at room temperature. The cells were then washed with PBS (5 times) before incubation with 0.5 μl/ml of a 30% H_2O_2 solution and 0.5 mg/ml diaminobenzidine in PBS for 1 hour at room temperature. The cells were then washed, scraped off the flasks, pelleted, and post-fixed with OsO_4, contrasted en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Epon. Sections were contrasted with lead citrate and uranyl acetate, before examination in a Phillips CM 100 electron microscope (Phillips, Eindhoven, The Netherlands).

RESULTS

Introduction of caveolae basolaterally in CaCo-2 cells does not alter the endocytosis of CT

To what extent caveolae represent dynamic structures in different cell types is not clear. We have therefore investigated the endocytic uptake of CT in a cell line without caveolae, and measured whether introduction of these specialized structures would alter the uptake of the toxin. As a model we chose a CaCo-2 cell line in which caveolae are formed basolaterally after transfection with caveolin-1 (Vogel et al., 1998). Wild type CaCo-2 cells and CaCo-2 cells transfected with control plasmid (clone 12-2) have nearly undetectable levels of caveolin-1 and few, if any caveolae, whereas transfected cells (clone 33-2) express high levels of caveolin-1, and caveolae are observed at the basolateral surface. To assess the endocytosis of CT in cells transfected with caveolin-1 and control plasmid, TAG- and biotin-labeled CT was added apically or basolaterally to polarized CaCo-2 cells grown on filters. As shown in Table 1, the introduction of caveolae basolaterally does not alter the endocytosis of CT apically nor basolaterally. The uptake of CT was measured after a short time since longer incubations might be influenced by recycling, transcytosis or degradation of the toxin.

It has previously been published that GM1 interacts directly with caveolin-1 (Fra et al., 1995a). It was therefore an interesting question whether transfection of CaCo-2 cells with caveolin-1 and introduction of caveolae basolaterally could lead to an altered distribution of GM1. To investigate this question, we measured the binding of TAG- and biotin-labeled CT to the apical and basolateral membrane of CaCo-2 cells transfected with caveolin-1 and control plasmid. There was no change in CT-binding neither apically nor basolaterally after transfection of the cells with caveolin-1 and introduction of caveolae basolaterally (data not shown).

Biological activity of CT in CaCo-2 cells transfected with caveolin-1 is unchanged

Although the endocytosis of CT in CaCo-2 cells transfected with caveolin-1 was unchanged, it could not be excluded that overexpression of caveolin-1 might lead to altered intracellular transport and action of CT. We therefore measured the amount of cAMP generated when CT was added either apically or basolaterally to cells transfected with caveolin-1 and control plasmid. As shown in Table 2, transfection with caveolin-1 did not increase the amount of cAMP generated in response to CT when the toxin is added either apically or basolaterally. Similar results were obtained also when the cells were incubated with lower amounts of CT (0.1 and 1 μg/ml) before cAMP measurements (data not shown).

In addition to CT-induced activation of adenylyl cyclase, the toxin stimulates arachidonic acid (AA) metabolism in some cells. It is known that the toxin can activate phospholipase A_2 (Burch et al., 1988; Peterson et al., 1999), and thereby increase hydrolysis of AA from membrane phospholipids. To investigate whether the AA metabolism was altered after transfection of the CaCo-2 cells with caveolin-1, the amount

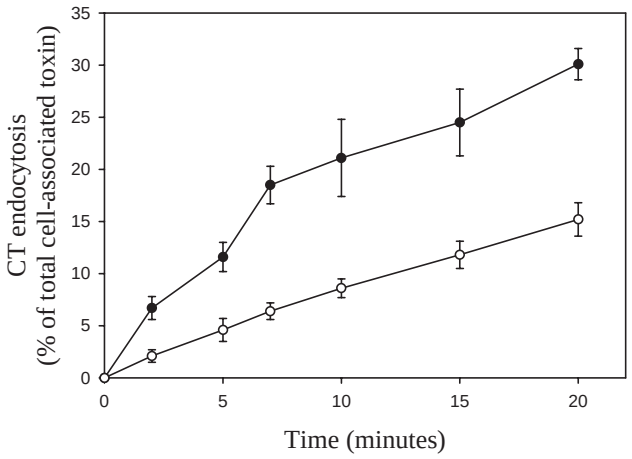

Fig. 1. Rate of internalization of CT in HeLa K44A cells, with (○) and without (●) induction of mutant dynamin. The cells were washed, and TAG- and biotin-labeled CT (9 ng/ml) in 0.2 ml of Hepes-buffered MEM medium was added to each well containing approximately 40,000 cells. The cells were incubated at 37°C for 2-20 minutes, and bound and endocytosed CT were quantified as described in Materials and Methods, and are presented here as percent of total cell-associated toxin (mean ± s.d., n=6). The amount of total cell-associated CT at 2 and 20 minutes was 0.11 and 0.38 ng/40,000 cells, respectively. Equal amounts of CT were bound to non-induced and induced cells.

Table 1. Endocytosis of CT added apically or basolaterally to polarized CaCo-2 cells transfected with caveolin-1 and control plasmid

	Endocytosed toxin (% of total cell-associated)	
	Control plasmid	Caveolin-1 plasmid
Apical side	31±4	30±7
Basolateral side	30±3	23±4

TAG- and biotin-labeled CT (12.5 ng/ml) was prebound to the cells at 0°C for 30 minutes in Hepes-buffered MEM medium. Then the cells were washed, and the incubation continued for 10 minutes at 37°C in the same medium. Bound and endocytosed toxin was quantified after 10 minutes of internalization at 37°C as described in Materials and Methods, and the results shown are the mean values±s.d. (n=4). The amount of total cell-associated CT after 10 minutes of endocytosis from the apical or the basolateral side was approximately 9 and 12 pg/filter, respectively.

