

G α Stimulates Transcytosis and Apical Secretion in MDCK Cells through cAMP and Protein Kinase A

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Abstract. Recent evidence suggests a role for heterotrimeric G proteins in vesicular transport. Cholera toxin, which activates G α by ADP-ribosylation, has been reported to stimulate both apical secretion (Pimplikar, S.W., and K. Simons. 1993. *Nature (Lond.)*. 352:456–458) and apically directed transcytosis (Bomsel, M., and K.E. Mostov. 1993. *J. Biol. Chem.* 268:25824–25835) in MDCK cells, via a cAMP-independent mechanism. Here, we demonstrate that apical secretion and apically directed transcytosis are significantly stimulated by agents that elevate cellular cAMP. Forskolin, which activates adenylyl cyclase directly, and 8BrcAMP augment both transport processes in MDCK cells. The increase is not limited to receptor-mediated transport (polymeric Ig receptor), since transcytosis of ricin, a galactose-binding lectin, is similarly stimulated. The effects of elevated cellular cAMP on apical secretion and transcytosis are apparently mediated via protein kinase A (PKA), as

they are inhibited by H-89, a selective PKA inhibitor. Experiments employing a 17°C temperature block indicate that cAMP/PKA acts at a late, possibly rate-limiting stage in the transcytotic pathway, after translocation of internalized markers into the apical cytoplasm. However, no significant stimulus of apical recycling was observed in the presence of FSK, suggesting that cAMP/PKA either affects transcytosis at a level proximal to apical early endosomes and/or specifically increases the efficiency by which transcytosing molecules are delivered to the apical plasma membrane. Finally, we overexpressed wild-type G α and a mutant, Q227L, which constitutively activates adenylyl cyclase, in MDCK cells. Although Q227L increased transcytosis more than wild-type G α , neither construct was as effective as FSK in stimulating transcytosis, arguing against a significant role of G α in transcytosis independent of cAMP and PKA.

TRANSCYTOSIS is the vesicular transport process by which polarized epithelial cells transport membrane constituents and soluble cargo from one plasma membrane domain to another. Epithelial cells utilize transcytosis in the biogenesis and maintenance of apical and basolateral plasma membrane domains, and as a mechanism for selective and efficient transport of macromolecules between these domains without perturbing the barrier function of the epithelium (6, 38).

Perhaps the best characterized example of transcytosis is the transport of the polymeric immunoglobulins IgA and IgM across mucosal epithelia, which is mediated by a cognate receptor, the polymeric immunoglobulin receptor (pIgR)¹ (1). This process has been reconstituted in MDCK

cells transfected with cDNA encoding rabbit pIgR (33). Newly synthesized receptors are transported vectorially from the TGN to the basolateral surface of the epithelium (14) where they may bind ligand, although ligand binding is not obligatory for subsequent transport steps (10, 15). The receptors are then internalized and enter an endosomal compartment where they are sorted away from other, recycling receptors, and transported to the apical cell surface (10). Upon arrival at this destination, the extracellular, ligand-binding domain of the receptor, referred to as secretory component (SC), is proteolytically cleaved and released along with bound ligand into mucosal secretions (34).

Receptors like the pIgR which serve specialized functions are sorted very differently from bulk membrane proteins and lipids. The transcytosis of bulk membrane has been studied using the toxic lectin ricin, which binds to terminal galactose residues on glycoproteins and glycolipids (37, 43). Using this marker, it has been shown that, although endocytosis of

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1. *Abbreviations used in this paper:* 8BrcAMP, 8-bromoadenosine 3':5'-cyclic monophosphate; CT, cholera toxin; DDF, 1,9-dideoxyforskolin; dIgA, dimeric IgA; FSK, forskolin; H-89, N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide; HA, influenza virus hemagglutinin;

IBMX, 3-isobutyl-1-methyl-xanthine; pIgR, polymeric immunoglobulin receptor; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; rt, room temperature; SC, secretory component; wt, wild type.

membrane is more efficient from the basolateral than from the apical domain in MDCK cells, apical to basolateral transcytosis—expressed as the fraction of transcytosed to internalized membrane—is more efficient than transcytosis in the basolateral to apical direction (37, 43). In this respect, the transport of ricin resembles that of the fluid-phase marker HRP (37, 44).

The regulation of vesicular trafficking is an issue of major importance in terms of understanding how epithelial polarity is generated, maintained, and lost. In this regard, considerable attention has been drawn to the family of small ras-like GTPases, or rabs, members of which have been shown to influence essentially all known vesicular transport processes in eukaryotic cells (3). However, recent evidence also suggests a role for the larger, heterotrimeric G proteins, traditionally associated with signal transduction pathways. These proteins have been implicated in both constitutive (4, 36, 41) and regulated (4) secretion, endocytosis (13), endosome-endosome fusion (18), and transcytosis (5, 8). In epithelia, it has been proposed that two members of this family, G_{α} and $G_{i\alpha}$, regulate membrane transport in the apical and basolateral directions, respectively. Pertussis toxin, which by ADP-ribosylation of the α subunit inactivates members of the Gi-Go subclass of heterotrimeric G proteins, has been reported to increase transport of newly synthesized protein to the basolateral surface of LLC-PK and MDCK cells (36, 41). Similarly, experiments with cholera toxin, which activates G_{α} (again by ADP-ribosylation), have implicated G_{α} in both apically directed secretion (36) and in basolateral to apical transcytosis of the pIgR in MDCK cells (5, 8). The role of G_{α} in transcytosis, however, remains unclear. Whereas Bomsel and Mostov (8) found that cholera toxin stimulated transcytosis of the pIgR, Barroso and Sztul (5) reported that transcytosis of the receptor was inhibited by cholera toxin. Both groups agreed, however, that G_{α} affected transcytosis independently of adenylyl cyclase, its classical downstream effector in signal transduction. Based on the involvement of G_{α} in transcytosis of the pIgR, Bomsel and Mostov proposed a model in which pIgR activates G_{α} and activated G_{α} mediates incorporation of the pIgR into transcytotic vesicles through direct interaction with a putative transcytotic sorter (7).

We have investigated a possible role for heterotrimeric G proteins and their downstream effectors in transcytosis in MDCK cells. The combined use of two markers, pIgR and ricin, has made it possible to distinguish between effects on receptor-mediated (pIgR) and bulk membrane (ricin) transport. Using this approach, we demonstrate that stimulation of transcytosis by cholera toxin is not limited to specific receptors but results from an increase in flow of bulk membrane along the transcytotic pathway. Contrary to previous reports, we show that G_{α} stimulates transcytosis by elevation of cellular cAMP through direct activation of adenylyl cyclase, as both forskolin and 8BrcAMP augment transcytosis. Moreover we show that the effect is exerted at a late stage in the pathway after translocation of transcytosing molecules into the apical cytoplasm. Interestingly, forskolin and 8BrcAMP also stimulate the apical secretion of an endogenous glycoprotein, gp 80. The effects of cAMP on transcytosis and apical secretion appear to be mediated via PKA as they are both strongly reduced in the presence of H-89, a specific inhibitor of the kinase. Finally, to further address whether G_{α}

stimulates transcytosis by a cAMP-independent mechanism, we generated stable MDCK cell lines overexpressing either the wt G_{α} , or a mutant (Q227L) which constitutively activates adenylyl cyclase. The mutant Q227L enhanced transcytosis significantly more than wt G_{α} , but neither construct was as efficient as raising cellular cAMP in control cells. These data combine to suggest that cholera toxin, and possibly Gs-coupled receptors, can stimulate both apically directed transcytosis and secretion via their activation of adenylyl cyclase, rather than through a direct interaction of G_{α} with the cellular sorting machinery.

Materials and Methods

Reagents

These were obtained from the following sources: 1,9-dideoxyforskolin (DDF), forskolin (FSK), 3-isobutyl-1-methyl-xanthine (IBMX), 8-bromo-adenosine 3':5'-cyclic monophosphate, sodium salt (8BrcAMP; B-7880) from Sigma Chemical Company (St. Louis, MO). Cholera toxin and pertussis toxin from List Biological Laboratories Inc. (Campbell, CA). Ro 20-1724 (4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone) from GIBCO BRL (Gaithersburg, MD). H-89 (N-[2-[p-bromocinnamylamino]ethyl]-5-isoquinolinesulfonamide) from LC Laboratories (Woburn, MA). DDF, FSK, IBMX, and H-89 were all dissolved in DMSO as a 1000 \times stock and stored at -20°C . 8BrcAMP was dissolved in PBS and stored at -20°C . Cholera toxin and pertussis toxin were reconstituted with sterile distilled water and stored at 4°C . Ro 20-1724 is supplied as an aqueous solution (2 mM with 0.7% DMSO) and was stored at room temperature (rt).

