Activators of Protein Kinase A Stimulate Apical but Not Basolateral Transport in Epithelial Madin-Darby Canine Kidney Cells*

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In polarized Madin-Darby canine kidney cells the newly synthesized plasma membrane proteins, on the exocytic path, are sorted in the trans-Golgi network (TGN) and delivered directly to the apical or basolateral surface. Forskolin, isobutylmethylxanthine, and dibutyryl cAMP, all known to activate protein kinase A, stimulated transport of influenza hemagglutinin (HA) from the TGN to the apical surface. The same reagents, however, did not affect the transport of HA from the endoplasmic reticulum to the Golgi complex nor did they affect transport of vesicular stomatitis virus G protein from the TGN to the basolateral surface. The addition of staurosporin, a general protein kinase inhibitor, did not affect the transport of HA in nontreated cells but blocked the stimulation caused by the above reagents. Apical transport of HA was also stimulated by phorbol ester, an activator of protein kinase C. Activation of apical transport by phorbol ester as well as aluminum fluoride (Pimplikar, S. W., and Simons, K. (1993) Nature 362, 456–458) was also negated by staurosporin. These results show that in polarized Madin-Darby canine kidney cells, protein kinase A and protein kinase C selectively stimulate the apical transport.

As we gain a better understanding of the process of intracellular membrane trafficking (1, 2), it is becoming evident that several of these steps are subjected to cellular regulation. For example, some specialized cells such as neutrophils, platelets, mast cells, etc. respond to external stimuli by accelerating secretory activity (3). Cells of neuroendocrine origin show a regulated secretory pathway in which proteins stored in secretory granules are released upon receiving external signals. The mechanism of regulated secretion has been extensively studied (4). Finally, as cells enter mitosis both the exocytic and endocytic transport steps are inhibited, and only at the completion of the cell cycle is membrane traffic resumed (5, 6). Together, these observations show that various steps in membrane trafficking pathways are regulated by the cell. This idea is further supported by recent observations that molecules of the signal transduction pathway are involved in one or more aspects of membrane trafficking. Thus, heterotrimeric G proteins have been shown to play a role at many stages of exocytic and endocytic pathways (7–11). A cascade of protein kinases has been implicated in modulating regulated secretion in chromaffin cells (12). A recent study has suggested that protein kinase C may play an important role in constitutive secretion of proteins by modulating membrane trafficking through the Golgi complex (13). Protein phosphorylation has been implicated in protein transport from the ER to the Golgi complex and within the Golgi complex and in endocytic vesicle fusion, although the identity of the kinases implicated in these studies remains unclear (14, 15).

We are interested in studying polarized membrane trafficking in epithelial cells. A distinguishing feature of epithelial cells is that their cell surface is divided into two domains. The luminal facing apical domain is separated by tight junctions from the basolateral domain, which rests on the underlying connective tissue. These two domains maintain distinct sets of lipids and proteins that are essential for the functional diversity. Madin-Darby canine kidney (MDCK) cells have provided a good model to study polarized membrane transport since they sort and deliver viral glycoproteins HA and VSV G to apical and basolateral surfaces, respectively (for reviews, see Ref. 16 and 17). We had earlier observed (9) that in MDCK cells, apical protein transport was regulated by a stimulatory class of G protein (Gs), which is also present in other epithelial cells. These observations show that various steps in membrane trafficking pathways are regulated by the cell.

This idea is further supported by recent observations that molecules of the signal transduction pathway are involved in one or more aspects of membrane trafficking. Thus, heterotrimeric G proteins have been shown to play a role at many stages of exocytic and endocytic pathways (7–11). A cascade of protein kinases has been implicated in modulating regulated secretion in chromaffin cells (12). A recent study has suggested that protein kinase C may play an important role in constitutive secretion of proteins by modulating membrane trafficking through the Golgi complex (13). Protein phosphorylation has been implicated in protein transport from the ER2 to the Golgi complex and within the Golgi complex and in endocytic vesicle fusion, although the identity of the kinases implicated in these studies remains unclear (14, 15).