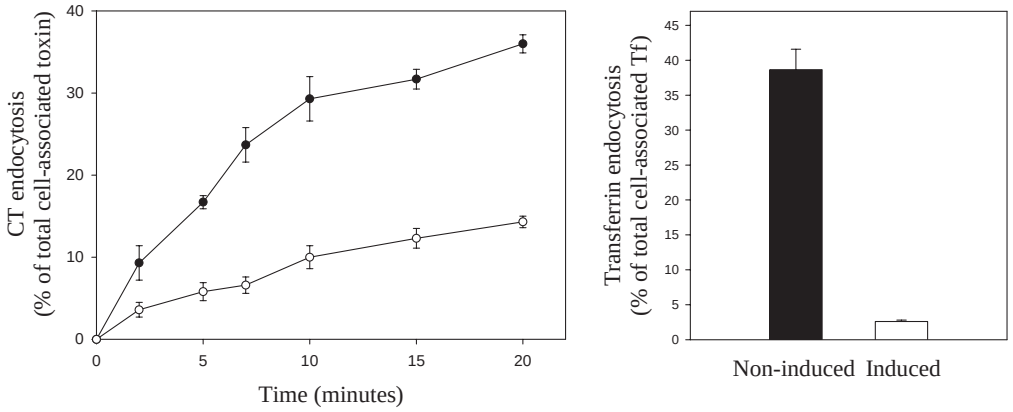

Fig. 2. (A) Rate of internalization of CT in non-induced (●) and induced (○) BHK cells with inducible expression of antisense CHC, which inhibits clathrin-dependent endocytosis. The cells were washed, and TAG- and biotin-labeled CT (9 ng/ml) in 0.2 ml of Hepes-buffered MEM medium was added to each well containing approximately 35,000 cells. The cells were incubated at 37°C for 2-20 minutes, and bound and endocytosed CT were quantified as described in Materials and Methods, and is presented here as percent of total cell-associated toxin (mean ± s.d., *n*=6). The amount of total cell-associated CT after 2 and 20 minutes of endocytosis was 0.058 and 0.47 ng/35,000 cells, respectively. (B) Endocytosis of ^{125}I-labeled transferrin in BHK cells with (open bar) and without (filled bar) induction of antisense CHC. Endocytosed transferrin was quantified after 5 minutes of internalization as described in Materials and Methods. The results are mean ± s.d. (*n*=6).

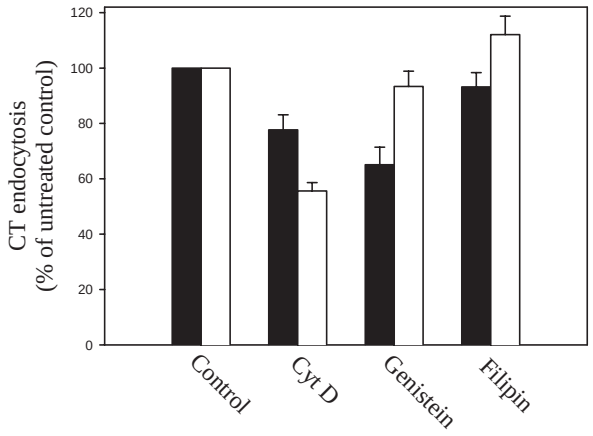

Fig. 3. Endocytosis of CT after treatment of non-induced (filled bars) and induced (open bars) BHK antisense CHC cells with different inhibitors. The cells were preincubated with cytochalasin D (10 μg/ml), genistein (100 μg/ml) and filipin (5 μg/ml) in Hepes-buffered MEM medium for 30 minutes at 37°C, before TAG-and biotin-labeled CT (9 ng/ml) was added, and the cells were incubated at 37°C for 20 minutes in the presence of the different inhibitors. Endocytosed CT was quantified as described in Materials and Methods, and is presented here as percent of untreated control. The values are mean ± s.d. (*n*=4). The amount of CT associated with the cells was unaffected by the inhibitors added. Absolute values of controls for non-induced cells were in the range 31-48%, and for induced cells 8-19%.

of released [^{3}H]-labeled AA-metabolites was measured after addition of CT to cells transfected with control plasmid and caveolin-1 and preincubated with [^{3}H]AA. The results revealed no significant differences between control and caveolin-transfected cells.

CT has also been found to have a proliferative effect on several cell types, an effect that is not mediated via the increasing cAMP concentration, but rather via increased levels of intracellular Ca^{2+} (Spiegel and Fishman, 1985; Okada et al., 1982; Yamaoka and Imamura, 1998; Uhal et al., 1998). To investigate whether this effect of CT was altered after transfection of the CaCo-2 cells with caveolin-1, the proliferation of the cells was measured after incubation with CT for 1-3 days. The results did not show significant proliferative effect of CT on the control CaCo-2 cells, and transfection with caveolin-1 did not induce any proliferative effect (data not shown).