Mutagenesis of Epitope-tagged G_{α} and Expression in MDCK Cells

cDNA encoding the 52-kD form of G_{α} tagged with an epitope from the hemagglutinin of influenza virus (29) was kindly provided in pCDNA-I by Dr. Frank Kolakowski (Massachusetts General Hospital East, Charlestown, MA). The HA epitope is comprised of residues 76–82 of the hemagglutinin (DVPDYAS) and is located within the 14-amino acid insert which distinguishes the 45 from the 52-kD form of G_{α} (29). The two forms do not differ substantially in function (29). The Q227L mutant, which has been previously shown to constitutively activate adenylyl cyclase (16, 35) was generated by PCR mutagenesis in which the glutamine codon (CAG) at position 227 in wt G_{α} was converted to a leucine codon (CTA). A 449-bp Eco RI, Bgl II fragment of the amplified sequence was ligated into place of the corresponding wt sequence. The cDNAs encoding normal and mutant HA- G_{α} were excised from pCDNA-I and ligated into the vector pCB7 which encodes a hygromycin B selectable marker. Finally, the sequence of the amplified fragment containing the Q227L mutation was confirmed by dideoxy sequencing (40).

MDCK cells expressing the rabbit pIgR (33) were transfected with wt G_{α} or Q227L (in pCB7) by the calcium phosphate coprecipitation method and stable transfectants isolated following selection with hygromycin B (Boehringer Mannheim Corp., Mannheim, Germany). Resistant clones were screened by Western blotting as well as by immunoprecipitation from metabolically labeled cells.

Cell Culture

MDCK II cells expressing rabbit pIgR alone or coexpressing either wt or Q227 G_{α} were grown in DME supplemented with 10% FCS and antibiotics, and maintained in 5% CO_2 at 37°C . The cells were cultured in 100-mm plates and split 1:10 three times in two weeks with one change of medium between each split. For transcytosis experiments, cells were plated at confluent density on Transwell polycarbonate filters with 0.4- μm pore size (Costar Corp., Cambridge, MA). One confluent 100-mm dish was used to plate 24 12-mm filters and the cells were cultured for 5 d before performing experiments, with two changes of the apical and basal medium in between. In experiments involving wt G_{α} or Q227L, all cells were induced with 10 mM sodium butyrate overnight. Confocal microscopy confirmed that the cells plated under these conditions formed a homogeneous monolayer. The polarity of all clonal cell lines was assessed by the predominantly apical secretion of an endogenous sulfated glycoprotein, gp 80 (28), determined es-

entially according to Urban et al. (42) except that metabolic labeling was performed with ³⁵S-Express (Dupont NEN, Boston, MA).

Transcytosis Experiments

Human dimeric IgA (dIgA) was prepared as previously described (9) from the serum of a patient with IgA myeloma, generously provided by Dr. Per Bratzaeg (Rikshospitalet, Oslo, Norway). dIgA, ricin (RCA60; Sigma Chem. Co.) and BSA were iodinated by the chloramine T method to a specific activity of $\sim 2 \times 10^4$ cpm/ng for dIgA and BSA, and 1×10^4 cpm/ng for ricin.

Filters were rinsed twice with Hank's Buffered Saline containing calcium, magnesium, and 0.6% BSA (HBSA) at 37°C prior to experiments. Transcytosis experiments were performed with 400 and 600 μ l HBSA in the apical and basal chambers, respectively. Treatment of cells with drugs or toxins (always in both chambers simultaneously) was performed as summarized in the figure legends. In continuous transcytosis experiments with dIgA, filters were incubated with 400 ng/ml ¹²⁵I-dIgA in the basal chamber for 60 min at 37°C. The basal side of the filters was then rinsed four times with ice-cold HBSA. Transcytosed ligand was recovered by collecting the apical medium together with 500 μ l ice-cold HBSA used to rinse the apical side of the filter once. The filters with cells containing the cell-associated ligand were next excised from their support. The medium containing transcytosed ligand and the filters were counted in a LKB 1275 mini gamma counter (Pharmacia LKB, Piscataway, NJ). Transcytosis of dIgA was expressed as the ratio of transcytosed counts to cell-associated counts.

In a basolateral to apical transcytosis experiment with ricin, 250 ng/ml ¹²⁵I-ricin was added to the basal chamber for 30–90 min at 37°C. The basal side of the filter was then rinsed six times with ice-cold HBSA and the apical medium containing soluble transcytosed ligand was harvested. Membrane-associated transcytosed ligand and ¹²⁵I-ricin bound to the basolateral membrane were removed by incubating 5 min at 37°C with 0.1 M lactose, which competes for galactose-binding domains on the ricin molecule (43). This treatment released $\geq 98\%$ of ricin bound to either surface in 1 h at 4°C. The apical media containing soluble and membrane-associated transcytosed ligand were pooled. The basal medium containing bound ¹²⁵I-ricin and the filter-grown cells were collected separately. Transcytosed ricin was expressed as the ratio of counts released into the apical medium plus counts removed from the apical membrane with 0.1 M lactose to counts removed from the basolateral membrane with 0.1 M lactose plus counts remaining in the cells. Apical to basolateral transcytosis was assessed similarly except for the reversed polarity.

17°C experiments were carried out with addition of 700 ng ¹²⁵I-dIgA or 400 ng ¹²⁵I-ricin to the basal chamber. In a typical experiment, filter-grown MDCK cells were pulsed with iodinated ligand added to the basal chamber for 4 h at 17°C to allow endocytosis and translocation of ligand into the apical cytoplasm (25). The basal side of filters was then rinsed six times and the cells were incubated with or without IBMX for the last 30 min and FSK for the last 15 min at 17°C. Apical release of internalized ligand was assessed by warming the cells to 37°C for either 15 or 60 min. In experiments with ¹²⁵I-ricin, the chase medium included 0.1 M lactose in the apical and basal chambers to prevent endocytosis of membrane-associated ricin from either surface. Transcytosis of ricin and dIgA was expressed as in continuous transcytosis experiments. More than 95% of transcytosed counts could be precipitated by TCA in continuous as well as in 17°C pulse-chase transcytosis experiments. Essentially no counts were recovered from the apical medium after 4 h at 17°C. It was verified that the number of counts inside the cells before the chase were identical whether the cells had been exposed to drugs or not. There are two technical points which should be addressed with regard to the 17°C experiments. First, the basal level of transport could vary by up to twofold between different experiments carried out according to this protocol. However there was always very significant stimulation in transport by IBMX+FSK which, expressed as % control, was essentially constant. Second, H-89 did not give consistent results when used at 17°C as opposed to 19.5 and 37°C. This property of H-89 is most likely explained by temperature-dependent changes in the physicochemical properties of lipid bilayers that prevent access of the lipophilic compound to PKA at low temperature.

Apical Recycling

Measurement of apically recycling dIgA was performed essentially as described by Breitfeld et al. (11). Briefly, filter-grown cells were allowed to internalize ¹²⁵I-dIgA (500 ng/ml) from the apical medium for 30 min at 37°C. Cells were then cooled to 4°C by addition of cold HBSA and in-

cubated for 60 min at this temperature in HBSA containing 20 μ g/ml trypsin to remove cell surface ligand. Proteolysis was stopped by washing filters three times in cold HBSA containing 50 μ g/ml soybean trypsin inhibitor. Cells were then warmed to 17°C with HBSA in the presence or absence of 500 μ M IBMX, 1 μ M FSK for 15 min, and next to 37°C with or without drugs. Apical and basal media were harvested at each time point and the filters cut from their holders for gamma counting.

To measure ricin recycling, ¹²⁵I-ricin (400 ng/ml) was internalized from the apical medium as above. Noninternalized ricin was then removed from the apical surface by incubation for 5 min with 0.1 M lactose. Cells were then incubated for 15 min at 17°C in the presence or absence of IBMX + FSK as above. Finally, cells were warmed to 37°C in the continuous presence of 0.1 M lactose plus or minus IBMX+FSK. Apical, basal media and cells were harvested at each time point and counted.

Preparation of Membranes and Cytosol from MDCK Cells

To determine the subcellular distribution of wt and Q227L G α , 100-mm confluent plates with MDCK cells expressing the pIgR alone or coexpressing either wt G α or Q227L were rinsed twice with PBS, the cells scraped off in 1 ml PBS, pelleted in a microfuge, and resuspended in 1 ml of ice cold 3 mM imidazole, 300 mM sucrose, pH 7.4. The resuspended cells were passed 40 times through a 25-gauge syringe. Virtually 100% of the cells were lysed under these conditions, as assessed by trypan blue staining. The nuclei were then pelleted by centrifugation at 14,000 g for 5 min in a tabletop centrifuge, and the supernatant further centrifuged at 105,000 g for 1 h in a Beckman tabletop ultracentrifuge using a TLA 100-3 rotor. The pellet and supernatant from this centrifugation were taken to represent membranes and cytosolic proteins, respectively. To prepare the samples for immunoprecipitation and Western blotting, the membranes were resuspended in 1 ml of SDS lysis buffer (0.5% SDS, 50 mM Tris, pH 8.1, 100 mM NaCl, 5 mM EDTA, and 0.2% Na₃N) by vortexing. The concentrations of Tris, SDS, NaCl, and EDTA in cytosolic fraction were adjusted to that of the SDS lysis buffer, and both sets of samples boiled for 3 min.

