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The effects of cytochalasin D and phorbol myristate acetate on the apical endocytosis of ricin in polarised Caco-2 cells

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SUMMARY

Apical endocytosis of ^{125}I-ricin in Caco-2 cells was inhibited >95% by hypertonic and/or acid media, consistent with the major uptake route being clathrin-mediated. The presence of apical cell surface bound ricin-gold in clathrin coated pits and vesicles was observed by electron microscopy. An electron microscopic investigation in which ricin-gold bound to the apical surface was quantitated, showed that cytochalasin D, which inhibits apical but not basolateral endocytosis, prevented movement of ricin-gold along the microvillar surface. This was consistent with an actin bound mechanochemical motor within microvilli driving the movement of membranous components towards the cell body. Cytochalasin D also caused an increase in the number of coated pits observed at the apical cell surface relative to the number observed in untreated cells. Stimulation of apical endocytosis of ricin by phorbol 12-myristate 13-acetate showed the characteristics of being mediated by protein kinase C, was not due to an effect on ricin movement along the microvillar surface, and may be explained by increases in formation and pinching off of clathrin coated pits at the apical cell surface.

Key words: Endocytosis, Actin, Polarised cell

INTRODUCTION

Endocytosis from the apical and basolateral surfaces of polarised epithelial cells can be distinguished by the differential requirement for an intact actin cytoskeleton on the apical side. Thus, cytochalasin D may be used to inhibit apical fluid phase endocytosis and the apical endocytosis of membrane bound ligands in a variety of polarised cell lines without having any effect on basolateral uptake (Gottlieb et al., 1993; Jackman et al., 1994). Domain specific differences in endocytic uptake mechanisms have also been suggested by different responses to agonists. Thus, in MDCK (Madin-Darby canine kidney) cells it has been found that the fungal metabolite brefeldin A (Prydz et al., 1992), agonists activating adenylyl cyclase (Eker et al., 1994), and phorbol esters probably acting via protein kinase C (Holm et al., 1995), stimulate apical but not basolateral endocytosis. In Caco-2 cells (a cell line derived from a human colon adenocarcinoma), it has also been reported that apical but not basolateral endocytosis is stimulated by phorbol esters (Holm et al., 1995; Shurety and Luzio, 1995).

A requirement for an intact actin cytoskeleton for efficient endocytosis appears to be evolutionarily conserved since actin mutant strains of the yeast *Saccharomyces cerevisiae* and mutants lacking the homologue of the mammalian actin-binding protein, fimbrin, have impaired endocytosis (Kübler and Riezman, 1993). A further similarity between yeast endocytosis and apical endocytosis in polarised epithelial cells has been demonstrated by expression of mammalian protein kinase C in *Saccharomyces pombe* showing that some isotypes can upregulate internalisation and the endocytic pathway (Goode et al., 1994). In non-polarised mammalian cells there have been reports of a requirement for an intact actin cytoskeleton for efficient endocytosis (Sandvig and van Deurs, 1990; Durrbach et al., 1996), although this may simply reflect the presence of some apical-like microdomains at the cell surface (Gottlieb et al., 1993).

The mechanism by which the actin cytoskeleton is involved in endocytosis at the apical surface of polarised epithelial cells is not known. In the present study we have extended our earlier investigations of the apical endocytosis of ricin in Caco-2 cells (Jackman et al., 1994; Shurety and Luzio, 1995), in order to discover more about this mechanism. Since there is evidence that both clathrin mediated and non-clathrin mediated apical endocytic uptake routes are inhibited by cytochalasin D (Gottlieb et al., 1993; Jackman et al., 1994), and that ricin uptake in a variety of cells is primarily mediated by a non-clathrin uptake mechanism, we investigated the effect of known inhibitors of different uptake routes (reviewed by Lamaze and Schmid, 1995) on apical endocytosis of ricin in these cells. In contrast to the apical endocytosis of ricin in MDCK cells (Sandvig and van Deurs, 1990; Eker et al., 1994), it was found that the properties of apical uptake in Caco-2 cells were consistent with the major route being via clathrin-coated pits. Phorbol ester stimulation of apical endocytosis of ricin also appeared to be a result of stimulation of the clathrin-mediated endocytic route probably by increasing both the formation and pinching off of coated pits. A quantitative electron microscopic study using ricin labelled with gold

indicated that cytochalasin D inhibited both the movement of membrane molecules binding ricin along the surface of apical microvilli and the pinching off of clathrin coated vesicles from the apical plasma membrane.

MATERIALS AND METHODS

Materials

$Na^{125}I$ was from Amersham International, Amersham, UK. Ricin-gold (ricin labelled with 10 nm gold) was from EY Laboratories Inc., San Mateo, CA, USA. The rabbit polyclonal anti-clathrin antibody was a gift from Dr Margaret S. Robinson (Department of Clinical Biochemistry, University of Cambridge, UK). Protein A labelled with 15 nm gold was from the Department of Cell Biology, University of Utrecht. Electron microscopy embedding materials and grids were from Agar Scientific Ltd, Stansted, Essex, UK. All other reagents were from Sigma-Aldrich Co. Ltd, Poole, Dorset UK. Stock solutions of PMA (phorbol 12-myristate 13-acetate, 1 mM), 4α-PDD (4α-phorbol 12,13 didecanoate, 1 mM), cytochalasin D (10 mM) and wortmannin (1 mM) were prepared in DMSO and stored in batches at –20°C. The activity of each batch of wortmannin was confirmed by inhibition of glucose uptake in 3T3-L1 adipocytes (Clarke et al., 1994; Shepherd et al., 1995a).

Cell culture and endocytosis

Caco-2 cells were cultured on filter supports as previously described (Jackman et al., 1994). All experiments were conducted in cells cultured for 14 days after seeding by which time all monolayers used showed a transepithelial electrical resistance of >400 Ω cm^2. Radiolabelling of ricin, binding to cells and measurement of endocytosis were carried out as previously described (Jackman et al., 1994). Briefly for ricin, ^{125}I-ricin (25 ng/ml; specific activity ~20,000 dpm/ng) was bound to either the apical or basolateral surface of a filter grown cell monolayer for 1 hour at 4°C. Cell monolayers were washed and then incubated for 30 minutes at 37°C except when time course experiments were conducted. Unless otherwise stated, PMA and 4α-PDD were added at the start of the 37°C incubation. Cytochalasin D treatment was as previously described (Jackman et al., 1994), with cell layers being pre-incubated for 30 minutes at 37°C with cytochalasin D before ricin binding and the maintenance of cytochalasin D in subsequent incubation media.

Ligand binding and endocytosis studies were normally carried out in isotonic Dulbecco's modified Eagle's medium, 0.6% bovine serum albumin, 20 mM Hepes, pH 7.4. Acid medium was Dulbecco's modified Eagle's medium without sodium bicarbonate, but containing 0.6% bovine serum albumin, 20 mM MES, 20 mM succinic acid, pH 5.7. Hypertonic medium was isotonic medium to which 0.45 M sucrose was added, and acid hypertonic medium was acid medium containing 0.45 M sucrose. When incubation in acid medium, hypertonic medium, or acid hypertonic medium was used to inhibit endocytosis, cells were preincubated for 5 minutes at 4°C in the relevant medium before ligand binding and uptake in the same medium. In acid, hypertonic and acid hypertonic medium, binding of ricin to the cell surface was approximately 50% of that observed in isotonic medium, pH 7.4.

