

Extraction of Cholesterol with Methyl- β -Cyclodextrin Perturbs Formation of Clathrin-coated Endocytic Vesicles

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The importance of cholesterol for endocytosis has been investigated in HEp-2 and other cell lines by using methyl- β -cyclodextrin (M β CD) to selectively extract cholesterol from the plasma membrane. M β CD treatment strongly inhibited endocytosis of transferrin and EGF, whereas endocytosis of ricin was less affected. The inhibition of transferrin endocytosis was completely reversible. On removal of M β CD it was restored by continued incubation of the cells even in serum-free medium. The recovery in serum-free medium was inhibited by addition of lovastatin, which prevents cholesterol synthesis, but endocytosis recovered when a water-soluble form of cholesterol was added together with lovastatin. Electron microscopical studies of M β CD-treated HEp-2 cells revealed that typical invaginated caveolae were no longer present. Moreover, the invagination of clathrin-coated pits was strongly inhibited, resulting in accumulation of shallow coated pits. Quantitative immunogold labeling showed that transferrin receptors were concentrated in coated pits to the same degree (approximately sevenfold) after M β CD treatment as in control cells. Our results therefore indicate that although clathrin-independent (and caveolae-independent) endocytosis still operates after removal of cholesterol, cholesterol is essential for the formation of clathrin-coated endocytic vesicles.

INTRODUCTION

Endocytosis occurs by clathrin-dependent as well as clathrin-independent mechanisms (van Deurs *et al.*, 1989; Sandvig and van Deurs, 1994, 1996). Clathrin-dependent endocytosis is the best-defined process so far and is responsible for the rapid uptake of, e.g., hormones, growth factors, and transport molecules such as EGF and transferrin. The interaction of the molecules involved in this process has been investigated both *in vivo* and *in vitro*, resulting in characterization of a number of important proteins such as clathrin, adaptors, and dynamin (Schmid, 1997). Less is known about clathrin-independent endocytosis, but different forms seem to exist; for instance, both dy-

namin dependent and dynamin-independent mechanisms have been reported (Damke *et al.*, 1994, 1995; Deirdre *et al.*, 1998; Henley *et al.*, 1998). Clathrin-independent endocytosis can clearly be different from uptake by invaginated caveolae. Thus, clathrin-independent endocytosis exists both in lymphocytes (Subtil *et al.*, 1994) and on the apical side of polarized Madin-Darby canine kidney (MDCK) cells (Eker *et al.*, 1994), which do not contain invaginated caveolae (Vogel *et al.*, 1998).

Earlier studies have suggested a role for cholesterol in endocytosis (Heiniger *et al.*, 1976); however, the type of endocytosis affected by cholesterol depletion was not investigated. Cholesterol present in the membrane of mammalian cells is required for normal cellular function (Bloch, 1991) and either is delivered from low-density lipoproteins internalized by recep-

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tor-mediated endocytosis via clathrin-coated pits followed by subsequent hydrolysis in lysosomes (Brown and Goldstein, 1980) or is synthesized in the endoplasmic reticulum (Reinhart *et al.*, 1987). A major fraction of cholesterol is present in the plasma membrane (Lange, 1991), and cholesterol is important for various processes at the cell surface. For instance, there seems to be a direct interaction between the oxytocin receptor and cholesterol (Klein *et al.*, 1995), and also the acetylcholine receptor was found to have a functional requirement for cholesterol (Criado *et al.*, 1982). Furthermore, it has been proposed that annexin II, which is involved in the endocytic pathway (Creutz, 1992; Gruenberg and Emans, 1993), serves as an interface between membranes containing a high amount of cholesterol and the actin cytoskeleton (Harder *et al.*, 1997), and it is clear that cholesterol is essential for the structure and function of invaginated caveolae, including the caveolae-dependent endocytosis that has been reported in some cell types (Rothberg *et al.*, 1990; Parton *et al.*, 1994; Parton, 1996; Schnitzer and Oh, 1994; Smaby *et al.*, 1996; Chang *et al.*, 1998; Hailstones *et al.*, 1998).

In the present article, we have studied the role of cholesterol in clathrin-dependent and clathrin-independent endocytosis by using methyl- β -cyclodextrin (M β CD)¹ to remove cholesterol from the plasma membrane. It has been shown that both β -cyclodextrin and M β CD remove cholesterol from cultured cells (Ohtani *et al.*, 1989; Kilsdonk *et al.*, 1995; Klein *et al.*, 1995), and we have here used the methylated form because it was found to be more efficient than β -cyclodextrin (Klein *et al.*, 1995). Cyclodextrins are cyclic oligomers of glucose that have the capacity to sequester lipophiles in their hydrophobic core (Pitha *et al.*, 1988). The water-soluble M β CD is known to form soluble inclusion complexes with cholesterol, thereby enhancing its solubility in aqueous solution (Pitha *et al.*, 1988; Irie *et al.*, 1992). In this article we demonstrate that cholesterol plays an important role in the invagination of clathrin-coated pits and therefore in clathrin-dependent endocytosis.

MATERIALS AND METHODS

Materials

M β CD (average degree of substitution: 10.5–14.7 methyl groups per molecule), β -cyclodextrin, α -cyclodextrin, γ -cyclodextrin, water-soluble cholesterol (with ~40 mg of cholesterol per gram solid; balance M β CD), lovastatin, pronase, HEPES, geneticin, lactose, BSA, ricin, and holo-transferrin (iron-saturated) were obtained from Sigma Chemical Co. (St. Louis, MO). [³H]leucine was obtained from the Radiochemical Center (Amersham, Buckinghamshire, UK). Na ¹²⁵I and ¹²⁵I-EGF were purchased from DuPont (Brussels, Belgium). Ricin and transferrin were ¹²⁵I-labeled according to Fraker and Speck (1978) to a specific activity of 2×10^4 – 6×10^4 cpm/ng.

¹ Abbreviations used: M β CD, methyl- β -cyclodextrin; TfR, transferrin receptor.

Cells

MDCK cells (strain II) transfected with the human transferrin receptor (TfR) (MDCK II hTfR) (from Dr. W. Hunziker, Lausanne, Switzerland) were maintained in DMEM (Flow Laboratories, Irvine, Scotland) supplemented with 5% FCS (Life Technologies, Paisly, Scotland), 100 μ g/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine (Life Technologies), and 0.25 mg/ml geneticin. HEP-2, A431, and NIH/3T3 cells (American Tissue Type Collection, Rockville, MD) were maintained in the same medium without the addition of geneticin and with different amounts of serum (5% FCS, 10% FCS, and 10% NCS, respectively). The HeLa cell line (tTA-HeLa), stably transfected with the cDNA for dyn^{K44A}, was cultured as described previously (Damke *et al.*, 1995). For experiments, cell lines were seeded into 24-well plates (Falcon, Franklin Lakes, NJ) at a density of 10^5 cells/well, or in Falcon 25 cm² flasks at a density of 1.25×10^6 1 or 2 d before the experiment. The experiments were performed in HEPES-buffered medium.

Measurement of Binding and Endocytosis of ¹²⁵I-Ricin

Binding of ricin was measured as the amount of cell-associated ¹²⁵I-ricin after a 30 min incubation at 0° or 37°C with ¹²⁵I-ricin (40 ng/ml) and four washes with cold HEPES-buffered medium. Endocytosed ¹²⁵I-labeled ricin was measured after 15 min at 37°C as the amount of toxin that could not be removed after one rapid wash, a 5-min incubation at 37°C, followed by three rapid washes, all with 0.1 M lactose in HEPES-buffered medium at 37°C (Sandvig and Olsnes, 1979). The endocytosed ricin was measured in a gamma counter (1261 Multigamma, Wallac, Gaithersburg, MD).

Measurement of Binding and Endocytosis of ¹²⁵I-Transferrin

Binding of transferrin was measured as the amount of cell-associated ¹²⁵I-transferrin after a 30 min incubation at 0° or 37°C with ¹²⁵I-transferrin (100 ng/ml) and four washes with cold HEPES-buffered medium. Endocytosed ¹²⁵I-labeled transferrin was measured after 5 min at 37°C and calculated as the percentage of total cell-associated (endocytosed and surface-bound) transferrin (Ciechanover *et al.*, 1983). Surface-bound transferrin was removed by 1-h incubation with HEPES medium containing 2 mg/ml pronase on ice. After pronase treatment the medium containing the cells was centrifuged for 2 min in an Eppendorf centrifuge (Madison, WI) before the radioactive contents in the cell pellet (endocytosed) and in the supernatant (surface bound) were measured with a gamma counter.

