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Transcytosis in Cultured Proximal Tubular Cells

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Summary. Studies were designed to examine fluid-phase pinocytosis in proximal tubular cells. Canine proximal tubules were obtained from the band IV of Percoll® gradient centrifugation of the dispersed renal cortex, and were seeded on collagen-coated polycarbonate membranes. Integrity of monolayers was confirmed by electrophysiologic measurements, and by scanning electron microscopy. At confluence cell monolayers were studied in Ussing chambers. The rate of transfer of a marker of fluid-phase pinocytosis, Lucifer Yellow CH, from the luminal to the basolateral bath was three times higher than that occurring in the opposite direction. Fluorescence microscopy demonstrated that Lucifer Yellow was trapped exclusively in the vesicular compartment. Electron microscopy of the monolayers incubated with cationized ferritin added to the luminal or to the basolateral bath revealed that endocytic vesicles were formed only at the luminal surface. Luminal-to-basolateral transfer of Lucifer Yellow was almost completely blocked at 0°C, and was significantly diminished by K^+ depletion. Transcytosis of Lucifer Yellow was stimulated twofold by 1-oleoyl-2-acetyl-glycerol. Transfer of quin-2 acetoxymethylester across the monolayer was used as a marker of the paracellular pathway, demonstrating the lack of directional selectivity of this transport route. In summary, vectorial fluid-phase pinocytosis in proximal tubular cells represents an additional mechanism contributing to fluid transport in this segment of the nephron.

Key Words pinocytosis · proximal tubule · Lucifer Yellow · quin-2 · 1-oleoyl-2-acetyl-glycerol

Introduction

Endocytic vesicles have recently attracted close attention as a ubiquitous vehicle for exchange between cell interior and the extracellular milieu (for review *see* Chistensen & Maunsbach, 1974; Maunsbach, 1976; Steinman et al., 1983; Willingham & Pastan, 1984; Schneider et al., 1985). Different pathways of endocytic vesicular shuttle have been classified: 1) absorptive pathway characterized by endosomal fusion with lysosomes before recycling; 2) secretory pathway, involving fusion of endocytic vesicles with the Golgi complex and lysosomes; and 3) transcellular pathway, characterized by the movement of pinocytotic vesicles between the opposite cell surfaces.

The handling of low molecular weight proteins by proximal tubular epithelium is well established (Maack et al., 1985). It has been demonstrated that various tracers entered proximal tubule cells by means of pinocytosis. Pinocytotic vesicles have been tracked and found to fuse with lysosomes. Hence the overall scheme of this pathway represents an absorptive pinocytosis. There is, however, a lack of information regarding the possibility of transcellular movement of endocytic vesicles in this segment of the nephron. If it exists, this pathway could represent an additional route of fluid exchange between luminal and basolateral environment. Thus, the major goal of this study was to examine the possibility of existence of transcellular fluid-phase pinocytosis in proximal tubular cells (PTC). PTC were grown on collagen-coated polycarbonate membranes and mounted in the Ussing chamber. This approach allowed us to study transfer of markers of fluid-phase pinocytosis across the monolayer. Existence of vectorial (luminal-to-basolateral) transcytosis in cultured PTC was documented in this study.

Materials and Methods

Preparation of Proximal Tubular Segments

Mongrel dogs of either sex were fed with standard Purina dog chow and had free access to water. Under pentobarbital anesthesia nephrectomy was performed, and the renal artery was immediately perfused with ice-cold saline. Isolated proximal tubular segments were prepared as described by Vinay et al. (1981). Superficial renal cortical slices were incubated in a Krebs-Henseleit buffer containing 0.1 g/dl collagenase (type 1, Sigma Chemical, St. Louis, MO), and 0.3 g/dl bovine serum albumin. The incubation continued for 45 min in a shaking water bath at

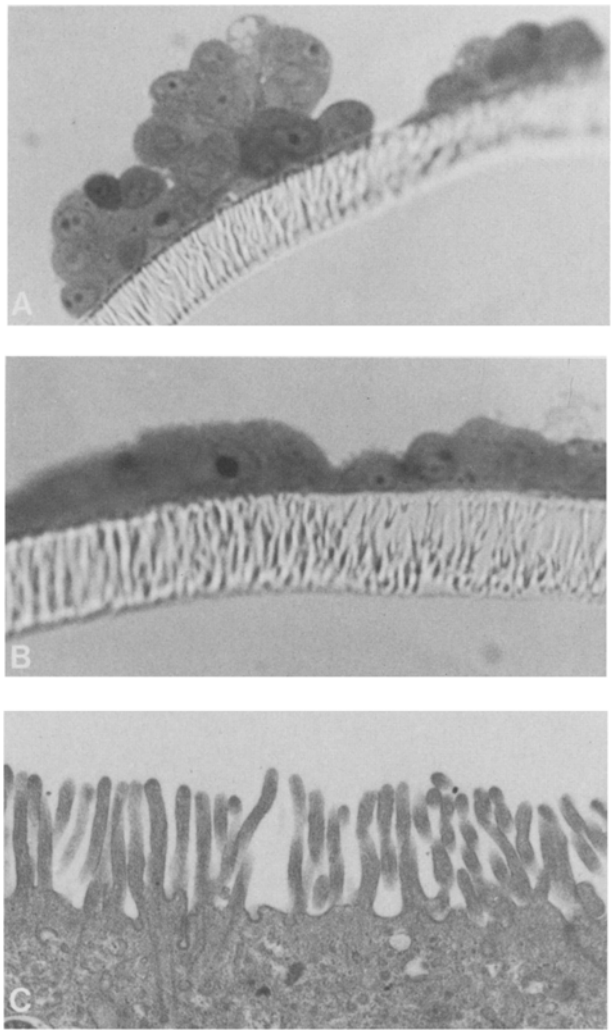

Fig. 1. Time-course of the formation of polarized monolayers following seeding of the proximal tubular fragments on collagen-coated polycarbonate membranes. (*A*) Five to seven days following the initiation of cultures, conglomerates of nonpolarized cells are seen; gaps in the monolayer are still present. 122×. (*B*) Two weeks after seeding cells are uniformly polarized, covering the entire membrane with a monolayer. 122×. (*C*) Electron micrograph of the apical portion of the 2-week-old proximal tubular cell grown on a collagen-coated membrane. 21,300×

