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Transport of apically but not basolaterally internalized ricin to the Golgi apparatus is stimulated by 8-Br-cAMP in MDCK cells

Alicia Llorente[a], Bo van Deurs[b], Kirsten Sandvig[a,*]

[a]*Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway*
[b]*Structural Cell Biology Unit, Department of Medical Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark*

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Abstract The plant toxin ricin has to be transported to the Golgi apparatus after endocytosis to exert its toxic effect. In this study we show that transport of apically endocytosed ricin to the Golgi apparatus is stimulated by 8-Br-cAMP in polarized MDCK cells. This stimulation is counteracted by the PKA inhibitor H-89. In contrast, there is no increase in the transport to the Golgi apparatus of ricin internalized from the basolateral membrane. These results suggest that protein kinase A selectively regulates endosome to Golgi transport in these cells.
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Key words: Ricin; Membrane traffic; Endosome; Golgi apparatus; cAMP; MDCK cell

1. Introduction

Polarized MDCK cells contain two compositionally and functionally different plasma membrane domains, the apical and the basolateral. Several transport pathways such as endocytosis, transcytosis and transport along the biosynthetic pathway are differentially regulated in polarized cells depending on the plasma membrane domain that is involved. In the biosynthetic pathway, heterotrimeric G_s proteins [1], protein kinase A (PKA) [2–4] and protein kinase C (PKC) [4] regulate constitutive transport from the Golgi apparatus to the apical plasma membrane, whereas the small GTPase Rab8 [5], heterotrimeric G_i proteins [1] and myosin II [6] are involved in vesicular traffic between the Golgi apparatus and the basolateral plasma membrane. Also transport from endosomes to the Golgi apparatus is under regulation, but much less is known about this transport step and whether it is differentially affected depending on the pole involved in endocytosis of the ligand. By using the plant toxin ricin we have earlier found that brefeldin A [7], monensin [8] and calmodulin antagonists [9] regulate endosome to Golgi transport in MDCK cells. We here present evidence that 8-Br-cAMP, an activator of PKA known to affect both endocytosis [10], transcytosis [2,10] and transport from the Golgi apparatus to the apical plasma membrane [2] in MDCK cells, also stimulates transport of ricin to the Golgi apparatus after apical endocytosis. In contrast, there is no significant effect on the transport of ricin to the Golgi apparatus after basolateral endocytosis. These results provide evidence for an additional role of PKA in vesicular transport, and demonstrate that endosome to Golgi transport is differentially regulated by cAMP depending on the pole from where endocytosis occurs.

*Corresponding author. Fax: (47) 22508692.
E-mail: ksandvig@radium.uio.no

Abbreviations: AKAPs, A-kinase anchoring proteins; BSA, bovine serum albumin; 8-Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; H-89, *N*-(2-[*p*-bromo cinnamylamino]-ethyl-5-isoquinolinesulfonamide; HRP, horseradish peroxidase; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; UDP-galactose, uridine diphosphogalactose

2. Materials and methods

2.1. Materials

8-Br-cAMP, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, horseradish peroxidase (HRP) type VI, *o*-dianisidine, HEPES, lactose, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Imidazole was obtained from Serva Biochemicals, Heidelberg, Germany. Sucrose was obtained from Merck, Darmstadt, Germany. H-89 was obtained from Seikagaku Corp., Tokyo, Japan. [^{3}H]Leucine, Na^{125}I and UDP-D-[6-^{3}H]galactose ammonium were obtained from the Radiochemical Centre, Amersham, UK. Nycodenz was obtained from Nycomed, Oslo, Norway. Ricin was labelled by the iodogen method [11].

2.2. Cell culture

MDCK cells (strain I) [12] were grown either in 175 cm^2 flasks and passaged every 3–4 days or on polycarbonate filters (Costar Transwell, pore size: 0.4 μm, diameter: 24.5 mm) at a density of 10^6 cells per filter and used 4–5 days later. The cells were maintained in DMEM (Flow Laboratories, Irvine, UK) supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. The transepithelial resistance of the monolayer was measured with a Millicell-ERS equipment (Millipore Corporation, Bedford, MA, USA) at the beginning of the experiments. Filters with a transepithelial resistance higher than 1000 Ω cm^2 were used. The experiments were carried out in HEPES (20 mM)-buffered MEM with 0.2% BSA.