The effects of amiloride (3 mM) or DMA (dimethyl amiloride, 0.1 mM) on endocytosis were investigated by pre-incubating cell layers for 5 minutes at 37°C with amiloride or DMA before ricin binding and then maintaining amiloride or DMA in subsequent incubation media. The effect of sodium-free medium on endocytosis was investigated following pre-incubation with serum-free Dulbecco's modified Eagle's medium for 1 hour at 37°C and then 30 minutes at 37°C in sodium-free medium (137 mM choline chloride, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mg/ml BSA and 20 mM Hepes, pH 7.4), before ricin binding. In these experiments control sodium containing medium was the same except that choline chloride was replaced with 137 mM NaCl. Endocytosis of surface bound ^{125}I-ricin was calculated by measuring accumulation of ^{125}I-ricin, resistant to lactose washes. Background values were calculated by measuring ^{125}I-ricin resistant to lactose washes in cells kept at 4°C and were subtracted from experimental values. Recycling of endocytosed ^{125}I-ricin was measured as previously described (Jackman et al., 1994). Values shown are expressed as % uptake compared to a control value of 100%. Endocytosis of HRP-transferrin (transferrin coupled to horseradish peroxidase) was measured as described by Shepherd et al. (1995b).

Transmission electron microscopy

Cell monolayers on filters were prepared for conventional transmission electron microscopy essentially as described by Glauert (1975). When required, ricin-gold was diluted tenfold from the manufacturer's stock solution into isotonic medium used for ligand binding, and bound to the apical cell surface for 1 hour at 4°C. Following washing and glutaraldehyde fixation, filters were excised, cut into 3 mm^2 pieces and post-fixed with osmium tetroxide. To provide good membrane contrast in the electron microscope, the cell monolayer was 'en bloc' stained with uranyl acetate before dehydration and embedding. Sections of the resin embedded cell monolayers were cut using a Reichert UltraCut S ultramicrotome (Leica, Milton Keynes, UK), collected on Formvar/carbon coated 200 mesh copper electron microsope grids and stained for 30 seconds with 10% uranyl acetate in methanol and 5 minutes in Reynolds' lead citrate (Reynolds, 1963). The number of surface-connected clathrin coated pits per 1 μm measured in a straight line across the bases of microvilli where they join the cell body was measured on between 27 and 36 micrographs of control cells and cells treated with cytochalasin D or PMA. The magnification on each 11 cm × 14 cm micrograph was ×48,000.

To quantitate movement of ricin-gold along microvilli, it was first bound to the apical surface of filter grown Caco-2 cells at 4°C. Cytochalasin D (10 μM) and PMA (1 μM) treatment were as described above. After incubation for 30 minutes at 37°C and preparation for electron microscopy, electron micrographs were taken of random areas of the apical domain. Ricin-gold particles along the surface of two hundred microvilli were counted for each experimental condition. The length of each microvillus was measured and divided into four equal sections along its length (tip, upper shaft, lower shaft, base), and the ricin-gold particles were counted in each section. Gold particle distribution was only measured on individual microvilli characterised by an intact membrane profile along the whole length of each microvillus from the tip to the cell body.

Ultrastructural immunocytochemistry on frozen ultrathin sections was performed essentially as described by Griffiths (1993) with immunolabelling of antigens performed using the Protein A-gold technique (Slot and Geuze, 1983) as described previously (Reaves et al., 1996).

Grids were observed in a Philips CM 100 transmission electron microscope and micrographs were taken according to conventional procedures.

Enzyme assay

PI (phosphoinositide) 3-kinase activity was assayed by TLC determination of ^{32}P incorporation into phosphatidylinositol as previously described (Jackson et al., 1992).

Statistics

All biochemical data are presented as mean ± s.e.m. (number of filters assayed) or ± range (when only two filters were assayed). The % distributions of ricin-gold particles in different sections of the surfaces of microvilli are presented as mean ± s.e.m. (200 microvilli). Differences in distribution were assessed by comparing data sets using Student's *t*-test and were only regarded as significant when $P<0.001$.

RESULTS

The effects of inhibitors of endocytic uptake routes on the apical endocytosis of ricin suggest that >95% of uptake occurs via clathrin-coated pits

Ricin binds to galactosylated membrane proteins and lipids and is convenient to use to measure their endocytosis, since any ricin remaining at the plasma membrane after an incubation period may be efficiently removed by lactose. Since ricin that enters the cytosol causes inhibition of protein synthesis, we have previously defined conditions where ricin uptake into Caco-2 cells can be measured over a 2 hour period before this inhibition occurs (Jackman et al., 1994). Our previous experiments did not identify the uptake routes taken by ricin at the apical or basolateral cell surface. In the present experiments, cells were incubated in hypertonic medium and/or under conditions causing cytosol acidification, since these treatments result in clathrin re-distribution from the plasma membrane and prevent clathrin mediated endocytosis (Davoust et al., 1987; Sandvig et al., 1987, 1988; Pelchen-Matthews et al., 1993; Cupers et al., 1994; Lamaze and Schmid, 1995). Accumulation of ricin after 30 minutes of uptake from the apical surface was inhibited >95% under each of these conditions (Fig. 1a), consistent with the major route of internalisation being via clathrin coated pits. Endocytosis of ricin from the basolateral surface was also greatly inhibited by incubation in hypertonic medium (Fig. 1b), as was basolateral endocytosis of HRP-transferrin (Fig. 1c), which is known to occur via the clathrin coated route in Caco-2 cells (Hughson and Hopkins, 1990). The extent of inhibition of intracellular accumulation of ricin in Caco-2 cells after treatment with hypertonic and/or acid medium was surprising in view of reports that in other cells (Sandvig and van Deurs, 1990), including polarised MDCK cells (Eker et al., 1994), the major part of ricin uptake occurs via non-coated pits.

Inhibitors of other endocytic uptake routes which do not affect clathrin mediated endocytosis are known. Macropinocytic uptake, which is stimulated by growth factors, has been reported to occur at the apical surface of polarised epithelial cells, particularly MDCK cells (Dowrick et al., 1993), as well as in non-polarised cell lines (West et al., 1989; Hewlett et al., 1994). It has been shown to be greatly reduced in the presence of amiloride or dimethylamiloride which are inhibitors of Na^+/H^+ exchange (West et al., 1989; Dowrick et al., 1993). At concentrations used to prevent macropinocytosis in A431 cells (West et al., 1989) neither of these agents inhibited intracellular accumulation of ricin after apical endocytosis in Caco-2 cells (Fig. 1a). Indeed, both agents caused greater accumulation than in control cells. Although incubation of the cells in Na^+-free medium resulted in a slight inhibition of apical endocytosis of ricin (Fig. 1a), the magnitude of this effect was also not consistent with macropinocytosis being a major route.