EXPERIMENTAL PROCEDURES

Materials

Forskolin and 1,9-dideoxyforskolin were from Calbiochem and were dissolved in ethanol and stored at –20 °C. Dibutyryl cAMP, isobutylmethylxanthine (IBMX), phorbol 12,13-dibutyrate (PDBu), and staurosporin (Sigma) were dissolved in dimethyl sulfoxide and stored at –20 °C.

Cell Culture and Viral Infections

MDCK cells, strain II (low resistance) were cultured as described previously (18). The cells were grown on permeable filter support (12-mm, 0.4-μm pore size Transwell polycarbonate filters from Costar Corp., Cambridge, MA) as described previously (9, 19). The cells were used 3–4 days after plating. Viral infections were performed as described earlier (9, 19).

Pulse-Chase Protocol

TGN to Cell Surface Transport—The cells were pulse-labeled with 25 μl of labeling medium methionine-free Eagle’s minimal essential me-

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containing 0.35 g/l filter sodium bicarbonate (instead of the usual 2.2 g/liter) and 10 mM HEPES pH 7.3, 0.2% (w/v) bovine serum albumin) containing 50 μCi of $^{35}$S-methionine exactly as described before (19) for 8 min at 37 °C. The pulse was terminated by washing the monolayers in chase medium (CM, labeling medium containing 40 μg/ml cycloheximide and 150 μg/ml cold methionine) and incubating for an additional 4 min at 37 °C. The filters were then transferred to fresh CM pre-warmed to 19.5 °C and further incubated at 19.5 °C for 75 min to block the viral glycoproteins in the TGN (20).

ER to Golgi Transport—The cells were pulse-labeled for 3 min, and after terminating the pulse, the cells were incubated for an additional 3 min at 37 °C in CM. The filters were immediately washed with ice-cold CM and further incubated at 4 °C for 30 min to accumulate the viral glycoprotein in ER.

**Transport Assays**

**TGN to Cell Surface Transport—**After 75 min at 19.5 °C, the medium was removed and the filters were placed on a 30-μl drop of warm CM (without cycloheximide) containing the given amount of a reagent. The apical side received 150 μl of the same medium, and the incubation was continued for 10 min at 19.5 °C. The transport reaction was initiated, without changing the medium, by transferring the filters to 37 °C for 20 min. The filters were then transferred to 4 °C and washing (x2) the monolayers with cold phosphate-buffered saline containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$. In some experiments the cells were treated with the given reagent for 15 min at 4 °C. In this case the transport was carried out by transferring the filters in the same medium to 37 °C for 30 min. Both protocols gave essentially the same results. The apical transport activity (arrival of HA at the apical surface) was measured by trypsinization and the basolateral transport activity (arrival of VSV G at the basolateral surface) by surface immunoprecipitation as described earlier (19). The apical transport activity is expressed as the ratio of HA2/HA0 and represents as a transport index with the HA2/HA0 ratio, which in control cells is arbitrarily normalized to 1. This method of calculation gave qualitatively similar results to a previous protocol (20) described earlier but is more sensitive to reflect the changes in transport activity. The basolateral transport index is expressed arbitrarily as the ratio of VSV G bound to protein A-Sepharose/total.

**ER to Golgi Transport—**After 30 min at 4 °C, the medium was removed, and the filters were placed on a 30-μl drop of cold CM (without cycloheximide) containing the given amount of a reagent. The apical side received 150 μl of the same medium, and the incubation was continued for 15 min at 4 °C. The transport reaction was initiated by transferring the filters in the same medium to 37 °C for 30 min and terminated as above. The extent of transport (amount of HA acquiring Endo H resistance) was measured as described earlier (9), and the transport index is expressed arbitrarily as the ratio of Endo H sensitive/Endo H resistant form.

