Rapid sequestration of DPP IV/CD26 and other cell surface proteins in an autophagic-like compartment in Caco-2 cells treated with forskolin

Laurent Baricault1, Jack A. M. Fransen2, Martine Garcia3,* Catherine Sapin1, Patrice Codogno1, Leo A. Ginsel2 and Germain Trugnan1,*

1Unité de Recherches sur la Neuroendocrinologie et la Biologie Cellulaire Digestives, INSERM U410, 16 Rue Henri Huchard 75018 Paris, France
2Department of Cell Biology and Histology, University of Nijmegen, PO Box 9101, NL 6500 HB Nijmegen, The Netherlands
3MRC Laboratory for Molecular Cell Biology, University College of London, Gower Street London, WC1 E6BT, UK
*Present address: Laboratoire de Biophysique, Université de Poitiers, 40 Avenue du Recteur Pineau, 86022 Poitiers, France
†Author for correspondence

SUMMARY

The enterocytic differentiation of Caco-2 cells, a human colon adenocarcinoma cell line, is accompanied by the transcriptionally regulated expression of a subset of proteins and their correct sorting towards the cell surface. In the present work we have explored the possibility that post-translational events may interfere with this process by investigating the short term effects of a potent adenylyl cyclase activator, forskolin, on cell surface expression of dipeptidyl peptidase IV. Previous works have shown that this protein is targeted towards the apical domain through either a direct or an indirect route. Domain specific biochemical experiments demonstrate that cell surface expression of neosynthesized dipeptidyl peptidase IV rapidly decreases after a 1 hour forskolin treatment. Both initial basolateral and apical dipeptidyl peptidase IV membrane delivery were altered by forskolin treatment. Decrease of dipeptidyl peptidase IV cell surface expression was not restricted to this protein, since membrane expression of '525' antigen, a basolateral protein and of sucrase-isomaltase, an apically targeted hydrolase, which unlike dipeptidyl peptidase IV mainly follows a direct route to the brush border membrane, also decreases. In addition endocytosis of proteins from the apical and from the basolateral domain was essentially unchanged, suggesting that forskolin's target may be located on the exocytic pathway. Confocal laser scanning microscopy and immune-electron microscopy studies demonstrate that, within 5 minutes of forskolin treatment, the cell surface proteins studied accumulate in intracellular vesicles which were co-labeled with a polyclonal antibody raised against Lamp-1, a lysosomal membrane marker. Electron microscopy studies show that these vesicles display an autophagic-like morphology. Finally, biochemical experiments indicate that dibutyryl cAMP does not mimic the forskolin effect, thus suggesting that it is a cAMP-independent phenomenon.

Key words: protein traffic, Caco-2 cell, forskolin, autophagy

INTRODUCTION

Caco-2 cells, originating from a human colon adenocarcinoma (Fogh et al., 1977; Pinto et al., 1983) are, together with MDCK cells and hepatocytes, among the most popular cell models used to study the protein sorting machinery in polarized epithelial cells (Hopkins, 1991; Le Bivic et al., 1990; Matter et al., 1990a; Mostov et al., 1992; Rodriguez-Boulan and Powell, 1992; Simons and Wandinger-Ness, 1990). These studies have demonstrated that in Caco-2 as in normal intestinal cells, apical (brush border) proteins follow a more complex sorting pathway as compared to other cell systems. Some proteins like sucrase-isomaltase (SI) or GPI-anchored proteins use mostly a direct pathway whereas other proteins, such as dipeptidyl peptidase IV (DPP IV) use at least in part an indirect pathway passing first through the basolateral membrane before reaching the apical membrane by transcytosis (Feracci et al., 1985; Garcia et al., 1993; Le Bivic et al., 1990; Matter et al., 1990b). A small pool of these proteins may be routed either directly or indirectly to lysosomes where they are degraded. From endosomes they may be recycled to the surface or to lysosomes (Fransen et al., 1988; Ginsel et al., 1988; Matter et al., 1990b).

A powerful approach to a better understanding of this complex phenomenon is the use of drugs that may act on trafficking and/or sorting events. This includes: (i) drugs known to inhibit glycosylation processing enzymes (Elbein, 1987; Ogier-Denis et al., 1990; Trugnan et al., 1991); (ii) drugs modifying structures and/or functions of subcellular compartments, as for example monensin (Akin et al., 1987; Stewart and Kenny, 1984; Tartakoff, 1983) or brefeldin A (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Low et al., 1992; Misumi et al., 1986; Pelham, 1991); and (iii) drugs acting on the cytoskeleton, especially on microtubules (Achler et al.,
VESTIGATE WHETHER CACO-2 CELLS, WHICH HAVE A DIFFERENT PATTERN OF PROTEIN INTRACELLULAR TRAFFIC AND SORTING AS COMPARED TO MDCK, MAY DISPLAY SIMILAR TRAFFIC PERTURBATIONS AFTER SHORT PERIODS (5 MINUTES TO 7 HOURS) OF FK TREATMENT.

