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Distinct role of clathrin-mediated endocytosis in the functional uptake of cholera toxin★

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The involvement of the clathrin-mediated endocytic internalization route in the uptake of cholera toxin (CT) was investigated using different cell lines, including the human intestinal Caco-2 and T84 cell lines, green monkey Vero cells, SH-SY5Y neuroblastoma cells and Madin-Darby canine kidney cells. Suppression of the clathrin-mediated endocytic pathway by classical biochemical procedures, like intracellular acidification and potassium depletion, inhibited cholera toxin uptake by up to about 50% as well as its ability to raise intracellular levels of cAMP. Also prior exposure of these cell types to the cationic amphiphilic drug chlorpromazine reduced the functional uptake of cholera toxin, even to a greater extent. These effects were dose- and cell type-dependent, suggesting an involvement of clathrin-mediated endocytosis in the functional uptake of cholera toxin. For a more straightforward approach to study the role of the clathrin-mediated uptake in the internalization of cholera toxin, a Caco-2^{eps-} cell line was exploited. These Caco-2^{eps-} cells constitutively suppress the expression of epsin, an essential accessory protein of clathrin-mediated endocytosis, thereby selectively blocking this internalization route. CT uptake was found to be reduced by over 60% in Caco-2^{eps-} paralleled by a diminished ability of CT to raise the level of cAMP. The data presented suggest that the clathrin-mediated uptake route fulfils an important role in the functional internalization of cholera toxin in several cell types.

Keywords: Cholera toxin, clathrin-mediated endocytosis, siRNA, internalization, chlorpromazine

INTRODUCTION

Cholera toxin (CT), the enterotoxin secreted by the *Vibrio cholerae* bacteria, classical as well as El Tor biotypes, is the major virulence factor causing acute diarrhoeal disease in humans (de Haan & Hirst, 2004; WHO, 2006). CT belongs to the superfamily of AB toxins. The toxin is an oligomeric protein composed of a heterodimeric A-subunit (CT-A, M_r 27400) and a homopentameric B-subunit (CT-B, M_r 58000). CT-B is responsible for binding to the cell surface receptor, monosialoganglioside GM_1 (Van Heyningen *et al*., 1971). CT-A consists of two distinct polypeptide chains (CT-A_1, M_r 22000 and CT-A_2, M_r 5400) linked by a single disulfide bridge. CT-A_1 is a catalytic polypeptide, displaying mono ADP-ribosyl transferase activity. Following ADP-ribosylation of the $G_{s\alpha}$ subunit of the stimulatory GTP-binding regulatory protein (G_s), the basolaterally located adenylate cyclase becomes constitutively activated.

★This paper is dedicated to Professor Tadeusz Chojnacki from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw on the occasion of the 50th anniversary of his scientific activity and 75th birthday.

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Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; BFA, brefeldin A; CAD, chloramphiphilic drug; CHC, clathrin heavy chain; CPZ, chlorpromazine; CT-A, -A_1, A_2 and -B, A subunit, A_1 peptide, A_2 peptide, and B subunit of cholera toxin; CT, cholera toxin; DIG, detergent insoluble glycolipid rich microdomain; FBS, foetal bovine serum; GM_1, monosialo-tetrahexosyl-ganglioside; HAc, acetic acid; IBMX, 3-isobutyl-1-methylxanthine; MDCK, Madin-Darby canine kidney cell; MβCD, methyl-β-cyclodextrin; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; TfR, transferrin receptor.

The resulting increase in the intracellular cAMP level causes net salt and water secretion manifested by severe secretory diarrhoea (Vanden Broeck & De Wolf, 2007).

Several pathways mediating the functional internalization of CT have been proposed. Initially, internalization *via* non-coated invaginations (Tran *et al*., 1987) was favoured, gradually changing into an uptake *via* caveolae-like, detergent-insoluble glycolipid rich microdomains (DIG's) or so-called lipid rafts (Fra *et al*., 1994; Harder & Simons, 1997; Orlandi & Fishman, 1998; Wolf *et al*., 2002; Fishman & Orlandi, 2003) supported by the observation that lipid rafts and caveolae are enriched in GM_1, by studies visualizing CT in caveolae and by experiments with drugs perturbing the cholesterol balance of the plasma membrane. On the other hand, several other lines of evidence (e.g., the time course of the intoxication process (De Wolf, 2000), CT response in cells lacking caveolae (Orlandi & Fishman, 1998), identification of CT in clathrin-coated vesicles (Parton, 1996), genetic approaches (Damke *et al*., 1994; Torgersen *et al*., 2001) and the effect of amphiphilic drugs (Shogomori & Futerman, 2001)) rather point to a role for clathrin-dependent endocytosis in the internalization of CT. Recently, even alternative internalization mechanisms have been proposed such as an entry route regulated by the small GTPase ARF6, which has been demonstrated to interact directly with CT-A_1 (Jobling & Holmes, 2000; Massol *et al*., 2004). In addition, simultaneous blocking of all three described internalization routes had no effect on the ability of CT to raise intracellular levels of cAMP, thereby suggesting an involvement of other unidentified endocytic pathways (Massol *et al*., 2004).

In the present study experimental evidence is presented in favour of an involvement of the clathrin-mediated endocytic machinery in the functional internalization of CT. To this end a number of more classical biochemical strategies including (i) cytosolic acidification, (ii) K^+ depletion and, (iii) preincubation in the presence of the cationic amphiphilic drug chlorpromazine, all known to specifically interfere with clathrin-mediated endocytosis, have been used. To provide more direct evidence the recently developed and powerful technique of RNA interference (RNAi) was applied targeting the mRNA encoding epsin, an accessory protein of coated pit formation, generating a Caco-2^{eps-} cell line with a highly reduced epsin expression and accordingly a severely impaired clathrin-mediated uptake (Vanden Broeck & De Wolf, 2006).

MATERIALS AND METHODS

Materials. Highly purified CT was obtained from List Biological Laboratories (Campbell, CA, USA). HEPES, BSA, methyl-β-cyclodextrin, chlorpromazine, IBMX, iodixanol were from Sigma (St. Louis, MO, USA). $Na^{125}I$ was obtained from MP Biochemicals & Reagents. ^{125}I-transferrin (diferric) and protease inhibitor cocktail were from Roche Diagnostics (Mannheim, Germany). Clathrin heavy chain (CHC) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). BCECF-AM was obtained from Invitrogen-Molecular Probes.