Immunoprecipitation and Western Blotting

1-ml samples lysed in 0.5% SDS lysis buffer were diluted with 0.5 ml 2.5% Triton dilution buffer (100 mM Tris, pH 8.6, 100 mM NaCl, 5 mM EDTA, 0.02% Na₃N), and tumbled with 50 μ l of a 10% slurry of protein A-Sepharose for 30 min at rt. The Sepharose beads were spun down and the supernatant incubated with 12CA5, a mouse monoclonal antibody to the HA-tag (IgG_{2b}/ κ ; BABCO, Berkeley, CA), diluted 1:500, for 2 h at rt with addition of 50 μ l 10% slurry of protein A-Sepharose for the last hour. The beads were then washed four times with mixed micelle buffer (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 8% sucrose 10 μ g/ml Trasylol, 20 mM triethanolamine HCl, pH 8.6) and two times with the same buffer without detergents. Precipitated proteins were resolved by SDS-PAGE (10% gels), and transferred to nitrocellulose. Membranes were blocked in Blotto (5% nonfat dry milk, 0.1% Triton X-100, and PBS), and probed with purified 12CA5, a kind gift from Dr. Michael Shia (Boston University, Boston, MA), diluted 1:2,000, overnight at 4°C. Finally, the membranes were incubated 1 h at rt with sheep anti-mouse/HRP (Amersham Corp., Arlington Heights, IL) 1:2,000, and antigens finally visualized on film by enhanced chemiluminescence (Renaissance; Dupont NEN). Results were quantitated by laser densitometry (Ultrascan XL; LKB).

Results

Cholera Toxin Stimulates Transcytosis of Ricin in MDCK Cells

In a recent report, Bomsel and Mostov (8) have presented evidence that cholera toxin (CT) stimulates transcytosis of the pIgR. To investigate whether this effect was selective for the pIgR or reflected a general stimulation in the flow of membrane along the transcytotic pathway, we examined the effects of CT on transcytosis of ¹²⁵I-ricin. Filter-grown MDCK cells expressing the pIgR were preincubated with CT (10 μ g/ml) for 60 min, followed by a 60-min incubation at 37°C with ¹²⁵I-ricin (200 ng/ml) in either the basal or apical

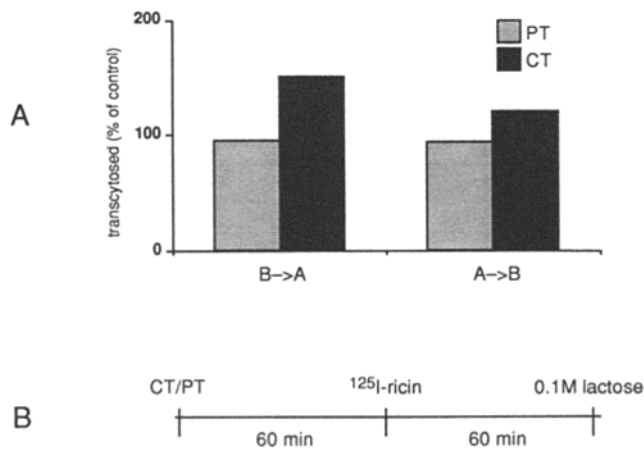


Figure 1. CT but not PT stimulates transcytosis of ricin. (A) Monolayers of filter-grown pIgR-expressing MDCK II cells were preincubated with 10 $\mu\text{g/ml}$ PT (gray bars) or CT (black bars) in both apical and basal chambers for 60 min at 37°C. ^{125}I -ricin (250 ng/ml) was then added to the basal or the apical side of the filter, and the monolayers incubated for another 60 min at 37°C in the continuous presence of toxins. The medium containing soluble transcytosed ^{125}I -ricin was harvested and membrane-associated ^{125}I -ricin recovered by incubating the apical or basolateral surface with 0.1 M lactose as described in Materials and Methods. Finally, the filters with cells were excised from their support and counted directly. (B→A) Basolateral to apical transcytosis; (A→B) apical to basolateral transcytosis. The data were first expressed as the ratio of transcytosed to cell associated (bound + internalized) counts and then normalized to nontreated cells (100%) from the same experiment. The results are representative of at least three separate experiments with a coefficient of variation less than 10% in the data shown. (B) Summary of the protocol for these experiments.

medium. As shown in Fig. 1, preincubation with CT for 60 min at 37°C increased basolateral to apical transcytosis of ^{125}I -ricin by 50% relative to untreated controls. Interestingly, CT also increased apical to basolateral ricin transport, although to a much smaller extent (~15%, Fig. 1). Treatment with an equivalent concentration of pertussis toxin, however, had no detectable effects on transcytosis in either direction (Fig. 1). To ensure that the increase in ricin transcytosis was not due to paracellular transport, we measured both the transepithelial electrical resistance and the permeability to a fluid phase marker, ^{125}I -BSA, in parallel monolayers. Neither parameter was affected by CT over the time course of the experiment (Fig. 2; Table I).

Stimulation of Transcytosis by Cholera Toxin Is Mediated by Adenylyl Cyclase

The best characterized downstream effector of $\text{G}\alpha$ is adenylyl cyclase, which responds to activated $\text{G}\alpha$ by stimulating cAMP synthesis. To determine whether the observed effects of CT were mediated by cAMP, we performed transcytosis assays in the presence of forskolin (FSK, 1 μM), a diterpene that activates adenylyl cyclase directly, bypassing $\text{G}\alpha$. To inhibit cAMP breakdown, cells were also treated with the phosphodiesterase inhibitor IBMX (500 μM). In a 1-h continuous transcytosis assay, treatment with IBMX alone stimulated apically directed transcytosis of ricin by 30–40%, while the combination of IBMX and FSK elicited an increase that ranged from 60–100%. Data from a repre-

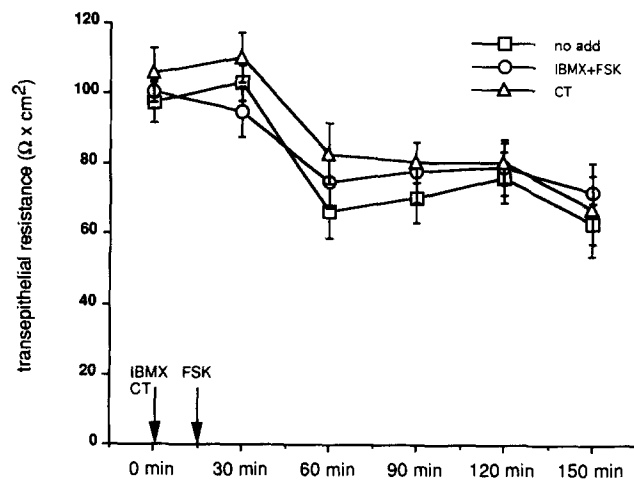


Figure 2. Transepithelial resistance in pIgR-expressing MDCK II cells in the absence (squares) or presence of 10 $\mu\text{g/ml}$ CT (triangles), or 500 μM IBMX and 1 μM FSK (triangles). The data are mean \pm SD ($n = 4$) from an experiment representative of three separate experiments.

sentative experiment are shown in Fig. 3 A. A time-course showed that the stimulation (in %) was similar after 30, 60, and 90 min of incubation with ^{125}I -ricin (data not shown). In agreement with the experiments described in Fig. 1, apical to basolateral transcytosis was stimulated to a lesser extent (20–40%; Fig. 3 B). Substitution of FSK with DDF, an analogue which does not activate adenylyl cyclase, did not stimulate transcytosis more than treatment with IBMX alone. This is an important control, as both FSK and DDF have been reported to antagonize the effects of brefeldin A on membrane traffic (30). Similarly, it was formally possible that IBMX, which is a xanthine derivative, could stimulate transcytosis by binding to adenosine receptors on the cell surface. This possibility was ruled out by substituting IBMX with 20 μM of the drug Ro 20-1724, a nonxanthine phosphodiesterase inhibitor which does not interact with adenosine receptors. In four separate experiments, the combination of 20 μM Ro 20-1724 and 1 μM FSK increased basolateral to apical transcytosis by 73, 100, 104, and 106%. Thus results obtained in experiments with Ro 20-1724 were similar to those utilizing IBMX.