USING BIOCHEMICAL, IMMUNOHISTOCHEMICAL AND IMMUNOELECTRON MICROSCOPY TECHNIQUES, WE SHOW THAT SHORT FK TREATMENT LEADS TO A DECREASED CELL SURFACE EXPRESSION OF NEWLY SYNTHESIZED DPP IV AND ITS CONCOMITANT ACCUMULATION IN INTRACELLULAR VESICLES. NEWLY SYNTHESIZED SUCRASE-ISOMALTASE (SI), AN APICALLY TARGETED PROTEIN THAT REACHES THE BRUSH BORDER MEMBRANE MOSTLY THROUGH A DIRECT PATHWAY (GARCIA ET AL., 1988), AND NEWLY SYNTHESIZED 525 ANTIGEN, A BASOLATERALLY TARGETED PROTEIN (LE BIVIC ET AL., 1987) ALSO DISPLAYS A REDUCED CELL SURFACE EXPRESSION ASSOCIATED WITH AN INTRACELLULAR ACCUMULATION AFTER 5 MINUTES TO 7 HOURS OF FK TREATMENT. ENDOCYTOSIS WAS DETERMINED FOR AN APICAL (DPP IV) AND A BASOLATERAL (525 ANTIGEN) PROTEIN FROM BOTH THE APICAL AND THE BASOLATERAL MEMBRANE AND WAS SHOWN TO BE ESSENTIALLY NOT MODIFIED, THEREBY INDICATING THAT THIS PROCESS IS NOT INVOLVED IN THE ABOVE OBSERVATIONS. MORPHOLOGICAL CHARACTERIZATION OF THE VESICLES IN WHICH PROTEINS ACCUMULATE INDICATES THAT THEY WERE CO-LABELED WITH ANTI-LAMP-1 ANTIBODIES, REPORTED TO RECOGNIZE A RESIDENT LISSOMAL MEMBRANE PROTEIN (CARLSSON ET AL., 1988). ELECTRON AND IMMUNO-ELECTRON MICROSCOPY STUDIES HAVE ALLOWED US TO SHOW THAT THESE VESICLES DISPLAY AN AUTOPHAGIC-LIKE MORPHOLOGY. THE POSSIBLE MECHANISMS UNDERLYING THESE OBSERVATIONS WILL BE DISCUSSED IN VIEW OF THE FACT THAT DIBUTYRYL cAMP DOES NOT MIMIC THE EFFECT OF FK, THEREBY SUGGESTING THAT IT IS cAMP-INDEPENDENT.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENTS

Caco-2 cells were obtained from J. Fogh (Memorial Sloan Kettering Cancer Center, Rye, NY) and were grown at 37°C in a 10% CO2/90% air atmosphere in Dulbecco's modified Eagle's minimal essential medium (Gibco Europe, Glasgow, Scotland) supplemented with 20% heat inactivated (36°C, 30 minutes) fetal calf serum (Gibco), 1% non essential amino acids (Gibco), and 1% penicillin-streptomycin (Gibco), as previously described (PINTO ET AL., 1983; ROIUSSET ET AL., 1985). This medium was changed 48 hours after seeding and then daily. For cell maintenance and for biochemical studies, cells were seeded at 1x10⁶ cells per cm² in 25 cm² plastic flasks (Corning Glassworks, Corning, NY). FOR DOMAIN SPECIFIC BIOCHEMICAL ASSAYS AND IMMUNOFLOUORESCENCE STUDIES, CELLS WERE SEEDED AT 1x10⁵ CELLS PER CM² ON TRANSWELLS (Costar Inc., Cambridge, MA) AS PREVIOUSLY DESCRIBED (LE BIVIC ET AL., 1990). CACO-2 CELLS (PASSENGES 65-85) WERE ROUTINELY PASSAGED EVERY 6 DAYS AND USED AFTER 15-20 DAYS OF CULTURE. FK WAS OBTAINED FROM CALBIOCHEM (LA JOYA, SAN DIEGO, CA) DILUTED 1:1000 IN ETHANOL AND USED AT 5X10⁻⁸ M. PREVIOUS WORK HAS SHOWN THAT THIS CONCENTRATION OF FK AND THE PRESENCE OF DILUTED ETHANOL IS WITHOUT EFFECT ON GROWTH AND VIABILITY OF CACO-2 CELLS (ROIUSSET ET AL., 1985). CACO-2 CELLS WERE TREATED WITH 5X10⁻⁸ M FK FOR 1 HOUR BEFORE STARTING THE LABELING PERIOD. IT HAS BEEN PREVIOUSLY SHOWN THAT IN FK-TREATED CACO-2 CELLS cAMP CONCENTRATION INCREASES IMMEDIATELY AND RISES TO ITS MAXIMAL VALUE WITHIN 30 MINUTES (ROIUSSET ET AL., 1985).