Radiolabelling. CT was radiolabelled following the Iodo-gen method as described by Fraker and Speck (1978).

Cell culture. Except for the green monkey Vero cell line, obtained from Flow Laboratories, all other cell lines were purchased from ATCC. T84 human intestinal epithelial cells were grown in a 1 : 1 (v/v) mixture of Ham's F12 and Dulbecco's modified Eagle's medium in the presence of 2.5 mM L-glutamine and 5% foetal bovine serum (FBS). Caco-2 human intestinal colon carcinoma cells were propagated in modified Eagle's medium supplemented with 1.0 mM sodium pyruvate, 0.1 mM non essential amino acids, 1.5 g/l sodium pyruvate and 20% FBS. Madin-Darby canine kidney (MDCK) cells were subcultured in modified Eagle's medium complemented with 2 mM L-glutamine, modified Earle's balanced salt solution (sodium bicarbonate free) and sterile water (to achieve a final concentration of 1.5 g/l sodium bicarbonate) next to 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% FBS. SH-SY5Y neuroblastoma cells were cultivated in a 1 : 1 (v/v) mixture of Eagle's minimum essential medium and Ham's F12 medium containing 10% FBS. Vero cells were grown in Medium 199 enriched with 5% FBS. Growth medium was changed twice a week and cells were passed at confluence using a 0.1% trypsin solution (Gibco BRL) in Dulbecco's phosphate-buffered Ca^{2+}- and Mg^{2+}-free saline. Cell numbers were counted in a haemocytometer (Bürker) and cell viability was controlled by the trypan blue exclusion method.

Inhibition of clathrin-mediated endocytosis

Acidification. Acidification of the cystosol was performed according to a method described by Sandvig *et al*. (1987).

K^+ depletion. The procedure for intracellular K^+ depletion was as described by Larkin *et al*. (1983).

Drugs. Cells were starved for 30 min in serum-free medium supplemented with 25 mM Hepes and 1% BSA, whereafter the incubation was continued at 37°C for 60 min in the same medium in the presence of increasing amounts of chlorpromazine (CPZ), known to interfere with clathrin-mediated en-

docytosis (Wang *et al*., 1993), followed by the evaluation of the effects elicited by CT.

Gene silencing. Highly selective blocking of clathrin-mediated endocytosis was accomplished by post-transcriptional gene silencing as described by Vanden Broeck and De Wolf (2006).

Binding of ^{125}I-CT. Treated and control cells, either in monolayer or in suspension, were placed on ice and ^{125}I-CT (10^6 cpm/ml; about 1 nM CT) was allowed to bind for at least 30 min at 4°C. After washing with ice-cold phosphate-buffered saline (PBS), radioactivity was measured in both growth medium and cells.

Internalization of ^{125}I-CT. Internalization of ^{125}I-CT was always monitored in cell suspensions. After binding of ^{125}I-CT, cells were washed with ice-cold serum-free medium buffered with 25 mM Hepes and subsequently incubated at 37°C for the indicated times in prewarmed serum-free medium. With each medium change effector concentrations or experimental conditions were readjusted as required. After incubation cells were washed with PBS. To remove surface-bound ligand, cells were placed for exactly 5 min at 4°C in a solution containing 0.5 M NaCl and 0.2 M acetic acid (pH = 2.5). Release of surface-bound CT reached an efficiency of about 90%, in accordance with the data of Sorkin and Carpenter (1991). After centrifugation, the cells were washed three times with ice-cold PBS followed by disruption overnight in 0.1 M NaOH. Radioactivity was assayed in both the acid supernatant and lysed cells.

Internalization of ^{125}I-transferrin. Internalization of ^{125}I-transferrin was monitored in cell suspensions. After binding of ^{125}I-transferrin (120 min, 4°C), cells were washed with ice-cold serum-free medium buffered with 25 mM Hepes and subsequently incubated at 37°C for the indicated times in prewarmed serum-free medium. With each medium change effector concentrations or experimental conditions were readjusted as required. After incubation, cells were cooled and washed again with PBS. Surface-bound and internalized ^{125}I-transferrin were determined as described for CT in the previous section.

Assay of cyclic AMP. CT-induced activation of adenylate cyclase and intracellular cAMP accumulation were determined as previously described (De Wolf, 2000).

Forskolin-induced activation of adenylate cyclase. In order to exclude direct effects on adenylate cyclase, cells were treated with forskolin, assumed to induce activation of the enzyme without intervening steps. The experiments were performed as described in section 'assay of cAMP' except that the cells were stimulated priorly by a fixed concentration of forskolin (50 μM) for 10 min at 37°C.

Generation of CT-A_1 and analysis by SDS polyacrylamide gel electrophoresis. Generation of CT-A_1 was monitored as described previously (De Wolf, 2000).

Measurement of intracellular pH. Measurements of intracellular pH were performed using the membrane-permeable pH-sensitive probe BCECF-AM (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester) (Molecular Probes) according to the method described by Rink *et al*. (1980). Calibration was done using the K^+-nigericine method (Thomas *et al*., 1979).

