Apical endocytosis of ricin in MDCK cells is regulated by the cyclooxygenase pathway

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SUMMARY

Addition of arachidonic acid or stimulation of arachidonic acid production by secretory phospholipase A2 selectively upregulated apical endocytosis of ricin in MDCK cells without affecting basolateral endocytosis. Electron microscopic studies revealed that MDCK cells treated with secretory phospholipase A2 and incubated with horseradish peroxidase had an increased number of normal appearing peroxidase-labeled endosomes and no sign of membrane ruffling. Moreover, inhibition of basal arachidonic acid release, either by decreasing the cytosolic phospholipase A2 activity or the diacylglycerol lipase activity, reduced the rate of apical endocytosis. Furthermore, indomethacin, an inhibitor of the cyclooxygenase pathway, counteracted the stimulation of endocytosis seen with both secretory phospholipase A2 and arachidonic acid, suggesting that formation of eicosanoids such as prostaglandins could be essential for the regulation. This idea was supported by the finding that prostaglandin E2, the predominant prostaglandin formed in kidney, also upregulated ricin uptake. The regulatory effect of the cyclooxygenase pathway on apical endocytosis of ricin was found to be independent of protein kinases A and C, which are known to selectively control apical clathrin-independent endocytosis in polarized cells.

Key words: Endocytosis, Ricin, MDCK cell, Cyclooxygenase pathway, Arachidonic acid, Prostaglandin

INTRODUCTION

Endocytosis of ricin from the apical surface in polarized Madin-Darby canine kidney (MDCK) cells seems to be under complex regulation (Fig. 1). Previous studies showed that apical clathrin-independent endocytosis is selectively increased by activation of protein kinase C (PKC) (Holm et al., 1995), protein kinase A (PKA) (Eker et al., 1994) and heterotrimeric G proteins (Eker et al., 1994). Under the same conditions there is no increase of basolateral endocytosis. To further elucidate the molecular mechanisms involved in regulation of apical endocytosis in MDCK cells we have now investigated whether arachidonic acid (AA) or its metabolites play any role in this regulation. AA might be a mediator of the activation of apical endocytosis by mastoparan since this compound was shown to stimulate release of AA via a heterotrimeric G protein in Swiss 3T3 cells (Gil et al., 1991). Also activation of PKC has been reported to be involved in stimulation of phospholipase A2 (PLA2; Nemenoff et al., 1993; Rao et al., 1994), an enzyme that liberates AA from phospholipids.

AA is important for regulation of membrane transport and fusion in many cell types (Lennartz and Brown, 1991; Prusch et al., 1989; Vult von Steyern et al., 1996), but it seems to vary as to whether there is a requirement for AA formation or if metabolites formed from AA by the action of cyclooxygenase (COX) or lipoxygenase (LO) are the active compounds. In Amoeba proteus phagocytosis can be induced both by AA and by prostaglandins (PG) (Prusch et al., 1989). However, in human monocytes IgG Fc receptor-mediated phagocytosis seems to be dependent only on formation of AA (Lennartz and Brown, 1991). In HeLa cells overexpressing the ARF6 GTPase, PLA2 activity is required for the increased macropinocytosis that occurs upon treatment with aluminium fluoride (Radhakrishna et al., 1996). PLA2 function has furthermore been reported to be important for endosome fusion (Mayorga et al., 1993) and for intra-Golgi protein transport in vitro and in vivo, as indicated by a decreased prolactin secretion in the presence of nordihydroguaiaretic (NDGA) (Tagaya et al., 1993). In addition, when the effect of this compound on the transport of the vesicular stomatitis virus glycoprotein was studied, a decreased protein transport from the endoplasmic reticulum to the Golgi apparatus and from the trans-Golgi network to the plasma membrane was found (Tagaya et al., 1996).

In this study we have investigated to what extent externally added secretory PLA2 (sPLA2), compounds that induce release or inhibit formation of AA, AA itself and PG can affect endocytosis of ricin in polarized MDCK cells. In addition we have studied whether some of the previously published
conditions for stimulation of apical endocytosis of ricin in MDCK cells do so via a stimulation of AA production and the COX pathway.