Antibodies


Cell labeling, pulse-chase experiments, domain specific biochemical assays, immunosolization and SDS-PAGE

Domainspecific biochemical assays on filter-grown Caco-2 cells were performed according to a previously published technique (Le Bivic ET AL., 1989). Briefly, cells grown on filters were incubated for 30 minutes in DMEM without methionine and cysteine, then labeled for 20 minutes using 8.5 mCi/ml of Trans-35S-label (ICN Biomedicals Inc. Costa Mesa, CA). After a wash with DMEM, cells were chased in DMEM containing 5x10⁻⁴ M Hepes and 0.2% BSA before biotinylation. Specific cell surface biotinylation with S-NHS biotin (Pierce, Rockford, IL) was carried out twice in succession for 20 minutes each. After biotinylation, cells were processed for immunoprecipitation using specific antibodies and Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Biotinylated antigens were recovered with streptavidinagarose beads (Pierce) as described (Le Bivic ET AL., 1989).

Immunopurified antigens were analyzed on 6-15% polyacrylamide slab gradient gels. FOR BIOSYNTHESIS AND MATURATION STUDIES, THE SAME AMOUNT OF CELLULAR PROTEIN WAS LOAD IN EACH LANE IN ORDER TO ALLOW QUANTITATIVE COMPARISON. 14C-LABELLED PROTEINS WERE USED AS STANDARD LOW-MASS MARKERS (AMERSHAM INTERNATIONAL plc, AMERSHAM, UK). AFTER ELECTROPHORESIS, GELS WERE IMPREGNATED WITH AMPLIFY (AMERSHAM) AND ANALYZED BY FLUOROGRAPHY. QUANTIFICATION OF IMMUNOPRECIPITATED ANTIGENS WAS ACHIEVED BY SCANNING THE FLUOROGRAMMS USING A DENSITOMETRIC SCANNER (MACINTOSH DRIVEN ONESCANER) AND IMAGE 1.45 AS A SOFTWARE. CONTROL IMMUNOPRECIPITATIONS WERE CARRIED OUT USING THE SAME PROTOCOL IN THE ABSENCE OF ANTIBODY. IN THIS CASE, NO SPECIFIC SIGNAL WAS DETECTABLE.

Endocytosis experiments

Cells grown on filters were pretreated with 5x10⁻⁵ M FK. After 30
minutes starvation in DMEM without cysteine and methionine, cells were pulsed for 20 minutes with 1 mCi/ml of $^{35}$S labeling mix (NEN, Boston, MA) and chased for 3 hours 30 minutes at 37°C in complete DMEM. After two rapid washes, anti-DPP IV or anti-525 antibodies were selectively applied to the apical or basolateral face at a concentration of 5 μg/ml and incubated for 1 hour at 37°C. All steps were performed with or without 5×10⁻⁵ M FK. Cells were then cooled at 4°C and washed four times with cold PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂.

Non internalized antibodies were removed by two successive 2 minute washes with cold elution buffer (0.2 M ascorbic acid, 0.15 M NaCl). After three washes with cold PBS, cells were lysed and immune complexes were recovered using Protein A-Sepharose beads (Pharmacia) precoated with rabbit polyclonal anti-mouse IgG (Dakopatt, Glastrup, Denmark). Analysis of immunoprecipitated samples was done on 6-15% polyacrylamide slab gradient gels and quantified as above. Endocytosis was calculated as the ratio of the radioactivity recovered for each protein on each face after acid treatment versus before acid treatment.

**RESULTS**

**Forskolin impairs both the apical and the basolateral DPP IV membrane delivery**

To study the short term effect of FK on DPP IV membrane delivery, domain specific biochemical assays were performed on filter-grown Caco-2 cells pretreated for 1 hour with 5×10⁻⁵ M FK before a 20 minute labeling pulse and various chase periods (1, 2, 3, 4, 6 hours and 23 hours) to follow the complete sorting process (Le Bivic et al., 1990; Matter et al., 1990a). Biotinylation was performed either on the apical or on the basolateral side of the cells and DPP IV was immunocolloidal from either the whole cell homogenate or from the apical or the basolateral membranes by using streptavidin-agarose beads. Results presented in Fig. 1 indicate that FK interferes with DPP IV delivery to the plasma membrane through both the apical and the basolateral pathway. Furthermore, it should be pointed out that, as previously shown for long term FK treatment (Baricault et al., 1993), FK does alter neither the overall biosynthesis of the protein nor its maturation kinetics. In the initial part of the chase (i.e. from 1 to 4 hours of chase) the rate of apical DPP IV delivery is decreased (Fig. 1A,C, left panels, and 1E). During the same chase period, the basolateral DPP IV delivery is also decreased as shown in Fig. 1A,C (left panels) and 1F. Contrary to this, for longer chase periods (i.e. 4 to 23 hours) both the apical appearance and the basolateral disappearance of DPP IV are comparable in control and FK-treated cells. It should be pointed out that FK never reverses the polarized expression of DPP IV, as shown in Fig. 1G, where the ratio of apical to basolateral is plotted versus the duration of chase. These results seem to indicate that FK blocks the initial delivery of DPP IV to both plasma membranes and raised several questions: (1) is the FK-induced blockade of DPP IV intracellular transport a more general phenomenon? (2) Does FK block the exocytosis or increase endocytosis from the apical and/or basolateral side? (3) Where do these proteins accumulate inside the cells? (4) What is the kinetics of this phenomenon? (5) Does FK act through its cAMP elevating effect?