Cytochalasin D and nocodazole have no effect on the endocytosis of CT in CaCo-2 cells transfected with caveolin-1

It has previously been shown that the actin cytoskeleton and microtubules can be important for endocytic mechanisms. Both actin-depolymerizing drugs and the microtubule-disrupting drug nocodazole, have been reported to inhibit clathrin-dependent endocytosis in some cell types, but not in others (Lamaze et al., 1997; Subtil and Dautry-Varsat, 1997; Fujimoto et al., 2000). To investigate whether the basolateral internalization of CT in CaCo-2 cells is dependent on actin filaments or microtubules, and to test whether transfection with caveolin-1 might induce a difference with respect to this requirement, we treated both control cells and caveolin-1-expressing cells with cytochalasin D and nocodazole before measuring CT-endocytosis. As shown in Table 3, disruption of

Table 2. cAMP generation after addition of CT to the apical or the basolateral side of CaCo-2 cells grown on filters with or without caveolae at the basolateral side

	Generated cAMP (pmol cAMP/OD)			
	Apical side		Basolateral side	
	Untreated	CT-treated	Untreated	CT-treated
Control plasmid	25±2	250±32	6±3	285±77
Caveolin-1 plasmid	16±3	202±26	4±2	252±80

The amount of cAMP was measured after incubation with CT (10 μg/ml for 60 minutes) as described in Materials and Methods. The results shown are the mean values±s.d. (*n*=4-8).

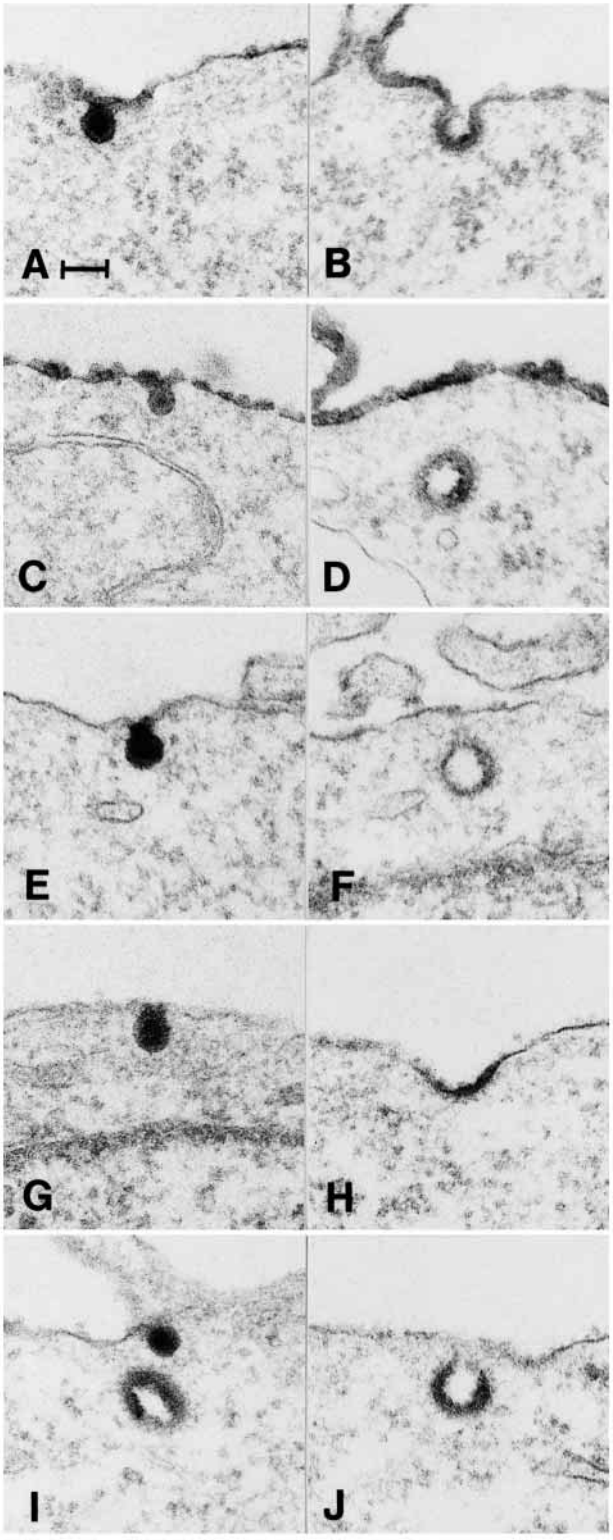

Fig. 4. Electron micrographs of HeLa K44A cells incubated with a CTB-HRP conjugate. In A-D the cells were incubated for 30 minutes at 0°C before fixation and processing for EM. The CTB-HRP conjugate labeling is shown on the cell surface and both in caveolae (A,C) and clathrin-coated pits (B,D). Incubation of the cells with CTB-HRP for 30 minutes at 37°C also labels both caveolae (E) and clathrin-coated pits (F), whereas the labeling of the non-specialized cell surface is strongly reduced. In G-J the cells were induced for mutant dynamin expression before incubation with CTB-HRP for 30 minutes at 37°C. Both caveolae (G,I) and particularly clathrin-coated pits, which were frequent (two- to threefold increase compared with controls), were distinctly labeled (H-J). Bar, 100 nm.

the actin cytoskeleton with cytochalasin D had little effect on the basolateral internalization of CT, and nocodazole treatment left the basolateral CT-uptake unaltered in both cell types. Thus, there is no evidence for the involvement of either actin or microtubules in basolateral endocytosis of CT in these cells.