Measurement of Binding and Endocytosis of ¹²⁵I-EGF

Binding of EGF was measured as the amount of cell-associated ¹²⁵I-EGF after a 30 min incubation at 0° or 37°C with ¹²⁵I-EGF (3–4 μ Ci/ml) and four washes with cold HEPES. Endocytosed ¹²⁵I-labeled EGF was measured after 10 min at 37°C and calculated as the percentage of total cell-associated EGF. Surface-bound EGF was measured as the amount of EGF that could be released by low pH after three rapid washes with PBS at 0°C. The cells were then incubated at 0°C for 6 min with low pH buffer (0.5 M NaCl and 0.2 M CH₃COOH in HEPES, pH 2.5), followed by one rapid wash with the same buffer. Endocytosed EGF was measured as the amount of EGF that could not be removed by this treatment (Sandvig *et al.*, 1987).

Measurement of the Potassium Content in Cells

The potassium content in cells was measured after the cells were washed three times with 100 mM MgCl₂, air-dried, and dissolved in

0.1 M NaOH as described by Larkin *et al.* (1983). Potassium was then determined by ion selective electrodes (Vitros 250; Johnson-Johnson Clinica Diagnostics, Rochester, NY).

Measurement of Protein Synthesis

Protein synthesis was measured by incubating the cells for 10 min in a HEPES-buffered medium with 1 $\mu\text{Ci/ml}$ [^3H]leucine. The medium was then removed, and 5% trichloroacetic acid was added. This solution was removed 10 min later, and the cells were washed twice with the same solution to remove free radioactive leucine. The precipitated protein was then dissolved in 0.1 M KOH, and the radioactivity associated with the cells was measured in a β -counter (MINAXI, TRI-CARB 4000 SERIES, United Technologies, Packard, Meriden, CT).

Electron Microscopy

HEp-2 cells for EM were preincubated with and without M β CD (10 or 15 mM) for 15 or 30 min at 37°C, washed with HEPES-buffered medium, and fixed with 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The cells were then washed, scraped off the flasks, pelleted, and post-fixed with OsO₄, contrasted en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanols, and embedded in Epon. Sections were further contrasted with lead citrate and uranyl acetate and examined in a Philips CM 100 electron microscope (Philips, Eindhoven, the Netherlands). In some experiments, control cells and cells treated with M β CD were fixed, washed with PBS, and incubated with anti-human TfR antibody B3/25 (Boehringer Mannheim, Mannheim, Germany), 2 $\mu\text{g/ml}$ PBS, for 1 h at room temperature. Then the cells remaining in monolayer in the culture flasks were washed with PBS and incubated for 2 h at room temperature with goat anti-mouse IgG coupled to 10 nm gold (Amersham), washed, scraped off, and processed for EM. Quantification of the immunogold labeling for TfRs was performed as described by Hansen *et al.* (1992).

Effect of M β CD on Cellular Cholesterol and Invaginated Caveolae

HEp-2 cells were preincubated in HEPES-buffered DMEM with 0.2% BSA for 10 min and then incubated for 15 min at 37°C in HEPES-buffered DMEM with 1 μCi (1 α ,25(OH)₂D₃)-cholesterol (toluene solution; Amersham) to label the cell surface (Lange, 1991; Debry *et al.*, 1997). To equilibrate with the cholesterol of intracellular compartments, cells were incubated for 20 h in DMEM containing 2.5% delipidated calf serum (Sigma C-1696) and 1 μCi [^3H]cholesterol (Debry *et al.*, 1997; Keller and Simons, 1998). In both instances, cells were subsequently washed thoroughly and incubated at 37°C with agitation in 1 ml DMEM without M β CD, or with 10 mM M β CD for 15, 30, and 60 min. After washing, the cells were lysed in 250 μl 2% NP-40, 0.2% SDS in distilled water, and the lysates were then centrifuged to remove insoluble material before aliquots were mixed with EcoLite+ scintillation mixture, 1:10, and counted in a scintillation counter (Beckman, Fullerton, CA).

In parallel experiments (i.e., same cell passage, same day, and same M β CD solution), HEp-2 cells were washed and incubated with 10 mM M β CD in DMEM for 0, 15, 30, and 60 min at 37°C, washed again, fixed, and further processed for electron microscopy as described above. The amount of characteristically invaginated caveolae on the plasma membrane (Vogel *et al.*, 1998) was subsequently quantitated.

RESULTS

Effect of M β CD on Endocytosis of Transferrin, EGF, and Ricin

To study whether incubation with M β CD would affect endocytosis of transferrin and EGF, cells were prein-

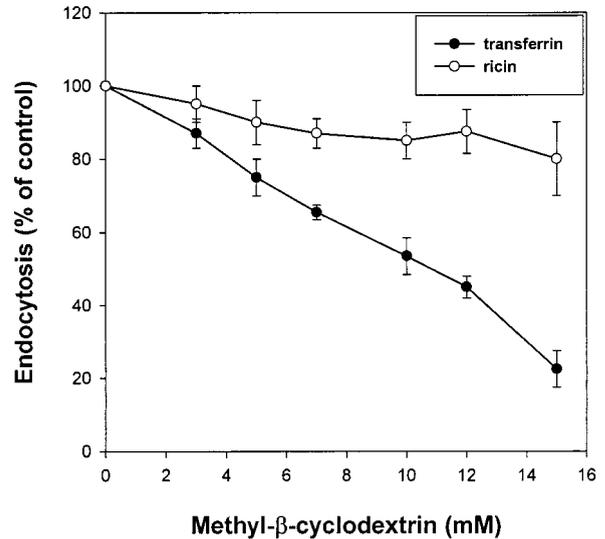


Figure 1. Effect of M β CD on transferrin (●) and ricin (○) endocytosis. MDCK II cells were washed briefly in HEPES-buffered medium before incubation with and without the indicated concentrations of M β CD for 15 min at 37°C. ^{125}I -labeled transferrin or ^{125}I -labeled ricin was then added to the cells, and the amounts of endocytosed transferrin or ricin were measured as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

cubated for 15 min with M β CD at 37°C, and then ^{125}I -transferrin or ^{125}I -EGF was added. Endocytosis of transferrin was strongly reduced with increasing concentrations of M β CD (50% reduction at 10 mM M β CD) (Figure 1). As shown in Figure 2, 10 mM

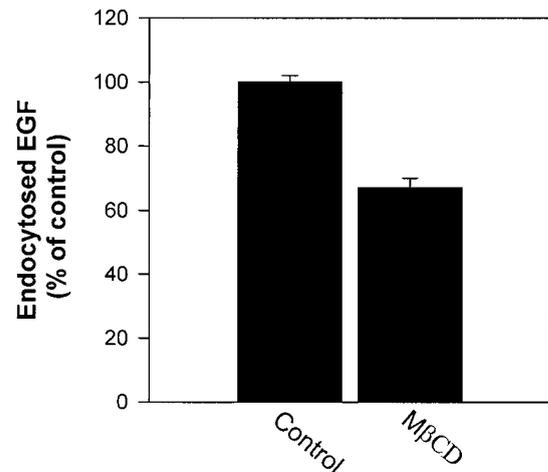


Figure 2. Effect of M β CD on endocytosis of EGF. HEp-2 cells were washed briefly in HEPES-buffered medium and incubated with and without M β CD (10 mM) for 15 min at 37°C. ^{125}I -labeled EGF was then added to the cells, and the amounts of endocytosed EGF were measured as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