**The forskolin-induced blockade of DPP IV intracellular transport is a more general phenomenon**

To address this question, domain specific biochemical assays were performed with 525 antigen, a strictly basolaterally targeted protein (Le Bivic et al., 1987), and sucrase-isomaltase, an apically targeted protein, which is mostly sorted to the apical membrane through the direct pathway (Garcia et al., 1993; Le Bivic et al., 1990; Matter et al., 1990a). Caco-2 cells were treated for 1 hour with 5×10⁻⁵ M FK prior to a 20 minute pulse and cells were then chased for 6 hours. Biotinylation was performed on both sides of the filters and labeled proteins were recovered from the plasma membrane compartments as described above, using immunoprecipitation and streptavidin. Results, shown in Fig. 2, indicate that the two proteins are correctly processed and are expressed as complex mature glycoproteins after a one hour FK pre-treatment and a six hour chase in the presence of the drug. In the meantime, FK reduces by about 50% the membrane delivery of 525 antigen and of SI. It should be noted that, in the particular conditions used in this study, SI biosynthesis was not affected, contrary to what has been observed previously (Rousset et al., 1985, 1989). This result suggested that FK may exert a general effect on the sorting of apically or basolaterally targeted proteins.

**FK does not increase endocytosis from the apical or the basolateral side**

Since the reduced protein cell surface expression may result either from a block on the exocytic route or from an increased endocytosis, experiments were carried out on filter-grown Caco-2 cells to measure the contribution of this later process. Endocytosis was analyzed from both the apical and the basolateral side for DPP IV and for 525 antigen. As shown in Fig. 3 and Table 1, endocytosis of DPP IV from the apical membrane of control Caco-2 cells is low, a result that confirms previous data (Klumpen et al., 1991; Matter et al., 1990c). Interestingly, FK does not significantly change the rate of DPP IV apical endocytosis. As shown in Fig. 4 and Table 1, basolateral endocytosis of 525 antigen, a strictly basolateral protein,
is low and remains insensitive to FK treatment. On the other hand, basolateral endocytosis of DPP IV is relatively high (Fig. 3 and Table 1), a result that may partly reflect the fact that DPP IV is actively transcytosed from the basolateral to the apical membrane (Le Bivic et al., 1990; Matter et al., 1990a). Again, FK was unable to significantly increase DPP IV basolateral endocytosis. Together these results favor the idea that, in the experimental model used here, FK does not interact with an endocytic process but rather interferes with a step located on the exocytic route.

**Fig. 1. Forskolin impairs both the apical and the basolateral DPP IV membrane delivery.** Filter-grown Caco-2 cells were treated for 1 hour either with 1:1000 ethanol (A and B) or with 5x10^-6 M FK (C and D). A 20 minute labeling pulse was started 60 minutes after the beginning of drug treatment. Cells were then chased in the continuous presence of the drug for 1, 2, 3, 4, 6 and 23 hours to follow the complete sorting process. At the end of the chase period, biotinylation of either the apical or the basolateral side of the filters was performed and apical (left panels) or basolateral (right panels) DPP IV was recovered using immunoprecipitation and streptavidin-agarose (A and C). A 1/10 aliquot was removed before the streptavidin agarose step in order to verify that the amount of total labeled DPP IV was not modified during the chase period (B and D). Total and cell surface DPP IV recovered after streptavidin agarose precipitation was analyzed by a 6-15% SDS-PAGE gradient. Cell surface delivery was quantified by scanning the gels. The apical delivery of DPP IV was calculated for each chase point relative to the maximal apical appearance (Control, 23 hours of chase) (E). The basolateral delivery of DPP IV was calculated for each chase point relative to the maximal basolateral appearance (Control, 3 hours of chase) (F). The amount of DPP IV detected on the apical surface over that on the basolateral surface (A/B ratio) was calculated for each chase point and was plotted as a function of the chase duration (G). FK decreases both the apical and the basolateral appearance of DPP IV, without changing the apical to basolateral ratio.
vesicular structures appear to be labeled with anti-DPP IV antibody. When Caco-2 cells were treated with FK for 7 hours, numerous intracellular vesicles became strongly stained (Figs 5D, 6C). These vesicles appear to be intracellular, as assessed by performing sections with CLSM. They also appear heterogeneous in size with some being very large. Similar results were obtained when Caco-2 cells were stained with an anti-SI antibody (Fig. 6B, D), except that as previously mentioned, SI labeling is much more heterogeneous in control Caco-2 cells as compared to DPP IV, due to a mosaic pattern of this protein (Beaulieu and Quaroni, 1991). Concomitantly, less vesicles were apparent with anti-SI antibody in FK-treated Caco-2 cells, as compared to DPP IV staining. Finally it was shown that FK also rapidly changes the cellular distribution of 525 antigen, with the emergence of numerous 525 positive vesicles in 7 hour FK-treated Caco-2 cells (Fig. 5F, H).