Effects of filipin and MβCD on basolateral CT-endocytosis

The drug filipin is known to bind cholesterol in the plasma membrane and impair the invagination of caveolae, thereby inhibiting caveolae internalization (Rothberg et al., 1990; Schnitzer et al., 1994; Rothberg et al., 1992). Here, we have investigated the effect of filipin on CT-endocytosis on the basolateral side in both our control CaCo-2 cells and in the caveolin-transfected cells. As shown in Table 3, the basolateral endocytosis of CT was reduced by only 13-17% after filipin treatment, and there was no significant difference in CT-uptake between control and caveolin-transfected cells with respect to the effect of filipin. This supports the idea that CT is not endocytosed via the newly introduced caveolae in the transfected cells. Also, since treatment of the CaCo-2 cells with filipin gave only a slight reduction in CT-uptake, we wanted to investigate whether MβCD, which extracts cholesterol from the membrane and inhibits both caveolae- and clathrin-dependent endocytosis (Rodal et al., 1999; Ohtani et al., 1989; Klein et al., 1995; Subtil et al., 1999), would give a stronger reduction in CT-uptake. As shown in Table 3, MβCD treatment of control and caveolin-1 transfected cells resulted in a 30-40% reduction of basolateral CT internalization. As with filipin treatment, there was little difference between the control and caveolin-transfected cells.

Table 3. Basolateral endocytosis of TAG- and biotin-labeled CT in control and caveolin-1 transfected CaCo-2 cells treated with different compounds

	Endocytosed CT (% of untreated control)	
Compound (concentration)	Control plasmid	Caveolin-1
Cytochalasin D (20 μM)	75±1	86±9
Nocodazole (33 μM)	97±8	91±15
Filipin (5 μg/ml)	83±2	87±7
MβCD (10 mM)	57±11	71±8

The cells were preincubated with the compounds listed in Hepes-buffered MEM medium at 37°C for 30 minutes, then TAG- and biotin-labeled CT (12.5 ng/ml) was added, and the incubation was continued for 20 minutes at 37°C in the presence of the different compounds. Bound and endocytosed CT was quantified as described in Materials and Methods. The results shown are the mean values±s.d. (n=4-6).

Endocytosis of CT is reduced in HeLa cells expressing mutant dynamin

CT has been visualized both in clathrin-coated pits and in caveolae, and vesicle formation from both types of structures has been reported to be dependent on the GTP-ase dynamin (Oh et al., 1998; Henley et al., 1998). Our quantification of CT uptake in CaCo-2 cells suggested that CT could enter cells by

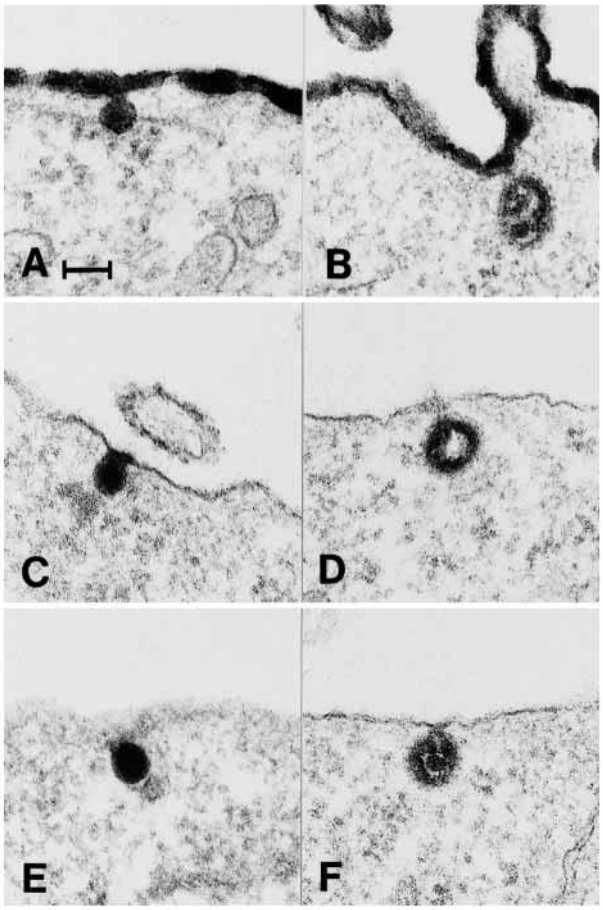

Fig. 5. Electron micrographs of BHK antisense CHC cells incubated with a CTB-HRP conjugate. In A,B the non-induced control cells were incubated for 30 minutes at 0°C before fixation and processing for EM. The CT conjugate labeled the entire cell surface, including caveolae (A) and clathrin-coated pits (B). C,D show non-induced cells incubated with CTB-HRP for 30 minutes at 37°C, and both caveolae (C) and clathrin-coated pits (D) were labeled, whereas the rest of the cell surface appeared largely unlabeled. In E,F, the BHK cells were induced for antisense CHC expression before incubation with CTB-HRP for 30 minutes at 37°C. The toxin conjugate was distinctly present in caveolae (E) and in clathrin-coated pits (F), which were frequent (two to fivefold increase compared with non-induced control cells). Bar, 100 nm.