37°C in an atmosphere of 95% O_2/5% CO_2. The tissue slices were dispersed and filtered through a small strainer. The homogenate was centrifuged at 620 × *g*, the pellet was resuspended in 15 ml of Krebs-Henseleit buffer and washed three times. After incubation in 5 g/dl of bovine serum albumin for 5 min at 4°C, the crude preparation of tubular segments was recentrifuged at 620 × *g*. The pellet was resuspended in 30 ml 50% Percoll and centrifuged for 30 min at 27,000 × *g* at 4°C.

Cell Culture Techniques

Isolated tubular segments from the band IV (proximal tubules) of the Percoll gradient were seeded at hyperconfluent density on collagen-coated polycarbonate membranes (0.8 μm pore size; Nucleopore Corp., Pleasanton, CA) glued to rubber rings with the Millipore cement #1 formulation (Millipore, Bedford, MA). The growth medium was initially 45% Dulbecco's MEM, 45% F-12 medium, 5% fetal calf serum and 5% donor horse serum. Cells were maintained in a constant 95% O_2/5% CO_2 atmosphere at 37°C. After 3 days, the growth medium was changed to serum-free 50% Dulbecco's MEM and 50% F-12 medium supplemented with 5 μg/ml transferrin, 3 μg/ml insulin, 25 ng/ml PGE_1, 5×10^{-8} M hydrocortisone, and 5×10^{-12} M triiodothyronine.

This preparation has been characterized in our previous study (Hruska et al., 1986) and by other investigators (Taub & Sato, 1980; Vinay et al., 1981; Chung et al., 1982; Chuman et al., 1982; Sakhrani et al., 1984; Yau et al., 1985). Morphological characterization of the primaries revealed formation of a cuboidal monolayer with microvilli covering the apical surface, formation of occluding junctions (Hruska et al., 1986), binding of rabbit anti-dog proximal tubular brush-border membrane antibody to the surface of cultured cells (Yau et al., 1985). Functionally, primary cultures of proximal tubular cells exhibit sodium-dependent glucose transport, enrichment in marker enzymes such as PEPCK and alkaline phosphatase (95 to 98% of the cells showed positive alkaline phosphatase staining) as compared to the preparations obtained from the whole cortex, and ability to convert 25-hydroxycholecalciferol to 24,25-dihydroxy-form (Sakhrani et al., 1984; Yau et al., 1985; Hruska et al., 1986).

Cells were used 14 to 21 days after initiation of cultures. At this time, the cells formed a confluent monolayer as determined by light and electron microscopy of fixed sections, and by the electrical characteristics of the monolayers. At confluence, PTC grown on a semipermeable support expressed electrical polarity with establishment of a lumen-negative transepithelial potential difference of 0.28 ± 0.09 mV and a resistance of 94.7 ± 9.3 ohm cm^2 (n = 23), both parameters characteristic of a leaky epithelium. Treatments used in this study did not affect the resistance of the monolayers, except when indicated in the text (a decrease of the transepithelial resistance following administration of EGTA, and an increase in the resistance upon exposure to 1-oleoyl-2-acetyl-glycerol).

Transfer of Lucifer Yellow CH Across the Monolayers

To study pinocytosis (or transcytosis), PTC monolayers were mounted in a modified Ussing chamber. Both sides were bathed in 5 ml of Krebs-Henseleit buffer, pH 7.4. Both hemichambers were water-jacketted to maintain the temperature of the solutions at 37°C. Solutions were gently mixed by constant bubbling of 95% O_2 + 5% CO_2. After 30-min equilibration, a marker of fluid-phase pinocytosis, Lucifer Yellow CH (LY), was added to one side of the membrane. Solution on the contralateral side (without added dye) was changed every hour, and sampled for fluorescence measurements. After 3 hr of fluorescence measurements, each side was thoroughly washed and LY was added to the opposite side. Fluorescence measurements were then continued for an additional 3 hr. LY was used regularly at a concentrations 0.25 mg/ml (LY transfer exhibited linear relationship with the concentrations of LY tested in the range 0.06 to 0.75 mg/ml). In a separate series of experiments quin-2 acetoxymethyl ester (quin-2/AM) was used at a concentration of 20 μM in place of or together with LY. Net transfer was calculated as an algebraic sum of transfer in both directions. Fluorescence of the samples was measured with a spectrofluorimeter SLM 4880 (SLM Instruments, Urbana, IL). For measurements of LY fluorescence,

samples were excited at 430 nm, and fluorescence intensity was monitored at 540 nm wavelength (Swanson et al., 1985). Fluorescence measurements of quin-2/AM containing samples were performed using excitation wavelength of 339 nm, and emitted light was collected at 430 nm, as described by Tsien et al. (1982).

Transmission and Scanning Electron Microscopy

Cultures growing on polycarbonate membranes were fixed in cold cacodylate-buffered 2% glutaraldehyde for 48 hr, and rinsed with cacodylate buffer, pH 7.4. Specimens for transmission electron microscopy were post-fixed for 1 hr with 1% osmium tetroxide in the same buffer, and then dehydrated through a graded ethanol series. After embedding in Epon, ultrathin sections were cut normal to the membrane surface in a Porter Blum MT-2 ultramicrotome. Sections were stained with uranyl acetate and lead citrate, and observed under a Philips 200 transmission electron microscope.