Cell surface proteins-containing vesicles are colabeled with Lamp-1, a resident lysosomal protein

Since the maturation of the three studied proteins has been completed in control as well as in FK-treated cells (see Figs 1, 2), this means that the protein must have reached the distal cisternae of the Golgi apparatus where terminal glycosylation occurs. In addition the absence of significant changes of endocytosis (Figs 3, 4 and Table I) suggested that the decreased cell surface expression of newly synthesized proteins may be due to an alteration of a step located on the exocytic route. Therefore the observed vesicles may be either part of the trans-Golgi or may correspond to post-Golgi compartments, i.e. trans-Golgi network, lysosomes or endosomes. Since we have previously shown that in long term FK-treated Caco-2 cells, DPP IV accumulates in Lamp-1 containing vesicles (Baricault et al., 1993), we looked for such a colocalization of DPP IV, SI or 525 antigen with Lamp-1 in the present experiments. We

Fig. 2. Forskolin impairs plasma membrane delivery of two other proteins. Filter-grown Caco-2 cells were treated for 1 hour either with 1:1000 ethanol (C, Control) or with 5x10-5 M FK (F). A 20 minute labeling pulse was started 60 minutes after the beginning of the drug treatment. Cells were then chased for 6 hours in the continuous presence of the drug. After biotinylation of the two sides of the filters, cell surface SI or 525 antigen were recovered using immunoprecipitation and streptavidin-agarose. Cell surface SI (A) and 525 antigen (D), recovered after streptavidin agarose precipitation were analyzed on a 6-15% SDS-PAGE gradient gel. A 1/10 aliquot was removed before the streptavidin agrose step in order to verify that the amount of total labeled proteins was not modified by drug treatment (B and E). Cell surface labeling was quantitated by scanning the gel and the intensity of each spot relative to the control was calculated. Results were expressed as the percentage of total membrane expression. FK reduces by about 50% the amount of SI (C) and by about 40% the amount of 525 antigen (F) delivered to the surface.

**Table 1. Quantification of endocytosis of DPP IV and 525 antigen from the apical (AP) and the basolateral (BL) side**

<table>
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<tr>
<th>Side</th>
<th>From AP</th>
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<tr>
<td></td>
<td>DPP IV</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>DPP IV</td>
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<tr>
<td>Control</td>
<td>4.17</td>
<td>n.d.</td>
</tr>
<tr>
<td>FK</td>
<td>4.25</td>
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Gels similar to those displayed in Fig. 3 and 4 were quantified using a densitometric scanner. The amount of endocytosis for one protein from one side was calculated relative to the amount on the corresponding membrane as described in the Materials and Methods. Results are mean of two independent experiments with less than 10% inter-experiment variation. n.d., not detectable.
Therefore performed a series of double staining using an anti-DPP IV mouse monoclonal antibody, or a rabbit polyclonal antibody raised against SI, or a mouse monoclonal antibody recognizing 525 antigen with either a rabbit polyclonal antibody or a mouse monoclonal antibody raised against Lamp-1, a protein which has been previously described to specifically label lysosomal structures (Carlsson et al., 1988). In control Caco-2 cells, the anti-Lamp-1 antibody stains small intracellular vesicles, randomly distributed inside the cell (Fig. 5A), which appear to be clearly distinct from the apical DPP IV staining (Fig. 5B). It should be noted, however, that a small number of vesicular structures are labeled with both anti-DPP IV and anti-Lamp-1 antibodies, a result that may reflect the presence of DPP IV in lysosomes at steady state (Kroyuden et al., 1992; Matter et al., 1990c). In FK-treated Caco-2 cells, Lamp-1 positive vesicles appear more heterogeneous in size than in control cells, with some very large structures (Fig. 5C). Comparison of DPP IV (Fig. 5D) and Lamp-1 stainings shows that the two proteins colocalized. It should be noted that all DPP IV-positive vesicles are also stained with anti-Lamp-1, whereas several Lamp-1-positive vesicles do not contain DPP IV. Quantification of the colocalization of DPP IV and Lamp-1 has been made in a previous series of experiments (Baricault et al., 1993) indicating that more than 60% of the two stainings colocalized in FK cells whereas less than 10% of colocalization was detected in control cells. The colocalization of a Golgi marker, CTR 314 (Jasmin et al., 1989), with DPP IV has also been performed and was shown not to change when Caco-2 cells were treated with FK. Similar results were obtained with the double staining using anti-SI and anti-Lamp-1 antibodies (not shown). When control and FK-treated Caco-2 cells were stained with 525 and Lamp-1 antibodies, it became apparent that FK also induces a redistribution of 525 antigen to Lamp-1 positive vesicles (Fig. 5E-H). Together these results are in line with the biochemical data displayed in Fig. 2, indicating that a 7 hour FK treatment of Caco-2 cells reduces the plasma membrane expression of cell surface proteins and reroutes these proteins to a Lamp-1 positive compartment. To further confirm the colocalization of these proteins in FK-treated Caco-2 cells, we performed another set of double staining experiments (Fig. 6). Cells were again treated for 7 hours with FK, then fixed and permeabilized and stained either with 4H3, a rat monoclonal antibody raised against DPP IV (Fig. 6A,C), and 359.7, a rabbit polyclonal antibody raised against SI (Fig. 6B,D), or with L 1650, an anti-DPP IV rabbit polyclonal antibody (Fig. 6E,G) and an anti-525 mouse monoclonal antibody (Fig. 6F,H). Results clearly show that either DPP IV and SI, two apically targeted proteins, or DPP IV, an apical protein, and 525 antigen, a basolateral protein, may be detected in the same intracellular vesicles after short FK treatment. Although the distribution of brush border hydrolases and especially of SI has been shown to be irregular (Beaulieu and Quaroni, 1991), this cannot account for the observed results, since DPP IV- and SI-containing vesicles may be detected in cells with a high level as well as in cells with a low level of hydrolase cell surface expression (see for example Fig. 6A-D or Fig. 7).