clathrin- and caveolae-independent endocytosis as well as by clathrin-dependent endocytosis. To investigate this question in more detail we chose HeLa K44A cells, which express mutant dynamin in an inducible manner, as a model. The K44A mutant of dynamin has impaired binding of GTP and is known to inhibit receptor-mediated endocytosis (Damke et al., 1994), and the similar K44E mutant was found to inhibit caveolae budding in cells expressing caveolin-1 (Oh et al., 1998). The HeLa K44A cells have a very low level of caveolin-1 and few distinguishable caveolae on the plasma membrane (Skretting et al., 1999). Further, a possible uptake from the few caveolae that might be present should be inhibited by mutant dynamin. Thus, the CT-endocytosis obtained upon expression of mutant dynamin is likely to reflect the amount of CT taken up by clathrin- and caveolae-independent endocytosis. The HeLa K44A cells were incubated with TAG- and biotin-labeled CT after 2 days of induction of mutant dynamin. Fig. 1 shows the kinetics of CT endocytosis in cells with wild-type and mutant dynamin. Overexpression of mutant dynamin reduced the endocytosis of CT to 32-50% of control cells, indicating that a relatively large fraction of CT can be taken up by a dynamin-independent process. Since neither nystatin nor filipin had any significant effect on CT-endocytosis in these cells, the dynamin-dependent uptake most likely reflects clathrin-dependent endocytosis of CT. In cells incubated with and without tetracycline, uptake of CT in the presence of nystatin (25 μg/ml) was 102±3% and 101±6%, respectively (mean values ± s.d., n=4). In the presence of filipin (5 μg/ml), corresponding numbers were 108±11% and 108±11%. The binding of CT to HeLa cells expressing mutant dynamin was unchanged (data not shown).

Uptake of CT in BHK cells with selective inhibition of clathrin-dependent endocytosis

Since the investigations of HeLa cells with mutant dynamin indicated that a large fraction of CT is taken up from clathrin-coated pits, we further investigated the role of clathrin-coated pits in the internalization of CT by using BHK cells with inducible expression of antisense CHC. Expression of antisense CHC leads to non-functional clathrin-coated pits (Llorente et al., 2001; Iversen et al., 2001). To investigate the initial CT-uptake, the cells were induced for antisense CHC expression for two days, then TAG- and biotin-labeled CT was added and internalized for 2 to 20 minutes. The percent endocytosis of CT with increasing time is shown in Fig. 2A. Inhibition of clathrin-dependent endocytosis resulted in approximately 60% reduction in CT-internalization throughout the time period studied. To verify that the clathrin-dependent endocytosis was inhibited after two days of induction of antisense CHC expression, the internalization of transferrin was measured in parallel with the CT experiments. As shown in Fig. 2B, the endocytosis of transferrin was reduced to background levels in induced cells.

Clathrin is important for several intracellular transport routes (Jackson, 1998; Le Borgne and Hoflack, 1998) in addition to uptake from the cell surface. Thus, the possibility existed that inhibition of CHC expression could affect the amount of GM1 at the cell surface. To assess whether the level of GM1 was altered after induction, the binding of CT was measured, and revealed a slight increase in CT-binding in induced cells (data not shown). These cells have, like the HeLa K44A cells, relatively few caveolae on the plasma membrane (Skretting et al., 1999), and filipin (5 μg/ml) had little effect on CT endocytosis (Fig. 3).

The possibility existed that CT was endocytosed via different mechanisms depending on surface occupancy and receptor crosslinking. This was investigated by measuring the CT-uptake at a high toxin concentration in both HeLa K44A and BHK antisense CHC cells. Endocytosis of TAG- and biotin labeled CT (9 ng/ml) was measured with or without the simultaneous addition of 0.5 μg/ml unlabeled CT in both cell types. In BHK antisense CHC cells, the ratio of CT endocytosis at high to low toxin concentration for cells grown in the presence and absence of tetracycline was 1.0±0.2 and 0.9±0.1, respectively (mean values ± s.d., n=4). In HeLa K44A cells, corresponding values were 1.1±0.1 and 0.9±0.1. Thus, there was no difference in CT-

