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Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D

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SUMMARY

Apical and basolateral endocytic pathways in polarised Caco-2 cells were investigated by following the uptake, recycling and transcytosis of the galactose-binding protein toxin ricin, as a membrane marker. Differences in the extent and kinetics of lectin uptake, recycling and transcytosis were observed at the apical and basolateral domains and altered with the age of the cell monolayer. Treatment of polarised Caco-2 cells with cytochalasin D showed a domain-specific, concentration-dependent inhibition of apical endocytosis of ricin. Inhibition of apical endocytosis by cytochalasin D was not due to a gross change in brush border morphology, although actin stress fibres within the cell body were disrupted. It is not clear whether inhibition of apical endocytosis in polarized epithelial cells by cytochalasin D is caused simply by disruption of a mechanochemical motor involving microvillar actin filaments. The cytochalasin D effect was also observed when measuring uptake of folate, suggesting apical domain-specific inhibition of caveolar, as well as clathrin-mediated, endocytic routes.

Key words: endocytosis, actin, brush border

INTRODUCTION

In polarised epithelial cells, endocytosis occurs from both the apical and basolateral domains with endocytosed ligands first entering domain-specific early endosome compartments and subsequently a common late endosome compartment. Such pathways have been shown both for MDCK (Bomsel et al., 1989; Parton et al., 1989; van Deurs et al., 1990) and Caco-2 (Hughson and Hopkins, 1990) cells grown on filter supports. Transcytosis also occurs in polarised epithelial cells from the basolateral to apical plasma membrane domains and in the reverse direction (Mostov and Simister, 1985; Mostov et al., 1992). In the present study we have used Caco-2 cells, which are derived from a human colon adenocarcinoma (Fogh et al., 1977a,b; Pinto et al., 1983), to investigate apical endocytosis, an essential first step in apical to basolateral transcytosis. Whereas basolateral endocytosis has been well studied in these cells, less is known about apical endocytosis, though there have been studies of apical fluid-phase uptake (Hidalgo et al., 1989), apical endocytosis and recycling of specific membrane hydrolases (Matter et al., 1990; Klumperman et al., 1991), apical endocytosis of concanavalin A (Hughson and Hopkins, 1990) and apical uptake and transcytosis of cobalamin (Dix et al., 1987, 1990). We have used ^{125}I-ricin, which binds to galactose residues of membrane glycoproteins and glycolipids, as a general membrane marker to quantify and characterise endocytosis at the apical and basolateral surfaces of Caco-2 cells, and to allow comparison with previous data obtained from MDCK cells (van Deurs et al., 1990). Recently there have been reports of differential effects of membrane traffic-disrupting agents on apical and basolateral endocytosis in MDCK cells. Prydz et al. (1992) showed stimulation of apical, but not basolateral, uptake of ricin and horseradish peroxidase by brefeldin A, and Gottlieb et al. (1993) observed inhibition of apical endocytosis of vesicular stomatatis virus G protein in transfected MDCK (strain II) cells by cytochalasin D. This latter effect was interpreted as being caused by disruption of microvillar actin filaments. The microvilli of the brush border of Caco-2 cells are much better developed than those of MDCK cells, allowing us to investigate this in more detail. We present evidence that cytochalasin D, at concentrations having no visible effect on gross microvillar morphology, can inhibit apical but not basolateral endocytosis in Caco-2 cells. Our data extend previous observations on the inhibition of apical endocytosis by cytochalasin D in polarised epithelial cells (Gottlieb et al., 1993) and provide the first evidence that this inhibition extends to caveolar as well as clathrin-mediated endocytosis. A preliminary report of some of these data has been published (Luzio et al., 1992).

MATERIALS AND METHODS

Reagents

[3′,5′,7,9-^{3}H]folate, D-[1-^{14}C]mannitol and Na^{125}I were from Amersham International, Amersham, UK. Unless otherwise stated, all other reagents were from Sigma Chemical Co.

Cell culture

Caco-2 cells were a gift from Dr M. Mackay and Dr I. Hassan of Ciba-

Geigy Pharmaceuticals, Horsham, Sussex, UK, and MDCK (strain II) cells were from Dr L. Muir of the Department of Clinical Biochemistry, University of Cambridge, UK. Cells were maintained and grown on polycarbonate Transwell filter chambers (Costar, Cambridge, MA) as previously described (Ellis et al., 1992a,b). Caco-2 cells grown on filters showed an increasing transepithelial electrical resistance (TEER) from 50 to 200 Ω cm^2 between 2 and 7 days and this further increased to a plateau of 400 Ω cm^2 by 12 days, which was maintained for at least an additional 12 days. Monolayer permeability was assessed by measuring the leakage of D-[^{14}C]mannitol (60 mCi/mmol, from Amersham) from the apical to the basolateral chamber as previously described (Ellis et al., 1992a). After 2 hours at 37°C, <1% of radiolabel leaked across either 7-day or 14-day monolayers. All experiments used Caco-2 cells between passages 82 and 138.

Ricin binding at the apical and basolateral membrane domains

Ricin (*Ricin communis* agglutinin$_{60}$) was radiolabelled using the iodogen (Pierce) technique (Fraker and Speck, 1978) to a specific activity of 7×10^3-14×10^3 cpm/ng. Prior to ricin binding, Caco-2 (or MDCK) cells grown on filters were washed 3× with ice-cold PBS^+ (PBS^+ supplemented with 0.5 M $CaCl_2$, 0.2 mM $MgCl_2$). The ^{125}I-labelled ricin (25 ng/ml) in ice-cold lectin-binding medium (LBM: DMEM, 0.6% bovine serum albumin, 20 mM HEPES, pH 7.4) was added to either the apical (1 ml) or basolateral (1.5 ml) chambers of the cultured Caco-2 cell monolayers. An identical volume of LBM was added to the opposite chamber. Ricin binding continued for 1 hour at 4°C, with horizontal shaking. Caco-2 cell monolayers were then washed 4× with ice-cold PBS^+ to remove unbound ricin. Under these conditions, using 7-day-old Caco-2 cell monolayers ~100,000 cpm ^{125}I-ricin bound to the apical surface and ~30,000 cpm bound to the basolateral surface. After washing either surface for 5× 5 minute with ice-cold PBS^+/0.2 M lactose washes, followed by 2× 2 minute washes with ice-cold PBS^+, <4% of initial radioactivity bound remained.