MATERIALS AND METHODS

Reagents
PLA2 from bee venom, bovine pancreas, Crotalus atrox venom, Naja naja venom, indomethacin, ionomycin, thapsigargin, NGDA, horseradish peroxidase (HRP) types II and VI-A, 0-dianisidine, diaminobenzidine, BSA (fraction V), Heps, bradykinin, Tris, ATP, 8-Br-cAMP, TPA, PGE1, PGE2, AA and mastoparan were obtained from Sigma Chemical Co., St Louis, MO, USA, MAFP, A23187 and RHC 80267 were from Bismol Research Laboratories, Plymouth Meeting, PA, USA. H89 was purchased from Seikagaku Corp., Tokyo, Japan. \(^{3}H\)AA was from NEN Research Products (Wilmington, DE, USA). Na\(^{125}\)I was from the Radiochemical Centre, Amersham, UK. Ricin and transferrin were iodinated by the iodoogen method (Fraker and Speck, 1978).

Cells
MDCK (strains I and II) cells, kindly provided by Dr Kai Simons, EMBL, Heidelberg, Germany, were grown in Costar (Badhoevedorp/ The Netherlands) or NUNC (Roskilde, Denmark) flasks. In most experiments, and when nothing else is indicated, MDCK I cells were used. MDCK cells were also seeded on polycarbonate filters (Costar Transwell, pore size 0.4 \(\mu\)m) of 24.5 mm or 12 mm diameter at a density of \(10^6\) and \(10^5\) per filter, respectively, and used for experiments 4 or 5 days later (van Deurs et al., 1990). All filters with MDCK I cells used for experiments had a transepithelial resistance of at least 1000 ohm.cm\(^2\) as measured with the Millicell-ERS equipment (Millipore Corporation, Bedford, MA, USA). The medium used was DMEM (3.7 g/l sodium bicarbonate; Flow Laboratories, Irvine, Scotland) containing 5% fetal calf serum, and 2 mM L-glutamine (Gibco). For MDCK II cells, 10% fetal calf serum was used. Endocytic uptake measurements of ricin and HRP in MDCK cells were performed in Heps (20 mM)-buffered DMEM with 2 mM glutamine without sodium bicarbonate (DMEM-H, Flow), supplemented with 0.2% BSA. HEP-2 cells were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland; A431 cells were from the American Type Culture Collection, Rockville, MD, USA; U-2OS cells were from Dr G. F. Vande Woude, National Cancer Institute, Frederick, MD, USA; NIH 3T3 cells were from the American Type Culture Collection, Rockville, MD, USA. The different cells were grown in the medium described above, except for A431 cells which were grown in 10% serum.

Release of \(^{3}H\)AA
Release of \(^{3}H\)AA was measured essentially as described (Balsinde et al., 1997; Barbour and Dennis, 1993; Felder et al., 1989). MDCK cells were incubated overnight in 24-well disposable trays or on polycarbonate filters with 0.25 \(\mu\)Ci/ml of \(^{3}H\)AA. The cells were then washed twice with Heps-buffered medium containing 0.2% BSA (fatty acid-free). Experimental agents were added as required, and the reaction stopped by removing the incubation medium, which was centrifugated to remove detached cells. The released radioactivity in the medium was measured by liquid scintillation counting. In control cells about 0.5% of the total \(^{3}H\)AA incorporated was released into the medium.

Measurements of endocytosis
Endocytosis of \(^{125}\)I-ricin (100-200 ng/ml; 30,000-40,000 cpm/ng) was measured as the amount of toxin that could not be removed by five washes, one of them for 5 minutes, with a 0.1 M lactose solution in Heps at 37°C (Sandvig and Olsnes, 1979). Endocytosis of \(^{125}\)I-transferrin was measured as described previously (Ciechanover et al., 1983). Briefly, cells were incubated with \(^{125}\)I-transferrin (50-150 ng/ml; 10,000-20,000 cpm/ng) at 37°C for the indicated times, washed three times with ice-cold PBS, and then treated for 1 hour at 0°C with serum-free medium containing 0.3% (w/v) Pronase. Then the cells and the medium were transferred to Eppendorf tubes and centrifuged for 2 minutes, and the radioactivity in the pellet and in the supernatant was measured.