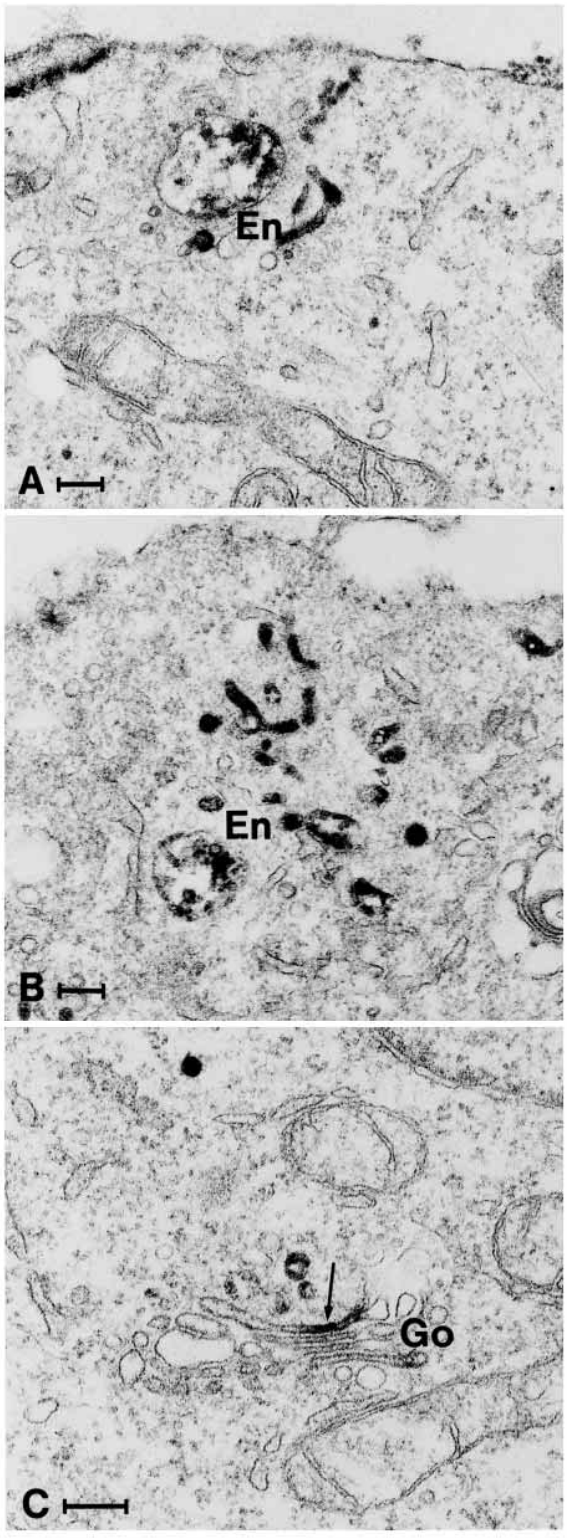

Fig. 6. Electron micrographs of mutant dynamin-expressing HeLa K44A (A) and induced BHK antisense CHC cells (B,C) showing internalized CTB-HRP conjugate. In A, HeLa K44A cells were incubated with CTB-HRP conjugate for 30 minutes at 37°C before processing for EM. The CTB-HRP labeling was seen in an endosome (En) and surrounding tubulo-vesicular structures. In B,C, BHK antisense CHC cells were similarly incubated with CTB-HRP conjugate for 30 minutes at 37°C. Labeling was seen in an endosome (En) and surrounding tubulo-vesicular structures (B) as well as in a cistern (arrow) of a Golgi complex (Go) (C). Bars, 200 nm.

uptake between non-induced and induced cells with increased toxin concentration in either cell type.

Importance of actin disruption and tyrosine kinase inhibition for clathrin-dependent and -independent endocytosis in BHK antisense CHC cells

To further characterize the internalization mechanisms employed by CT, we treated BHK antisense CHC cells with different inhibitors before measuring CT-uptake. Actin has been shown to be involved in endocytosis in several cell types (Lamaze et al., 1997; Durrbach et al., 1996; Gottlieb et al., 1993; Sandvig and van Deurs, 1990), although the effect of the different drugs interfering with actin polymerization seems to be dependent on the cell line and also the experimental protocol. In this study, BHK antisense CHC cells were treated with cytochalasin D (10 μg/ml) before endocytosis of CT in both non-induced and induced cells. The results showed a 22% reduction in endocytosis of CT in non-induced cells, while the induced cells were inhibited by 44% (Fig. 3), indicating that actin is more involved in the clathrin-independent uptake of CT than in the clathrin-dependent uptake of the toxin.

The mechanisms behind uptake of CT by different endocytic processes are not known, but somehow phosphorylation of membrane components might be involved (Friant et al., 2000; Lamaze et al., 1993). Interestingly, as shown in Fig. 3, when BHK antisense CHC cells were incubated with the tyrosine kinase inhibitor genistein (100 μg/ml), the uptake of CT was reduced by 35% in non-induced control cells, while the uptake was unaltered in induced cells. This indicates that tyrosine kinase activity is selectively involved in clathrin-dependent uptake of CT, whereas it does not seem to play a role in the clathrin-independent uptake of CT.

Labeling of membrane domains and endocytic vesicles in HeLa K44A and BHK antisense CHC cells with a CTB-HRP conjugate

To study, at the ultrastructural level, the ability of CT to label various membrane domains in HeLa K44A and BHK antisense CHC cells, these cells were incubated with a CTB-HRP conjugate at 0°C and 37°C and analyzed by EM. The CT-labeling of HeLa K44A cells is shown in Fig. 4. After 30 minutes incubation at 0°C, the CT-conjugate strongly labeled the entire cell surface, including caveolae (Fig. 4A,C) and clathrin-coated pits (Fig. 4B,D). After 30 minutes of incubation at 37°C, the labeling of the cell surface was significantly reduced owing to internalization; however, the toxin conjugate could still be found in both caveolae (Fig. 4E) and clathrin-coated pits (Fig. 4F). Also, when dynamin-mediated endocytosis was inhibited by expression of mutant dynamin, the surface labeling was reduced due to internalization after incubation at 37°C. Moreover, both caveolae (Fig. 4G,I) and particularly clathrin-coated pits (Fig. 4H-J), were distinctly labeled. In addition, there was a two- to threefold increase in the number of clathrin-coated pits on the cell surface compared with numbers in non-induced control cells. Fig. 5 shows electron micrographs of CTB-HRP-treated BHK cells with inducible expression of antisense CHC. As for the HeLa K44A

cells, the toxin conjugate strongly labeled the entire cell surface including caveolae (Fig. 5A) and clathrin-coated pits (Fig. 5B) after incubation at 0°C, and incubation at 37°C resulted in markedly reduced surface labeling due to internalization. However, as for the HeLa cells, the toxin conjugate could still be found in both caveolae (Fig. 5C) and clathrin-coated pits (Fig. 5D). Also, after inhibition of clathrin-dependent endocytosis by expression of antisense CHC, the toxin conjugate was distinctly present in caveolae (Fig. 5E) and particularly in clathrin-coated pits (Fig. 5F), which were frequent (two- to fivefold increase compared with non-induced cells). Fig. 6 shows electron micrographs of internalized CTB-HRP after 30 minutes of endocytosis at 37°C in HeLa K44A (Fig. 6A) and BHK antisense CHC cells (Fig. 6B,C). CTB-HRP could be seen in endosomes and surrounding tubulo-vesicular structures even after expression of mutant dynamin (Fig. 6A) or antisense CHC (Fig. 6B). Furthermore, CTB-HRP could be visualized in the Golgi apparatus (Fig. 6C) after expression of antisense CHC.