Sequestration of cell surface proteins in Lamp-1 positive vesicles is a very rapid process

We have tried to show that the rerouting of DPP IV and SI to these vesicles may also be rapid. Caco-2 cells were treated for 5 minutes, 30 minutes and 7 hours with FK and were then processed for immunofluorescence and CLSM. This experiment shows that even after a 5 minutes FK treatment numerous vesicles become strongly labeled with the anti-DPP IV antibody. The membrane of the same vesicles was selectively applied to the apical or basolateral face at a concentration of 5 μg/ml. (A) A 24 hour exposure time of the gel, and (B) the same gel after 5 days exposure. Endocytosis was calculated as the ratio of the radioactivity recovered for 525 antigen on each face after acid treatment versus before acid treatment.

Dibutyryl cAMP does not mimic the effect of FK on DPP IV cell surface expression

Forskolin is known to strongly and permanently stimulate
adenyl cyclase leading to high intracellular concentration of cAMP (Darmoul et al., 1991a; Roussel et al., 1985; Seamon and Clark, 1985). However, FK is also known to display several cAMP-independent effects (Coombs and Thompson, 1987; Grussi et al., 1987; Hoshi et al., 1988; McHugh and McGee, 1986; Middelton et al., 1986; Sergeant and Kim,

Fig. 5. Apical (DPP IV) and basolateral (525 antigen) proteins are sequestered in Lamp-1 positive vesicles after a 7 hour FK treatment. Cell culture medium was replaced either with fresh medium alone (Control, A,B,E,F) or with fresh medium containing 5x10⁻⁵ M FK (FK, C,D,G,H). Cells were incubated for 7 hours and were then fixed, permeabilized and stained as described in Materials and Methods. The same fields were analysed by conventional indirect immunofluorescence for Lamp-1 (A,C) and DPP IV (B,D) or for Lamp-1 (E,G) and 525 antigen (F,H). In FK-treated Caco-2 cells, several vesicles appear to contain both Lamp-1 and DPP IV or Lamp-1 and 525 antigen (see arrowheads). Bar, 7.5 μm for A-D and 10μm for E-H.
Therefore, we have performed experiments using dibutyryl cAMP, a stable and permeant analogue of cAMP known to mimic the cAMP effect in Caco-2 cells (Mahraoui et al., 1992). Filter-grown Caco-2 cells were treated for 1 hour with either $5 \times 10^{-5}$ M FK or up to $10^{-8}$ M dbcAMP, before a 20 minute pulse and a 6 hour chase. Apical and basolateral

![Image of colocalization experiments](image-url)

**Fig. 6.** Colocalization experiments with SI and DPP IV and DPP IV and 525 antigen. Cells were treated as in Fig. 5. Cell culture medium was replaced either with fresh medium alone (Control, A,B,E,F) or with fresh medium containing $5 \times 10^{-5}$ M FK (FK, C,D,G,H). The same fields were analysed by indirect immunofluorescence, using a Bio-Rad MRC 1000 laser scanning confocal microscope, for DPP IV (A,C) and SI (B,D) or for DPP IV (E,G) and 525 antigen (F,H). Results indicate that the two apical proteins colocalized in intracellular vesicles (see arrowheads) and that an apical and a basolateral protein are found in the same structures in cells grown for 7 hours with FK (see arrowheads). Bar, 25 μm.
Forskolin perturbs surface protein delivery in Caco-2 cells

sides of the filters were both biotinylated and the overall cell surface expressed DPP IV was recovered by specific immunoprecipitation followed by streptavidin. As shown in Fig. 9, dbcAMP was without effect on cell surface expression of DPP IV, whereas, FK inhibits by 50% this membrane expression. Therefore, we conclude that FK interferes with a trafficking and/or a sorting event through a cAMP independent mechanism.