2.3. Subcellular fractionation

Subcellular fractionation after incubation with ^{125}I-ricin and HRP was carried out essentially as described by Sandvig et al. [13]. Filters with polarized cells were cut out of their plastic holders and, in those cases where ^{125}I-ricin was present, washed with constant shaking four times for 15 min with a cold PBS solution containing 0.1 M lactose and 0.2% BSA to remove surface-bound toxin [14]. Homogenization buffer (H-buffer) (0.3 M sucrose, 3 mM imidazole, pH 7.4) (1 ml) was then added to each filter and the cells were scraped off with a rubber policeman. This procedure was repeated and finally H-buffer (1 ml) was added to wash the filters. For each gradient cells from two or three filters were collected and centrifuged for 10 min at 100×*g* at 4°C. The supernatant was removed and the pellet was resuspended in H-buffer (1 ml) and homogenized by passing it 3–4 times up and down through a 1 ml tip, followed by 3–4 times through a 1-ml syringe with a 22G×1.5″ needle. The homogenate was centrifuged for 10 min at 2500 rpm in an Eppendorf centrifuge (model 5415). Postnuclear supernatant (0.660 ml) was mixed with a 2 M sucrose solution (0.880 ml) and then added to a SW 40 tube containing 4 ml of a 1.15 M sucrose, 15 mM CsCl solution (light solution) and 1.5 ml of a 1.15 M sucrose, 15 mM CsCl, 15% Nycodenz (w/v) solution (heavy solution) mixed in a Biocomp Gradient Master, Nycomed, Oslo, Norway (angle 74, speed 16, time 2′ 45′). Then, 3 ml of a 0.9 M sucrose solution was added and the tube was filled with 1.5–2 ml H-buffer. The gradients were centrifuged for 4.5 h at 33 000 rpm at 4°C and fractionated into 24–26 portions.

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2.4. Subcellular fractionation analysis

The amount of ^{125}I-ricin in the Golgi apparatus was calculated from the radioactivity found in the fractions that were shown to contain this organelle by measuring UDP-galactose/glycoprotein galactosyl transferase activity according to Brandli et al. [15]. The activity of the lysosomal enzyme β-*N*-acetyl-glucosaminidase in each fraction was determined as described by Beaufay et al. [16]. HRP activity was determined according to Steinman et al. [17].

3. Results

The goal of this study was to investigate whether transport of ricin from endosomes to the Golgi apparatus is regulated by cAMP in polarized MDCK cells. Ricin does not dissociate at low endosomal pH, and it has previously been shown to be transported to the Golgi apparatus in a number of cell lines [18], including polarized MDCK cells [19]. In these cells, ricin is endocytosed from both the apical and the basolateral plasma membrane after binding to glycoproteins and glycolipids containing terminal galactose [19]. In the present study polarized MDCK cells were incubated with ^{125}I-ricin in the absence or in the presence of 8-Br-cAMP, and Golgi-enriched fractions were isolated by subcellular fractionation. As previously described [13,20], the method used here allows us to distinguish three different zones: zone I (top of the gradient) containing Golgi-enriched fractions, zone II where the sample was loaded, and zone III (bottom of the gradient) containing

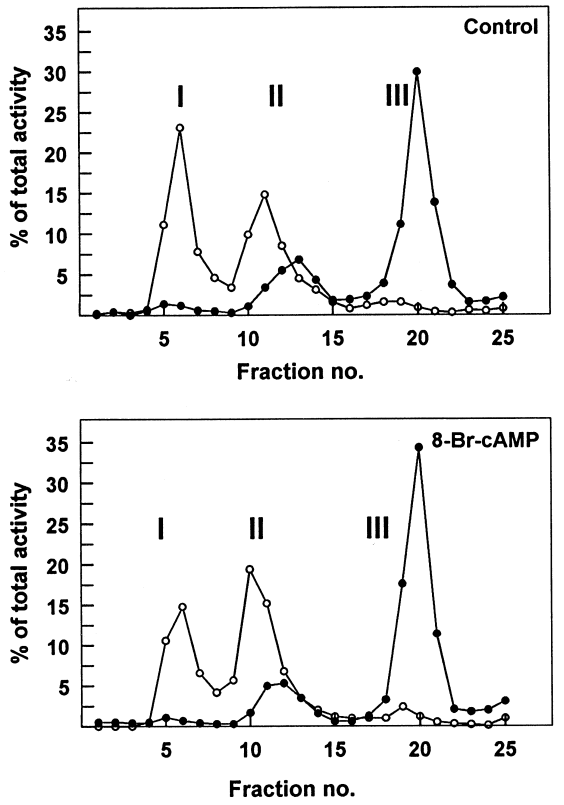


Fig. 1. UDP-galactosyltransferase and β-*N*-acetyl-glucosaminidase activity profiles in subcellular fractions of MDCK cells. Polarized MDCK cells were incubated for 90 min without or with 8-Br-cAMP (1 mM) and then homogenized and fractionated. The activities of UDP-galactosyltransferase (○) and β-*N*-acetyl glucosaminidase (●) were measured in each fraction as described in Section 2. Zone I: fractions enriched in the Golgi apparatus. Zone II, load zone. Zone III, fractions enriched in endosomes and lysosomes.