DISCUSSION

In this study we have investigated pathways for the internalization of CT in three different cell types. We show that both clathrin-dependent and clathrin-independent endocytosis, which is caveolae- and dynamin-independent, constitute major routes for CT-uptake in the cell types studied.

Overexpression of caveolin-1 has previously been shown to induce formation of caveolae in several cell types (Engelman et al., 1997; Fra et al., 1995b; Hatanaka et al., 1998). CaCo-2 cells express undetectable levels of caveolin-1 and show few, if any invaginated caveolae on the plasma membrane, while transfection with caveolin-1 induces formation of caveolae that localizes preferentially to the basolateral surface (Vogel et al., 1998), like caveolae normally present in MDCK cells (Mora et al., 1999). Since internalization of CT has previously been reported to be mediated by caveolae, and CT is often regarded as a marker for this endocytic mechanism, it was of interest to investigate whether introduction of caveolae would alter the endocytosis of CT or the ability of the toxin to increase the amount of cAMP in the CaCo-2 cells. As shown here, formation of caveolae neither increased the uptake of CT nor the effect of the toxin, suggesting that CT is not endocytosed via the newly formed caveolae to any significant extent.

Treatment of both control- and caveolin-1-transfected CaCo-2 cells with the cholesterol-binding drug filipin (Rothberg et al., 1990; Rothberg et al., 1992) resulted in only a slight reduction in CT-uptake, indicating that neither caveolae nor DIGs, which have been proposed to be involved in CT-uptake in cells without caveolae (Fra et al., 1994; Fra et al., 1995b; Harder and Simons, 1997), are of any major importance for CT-uptake in these cells. By contrast, treatment of the control CaCo-2 cells with MβCD, which not only removes caveolae, but also inhibits clathrin-dependent endocytosis (Klein et al., 1995; Ohtani et al., 1989; Rodal et al., 1999; Subtil et al., 1999), resulted in a 30-40% reduction in CT-uptake, suggesting that clathrin-dependent endocytosis is involved in CT-uptake in these cells. The fact that MβCD extracts cholesterol from the plasma membrane whereas filipin forms a complex with the lipid in the membrane might be the reason for the lack of effect of filipin on clathrin-dependent endocytosis.

The results shown here are apparently not in agreement with previously published results (Orlandi and Fishman, 1998), where CT seemed to be taken in mainly by caveolae or caveolae-like domains in CaCo-2 cells, since filipin-treatment resulted in a significant reduction (58%) of CT-uptake. The apparent discrepancy might be due to methodological differences. In this study we have used TAG- and biotin-SS-labeled CT to differentiate between truly pinched off endocytic vesicles and surface-connected structures. Labeled toxin molecules on the plasma membrane that are clustered in invaginations, even with narrow necks, become undetectable in our assay after treatment with the reducing agent MESNa. Surface connections of invaginated membrane are in some cases so narrow that the membrane-bound molecules in the invagination may not be reached by antibodies (Smythe et al., 1992). The principle of different neck openings has in fact previously been used to determine the different steps in budding of clathrin-coated pits (Smythe et al., 1992; van der Bliek et al., 1993). Narrowing of the neck opening results in reduced antibody detection of transferrin, but unaltered reduction of a biotin-SS-label on transferrin. Only complete budding of the coated pit prevents reduction of the SS-biotin. The reported caveolae-mediated endocytosis of CT has been measured as loss of surface-associated CT detectable by an anti-CT-A_1 antibody (Orlandi and Fishman, 1998). One might speculate that CT is clustered in caveolae with different diameters in the neck openings. This could exclude the anti-CT-A_1 antibody from some of the caveolae, and the results would be interpreted as endocytosis of CT, although no budding of caveolae had occurred. Treatment with filipin might somehow alter the diameter of the openings, leaving the caveolae more open and susceptible to antibody-detection, which in turn would indicate a reduction in CT-uptake.

The apparent discrepancy in results could also be explained by cell differences. CaCo-2 cells grown in different laboratories vary, and moreover, the reported studies of CT were performed on non-polarized CaCo-2 cells, while in this paper we use polarized cells grown on filters. Several important changes occur in the cells during the polarization process (Massey-Harroche, 2000), changes that might explain the apparent discrepancy. Although CT-uptake in the non-polarized CaCo-2 cells was reported to occur via caveolae and caveolae-like domains (Orlandi and Fishman, 1998), the role of dynamin in the uptake was not investigated. Whether clathrin-independent endocytosis occurs independently of dynamin, might be used to differentiate between mechanistically different processes. Finally, the CaCo-2 cells (Orlandi and Fishman, 1998) were suggested to have caveolae on the plasma membrane based on the fact that the cells express small amounts of caveolin-1. However, the structures were not visualized by electron microscopy and there might be a threshold value of caveolin-1 that determines the generation of functional caveolae. Consequently, the uptake of CT in these cells might be mediated via other internalization mechanisms.