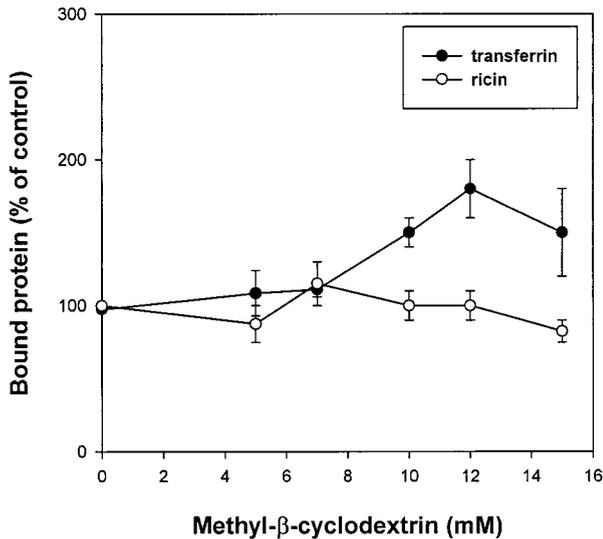


Figure 3. Effect of MβCD on binding of transferrin (●) and ricin (○). MDCK II cells were incubated with and without the indicated concentrations of MβCD in HEPES-buffered medium at 37°C for 15 min. Then the cells were cooled down to 0°C, ¹²⁵I-labeled transferrin or ¹²⁵I-labeled ricin was added, and binding was measured after 30 min at 0°C as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

MβCD also gives a strong reduction of ¹²⁵I-EGF endocytosis (~40%). In agreement with the result that MβCD inhibits transferrin endocytosis, pretreatment of cells with 10 mM MβCD for 15 min at 37°C increased the cell surface binding of ¹²⁵I-transferrin two-fold (Figure 3). To further investigate the effect of MβCD on clathrin-dependent endocytosis, uptake of ¹²⁵I-transferrin was measured in different cell lines preincubated with 10 mM MβCD for 15 min at 37°C. As shown in Figure 4, this concentration of MβCD inhibited endocytosis of transferrin in all cell lines by ~50%.

To test whether MβCD would affect all endocytic activity, we studied the uptake of a molecule that does not only enter by clathrin-dependent endocytosis. Experiments performed with increasing concentrations of MβCD showed that the endocytosis of the general membrane marker ricin (Sandvig and Olsnes, 1979; Sandvig and van Deurs, 1996) was less affected by increasing concentrations of MβCD than that of transferrin in MDCK II cells (Figure 1). In addition, there was no effect on the binding of ¹²⁵I-ricin (Figure 3). Endocytosis of ¹²⁵I-ricin was also measured in different cell lines preincubated with 10 mM MβCD for 15 min at 37°C. As shown in Figure 4, MβCD inhibited endocytosis of ricin in the various cell types by ~20%. Because MβCD strongly decreased the number of invaginated caveolae (see below), the data suggest that there is another clathrin-independent form of endocytosis that is resistant to removal of cholesterol.

The data described above suggest that removal of cholesterol has a much stronger effect on clathrin-dependent than on clathrin-independent endocytosis. To further investigate this question we studied the effect of MβCD on transferrin and ricin endocytosis in HeLa K44A cells. These cells express mutant dynamin on removal of tetracycline, and clathrin-dependent endocytosis will then become inhibited (Damke *et al.*, 1994). HeLa K44A cells grown with and without tetracycline were incubated with and without MβCD for 15 min at 37°C, and then ¹²⁵I-transferrin or ¹²⁵I-ricin was added. As shown in Figure 5A, ricin endocytosis was essentially unaffected by MβCD in the absence of tetracycline (mutant dynamin is expressed); however, MβCD decreased the endocytosis of ricin by ~20% in the presence of tetracycline (both clathrin-dependent and clathrin-independent endocytosis are functioning) (Figure 5A). As shown in Figure 5B, endocytosis of transferrin is inhibited by MβCD to more than half in the presence of tetracycline, whereas transferrin endocytosis is blocked (>80%), as expected, when mutant dynamin is expressed and inhibits the clathrin-dependent pathway.

The Effect of MβCD on Endocytosis Is Related to Removal of Cholesterol

In agreement with previous studies (Neufeld *et al.*, 1996; Keller and Simons, 1998) we found that MβCD efficiently extracts cholesterol from HEP-2 cells (Figure 6A). Caveolae with the characteristic deeply invaginated shape were frequently observed in control HEP-2 cells. As an alternative approach to evaluating the effect of MβCD on plasma membrane cholesterol, we used the observation that typically invaginated caveolae (Figure 6B) can only be maintained in the presence of cholesterol (Rothberg *et al.*, 1990; Chang *et al.*, 1998; Hailstones *et al.*, 1998). Figure 6C shows that MβCD extraction of cholesterol has a pronounced effect on the number of invaginated caveolae.

Although previous studies have shown that MβCD specifically removes cholesterol from the plasma membrane (Ohtani *et al.*, 1989; Kilsdonk *et al.*, 1995; Klein *et al.*, 1995; Neufeld *et al.*, 1996; Keller and Simons, 1998), we investigated whether the decreased transferrin endocytosis in the presence of MβCD was due to loss of cholesterol from the plasma membrane. Two other cyclodextrins, α-cyclodextrin and γ-cyclodextrin, do not bind cholesterol in the same way as MβCD (Ohtani *et al.*, 1989), and we therefore tested whether they would affect transferrin endocytosis. In contrast, β-cyclodextrin, like MβCD, will extract cholesterol from the plasma membrane (Ohtani *et al.*, 1989; Klein *et al.*, 1995). HEP-2 cells were preincubated with 10 mM α-, β-, methyl-β-, or γ-cyclodextrins for 15 min at 37°C, before the addition of ¹²⁵I-transferrin. Five minutes later, the amount of endocytosed trans-

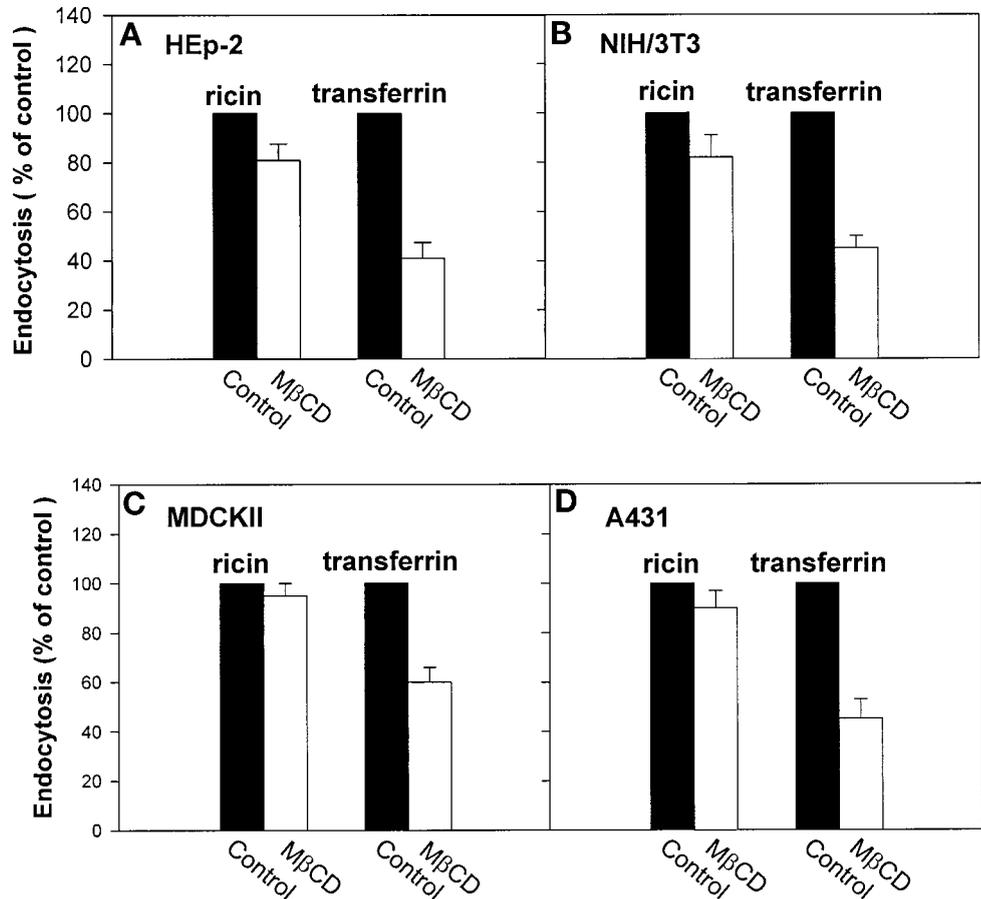


Figure 4. Effect of M β CD on transferrin and ricin endocytosis in different cell lines. HEp-2, NIH/3T3, MDCK II, and A431 cells were washed briefly in HEPES-buffered medium and incubated with and without M β CD (10 mM) for 15 min at 37°C. 125 I-labeled transferrin or 125 I-labeled ricin was then added to the cells, and the amounts of endocytosed transferrin or ricin were measured as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

ferrin was measured. As shown in Figure 7, both M β CD and β -cyclodextrin inhibited endocytosis of transferrin (>50%), whereas neither α - nor γ -cyclodextrin had any significant effect. Furthermore, when transferrin endocytosis was measured after preincubation of the cells with a M β CD-complex that was already saturated with cholesterol before it was added to the cells, there was no reduction in the endocytosis of transferrin (our unpublished results).