Preparation of clathrin-coated vesicles. Isolation of clathrin-coated vesicles was according to the procedure of Pearse (1982) for isolating this type of vesicles from placenta. Caco-2 or Caco-2^{eps-} cells (1.5×10^8 cells) were washed three times with PBS and further incubated at 4°C for 2 h with 0.5 μg/ml ^{125}I-transferrin (1 μg/ml, about 2.5×10^6 cpm) or for 1 h with 0.5 μg/ml ^{125}I-CT (1 μg/ml, about 1.2×10^6 cpm) in binding medium (DMEM containing 20 mM Hepes, (pH 7.4) and 0.1% (w/v) BSA). After washing with PBS, cells were warmed up for 2 min at 31°C in binding medium and washed four times in vesicle buffer (140 mM sucrose, 75 mM potassium acetate, 10 mM Mes, pH 6.6, 1 mM EGTA, 0.5 mM magnesium acetate). After draining the dishes, the cells were scraped with a rubber policeman and a protease inhibitor cocktail (Roche) was added. Cells were homogenized on ice in a Potter-Elvehjem homogenizator by passing the suspension 10 times through a 0.2540 inch bore containing a 0.2530 inch diameter ball. Subsequently a post-nuclear supernatant was prepared by centrifugation at $3\,000 \times g$ for 10 min. To this supernatant self gradient forming Iodixanol (Sigma) was added to obtain a final concentration of 12% (v/v). Nine milliliters of diluted supernatant were applied to 1.5 ml 15% (v/v) Iodixanol in Sorvall vertical centrifugation tubes. Centrifugation was continued until equilibrium (Centrikon T2060 (Kontron Instruments) analytical ultracentrifuge; Sorvall TV-850 vertical rotor (Sorvall); $45\,000 \times g$ for 30 min at 4°C), whereafter 0.5 ml fractions were collected and further assayed for radioactivity in a Beckman scintillation counter. Optical density of isolated fractions was determined with a Carl Zeiss Jena refractometer.

Western blotting. Fractions were used immediately or stored at –80°C. Protein concentrations were determined by the BCA® method (Pierce Chemicals, Rockford, IL, USA). Fractions were suspended in NuPage LDS Sample Buffer (Invitrogen) before loading on a NuPAGE 12% bis/Tris polyacrylamide gel (Invitrogen) and processed as described previously (Vanden Broeck & De Wolf, 2006).

RESULTS

Effect of acidification of the cytosol on CT action

Measurement of intracellular pH. To check the effect of the acidity of the incubation medium on the intracellular pH, T84 cells were incubated in a series of media covering a pH range from 5.0 to 7.5. As can be read from the inset in Fig. 2, the outer cellular pH was paralleled by a similar but less pronounced decrease of the intracellular pH, e.g. an extracellular pH = 6.0 (commonly used throughout the experiments) corresponded to the intracellular pH = 6.4. This pH difference can be explained by the cells' capacity to resist cytosolic acidification. At more physiological pH values, the divergence was less pronounced, with nearly identical inner and outer pH values.

Under all experimental conditions no difference in viability was found between control and treated cells, even at the lowest pH tested of 5.0 (not shown).

Binding of cholera toxin. As shown in Table 1, preincubation of Caco-2, T84, SH-SY5Y, MDCK and Vero cells in medium supplemented with HAc (5 mM) to tune the intracellular acidity to pH = 6.5 did not alter the ^{125}I-CT binding capacity appreciably. The binding capacity was measured for cells in suspension as well as in monolayer with a general tendency of a somewhat decreased binding potential of acidified cells in monolayer.

Internalization of cholera toxin. In contrast to the minimal pH effect on the CT binding, a diminution of CT internalization by about 50% was observed in all cell types tested when they were treated with 5 mM HAc (Fig. 1A). Surface-bound toxin removal occurred initially rapidly and then decreased, accompanied by a concurrent increase of internalized ^{125}I-CT.

Accumulation of cAMP and adenylate cyclase activation. Incubation of intact cells in acidified medium resulted in a strong suppression of CT-induced cAMP accumulation. All cell lines tested displayed a similar sensitivity to this treatment. As shown in Fig. 1B acidification of the cytosol down to pH = 6.0 suppressed the CT action on the level of cAMP accumulation in all cell lines by 60 to 65%.

The impact of acidification was investigated in a pH range from 5.0 to 7.4. As evidenced from Fig. 2, a maximal inhibitory effect on the ability of CT to raise intracellular cAMP was found below pH = 6 for the three cell types tested after 60 min of incubation. Already from pH = 7.0 a significant decrease of CT action amounting to 50% *vs*. control was observed. To achieve an optimal balance between the efficient reduction of CT action and cytosolic acidification most experiments were performed in a medium supplemented with 5 mM HAc adjusted to pH = 6.0.

When investigating the time dependence of the inhibitory effect of cytosolic acidification (not shown), it was found that the diminished cAMP accumulation was virtually independent of the moment of acidification, i.e. prior to or upon simultaneous CT administration. All cell types tested showed a similar acidification response.

The inhibitory effect of intracellular acidification appeared to be a partially reversible phenom-

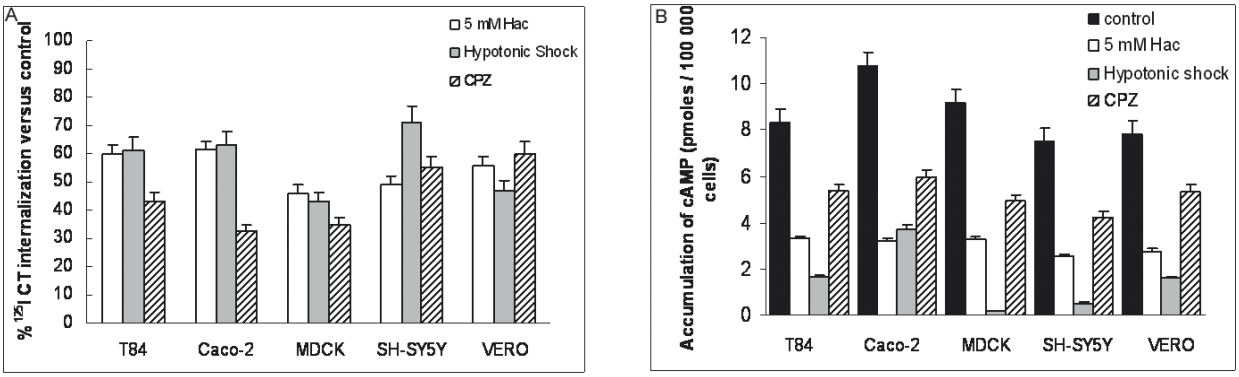

Figure 1. Internalization of ^{125}I-CT and CT-induced cAMP accumulation in different cell types.
Panel A. Suspensions of cultured cells were preincubated in medium containing 5 mM HAc, adjusted to pH about 6.0 for 10 min at room temperature (open bars) or washed with a K^+-free isotonic buffer and shocked with a hypotonic K^+-free buffer diluted 1 : 1 with H_2O for 5 min at room temp. (closed bars) or incubated for 60 min at 37°C with 50 μM CPZ (hatched bars) in control medium prior to binding of ^{125}I-CT. After treatment, ^{125}I-CT was allowed to bind for 60 min at 4°C, cells were washed with ice cold PBS and temperature shifted to 37°C for 40 min. Cells were further assayed for surface-associated ^{125}I-CT. *Panel B.* Suspensions of cultured cells were pretreated as described under A. After treatment, cells were placed at 37°C for 15 min after which cholera toxin (1 μg/ml) was added and the cells further incubated for 60 min at 37°C. The intracellular concentration of cAMP was measured. Cells under control conditions are depicted by filled bars. Values are means ± S.D. of triplicate assays from one of at least three similar experiments.