DISCUSSION

Recent works have used FK to study the potential relationship between adenylyl cyclase activation and protein traffic in a polarized cell line, MDCK (Eker et al., 1994; Hansen and Casanova, 1994; Pimplikar and Simons, 1994). Results obtained indicated that, in this cell line, FK was able to stimulate both the exocytic and the endocytic route through a cAMP-dependent mechanism. We previously showed that FK applied to Caco-2, another polarized cell line, for a longer period of time produces an inhibition of protein cell surface expression (Barcault et al., 1993; Rousset et al., 1989). In the present work we studied whether a short term activation of adenylyl cyclase by forskolin in this cell line may alter the intracellular traffic and sorting of cell surface proteins. Indeed, we showed at the biochemical and the morphological levels that 0-24 hours treatment of Caco-2 cells with this drug does perturb protein traffic. However, alterations were largely divergent from those observed in MDCK cells since FK rapidly induces a decrease of cell surface expression of three newly

![Fig. 7. Intracellular sequestration of DPP IV and SI is a rapid event. Filter grown Caco-2 cells were cultured for 0 minute (A,B), 5 minutes (C,D) and 7 hours (E,F) in the presence of 5x10^{-9} M FK. Cells were fixed and permeabilized as above and stained with either DPP IV (A,C,E) or SI (B,D,F). Sections were analysed using a Bio-Rad MRC 1000 laser scanning confocal microscope. Sections corresponding to the vesicles layer are displayed. Results indicate that the sequestration of proteins in intracellular structures is a rapid event. Note that some vesicles are colabelled by the two markers (see arrowheads), whereas other vesicles are only stained with DPP IV (see arrow). Bar, 25 μm.](image)
synthesized membrane proteins and their sequestration in intracellular vesicles, related to the lysosomal system. Endocytosis from either the apical or the basolateral face does not contribute to this process. The phenomenon is rapid, appears to be general and concerns both the basolateral and the apical pathways, leading to the accumulation of membrane proteins in intracellular structures that resemble autophagic vesicles. We also showed that FK probably acts through a cAMP-independent mechanism, since dibutyryl cAMP does not reproduce these perturbations.

From the results presented here, it appears that, in Caco-2 cells, FK does not interfere with the biosynthetic rate of the proteins under study. In particular, it is interesting to note that FK does not inhibit the neosynthesis of SI with the protocol used here. This contrast with previous results which have shown that SI biosynthesis was reduced after long exposure of Caco-2 cells to FK (Darmoul et al., 1991a; Rousset et al., 1985, 1989). It is also important to note that FK does not interfere with the maturation of the proteins studied, since biochemical experiments clearly show that after 7 hours FK treatment, SI, DPP IV and 525 antigen appear as mature proteins on SDS-PAGE. This means that the proteins have reached the distal part of the Golgi, where the terminal glycosylation occurs. In addition endocytosis is not significantly altered. Together, these results indicate that the decrease of protein cell surface expression must be ascribed to an alteration of the intracellular traffic, which will take place on the exocytic route, either in the TGN or in a later compartment.

The precise step to be considered as a target of FK on the exocytic pathway remains to be defined. Domain specific biochemical assays indicate that after FK treatment the initial rates of DPP IV appearance at the basolateral and the apical plasma membranes are decreased, suggesting that both the direct and the indirect pathway of DPP IV to the apical membrane are slowed down. The same experiments show that the later sorting steps are less affected, since the rates of DPP IV disappearance from the basolateral and late appearance to the apical membrane are similar in control and treated cells. The ratio of

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**Fig. 8.** Transmission electron microscopy of control and FK treated Caco-2 cells. Filter grown Caco-2 cells were treated for 0 minute (A), 5 minutes (B) and 30 minutes (C,D) with 5x10^{-5} M FK. Cells were prepared for electron microscopy and immunogold labeling as described in Materials and Methods. The polyclonal DPP IV antibody was used but similar results were obtained with the mouse monoclonal antibody. Gold particles may be detected in vesicular structures after 5 minutes FK treatment. Heterogeneous pattern of DPP IV containing vesicles are displayed. Some vesicles appear as dense granules (C) that resemble lysosomes, whereas other vesicles appear to contain intracellular membranes and cellular debris (D), strongly suggesting that they are part of the autophagic system. Bar, 0.5 μm.
apical to basolateral DPP IV delivery is essentially unchanged by FK treatment, thus confirming that the drug acts in a similar manner on the two sorting pathways and probably does not interfere with the transcytotic route. Interestingly, these biochemical studies also indicate that the decrease of cell surface appearance was similar for DPP IV, a protein which uses both the direct and the indirect routes to the apical membrane, for SI, which is known to reach the brush border membrane mainly through a direct route and for 525 antigen which is known to follow a direct route to the basolateral membrane. This implies that FK interferes with a traffic step which must be common to the pathways starting from the TGN to apical and basolateral domains.