Measurement of cAMP
The content of cAMP in cells was measured by a \([8-^{3}H]\)cyclic AMP assay system from Amersham. In principle, 2x10^6 cells growing in Petri dishes (diameter 5 cm) were washed twice in PBS and then dissolved in 750 \(\mu\)l of ice-cold HCl (10 mM) in ethanol (96%). After a 5 minute incubation at 0°C, the cells were removed with a rubber policeman and the cell suspension was centrifuged for 10 minutes in an Eppendorf centrifuge. The supernatant was freeze-dried and the pellet was dissolved in 2 ml 0.2 M KOH. The OD at 280 nm of this solution was used as a measurement of the amount of cells used. The freeze-dried supernatant was dissolved in 250 \(\mu\)l sodium acetate (0.5 M, pH 6.2). This solution was then used in the Amersham cAMP kit to measure the concentration of cAMP in the cells.

Electron microscopy
Filter-grown MDCK cells were rinsed twice with DMEM-H and incubated with HRP (10 mg/ml) as described in the text. Fixation was carried out using 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Following fixation, cells were processed for DAB-cytochemistry immediately and then contrasted en bloc with uranyl acetate and embedded in Epon as previously described (van Deurs et al., 1993). Sections were examined in a Jeol 100 CX electron microscope.

RESULTS
AA selectively increases apical endocytosis of ricin in MDCK cells
To test whether externally added AA could affect endocytosis of ricin, polarized MDCK cells were incubated in the presence of increasing amounts of AA. Membrane internalization was then measured as endocytosis of ricin both at the apical and the basolateral side of the filter-grown cells. As shown in Fig. 2, AA treatment clearly increased the uptake of ricin at the apical side, whereas it did not affect endocytosis from the basolateral side. AA stored at the sn-2 position of phospholipids can be liberated by the action of PLA2. The PLA2 enzymes constitute a large and diverse family (for reviews, see Dennis, 1997, 1994; Kramer and Sharp, 1997; Leslie, 1997; Tischoff, 1997), which can be classified by location into sPLA2 and cytosolic PLA2 (cPLA2) enzymes. Thus we decided to study whether addition of sPLA2 stimulated AA release and apical endocytosis of ricin in MDCK cells. Bee venom sPLA2 (25 units/ml) added to both sides of polarized MDCK cells caused a two- to threefold stimulation of AA release into both the apical and the basolateral media (Fig. 3A), but stimulated endocytosis of ricin only from the apical domain in polarized MDCK I cells (Fig. 3B). Preincubation of MDCK II cells, which differ somewhat from MDCK I cells (Richardson et al., 1981), with bee venom sPLA2 also stimulated apical endocytosis of ricin. In contrast, the enzyme did not affect endocytosis of the toxin in nonpolarized cells such as HEP-2 cells, NIH 3T3 cells, U20S cells or A431 cells (data not shown). sPLA2 enzymes are divided into several groups (Dennis, 1997). Be venom PLA2 belongs to the group III sPLA2s. However, sPLA2s belonging to other groups such as...
The COX pathway regulates apical endocytosis

Naja naja venom (group IA), pancreas (group IB), and Crotalus atrox (group IIA) sPLA2s also affected apical endocytosis in MDCK I (data not shown). The possibility that sPLA2 might affect recycling of ricin was investigated by allowing bee venom sPLA2-treated cells to internalize ricin, then removing the surface-bound ricin with lactose and finally measuring the rate of ricin recycling. Only a slight reduction of ricin recycling was observed, from 100% in control cells to approximately 90% after 20 minutes, thus indicating that sPLA2 increases the rate of ricin uptake. Furthermore, PLA2-treatment stimulated endocytosis of ricin even when the cytosol was acidified to block clathrin-dependent endocytosis (Sandvig et al., 1987), indicating that sPLA2 is able to upregulate clathrin-independent endocytosis (data not shown).

We have previously demonstrated that selective upregulation of apical endocytosis of ricin by addition of mastoparan and by stimulation of PKA and PKC leads to an increased number of endocytic vesicles (Eker et al., 1994; Holm et al., 1995), but there is no sign of induced ruffling and macropinocytosis. This has been studied by using the fluid-phase marker HRP, since one would expect that uptake of this marker is increased when uptake of the membrane marker ricin is increased. To investigate whether the same was the case after sPLA2 treatment, we decided to do EM studies of cells that had internalized HRP. MDCK cells were treated with bee venom sPLA2, HRP was then added to the apical side, and finally HRP-labeled endosomes were studied by electron microscopy (Fig. 4 and Table 1). Quantification showed a clear increase in the number of HRP-labeled endosomes (Table 1) in the sPLA2-treated cells, while the diameter of HRP-labeled endosomes larger than 200 nm was not increased. The quantification was limited to endosomes larger than 200 nm in diameter because smaller, tubulovesicular endosomes are very difficult to quantify. Furthermore, after sPLA2 treatment there was no sign of ruffling, the area of the apical membrane was not changed (Fig. 4 and Table 1), and no visible difference in the location of labeled endosomes was observed.