Table 1
Effect of 8-Br-cAMP on transport of endocytosed ricin to the Golgi apparatus

Toxin administration		Ricin in Golgi fractions[a] (% of control, no additions)
Apical	−8-Br-cAMP	100
	+8-Br-cAMP	143.8 ± 15.8 (n = 6)
Basolateral	−8-Br-cAMP	100
	+8-Br-cAMP	87.1 ± 6.4 (n = 8)

Filter-grown MDCK cells were incubated with or without 8-Br-cAMP (1 mM) for 30 min before ^{125}I-ricin (∼200 ng/ml, 30 000–40 000 ng/ml) was added to the apical or the basolateral plasma membrane. The incubation was continued for 1 h and then surface-bound toxin was removed. Homogenization and subcellular fractionation were performed as described in Section 2. The results are given as the mean of 6–8 experiments ± S.D.

[a]Calculated in percent of the amount of ricin endocytosed.

fractions enriched in lysosomes and endosomes. This is demonstrated in Fig. 1, where the activity profiles of the Golgi marker UDP-galactosyl transferase (open symbols) and the lysosomal marker β-*N*-acetyl-glucosaminidase (closed symbols) in control cells and in cells treated with the membrane permeant cAMP analogue, 8-Br-cAMP (1 mM), for 90 min are shown. A considerable percentage of the total UDP-galactosyl transferase activity and an insignificant percentage of the total β-*N*-acetyl-glucosaminidase activity of the gradient are found in zone I, indicating that zone I contains Golgi-enriched fractions which are not contaminated by lysosomal content, which is mainly found in zone III. A slightly lower percentage of the total activity of the Golgi enzyme seems to be present in zone I in cells treated with 8-Br-cAMP than in control cells. This may be due to some loss of intact Golgi stacks during homogenization. In any case, the profiles of the two enzymes investigated do not seem to be altered by 8-Br-cAMP. Also, we have previously found that the Golgi-enriched fractions are essentially free of early and late endosomes [13]. When MDCK cells were incubated with HRP for 10 min to label early endosomes or for 10 min followed by a 40 min chase to label late endosomes/lysosomes, only 0.4% of the total HRP activity was associated with zone I (Golgi-enriched fractions) [13]. In this study we have also used HRP to measure a possible contamination of the Golgi-enriched fractions with early and late endosomes in the absence and in the presence of 8-Br-cAMP. To label early and late endosomes MDCK cells were incubated with HRP (2 mg/ml) for 90 min. The cells were then washed, homogenized and fractionated. Measurements of the HRP activity in the different fractions of the gradient confirmed that the contamination of the Golgi-enriched fractions with endosomes was very low. Furthermore, the HRP activity in these fractions was not increased by 8-Br-cAMP treatment (data not shown).

To investigate the effect of 8-Br-cAMP on transport of ricin to the Golgi apparatus, filter-grown MDCK cells were preincubated with or without 8-Br-cAMP (1 mM) for 30 min before ^{125}I-ricin (∼200 ng/ml, 30 000–40 000 cpm/ng) was added. One hour later the cells were washed with PBS containing lactose (0.1 M) and BSA (0.2%) to remove surface-bound toxin and then homogenized and fractionated as described in Section 2. The results obtained in several experiments are summarized in Table 1, and the data show that 8-Br-cAMP increases the transport of ricin to the Golgi appa-