IBMX+FSK had no detectable effect on the amount of ricin internalized from the basolateral surface during the 60-

Table I. Permeability of Filter-grown MDCK Cells to ^{125}I -BSA

Treatment	cpm in apical medium
No addition	103 \pm 25
CT	113 \pm 80
IBMX + FSK	89 \pm 10
EGTA	32,800 \pm 1,700

pIgR-expressing MDCK cells cultured 5 d on filters were preincubated at 37°C with either HBSA only, or HBSA containing 10 $\mu\text{g/ml}$ CT for 60 min, or 500 μM IBMX for 15 min followed by 500 μM IBMX and 1 μM FSK for another 15 min, or 5 mM EGTA for 30 min. Next, 250 ng/ml (2×10^4 cpm/ng) ^{125}I -BSA was added to the basal medium (600 μl) of each filter and the cells incubated for 60 min at 37°C. Finally, the apical medium (400 μl) was collected and counted directly to determine the effect of additives on the permeability of the monolayers to ^{125}I -BSA.

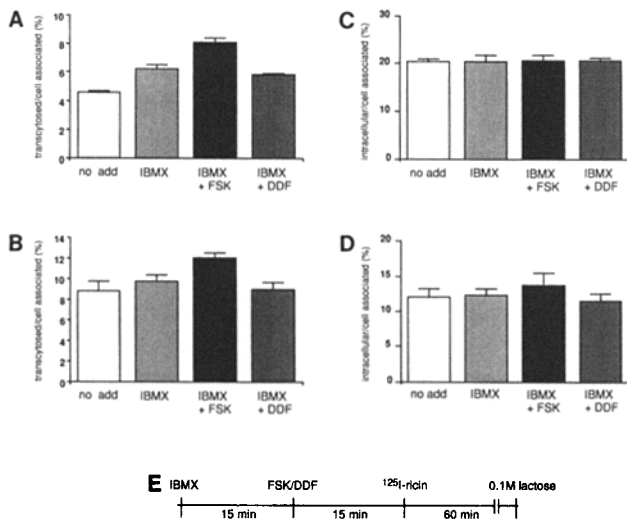


Figure 3. Effect of IBMX, FSK, and DDF on transcytosis of ricin. (A–D) Monolayers of filter-grown pIgR-expressing MDCK II cells received either no pretreatment (white bars) or 500 μ M IBMX for 15 min at 37°C followed by either IBMX alone, or in combination with either 1 μ M FSK or 1 μ M DDF for another 15 min at 37°C. Next, 250 ng/ml ¹²⁵I-ricin was added to the basal or the apical side of the filter and the cells incubated for 60 min at 37°C. Soluble, membrane-bound and intracellular ¹²⁵I-ricin were measured as in Fig. 1. Data are expressed as the ratio of transcytosed to cell-associated (bound + internalized) counts. (A) Basolateral to apical transcytosis. (B) Apical to basolateral transcytosis. (C) Intracellular accumulation of ¹²⁵I-ricin administered from the basal side. (D) Accumulation of ¹²⁵I-ricin administered from the apical side. The data shown are mean \pm SD ($n = 3$), from an experiment representative of at least three separate experiments. (E) Summary of the protocol for these experiments.

min assay period. However, apical internalization appeared to be stimulated slightly (Fig. 3, C and D), suggesting that apical and basolateral endocytosis may be differentially regulated. Treatment of cells with IBMX+FSK also increased transcytosis of ¹²⁵I-dIgA by 50% (Fig. 4), demonstrating that the stimulatory effect was similar for both receptor-mediated and bulk membrane transcytosis. If anything the increases observed for ricin were larger than those obtained with dIgA. The increase in transcytosis of ricin and dIgA in MDCK cells mediated by IBMX+FSK clearly distinguishes this effect from that of BFA, which inhibits transcytosis of dIgA (26) but stimulates transport of ricin (37).

To obtain direct evidence for a stimulatory role of cAMP in transcytosis, cells were incubated with increasing concentrations of 8BrcAMP, a membrane-permeable analogue of cAMP. In the presence of IBMX, 100–500 μ M 8BrcAMP stimulated transcytosis to the same extent as FSK (Fig. 5), with no additional increase observed at higher concentrations (data not shown). It should be noted that in order to obtain a reproducible stimulation in transcytosis with 8BrcAMP of the magnitude reported here, it was crucial to use the sodium salt as opposed to the free acid form of the drug. In initial experiments with the free acid, no stimulus in transport was observed at concentrations \leq 500 μ M. At higher concentrations, an inhibition of transport was observed, probably resulting from acidification of the cytosol, which is known to inhibit other vesicular transport processes

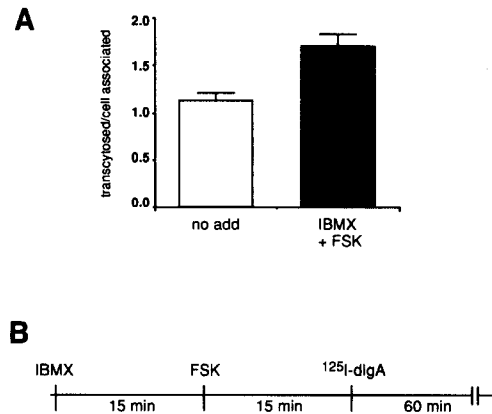


Figure 4. IBMX and FSK stimulate transcytosis of dIgA. (A) Monolayers of filter-grown pIgR-expressing MDCK II cells received either no pretreatment or 500 μ M IBMX for 15 min at 37°C followed by IBMX in combination with 1 μ M FSK for another 15 min at 37°C. Next, 400 ng/ml ¹²⁵I-dIgA was added to the basal chamber and the cells incubated for 60 min at 37°C before harvesting of the apical media. Filters were washed and cut from their holders for counting. The data are expressed as the ratio of transcytosed and cell-associated (bound + internalized) counts, and are the mean \pm SD ($n = 3$) from an experiment representative of at least three separate experiments. (B) Summary of the protocol for these experiments.

including clathrin-dependent endocytosis and transport from the TGN to the cell surface (19, 23, 24, 39).

The data presented above combine to suggest that the previously reported stimulatory effect of CT on transcytosis is mediated solely through elevation of cellular cAMP. The larger increases observed in the present work with IBMX+FSK as compared to CT alone are most likely due to inhibition of phosphodiesterase activity, since CT in combination with IBMX was equally efficient as IBMX+FSK in stimulating transcytosis (data not shown). The data presented here further show that the effects of CT and cAMP on transcytosis are not specific for the pIgR but due to a more general increase in membrane flow along the transcytotic pathway. Moreover, this effect was polarized, as apically directed transport was enhanced to a much larger extent than apical to basolateral transcytosis.

cAMP Stimulates Transcytosis via Protein Kinase A

One of the primary effectors of cellular cAMP is the cAMP-dependent protein kinase, PKA (20). To address whether this kinase mediates the stimulation in transcytosis observed after elevation of cellular cAMP, cells were incubated with the isoquinolinesulfonamide drug H-89, a selective inhibitor of PKA (17), before and during incubation with IBMX+FSK. At a concentration of 30 μ M, H-89 almost completely abolished the increase in transcytosis of ricin obtained in the presence of IBMX+FSK (Fig. 6). This result strongly suggests that activation of PKA is required to stimulate transcytosis. It should be noted that treatment of cells with H-89 alone did not significantly affect the basal level of transcytosis, suggesting that PKA is not a major component of the constitutive transcytotic machinery.

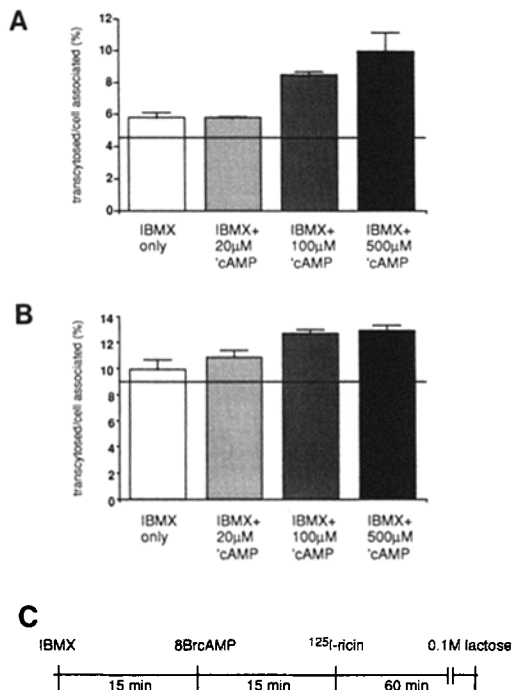


Figure 5. 8BrcAMP stimulates transcytosis of ricin. (A and B) Monolayers of filter-grown pIgR-expressing MDCK II cells received 500 μM IBMX for 15 min at 37°C followed by either IBMX alone or 500 μM IBMX in combination with 20, 100, or 500 μM 8BrcAMP for 15 min at 37°C. Next, 250 ng/ml ¹²⁵I-ricin was added to the basal or the apical side of the filter and the cells incubated for 60 min at 37°C before treatment with 0.1 M lactose to recover bound, internalized, and transcytosed ¹²⁵I-ricin as described in Fig. 1. The horizontal line represents the value for transcytosis in untreated cells (Fig. 4). (A) Basolateral to apical transcytosis. (B) Apical to basolateral transcytosis. The data shown are mean ± SD (n = 3) from an experiment representative of at least three separate experiments. (C) Summary of the protocol for these experiments.