**Analytical Techniques**

VSV G or influenza HA samples obtained after the transport reactions were analyzed by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel (21), and in some cases the gels were fluorographed using Entensify (DuPont NEN). The dried gels were exposed to X-Omat AR film (Eastman Kodak Co.) at -70 °C for 2–4 days.

**RESULTS**

**Forskolin Stimulates Transport of HA from the TGN to Apical Surface—**We had earlier shown that the apical transport of HA was regulated by a G, protein (9). In our initial studies we had observed that 100 μM dibutyryl cAMP had no significant effect on the transport step (9). As an extension of those studies we examined the effect of forskolin on the transport of HA. Forskolin is known to activate the enzyme adenylyl cyclase, leading to increased intracellular levels of cAMP. Influenza virus-infected MDCK cells were pulse-labeled with $^{35}$S-methionine, and the pulse-labeled HA was accumulated in the TGN by incubation at 19.5 °C. The cells were treated with increasing concentrations of forskolin, and the amount of HA arriving at the surface was assessed by surface trypsinization. Compared with nontreated control cells, the forskolin-treated cells showed a decrease in HA0 and a corresponding increase in HA2 band intensity (Fig. 1a, compare lanes 3 and 4 with lanes 5 and 6) suggesting an increased transport of HA to the cell surface. If trypsin treatment was omitted following the transport reaction, forskolin-treated cells showed the same amounts of HA0 and HA2 as the control cells left at 4 °C (data not shown). This shows that forskolin treatment did not somehow cause increased endogenous cleavage of HA resulting in an erroneous increase in the transport signal. Fig. 1b shows the quantitation of transport carried out in the presence of various concentrations of forskolin. The stimulation of HA transport was proportional to the concentration of the reagent. Maximal stimulation was observed at a concentration of 50 μM, and a further increase up to 250 μM did not cause further stimulation (data not shown).

**Several Activators of PKA Stimulate Apical Transport of HA—**Forskolin, in addition to stimulating the enzyme adenylyl cyclase and increasing cAMP levels, also exhibits several cAMP-independent effects (22). 1,9-Dideoxyforskolin, which is unable to stimulate adenylyl cyclase, shows only the latter effects and has been used in distinguishing between these two effects of forskolin. Whereas treatment with forskolin resulted in stimulation of apical transport, treatment with 1,9-dideoxyforskolin showed no such effect (Fig. 2a). We also tested the effect of IBMX, which inhibits the enzyme phosphodiesterase and...
increases cAMP levels. Addition of IBMX to cells potentiated the effect of forskolin but did not cause an additional stimulation that could be attributed to 1,9-dideoxyforskolin. The modest stimulation seen was due to IBMX alone since the addition of IBMX to control cells also resulted in stimulation of transport (data not shown). The stimulation by forskolin and the lack of stimulation by 1,9-dideoxyforskolin together with the effect of IBMX strongly suggest that the stimulation of apical transport was mediated through cAMP.

Considering the new evidence, we reinvestigated the effect of increasing concentrations of dibutyryl cAMP on apical transport. As reported in the earlier studies (9), concentrations of up to 100 μM had no significant effect on apical transport (Fig. 2b), but there was significant stimulation with 500 μM and even higher stimulation with 5 mM dibutyryl cAMP. However, even at the maximal concentration of dibutyryl cAMP, the level of stimulation was lower than that obtained by forskolin. We have consistently observed that, compared with forskolin, dibutyryl cAMP is a weaker stimulator of apical transport. These data suggest that the transport of HA from the TGN to the apical surface is stimulated by agents that increase intracellular concentrations of cAMP resulting in activation of protein kinase A (PKA).