In our previous experiments, the observation that cytochalasin D inhibited apical uptake of folate in Caco-2 cells as well as ricin uptake had prompted the suggestion that some ricin uptake might occur via caveolae (Jackman et al., 1994). Internalisation of caveolae has been shown to be inhibited by cytochalasin D in other cell types (Parton et al., 1994) and it has been reported that PMA prevents the internalisation of caveolae in some cells (Smart et al., 1994). The effect of PMA on apical endocytosis of ricin in Caco-2 cells was stimulatory (Fig. 1a; Holm et al., 1995; Shurety and Luzio, 1995).

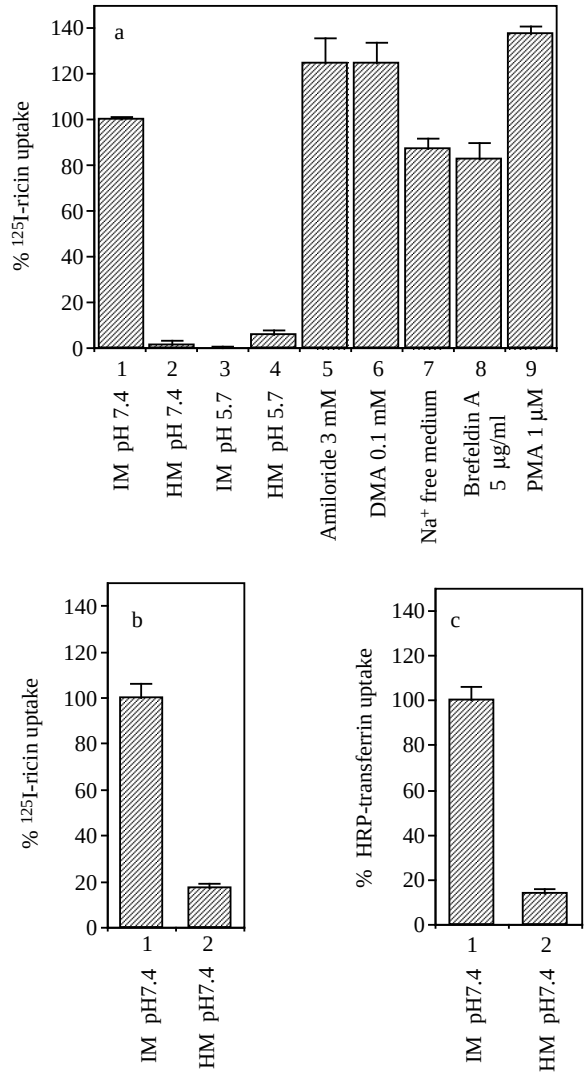

Fig. 1. The effect of known inhibitors of endocytic uptake routes on endocytosis in Caco-2 cells. (a) Apical endocytosis of ^{125}I-ricin. (b) Basolateral endocytosis of ^{125}I-ricin. (c) Basolateral endocytosis of HRP-transferrin. IM, isotonic medium; HM, hypertonic medium. The bars show accumulation of radioactivity in cells after 30 minutes at 37°C as % control values. Each bar shows the mean of triplicate samples ± s.e.m.

Since brefeldin A has been shown to stimulate apical endocytosis of ricin in MDCK cells (Prydz et al., 1992), the effect of this fungal metabolite on the apical endocytosis of ricin in Caco-2 cells was examined. Whereas we were able to reproduce the published stimulatory effect of brefeldin A on MDCK cells (19±1.5% (n=3) stimulation of apical endocytosis of ricin by 5 μg/ml brefeldin A in MDCK II cells), some inhibition of apical endocytosis of ricin was observed in Caco-2 cells (Fig. 1a).

After binding of ricin-gold to the apical cell surface, gold particles can be observed on the surface of apical microvilli and in clathrin-coated pits

Following binding of ricin-gold to the apical cell surface of filter-grown Caco-2 cells at 4°C, and processing for electron microscopy, gold particles were observed along the surface of