cAMP Acts at a Late Stage in Transcytosis

To localize the cellular site at which cAMP/PKA acts to stimulate transcytosis, we utilized a 17°C temperature block (25). At this temperature, dIgA is internalized from the basolateral plasma membrane, passes through basolateral endosomes and is transported to the apical cytoplasm. However, exocytosis at the apical surface is markedly reduced, resulting in the accumulation of ligand in the apical region of the cell (5, 25). In our experiments, pIgR-expressing MDCK cells cultured on filter supports were incubated with either ¹²⁵I-dIgA or ¹²⁵I-ricin from the basal side for 4 h at 17°C. The monolayers were then washed, incubated with or without IBMX+FSK, and then warmed to 37°C for either 15 or 60 min in the presence or absence of IBMX+FSK. After 15 min at 37°C, apical release of both IgA (Fig. 7 A) and ricin (Fig. 7 B) was stimulated nearly threefold in cells treated with IBMX+FSK, compared to nontreated controls. The rapid rate of release, combined with the previously demonstrated accumulation of ligand in the apical cytoplasm strongly suggests that cAMP acts at a late stage in transcytosis. The effects of IBMX+FSK under both sets of conditions were much less pronounced at the later time point, 60 min

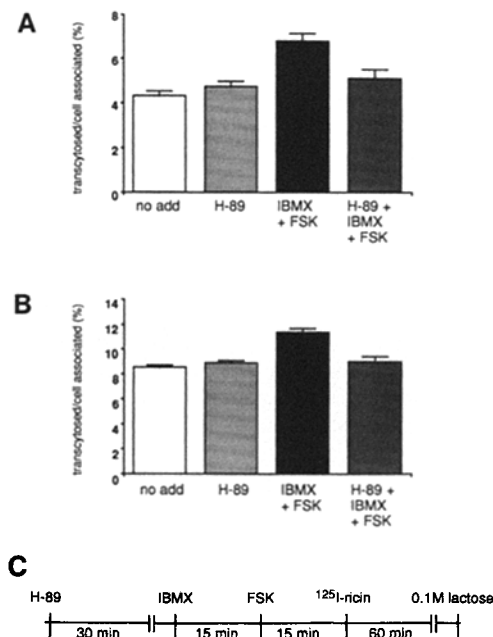


Figure 6. cAMP stimulates transcytosis of ricin through PKA. (A and B) Monolayers of filter-grown pIgR-expressing MDCK II cells received either no pretreatment, pretreatment with 500 μM IBMX and 1 μM FSK, or 30 μM H-89 for 30 min at 37°C followed by either H-89 only, or H-89 in combination with 500 μM IBMX and 1 μM FSK for an additional 30 min at 37°C. Next, 250 ng/ml ¹²⁵I-ricin was added to the basal or the apical side of the filter and the cells incubated for 60 min at 37°C in the continuous presence of the drugs. Bound, internalized, and transcytosed ¹²⁵I-ricin was assayed as described in Fig. 1. (A) Basolateral to apical transcytosis. (B) Apical to basolateral transcytosis. The data shown are mean ± SD (n = 3) from an experiment representative of at least three separate experiments. (C) Summary of the protocol for these experiments.

(Fig. 7), suggesting that the primary effect of IBMX+FSK treatment in pulse-chase experiments was to increase the rate rather than the amount of release. Basolateral recycling of ¹²⁵I-dIgA under these conditions was identical both in the presence and absence of IBMX+FSK (data not shown).

One possible interpretation of these data is that cAMP/PKA serves to regulate the fusion of transcytotic vesicles directly with the apical plasma membrane. However, it has recently been proposed that transcytosis of dIgA occurs indirectly (2, 5), involving the fusion of transcytotic vesicles with apical endosomes, after which transport to the apical surface is accomplished via recycling vesicles. If cAMP/PKA were acting on either budding of recycling vesicles from apical endosomes, or fusion of these vesicles with the apical membrane, one would predict that transcytosis and apical recycling would be stimulated to the same degree. Just such a phenomenon has been observed in MDCK cells treated with the phorbol ester PMA (12). To determine whether this is also true for cAMP-mediated stimulation, we measured the recycling of both dIgA and ricin, internalized for 30 min from the apical cell surface at 37°C. As described previously (11, 12), a substantial pool of uncleaved pIgR molecules exists at the apical plasma membrane in these cells, and can be used to efficiently internalize dIgA. Surprisingly, apical exocytosis of both ¹²⁵I-dIgA (5%; Fig. 8, A

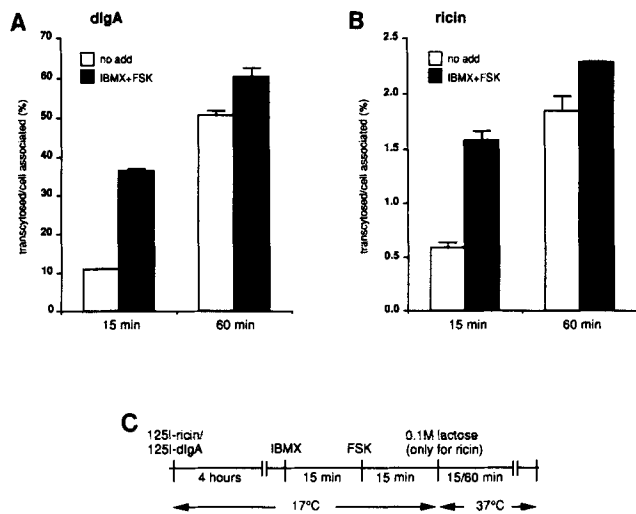


Figure 7. IBMX and FSK stimulate transport at a late stage in the transcytotic pathway. Monolayers of filter-grown pIgR-expressing MDCK II cells were pulsed with either 700 ng/ml ^{125}I -dIgA (A) or 400 ng/ml ^{125}I -ricin (B) in the basal chamber for 4 h at 17°C. The basal side was then rinsed extensively, and the cells incubated in medium alone or treated with 500 μM IBMX for 15 min at 17°C followed by another 15 min with IBMX and 1 μM FSK. The monolayers were next chased for 15 or 60 min at 37°C in prewarmed medium. In experiments with ^{125}I -ricin, the chase was performed with medium containing 0.1 M lactose (in both chambers). Finally, the internalized, transcytosed, and in the case of ^{125}I -dIgA, recycled ligand was recovered. The data shown are mean \pm SD ($n = 3$) from an experiment representative of at least three separate experiments. (C) Summary of the protocol for these experiments. It should be noted that similar results were obtained when IBMX+FSK were added simultaneously at 17°C.

and B) and ^{125}I -ricin (20%; Fig. 8, C and D) were stimulated to a much smaller degree following apical endocytosis than when the same ligands were internalized from the basolateral surface ($\sim 200\%$; Fig. 7). These findings show that the effect of cAMP/PKA on transcytosis cannot simply be explained by a stimulation in budding of recycling vesicles from apical endosomes or fusion of these recycling vesicles with the apical plasma membrane.

Overexpression of G α

To further test the possibility that G α stimulates transcytosis by a cAMP-independent mechanism we generated stable MDCK cell lines coexpressing the pIgR and either the wt G α subunit or a constitutively active mutant Q227L (16, 35). Both G α constructs contained an epitope tag consisting of seven amino acids from the hemagglutinin of the influenza virus (29), which allows detection of the exogenous protein against the background of endogenous G α subunits.