To further investigate the dependence of endocytosis on cholesterol, we studied whether the inhibition of transferrin endocytosis by M β CD was reversible. First we tried to compensate for the loss of plasma membrane cholesterol by adding cholesterol to the cells. The cells were preincubated with M β CD for 15 min at 37°C, and then HEPES-buffered medium with or without 5% fetal calf serum or 400 μ g/ml water-soluble cholesterol was added before endocytosis of 125 I-transferrin was measured. As shown in Figure 8A, there was a time-dependent recovery of the transferrin endocytosis. The uptake of transferrin was still inhibited by ~20% after a 1-h incubation even when serum or cholesterol was added; however, after 3-h incuba-

tion, the inhibition by M β CD was totally reversed. The finding that transferrin endocytosis recovered even in the absence of added serum or cholesterol suggested that the recovery could be due to cholesterol synthesis in the cells. This hypothesis was strengthened by the finding that lovastatin, an inhibitor of cholesterol synthesis, did inhibit the recovery of transferrin endocytosis unless the water soluble form of cholesterol was added (Figure 8B). Unless the cells were pretreated with M β CD, lovastatin only decreased transferrin endocytosis by ~10%, suggesting that there is only a small decrease in the level of plasma membrane cholesterol during 3 h in the absence of cholesterol synthesis. Thus, our results clearly indicate that the effect of M β CD observed is due to extraction of cholesterol.

It was recently reported that removal of cholesterol with M β CD did not induce release of lactate dehydrogenase, suggesting that there are no large changes in the permeability of the plasma membrane (Keller and Simons, 1998). This is in agreement with our finding that up to 10 mM M β CD did not reduce protein synthesis in our cells to any significant extent (Figure 9A). Also, as shown in Figure 9B, 10 mM M β CD did not

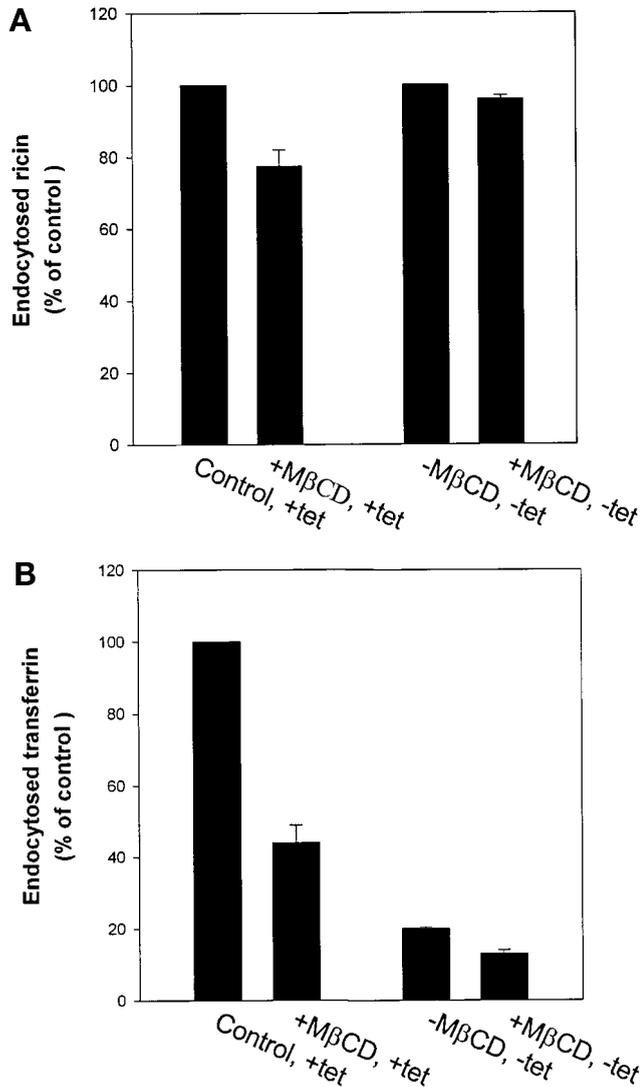
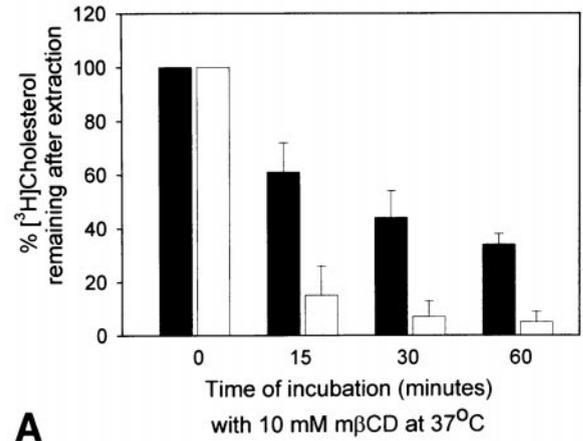


Figure 5. Effect of MβCD on endocytosis of ricin (A) and transferrin (B) in HeLa cells stably transfected with the cDNA for mutant dynamin (dyn^{K44A}). The cells were washed briefly in HEPES-buffered medium and incubated with and without MβCD (10 mM) for 15 min at 37°C. ¹²⁵I-labeled transferrin or ¹²⁵I-labeled ricin was then added to the cells, and the amounts of endocytosed transferrin or ricin were measured as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

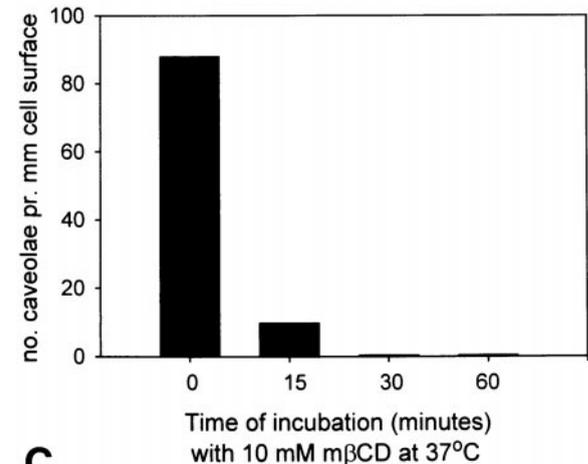
cause any significant membrane leakage of potassium. Furthermore, in MDCK II cells there was no increase in the cell-associated amount of ⁴⁵Ca²⁺ even when the isotope was added to cells in a Ca²⁺-free buffer (our unpublished results). In HEP-2 cells there was a small increase (20%) under the same conditions, but there was no significant change when the isotope was added to a buffer containing normal amounts of CaCl₂ (2 mM) (our unpublished results). Thus, in agreement with the data published by Keller and Simons (1998),



A



B



C

Figure 6. Effect of MβCD on cellular cholesterol and invaginated caveolae. A shows the percentage of [³H]cholesterol remaining in HEP-2 cells after extraction with MβCD as indicated. Black bars represent cells incubated with [³H]cholesterol for 20 h, whereas open bars are cells incubated for 15 min. Data from three independent experiments with samples performed in quadruplicate. Mean ± SD; n = 3. B shows characteristic invaginated caveolae (arrowheads) as observed in HEP-2 cells (without incubation with MβCD). Bar, 100 nm. In C, the frequency of such caveolae has been quantified after cholesterol extraction with MβCD as indicated; pooled data from two of the three experiments shown in A.