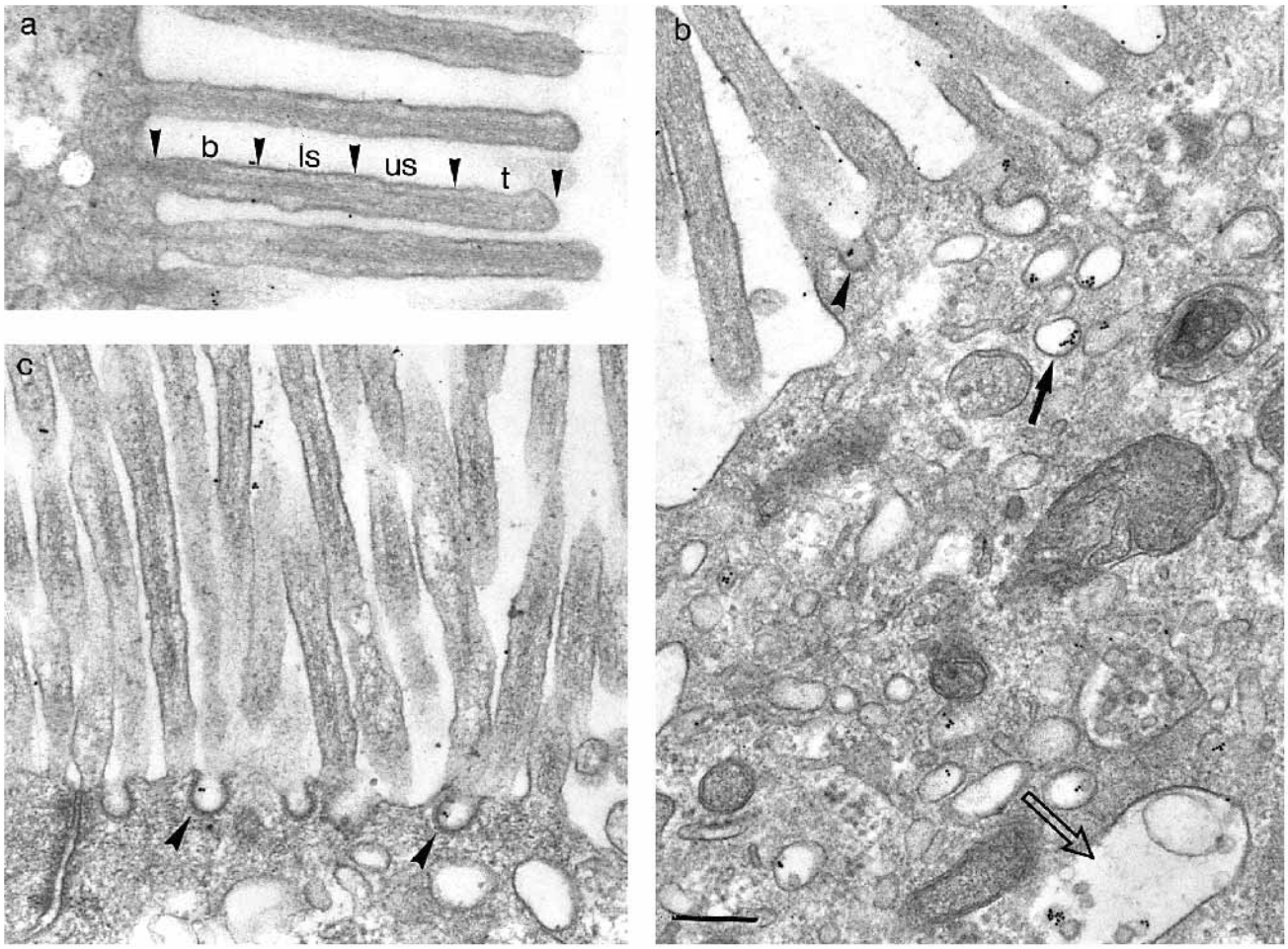

Fig. 2. Transmission electron micrographs of Caco-2 cells after ricin-gold binding and uptake. (a) Ricin-gold bound to apical microvilli at 4°C. The division of a microvillus into tip (t), upper shaft (us), lower shaft (ls) and base (b) is shown by arrowheads. (b) Ricin-gold distribution after endocytosis from the apical surface for 30 minutes at 37°C. Arrowhead, ricin-gold in coated pit; filled arrow, ricin-gold in uncoated peripheral endosome; open arrow, ricin-gold in apical multivesicular body. (c) Ricin-gold in coated pits (arrowheads) after apical endocytosis for 30 minutes at 37°C in the presence of 10 μM cytochalasin D. Bar, 250 nm.

the microvilli (Fig. 2a). After warming the cells at 37°C for 30 minutes, gold particles were clearly observed in clathrin coated pits at the base of microvilli, in non-coated vesicular endocytic structures and in sub-apical multivesicular bodies (Fig. 2b). In the latter structures gold particles were often observed associated with the intravesicular membrane profiles. The localisation of gold particles in coated pits at the base of microvilli was more easily observed in cytochalasin D treated cells (Fig. 2c). Co-localisation of ricin-gold and clathrin in the same apical coated pits/vesicles was demonstrated in ultrathin frozen sections (Fig. 3).

Phorbol ester-stimulated apical ricin uptake occurs via clathrin coated pits

In agreement with a previous study, it was found that PMA, at concentrations in the micromolar range, stimulated intracellular accumulation of ricin endocytosed from the apical plasma membrane without having any effect on basolateral endocytosis (Fig. 4a). The stimulatory effect of PMA was observed at all time points measured (Fig. 4b) and could not be explained by inhibition of recycling to the apical surface since in experiments to measure recycling of ricin, 25.9±3.7% (n=3) stimulation was observed over 30 minutes in the presence of 1 μM PMA. Although PMA has been reported to alter the paracellular permeability of polarised epithelial cell monolayers (Stenson et al., 1993), no significant effects on transepithelial electrical resistance were observed in the present experiments after addition of PMA (0.01-10 μM) to Caco-2 cell monolayers (data not shown). Intracellular accumulation of ^{125}I-ricin as a result of apical endocytosis in the presence of PMA was essentially abolished when the cells were incubated in hypertonic and/or acid medium, consistent with PMA stimulating the clathrin mediated uptake route (Fig. 4c). The effect of PMA on apical endocytosis was probably mediated by protein kinase C since the PMA analogue, 4α-PDD, which cannot activate protein kinase C caused no significant stimulation (Table 1), and PMA was unable to stimulate apical uptake of ricin after treating cells overnight with 16 μM PMA (Table 1), a treatment which causes down regulation of several PKC (protein kinase C) isoforms (Cardone et al., 1994).

In view of reports that PMA can activate PI 3-kinase (Navé et al., 1995), that PI 3-kinase products can activate some isoforms of PKC (Nakanishi et al., 1993; Toker et al., 1994), and that PI 3-kinase may play a role in regulation of several

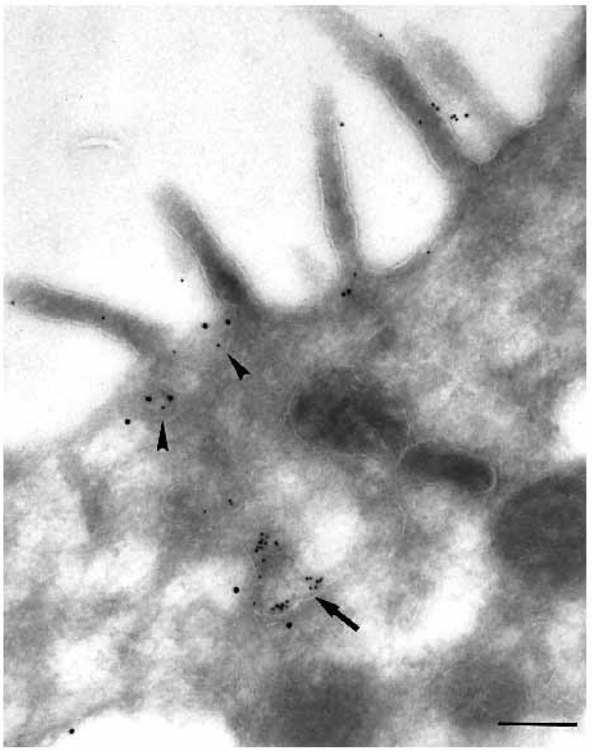

Fig. 3. Immunoelectron microscopic analysis of the apical surface of Caco-2 cells. Ricin-gold (10 nm) was endocytosed for 30 minutes at 37°C. Ultrathin frozen sections were labelled with rabbit anti-clathrin antibodies followed by Protein A-gold (15 nm). Clathrin coated structures (arrowheads) are present at the base of the microvilli and some contain ricin-gold. Arrow, ricin-gold in apical multivesicular body with some associated clathrin. Bar, 200 nm.

intracellular membrane traffic pathways (for review see Shepherd et al., 1996), we examined the effect of the PI 3-kinase inhibitor wortmannin on endocytosis in Caco-2 cells. Some inhibition of ricin endocytosis was observed, but without cell surface domain specificity (Fig. 5). Inhibition of endocytosis continued to increase at concentrations of wortmannin greater than 100 nM, i.e. when PI 3-kinase activity in cell homogenates was completely inhibited (Fig. 5) and when other kinases including myosin light chain kinase and PKC would be expected to be affected (Nakanishi et al., 1992).

Cytochalasin D inhibits the movement of ricin-gold along the surface of microvilli and causes an increase in the number of clathrin coated pits at the base of the microvilli

Treatment of Caco-2 cells with 10 μM cytochalasin D caused >50% inhibition of apical endocytosis of ricin with no significant effect on ricin recycling or basolateral endocytosis (Fig 4c; Jackman et al., 1994). Whereas in control cells, 1 μM PMA stimulated apical endocytosis by 35.8±4.7% (n=3), stimulation was 27.3±4.6% in cytochalasin D treated cells (Fig. 4c). The ability to observe ricin-gold particles on the surface of apical microvilli together with the biochemical evidence suggesting that apical uptake of ricin occurred mainly through clathrin coated pits allowed us to devise experiments to test the effects of cytochalasin D on movement along the surface of the microvilli and the pinching off of the coated pits.