Inhibitors of AA production decrease apical endocytosis of ricin in MDCK cells

In mammalian cells AA stored at the sn-2 position of phospholipids can mainly be liberated by two pathways, either directly by the action of PLA2 or by the concerted action of PLC and DAG lipases. To investigate the possibility that a decrease in basal AA production might cause an inhibition of apical endocytosis of ricin we decided to treat polarized MDCK cells with methyl arachidonyl fluorophosphonate (MAFP; 15 \( \mu \)M), an inhibitor of cPLA2, or with RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A). Also, endocytosis of ricin from the apical domain of MDCK cells was reduced by 15-20% both in the presence of MAFP or RHC 80267 (25 \( \mu \)M), an inhibitor of DAG lipase. Under these conditions the basal AA release was somewhat decreased (Fig. 5A).

**Effect of inhibitors of COX and LO on the ability of PLA2 and AA to stimulate apical endocytosis of ricin**

As discussed in the Introduction, several studies where AA was shown to play a role in endocytosis and intracellular transport demonstrated that metabolites formed by either COX or LO rather than AA as such were the active compounds. To investigate whether this was the case for stimulation of apical endocytosis of ricin, the effects of indomethacin (30 \( \mu \)M), which inhibits the COX pathway, and NDGA (20 \( \mu \)M), which inhibits the LO pathway, on the stimulation of apical endocytosis of ricin by sPLA2 or AA were investigated. As shown in Fig. 6A, indomethacin strongly counteracted the effect of PLA2 and AA on endocytosis of ricin from the apical domain, suggesting that eicosanoids formed by the COX pathway regulate apical endocytosis.

![Fig. 1. Schematic drawing of the various pathways leading to increased apical clathrin-independent endocytosis of ricin in MDCK cells.](image1)

![Fig. 2. Exogenously added AA stimulates apical endocytosis of ricin.](image2)
pathway are important for the stimulation. As also shown in Fig. 6A, indomethacin by itself inhibited endocytosis of ricin to some extent. This was not the case when ricin was added to the basolateral side (data not shown). In contrast to the results with indomethacin, NDGA did not affect uptake of ricin after treatment with PLA2 and AA (Fig. 6B).

Fig. 3. Bee venom PLA2 stimulates AA release and apical endocytosis of ricin. (A) Polarized MDCK cells were incubated with [3H]AA overnight as described in Materials and Methods. Then the cells were washed and treated with or without PLA2 (25 U/ml) for 60 minutes. The medium was removed, and the amount of released [3H]AA to the apical and basolateral medium was measured. The figure shows deviations between duplicates in a representative experiment. (B) Polarized MDCK cells were preincubated with bee venom sPLA2 (25 u/ml) for 60 minutes at 37°C before addition of 125I-ricin at the apical or basolateral side. The amount of endocytosed ricin was measured after 15 minutes. Values are means + s.d. (n=5).

PGs selectively increase apical endocytosis of ricin in MDCK cells
Since eicosanoids formed by the COX pathway seem to be important for the stimulation of endocytosis of ricin, we investigated whether PGE2 (5 μM), the predominant arachidonate metabolite in MDCK cells (Lewis and Spector, 1981), and another prostaglandin, PGE1 (5 μM), were able to stimulate apical endocytosis in MDCK cells. As shown in Fig. 7A, both PGs stimulated the endocytosis of ricin from the apical side. As expected, PGE1 and PGE2 did not stimulate
The COX pathway regulates apical endocytosis of ricin (Fig. 7B). Also the endocytosis of HRP was stimulated by PGE2 (data not shown). The COX pathway and the PKA pathway regulate apical endocytosis of ricin independently Previous studies show that 8-Br-cAMP stimulates apical endocytosis of ricin via PKA in polarized MDCK cells (Eker et al., 1994). Since PGs have been shown to increase the concentration of cAMP in some MDCK cells (Hassid, 1983) we decided to investigate whether the regulatory effect of the COX pathway on apical endocytosis of ricin could be mediated by PKA. When cAMP levels were measured after treatment with bee venom sPLA2, the cAMP level was increased to 140±20% (± s.d., n=5 experiments) of that in control cells. To test whether this small increase could be responsible for the increased endocytosis of ricin, uptake was measured after PLA2 treatment in the presence of H89 (10 μM), an inhibitor of PKA. As shown in Fig. 8, H89 did not inhibit the stimulatory effect of PLA2 indicating that the slightly increased level of cAMP was not responsible for the increased endocytosis of ricin. Control experiments showed that H89 was able to counteract the stimulatory effect of 8-Br-cAMP (2 mM) on endocytosis of ricin (Eker et al., 1994) (Fig. 8).