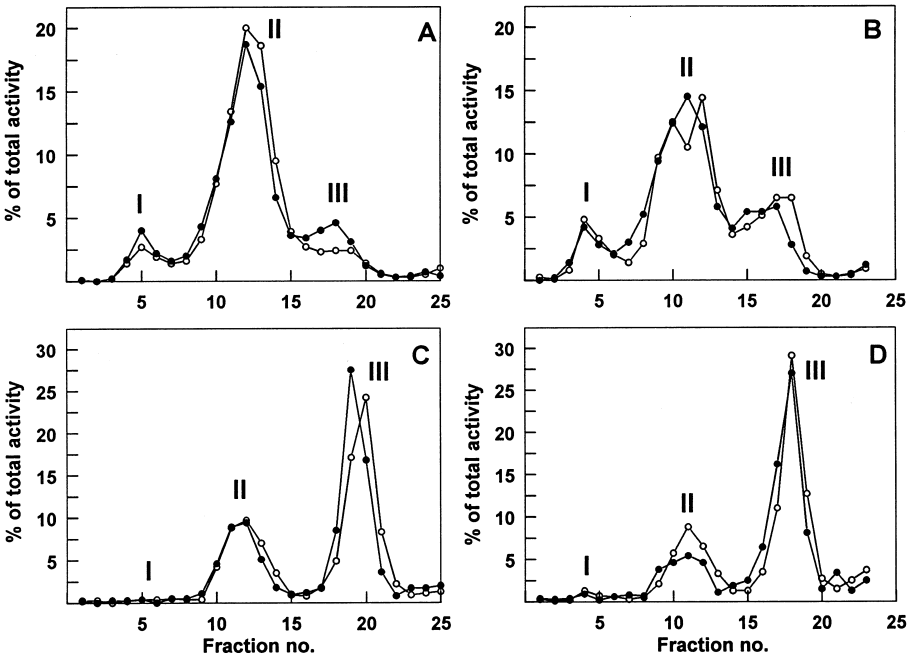


Fig. 2. ^{125}I-Ricin (A,B) and β-*N*-acetyl-glucosaminidase (C,D) activity profiles from cells incubated without or with 8-Br-cAMP. Filter-grown cells were incubated without (○) or with (●) 8-Br-cAMP (1 mM) for 30 min at 37°C. Then ^{125}I-ricin (~200 ng/ml, 30 000–40 000 cpm/ng) was added either to the apical (A) or to the basolateral (B) side. After 1 h surface-bound ricin was removed with 0.1 M lactose and subcellular fractionation was performed as described in Section 2. Profiles A and B: ^{125}I-ricin radioactivity after apical and basolateral internalization respectively. Profiles C and D: β-*N*-acetyl-glucosaminidase activity corresponding to the fractions in A and B. Zone I, fractions enriched in the Golgi apparatus. Zone II, load zone. Zone III, fractions enriched in endosomes and lysosomes.

ratus after apical internalization to 140%. It should be noted that the amount of ricin in the Golgi apparatus is always calculated in percent of the total amount of ricin endocytosed to correct for a change in the rate of endocytosis. The stimulation is restricted to toxin entry from the apical side. After basolateral addition the transport of ricin to the Golgi apparatus seems to be slightly inhibited. Fig. 2A,B shows the percentage of the total radioactivity found in each fraction of the gradient after apical (A) or basolateral (B) addition of ricin in the absence (open symbols) and in the presence (closed symbols) of 8-Br-cAMP of a representative experiment. The Golgi-enriched fractions of these gradients are also shown in Fig. 3. As described above the Golgi-enriched fractions were not significantly contaminated with early or late endosomes. Also, to demonstrate that there was no lysosomal contamination in zone I, the profiles of the lysosomal enzyme β-*N*-acetyl-glucosaminidase activity are represented in Fig. 2C,D (same fractions as in Fig. 2A,B respectively). Interestingly, the amount of ^{125}I-ricin (% of total radioactivity) found in the Golgi apparatus after apical endocytosis (Figs. 2A and 3A) is increased by 8-Br-cAMP. In contrast, 8-Br-cAMP does not increase the transport of ricin to the Golgi apparatus when the toxin is internalized from the basolateral side (Figs. 2B and 3B). It has previously been shown that ricin does not reach the Golgi apparatus at 17°C [21] and, as shown in Fig. 3, both after apical (A) and basolateral (B) uptake of ^{125}I-ricin in the absence (open symbols) or in the presence (closed symbols) of 8-Br-cAMP, the amount of labelled toxin found in the Golgi-enriched fractions (zone I) was significantly lower at 17°C than at 37°C. Importantly, at 17°C there was no difference in the amount of ricin found in the Golgi-enriched fractions between cells incubated with or without 8-Br-cAMP. These results are in agreement with the idea that the radioactivity found in zone I at 37°C is mainly due to ^{125}I-ricin that has reached the Golgi apparatus and not due to endosomal or lysosomal contamination.