Cells expressing either the pIgR alone, or coexpressing the wt or constitutively active (Q227L) G α subunit were allowed to internalize ^{125}I -ricin or ^{125}I -dIgA from the basolateral surface at 17°C as described above. As shown in Fig. 9 A, overexpression of wt G α had a moderate stimulatory effect on transcytosis of both ligands ($\sim 30\%$). This was expected since it had previously been shown that overexpression of G α stimulates cAMP synthesis in both COS cells and NIH 3T3 cells (16, 35). Moreover, the Q227L mutation

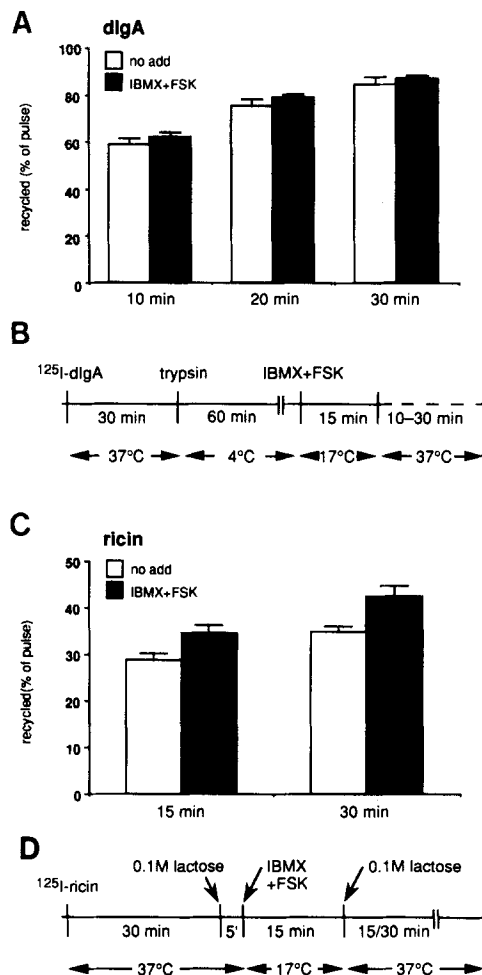


Figure 8. Effect of IBMX and FSK on apical recycling. (A) Monolayers of filter-grown pIgR-expressing MDCK II cells were loaded with 700 ng/ml ^{125}I -dIgA from the apical side for 30 min at 37°C, then washed and cooled to 4°C, and incubated with trypsin for 1 h to remove noninternalized ligand. Cells were then warmed to 17°C and treated with or without a combination of IBMX (500 μM) and FSK (1 μM) for 15 min. Finally, filters were warmed to 37°C in the continuous presence or absence of the drugs. The apical and basal media and cells were harvested at each time point and counted. (B) Cells were loaded with 400 ng/ml ^{125}I -ricin from the apical side for 30 min at 37°C, then noninternalized lectin removed with 0.1 M lactose. Cells were then treated with or without IBMX and FSK for 15 min at 17°C and chased in medium containing 0.1 M lactose for 15–30 min at 37°C in the presence or absence of IBMX and FSK. Finally, the recycled, transcytosed, and intracellular ligand was recovered. The data are presented as a percentage of the total internalized cpm. Shown are the means \pm SD ($n = 3$) from an experiment representative of at least three separate experiments. B and D summary of the protocol for the experiments in A and C, respectively.

increased transepithelial transport of both ^{125}I -ricin and ^{125}I -dIgA approximately twofold more than cells expressing an equivalent amount of wt G α (Fig. 9 A). Neither cell line, however, exhibited an increase in transcytosis equivalent to that observed in cells treated with IBMX+FSK. This was confirmed in continuous transcytosis experiments at 37°C where the Q227L mutant increased transcytosis of ^{125}I -ricin by 30% whereas overexpression of wt G α had no effect on

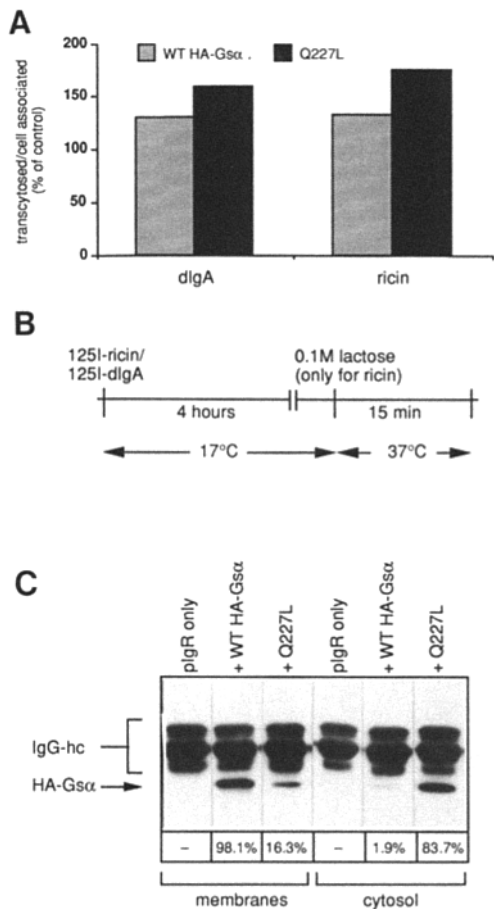


Figure 9. Overexpression of G α subunits. (A) MDCK II cells expressing either rabbit plgR only or coexpressing either wt HA-G α or the Q227L mutant were subjected to a 17°C transcytosis assay essentially as described in Fig. 7. The data were first expressed as the ratio of transcytosed to cell associated (bound + internalized) counts and then normalized to cells expressing plgR only (100%) from the same experiment. The results are representative of three separate experiments with 125 I-ricin and 125 I-dIgA, respectively, with a coefficient of variation less than 10% in the data shown. (C) Membrane and cytosolic fractions prepared from MDCK II cells expressing rabbit plgR alone, or coexpressing either wt HA-G α or Q227L HA-G α were suspended in detergent-containing buffer and the epitope tagged subunits recovered by immunoprecipitation using a mouse monoclonal antibody to the HA-tag (see Materials and Methods for details). Immunoprecipitates were resolved by SDS-PAGE and detected by Western blotting using the same antibody. The content of HA-G α was quantitated by densitometry. The total amount of HA-G α (membranes + cytosol) differed by less than 15% between wt G α and Q227L, and the total amount of protein varied by less than 10%.

transport (data not shown). These data suggest that G α does not play a significant role in transcytosis that is independent of adenylyl cyclase.

Interestingly, we found that the wt G α and the Q227L mutant were distributed reciprocally in membranes and cytosol prepared from transfected MDCK cells (Fig. 9 C). The epitope-tagged subunits were recovered from membrane and cytosolic fractions by immunoprecipitation as described in Materials and Methods. Immunoprecipitates were then transferred to nitrocellulose and probed with the same

monoclonal antibody. Whereas the wild-type protein was found almost exclusively (98%) in the membrane fraction, the majority (84%) of Q227L was recovered from the cytosol (Fig. 9 C). This distribution is consistent with the findings of Levis and Bourne (29) who previously showed that a different constitutively active mutant, R201C, was also predominantly cytosolic.

Apical Secretion of an Endogenous Glycoprotein Is Also Stimulated by cAMP

G α has recently been implicated in apically directed secretion in MDCK cells (36). In that study, cholera toxin and aluminum fluoride were shown to stimulate the apical secretion of endogenous proteins and the transport of the influenza virus hemagglutinin (HA) to the apical cell surface, respectively. This stimulus was reported to occur independently of cAMP, based on the observation that 100 μ M dibutyryl cAMP did not stimulate transport.

As apically directed transcytosis was stimulated by treatment of cells with IBMX+FSK, it was of interest to determine whether apically directed secretion was similarly affected. MDCK cells constitutively secrete an endogenous, sulfated glycoprotein termed gp 80 (42). This protein is synthesized as an 80-kD preprotein that undergoes intracellular proteolytic processing and is secreted as a group of 35–45-kD peptides, predominantly from the apical pole of the cell (28).

To determine the effect of elevation of cellular cAMP on apical secretion of gp 80, filter-grown MDCK cells were pulse-labeled with a mixture of [35 S]methionine and [35 S]cysteine for 15 min followed by incubation at 20°C for 2 h to accumulate labeled protein in the TGN (22, 31). During the last 30 min at 20°C, the cells were incubated in the presence or absence of 500 μ M IBMX and, for the final 15 min at 20°C, with or without 500 μ M 8BrcAMP. The cells were then warmed to 37°C for 15 or 30 min in the continuous presence or absence of the drugs. As the gp 80 peptides are the predominant apical secretory products of MDCK cells, samples of media were run directly on SDS-PAGE gels without further purification. As shown in Fig. 10, A and B, at the 15-min time point, apical secretion of gp 80 was stimulated more than fourfold in cells treated with IBMX+8BrcAMP (lane 2) relative to untreated controls (lane 1). This increase was reduced to less than 1.5-fold after 30 min (Fig. 10, A and B, lanes 3 and 4) again suggesting that cAMP enhances the rate rather than the amount of transport in pulse-chase experiments. Essentially identical results were observed when cells were treated with 500 μ M IBMX + 1 μ M FSK (Fig. 9, D and E, lanes 1 and 3). Similar to the effect on transcytosis, this stimulus was completely abrogated in the presence of 30 μ M of the PKA inhibitor H-89 (Fig. 9, D and E, lanes 2 and 4), indicating that the effects of cAMP on apical secretion are also mediated by PKA.