In a series of experiments cationized ferritin (Sigma Chemical, St. Louis, MO) was used as a marker of pinocytosis. Horse spleen ferritin coupled with N,N-dimethyl-1,3-propanediamine at a concentration of 1 mg/ml was added to the luminal or to the contraluminal side of the Ussing chamber for 3 to 30 min. Then cell monolayers were washed in Krebs-Henseleit buffer and fixed as described (*vide supra*). Preparations were processed as for transmission electron microscopy, except that they were observed unstained.

For the scanning electron microscopy (performed in collaboration with Dr. D. Menton, Department of Anatomy and Neurobiology, Washington University School of Medicine), specimens were fixed in 2% glutaraldehyde for 24 hr, dehydrated in a graded ethanol series, and dried out of liquid CO_2 by the critical point method in a Denton DCP-1 apparatus. After drying, the polycarbonate membranes with the cells were glued to specimen studs with the Millipore cement #1 formulation, and coated with approximately 15 nm of gold in a Denton Desk-1 cold sputter unit. All specimens were examined and photographed with a Philips 501 scanning electron microscope.

Fluorescence Microscopy

PTC were grown on coverslips as described above, with the only exception that they were used in studies 5 to 7 days following seeding. Cells were incubated in Krebs-Henseleit buffer with an addition of 1 mg/ml of Lucifer Yellow CH. The distribution of the dye was examined with a Zeiss IM-35 microscope equipped for epifluorescence. Photographs were taken with Kodak Tri-X pan 400 ASA film.

Analysis of the Data

The data are presented as mean ± SEM. The statistical comparison was performed using paired Student's *t*-test.

Other Materials

Cell culture medium was obtained from KC Biological. Quin-2/AM, 1-oleoyl-2-acetyl-glycerol and Lucifer Yellow CH were from Sigma Chemical.

Fig. 2. Scanning electron microscopy panoramic view of proximal tubular cells forming a monolayer on a collagen-coated polycarbonate membrane. Bar = 10 μm

Results

Validity of the Model

The prerequisite to study a transfer across the monolayer in the Ussing chamber is the integrity of the cell monolayers. For this reason light microscopy and scanning electron microscopy of randomly selected preparations in every batch of cultured primaries of PTC were performed routinely. In the preliminary experiments, the time required for proximal tubule fragments seeded upon polycarbonate membranes to form uniformly polarized monolayers was studied. Conglomerates of proximal tubule fragments with collapsed lumens were found up to one week following seeding (Fig. 1*A*). In the two-week-old cultures the monolayers were confluent, and the polarization of the cells on a collagen-coated membrane was accomplished (Fig. 1*B*,*C*). This was the reason all the studies reported below were performed using 2 to 3-week-old monolayers. This time-course of the polarization of the seeded cells is comparable with that one observed by Madara and Dharmsathaphorn (1985) in cultured T_{84} cells. Figure 2 demonstrates a low-power scanning electron-microscopy image of the confluent monolayer grown on a polycarbonate membrane coated with collagen (Vitrogel®). The integrity of the monolayer two weeks following seeding was beyond doubt. Furthermore, in each experiment transfer of fluorescent markers was examined in both directions, as described below, and these data provided an additional confirmation of confluence of the cell monolayers. Finally, monitoring the transepithelial resistance provided an additional clue to assess the integrity of the monolayers.