**PKA Activators Affect neither ER to Golgi Transport of HA nor TGN to Basolateral Transport of VSV G**—We further studied whether the PKA activators caused a general stimulation of the exocytic transport pathway or whether their effect was selective for the apical transport step. We studied ER to Golgi transport of HA by allowing the pulse-labeled protein to accumulate in the Endo H-sensitive form and monitoring its conversion to the Endo H-resistant form (9). After a short pulse (3 min) and chase (5 min) at 37 °C, the labeled HA was accumulated in the ER for 30 min at 4 °C. The cells were treated with various drugs for 15 min at 4 °C, and the temperature was raised to 37 °C for 30 min to allow the transport of HA to Golgi complex. If the cells were left at 4 °C throughout the assay, most of the HA was present as a sharp band (Fig. 3a, upper panel, lanes 1 and 2), which was completely sensitive to Endo H treatment (lower panel). Upon raising the temperature to allow protein transport, nearly half of the protein migrated as a diffuse band with a slower mobility (upper panel, lanes 3 and 4), which was resistant to Endo H treatment (lower panel). Cells treated with dibutyryl cAMP (lanes 5 and 6), IBMX (lanes 7 and 8), or forskolin (lanes 9 and 10) did not show any deviation from nontreated control cells (lanes 3 and 4). Quantitation of the autoradiogram (Fig. 3b) shows that the reagents that caused a significant stimulation of HA transport from the TGN to the apical surface did not stimulate its passage from the ER to the Golgi complex. The lack of stimulation cannot be due to the drug treatment at 4 °C (to keep the protein in ER) since the drug treatment either at 4 or 19.5 °C stimulated the TGN to apical transport of HA to a similar extent (data not shown). It
PKA Activators Stimulate Apical Transport in MDCK Cells

is possible that the conditions optimal for stimulation of apical transport may not be optimal for the stimulation of ER to Golgi transport. Though we cannot completely rule out a role for PKA activators in the early steps of the exocytic pathway, clearly the conditions that stimulate the transport of HA from TGN to apical surface do not do so for the same protein as it passes from the ER to Golgi.

Since the PKA activators seem to stimulate a transport step that originates at the TGN, we asked whether these reagents would also stimulate a transport step that originates at the same site but is directed toward another destination. To answer this question we made use of the fact that MDCK cells infected with VSV sort the VSV G protein in the TGN and deliver it to the basolateral surface. Filter-grown MDCK cells were infected with VSV, and the pulse-labeled VSV G was accumulated in the TGN. Cells were treated with various reagents as described for the apical transport experiment, and the amount of VSV G delivered to the basolateral surface was determined by surface immunoprecipitation. Fig. 4a shows that whereas the control cells showed the presence of VSV G at the basolateral surface (upper panel, lanes 3 and 4), very little VSV G reached the surface in the cells left at 4 °C (lanes 1 and 2). Treatment of cells with dibutyryl cAMP (lanes 5 and 6), IBMX (lanes 7 and 8), or forskolin (lanes 9 and 10) did not show increased accumulation of VSV G at the surface as compared with cells treated with only dimethyl sulfoxide (lanes 3 and 4). The relative amount of VSV G transported to the surface was calculated after normalizing for recoveries for the total amount of VSV G in cells (Fig. 4a, lower panel). Quantitation of this autoradiogram (Fig. 4b) shows that the drugs that caused a significant stimulation of protein transport from the TGN to the apical surface did not stimulate the TGN to the basolateral pathway.

Previous observations have demonstrated that VSV G is rapidly internalized when implanted directly in the cell surface (23). However, recent data have shown that when delivered to the plasma membrane by the normal biosynthetic pathway, VSV G is internalized slowly (24). Nevertheless, we checked whether or not the lack of stimulation of VSV G transport was because it was masked by increased internalization of the protein after delivery to the basolateral surface. We performed the transport experiment in the continuous presence of anti-VSV G antibodies. Fig. 4c shows that even under these conditions there was no significant increase in the amount of VSV G bound to the antibodies suggesting that basolateral transport is indeed not stimulated by PKA activators to any significant extent. These results show that of the three steps of the exocytic pathway studied here, the PKA activators selectively stimulated the TGN to apical pathway.