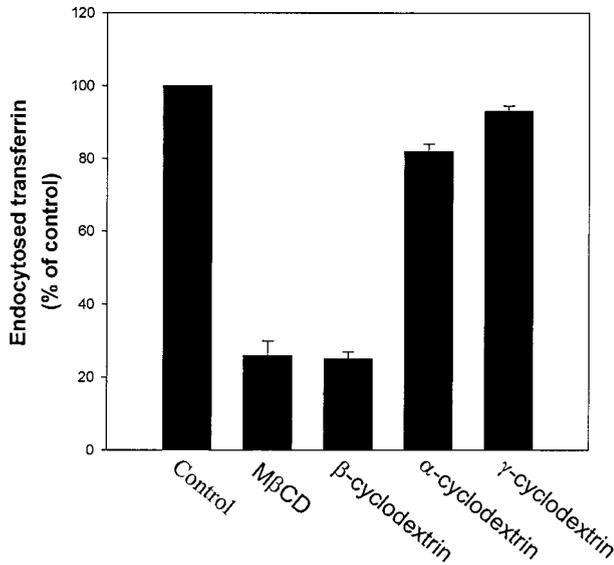


Figure 7. Effect of α -, β -, methyl- β -, and γ -cyclodextrins on transferrin endocytosis. HEP-2 cells were washed briefly in HEPES-buffered medium and incubated with and without α -, β -, methyl- β -, or γ -cyclodextrins (10 mM) for 15 min at 37°C. ^{125}I -labeled transferrin was then added to the cells, and the amounts of endocytosed transferrin were measured as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

our data indicate that M β CD strongly decreases clathrin-dependent endocytosis without causing any large permeability changes.

Effect of M β CD on Clathrin-coated Pits and TfR Distribution

The inhibition of endocytosis of transferrin (and EGF) by M β CD could reflect that removal of cholesterol prevented clathrin-coated pit formation, i.e., assembly of clathrin coats on the cytoplasmic face of the membrane; however, clathrin-coated pits were readily observed by electron microscopy in M β CD-treated cells, although they clearly appeared more flattened than in control cells. To investigate how M β CD affected the morphological appearance of clathrin-coated pits quantitatively, we classified the pits as follows: shallow pits, which are flattened or only slightly invaginated (Figure 10, A and B); invaginated pits, that is, when the pits are clearly invaginated but the necks connecting them with the exterior are still open wide (slightly wider or narrower than the diameter of the pit itself) (Figure 10C); and pits that are almost or completely pinched off, where the neck is very narrow (Figure 10D), or absent in the particular plane of sectioning (which could very well mean that the pit is surface-connected in another plane of sectioning; see Petersen and van Deurs [1983]) (Figure 10E). These three categories of clathrin-coated pits are shown

schematically in Figure 10F and will be referred to below as type 1, 2, or 3. When control HEP-2 cells and cells treated with 10 mM M β CD for 15 min at 37°C were compared with respect to the relative frequency of the three types of clathrin-coated pits (Figure 10G), the effect of M β CD was striking: type 2 and in particular type 3 dominated in control cells, whereas M β CD caused a clear predominance of type 1 pits.

Having shown that M β CD does not affect endocytosis in general or does not prevent assembly of clathrin-coated pits at the cell surface but interferes with the ability of flattened pits to invaginate, it was important to determine whether TfRs could still accumulate in the pits of M β CD-treated cells. We therefore used an immunogold-labeling protocol to detect TfRs on the surface of HEP-2 cells (Hansen *et al.*, 1992). As shown in Figure 11A, TfR gold labeling was distinct in the shallow, clathrin-coated pits mostly found in the M β CD-treated cells. Indeed, gold labeling was also seen on the cell surface outside clathrin-coated pits and on microvilli (Figure 11, B and C). In control HEP-2 cells, 8.7% of the TfRs as detected by immunogold labeling (using 2 $\mu\text{g}/\text{ml}$ of the B3/25 anti-TfR antibody on prefixed cells) are localized to coated pits that occupy 1.1–1.4% of the total surface area of these cells (Hansen *et al.*, 1992). Accordingly, TfRs are concentrated approximately sevenfold in the coated pits (Table 1). After incubation with 10 mM M β CD, we found that 11.5% of the TfR gold labeling was localized to coated pits (Table 1); however, because the surface area now occupied by coated pits had increased by 25% (presumably reflecting the fact that efficient invagination and pinching off are perturbed), the amount of golds in coated pits still corresponds to a sevenfold TfR concentration (Table 1). It should also be noted that we found a 60% increase in the total TfR cell-surface labeling in M β CD-treated cells as compared with control cells (Table 1), in agreement with the biochemical data reported above. Thus M β CD treatment does not interfere with the ability of TfRs to become concentrated efficiently in coated pits. Taken together, our findings therefore indicate that rather than blocking all types of endocytosis or preventing TfRs from entering clathrin-coated pits, M β CD perturbs invagination of the coated pits.

DISCUSSION

In the present study we have investigated the role of cholesterol in endocytosis by using M β CD, which specifically removes cholesterol from the plasma membrane (Ohtani *et al.*, 1989; Kilsdonk *et al.*, 1995; Klein *et al.*, 1995; Neufeld *et al.*, 1996). Our results show that endocytosis of transferrin and EGF was strongly reduced by addition of M β CD. This reduction could be due 1) to a general inhibition of all endocytic activity, 2) to a perturbation of the ability of clathrin-coated