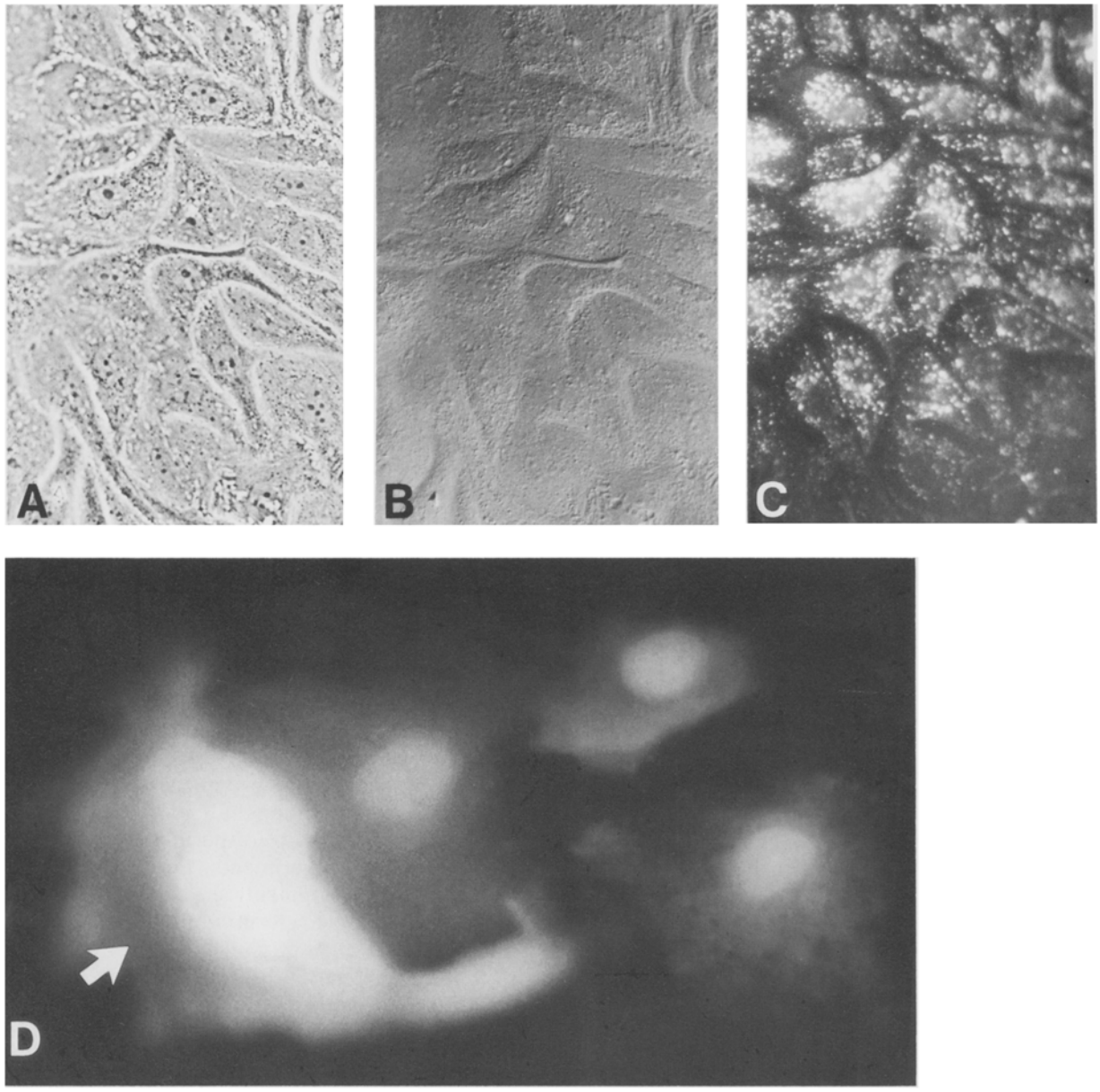

Fig. 3. Differential staining patterns of proximal tubular cells loaded with LY by fluid phase pinocytosis (*C*) or by microinjection of LY (*D*). (*A–C*) Phase-contrast, DIC and fluorescence microscopy of PTC grown on a glass cover slip. 0.5 mg/ml LY was added to the medium for 15 min, washed out, and the cells were photographed using phase-contrast (*A*), DIC (*B*) and fluorescence optics (*C*). Note punctate fluorescence throughout the cytoplasm; nuclei remained unstained. 400×. (*D*) The cell indicated with an arrow was microinjected with LY and observed under a fluorescence microscope. Note homogeneous fluorescence of the cytoplasm of this and of three neighboring cells (cell-to-cell communication) with a predominant staining of nuclei. 1,200×

Validity of a Marker of Fluid-Phase Pinocytosis, Lucifer Yellow CH

In our preliminary experiments it was found that LY in concentrations up to 2 mg/ml did not affect PTC viability, as assessed by trypan blue exclusion. More than 95% of the cells excluded the dye upon exposure to 0.1 to 2.0 mg/ml of LY. This is in agreement with the data obtained in macrophages, where LY did not affect cell viability at concentrations up to 6 mg/ml (Swanson et al., 1985).

It was important to assess the distribution of LY in cultured PTC. Fluorescence microscopy of cells preloaded with LY for 5 to 10 min revealed a

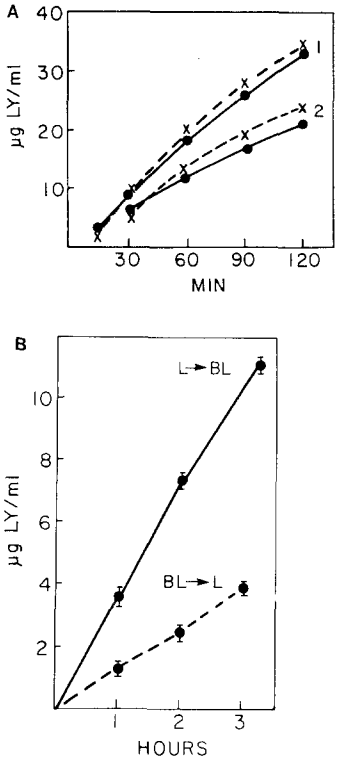

Fig. 4. (*A*) Diffusion of LY across a collagen-coated polycarbonate membrane without the cells (1), and across the membrane covered with cell monolayer which was treated with 2.5 mM EGTA (2). LY in the concentration of 0.25 mg/ml was added to either luminal or basolateral bath, and its transfer to the opposite side was monitored. The addition of EGTA to the cell monolayers (2) resulted in a 60 ± 7% (n = 4) drop in the transepithelial electrical resistance. Here and in subsequent figures, solid line represents luminal-to-basolateral transfer; dotted line = basolateral-to-luminal transfer of LY. (*B*) Bidirectional transfer of LY across the proximal tubular cell monolayers. Conditions are similar to those described in Fig. 4*A*. In contrast to Fig. 4*A*, the rate of the luminal-to-basolateral transfer of LY is higher than that occurring in the opposite direction

sparse punctate fluorescence within the cytoplasm, which itself remained unstained (Fig. 3*C*). This spatial compartmentalization of LY was compatible with the requirements for markers of fluid-phase pinocytosis: 1) impermeability across the plasma membrane, and 2) access to the cell interior exclusively via pinocytosis. In contrast to these findings, when LY was microinjected into the cell (in order to bypass the plasma membrane and get access to the cytoplasm), this resulted in a completely different phenomenon (Fig. 3*D*). The cytoplasm was homogeneously fluorescent with preferential staining of the nucleus (note that nucleus was unstained when LY was applied to the medium, Fig. 3*C*). Fig. 3*D* also demonstrates cell-to-cell communication, which is a specific feature of the proximal nephron.

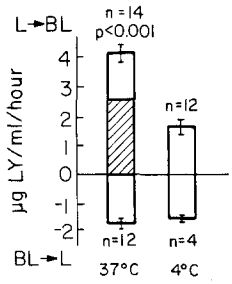

Fig. 5. Rate of bidirectional transfer of LY across the proximal tubular cell monolayers at 37 and at 4°C. The shaded area represents net transfer of LY. Note the disappearance of net transfer of LY at 4°C

Together with the data on LY published previously (Swanson et al., 1985) this study demonstrated that the dye represented an excellent marker of fluid-phase pinocytosis in cultured PTC.