Table 1. Down regulation of protein kinase C prevents PMA stimulation of apical endocytosis and 4 α-PDD does not stimulate apical endocytosis

	Pre-treatment of cells (16 hours)	
	None	PMA (16 μM)
Control	100.0±1.5	87.3±0.7
PMA, 1 μM	129.4±2.8	91.1±2.9
4α-PDD, 1 μM	106.0±2.0	-

Data presented as % of mean control value ± s.e.m. (n=4), in isotonic medium, pH 7.4

The distribution along the microvillar surface of ricin-gold bound to the apical surface of Caco-2 cells was quantitated. Gold particle distribution was measured on individual microvilli divided into four equal segments: tip, upper shaft, lower shaft and base as shown in Fig. 2a. Gold particles were counted on two hundred intact microvilli of cells kept at 4°C or incubated at 37°C for 30 minutes with or without 1 μM PMA or 10 μM cytochalasin D. The total number of gold particles counted was 872 on control cells kept at 4°C, 831 on control cells incubated at 37°C, 695 on PMA treated cells and 856 on cytochalasin D treated cells. The number of gold particles counted per microvillus varied, with >97% of microvilli measured having from 1-12 gold particles. The distribution of the number of gold particles per microvillus in cells kept at 4°C or incubated for 30 minutes at 37°C was not significantly different (data not shown), presumably reflecting the fact that after incubation for 30 minutes at 37°C only a small proportion of surface bound ricin (<5%) would have been internalised (Jackman et al., 1994).

The distribution of gold particles along the length of each microvillus was calculated as a % and the collated data is shown in Table 2. In the control cells kept at 4°C (Table 2; 0 minutes at 37°C), the majority of the ricin-gold was found at the tip of the microvilli (48.7%). After 30 minutes at 37°C, significantly less ricin-gold remained at the tip (30.7%) with significantly more at the base (P<0.001). There was no significant difference in the % of gold particles in the upper and lower shaft regions in the two populations of microvilli.

The distribution of ricin-gold along the microvilli of cytochalasin D pretreated cells at the 0 minutes time point or 30 minutes time point was not significantly different from the distribution in control cells at 0 minutes (Table 2). The distribution of ricin-gold along the microvilli of cytochalasin D treated cells after 30 minutes at 37°C was significantly

Table 2. The effect of PMA and cytochalasin D on the distribution of ricin-gold particles along individual microvilli

Section	Control 0 minutes % ± s.e.m.	Control 30 minutes % ± s.e.m.	PMA 30 minutes % ± s.e.m.	Cyt. D 0 minutes % ± s.e.m.	Cyt. D 30 minutes % ± s.e.m.
Tip	48.7±4.2	30.7±3.2	38.6±4.0	54.9±2.9	55.7±6.0
Upper shaft	16.1±1.7	21.1±1.9	17.7±1.9	20.3±1.5	20.7±3.5
Lower shaft	18.8±2.0	22.0±2.0	23.4±2.2	13.7±1.2	10.7±1.9
Base	16.4±1.7	26.2±2.4	20.3±2.0	11.1±1.0	12.9±3.0

Results are expressed as the percentage of gold particles in each section of a microvillus ± s.e.m. Cyt. D, cytochalasin D.

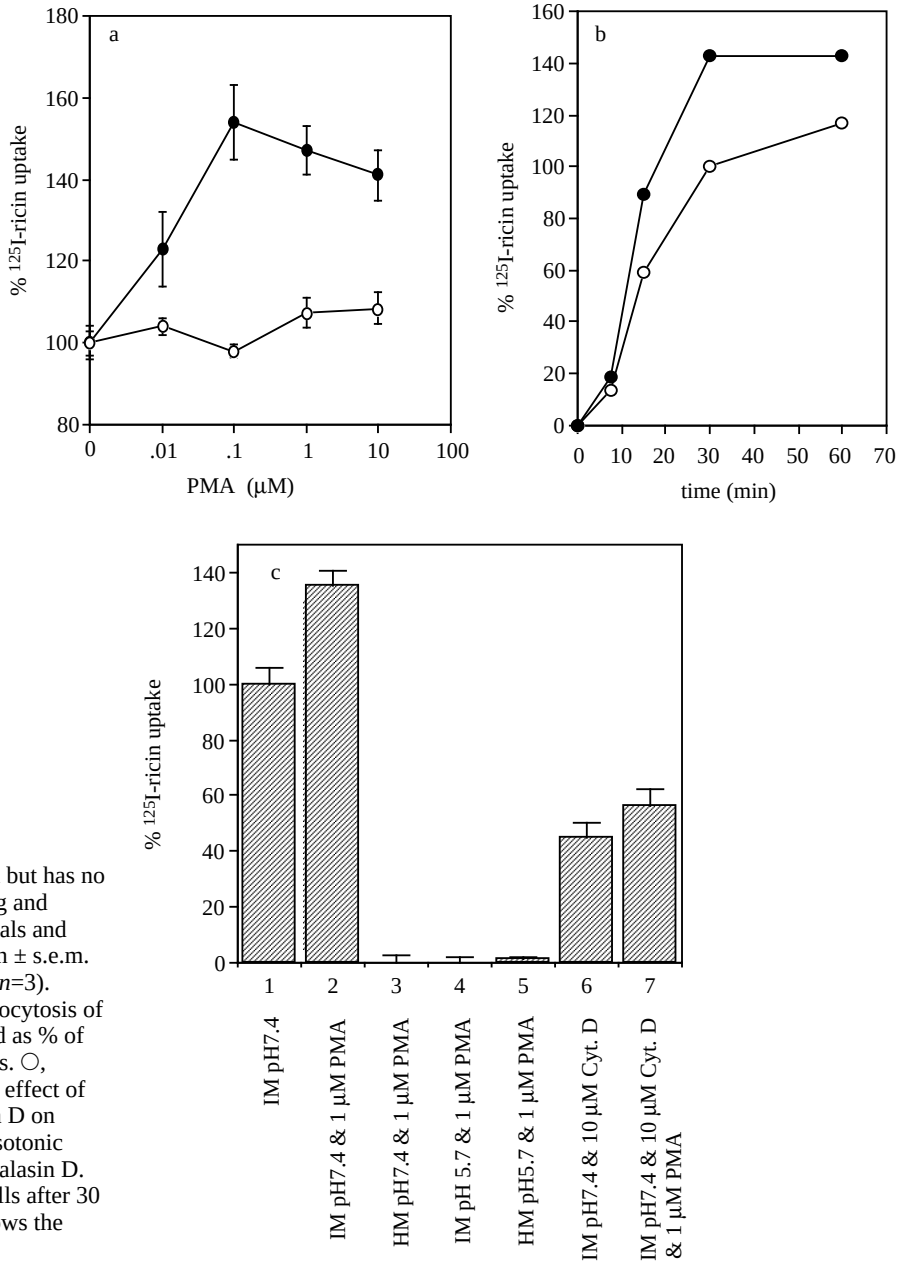

Fig. 4. PMA stimulates apical endocytosis of ricin but has no effect on basolateral endocytosis. (a) Ricin binding and endocytosis were measured as described in Materials and Methods. Data points, ●, apical endocytosis, mean ± s.e.m. (n=6); ○, basolateral endocytosis, mean ± s.e.m. (n=3). (b) Time course of PMA stimulation of apical endocytosis of ricin. Data (each point mean of 2 filters), presented as % of mean control value after 30 minutes of endocytosis. ○, control; ●, in the presence of 1 μM PMA. (c) The effect of hypertonic medium, acidification and cytochalasin D on PMA stimulated apical endocytosis of ricin. IM, isotonic medium; HM, hypertonic medium; Cyt. D, cytochalasin D. The bars show accumulation of radioactivity in cells after 30 minutes at 37°C as % control values. Each bar shows the mean of triplicate samples ± s.e.m.