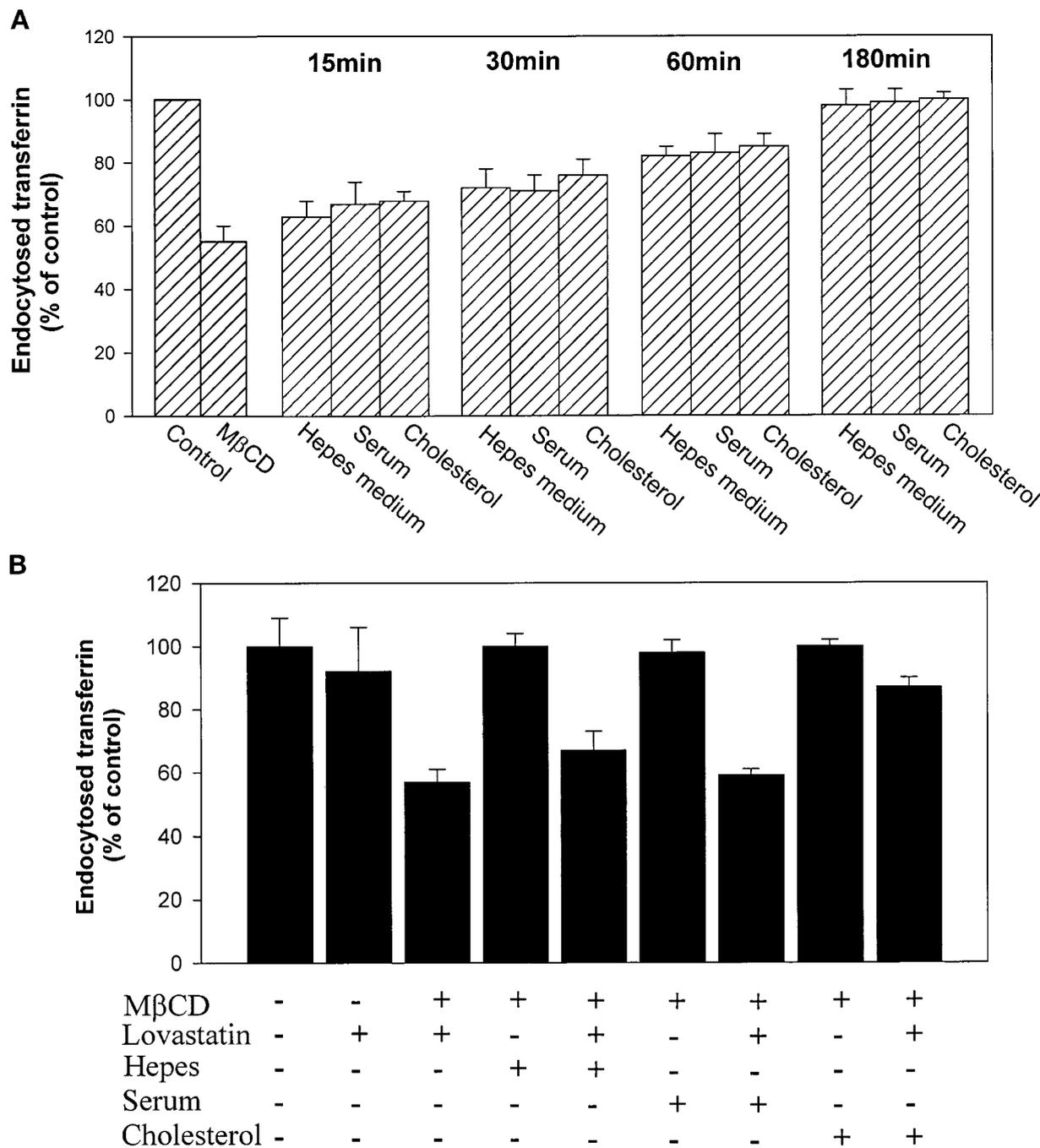


Figure 8. Reversal of MβCD-induced inhibition of transferrin endocytosis in the absence (A) or presence (B) of lovastatin. In both cases MDCK II cells were washed briefly in HEPES-buffered medium and incubated with and without MβCD (10 mM) for 15 min at 37°C. The cells were then incubated in HEPES-buffered medium with and without 5% FCS or water-soluble cholesterol (400 μg/ml) in the presence or absence of lovastatin (5 μg/ml) from 15 min to 3 h (A) or for 3 h (B) before ¹²⁵I-labeled transferrin was added. The amounts of endocytosed transferrin were measured as described in MATERIALS AND METHODS. The error bars show deviations between duplicates.

membrane areas to form, invaginate, or pinch off to form free, coated vesicles, or 3) to an exclusion of the receptors from clathrin-coated pits. The first possibility can be excluded because the general membrane

marker ricin is still internalized. Furthermore, MβCD clearly reduced ricin endocytosis to a much larger extent when both clathrin-dependent and clathrin-independent endocytosis were operating than when

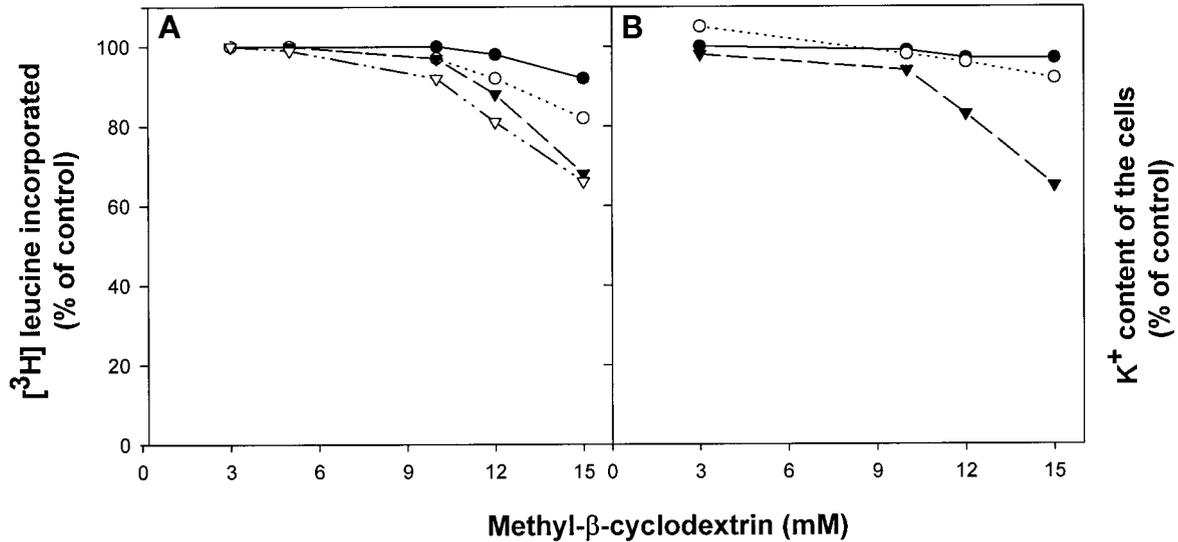


Figure 9. Effect of M β CD on protein synthesis (A) and on the intracellular potassium content (B). (A) HEp-2 (○), NIH/3T3 (▽), MDCK II (●), and A431 (▼) cells were washed briefly in HEPES-buffered medium and incubated with and without M β CD (10 mM) for 15 min at 37°C. [³H]leucine was then added to the cells, and after 15 min further incubation the amounts of incorporated [³H]leucine were measured as described in MATERIALS AND METHODS. (B) HEp-2 (●), MDCK II (○), and A431 (▼) cells were washed briefly in HEPES-buffered medium and incubated with and without M β CD (10 mM) for 15 min at 37°C. The cells were then washed with MgCl₂, and the amounts of intracellular potassium were measured as described in MATERIALS AND METHODS.

only clathrin-independent endocytosis was functioning. The results indicate that M β CD strongly reduces clathrin-dependent endocytosis, whereas there is a clathrin-independent endocytic pathway that is less affected. Because invagination of caveolae also is affected by M β CD, this clathrin-independent endocytosis seems to be caveolae-independent. It has previously been found that a downregulation of clathrin-dependent endocytosis can be associated with an upregulation of clathrin-independent endocytosis (Damke *et al.*, 1995), and there is a possibility that this might occur also on treatment with M β CD. Second, EM revealed that coated pits were indeed formed in M β CD-treated cells, but a marked flattening of these structures had taken place. Third, the quantitative immunogold data clearly show that the TfRs are not prevented from being concentrated in (the shallow) coated pits after M β CD treatment. A concentration of TfRs in clathrin-coated pits of approximately sevenfold both in M β CD-treated cells and in control cells is in agreement with previous data (Hansen *et al.*, 1992).

TfRs undergo endocytosis and recycling, and the number of TfRs on the surface is consequently dependent on both the amount of receptors which is internalized, and the amount that is recycled. The finding that binding of transferrin to the cell surface was increased nearly twofold by M β CD indicates that M β CD inhibits endocytosis without reducing the recycling of the TfRs to the same extent. There seems to be a somewhat stronger effect of M β CD on the morphology of the clathrin-coated pits than on the uptake

of transferrin. This might be due to the formation of some invaginated coated pits that rapidly pinch off. Our results strongly suggest that removal of cholesterol perturbs clathrin-coated pit invagination and, therefore, coated vesicle formation.

It has previously been reported that the membrane in coated pits is not disrupted by the sterol-binding drugs filipin or digitonin, although the membrane adjacent to these specialized domains readily binds these agents (Montesano *et al.*, 1979). On the other hand, endosomal membrane becomes susceptible to filipin disruption as soon as the clathrin coat is released (McGookey *et al.*, 1983), suggesting that the clathrin coat directly or indirectly binds cholesterol and prevents access to filipin. In agreement with those results, nystatin was reported not to affect transferrin endocytosis (Deckert *et al.*, 1996), and our own unpublished data showed that filipin has no effect on transferrin endocytosis in HEp-2 cells. Also, it is not clear that complexes between cholesterol and sterol-binding drugs would affect endocytosis in the same way as removal of cholesterol by M β CD.