Table 1. Quantitation of EM data obtained from MDCK cells treated without or with PLA2

<table>
<thead>
<tr>
<th></th>
<th>Brush border density per cell profile*</th>
<th>Diameter of HRP-labeled endosomes† (nm)</th>
<th>Number of HRP-labeled endosomes‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.97±0.25 (10)</td>
<td>44±124 (50)</td>
<td>1.4±0.2 (50)</td>
</tr>
<tr>
<td>PLA2</td>
<td>3.85±0.38 (10)</td>
<td>46±182 (50)</td>
<td>2.1±0.4 (50)</td>
</tr>
</tbody>
</table>

Polarized MDCK cells were washed with DMEM and incubated at 37°C for 60 minutes in DMEM alone (control) or in the presence of PLA2 (25 U/ml), followed by 30 minutes at 37°C with HRP (10 mg/ml) added to the apical medium. After processing for electron microscopy, sections were cut perpendicular to the filter and the quantitative analysis performed. Values are means ± s.d. (n).

*Microvillar membrane length per cell surface length (μm/μm).
‡Endosomes of diameter >200 nm only were counted.

Fig. 6. Indomethacin but not NDGA inhibits the stimulation of apical endocytosis of ricin induced by PLA2 and AA. Polarized MDCK cells grown on filters were incubated with and without indomethacin (Ind., 30 μM) for 30 minutes (A), or with and without NDGA (20 μM) for 10 minutes (B). The cells were then incubated with and without PLA2 (25 U/ml) for 60 minutes or AA (100 μM) for 30 minutes, before 125I-ricin was added. The amount of endocytosed ricin was measured after 15 minutes in the presence of the toxin. Values are means + s.d. (n=5-9).

Fig. 7. Exogenously added PGE1 and PGE2 stimulate apical endocytosis of ricin in MDCK cells. The cells were incubated for 20 minutes with and without PGE1 (5 μM) or PGE2 (5 μM) before 125I-ricin was added at the apical (A) or basolateral (B) side. The endocytosed amounts of toxin were measured 15 minutes later as described in Materials and Methods. Values are means + s.d. (n=3 experiments).
Activation of PKC by TPA or activation of heterotrimeric G proteins by mastoparan do not increase apical endocytosis of ricin via the COX pathway

Another possibility was that PKC or heterotrimeric G proteins were involved in the COX-dependent regulation of endocytosis of ricin, since activation of these proteins by TPA or mastoparan, respectively, increases apical endocytosis in MDCK cells (Eker et al., 1994; Holm et al., 1995). However, TPA-induced stimulation of apical endocytosis of ricin could already be observed after 5 minutes of preincubation, whereas a significant stimulation of AA release did not appear until 30 minutes later (Fig. 9A). This experiment indicates that by the time PKC stimulates AA release endocytosis of ricin is already increased, thus suggesting that PKC does not affect endocytosis of ricin through the activation of the COX pathway. Importantly, this idea was supported by the finding that neither indomethacin (Fig. 9B) nor NDGA (data not shown) reduced the stimulatory effect of TPA on endocytosis of ricin.

Mastoparan also increased the release of AA in MDCK cells (data not shown). However, indomethacin did not decrease the ability of mastoparan to increase endocytosis of ricin (data not shown), indicating that parallel pathways lead to increased endocytosis of ricin. On the other hand, mastoparan strongly reduced endocytosis of transferrin both in permeabilized A431 (Carter et al., 1993) and in intact MDCK cells (Eker et al., 1994). We therefore tested whether this effect could be mediated by PLA2. However, addition of PLA2 only slightly reduced endocytosis of transferrin in subconfluent non-polarized MDCK cells, supporting the view that mastoparan affects endocytosis by mechanisms that do not only involve stimulation of PLA2 and the COX pathway.