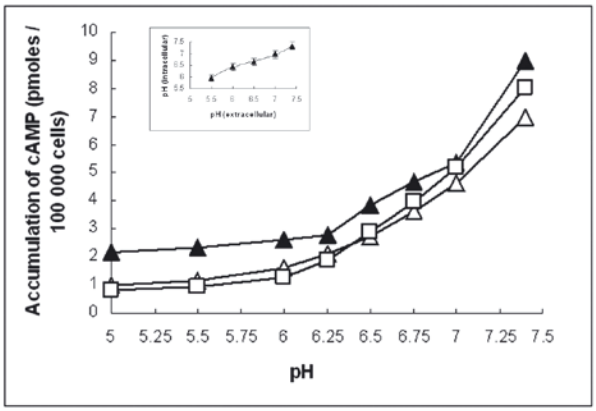

Figure 2. Inhibition of cholera toxin-induced accumulation of cAMP as a function of pH.
Human intestinal T84 cells (closed triangle), SH-SY5Y neuroblastoma cells (open triangles) and Vero cells (open squares) were incubated at 37°C for 60 min with medium at the indicated pH. Subsequently, cholera toxin (1 μg/ml) was allowed to bind at 4°C, excess CT washed away and cells were further incubated for 60 min at 37°C. The intracellular concentration of cAMP was measured. Values are means of triplicate assays from one of three independent experiments. S.D. values were always below 10%.

enon. Following incubation of the cells for 1 h at pH = 6.0 and substitution of the acidic medium by regular medium before CT administration, the CT effect as evaluated by cAMP accumulation was delayed, the lag phase being prolonged by nearly 20 min; also the extent of cAMP production was somewhat reduced (Fig. 3).

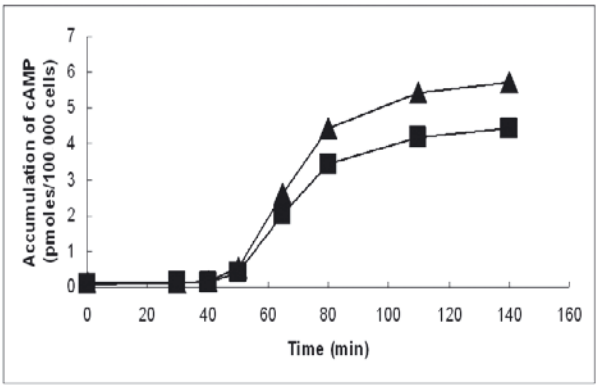

Figure 3. Reversibility of the inhibitory effect of intracellular acidification on CT action.
Human intestinal T84 (triangles) or Caco-2 cells (squares) in suspension were incubated at 37°C for 60 min in medium with 5 mM HAc (pH = 6.0). After washing with salt solution, cells were resuspended in normal medium at pH = 7.4, cholera toxin (1 μg/ml) added and cells incubated for the indicated times, whereafter intracellular cAMP levels were measured. Values are means of triplicate assays from one of three independent experiments. S.D. values were always below 10%.

To exclude any direct effects of acidification on adenylate cyclase activation, intact cells were incubated in the presence of forskolin (50 μM), a cell-permeable diterpenoid known to stimulate the adenylate cyclase directly. No significant difference between control and pH-lowered cells was noted (not shown). This observation was confirmed when membranes isolated from control and HAc- treated cells were exposed to *in vitro* generated CT-A_1, the enzymatically active subunit of the holotoxin which finally activates the adenylate cyclase. In both acidified and control cells, no significant difference in the extent of activation was observed (not shown).

Effect of acidification of the cytosol on the generation of active CT-A_1. Small amounts of CT-A_1 appeared in the cytoplasm after a delay of about 15–20 min, increasing in a time-dependent manner and reaching a value of about 4.5% under control conditions (37°C, 60 min). In acid-treated cells the generation of catalytically active CT-A_1 was nearly completely suppressed; after 1 h incubation only 0.5% of CT-A was converted into CT-A_1 (Fig. 4). The initial amount at time zero of cell-associated CT-A converted to CT-A_1 was subtracted as background value and is probably due to non-specific reduction of CT-A despite the presence of NEM.

Effect of K^+ depletion on CT action

Measurement of intracellular pH. Under conditions of K^+ depletion followed by further incubation with potassium-free medium (pH = 7.4), only a minor decrease of intracellular pH was found, excluding any side effects of hypotonic shock at the level of intracellular acidity (not shown). Viability of the shocked cells as determined by the trypan blue exclusion method amounted to 98 ± 0.9%.

Binding of cholera toxin. As observed after intracellular acidification, also after K^+ depletion and incubation in K^+-free medium CT binding was not altered to any appreciable extent in Caco-2, T84, MDCK, SH-SY5Y and Vero cells (Table 1).

Internalization of cholera toxin. The effect of hypotonic shock followed by K^+ depletion on CT internalization was strongly cell-type-dependent (Fig. 1A). In MDCK cells the uptake of CT was reduced down to 40%, while in the other cells a gradual but varying decrease of cell-surface CT was observed in function of incubation time, the CT uptake being the least affected in the SH-SY5Y cell line.

Accumulation of cAMP and adenylate cyclase activation. In contrast to the effect of acidification, the response of the different cell types to K^+ depletion was much more diverse with regard to the ability of CT to raise intracellular levels of cAMP (Fig. 1B). In T84 and Vero cells, but even more in

Table 1. CT binding to various cell types.