Polarity of Lucifer Yellow CH Transfer Across the PTC Monolayers

The study of diffusion of LY across collagen-coated polycarbonate membranes free of cells demonstrated that LY appearance on the contralateral side was characterized by a high rate of the transfer (17.15 ± 1.14 μg/ml/hr), and was independent of sidedness of addition (Fig. 4*A*). This process was close to linearity, suggesting that under the existing concentration gradient the effect of back-diffusion is negligible. Similarly, when cell monolayers were maximally permeabilized by EGTA (Fig. 4*A*), the rate of diffusion of LY was independent of sidedness of the addition. In contrast to this, PTC monolayers revealed selectivity of the transfer depending on the side of the addition of LY (Fig. 4*B* and Fig. 5). The rate of transfer across the monolayers was stable up to 9 hr of incubation. The luminal-to-basolateral transfer of LY averaged 4.228 ± 0.265 μg/ml/hr (n = 14), while the basolateral-to-luminal transfer occurred at a rate of 1.535 ± 0.126 μg/ml/hr (n = 12; $P < 0.001$). The resulting net transfer averaged 2.693 μg/ml/hr (Fig. 5). Thus, the luminal-to-basolateral transfer of LY was almost three times higher than that occurring in the opposite direction.

Physical Factors Affecting Net Transfer of Lucifer Yellow CH

It is known that pinocytosis is inhibited almost completely at 0°C (Leslie, 1980; Swanson et al., 1985). Since luminal-to-basolateral transfer of LY occurred in part via transcytosis, it was essential to

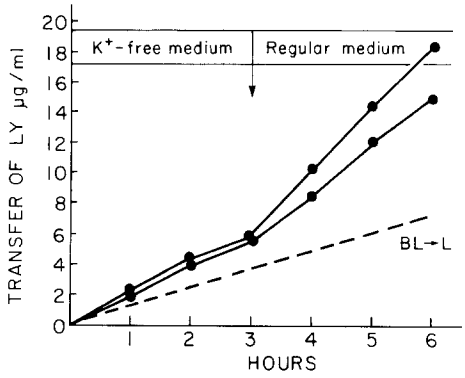

Fig. 6. Effect of K^+ omission in the incubation medium on the rate of bidirectional transfer of LY across cell monolayers. Note that basolateral-to-luminal transfer of LY is not affected by the K^+-free medium incubation, while luminal-to-basolateral transfer of the dye is significantly, but reversibly, reduced

examine the temperature-sensitive component of this transfer. When water-jacketted Ussing chambers were exposed to 4°C, a significant decline in the rate of the luminal-to-basolateral transfer of LY was observed (Fig. 5). Under these conditions, LY transfer averaged 1.581 ± 0.202 µg/ml/hr (n = 12), the value similar to the basolateral-to-luminal transfer (1.430 ± 0.07 µg/ml/hr). This resulted in a virtually complete annihilation of net transfer of LY. The data showed that the temperature-sensitive component of the luminal-to-basolateral transfer of LY was equal to the net transfer, which, hence, could reflect transcytosis of LY.

To further ascertain the transcytotic origin of net transfer of LY, the following series of experiments was performed. PTC monolayers were incubated in K^+-free Krebs-Henseleit buffer for 3 hr; then KCl was replenished, and experiments continued for an additional 3 hr. In two separate experiments (each performed in duplicate) luminal-to-basolateral transfer of LY during the K^+-free incubation period was reduced to 1.958 ± 0.119 µg/ml/hr (Fig. 6). Restoration of the regular Krebs-Henseleit buffer resulted in an increase of the luminal-to-basolateral transfer to 3.618 ± 0.359 µg/ml/hr. Thus, potassium depletion was associated with a 50% decrease in the luminal-to-basolateral transfer of LY. Since potassium depletion inhibited the rate of pinocytosis in cultured fibroblasts and hepatocytes (Larkin et al., 1985), this datum suggested that a significant fraction of the luminal-to-basolateral transfer of the marker of fluid-phase pinocytosis was represented by transcytosis. This complemented the data on the temperature-sensitive fraction of the luminal-to-basolateral transfer of LY. Collectively, the results of these series of experiments indicated that the net transfer of the marker of fluid-phase pinocytosis, LY, occurred mostly via vesicular transcellular movement from the apical to the basolateral cell surface.

Contribution of the Paracellular Pathway to the Transfer of Lucifer Yellow CH

The nature of the basolateral-to-luminal transfer of LY was not clear, however. Theoretically, it could be due to either paracellular pathway, or to the slow rate of transcytosis in the direction basolateral-to-luminal surface, or to both. Three series of experiments dealing with this question are presented below.

In order to examine qualitatively the routes of LY transfer, experiments using cationized ferritin (CF) were performed. CF was added either to the luminal or to the contraluminal bath for periods of 3 to 30 min. Then the PTC monolayers were rinsed and immediately fixed, as described in Materials and Methods. After 3 to 5 min of luminal exposure to CF, it was found on the apical surface of the PTC, within the invaginations of the brush border, within the multiple vesicular and vacuolar structures throughout the cytoplasm, and in the intercellular space (Fig. 7*A–D*). This distribution was repeatedly observed following 30-min exposure to CF. In contrast to this, basolateral exposure to CF for 3 to 5 min and up to 30 min resulted exlusively in labeling of the intercellular spaces below the occluding junctions, without any detectable labeling of the vesicular structures (Fig. 7*E*). The data indicated that basolateral addition of CF resulted in the penetration of the marker to the intercellular spaces, which was limited by the occluding junctions, and did not involve any intracellular traffic. If the data obtained with CF can be extrapolated to the transfer of LY, it would indicate that the basolateral-to-luminal transfer occurred only via the paracellular pathway, whereas the luminal-to-basolateral translocation of LY employed both the transcytotic and the paracellular routes. Thus, the net transfer of the dye was indeed represented by transcytosis.