different from control cells after 30 minutes at 37°C (Table 2). There was more ricin-gold at the microvilli tips in the cytochalasin D treated cells (P<0.001). No significant differences in distribution of gold particles along the surface of the microvilli was seen after PMA treatment compared to control cells.

To ensure that no bias had been introduced when counting gold particles on individual microvilli for the above quantitation, the microvillus area of each micrograph on which individual microvilli had been selected for quantitation was divided into four sections and the number of ricin-gold particles counted in each. The data collected were processed and found to be consistent with those shown in Table 2 (data not shown).

The number of clearly visible, surface-connected, clathrin coated pits per 1μm, measured in a straight line across the bases of the microvilli where they join the cell body was 0.46±0.07 (n=27) in control cells kept at 4°C, 0.35±0.06 (n=36) in control cells incubated for 30 minutes at 37°C, 0.92±0.11 (n=27) in cytochalasin D pre-treated cells kept at 4°C, 0.82±0.11 (n=34) in cytochalasin D pre-treated cells incubated for 30 minutes at 37°C in the continued presence of cytochalasin D and 0.43±0.08 (n=27) in cells incubated for 30 minutes at 37°C in the presence of PMA.

DISCUSSION

In contrast to polarised MDCK cells where the majority of apical endocytosis of ricin occurs via clathrin independent mechanisms (Sandvig and van Deurs, 1990; Eker et al., 1994), the present data suggest that in Caco-2 cells the major route of apical endocytosis of ricin is via clathrin coated pits, from

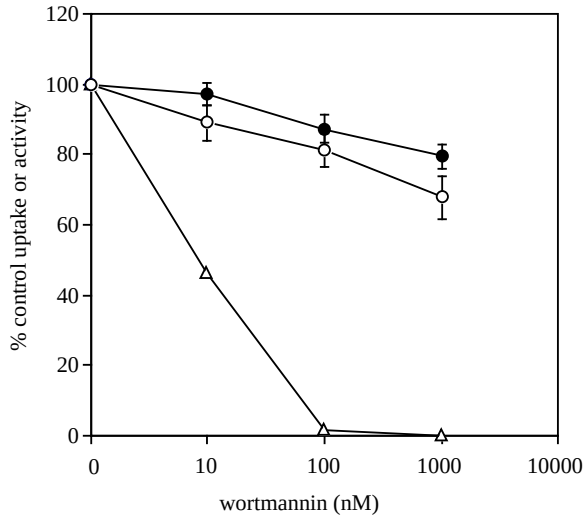

Fig. 5. Inhibition of ricin endocytosis and PI 3-kinase activity by wortmannin. ●, apical endocytosis; ○, basolateral endocytosis; △, PI 3-kinase activity. All data presented as mean of 100% control values in the absence of wortmannin. For endocytosis experiments, data points are mean ± s.e.m. (n=6), and for the PI 3-kinase experiment a representative inhibition curve is shown.

where the ricin is delivered to a sub-apical endosomal compartment with the morphology of multi-vesicular bodies. This compartment has the localisation and morphology of a previously described sub-apical endosomal compartment in Caco-2 cells that can receive endocytosed tracers and membrane proteins delivered from both the apical and basolateral cell surfaces and from which traffic occurs to various cell surface and intracellular destinations (Knight et al., 1995).

The inhibitory effects of hyperosmolarity and low pH separately or together were consistent with clathrin mediated endocytosis being the major apical uptake route for ricin in Caco-2 cells. However, it should be noted that cytosolic acidification and hypertonicity work by ill defined mechanisms, show cell-type variation and have other pleiotropic effects (Hansen et al., 1993; Heuser, 1989; Heuser and Anderson, 1989; Daukas and Zigmond, 1985). There are many other cellular processes apart from clathrin-dependent endocytosis that these treatments affect. For example, addition of hypertonic medium has been shown to block membrane traffic from the endoplasmic reticulum to the Golgi complex and from the *trans*-Golgi network to the cell surface (Docherty and Snider, 1991). Additional support for the importance of clathrin coated pits in ricin uptake at the apical domain of Caco-2 cells has come from electron microscopic experiments. Apically added gold-labelled ricin was shown to be present in clathrin-coated pits at the base of individual microvilli. Immunoelectron microscopy showed co-localisation of clathrin with ricin-gold at these apical plasma membrane invaginations. We cannot completely exclude the possibility that an unidentified coat, which is sensitive to hyperosmolarity and low pH, may be present on some invaginations at the apical domain of Caco-2 cells, but this is unlikely to account for more than a very small part of apical ricin uptake.

Inhibition of apical, clathrin-mediated endocytosis of ricin in Caco-2 cells by cytochalasin D is consistent with the inhibition of uptake of the apical clathrin coated route in MDCK cells reported by Gottlieb et al. (1993). The number of clathrin coated pits present at the apical surface of cytochalasin D treated Caco-2 cells was increased compared to control cells in agreement with the previous findings of Gottlieb et al. (1993) using MDCK cells and suggesting that cytochalasin D suppresses the pinching off of coated pits to form coated vesicles. In the present study comparison of the ricin-gold distribution on microvilli in control Caco-2 cells after binding at 4°C with the ricin-gold distribution after a 30 minutes incubation at 37°C, indicated that ricin-gold moves from the tip of a microvillus to the base. Cytochalasin D inhibited this movement down the microvillus such that the distribution of ricin-gold particles along the microvilli in cytochalasin D pretreated cells incubated at 37°C in the presence of cytochalasin D was the same as the distribution of ricin-gold particles in cells kept at 4°C after ricin-gold binding. This provides strong evidence in favour of an actin bound mechanochemical motor being involved in the movement of plasma membrane components down the microvillar surface as proposed by Gottlieb et al. (1993). However, we cannot rule out the possibility that inhibition of movement of ricin-gold down the microvilli by cytochalasin D is a secondary consequence of failure to pinch off coated pits and internalise ricin.