Treated and control cells, either in monolayer (ML) or in suspension (S), were placed on ice and ^{125}I-CT (10^6 cpm/ml; ~ 1 nM CT) was allowed to bind for at least 30 min at 4°C. After washing cell-bound radioactivity was measured.

	Caco-2		T84		SH-SY5Y		MDCK		Vero	
	ML (%)	S (%)	ML (%)	S (%)	ML (%)	S (%)	ML (%)	S (%)	ML (%)	S (%)
Control	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Acidification	93.7	94.3	86.3	103.9	78.6	95.4	87.1	83.7	86.2	106.5
K^+ depletion	90.3	96.3	92.9	99.3	90.5	93.0	93.3	84.7	83.7	98.8
Chlorpromazine	95.5	94.7	88.8	92.5	93.7	95.9	96.8	81.6	79.0	113.9
Epsin-deficient	100.4	104.9	/	/	/	/	/	/	/	/

All experiments were performed at least in triplicate with an overall S.D. of less than 7%.

MDCK and SH-SY5Y cells, the inhibitory effect was much more pronounced after K^+ depletion than after acidification. On the other hand, in Caco-2 cells a nearly similar inhibition was observed upon K^+ depletion and acidification.

A direct effect of K^+ depletion on adenylate cyclase activation was excluded based on experiments with forskolin. Also activated CT (CT-A_1)-induced stimulation of adenylate cyclase after preparing crude membranes displayed no differences between control and potassium-depleted cells under the conditions used (not shown).

Effect of chlorpromazine on CT action

Binding of cholera toxin. As shown in Table 1, administration of CPZ did not elicit any effect at the level of CT binding in all cells investigated, either in monolayer or in suspension, even at the highest CPZ concentration of 100 μM tested.

Internalization of cholera toxin. All experiments were performed at a final concentration of 50 μM CPZ. The response at the level of CT internalization varied between the different cell types. Caco-2 and MDCK cells were the most CPZ-sensitive (approx. 65% reduction), Vero and SH-SY5Y cells the most resistant (about 40–45% reduction) and T84 cells occupied an intermediate position (about 55% reduction) (Fig. 1A).

Accumulation of cAMP and adenylate cyclase activation. The sensitivity to the drug varied depending on the cell type (Fig. 1B). As shown in Fig. 5A, to reduce the cAMP accumulation in intestinal T84 cells down to 50%, a concentration of 20 μM CPZ was needed. To achieve the same reduction in Caco-2 and MDCK cells up to about 60 μM CPZ had to be administered to the cells. SH-SY5Y and Vero cells were more resistant to the drug. In Vero cells, even at 100 μM CPZ, 50% reduction of cAMP accumulation could not be attained.

Interestingly, in contrast to the findings after acidification or K^+ depletion, when verifying the CPZ effect on forskolin-activated adenylate cyclase, dramatic reductions of the enzymatic activity were registered, the phenomenon being strongly cell-type-dependent (Fig. 5B). At 50 μM CPZ *quasi nihil* or only limited inhibition was observed in T84, MDCK and Vero cells, while in Caco-2 and SH-SY5Y cells an inhibitory effect of over 60% was found. At 100 μM CPZ forskolin-activated adenylate cyclase was suppressed severely in all cell types: by about 60% of the control in Vero and MDCK cells, over 80% in T84 cells and over 90% up in SH-SY5Y and Caco-2 cells.

Activated CT (CT-A_1)-induced stimulation of adenylate cyclase in crude membranes displayed no

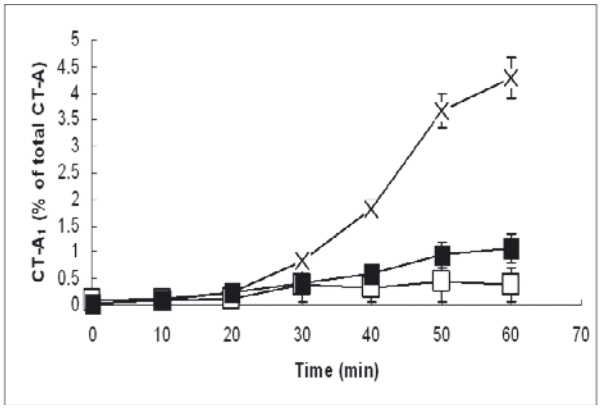

Figure 4. Effect of acidification and epsin-deficiency on the generation of CT-A_1.
Vero cells were grown in Petri dishes and preincubated for 10 min at room temp. in medium containing 5 mM HAc (pH = 6.0). After replacement by ice-cold serum-free medium containing ^{125}I-CT supplemented with 5 mM HAc (pH = 6.0) (open squares), cells were further incubated for the indicated times at 37°C. Control cells (crosses) were also preincubated for 10 min at room temp. in normal serum-free medium, whereafter also the CT-A_1 generation was measured following ^{125}I-CT administration. Caco-2^{eps-} cells (closed squares) were grown till confluence and used directly. Cells were scraped and collected by low speed centrifugation. Generation of CT-A_1 was determined after separation on SDS/PAGE measuring the amount of radioactivity in the CT-A_1 lanes as described in the Materials and Methods section. Values are means ± S.D. of triplicate assays from one of three independent experiments.