If the above conclusion regarding the paracellular pathway as a route for the basolateral-to-luminal transfer of LY is correct, one would expect that experimentally induced changes in its permeability would result in corresponding changes in the transfer of LY. In the next series of experiments the resistance of the monolayers was continuously monitored during successive additions of EGTA to the luminal bath, a maneuver known to desintegrate the occluding junctions (Pitelka et al., 1983). LY was added to the basolateral bath, and its transfer to the luminal side was monitored in parallel with the

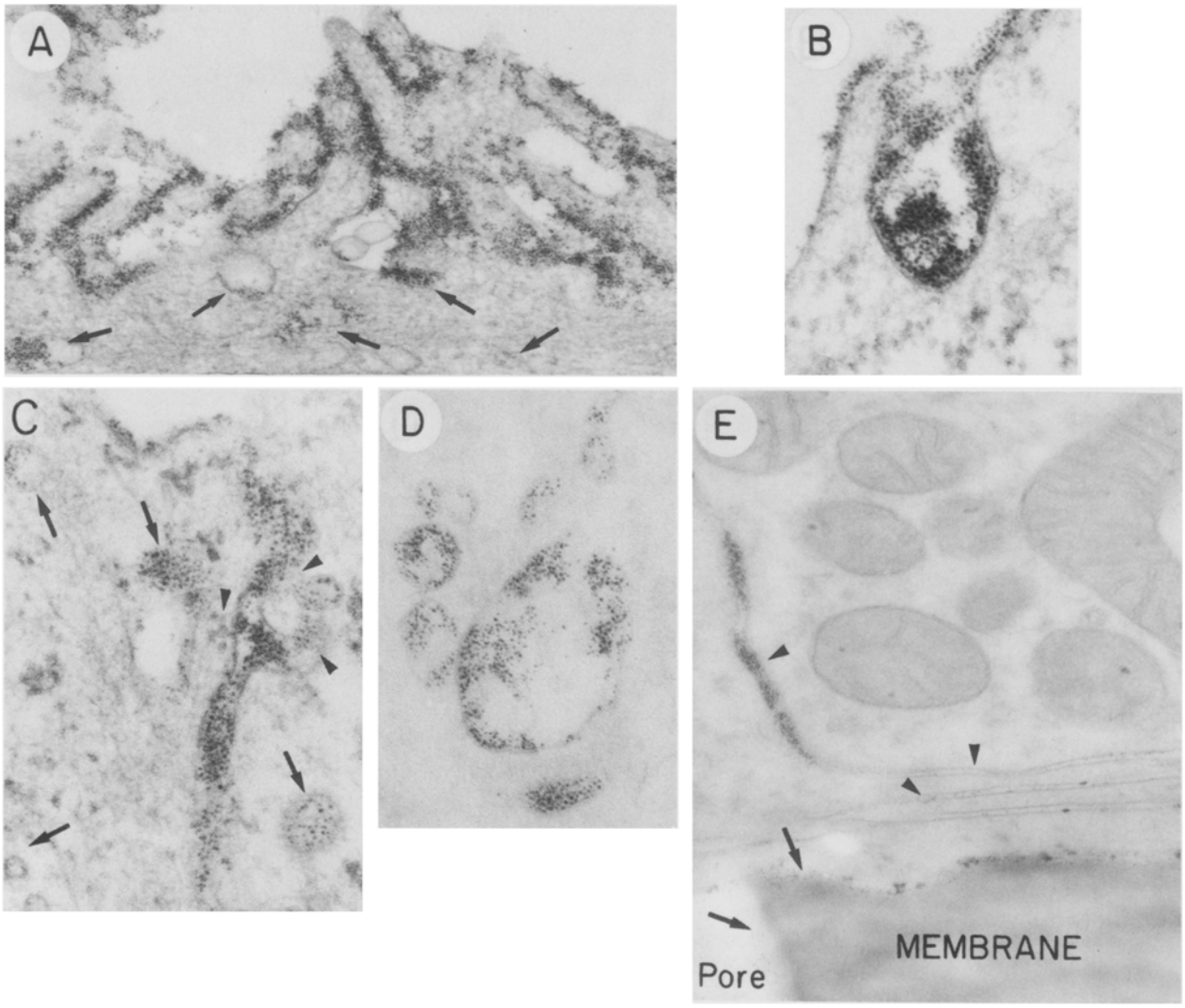

Fig. 7. Transmission electron microscopy of proximal tubular cell monolayers exposed to cationized ferritin: in *A–D* cationized ferritin was added to the luminal bath; in *E*, cationized ferritin was added to the basolateral bath. (*A*) Apical surface of the cells with cationized ferritin surrounding microvilli and being trapped by endocytic vesicles (arrows). Time: 5-min exposure; 52,900×. (*B*) Detailed view of the invagination of the plasma membrane with trapped cationized ferritin. Time: 5 min; 82,100×. (*C*) Two adjacent cells with multiple endocytic vesicles containing cationized ferritin (arrows) throughout the cytoplasm. Note that some have communication with and are emptying their contents into the basolateral space (arrowheads). Time: 5 min; 82,100×. (*D*) High magnification view of endocytic vesicles and an endosome containing cationized ferritin. 82,100×. (*E*) Proximal tubular cells exposed to cationized ferritin from the basolateral side. A part of a porous polycarbonate membrane with adherent cationized ferritin is shown (arrows). Note the appearance of cationized ferritin in the basolateral infoldings

changes in the resistance of the monolayers. Figure 8 summarizes the results obtained. Additions of EGTA resulted in a stepwise decrease of the transepithelial resistance and of the short-circuit current. This process was accompanied by an increase in LY transfer to the luminal bath. When the transepithelial resistance dropped to 54 ± 5% of the control level (concentration of EGTA of 1 mM; $P < 0.05$), the transfer of LY increased more than twofold ($P < 0.001$). This was not due to a denudation of the collagen-coated membrane and cell desquamation, since we could not harvest cells from the luminal bathing solution. Thus, the maneuver leading to an increase in the permeability of the paracellular pathway (increase in the conductivity of the monolayer), resulted in a significant elevation of the rate of LY transfer from the basolateral to the luminal bath. This is in concert with the conclusion that the bulk transfer of LY in this direction occurs via a paracellular pathway.