It was an interesting question whether transfection of the CaCo-2 cells with caveolin-1 would lead to an altered distribution of GM1, since a direct interaction between caveolin-1 and GM1 has been reported (Fra et al., 1995a). Caveolin is found along several transport routes (Scheiffele

et al., 1998; Smart et al., 1996), thus interaction of the overexpressed caveolin with GM1 could alter the intracellular transport of the glycosphingolipid. Furthermore, caveolin binds directly to cholesterol, which is important for regulation of intracellular transport (Smart et al., 1996). However, the results showed that overexpression of caveolin-1 in the CaCo-2 cells did not alter the distribution of GM1 or the effects of CT. Inhibition of actin polymerization with cytochalasin D has been shown to inhibit receptor-mediated and fluid-phase endocytosis at the apical, but not the basolateral side of polarized MDCK cells (Gottlieb et al., 1993) and CaCo-2 cells (Jackman et al., 1994). The importance of cytoskeletal elements in CT-uptake is less clear. The results presented here suggest that neither actin nor microtubules are required for basolateral endocytosis of CT in polarized CaCo-2 cells, regardless of the presence of caveolae. This finding is in accordance with data from other cell types studied (Jackman et al., 1994; Gottlieb et al., 1993; Schapiro et al., 1998).

To further elucidate the role of dynamin and clathrin in the uptake of CT we have used two different cell lines, HeLa K44A, which can express dominant negative dynamin, and BHK, which can express antisense CHC upon induction. It has recently been reported that the GTPase dynamin, known to mediate the scission of clathrin-coated pits, is also involved in the budding of caveolae (Oh et al., 1998; Henley et al., 1998). HeLa K44A mutant dynamin cells have relatively few caveolae on the plasma membrane (Skretting et al., 1999) but, as shown here, still endocytose significant amounts of CT. The results show a marked reduction in CT-uptake when mutant dynamin was expressed, implying the involvement of dynamin-dependent pathways in CT-uptake. Owing to the fact that these cells express few caveolae on the plasma membrane, the inhibitory effect observed upon overexpression of mutant dynamin most likely reflects the involvement of clathrin-coated pits in the CT-uptake. This notion is supported by the finding that neither nystatin nor filipin had any effect on the uptake of CT in K44A cells, and is in agreement with recent reports indicating that clathrin-dependent endocytosis is involved in the uptake of CT in neurons (Shogomori and Futerman, 2001) and in the uptake of CT-B in COS-7 cells transiently transfected with an eps 15 mutant (Nichols et al., 2001).

Remarkably, a relatively large fraction (30-50%) of the CT was taken in via dynamin-independent pathways. The existence of dynamin-independent endocytosis in these cells has previously been observed using the plant toxin ricin (Llorente et al., 1998), the GPI-linked diphteria toxin receptor (Skretting et al., 1999) and by studies of fluid uptake (Damke et al., 1995). The involvement of clathrin-coated pits in CT-uptake was documented even more directly using BHK antisense CHC cells. Clathrin-dependent endocytosis was essentially abrogated after two days induction of antisense CHC in these cells, as shown by complete inhibition of transferrin-uptake. This resulted in approximately 60% reduction in CT-uptake throughout the time period, clearly implying clathrin-coated pits in rapid CT-uptake in these cells, but also other, clathrin-independent, mechanisms since the inhibition was not complete. In addition, there was no evidence in these cells of involvement of caveolae or DIGs in CT-uptake, since filipin-treatment gave only a slight reduction in CT-uptake (Fig. 3).

Electron micrographs of HeLa K44A cells and BHK antisense CHC cells treated with a CTB-HRP conjugate at 0°C show a strong labeling of the entire cell surface, including clathrin-coated pits and the relatively few caveolae present. This distribution of CT supports the notion that different endocytic mechanisms can be involved in uptake of CT. However, as mentioned above, the visualization of a ligand in one type of membrane invagination cannot be used to evaluate the role of this structure in the uptake. Importantly, the EM studies support the biochemical data showing that CT is internalized even when the dominant negative mutant of dynamin and antisense CHC are expressed.

Inhibition of clathrin-dependent endocytosis in both K44A mutant dynamin cells and in BHK antisense CHC cells leads to increased surface-binding of transferrin (Damke et al., 1994) (data not shown). This is as expected since transferrin-receptors are aggregated in coated pits even in the absence of ligand, and the endocytic uptake seems to be inhibited without any concomitant effect on recycling. By contrast, there was no significant increase in binding of CT, suggesting that the GM1 are not preclustered in coated pits. The efficient uptake of CT by clathrin-dependent endocytosis suggests that there might be a toxin-induced translocation of receptors to clathrin-coated pits. A similar translocation to clathrin-coated pits has previously been shown for another glycolipid-binding toxin, Shiga toxin (Sandvig and van Deurs, 1996). It is not known how these toxins enter clathrin-coated pits, but there might be interactions with proteins entering by this route. The fact that the tyrosine phosphorylation inhibitor genistein counteracts CT uptake from clathrin-coated pits suggests involvement of phosphorylation. Interestingly, Shiga toxin has been found to activate tyrosine kinases (Katagiri et al., 1999; Mori et al., 2000).

In conclusion, our results demonstrate that CT in several cell types are endocytosed by different endocytic mechanisms, both clathrin-dependent and clathrin-independent mechanisms that do not require dynamin, and that caveolae, although they contain GM1, may not necessarily be mobile structures actively involved in endocytosis of CT.

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