Sterol-binding drugs such as filipin, nystatin, and digitonin have been found to inhibit endocytosis from invaginated caveolae and caveolae-like domains (Schnitzer *et al.*, 1994; Deckert *et al.*, 1996; Orlandi and Fishman, 1998). Invaginated caveolae disappear in cells that are depleted of cholesterol or exposed to sterol-binding agents (Rothberg *et al.*, 1990, 1992; Schnitzer *et al.*, 1994). In agreement with those data, our results clearly show that M β CD leads to disap-

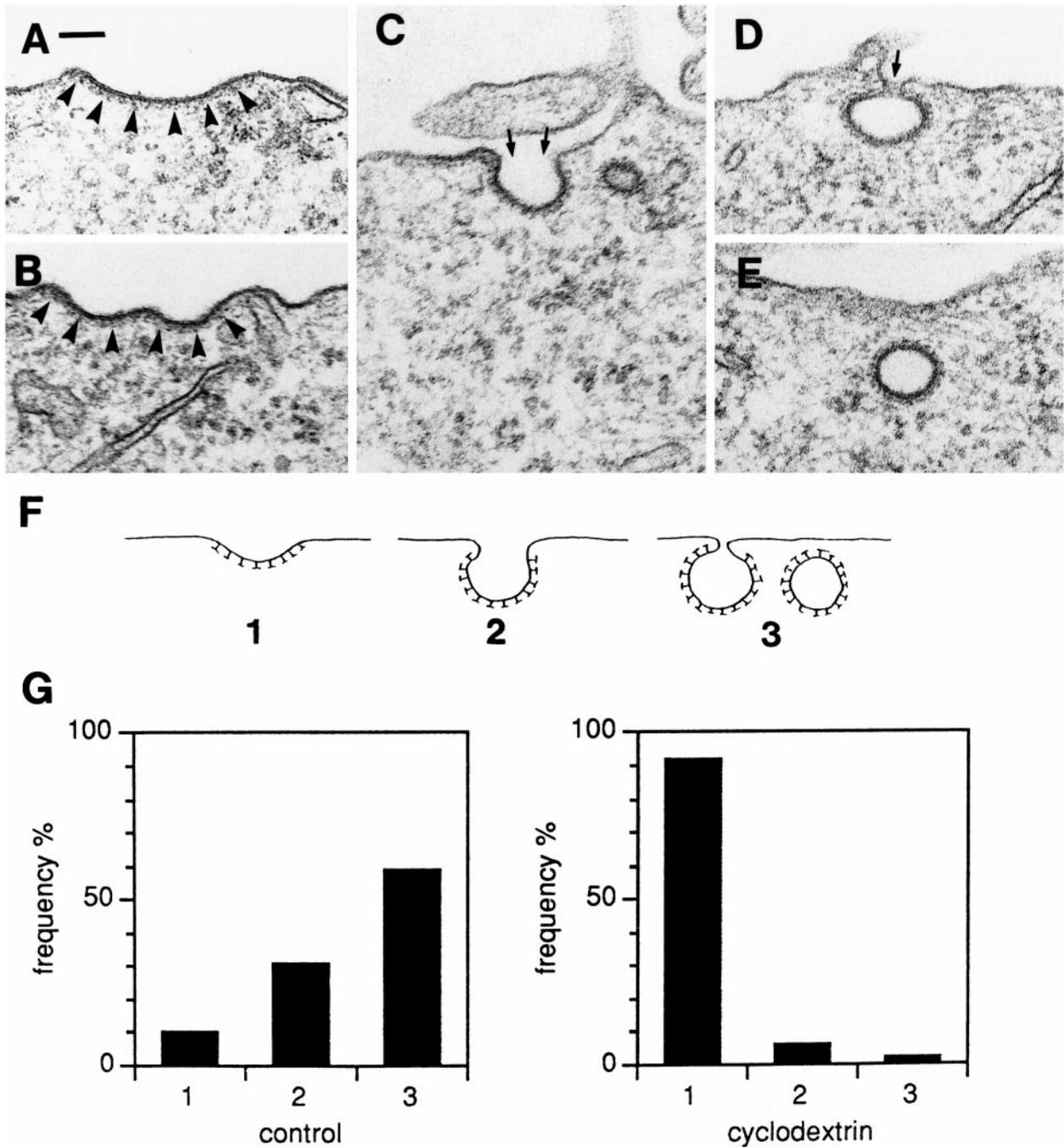


Figure 10. Examples of clathrin-coated pits at various degrees of invagination (A–D); E shows an apparently free, clathrin-coated vesicular profile that, however, may be surface-connected in another plane of sectioning. The clathrin coat is indicated by arrowheads in A and B. The small arrows in C and D indicate the necks connecting the pits to the exterior. Bar, 100 nm. (F) For quantification, the coated pits and apparently free vesicles (A–E) were subdivided into three types. (G) Coated pits in control HEp-2 cells and cells treated with 10 mM M β CD for 15 min (approximately 200 coated pits in each experiment) were scored at random and classified as 1, 2, or 3 as defined in F. The relative frequency of the different types of coated pits is shown. Bar, 100 nm.

pearance of invaginated caveolae in HEp-2 cells. That M β CD treatment leads to loss of invaginated caveolae is in agreement with the recent results of Hailstones *et*

al. (1998) showing that different cholesterol depletion treatments, including β -trimethyl cyclodextrin treatment, remove morphologically recognizable invagi-

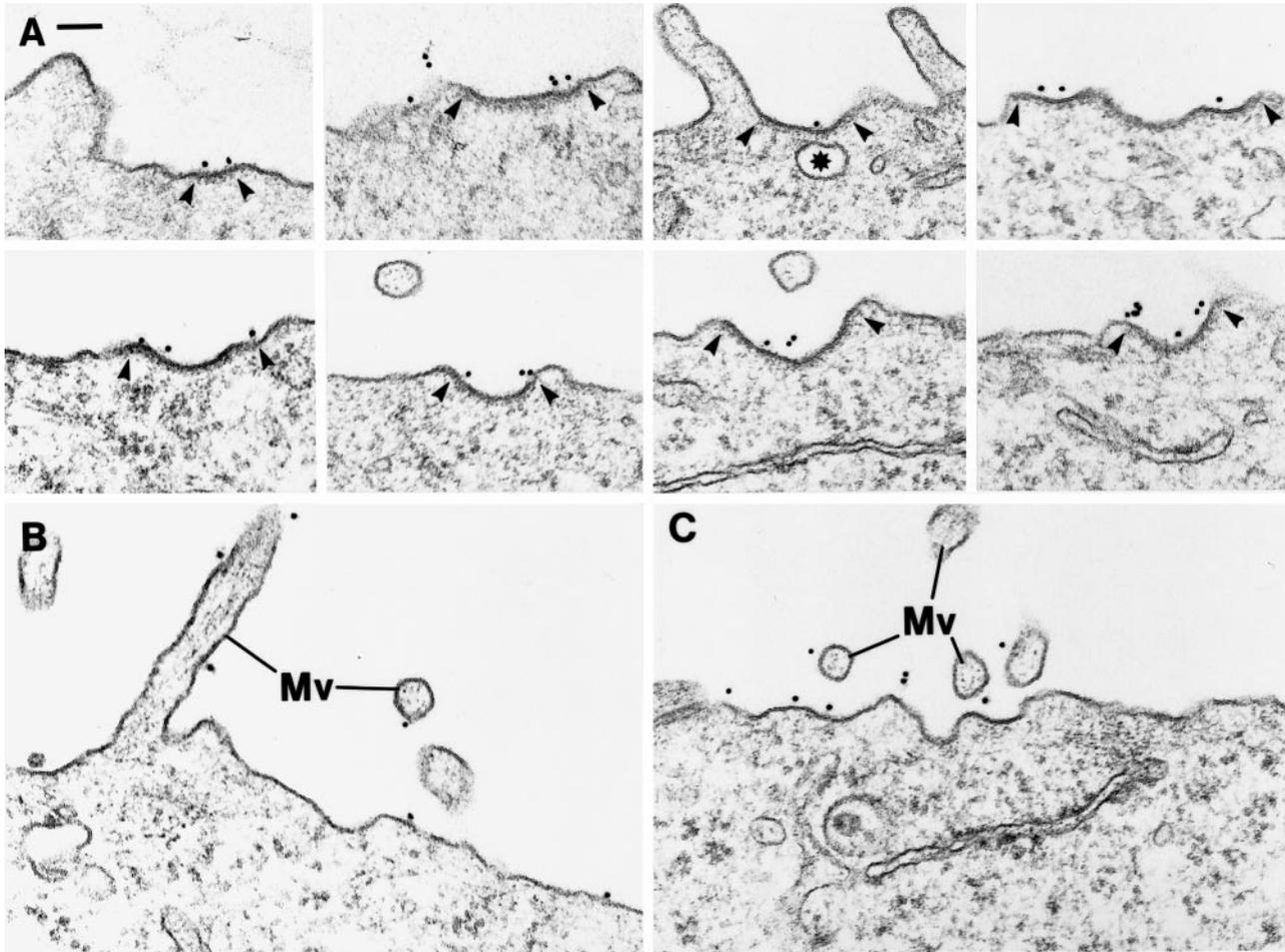


Figure 11. Effect of $M\beta CD$ on clathrin-coated pits and TfR distribution. (A) Examples of shallow coated pits characteristic of $M\beta CD$ -treated cells and of immunogold labeling for TfRs in the coated pits. Arrowheads mark the extension of the pits. Asterisk marks for comparison a clearly noncoated vesicular profile. (B and C), Examples of TfR immunogold labeling of portions of the cell surface without coated pits and of microvilli (Mv). HEP-2 cells were incubated for 15 or 30 min at $37^{\circ}C$ with 10 mM $M\beta CD$, fixed, incubated with B3/25 anti-TfR antibody and then with goat anti-mouse antibody conjugated to 10 nm gold, and processed for EM. Bar, 100 nm.