Endocytosis of ricin at the apical and basolateral membrane domains

After binding ricin to the apical or basolateral cell surface, Caco-2 cell monolayers were incubated for various times at 37°C in LBM (1 ml added apically, 1.5 ml added basolaterally). After these incubations, non-internalised ricin was removed with 5× 5 minute ice-cold PBS^+/0.2 M lactose washes, followed by 2× 2 minute washes with ice-cold PBS^+. Filters were then excised and radioactivity determined. Throughout the experiments the amounts of endocytosed ^{125}I-ricin at the apical and basolateral membrane domains were normalised to % of ^{125}I-ricin bound to that membrane domain. The requirement of energy for ricin uptake in Caco-2 cells was shown by pretreating Caco-2 cells with DMEM/10% FCS containing the metabolic inhibitors, 2-deoxyglucose (50 mM) and NaN_3 (10 mM), for 30 minutes at 37°C. The inhibitors were also present in the LBM during ricin binding. To study the effect of cytochalasin D upon ricin endocytosis in Caco-2 cells, prewarmed DMEM/10% FCS supplemented with cytochalasin D (from 5 mg/ml stock solution in DMSO) was added to both the apical (1 ml) and basolateral (1.5 ml) domains of Caco-2 cell monolayers. Cells were pretreated with cytochalasin D for 30 minutes at 37°C, before the medium was aspirated off. Ricin endocytosis studies were then carried out as described above. Cytochalasin D was maintained in all media throughout ricin binding and endocytosis, unless otherwise stated. Control cells were mock treated with medium supplemented with DMSO only, at the concentrations used in cytochalasin-D-supplemented media. The effects of microtubule depolymerisation on basolateral and apical endocytosis were investigated by treating cells with 10 μM colchicine (dissolved in DMEM/10% FCS) for 30 minutes at 4°C, then incubating at 37°C for 4 hours in the presence of the drug. Ricin endocytosis was then measured as above. The irreversible action of colchicine negated the need for the drug to be present during ricin binding and endocytosis.

Recycling of ricin at the apical and basolateral domains

Caco-2 cells were allowed to endocytose ^{125}I-ricin for 30 minutes at 37°C, as described above. Non-internalised ^{125}I-ricin was then washed from the monolayers at 4°C for 5 minutes with LBM/0.2 M lactose. Cells were then reincubated at 37°C with LBM/0.2 M lactose. Radioactivity released into the medium was measured.

Transcytosis of ricin

TEER was measured or monolayer permeability studies were carried out prior to transcytosis experiments in order to assess the integrity of the monolayer. Caco-2 cell monolayers showing a TEER of less than 300 Ω cm^2, or transport of >1% [^{14}C]mannitol across the cell monolayer after 2 hours at 37°C, were not used. Cell monolayers were washed, ^{125}I-ricin-bound and endocytosed as described above. The amount of transcytosed ^{125}I-ricin was calculated from the amount of trichloroacetic acid (10%, w/v)-precipitable radioactivity recovered from the medium in the chamber opposite that to which the ^{125}I-ricin had been added, plus the amount of ^{125}I-ricin released from the appropriate plasma membrane domain during a 5 minute incubation with PBS^+/0.2 M lactose at 37°C.

Folate uptake

Caco-2 cells were routinely grown in folate-free growth medium (prepared by using folate-free DMEM and FCS stripped with charcoal, as described by Kamen and Capdevila, 1986), to maximise [^{3}H]folate binding. Filter-grown Caco-2 cells cultured in folate-free DMEM/5% FCS were washed 2× 2 minutes with ice-cold PBS^+. Folate-free DMEM, pH 7.1, supplemented with 20 nM [^{3}H]folate (1 mCi/ml, 20 Ci/mmol), was then added to either the apical (1 ml) or basolateral (1.5 ml) filter chambers, and corresponding amounts of folate-free DMEM were added to the opposite chamber. The cells were then incubated at 4°C with horizontal shaking for 1 hour. After folate binding, the medium was aspirated from the filter chambers and unbound folate was washed from the Caco-2 cell monolayers with 4× 2 minute washes with ice-cold PBS^+ (adjusted to pH 7.1 with HCl). Prewarmed folate-free DMEM was added to Caco-2 cell monolayers (1 ml apically, 1.5 ml basolaterally), which were then incubated at 37°C for various times. Following internalisation of [^{3}H]folate, cell surface [^{3}H]folate was removed by 2× 1 minute washes with an ice-cold acid solution (0.15 M NaCl adjusted to pH 3.0 with acetic acid; 2 ml apically, 2 ml basolaterally), followed by 2× 2 minute washes with ice cold PBS^+ (pH 7.1). Filters were excised and after addition of 10 ml Optiphase II scintillant, acid-resistant [^{3}H]folate was measured on a Packard 1500 Tri-Carb liquid scintillation counter. Specific binding of folate was calculated by subtracting the amount of [^{3}H]folate that bound to Caco2 cells in the presence of a 100-fold excess of unlabelled folic acid (non-specific binding), from the amount of [^{3}H]folate that bound to Caco-2 cell monolayers in the absence of unlabelled folate (total binding). Unlabelled folic acid was added from a freshly prepared 2 mM stock of folic acid in 0.1 M bicarbonate buffer, pH 8.0.

Microscopy

To assess the actin depolymerising effects of cytochalasin D, Caco-2 cells grown for 14 days on filter supports were treated with 10 μM cytochalasin D and subsequently permeabilised with detergent, fixed and stained with TRITC-phalloidin (1 μg/ml in PBS/0.1% bovine serum albumin, 1 hour at 20°C), prior to confocal microscopy. Permeabilisation, pH shift/formaldehyde fixation and mounting were as described by Bacallao et al. (1990), with 1% Triton X-100 added for 5 minutes prior to addition of formaldehyde. For electron microscopy, Caco-2 cells grown for 14 days on filter supports and treated with cytochalasin D were fixed, stained and processed as previously described (Ellis et al., 1992b).

RESULTS

The broad specificity of ricin binding to many galactosylated membrane lipids and proteins (Turpin et al., 1984), and its efficient removal from the plasma membrane by lactose, enabled this lectin to be used as a marker to measure both apical and basolateral endocytosis in Caco-2 cells. It has been shown previously that ricin binds avidly to the surface of Caco-2 cells (3.5×10^7 binding sites per cell with a K_A of 1.25×10^7 M^{-1}; Luzio et al., 1992). Ricin binding was greater to the apical than to the basolateral surface of polarized Caco-2 cell layers, the ratio of apical:basolateral-bound ricin increasing with the age of the culture, from ~3.5 in 7-day-old cells to ~4.7 in 14-day-old cells. When ^{125}I-ricin was endocytosed from either side of the Caco-2 cell monolayer, biphasic accumulation was observed, the amount of accumulation from each side being dependent on the age of the monolayer. The initial phase provides an indication of the amount of ricin being taken up into the cell, whereas the later phase reflects an equilibrium between uptake and recycling. In Caco-2 cells grown for 7 days on filters, normalised uptake of ^{125}I-ricin was found to be greater from the basolateral than the apical membrane domain (Fig. 1). However, both apical and basolateral endocytosis of ^{125}I-ricin decreased as the Caco-2 cell culture aged. The decrease in endocytosis was most dramatic at the basolateral domain. Consequently, in 14-day-old Caco-2 cells, efficiencies of apical and basolateral ^{125}I-ricin endocytosis were very similar (Fig. 1). Ricin uptake was abolished when cells were kept on ice and inhibited >85% when cells were incubated with the metabolic inhibitors NaN_3 and 2-deoxyglucose (data not shown). All endocytosis experiments were confined to a maximum duration of 2 hours at 37°C, since it was found that ricin inhibition of [^{14}C]leucine incorporation into cell protein did not occur until >2 hours after exposure to the lectin at concentrations up to 25 ng/ml (data not shown).