Discussion

In an attempt to identify molecules involved in regulation of vesicular trafficking in polarized epithelial cells, we have addressed the possible involvement of heterotrimeric G proteins in transcytosis in MDCK cells. Our findings may be summarized as follows: first, we found that cholera toxin, but not pertussis toxin enhances transcytosis in MDCK cells,

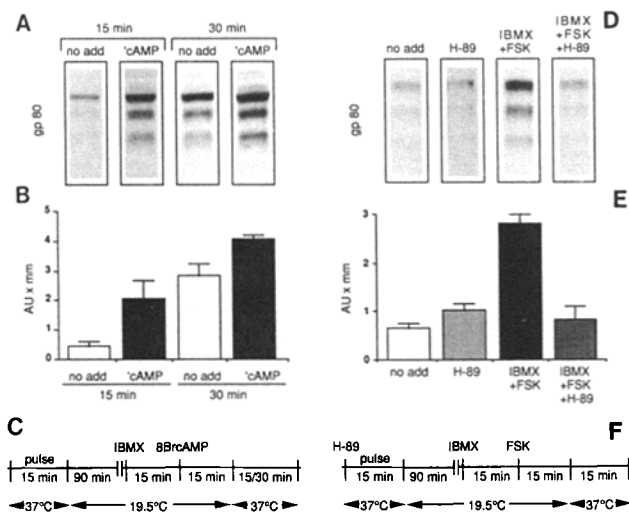


Figure 10. Secretion of gp 80 is stimulated by cAMP and PKA. (A) Monolayers of filter-grown pIgR-expressing MDCK II cells were starved for 20 min at 37°C, pulse-labeled with ³⁵S-Express label (1 mCi/ml) for 15 min at 37°C, and chased for 2 h at 19.5°C in the presence or absence of 500 μM IBMX for the last 30 min and 500 μM 8BrcAMP for the last 15 min at 19.5°C. The cells were then warmed to 37°C for 15 or 30 min at 37°C medium with or without IBMX and 8BrcAMP. The apical medium was harvested and aliquots subjected to SDS-PAGE and fluorography. Under reducing conditions, gp 80 appears as three polypeptides of 35–45 kD. The amount of secreted gp 80 (mean ± SD; n = 3) was quantified by densitometry (B). (C) Summary of the protocol for this experiment. (D) Secretion of gp 80 in cells exposed to medium only, 30 μM H-89, 500 μM IBMX, and 1 μM FSK, or IBMX and FSK in the presence of H-89. The densitometry data shown in E are mean ± SD (n = 3) from an experiment representative of three separate experiments. (F) Summary of the protocol for these experiments.

confirming a stimulatory role for G α , but not G β in apically directed transcytosis. Second, we determined that this stimulus was due to the activation by G α of downstream effectors of the cAMP cascade. Forskolin, which activates adenylyl cyclase directly, or 8BrcAMP, in combination with the phosphodiesterase inhibitor IBMX, stimulated transcytosis to a higher degree than did cholera toxin alone. Furthermore, H-89, a specific inhibitor of protein kinase A, inhibited the effects of FSK on transcytosis, but had no effect on the basal level of transport. Third, transport of both ¹²⁵I-dIgA, a marker for receptor-mediated transcytosis, and ¹²⁵I-ricin, a nonselective marker of membrane glycoproteins and glycolipids with terminal galactose, was increased to a similar extent. Fourth, cAMP appears to act at a late, possibly rate-limiting step in the transcytotic pathway, as demonstrated using a 17°C block. Fifth, recycling of apically internalized ligand was not significantly stimulated. Sixth, cells overexpressing either the wt G α or a constitutively active mutant, Q227L, exhibited enhanced transcytosis, but neither was stimulated to the same degree as cells treated with IBMX+FSK. Finally, treatment of cells with IBMX+FSK or 8BrcAMP stimulated apical secretion of an endogenous MDCK glycoprotein, gp 80, suggesting that secretion and transcytosis may be regulated by similar mechanisms.

In two recently published studies, treatment of MDCK cells with CT has been reported to either inhibit (5) or augment (8) transcytosis of the pIgR. In both studies, it was

reported that the effects of CT on transcytosis did not involve the activation of adenylyl cyclase, but rather, invoked a direct role of G α in vesicular transport. Results obtained in the present study agree with those of Bomsel and Mostov, in that CT increases transcytosis of the pIgR. However, in direct contrast, our data show that effectors downstream of G α in the signal transduction pathway, namely adenylyl cyclase, cAMP, and protein kinase A, mediate the observed stimulus of transcytosis. Moreover, our data show that this stimulus is exerted, not by a direct interaction between G α and the pIgR as previously speculated (8), but rather via an increased flow of membrane along the transcytotic pathway. One possible reason for the discrepancy between the data presented here and those of Bomsel and Mostov is our use of phosphodiesterase inhibitors, which were necessary to maximize stimulation. However, in our hands, FSK alone increased transcytosis with at least the same efficiency as cholera toxin. In any case, the results obtained in this work demonstrate that agents that augment cellular cAMP significantly increase transcytosis in a continuous transcytosis assay, suggesting that a rate-limiting step in transcytosis may be regulated by extracellular cues. In support of this hypothesis, cholinergic agonists such as cholecystokinin and bombesin have been shown to stimulate dIgA secretion in rat intestine (21, 27, 45).

Classically, transcytotic vesicles containing pIgR have been thought to bud from basolateral endosomes and, following transport along microtubules into the apical cytoplasm, fuse directly with the apical plasma membrane (32). Two recent studies, however, have suggested that transcytosing molecules are first routed to an early apical endosomal compartment and subsequently delivered to the apical membrane by recycling vesicles (2, 5). This hypothesis seems to be supported by the finding that phorbol esters, which stimulate the transcytosis of dIgA, also apparently stimulate recycling from apical endosomes (12). However, in our study, we have been unable to demonstrate a significant increase in apical recycling in the presence of elevated cAMP, suggesting that PKA stimulates transcytosis at a site distinct from the site of PKC-mediated stimulus. Similarly, Barroso and Sztl reported that brefeldin A inhibited apical release of pIgR that had been preinternalized from the basolateral surface at 17°C, but did not inhibit recycling of apically internalized ligand (5). It is possible that recycling and transcytotic cargo become segregated into separate vesicle populations whose budding from apical endosomes is differentially regulated by protein kinases. Thus, according to this model, protein kinase A would promote formation of vesicles from apical endosomes specifically carrying transcytosing molecules, whereas protein kinase C would stimulate budding of recycling vesicles. A second possibility is that protein kinases A and C facilitate incorporation of transcytosing and recycling molecules, respectively, into a single population of vesicles budding from apical endosomes and destined for fusion with the apical membrane. A third and simpler model to account for our data is that cAMP/PKA stimulates the fusion of transcytotic vesicles either with apical endosomes, and/or directly with the apical plasma membrane. Clearly, the pathway traversed by transcytosing molecules needs to be dissected in greater detail before the exact site at which cAMP/PKA stimulates transport can be identified.

We have also found that elevation of cellular cAMP stimu-

lates apically directed secretion of an endogenous MDCK glycoprotein, gp 80. In a recent study, Pimplikar and Simons (36) reported that CT stimulated apical secretion via a mechanism that did not involve cAMP. This assumption was based on the observation that addition of dibutyryl cAMP at concentrations up to 100 μ M had no effect on secretion. Again, a likely reason for the discrepancy between their data and ours is their omission of a phosphodiesterase inhibitor from the assay. Furthermore, neither forskolin nor 8BrcAMP was tested in that study.

Our finding that both apically directed transcytosis and apical secretion are stimulated by cAMP suggest that the two pathways share elements of the machinery involved in regulation of vesicular trafficking in polarized epithelial cells. Classically, both transcytotic and exocytic vesicles have been assumed to fuse directly with the apical plasma membrane, and it is possible that the fusion of both classes of vesicles are regulated by cAMP/PKA. An alternative model is that transcytotic and exocytic vesicles intersect at the level of apical early endosomes, and it is the fusion of these vesicles with the endosomal compartment that is regulated by cAMP/PKA. A third alternative is that cAMP/PKA regulates entirely different steps in the two pathways. For example, budding of exocytic vesicles from the TGN might be stimulated in secretion while a fusion step might be regulated in transcytosis. At present our data do not allow us to distinguish between these possibilities.

To further address a possible cAMP-independent role for G α in transcytosis, we overexpressed epitope-tagged wt G α and a mutant, Q227L, which constitutively activates adenylyl cyclase, in MDCK cells. For the experiments described here, we utilized clonal cell lines in which the levels of expression of the recombinant protein were equivalent. We found that, although transcytosis in 17°C experiments was elevated in cells expressing wt G α , transport was stimulated twofold more in cells expressing an equivalent level of Q227L. However, only 16% of the constitutively active mutant was found to be associated with the membrane fraction as opposed to almost 100% in the wild type. This distribution for Q227L is similar to that observed by Levis and Bourne (29) for a different constitutively active mutant, R201C, in S49 cells. As these authors have proposed, this small membrane-associated fraction may still be sufficient to saturate available adenylyl cyclase molecules. The moderate stimulation observed for wt G α in 17°C experiments is most likely due to its sheer abundance in the membranes, which may be in excess of available $\beta\gamma$ subunits. Neither construct, however, increased transcytosis to the extent observed following treatment with IBMX+FSK. The hierarchy of IBMX+FSK > Q227L > wt G α in stimulating transcytosis is a strong argument against a significant cAMP-independent role for G α in this process. It will be of interest in future work to examine to what extent other vesicular transport pathways, in particular those that have been reported to be influenced by heterotrimeric G proteins, are affected by agents that augment cellular cAMP and by overexpression of heterotrimeric G proteins.