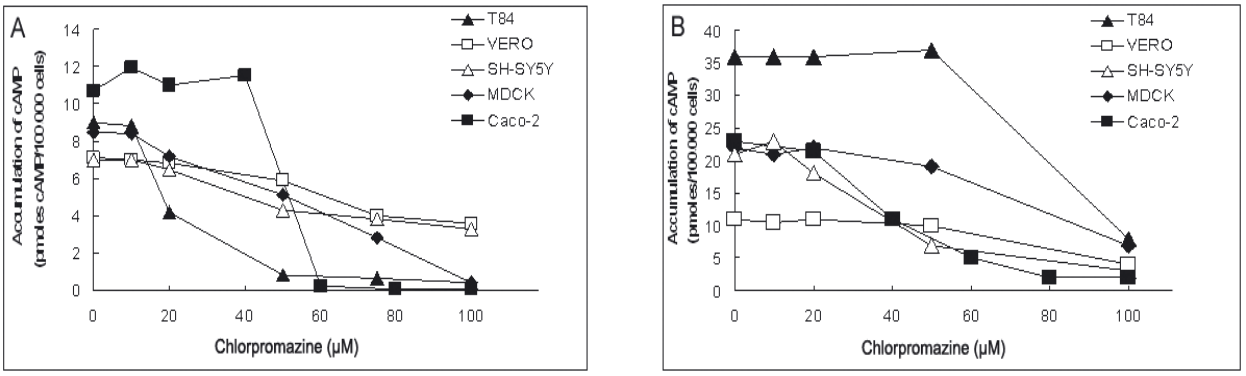

Figure 5. Effect of different concentrations of chlorpromazine on CT- and forskolin-induced cAMP accumulation in different cell types.
Panel A. Suspensions of human intestinal T84 (closed triangles) and Caco-2 (closed squares) cells, SH-SY5Y (open triangles) cells, MDCK (closed diamonds), or Vero (open squares) cells were preincubated for 60 min at 37°C with different concentrations of CPZ as indicated. After treatment, cholera toxin (1 μg/ml) was added, allowed to bind and cells were further incubated for 60 min at 37°C, whereafter intracellular levels of cAMP were measured. *Panel B.* Cells were also stimulated with forskolin for 30 min at 37°C after preincubation with the indicated concentrations of CPZ. Values are means of triplicate assays from one of three independent experiments. S.D. values were always below 10%.

differences under the working conditions between control and CPZ-treated cells (not shown).

Inhibition of clathrin-mediated endocytosis by shRNA targeting epsin

Binding of cholera toxin. The binding of ^{125}I-CT to Caco-2^{eps-} cells in monolayer or suspension was not affected as a result of cell transformation with an shRNA-encoding construct directed against epsin mRNA (Table 1).

Internalization of cholera toxin. In parallel with the decreased transferrin internalization (Fig. 6B) also a reduction of CT uptake amounting to about 60% was observed in the Caco-2^{eps-} cells as compared to the control Caco-2 cell line (Fig. 6A).

Accumulation of cAMP and adenylate cyclase activation. As depicted in Fig. 7, the decreased CT uptake was paralleled by a nearly equal, also up to about 65%, lowering of the CT-induced cAMP accumulation *versus* control cells (wild type Caco-2 cells and Caco-2^{ss} cells, transfected with a functional, non-targeting scrambled sequence).

Incubation of Caco-2^{eps-} cells with 50 μM forskolin showed no significant difference in adenylate cyclase activation compared to control Caco-2 cells, excluding a direct effect of the genetic modification on the activity of adenylate cyclase (not shown).

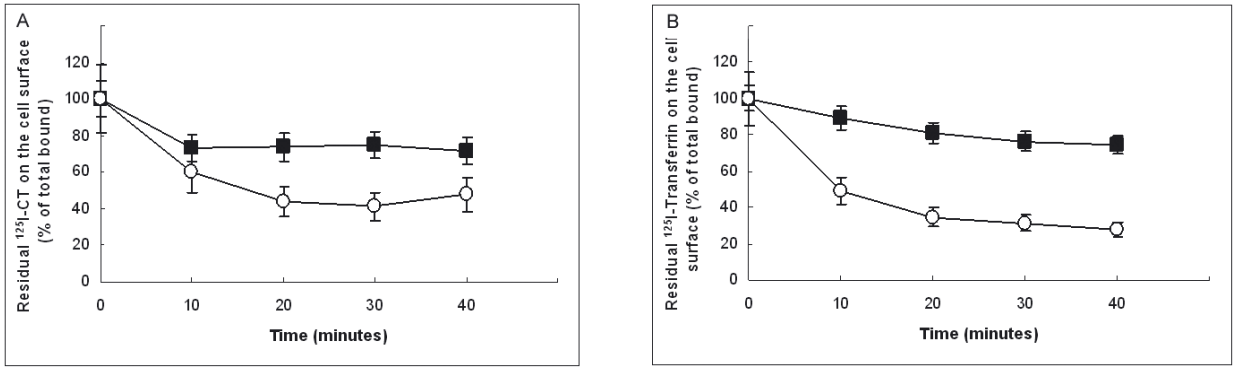

Figure 6. Effect of reduced epsin expression on the internalization of CT.
Panel A. Caco-2 (circles) and Caco-2^{eps-} cells (squares) in suspension were cooled on ice and ^{125}I-CT was allowed to bind for 60 min. Cells were washed 3 times with ice-cold PBS, further incubated at 37°C for the indicated times and temperature shifted to 4°C to inhibit endocytosis. Surface-bound ^{125}I-CT was removed using 0.1 M HAc (pH = 2.5) and samples were assayed for radioactivity. *Panel B.* Caco-2 (circles) and Caco-2^{eps-} cells (squares) in suspension were preincubated for 120 min on ice in medium containing ^{125}I-transferrin and further processed as described. Values are means ± S.D. of duplicate assays from one of three independent experiments.

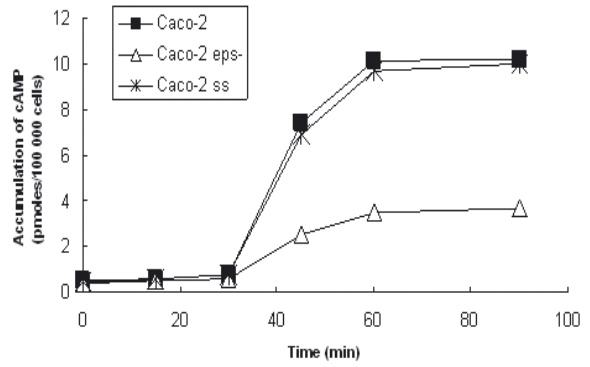

Figure 7. Effect of reduced epsin expression on the ability of cholera toxin to raise the intracellular concentration of cAMP.
Suspensions of cultured wild type Caco-2 cells (closed squares), Caco-2^{ss} (crosses) and Caco-2^{eps-} (open triangles) were incubated with CT (1 μg/ml) on ice for 30 min, washed with ice-cold PBS and incubated at 37°C for the indicated times. The Caco-2^{ss} cell line, engineered by transfection with a functional, non-targeting scrambled shRNA sequence and wild type Caco-2 cells were used as negative controls. The intracellular concentration of cAMP was monitored. Values are means of triplicate assays from one of three independent experiments. S.D. values were always below 10%.