Recycling of endocytosed ^{125}I-ricin was measured and was found to occur to a lesser extent at the apical than the basolateral domain. Over 1 hour, Caco-2 cells incubated with lactose present in the medium released ~60% of basolaterally internalised ^{125}I-ricin into the basolateral chamber, and ~35% of apically internalised ^{125}I-ricin into the apical chamber (Fig. 2). Recycling of endocytosed ricin at the basolateral domain did not change in magnitude upon ageing of Caco-2 cells and there was only a slight decrease in recycling at the apical domain (Fig. 2).

Transcytosis of ^{125}I-ricin was also measured in polarized Caco-2 cells. When ^{125}I-ricin was bound to either the apical or basolateral membrane domains of cultured Caco-2 cells, and incubated for 2 hours at 37°C, a small fraction of bound ^{125}I-ricin could be detected attached to the opposing membrane domain or in the medium bathing the opposing membrane domain (Fig. 3). At all ages of Caco-2 cell culture, more ^{125}I-ricin was transcytosed in the basolateral to apical direction than the apical to basolateral direction (Fig. 3). ^{125}I-ricin transcytosis in both directions increased with the ageing of Caco-2 cell

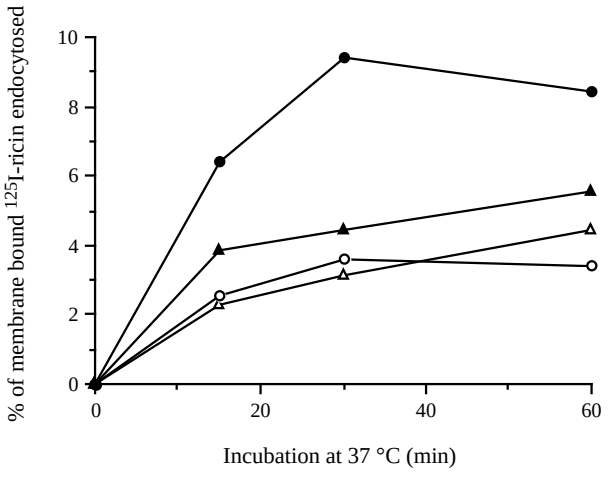


Fig. 1. Endocytosis of ^{125}I-ricin by Caco-2 cells grown as monolayers for 7 and 14 days on permeable filters. ^{125}I-labelled ricin (25 ng/ml, specific activity 8,000 to 10,000 cpm/ng) was added to the apical surface of Caco-2 cells grown on polycarbonate filters and allowed to bind for 1 hour at 4°C. Endocytosis of bound ligand was then measured by measuring lactose wash-resistant ^{125}I-ricin. Background values from Caco-2 cell monolayers pretreated with 10 mM NaN_3 and 50 mM 2-deoxyglucose were subtracted from experimental values. Endocytosis at each domain was normalised to % of total lactose displaceable ricin binding to that domain. Endocytosis is shown from the basolateral (●,○), and apical (▲,△), domains of cell layers cultured for 7 (●,▲), and 14 (○,△), days. Each point is the mean of tripicate samples; all s.d.values were <10% of data point values.

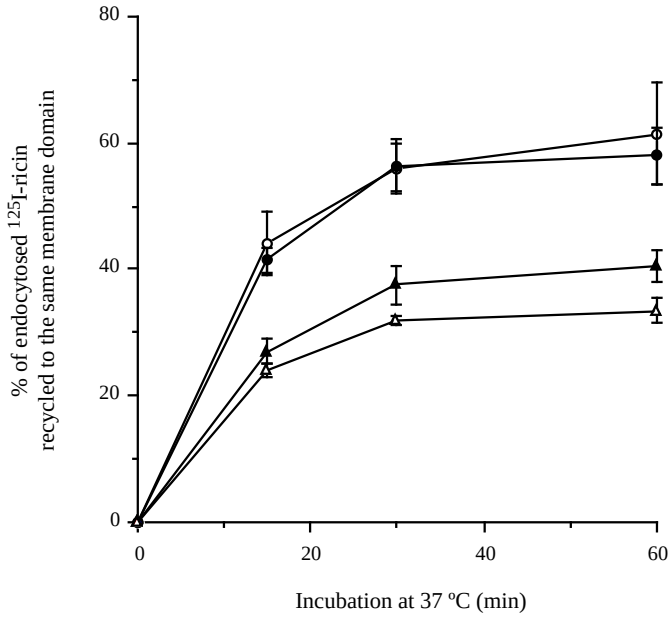


Fig. 2. Endocytotic recycling of ^{125}I-ricin in Caco-2 cells. Caco-2 cells grown on filters were allowed to endocytose ^{125}I-ricin for 30 minutes at 37°C. Non-internalised ^{125}I-ricin was then washed from the Caco-2 cell monolayers at 4°C with 0.2 M lactose. Caco-2 cells were then reincubated at 37°C in the presence of 0.2 M lactose. Greater than 95% of radioactivity released from the cells after 1 hour was precipitated by 10% (w/v) trichloroacetic acid. Recycling is shown after endocytosis from the basolateral (●,○), and apical (▲,△), domains of cell layers cultured for 7 (●,▲), and 14 (○,△), days. Values shown are the mean ± s.d. of triplicate samples of trichloroacetic acid-precipitable radioactivity released into the apical or basolateral medium.