Effect of AA-mobilizing compounds on apical endocytosis of ricin in MDCK cells

Since a number of compounds are known to induce release of AA in different cell lines we tested whether some of these might stimulate AA release and apical endocytosis of ricin in MDCK cells. Calcium ionophores can mobilize AA by increasing the cytosolic level of free Ca\(^{2+}\) and thereby inducing the association of PLA2 with cellular membranes (Glover et al., 1995; Schievella et al., 1995). Interestingly, two calcium ionophores, A23187 and ionomycin, caused liberation of arachidonic acid and stimulated apical endocytosis of ricin (Table 2). Furthermore, the stimulatory effect of ionomycin on endocytosis of ricin could be partially reduced by indomethacin (data not shown). When the cytosolic levels of Ca\(^{2+}\) were increased by mobilizing Ca\(^{2+}\) from intracellular stores with thapsigargin, AA release, but not apical endocytosis.
of ricin, was stimulated in MDCK cells. Receptor-mediated activation of PLA2 with the peptide bradykinin gave a similar result. This could be due to lack of PG production in response to AA release (see Discussion). On the other hand, when the nonpeptide agonist ATP, which interacts with purinergic receptors, was used, an increase of both AA release and apical endocytosis of ricin was observed. However, indomethacin did not inhibit the ATP-induced stimulation of apical endocytosis, suggesting that ATP is either not affecting endocytosis by the COX pathway or that additional pathways are activated, thereby permitting the stimulation of endocytosis to continue even in the presence of indomethacin.

DISCUSSION

We have shown here that the COX pathway is important for regulation of apical endocytosis of ricin in MDCK cells. Addition of PGs stimulates apical endocytosis of the toxin and it is known that the rate of PG synthesis is controlled by the availability of free AA (Lands and Samuelsson, 1968). When the cellular free AA level was increased either by exogenously added AA or by sPLA2 from different sources, a stimulation of apical endocytosis of ricin that could be inhibited with the COX inhibitor indomethacin was observed. Formation of arachidonic acid (AA) by AA release and apical endocytosis of ricin, did not reduce the stimulatory action of AA or sPLA2. Basolateral endocytosis of ricin was unchanged in the presence of both sPLA2 and AA.

Several forms of PLA2 have been found in the kidney (Bonventre, 1992; Portilla and Dai, 1996; Smith, 1992). A calcium-dependent cPLA2 of high molecular mass, as well as a 14 kDa sPLA2, have been characterized (Smith, 1992), and recently the purification of a calcium-independent cPLA2 from rabbit kidney was also reported (Portilla and Dai, 1996). We have therefore addressed the question: Do the endogenous PLA2s affect endocytosis of ricin in polarized MDCK cells? Interestingly, the cPLA2 inhibitor MAFP reduced basal AA release and apical endocytosis of ricin in MDCK cells. The findings that calcium ionophores were able to increase the release of AA and apical endocytosis of ricin are in agreement with an involvement of the calcium-dependent cPLA2 in the regulation of apical endocytosis of ricin. Furthermore, free AA can also be obtained by the PLC/DAG pathway. Inhibition of this pathway with an inhibitor of DAG lipase also reduced the basal AA release and apical endocytosis of ricin, suggesting that other enzymes controlling the basal production of AA in MDCK cells also can regulate apical endocytosis.

As mentioned above, calcium ionophores were able to stimulate apical endocytosis of ricin. However, not all of the AA mobilizing agents we used stimulated apical endocytosis. Although we do not have any direct evidence, the activity of PG synthesizing enzymes could be inhibited by the stimulatory agent, or the agent might in itself affect the stimulation of endocytosis, even though it did not affect the basal level of endocytosis of ricin. Under the various conditions used, AA might also end up in different compounds, giving rise to different responses in endocytosis of ricin. Furthermore, one AA mobilizing agent we used, ATP, did stimulate apical endocytosis of ricin, but the stimulation could not be inhibited by indomethacin. The explanation for this is not known, but the possibility exists that more than one pathway involved in stimulation of endocytosis of ricin is activated by this compound. It is clear that a number of ligands regulate several cellular enzymes and pathways at the same time. Indeed, it has been shown that P2-purinergic receptors expressed on MDCK-D1 cells also respond to extracellular nucleotides by increasing the activity of PLC, PLD, PKC and the cAMP level (Post et al., 1998).