Effect of reduced epsin expression on the generation of catalytically active CT-A_1. In Caco-2^{eps-} cells, the generation of catalytically active CT-A_1 was strongly suppressed; after 1 h incubation, only about 1.0% of CT-A was converted into CT-A_1 (Fig. 4).

Effect of epsin depletion on the association of CT with clathrin-coated vesicles. Figure 8 represents the distribution of internalized ^{125}I-CT (panel A) and ^{125}I-transferrin (panel B) over the Iodixanol gradient after incubating Caco-2 and Caco-2^{eps-} cells with these ^{125}I-labelled ligands. All radioactivity profiles show a peak equilibrating at a density of 1.05, matching the density of clathrin-coated vesicles as reported by Pearse (1982). In addition, these ^{125}I-labelled vesicles cosedimented with the clathrin heavy chains in the gradient as illustrated by Western blotting (Fig. 8). From Fig. 8B, a dramatic reduction of transferrin-loaded coated vesicle generation (about 50%) becomes obvious. Repeating the experiments with ^{125}I-CT resulted in similar distribution profiles with again a striking depletion of coated vesicle formation by about 40% in Caco-2^{eps-} cells (Fig. 8A).

DISCUSSION

For the functional internalization of CT different entry routes have been proposed. The CT receptor, GM_1, being enriched in detergent-insoluble microdomains or lipid rafts, a number of researchers have focussed on the potential role of lipid raft/caveolae-assisted uptake of CT. These studies were mainly based on the use of pharmaceutical inhibitors assumed to affect selectively DIG's by interfering with the cholesterol content of the plasma membrane. For instance cyclodextrins (Ilangumaran & Hoessli, 1998) are capable of extracting cholesterol out of the membrane, whereas filipin/nystatin binds and complexes cholesterol (Schnitzer *et al*., 1994), in this way perturbing normal membrane function. In all those studies, administration of the cholesterol-interacting drugs resulted in a significant reduction of internalization of CT (Ilangumaran & Hoessli, 1998), leading to the conclusion that CT was internalized *via* a non-clathrin-mediated route into the cell. However, it should be emphasized that other

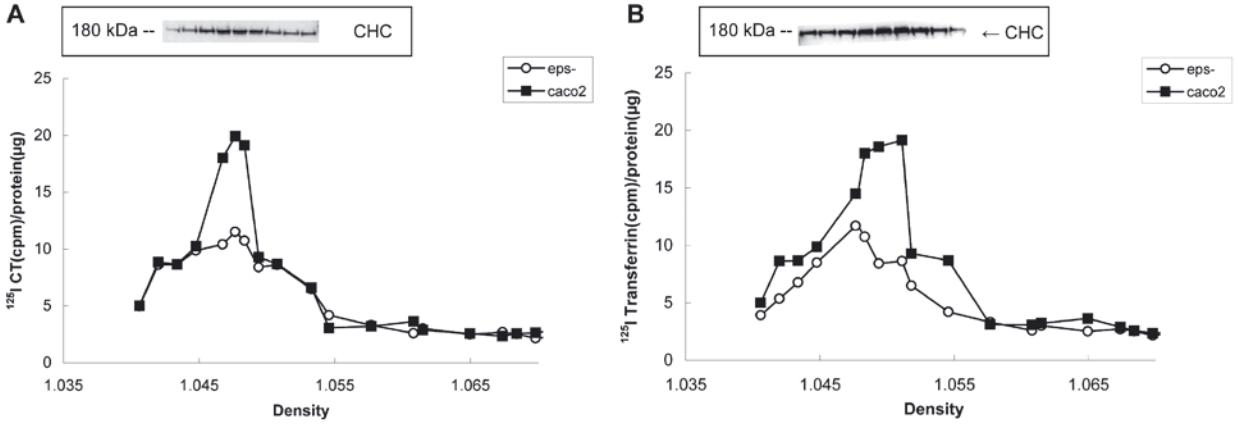

Figure 8. Isolation of clathrin-coated endocytic vesicles and Western blotting.
^{125}I-CT (A) or ^{125}I-transferrin (B) was bound to Caco-2 (closed squares) or Caco-2^{eps-} (open circles). Cells were homogenized and a post-nuclear supernatant prepared before OptiPrep density gradient centrifugation. Fractions (500 μl) were sampled for radioactivity and assayed for clathrin heavy chain by semi-quantitative Western blotting. The figure displays one representative experiment out of three independent ones.

groups have reported that cholesterol depletion with cyclodextrins not only disrupts raft structures, but also flattens clathrin lattices and reduces the proportion of deeply invaginated pits (Rodal *et al*., 1999). Therefore, the drug-induced inhibition of CT internalization could reflect either a disruption of lipid rafts/caveolae and/or a perturbation at the level of clathrin lattices. Accordingly, the data collected in experiments with cholesterol-affecting drugs must be carefully interpreted. Studying functional internalization is even more complicated since intracellular transport of CT is MβCD-sensitive (Shogomori & Futerman, 2001).

Another argument against caveolae-mediated endocytosis of CT comes from the time course of the CT-induced cAMP accumulation in several cell types (De Wolf, 2000), implying fast binding, internalization and intracellular transport of CT from the cell surface towards its intracellular target, the stimulatory G protein of adenylate cyclase. Indeed, within about 20 min after CT intoxication, the toxic effect, i.e. the increase of intracellular cAMP, can be observed. Also internalization of the transferrin receptor, a marker for clathrin-mediated endocytosis, is a fast phenomenon (Moya *et al*., 1985), whereas caveolae-mediated internalization is described as a slow process (about 3 h). Moreover, caveolae appear to be very stable structures of the plasma membrane (Thomsen *et al*., 2002; Carver & Schnitzer, 2003).

In this paper, we report on the perturbation of the clathrin-mediated endocytic system in several cell lines using different experimental approaches and evaluate the effects of blocking this entry route on the internalization and toxicity of CT.