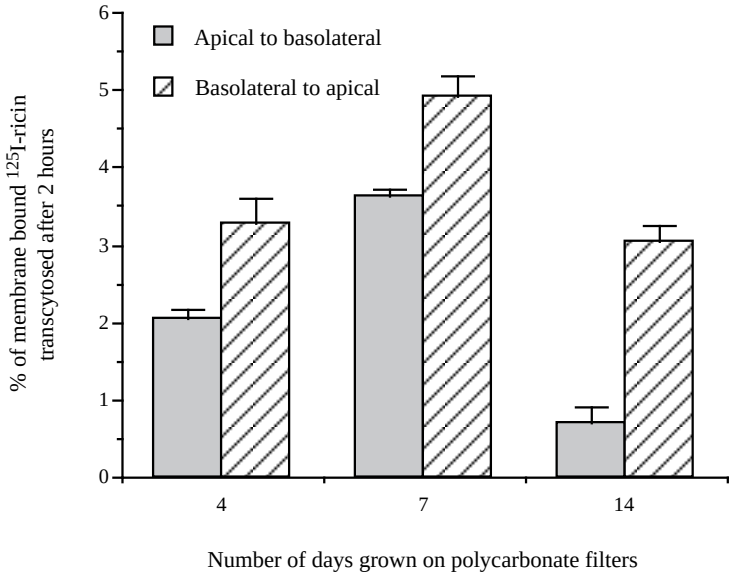


Fig. 3. Transcytosis of ricin across Caco-2 cell monolayers. ^{125}I-ricin was bound to either the apical or basolateral plasma membranes of Caco-2 cells for 1 hour at 4°C. The amount of ricin transcytosed after 2 hours at 37°C was calculated by totalling the amount of radioactivity released into the medium in the opposite filter chamber and the amount that could be released after washing the opposing membrane domain with 0.2 M lactose. Greater than 90% of transcytosed radioactivity was precipitated by 10% (w/v) trichloroacetic acid. Values for transcytosis were normalised by expressing the amount of ricin transcytosed as a % of ricin bound to the domain to which it was added after subtracting the amount crossing the cell monolayer at 4°C. Each value is the mean ± s.d. of triplicate samples.

cultures from 4 to 7 days, but fell when Caco-2 cell cultures were allowed to age from 7 to 14 days. The reduction in the amount of apically to basolaterally transcytosed ^{125}I-ricin, seen when comparing 14-day-old Caco-2 cell cultures to 7-day-old Caco-2 cell cultures, was so great as to make transcytosis of ^{125}I-ricin in this direction statistically insignificant in 14-day-old Caco-2 cells. Movement of ^{125}I-ricin across the 7-day cultured monolayers was almost abolished by preincubating with the metabolic inhibitors, NaN_3 and 2-deoxyglucose, or keeping the cells at 4°C (data not shown). An overall summary of the endocytic routes of surface bound ricin in 7-day cultured, polarized Caco-2 cells is shown in Fig. 4 and compared with data for MDCK cells taken from van Deurs et al. (1990).

Treatment of polarized Caco-2 cells with the actin filament depolymerising agent cytochalasin D showed no effect on cell surface binding of ^{125}I-ricin, little effect on basolateral endocytosis, but a concentration-dependent inhibition of apical endocytosis (Fig. 5). This domain-specific inhibition of endocytosis by cytochalasin D was totally reversible (Fig. 6) and inhibition was dependent on the age of the cell culture, increasing ~2-fold between 5 and 14 days. In agreement with the lack of effect of cytochalasin D on basolateral endocytosis of ricin, receptor-mediated endocytosis of transferrin, which occurs specifically from the basolateral domain (Hughson and Hopkins, 1990), was also unaffected (data not shown). In transfected MDCK cells, it has been reported that 50 μM cytochalasin D can inhibit apical endocytosis of vesicular stomatatis virus G protein without any effect on basolateral endocytosis (Gottlieb et al., 1993). In the present study endocytosis of ^{125}I-ricin was also measured in cytochalasin-D-treated MDCK cells. Although apical endocytosis was inhibited by cytochalasin D in MDCK cells (Fig. 7), the dose-response curve was significantly shifted, with a greater concentration of cytochalasin D being required to cause equivalent inhibition (cf. Fig. 5). There was a small increase in basolateral endocytosis in both cell types after treatment with 1-10 μM cytochalasin D. The amount of ricin accumulated at any given time is dependent upon both its rate of internalisation, and the rate at which it is recycled to the plasma membrane. The reduced endocytosis of ^{125}I-ricin measured upon cytochalasin D treatment could be due to an increase in the rate of ^{125}I-ricin recycling. However, no significant difference was observed between ^{125}I-ricin recycling in untreated Caco-2 cells and that in cells treated with 10 μM cytochalasin D.

In some cell types other than Caco-2, internalisation of ricin has been shown to be mediated by both clathrin- and non-clathrin-dependent endocytosis (Moya et al., 1985; Sandvig

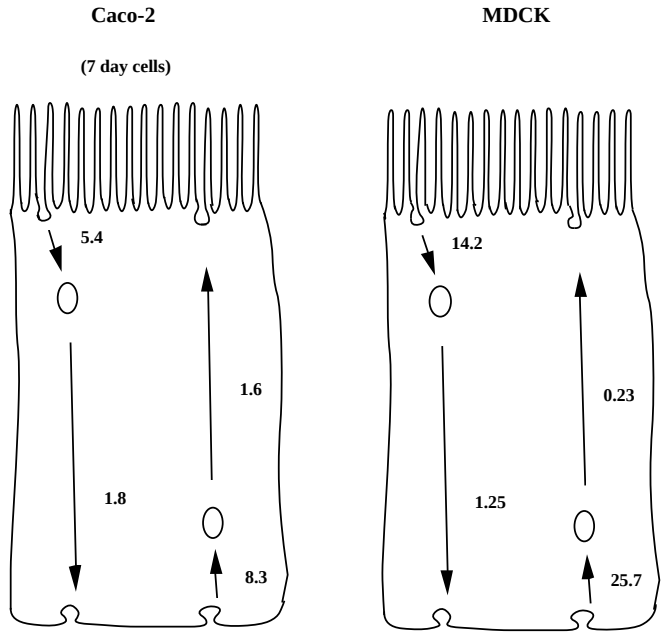


Fig. 4. Comparison of the endocytotic routes of surface-bound ricin in Caco-2 and MDCK cells. Numbers shown are % of surface-bound ricin travelling on a particular route 1 hour after warming cells to 37°C. Data for Caco-2 cells are shown for cell monolayers cultured for 7 days and are calculated from experiments shown in Figs 1-3. The drawing of endocytotic routes in MDCK cells is based on data from van Deurs et al. (1990).