In line with these results we observed that FK induces a rapid accumulation of the three cell surface proteins studied in intracellular vesicles that were colabeled with a lysosomal membrane marker. Different routes are known to address proteins from the TGN to lysosome containing structures. Proteins from the plasma membrane may be addressed to lysosomes following the endocytic route through early and late endosomes (Gruenberg and Clague, 1992). This pathway does not seem to be involved here, since endocytosis either from apical or basolateral membranes does not change. Another route exists for proteins bearing the mannose-6-phosphate signal which are addressed to lysosomes through a mannose-6-phosphate independent route (Carlsson and Fukuda, 1992; Dahms et al., 1989; Fukuda, 1991; Williams and Fukuda, 1990). This cannot be applied here since none of the proteins studied have been shown to display these signals. It has also been shown in Caco-2 cells that DPP IV and SI may reach lysosomes without passing first through the brush border membrane (Matter et al., 1990a). In control Caco-2 cells this pathway accounts for about 7-9% of the total neosynthesized molecules. This may correspond to the observation described in this paper that a small number of Lamin and DPP IV double labeled vesicles are detectable in control Caco-2 cells. However, the way by which these proteins reach lysosomes has not been described. It is known that proteins may reach lysosome containing structures through the autophagic process. Autophagy has been primarily described as a phenomenon that starts from the RER, leading to the sequestration of cytosol and various intracellular organelles (Aristila and Trump, 1968; Dunn, 1990a; Gordon et al., 1989; Holtzman, 1989; Seglen and Bohley, 1992). However, autophagic vesicles may also receive material from the Golgi apparatus (Dunn, 1990b). In this latter route, recently described by Dunn, it has been shown that immature autophagic vacuoles (AVi) may fuse with vesicles budding from the TGN, thus giving intermediate autophagic vacuoles (AVid) which then are transformed in mature autophagic vacuoles (AVd) by the import of acid hydrolases from either lysosomes or late endosomes. Our results may fit very well with an autophagic process that will include mature proteins from the TGN, since we found that FK rapidly induces the accumulation of mature cell surface proteins in vesicles that contain a lysosomal membrane marker. The fact that we observed at the EM level that the vesicles in which these proteins are rapidly sequestered display an autophagic-like structure is strong evidence for our conclusion.

Autophagy is a rapid and non selective bulk process by which cells may degrade intracellular components, including proteins and RNA (Lardeux et al., 1987), to fulfill cellular metabolic needs in various situations such as nutrient deprivation, cellular remodeling that occurs during differentiation, aging and transformation (Auteri et al., 1983; De Duve and Wattiaux, 1966; Mortimore et al., 1989; Schwarze and Seglen, 1985). The successive steps of the autophagic process may be controlled by nutritional and hormonal agents. This has been shown in perfused liver (Lardeux and Mortimore, 1987), as well as in isolated hepatocytes (Seglen and Bohley, 1992). Autophagy also depends on the cellular energetic status since a decreased ATP concentration may reduce autophagy (Plomp et al., 1989). Dibutyryl cAMP and deacetyl forskolin, an active analogue of FK, have also been shown to inhibit autophagy (Seglen and Bohley, 1992). In our experiments we show that FK stimulates a protein sequestration process and that at least DPP IV is not degraded over a 24 hour chase period. We also note that the intracellular vesicles display some features of autophagic vacuoles (Dunn, 1990b). We show that this process is cAMP-independent. Therefore this lead to the conclusion that FK acts through one of its several cAMP-independent effects (Coombs and Thompson, 1987; Grassi et al., 1987; Hoshi et al., 1988; McHugh and McGee, 1986; Middleton et al., 1986; Sergeant and Kim, 1985). Among these effects, some cAMP-independent metabolic changes have been shown to take place in Caco-2 cells. This includes a high rate of glycogenolysis and an increased glucose consumption (Darmoul et al., 1991a; Rousset et al., 1985). Whether these metabolic perturbations may cause an increased protein

Fig. 9. Dibutyryl cAMP does not mimic the effect of forskolin.
Filter grown Caco-2 cells were treated and labeled as described in Fig. 1. FK was added at 5x10^-5 M and dbcAMP was used at 1x10^-3 M. After a 20 minute pulse, cells were chased for 6 hours. Both the basolateral and the apical side of the filters were biotinylated in order to collect the whole cell surface fraction (upper panel). The ratio of cell surface delivered DPP IV to total DPP IV was calculated from scanning data and was plotted as a percentage of the control value (lower panel). Results are the means of three independent experiments.

Forskolin perturbs surface protein delivery in Caco-2 cells 2119
sequestration related to the autophagic process requires a direct demonstration. Interestingly, it has been recently shown that in another colon cancer cell line, namely HT-29 cells, a biochemically characterized autophagic process takes place when these cells display an undifferentiated phenotype (Houri et al., 1993) which is associated with glucose metabolism perturbations comparable to those observed in FK-treated Caco-2 cells (Wice et al., 1985; Zweibaum et al., 1985).

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