Since this point was essential for understanding of the traffic routes in cultured PTC monolayers, we

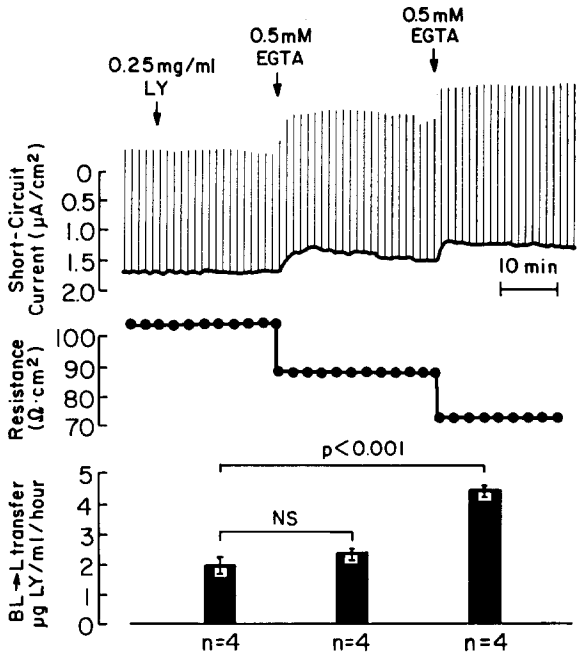

Fig. 8. Simultaneous measurements of the short-circuit current (upper tracing), the transepithelial electrical resistance of the monolayers (the tracing in the middle represents the typical example), and of the basolateral-to-luminal transfer of LY (lower panel). 0.5 mM EGTA caused a 16 ± 6% decrease in the transepithelial resistance. At the final concentration of EGTA of 1 mM the resistance dropped to 54 ± 5% ($n = 4$) of the control level ($P < 0.05$). This was accompanied by a significant increase in the permeability of the monolayers to LY

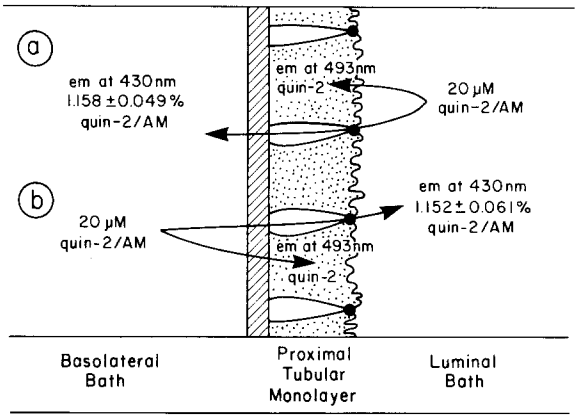

Fig. 9. Transfer of quin-2/AM (emission maximum at 430 nm) across the monolayers occurs exclusively via the paracellular pathway; when trapped in the cell, quin-2/AM is converted to quin-2, with a concomitant shift of emission maximum to 493 nm. Leakage of quin-2 out of the cells would not invalidate the measurements of the transfer of quin-2/AM by virtue of their different emission spectra. Luminal-to-basolateral and basolateral-to-luminal transfer of quin-2/AM across the monolayers are equal

attempted to obtain an additional evidence of distinct pathways involved in this process by means of a different approach, using another fluorescent dye, quin-2 acetoxymethyl ester (Tsien et al., 1982). This compound is membrane-permeant with an emission maximum at 430 nm, and undergoes de-esterification to free quin-2 in the cytoplasm of the cell. Free quin-2 is relatively membrane impermeant, and is characterized by an emission maximum at 493 nm (Tsien et al., 1982). Thus, quin-2/AM can reach the opposite side of the monolayer only via the paracellular pathway, as that which enters the cell will be converted to free quin-2 with a different emission maximum. Thus, quin-2/AM represents a convenient probe to study this pathway of transfer across the monolayers. In the following series of experiments quin-2/AM was added to one side of the monolayers for 3 hr, then thoroughly washed out and added to the opposite side for another 3 hr. Sampling of the bathing solutions was performed each hour. Figure 9 summarizes the results obtained in three separate experiments. Transfer of quin-2/AM, occurring exclusively via the paracellular pathway, averaged 1.158 ± 0.049% and 1.152 ± 0.061% of the contralateral pool for the luminal-to-basolateral and the basolateral-to-luminal vectors, respectively. Thus, the rates of the transfer were similar in both directions. This approach provided an additional evidence that paracellular transfer was nonselective, and occurred at the same rate independently of the direction of transfer. Hence, the net transfer of LY indeed represented a fraction of transfer which was contributed mostly, if not exclusively, by transcytosis.