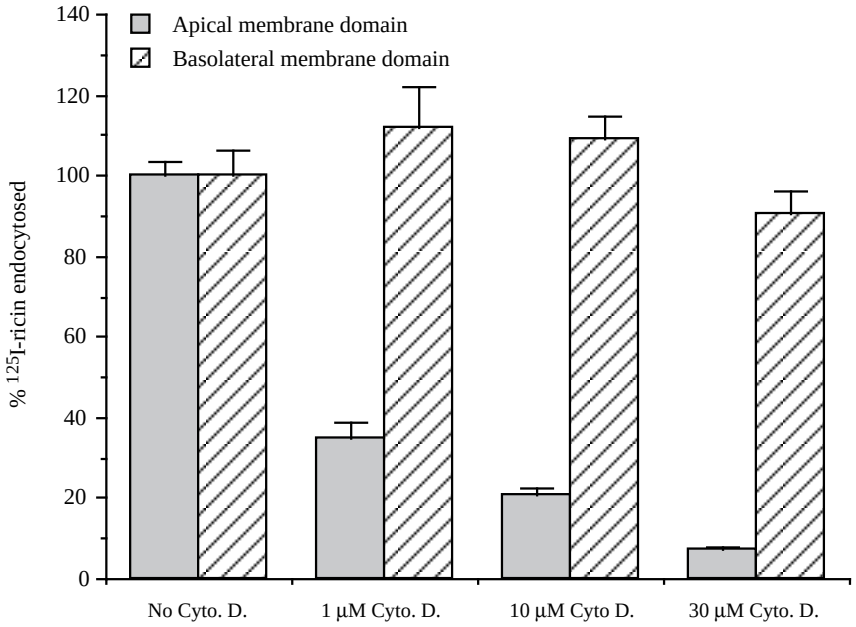


Fig. 5. The effect of cytochalasin D on ricin uptake by Caco-2 cells. Caco-2 cells grown on polycarbonate filters for 14 days were incubated for 30 minutes at 37°C, with various concentrations (0-30 μM) of cytochalasin D. ^{125}I-labelled ricin (25 ng/ml, specific activity 8,000 to 10,000 cpm/ng) was added to the apical or basolateral surface of the cells, and allowed to bind for 1 hour at 4°C. Endocytosis of bound ligand after 1 hour of incubation at 37°C was then calculated by measuring ^{125}I-ricin accumulation resistant to a lactose wash. Cytochalasin D was maintained in all media in which cytochalasin-D-pretreated cells were subsequently incubated. Background values from Caco-2 cell monolayers treated with 10 mM NaN_3 and 50 mM 2-deoxyglucose, were subtracted from experimental values. Inhibition of endocytosis is expressed as % of domain-specific ^{125}I-ricin endocytosis measured in the absence of cytochalasin D. Each value is the mean of triplicate samples, error bars indicate s.d.

and van Deurs, 1990). There are probably several non-clathrin-dependent endocytic routes for uptake of molecules into mammalian cells (van Deurs et al., 1993), but the best characterised is that taken by folate, which is internalized via caveolae after binding to its glycosylphosphatidylinositol (GPI)-anchored receptor (Lacey et al., 1989; Rothberg et al., 1990, 1992). In the present study it was found that there was ~3-fold more binding of [^{3}H]folate to the apical surface of filter-grown Caco-2 cells than to the basolateral surface (data not shown). Cytochalasin D (10 μM) inhibited [^{3}H]folate uptake at the apical domain, but not at the basolateral domain (Fig. 8). Interestingly, the inhibition of [^{3}H]folate uptake after 1 hour by 10 μM cytochalasin D (Fig. 8) was greater than that of ^{125}I-ricin uptake (Fig. 5). The effect of cytochalasin D on apical endocytic uptake was also observed with the fluid-phase marker [^{14}C]mannitol (~60% inhibition with 1 μM cytochalasin D).

The effect of cytochalasin D on apical endocytosis was not simply due to general disruption of cell integrity or of the cytoskeleton, since the TEER of Caco-2 cells did not significantly change following treatment with up to 10 μM cytochalasin D. Moreover, 10 μM colchicine showed <20% inhibition of ^{125}I-ricin endocytosis when uptake was measured for 30 minutes. The actin-filament-depolymerising effect of cytochalasin D in Caco-2 cells was visualised by fluorescence microscopy after staining with TRITC-phalloidin. Treatment of Caco-2 cells with 10 μM cytochalasin D caused almost complete depolymerisation of actin stress fibres without affecting TRITC-phalloidin-stained microvilli (Fig. 9). Cytochalasin D (10 μM) did not cause a gross change in Caco-2 cell brush border morphology and thin filaments running longitudinally down the microvilli (most likely actin filaments) were still visible in electron micrographs of cytochalasin-D-treated cells (Fig. 10).

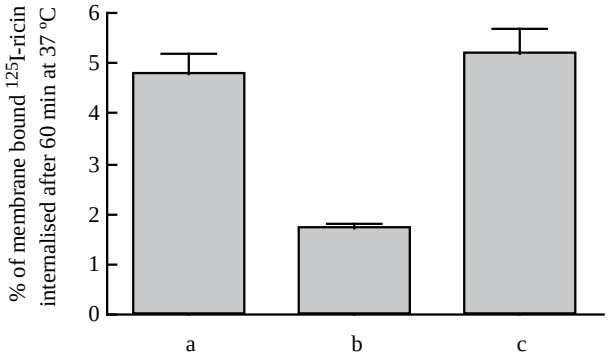


Fig. 6. Reversibility of the effect of cytochalasin D on apical endocytosis. Caco-2 cells grown for 14 days on polycarbonate filters were pre-incubated without (a) or with (b,c) 20 μM cytochalasin D, for 30 minutes at 37°C. Apical endocytosis of ^{125}I-ricin was then measured after 1 hour of incubation in the presence (b) or absence (a,c) of 20 μM cytochalasin D. Background values obtained with Caco-2 cells treated with metabolic inhibitors were subtracted from experimental values. Each value is the mean of triplicate samples, error bars indicate s.d.

DISCUSSION

In the present study we first quantified the endocytic pathways taken by ricin when internalised from the apical and basolateral domains of polarised monolyers of Caco-2 cells. We then investigated the effects of cytochlasin D on endocytosis from the two domains. The initial rate of ^{125}I-ricin accumulation from either side of the monolayer decreased with the age of the Caco-2 cell cultures. The decrease in basolateral endocytosis seen in 14-day-old compared to 7-day-old cultures was ~3-fold greater than for apical endocytosis over the same period. The decrease and the lower rate of basolateral endocytosis observed after 14 days of culture may reflect a reduction in the growth rate of Caco-2 cells, and thus a reduction in the requirement to take up nutrients by this route and/or a reduced