Basic biochemical methods like acidification of the cytosol or K^+ depletion are still commonly applied to study clathrin-mediated internalization processes. Both experimental approaches have been reported to be more selective for the clathrin-mediated internalization route than to the other entry routes (Brech *et al*., 1998). Although both acidification of the cytosol and K^+ depletion inhibit the uptake of TfR, their modes of action are different. Acidification of the cytosol inhibits coated pits from pinching off from the cell surface (Sandvig *et al*., 1987), while K^+ depletion prevents the formation of coated pits at the inner cell surface by interfering with the clathrin triskelions/AP-2 adaptor interaction (Larkin *et al*., 1983). However, it should be stressed that when using these basic biochemical techniques, side effects can never be excluded.

Nevertheless, our data exploiting these techniques demonstrate that, although CT binding to most cell types was hardly affected, CT uptake was highly reduced and accompanied by a strongly suppressed toxin action, supporting an involvement of coated vesicles in CT internalization. The effects of both treatments were similar in all cell lines tested reflecting the specificity of these two experimental approaches. As no direct inhibition of adenylate cyclase could be excluded, forskolin-induced stimulation of the enzyme was measured in both acidified and K^+ depleted cells. In either condition, no differences in activation degree were noticed *versus* the controls. Also with crude membranes isolated from HAc-treated cells, the same activation of adenylate cyclase was found upon exposure to *in vitro* generated CT-A_1. Moreover, the pH activity curve of adenylate cyclase displays a broad pH optimum ranging from pH = 6.5 to 9.5 (Sanders *et al*., 1986). As in our experiments the cytosolic pH was maintained at pH = about 6.5, theoretically no direct effects on adenylate cyclase at this lower pH were to be expected. As a conclusion, neither acidification nor K^+ depletion elicited any direct effect on adenylate cyclase.

As a third alternative experimental approach to suppress clathrin-assisted uptake, we used the drug CPZ that has been shown to exert specific inhibitory effects on the clathrin-mediated endocytic process (Sofer & Futerman, 1995). Treatment with CPZ causes clathrin lattices to assemble on endosomal membranes, simultaneously preventing coated pit formation at the plasma membrane (Wang *et al*., 1993).

Our data reveal that CPZ suppresses the internalization of CT in all cell types tested, further supporting the involvement of classical coated vesicles in the endocytic process. Interestingly, a prominent cell-dependent sensitivity was noted; e.g., in T84 cells only 20 μM CPZ was required to achieve a 50% reduction of cAMP accumulation, while Vero cells were much more resistant, needing more than 100 μM CPZ to attain the same degree of reduction. This cell CPZ-sensitivity and accompanying variability for CT internalization have also been reported by other authors. In hippocampal neurons (Sofer & Futerman, 1995), 25 μM CPZ was sufficient to block the internalization process of CT by about 50%, while according to Orlandi and Fishman (1998) relatively high concentrations of CPZ are required for inhibition of CT uptake by Caco-2 cells, in agreement with the results in this study. On the other hand, according to Ma and Lim (2003) much lower CPZ concentrations are sufficient to inhibit significantly the internalization process in the same cell line, although it should be noted that those authors studied the uptake of chitosan and associated insulin instead of CT.

It should be mentioned that CPZ also generates direct effects on the intracellular CT target, i.e. adenylate cyclase, as indicated by the inhibition of forskolin-stimulated adenylate cyclase. This direct effect of CPZ on adenylate cyclase therefore complicates the quantitative assessment of the func-

tional internalization based on changes of cAMP accumulation.

As a final experimental approach, a Caco-2^{eps-} cell line was developed, constitutively expressing shRNA targeting the mRNA coding for epsin (Vanden Broeck & De Wolf, 2006). Epsin is an intrinsic regulatory component of clathrin-mediated endocytosis and to our knowledge the *EPN1* gene has been proven to be highly specific for this type of internalization process. The protein is involved in the induction of membrane curvature, clathrin polymerization and recruitment of the AP-2 complex (Ford *et al*., 2002). The cell line is characterized by a severe depletion of epsin expression, resulting in a dramatic reduction of clathrin-mediated endocytosis as evidenced from the attenuated transferrin internalization. We have shown that in this Caco-2^{eps-} cell line, the CT uptake was also severely suppressed, paralleled by a nearly equal lowering of the ability of CT to raise cytosolic cAMP levels.

Summarizing, all our data point to an involvement of clathrin-coated pits in the functional uptake of CT. On the other hand, one cannot negate the experimental evidence obtained with cholesterol-interacting drugs by others (Orlandi & Fishman, 1998) and also in our laboratory (unpublished), suggesting CT uptake *via* non-clathrin-mediated endocytic mechanisms. These divergent opinions might be reconciled by the hypothesis that there is not always a strict segregation of clathrin-dependent and raft-dependent endocytic pathways and that CT uptake could rely on a crosstalk between signalling and internalization components. In other words, the site of CT binding does not necessarily coincide with the site of internalization. Such a model has already been proposed by Shogomori and Futerman (2001) for uptake of CT by hippocampal neurons, assuming initial binding of the toxin occurring in a GM_1 hotspot (lipid raft) followed by internalization *via* a clathrin-dependent mechanism. This CT/raft complex could either diffuse directly into the coated pit and be internalized, as the dimensions of a standard coated pit (70 nm to 100 nm) (Ehrlich *et al*., 2004) are sufficient to accept an intact lipid raft. Nevertheless, it has been described that coated pits are depleted in raft components (Nichols, 2003). Alternatively, the CT/GM1 complexes might move laterally from the raft, subsequently being internalized *via* the clathrin-mediated pathway.

In conclusion, all our data resulting from experiments applying different approaches to affect clathrin-assisted endocytosis confirm that functional internalization of CT occurs, at least partially, *via* the clathrin-mediated uptake system. The most convincing data were gathered from the development of a Caco-2^{eps-} cell line by RNA interference which was severely depleted in epsin, a regulatory protein assumed to be exclusively associated with the clathrin-mediated endocytosis at the level of the plasma membrane. The lowered epsin levels in this cell line resulted in a firm decrease in TfR uptake, as well as of CT. The latter effect was paralleled by a drastic reduction of the physiological effects generated intracellularly as a consequence of CT intoxication.

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