Stimulation of Apical Transport Is Prevented by a Protein Kinase Inhibitor—Activation of PKA should result in phosphorylation of proteins that lie downstream of PKA in the signaling pathway. Since the activation of PKA leads to stimulation of apical transport, it is reasonable to assume that protein phosphorylation is needed for stimulation to occur. If so, the reagents that interfere with the phosphorylation reaction would be expected to block the stimulation of apical transport caused by PKA activators. To address this question we added staurosporin, a potent kinase inhibitor known to inhibit both PKA and protein kinase C, to cells either alone or in combination with PKA activators and studied the arrival of HA at the apical surface. Fig. 5a shows that in the absence of staurosporin both cAMP and forskolin stimulate the apical transport. In the presence of the kinase inhibitor, however, this stimulation was

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**FIG. 4. Effect of various PKA stimulators on VSV G transport from the TGN to basolateral surface.** a, VSV-infected MDCK cells were pulse-labeled as indicated in the legend to Fig. 1a and were incubated in the absence (lanes 1-4) or presence of 500 μM dibutyryl cAMP (lanes 5 and 6) or 500 μM IBMX (lanes 7 and 8) or 50 μM forskolin (lanes 9 and 10) for 10 min at 19.5 °C at the end of "TGN block." The cells were then incubated at 4 °C (lanes 1 and 2) or 37 °C (lanes 3-10) in the continued presence of the reagents for 20 min, and the amount of VSV G arriving at the basolateral surface was measured by surface immunoprecipitation. Material bound to protein A-Sepharose (top panel) and one-tenth of cell lysates (bottom panel) were separated by SDSPolyacrylamide gel electrophoresis. b, the intensity of VSV G band was quantitated on a PhosphorImager from the above gel. Each column represents the amount of VSV G detected at the surface and is normalized for the sample recovery. The cell lysate material was not immunoprecipitated (to check the sample recovery from each filter), and therefore the precise percent of VSV G transport cannot be calculated. c, basolateral transport of VSV G was performed as described above except that anti-VSV G antibodies (together with 1% bovine serum albumin to prevent nonspecific binding) were added to the basolateral side during the drug treatment (15 min at 19.5 °C) and were present during the transport reaction (30 min at 37 °C). The transport reaction was terminated by replacing the apical medium with cold CM (without removing the basolateral medium), and the cells were incubated for an additional 30 min on ice to allow antibodies to continue binding to surface VSV G. The columns represent the mean of the triplicate (con-
HA Caused by Activators
tory effects of PKA activators on the apical transport of HA, we
that observed for forskolin. Again, the addition of staurosporin
stentially varied reagents such as forskolin, IBMX, and dibutyryl

C, like PKA, is also a serine/threonine kinase, and in some
instances these two kinases elicit similar physiological re-

Staurosporin Also Blocks Stimulation of Apical Transport of
HA Caused by Activators of PKC and AIF_{3-5}—Protein kinase
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instances these two kinases elicit similar physiological re-

We had earlier shown that TGN to apical surface was stimulated by treatment with AIF_{3-5} and that the apical pathway was regulated by a G_{o} activity in MDCK cells. Since staurosporin could antagonize the stimula-
tory effects of PKA activators on the apical transport of HA, we
wondered if it would do the same to the stimulatory effect of
AIF_{3-5}. Fig. 5c shows that while the addition of AIF_{3-5} stimu-
lation of apical transport was performed in the absence or presence of 1 
mu/ml PDBu with or without 2 
mu staurosporin as described above. The percent of HA transported in the control cells was 36.5 for both experiments. c, apical transport was performed in the absence or presence of 50 
mu ammonium aluminum sulfate plus 10 
M potassium
dide. Each column represents the mean of the triplicate values and the error bars indicate the deviation from the mean. The percent of HA transported in the control cells was 23.1 in this experiment.