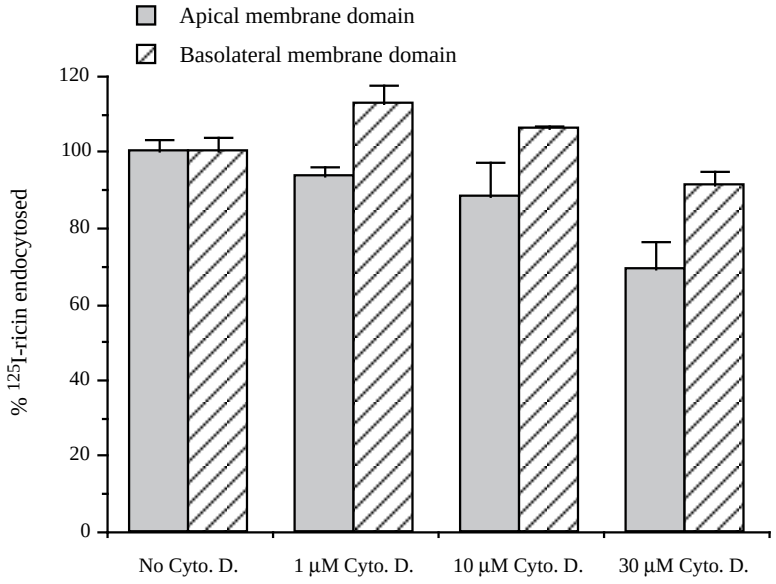


Fig. 7. The effect of cytochalasin D on ricin uptake by MDCK cells. MDCK cells grown on polycarbonate filters for 14 days were incubated for 30 minutes at 37°C, with various concentrations (0-30 μM) of cytochalasin D. ^{125}I-labelled ricin (25 ng/ml, specific activity 8,000 to 10,000 cpm/ng) was added to the apical or basolateral surface of the cells, and allowed to bind for 1 hour at 4°C. Endocytosis of bound ligand after 1 hour of incubation at 37°C was then calculated by measuring ^{125}I-ricin accumulation resistant to a lactose wash. Cytochalasin D was maintained in all media in which cytochalasin-D-pretreated cells were subsequently incubated. Background values from MDCK cell monolayers treated with 10 mM NaN_3 and 50 mM 2-deoxyglucosewere subtracted from experimental values. Inhibition of endocytosis is expressed as % of domain-specific ^{125}I-ricin endocytosis measured in the absence of cytochalasin D. Each value is the mean of triplicate samples, error bars indicate s.d.

requirement for the basolateral to apical transcytotic route to deliver indirectly targeted, newly synthesised apical plasma membrane proteins as the apical domain becomes better established. The lower uptake and accumulation of ^{125}I-ricin from the apical surface compared to the basolateral surface in 7-day cultured cells is consistent with studies on polarized MDCK cells where endocytosis has been found to be slower at the apical surface for both ricin (van Deurs et al., 1990) and other ligands (Bomsel et al., 1989; Brandli et al., 1990). The amount of endocytic recycling of ricin at the apical domain of Caco-2 cells was about half that seen at the basolateral domain, which was equivalent to ricin recycling previously observed in Vero (Sandvig and van Deurs 1990), and EJ bladder cells (McIntosh et al., 1990). This may be a consequence of greater trafficking of apically internalised ricin to late endosomal compartments, from which recycling is less likely to occur (Mayor et al., 1993). In Caco-2 cells, the late endosomal compartment is common to ligands endocytosed from either surface domain, but is more rapidly loaded from the apical domain (Hughson and Hopkins, 1990). In the present experiments it is probable that after 1 hour of internalisation, the majority of ^{125}I-ricin was not transported beyond this compartment to lysosomes,

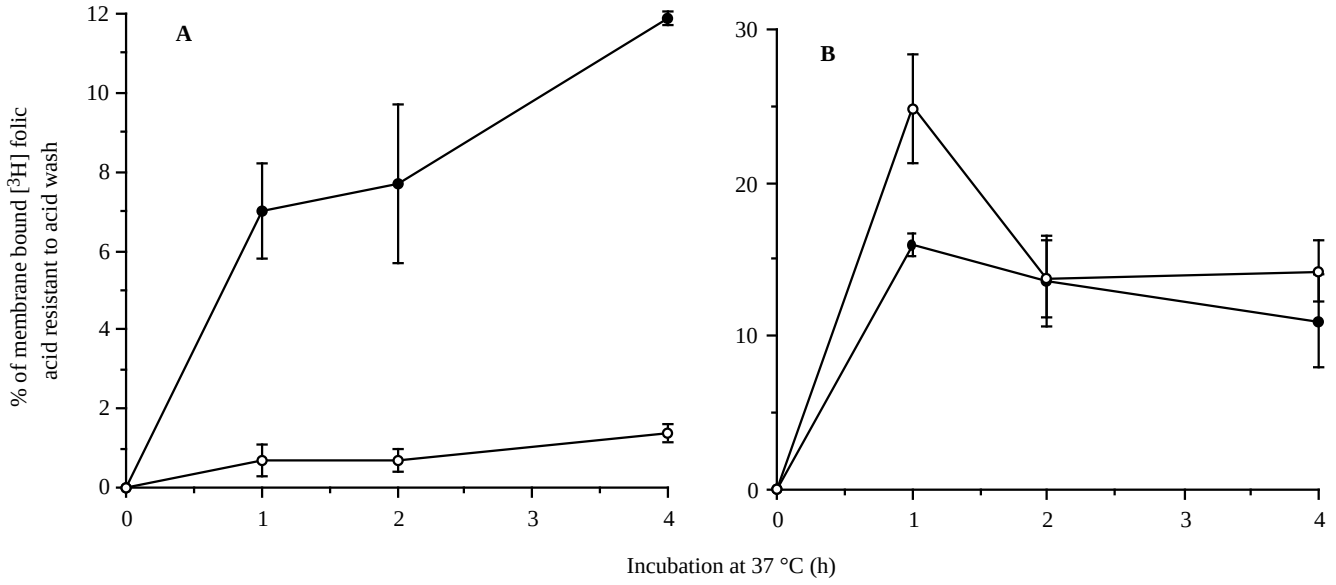


Fig. 8. The effect of cytochalasin D on folate uptake by Caco-2 cells. Caco-2 cells grown on polycarbonate filters for 14 days in folate-free media were preincubated with folate-free medium in the absence (●) or presence (○) of 10 μM cytochalasin D. Following binding of [^{3}H]folate to the apical (A) or basolateral (B) surfaces of the monolayers, the cells were incubated at 37°C in the absence (●) or presence (○) of cytochalasin D. Surface-bound [^{3}H]folate resistant to acid washing was determined. Values are the mean ± s.d. of triplicate samples and are corrected for non-specific binding by subtracting counts obtained with 100-fold excess non-radioactive folate.