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References

- Apodaca, G., M. Bomsel, J. Arden, P. P. Breitfeld, K. Tang, and K. E. Mostov. 1991. The polymeric immunoglobulin receptor: a model protein to study transcytosis. *J. Clin. Invest.* 87:1877-1882.
- Apodaca, G., L. A. Katz, and K. E. Mostov. 1994. Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. *J. Cell Biol.* 125:67-86.
- Balch, W. E. 1990. Small GTP-binding proteins in vesicular transport. *Trends Biochem. Sci.* 15:473-477.
- Barr, F. A., A. Leyte, and W. B. Huttner. 1992. Trimeric G proteins and vesicle formation. *Trends Cell Biol.* 2:91-93.
- Barroso, M., and E. S. Szul. 1994. Basolateral to apical transcytosis in polarized cells is indirect and involves BFA and trimeric G protein sensitive passage through the apical endosome. *J. Cell Biol.* 124:83-100.
- Bartles, J. R., and A. L. Hubbard. 1988. Plasma membrane protein sorting in epithelial cells: do secretory pathways hold the key? *Trends Biochem. Sci.* 13:181-184.
- Bomsel, M., and K. Mostov. 1992. Role of heterotrimeric G proteins in membrane traffic. *Mol. Biol. Cell.* 3:1317-1328.
- Bomsel, M., and K. E. Mostov. 1993. Possible role of both the α and $\beta\gamma$ subunits of the heterotrimeric G protein, Gs, in transcytosis of the polymeric immunoglobulin receptor. *J. Biol. Chem.* 268:25824-25835.
- Brantzaeg, P., and K. Baklien. 1977. Characterization of the IgA immunocyte population and its product in a patient with excessive intestinal formation of IgA. *Clin. Exp. Immunol.* 30:77-88.
- Breitfeld, P. P., J. M. Harris, and K. M. Mostov. 1989. Postendocytotic sorting of the ligand for the polymeric immunoglobulin receptor in Madin-Darby canine kidney cells. *J. Cell Biol.* 109:475-486.
- Breitfeld, P. P., W. C. McKinnon, and K. E. Mostov. 1990. Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells. *J. Cell Biol.* 111:2365-2373.
- Cardone, M. H., B. L. Smith, W. Song, D. Mochly-Rosen, and K. E. Mostov. 1994. Phorbol myristate acetate-mediated stimulation of transcytosis and apical recycling in MDCK cells. *J. Cell Biol.* 124:717-727.
- Carter, L. L., T. E. Redelmeier, L. A. Woollenweber, and S. L. Schmid. 1993. Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. *J. Cell Biol.* 120:37-45.
- Casanova, J. E., G. Apodaca, and K. E. Mostov. 1991. An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor. *Cell.* 66:65-75.
- Casanova, J. E., P. P. Breitfeld, S. A. Ross, and K. E. Mostov. 1990. Phosphorylation of the polymeric immunoglobulin receptor required for its efficient transcytosis. *Science (Wash. DC)* 248:742-745.
- Chen, J., and R. Iyengar. 1994. Suppression of ras-induced transformation of NIH 3T3 cells by activated G α s. *Science (Wash. DC)* 263:1278-1281.
- Chijiwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.* 265:5267-5272.
- Colombo, M. I., L. S. Mayorga, P. J. Casey, and P. D. Stahl. 1992. Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. *Science (Wash. DC)* 255:1695-1697.
- Cosson, P., I. de Curtis, J. Pouyssegur, G. Griffiths, and J. Davoust. 1989. Low cytoplasmic pH inhibits endocytosis and transport from the trans-Golgi network to the cell surface. *J. Cell Biol.* 108:377-387.
- Edelman, A. M., D. K. Blumenthal, and E. G. Krebs. 1987. Protein serine/threonine kinases. *Annu. Rev. Biochem.* 56:567-613.
- Freier, S., M. Eran, and J. Faber. 1987. Effect of cholecystokinin and of its antagonist, of atropine, and of food on the release of immunoglobulin A and immunoglobulin G specific antibodies in the rat intestine. *Gastroenterology* 93:1242-1246.
- Griffiths, G., S. Pfeiffer, K. Simons, and K. Matlin. 1985. Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol.* 101:949-964.
- Hansen, S. H., K. Sandvig, and B. van Deurs. 1993. Clathrin and HA2 adaptors: effectors of potassium depletion, hypertonic medium and cytosol acidification. *J. Cell Biol.* 121:61-72.
- Heuser, J. 1989. Effects of cytoplasmic acidification on clathrin lattice morphology. *J. Cell Biol.* 108:401-411.
- Hunziker, W., P. Male, and I. Mellman. 1990. Differential microtubule re-

- quirements for transcytosis in MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3515-3525.
26. Hunziker, W., J. A. Whitney, and I. Mellman. 1991. Selective inhibition of transcytosis by Brefeldin A in MDCK cells. *Cell.* 67:617-627.
 27. Jin, G.-F., Y. S. Guo, and C. W. Houstin. 1989. Bombesin: an activator of specific *Aeromonas* antibody secretion in rat intestine. *Dig. Dis. Sci.* 34:1708-1712.
 28. Kondor-Koch, C., R. Bravo, S. D. Fuller, D. Cutler, and H. Garoff. 1985. Exocytic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. *Cell.* 43:297-306.
 29. Levis, M. J., and H. R. Bourne. 1992. Activation of the α subunit of G_i in intact cells alters its abundance, rate of degradation, and membrane avidity. *J. Cell Biol.* 119:1297-1307.
 30. Lippincott-Schwartz, J., J. Glickman, J. G. Donaldson, J. Robbins, T. E. Kreis, K. B. Seamon, M. P. Sheetz, and R. D. Klausner. 1991. Forskolin inhibits and reverses the effects of brefeldin A on Golgi morphology by a cAMP-independent mechanism. *J. Cell Biol.* 112:567-577.
 31. Matlin, K. S., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233-243.
 32. Mostov, K., G. Apodaca, B. Aroeti, and C. Okamoto. 1992. Plasma membrane protein sorting in polarized epithelial cells. *J. Cell Biol.* 116:577-583.
 33. Mostov, K. E., and D. L. Deitcher. 1986. Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA. *Cell.* 46:613-621.
 34. Musil, L., and J. Baenziger. 1987. Cleavage of membrane secretory component to soluble secretory component occurs on the cell surface of rat hepatocyte monolayers. *J. Cell Biol.* 104:1725-1733.
 35. Osawa, S., and G. L. Johnson. 1991. A dominant negative G α s mutant is rescued by secondary mutation of the α chain amino terminus. *J. Biol. Chem.* 266:4673-4676.
 36. Pimplikar, S. W., and K. Simons. 1993. Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein. *Nature (Lond.).* 362:456-458.
 37. Prydz, K., S. H. Hansen, K. Sandvig, and B. van Deurs. 1992. Effects of brefeldin A on endocytosis, transcytosis and transport to the Golgi complex in polarized MDCK cells. *J. Cell Biol.* 119:259-272.
 38. Rodriguez-Boulan, E., and W. J. Nelson. 1989. Morphogenesis of the polarized epithelial phenotype. *Science (Wash. DC).* 245:718-725.
 39. Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105:679-689.
 40. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.
 41. Stow, J. L., J. B. de Almeida, N. Narula, E. J. Holtzman, L. Ercolani, and D. A. Ausiello. 1991. A heterotrimeric G protein, G α i-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. *J. Cell Biol.* 114:1113-1124.
 42. Urban, J., K. Parczyk, A. Leutz, M. Kayne, and C. Kondor-Koch. 1987. Constitutive apical secretion of an 80-kD sulfated glycoprotein complex in the polarized epithelial Madin-Darby Canine Kidney cell line. *J. Cell Biol.* 105:2735-2743.
 43. van Deurs, B., S. H. Hansen, O. W. Petersen, E. Løkken Melby, and K. Sandvig. 1990. Endocytosis, intracellular transport and transcytosis of the toxic protein ricin by a polarized epithelium. *Eur. J. Cell Biol.* 51:96-109.
 44. von Bonsdorff, C. H., S. D. Fuller, and K. Simons. 1985. Apical and basolateral endocytosis in Madin-Darby canine kidney (MDCK) cells grown on nitrocellulose filters. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2781-2782.
 45. Wilson, I., R. D. Soltis, R. E. Olson, and S. E. Erlandsen. 1982. Cholinergic stimulation of immunoglobulin A secretion in rat intestine. *Gastroenterology.* 83:881-888.