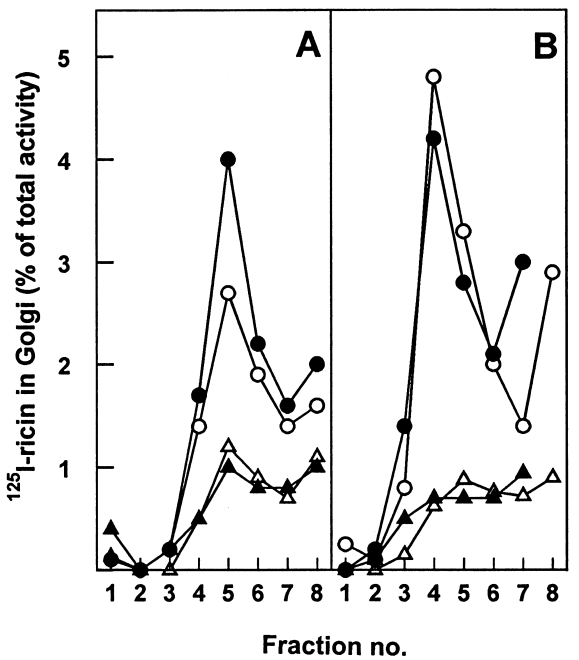


Fig. 3. Effect of 8-Br-cAMP and temperature on the amount of ricin found in Golgi-enriched fractions after apical (A) or basolateral (B) internalization. No additions 37°C, (○); 8-Br-cAMP 37°C (●); no additions 17°C (△); 8-Br-cAMP 17°C (▲). Only zone I is shown.

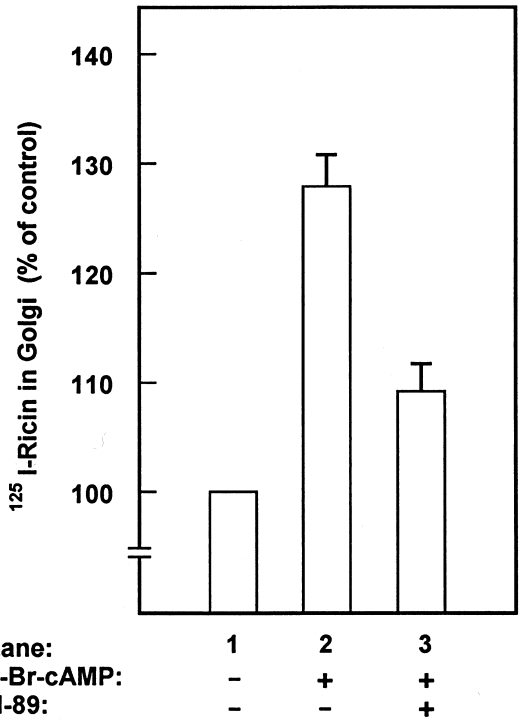


Fig. 4. Ability of H-89 to counteract the 8-Br-cAMP-induced stimulation of the transport of ricin to the Golgi apparatus. Filter-grown MDCK cells were incubated in HEPES medium with 0.2% BSA (lane 1), in the same medium with 8-Br-cAMP (1 mM) for 30 min (lane 2), or with 8-Br-cAMP (1 mM) after a 30 min preincubation with H-89 (20 μM) (lane 3) before ^{125}I-ricin was added. After 1 h at 37°C surface-bound ricin was removed and the experiment was continued as described in Section 2. The error bars show deviation between duplicates.

Finally, to investigate whether PKA is involved in the 8-Br-cAMP-induced stimulation of apical endocytosis, filter-grown cells were incubated with the PKA inhibitor H-89 [22] (20 μM) for 30 min before 8-Br-cAMP and ^{125}I-ricin were added. The experiment was continued as described above. As shown in Fig. 4, H-89 significantly decreased the stimulation caused by 8-Br-cAMP, suggesting that PKA is involved in the regulation of endosome to Golgi transport.

4. Discussion

In the present study the effect of the PKA activator 8-Br-cAMP on the transport of ricin internalized from both the apical and the basolateral plasma membrane of MDCK cells to the Golgi apparatus has been investigated. Subcellular fractionation studies show that 8-Br-cAMP selectively stimulates transport of ricin from the endosomes to the Golgi apparatus after apical endocytosis. The stimulation is counteracted by a PKA inhibitor, H-89, indicating that the effect of 8-Br-cAMP is mediated via this enzyme. cAMP has previously been shown to stimulate apical endocytosis [10], transcytosis in both directions [2,10], and transport from the trans-Golgi network to the apical plasma membrane [2,3] in MDCK cells presumably via PKA. In fact, cAMP regulates Golgi to apical plasma membrane transport to the same extent [1,4] (approximately to 140% of the control) as endosome to Golgi transport. Thus, although it has previously been shown that only a small fraction of ricin that is transported to the Golgi apparatus is exported back to the medium [23], the stimulatory effect of 8-Br-cAMP on ricin transport to the Golgi apparatus cannot be explained by a reduced export of the toxin.