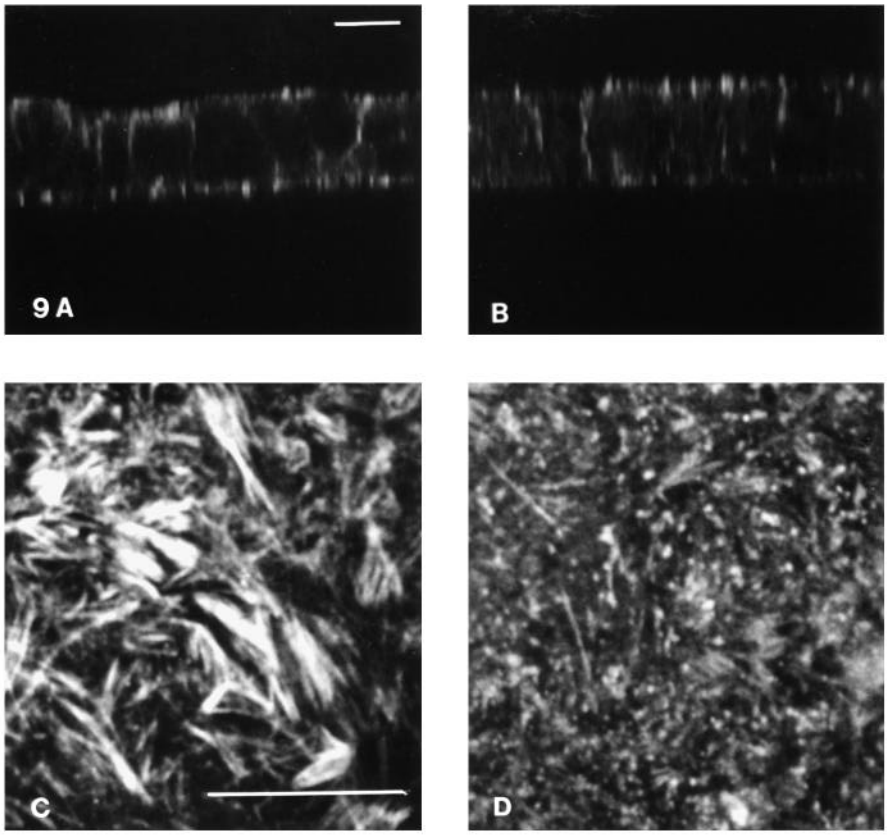
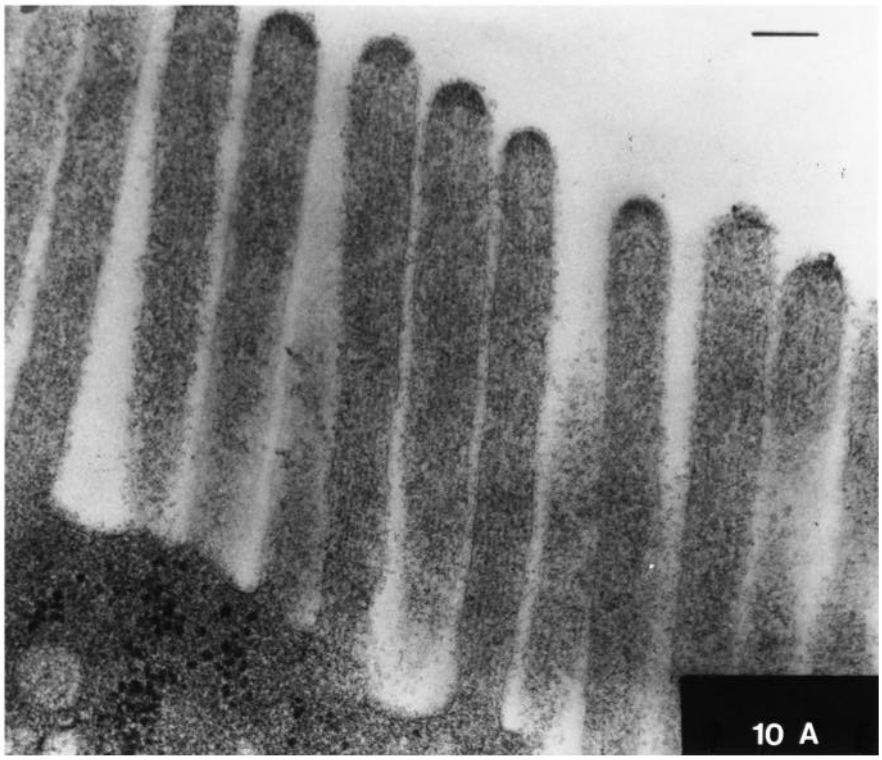


Fig. 9. Confocal microscopy of TRITC-phalloidin-stained, fixed, permeabilised Caco-2 cells. Caco-2 cells were grown on polycarbonate filters for 14 days before fixation and confocal microscopy. (A and B) *XZ* sections taken in 1 μm steps through the cells at 90° to *XY* sections. (C and D) Superimposed images of the three 1 μm *XY* sections closest to the filter. (A and C) Control cells; and (B and D), cells treated with 10 μM cytochalasin D for 30 minutes before fixation. Bars, 10 μm.

since >90% of accumulated radioactivity was acid precipitable. Transcytosis of ^{125}I-ricin was observed in both directions in Caco-2 cells, with the maximal basolateral to apical ricin transcytosis observed in 7-day cell cultures being greater than that seen in MDCK cells, which also transcytose this lectin (van Deurs et al., 1990). This is consistent with the absence in MDCK cells of a major indirect biosynthetic pathway for apical membrane proteins (Hubbard et al., 1989). Apical to basolateral transcytosis of ricin was severely reduced as the Caco-2 cell monolayer aged between 7 and 14 days, but it was not possible to determine whether this endocytic route still exists in fully differentiated cells.

Cytochalasin D, a fungal metabolite that disrupts actin polymerisation (Cooper, 1987), inhibited the endocytic uptake of ricin from the apical but not the basolateral, surface of Caco-2 cells. This result is in agreement with the studies of Gottlieb et al. (1993), who showed cytochalasin D inhibition of vesicular stomatatis virus G protein from the apical, but not basolateral, surface of MDCK cells. These authors also reinterpreted an earlier observation by Sandvig and van Deurs (1990), who found that cytochalasin D inhibited ricin endocytosis in Vero cells, by suggesting that this was due to partial polarisation of these cells with consequent inhibition at the apical domain. Gottlieb et al. (1993) used a single concentration (50 μM) of cytochalasin D in their experiments, considerably greater than that required to cause inhibition of apical ricin endocytosis in Caco-2 cells, but consistent with the observation in the present experiments that >10-fold higher concentrations of cytochalasin D were required to cause equivalent inhibitions of ricin endocytosis in MDCK cells. Gottlieb et al. (1993) also showed that cytochalasin D inhibited fluid-phase uptake of Lucifer Yellow at the apical surface of Caco-2 cells, demonstrating that the effect they had observed on apical endocytosis was not restricted to MDCK cells. Cytochalasin D was shown to inhibit pinching off of coated vesicles in MDCK cells (Gottlieb et al., 1993), but this may not be a sufficient explanation of inhibition of ricin endocytosis, since there is evidence from several cell types that non-clathrin-coated pit mechanisms are involved in ricin uptake (Moya et al., 1985; Sandvig and van Deurs, 1990).

In the present experiments it was found that in Caco-2 cells cytochalasin D also inhibited apical but not basolateral uptake of folate, demonstrating that the apical-specific cytochalasin D effect was also apparent on caveolae as well as clathrin-coated pits. Indeed, inhibition of folate uptake was even greater than that of ricin at a given cytochalasin D concentration. Although binding of [^{3}H]folate to Caco-2 cells has been demonstrated previously (Vincent et al., 1985), the present study is the first to investigate uptake. The almost complete inhibition of [^{3}H]folate internalisation at the apical domain by cytochalasin D strongly suggests that the mechanism of [^{3}H]folate uptake is different at the apical and basolateral surfaces. It is possible, but most unlikely, that the difference may be explained solely by a different route of uptake at the two domains, since caveolae, and their associated integral membrane protein caveolin, have been observed on both surface domains of polarised epithelial cells (Dupree et al., 1993). The incomplete polarisation of folate binding (3:1, apical:basolateral) observed in the present study presumably reflects the distribution of the GPI-anchored folate receptor and was not unexpected, being similar to the distribution of the GPI-anchored protein alkaline phosphatase (Le Bivic et al., 1990; Ellis et al., 1992b) in Caco-2 cells.