In the present study using Caco-2 cells, we provide evidence for apical specific stimulation by PMA of clathrin mediated endocytosis of ricin. This is in contrast to data from MDCK cells suggesting that PMA stimulates the apical uptake of ricin through a non-clathrin coated route (Holm et al., 1995). It is possible that the differences observed in these two polarised cell types do not reflect underlying mechanistic differences but differences in the galactosylated proteins and lipids to which ricin binds. Thus, ricin binding molecules on the cell surface of one cell type may be more likely to be internalised via a non-clathrin coated route, but in another cell type via clathrin coated structures. The stimulation of endocytosis by PMA observed in Caco-2 cells cannot be explained as a result of an effect on recycling, since this was also stimulated, and must be due to a direct effect on the uptake process. PMA did not affect the uptake of ricin by increasing the movement of ricin-gold along the microvilli suggesting that its effect may be exerted on the formation and pinching off of clathrin coated pits. If both processes are stimulated by PMA then the number of coated pits observed at the apical cell surface would be the same as in control cells as was observed in the present study.

A variety of membrane traffic pathways have been shown to be stimulated by PMA. These include internalisation of the transferrin receptor in a lymphoblastoid cell line (Eichholtz et al., 1992), fluid phase pinocytosis in polymorphonuclear leucocytes (Keller and Niggli, 1993), down regulation of CD4 from the surface of lymphocytic cells (Pelchen-Matthews et al., 1993), basolateral to apical transcytosis and apical recycling of the polymeric Ig receptor in transfected MDCK cells (Cardone et al., 1994), glucose transporter translocation (Gibbs et al., 1986) and membrane protein export from the endoplasmic reticulum (Fabbri et al., 1994). Most effects of PMA on membrane traffic pathways have been interpreted as being due to activation of PKC which has been suggested to play an important role in regulated exocytosis, movement of receptors through the endocytic pathway and constitutive transport of proteins through the Golgi complex (for summaries and reviews see Backer and King, 1991; Fabbri et al., 1994).

However, for PMA effects on protein export from the endoplasmic reticulum the catalytic activity of PKC does not seem to be required (Fabbri et al., 1994). Although PKC consists of a family of isoenzymes (Nishizuka, 1992; Hug and Sarre, 1993), specific involvement of particular isoenzymes with membrane traffic events in mammalian cells has yet to be proven.

The apical domain specific effect of PMA on endocytosis observed in the present study appeared to be mediated via PKC since down regulation of the enzyme prevented stimulation and the analogue 4α-PDD, which cannot activate PKC, had no effect. The stimulation of apical endocytosis by PMA was reduced by cytochalasin D treatment. It is possible that the PMA effect is mediated via the actin cytoskeleton, perhaps via actin associated proteins. Recently several unconventional myosins have been identified in Caco-2 cells (Bement et al., 1994) which may act as molecular motors and are candidates for proteins required for apical-specific endocytosis. Another possibility is that the MARCKS (myristoylated alanine-rich C kinase substrate) protein is involved in apical endocytosis. In Caco-2 cells this protein is a major substrate for PMA activated PKC (Stenson et al., 1993). Interestingly, in other cell types, phosphorylation of MARCKS has been shown to regulate its activity as a crossbridge between the actin cytoskeleton and the plasma membrane (Hartwig et al., 1992). A third possibility is that an actin associated PKC plays a role in apical endocytosis analogous to that suggested for PKC_{ε} in glutamate exocytosis in nerve terminals (Prekeris et al., 1996). A fourth possibility is that the PMA effect may be mediated via a signalling pathway involving the small ras-related G proteins rho and rac which are required for receptor mediated endocytosis in the non-polarised cell line A431 (Lamaze et al., 1996), and control assembly of the actin cytoskeleton (Ridley and Hall, 1992; Ridley et al., 1992). Whilst the exact mechanism of the PMA effect on apical endocytosis in Caco-2 cells remains unclear, our data are consistent with a protein kinase C mediated upregulation of both the formation and pinching off of clathrin coated pits. Further experiments, for example using a permeabilised cell system, will be necessary to test this hypothesis.

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REFERENCES

Backer, J. M. and King, G. L. (1991). Regulation of receptor mediated endocytosis by phorbol esters. *Biochem. Pharmacol.* **41**, 1267-1277.

Bement, W. M., Hasson, T., Wirth, J. A., Cheney, R. E. and Mooseker, M. S. (1994). Identification and overlapping expression of multiple unconventional myosin genes in vertebrate cell types. *Proc. Nat. Acad. Sci. USA* **91**, 6549-6553.

Cardone, M. H., Smith, B. L., Song, W., Mochly-Rosen, D. and Mostov, K. E. (1994). Phorbol myristate acetate-mediated stimulation of transcytosis and apical recycling in MDCK cells. *J. Cell Biol.* **124**, 717-727.

Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M. and Holman, G. D. (1994). Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. *Biochem. J.* **300**, 631-635.

Cupers, P., Veithen, A., Kiss, A., Baudhuin, P. and Courtoy, P. J. (1994). Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. *J. Cell Biol.* **127**, 725-735.

Daukas, G. and Zigmond, S. H. (1985). Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. *J. Cell Biol.* **101**, 1673-1679.

Davoust, J., Gruenberg, J. and Howell, K. E. (1987). Two threshold values of low pH block endocytosis at different stages. *EMBO J.* **6**, 3601-3609.

Docherty, P. A. and Snider, M. D. (1991). Effects of hypertonic and sodium-free medium on transport of a membrane glycoprotein along the secretory pathway in cultured mammalian cells. *J. Cell Physiol.* **146**, 34.

Dowrick, P., Kenworthy, P., McCann, B. and Warn, R. (1993). Circular ruffle formation and closure lead to macropinocytosis in hepatocyte growth factor/scatter factor-treated cells. *Eur. J. Cell Biol.* **61**, 44-53.

Durrbach, A., Louvard, D. and Coudrier, E. (1996). Actin filaments facilitate two steps of endocytosis. *J. Cell Sci.* **109**, 457-465.

Eichholtz, T., Vossebeld, P., van Overveld, M. and Ploegh, H. (1992). Activation of protein kinase C accelerates internalization of transferrin receptor but not of major histocompatibility complex class I, independent of their phosphorylation status. *J. Biol. Chem.* **267**, 22490-22495.

Eker, P., Holm, P. K., van Deurs, B. and Sandvig, K. (1994). Selective regulation of apical endocytosis in polarized Madin-Darby Canine Kidney cells by mastoparan and cAMP. *J. Biol. Chem.* **269**, 18607-18615.

Fabbri, M., Bannykh, S. and Balch, W. E. (1994). Export of protein from the endoplasmic reticulum is regulated by a diacylglycerol/phorbol ester binding protein. *J. Biol. Chem.* **269**, 26848-26857.

Gibbs, E. M., Lienhard, G. E., Appleman, J. R., Lane, M. D. and Frost, S. C. (1986). Insulin stimulates fluid phase endocytosis and exocytosis in 3T3-L1 adipocytes. *J. Biol. Chem.* **261**, 3944-3951.

Glauert, A. M. (1975). *Fixation, Dehydration and Embedding of Biological Specimens*. pp. 207. North Holland/American Elsevier, New York.