To investigate whether other activators of apical endocytosis of ricin might exert their effect via stimulation of the COX pathway we measured their ability to induce AA release. TPA has been shown to stimulate apical endocytosis of ricin in polarized MDCK cells via activation of PKC (Holm et al., 1995). However, the data shown in this article suggest that the ability of TPA-activated PKC to increase apical endocytosis of the toxin is not mediated via the COX pathway. Concentrations of TPA that increased endocytosis of ricin after 10 minutes had no significant effect on the release of AA, and indomethacin did not counteract the TPA-induced stimulatory effect. However, TPA was able to stimulate AA release after 30 minutes. These results are in agreement with a previous report showing that TPA did not cause significant release of fatty acids in MDCK cells within 1 hour (Beaudry et al., 1983). In fact, the ability of TPA to stimulate AA release seems to be cell-line dependent. Addition of TPA to CHO cells only induced a slight stimulation of AA release (Lin et al., 1992), and recent results published by Balsinde et al. (1997) demonstrated that PKC activation does not lead to enhanced AA release in P388D1 macrophages.

Our results also indicate that although mastoparan stimulates release of AA, this is not the whole explanation for its effect on endocytosis. Since indomethacin did not reduce the ability of mastoparan to stimulate endocytosis of ricin, and PLA2 did not reduce endocytosis of transferrin to the same extent as mastoparan (Eker et al., 1994), the G protein(s) activated by mastoparan must affect endocytosis of ricin by additional mechanisms. Recent data demonstrate that the subunits of G proteins can inhibit the activity of the dynamin 1-GTPase (Lin and Gilman, 1996). Such an inhibition of dynamin might explain the effect of mastoparan on endocytosis of transferrin and the finding that PLA2 does not have a similar inhibitory effect.

It was recently reported that aluminium fluoride stimulates formation of surface protrusions and macropinocytosis in cells overexpressing the ARF6 GTPase (Radhakrishna et al., 1996). This stimulation was dependent on the activity of PLA2 and the LO pathway since it could be inhibited by NDGA. Indomethacin, which inhibits COX and the formation of PG, had no effect (Radhakrishna et al., 1996). Thus, the stimulatory effect of PLA2 on MDCK cells seems to be mediated by a different mechanism, which also differs from the mechanism of PLA2-induced IgG-mediated phagocytosis (Lennartz and Brown, 1991) and from rac-induced ruffling (Peppelenbosch et al., 1993), where inhibitors of COX and LO have no effect. On the other hand, angiotensin II-induced fluid-phase endocytosis in primary cultures of brain microvessel endothelial cells is inhibited by indomethacin, suggesting that PG are involved in the regulation (Guillot and Audus, 1990).

How do PGs affect endocytosis? Signalling from cell surface receptors for PGs (Negishi et al., 1995; Smith, 1992) could
play a role for the regulation. PGs induce changes in the actin cytoskeleton (Peppelenbosch et al., 1993), but whether that is important for increased endocytosis is not known. AA, leukotrienes and PGs have been shown to inhibit or stimulate different GTPase-activating proteins in vitro (Golubic et al., 1991; Han et al., 1991; Tsai et al., 1991; Yu et al., 1990), possibly by direct physical associations (Tsai et al., 1991). Regulation of Ras-related proteins could be involved in the regulation of endocytosis of ricin observed here. It was recently published that Rho is involved in clathrin-independent endocytosis in oocytes (Schmalzing et al., 1995). The same actually seems to be the case for apical endocytosis in MDCK cells, where Clostridium botulinum exoenzyme C3, which inactivates Rho, inhibits apical clathrin-independent endocytosis of ricin (our unpublished data). On the other hand, it has been shown that active Rho inhibits receptor-mediated endocytosis (Lamaze et al., 1996). Since leukotrienes and PGs may have different effects on GTPase-activating proteins, it is possible that the relative amounts and not the absolute concentrations of these compounds are important. Thus, the apparently different results obtained with regard to the importance of leukotrienes and PGs may not be as different as they seem at present. Furthermore, GTPase-activating proteins or other potential target enzymes for PGs might be enriched in a domain specific fashion, thus explaining the selective regulation at the apical domain.

Activation of PLA2 and the COX pathways is important for normal kidney function. The metabolites can, for instance, modulate water and salt reabsorption (Smith, 1992). Although we cannot extrapolate from MDCK cells to kidney tubule cells in vivo, and although we do not know whether an endogenous or an exogenous PLA are involved in regulation of endocytosis under physiological conditions, it seems likely that such a regulation can also occur in vivo.

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