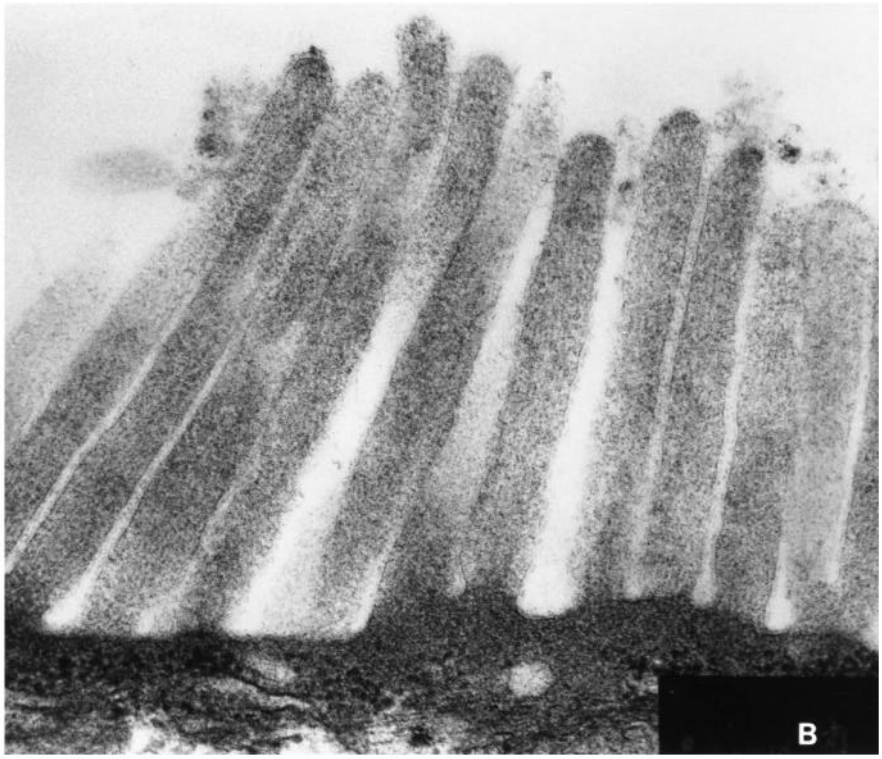


Fig. 10. Electron micrographs of Caco-2 cells. Caco-2 cells were grown on polycarbonate filters for 14 days. Micrographs show transverse sections through the apical surface of a control cell monolayer (A), and a monolayer treated with 10 μM cytochalasin D for 30 minutes before fixation (B). Bar, 0.1 μm.

The involvement of actin in mammalian cell endocytosis is not surprising, given the evidence of impaired endocytosis in actin mutant strains of yeast and mutants lacking the homologue of the mammalian actin-binding protein, fimbrin (Kubler and Riezman, 1993). However, the mechanism by which actin affects endocytosis and the restriction of the effect to the apical surface of polarised mammalian cells are not understood. It has been suggested that the actin filaments in the microvilli of polarised epithelial cells are part of a mechanochemical motor that moves membrane components along the microvillar surface towards intermicrovillar spaces, or provides the force required for converting a membrane

invagination or pit into an endocytic vesicle within the cytoplasm (Gottlieb et al., 1993). The greater sensitivity of apical endocytosis to cytochalasin D in Caco-2 cells compared to MDCK cells is consistent with the view that the effect is due to inhibition of microvillar actin filament function, since the microvillar brush border on Caco-2 cells is much better developed than that on MDCK cells. It is also the site of much fimbrin (Louvard et al., 1992). However, if the structural integrity of a mechanochemical motor is required for apical endocytosis, then it is surprising that cytochalasin D inhibition of apical endocytosis was observed without any gross change in brush border morphology or microvillar actin despite almost complete depolymerisation of actin stress fibres. It may be that the site of the cytochalasin D effect on apical endocytosis is the actin terminal web occurring at the base of the microvilli and connected to the tight junctions, since this was difficult to visualise in a way that would allow detection of changes in its morphology. Additionally or alternatively, actin-associated proteins may be required for efficient apical endocytosis. Other workers have shown a role for the rac family of small GTP-binding proteins in connecting growth factor stimulation signals to the function of actin in causing increased membrane ruffling and pinocytosis (Ridley et al., 1992). In this context it is of interest to note that growth factors have been reported to increase ricin and fluid-phase uptake in many cell lines, perhaps as a result of an increase in membrane ruffling leading to an increase in non-clathrin-mediated endocytosis (Haigler et al., 1979; Tushinski et al., 1982; Gibbs et al., 1986; Miyata et al., 1989; Sandvig and van Deurs, 1990).

Simple mechanical coupling may also transmit the effect of cytoskeletal disruption to the caveolar system, since in biochemical experiments caveolin fractionates with the cytoskeleton (Glenney and Zokas, 1989; Glenney and Soppet, 1992), implying a close association between the two. Although the molecular basis for such an association is not understood, further evidence comes from the relative immobility of GPI-anchored plasma membrane proteins (Hannan et al., 1993). Following biosynthesis these are clustered into caveolin-containing transport vesicles in the *trans*-Golgi network (TGN) for delivery to the plasma membrane where they are enriched in caveolae (Dupree et al., 1993). The relative immobility of GPI-anchored proteins when they reach the cell surface has suggested association with a cytoskeletal component, presumably via interaction with a transmembrane protein (Hannan et al., 1993). There is conflicting evidence as to whether caveolae are capable of budding off the plasma membrane, and a cycle of opening and closing while still attached to the cell surface has been described (Rothberg et al., 1992; Smart et al., 1994). This allows the creation of a caveolar environment for potocytosis, in which, for example, folate dissociates from its receptor and is transported into the cytosol. The recent discovery that activators of protein kinase C are potent inhibitors of potocytosis should provide reagents to investigate not only the function of caveolae but also any interactions with components of the cytoskeleton (Smart et al., 1994).

Domain-specific modulation of endocytosis appears to be applicable to several polarised epithelial cells, though the endocytic mechanisms may be differentially sensitive to different drugs in different cell types. In MDCK cells brefeldin A causes domain-specific stimulation of ricin endocytosis (Prydz et al., 1992), though we have not been able to observe this effect on Caco-2 cells. However, we have found that cytochalasin D can reverse the effect of brefeldin A on MDCK cells (data not shown), suggesting that a common apical endocytic mechanism is affected by these two fungal metabolites. Further experiments on the mechanism of action of these drugs should enable us to learn more about the specific proteins involved in modulation of apical endocytosis.

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