nated caveolae. Interestingly, they found that invaginated caveolae only form when the cholesterol level is $>50\%$ of control values. Also, Orlandi and Fishman (1998) found that the activity of cholera toxin was completely inhibited by β -cyclodextrin, and they therefore assumed that productive entry of cholera toxin occurs from caveolae. Furthermore, they reported that diphtheria toxin retained 80% of its toxic activity in the presence of β -cyclodextrin, and they therefore assumed that entry from clathrin-coated pits was essentially normal. This apparently contradicts our results; however, no direct measurement on uptake from clathrin-coated pits after β -cyclodextrin treatment was performed by Orlandi and Fishman (1998), and the apparent discrepancy can be explained if removal of cholesterol both reduces uptake from coated pits and at the same time facilitates membrane translocation of the toxin. In fact, filipin, which does not affect uptake

from clathrin-coated pits, increased the toxicity of diphtheria toxin (Orlandi and Fishman, 1998).

Heiniger *et al.* (1976) reported that L cells, in which hydroxymethylglutaryl-CoA reductase activity and cholesterol synthesis were inhibited by addition of 25-hydroxycholesterol, had a reduced rate of endocytosis of the fluid-phase marker HRP. The uptake of HRP was restored by the addition of cholesterol (Heiniger *et al.*, 1976). HRP is endocytosed, however, by both clathrin-dependent and clathrin-independent endocytosis (Oliver, 1982), and the fact that cholesterol depletions of L cells seem to have a stronger effect on total endocytosis in these cells (50%) than the effect we observed on HRP endocytosis in the cell types we have tested could be due to a larger proportion of uptake by clathrin-dependent endocytosis in L cells. Interestingly, Chang *et al.* (1992) reported that removal of cholesterol drastically increased the number of low-

Table 1. Concentration efficiency of TfR in clathrin-coated pits in HEp-2 cells as determined by immunogold labeling^a

	% of surface area occupied by clathrin-coated pits	% of TfR-gold in clathrin-coated pits	Concentration of TfR-gold in clathrin-coated pits; fold
Control cells	1.1–1.4	8.7	~7×
Cells treated for 30 min with 10 mM M β CD	1.4–1.8	11.5	~7×

HEp-2 cells were fixed before incubation with 2 μ g/ml B3/25 anti-TfR antibody. Forty large cell profiles (with a total of 2990 and 4812 golds, respectively) were included in the quantification from each experiment.

^a Data and calculations based on Hansen *et al.*, 1992.

density lipoprotein receptors in MA104 cells. The authors reported a slightly reduced internalization index, but because the calculation of such an index only takes into account the amount of surface-bound ligand versus internalized ligand, in this case at the end of a long incubation, the data might actually be in agreement with a reduced rate of endocytosis of the receptor from clathrin-coated pits. Two other cyclodextrins (Ohtani *et al.*, 1989)— α -cyclodextrin, which has been reported to remove phospholipids from the plasma membrane, and γ -cyclodextrin, which is also not specific for cholesterol—had much less effect on transferrin endocytosis than M β CD. This supports the notion that it is the removal of cholesterol per se that is essential for the inhibitory effect on clathrin-dependent endocytosis in the M β CD-treated cells. On removal of M β CD, the transferrin endocytosis was fully restored by continued incubation of the cells, even in serum-free medium. The recovery was inhibited by addition of lovastatin, suggesting that the cholesterol level in the plasma membrane was restored by newly synthesized cholesterol. This idea is supported by the finding that endocytosis did recover when a water-soluble form of cholesterol was added together with lovastatin. When lovastatin is used to inhibit cholesterol synthesis, fatty acid modification of intracellular proteins also is inhibited (Alberts *et al.*, 1980; Brown and Goldstein, 1980), and it is therefore essential for the conclusion, that cholesterol is required for clathrin-dependent endocytosis, that addition of cholesterol can counteract the effect of treatment with lovastatin.

It was reported recently that extraction of cholesterol with M β CD did not affect the amounts of lactate dehydrogenase in baby hamster kidney cells (Keller and Simons, 1998). In agreement with those findings, our results show that treatment with 10 mM M β CD had no effect on the intracellular potassium content nor on the protein synthesis, indicating that the plasma membrane permeability was not strongly affected. Furthermore, the lack of change in transepithelial resistance in M β CD-treated MDCK I cells (our unpublished results) and MDCK II cells (Keller and

Simons, 1998), together with our finding that binding of ricin and Shiga-toxin to the cell surface was unaffected by M β CD (our unpublished results), indicates that there are no large changes in the bilayer structure in cells treated with M β CD compared with the control cells. All of these data indicate that the flattening of the clathrin-coated pits is directly correlated to the removal of cholesterol.

It has been suggested that annexin II, which seems to be involved in both the regulated exocytic pathway (Creutz, 1992) and the endocytic pathway (Gruenberg and Emans, 1993), interacts directly with cholesterol-rich domains of the bilayer, and perhaps with cholesterol itself (Harder *et al.*, 1997). Such a direct protein-cholesterol interaction was recently revealed for the membrane protein VIP21-caveolin (Murata *et al.*, 1995). The cholesterol-binding drug filipin induces dissociation of annexin II and actin-binding proteins from endosomes (Harder *et al.*, 1997). In contrast, there is no evidence that cholesterol is important for binding of clathrin. As shown here, the clathrin coat stays at the plasma membrane after treatment of cells with M β CD. It is only the curvature that is affected. Actin was recently reported to be involved in clathrin-dependent endocytosis, because endocytosis was inhibited by perturbation of the actin cytoskeleton (Parton *et al.*, 1994; Schnitzer and Oh, 1994; Deckert *et al.*, 1996). Nevertheless, actin does not seem to be required for the formation of the coated-pit invagination (Lamaze *et al.*, 1997). Thus, the effect of M β CD described here is probably not due to an effect on the actin cytoskeleton; however, cholesterol could be important for the function of a protein involved in inducing curvature of clathrin-coated pits, such as affecting the activity of GTP-binding proteins that regulate coated-pit function (Schmid, 1997). Also, removal of cholesterol from the membrane might affect association of other molecules with the clathrin-coated pits, it might affect the interaction of membrane-associated proteins with each other, or it might somehow directly affect the ability of the other lipids in the clathrin-coated membrane domains to form an invaginated structure. As mentioned in the INTRODUC-

TION, cholesterol is important for the structure and function of membrane proteins (Muller and Shinitzky, 1979; Criado *et al.*, 1982; Bloch, 1991; Kilsdonk *et al.*, 1995), and furthermore, a change in the cholesterol content will affect the membrane viscosity, the exposure of membrane proteins to the surroundings, and therefore also the interaction between membrane proteins and cytosolic proteins (Shinitzky and Inbar, 1974; Shinitzky and Rivnay, 1977). Such changes might be responsible for the inhibition of formation of clathrin-coated pits observed here.

In conclusion, our results clearly indicate that the concentration of cholesterol in the plasma membrane of mammalian cells is essential for clathrin-dependent endocytosis, and it will be important for the understanding of this pathway to investigate why this is the case.

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