Multiple forms of both regulatory and catalytic subunits of PKA are known. The discovery of a large family of proteins denominated A-kinase anchoring proteins (AKAPs) (for review see [24,25]) that serve to anchor PKA to different cellular targets has been a step forward to understand the presence of PKA at different locations and the localized action of cAMP. AKAPs have been found in association with the endoplasmic reticulum, Golgi apparatus, plasma membrane, centrosomes, microtubules, actin cytoskeleton, nuclear matrix, mitochondria, and peroxisomes [25]. So far, AKAPs have not been identified in endosomes, but the possibility exists that one of these proteins attaches PKA to endosomes where the enzyme may phosphorylate a substrate involved in the budding of vesicles destined to the Golgi apparatus. Alternatively, PKA could modify the vesicle itself or the Golgi apparatus, increasing the probability or the rate of fusion with the Golgi apparatus.

In MDCK cells endosomes originating from the apical and the basolateral pole are two different populations that do not fuse with each other. Their contents seem to meet in late endosomes [26]. It is not established whether ricin is transported to the Golgi apparatus only from late endosomes or whether it can be transported to the Golgi apparatus also from an earlier compartment. The fact that the transport of ricin to the Golgi apparatus after apical and basolateral endocytosis is independently regulated is in agreement with the latter hypothesis. In fact, it is not only 8-Br-cAMP that causes a differential regulation of ricin transport to the Golgi apparatus from the two poles of polarized MDCK cells [7–9]. Furthermore, increased transport to the Golgi apparatus is not necessarily associated with an increased or changed endocytosis. We have previously found that monensin increases apical endocytosis without changing transport of apically internalized ricin to the Golgi apparatus. Also, monensin increases the transport of basolaterally internalized ricin to the Golgi apparatus without affecting toxin endocytosis at the basolateral pole [8]. On the other hand, brefeldin A [7] and calmodulin antagonists [9] decrease the transport of basolaterally internalized ricin to the Golgi apparatus without changing basolateral endocytosis of the toxin. The data discussed above furthermore illustrate that an apparent change in Golgi transport is not an artefact due to a changed amount of ricin in endosomes/lysosomes. We cannot exclude that 8-Br-cAMP stimulates a step, for instance between endosomes, preceding transport to the Golgi apparatus since we found an increased net flux from endosomes to this organelle. However, endosome fusion has been shown to be inhibited by cAMP in a cell-free assay prepared from BHK-21 cells [27]. Furthermore, transcytosis of ricin is stimulated by 8-Br-cAMP in MDCK cells in both directions [2,10], and not only from the apical side. In fact, the stimulatory effect of the cAMP analog is larger for basolateral to apical transcytosis than in the other direction. Thus, there is no correlation between the effect of 8-Br-cAMP on transcytosis and Golgi transport.

The localization of different coats (clathrin and COP-I) on endosomes has recently been published [28,29], and one might imagine that these coats could be involved in transport from endosomes to the Golgi apparatus, possibly via another compartment. It has been shown that brefeldin A inhibits the

binding of COP-I to endosomes from CHO cells in vitro [30], the formation of clathrin-coated buds on endosomes in A431 cells [29], and possibly also binding of coats to endosomes in MDCK cells is inhibited since brefeldin A affects endosomal morphology in these cells [31]. However, in MDCK cells the Golgi is resistant to brefeldin A, and this drug stimulates transport of ricin to the Golgi apparatus after apical endocytosis [7], thus suggesting that the endosomal coats may not be required for the transport of ricin from endosomes to the Golgi. Also, recruitment of COP coats to endosomes has been reported to require low pH [28], whereas ricin transport to the Golgi apparatus in MDCK cells is not dependent on low endosomal pH [8]. cAMP has so far been shown to regulate vesicular transport that does not originate in clathrin-coated pits, such as clathrin-independent apical endocytosis and constitutive transport from the Golgi apparatus to the apical pole of polarized MDCK cells [2,3,10]. In analogy, cAMP might regulate a clathrin-independent step between endosomes and the Golgi apparatus. Clearly, further work is required to understand the different pathways involved in ricin transport.

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