DISCUSSION
The results described above show that, in polarized cells, the
apical pathway is stimulated by activators of PKA. This stimu-
lation does not seem to be a general effect of these reagents
since they did not affect the two other transport steps of the
polarized exocytotic pathway. The stimulation is caused by struc-
turally varied reagents such as forskolin, IBMX, and dibutyryl
cAMP. A common property of these reagents is to activate PKA
suggesting that the stimulation of apical transport is mediated
through PKA. The data presented above demonstrate that ac-
tivation of PKC by PDBu also results in stimulation of apical
transport. At present the molecular connection between activa-
tion of kinases and stimulation of apical transport is not clear.
Since staurosporin blocks the stimulation induced by PKA and
PKC, it seems that protein phosphorylation is essential for the
stimulation of transport. Both PKA and PKC are serine/threonine kinases and may phosphorylate the same protein substrate.
The identity of such a substrate is at present unknown, but the selective stimulation of the apical pathway suggests that it must be restricted to the apical transport pathway.

We had earlier shown that TGN to apical transport of pro-
teins was stimulated by AIF_{3-5}, most probably through a G_{o}
protein. The observation that staurosporin also blocked the
stimulation of HA transport caused by AIF_{3-5} suggests that
protein phosphorylation is involved in the G_{o} stimulation of
apical transport. Since staurosporin did not inhibit the apical
transport in control cells, it is possible that the cycle of phos-
phorylation and dephosphorylation is not involved in the nor-
mal transport process. However, the possibility that a kinase,
resistant to staurosporin, is involved in the normal apical
transport process is not ruled out. Adenyl cyclase is a classical
downstream effector of C_{o}, and it is possible that the G_{o}
stimulation of apical transport is mediated through the adenyl
cyclase-PKA pathway. However, a direct proof that G_{o} protein-
adenyl cyclase coupling is responsible for AIF_{3-5} stimulation of
apical transport is not yet available. It should be noted that G
proteins activate a number of protein kinases including PKC
(27). Whatever the identity of kinase downstream of the G_{o}
protein, it seems that G_{o} stimulation of apical transport in
MDCK cells is mediated by protein phosphorylation.

These results also support the growing evidence that the so
called "constitutive" transport steps may be modulated by
physiological cues. It has long been recognized that there exists
a special "regulated" secretory pathway, which is characterized
by the stimulated release of secretory products stored in secre-
tory granules in response to extracellular signal (4). Recent
evidence indicates that in many cells the constitutive nonpo-
larized exocytosis can be stimulated either by ligands or by
activation of signal transduction pathway (28). The data pre-
sented above show that this is also true for constitutive secre-
tion in polarized cells. The novel finding is that, of the two
pathways of polarized exocytosis (apical and basolateral), only
the apical pathway is modulated by activation of PKA and PKC.
It is conceivable that some other kinases might do the same for the basolateral pathways. Unique serine/threonine protein kinases, other than PKA or PKC, have been implicated in the ER to Golgi transport step and in endosome-endosome fusion (14, 15).

Besides the direct route from the TGN to apical surface (direct pathway), polarized cells have a transcytotic pathway that leads from the basolateral to the apical surface. There is evidence that these two pathways share many common characteristics. They show similar sensitivity to the drug brefeldin A, exhibit similar dependence on microtubules (reviewed in Ref. 17), and share the same or similar machinery for sorting of apical and basolateral proteins (29). Both pathways are also stimulated by AlF$_3$ and cholera toxin and seem to be regulated by the G, class of G proteins (9, 10). It has recently been reported that in MDCK cells the transcytotic delivery of proteins from the basolateral to apical surface is stimulated by PKA (31). Our observation that TGN to apical transport is also stimulated by PKA and PKC activators shows that the regulatory mechanisms seem to be shared by these two pathways. With the availability of in vitro transport systems to study polarized transport (9, 19, 32) and a better understanding of various connections in signal transduction pathways, it is hoped that the role of signal-transducing molecules in membrane trafficking will become clear.

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