Goode, N. T., Hajibagheri, N., Warren, G. and Parker, P. J. (1994). Expression of mammalian protein kinase C in *Schizosaccharomyces pombe*: Isotype-specific induction of growth arrest, vesicle formation, and endocytosis. *Mol. Biol. Cell.* **5**, 907-920.

Gottlieb, T. A., Ivanov, I. E., Adesnik, M. and Sabatini, D. D. (1993). Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. *J. Cell Biol.* **120**, 695-710.

Griffiths, G. (1993). *Fine Structure Immunocytochemistry*. Springer-Verlag, Berlin. pp. 1-459.

Hansen, S. H., Sandvig, K. and van Deurs, B. (1993). Clathrin and HA2 adaptors: Effects of potassium depletion, hypertonic medium, and cytosol acidification. *J. Cell Biol.* **121**, 61-72.

Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C. and Aderem, A. (1992). MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* **356**, 618-622.

Heuser, J. (1989). Effects of cytoplasmic acidification on clathrin lattice morphology. *J. Cell Biol.* **108**, 401-411.

Heuser, J. E. and Anderson, R. G. W. (1989). Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell Biol.* **108**, 389-400.

Hewlett, L. J., Prescott, A. R. and Watts, C. (1994). The coated pit and macropinocytic pathways serve distinct endosome populations. *J. Cell Biol.* **124**, 689-703.

Holm, P. K., Eker, P., Sandvig, K. and van Deurs, B. (1995). Phorbol myristate acetate selectively stimulates apical endocytosis via protein kinase C in polarized MDCK cells. *Exp. Cell Res.* **217**, 157-168.

Hug, H. and Sarre, T. F. (1993). Protein kinase C isozymes: divergence in signal transduction? *Biochem. J.* **291**, 329-343.

Hughson, E. J. and Hopkins, C. R. (1990). Endocytic pathways in polarized Caco-2 cells: identification of an endosomal compartment accessible from both apical and basolateral surfaces. *J. Cell Biol.* **110**, 337-348.

Jackman, M. R., Shurety, W., Ellis, J. A. and Luzio, J. P. (1994). Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D. *J. Cell Sci.* **107**, 2547-2556.

Jackson, T. R., Stephens, L. R. and Hawkins, P. T. (1992). Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. *J. Biol. Chem.* **267**, 16627-16636.

Keller, H. U. and Niggli, V. (1993). The PKC-inhibitor Ro 31-8220 selectively suppresses PMA- and diacylglycerol-induced fluid pinocytosis and actin polymerization in PMNS. *Biochem. Biophys. Res. Commun.* **194**, 1111-1116.

Knight, A., Hughson, E., Hopkins, C. R. and Cutler, D. F. (1995). Membrane protein trafficking through the common apical endosome compartment of polarized Caco-2 cells. *Mol. Biol. Cell.* **6**, 597-610.

Kübler, E. and Riezman, H. (1993). Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J.* **12**, 2855-2862.

Lamaze, C. and Schmid, S. L. (1995). The emergence of clathrin-independent pinocytic pathways. *Curr. Opin. Cell Biol.* **7**, 573-580.

Lamaze, C., Chuang, T. H., Terlecky, L. J., Bokoch, G. M. and Schmid, S. L. (1996). Regulation of receptor-mediated endocytosis by rho and rac. *Nature* **382**, 177-179.

Nakanishi S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, S., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., Hashimoto, Y. and Nonomura, Y. (1992). Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J. Biol. Chem.* **267**, 2157-2163.

Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993). Activation of the ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-triphosphate. *J. Biol. Chem.* **268**, 13-16.

Navé, B. T., Shepherd, P. R. and Siddle, K. (1995). Effect of phorbol esters on phosphatidylinositol 3-kinase activity in adipocytes. *Biochem. Soc. Trans.* **23**, 183S.

Nishizuka, Y. (1992). Intracellular signalling by hydrolysis of phospholipid and activation of protein kinase C. *Science* **258**, 607-614.

Parton, R. G., Joggerst, B. and Simons, K. (1994). Regulated internalization of caveolae. *J. Cell Biol.* **127**, 1199-1215.

Pelchen-Matthews, A., Parsons, I. J. and Marsh, M. (1993). Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p561ck, increased association with clathrin-coated pits, and altered endosomal sorting. *J. Exp. Med.* **178**, 1209-1223.

Prekeris, R., Mayhew, M. W., Cooper, J. B. and Terrian, D. M. (1996). Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *J. Cell Biol.* **132**, 77-90.

Prydz, K., Hansen, S. H., Sanvig, K. and Deurs, B. (1992). Effects of brefeldin A on endocytosis, transcytosis and transport to the Golgi complex in polarized MDCK cells. *J. Cell Biol.* **119**, 259-272.

Reaves, B. J., Bright, N. A., Mullock, B. M. and Luzio, J. P. (1996). The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins suggests a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway. *J. Cell Sci.* **109**, 749-762.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208-212.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410.

Sandvig, K., Olsnes, S., Petersen, O. W. and van Deurs, B. (1987). Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* **105**, 679-689.

Sandvig, K., Olsnes, S., Petersen, O. W. and van Deurs, B. (1988). Inhibition of endocytosis from coated pits by acidification of the cytosol. *J. Cell. Biochem.* **36**, 73-81.

Sandvig, K. and van Deurs, B. (1990). Selective modulation of the endocytic uptake of ricin and fluid phase markers without alteration of transferrin endocytosis. *J. Biol. Chem.* **265**, 6382-6388.

Shepherd, P. R., Nave, B. T. and Siddle, K. (1995a). Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-56 kinase. *Biochem. J.* **305**, 25-28.

Shepherd, P. R., Soos, M. A. and Siddle, K. (1995b). Inhibitors of phosphoinositide 3-kinase block exocytosis but not endocytosis of transferrin receptors in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* **211**, 535-539.

Shepherd, P. R., Reaves, B. J. and Davidson, H. W. (1996). PI 3-kinases and membrane traffic. *Trends Cell Biol.* **6**, 92-97.

Shurety, W. and Luzio, J. P. (1995). Differential modulation of apical and basolateral endocytosis in Caco-2 cells. *Biochem. Soc. Trans.* **23**, 184S.

Slot, J. W. and Geuze, H. J. (1983). The use of Protein A-colloidal gold (PAG) complexes as immunolabels in ultrathin frozen sections. In *Immunohistochemistry* (ed. A. C. Cuello), pp. 323-346. John Wiley & Sons, Chichester.

Smart, E. J., Foster, D. C., Ying, Y.-S., Kamen, B. A. and Anderson, R. G. W. (1994). Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalisation of caveolae. *J. Cell Biol.* **124**, 307-313.

Stenson, W. F., Easom, R. A., Riehl, T. E. and Turk, J. (1993). Regulation of paracellular permeability in Caco-2 cell monolayers by protein kinase C. *Am. J. Physiol.* **265**, G955-G962.

Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M. and Cantley, L. C. (1994). Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3, 4-P2 and PtdIns-3, 4, 5-P3. *J. Biol. Chem.* **269**, 32358-32367.

West, M. A., Bretscher M. S. and Watts, C. (1989). Distinct endocytic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J. Cell Biol.* **109**, 2731-2739.

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