Protein Kinase C-Induced Stimulation of Transcytosis

Protein kinase C is implicated in the regulation of pinocytosis in the different cells (Haigler et al., 1979; Phaire-Washington et al., 1980; Swanson et al., 1985). The role of C-kinase activation as it pertains to the cultured PTC was assessed in further experiments. Cell monolayers were exposed to a membrane-permeant analog of diacyl-glycerol, 1-oleoyl-2-acetyl-glycerol (OAG) during the typical protocol for LY transfer study. Addition of 50 μg/ml of OAG to the luminal bath was accompanied by an increase in the transepithelial resistance by 8.6 to 15% (*data not shown*). Despite this fact, OAG resulted invariably in a twofold increase in the luminal-to-basolateral transfer of LY (Fig. 10), without any effect on the basolateral-to-luminal transfer of the dye (data from four separate experiments). Thus, activation of protein kinase C under physio-

logic conditions may represent a powerful mechanism of stimulation of transcytosis in PTC.

Discussion

Fluid-phase pinocytosis is a nonselective process which does not require specific binding of substances to particular membrane domains, and which is almost completely blocked at low temperature (Silverstein et al., 1977; Steinman et al., 1983; Schneider et al., 1985). This process has been demonstrated in a wide variety of cell types (for review *see* Schneider et al., 1985). There is a strong evidence that the similar process takes place in the proximal tubule. In a series of studies employing cationized ferritin and dextran microinjection into the lumen of the proximal tubule, these substances underwent pinocytosis, and have been demonstrated within the endocytic vesicles (Christensen et al., 1981; Christensen & Maunsbach, 1981; Christensen, 1982; van Deurs & Christensen, 1984). An additional morphologic evidence of the recycling of endocytic vesicles has been recently presented by Russo et al. (1985). The data described in this study demonstrate that fluid-phase pinocytosis in cultured proximal tubular cells is a vectorial process, resulting in the luminal-to-basolateral translocation of the specific marker, Lucifer Yellow CH.

Several lines of evidence support this conclusion. When LY is translocated from the basolateral to the luminal bath, this process occurs exclusively via the paracellular pathway (data on CF, experiments with EGTA, data obtained with quin-2/AM). When LY is added to the luminal bath, its transfer to the opposite side occurs via two distinct routes: 1) paracellularly, and 2) transcellularly. The paracellular route does not express any directional selectivity (data obtained with quin-2/AM). In contrast, pinocytosis occurs only from the apical membrane (CF data), and is characterized by transcellular movement of endocytic vesicles (transcytosis), which eventually fuse with the basolateral membrane liberating their contents (data obtained with CF). Consequently, net transfer of LY (the difference between the luminal-to-basolateral and the basolateral-to-luminal transfer rates) represents the fraction of the dye that underwent transcytosis. It is of interest that a similar vectorial movement of endocytic vesicles has been recently described in hepatocytes (Lake et al., 1985). Very important results have been obtained in the study of the kinetics of tritiated albumin absorption and catabolism by isolated perfused proximal convoluted tubules (Park & Maack, 1984). These investigators demonstrated the dual kinetics of albumin absorption, and suggested that it was due to a combination of adsorptive endocytosis (low capacity system) and fluid-phase pinocytosis of albumin (high capacity system). This study provided the first strong evidence for the existence of fluid-phase transcytosis in this particular segment of the nephron.

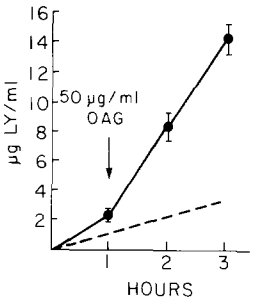

Fig. 10. The effect of the cell-permeant analog of diacylglycerol, 1-oleoyl-2-acetyl-glycerol (50 μg/ml) on LY transfer across the monolayers, demonstrating a twofold stimulation of the luminal-to-basolateral transcytosis

Different maneuvers known to modulate the rate of pinocytosis were tested in the present study. Low ambient temperature has been shown to block pinocytosis (Silverstein et al., 1977). The temperature-sensitive component of the luminal-to-basolateral transfer of LY in PTC monolayers was equal to the net transfer of the dye. This finding again suggested that the net transfer of LY is due to transcytosis. This is further supported by the results obtained using K^+-free Krebs-Henseleit buffer, the maneuver resulting in an inhibition of endocytosis (Larkin et al., 1985). In our experiments incubation of PTC monolayers in K^+-free medium resulted in a 50% decrease in the rate of transcytosis. Finally, activation of protein kinase C led to a twofold increase in the rate of transcytosis in PTC. This finding is in agreement with the data on stimulation of endocytosis by various growth factors and phorbol esters in the different cell types (Haigler et al., 1979; Phaire-Washington et al., 1980; Swanson et al., 1985). Thus, the maneuvers known to modulate the rate of endocytosis and tested in this study, resulted in the respective changes of net transfer of LY across PTC monolayers, in fair agreement with the transcytotic nature of LY transfer.

It is possible that transcytosis in PTC can contribute to fluid transport in this segment of the nephron. Russo et al. (1985) have demonstrated that ouabain-resistant regulation of the cell volume following swelling depended on exocytosis. The similar process has been described in hepatocytes during volume recovery (van Rossum & Russo,

1981). It is an unenviable task to attempt to estimate the relative importance of this route in fluid transport across PTC monolayers. This would necessarily require (for our experimental design) an assumption that the concentration of LY within pinocytotic vesicles, as well as their volume remain constant during their movement across the cell. Regarding this simplified situation, the following estimates have been performed. The number of cells on the polycarbonate membrane in our experiments averaged 450,000, or 60 ± 3 cells/10^4 μm^2, as calculated from our scanning electron micrographs. Net transfer of LY equals 0.028 ng/cell/hr, which will result in net fluid transcytosis of 0.112 nl/cell/hr for this simplified model.

It is interesting to compare our results obtained in PTC with those of Von Bonsdorff et al. (1985) obtained in MDCK cells. The latter express characteristics of the distal nephron and collecting tubule epithelium. In MDCK cells grown as monolayers, fluid phase transcytosis occurs at the same rate in both directions. Furthermore, the rate of fluid phase transcytosis in MDCK cells is significantly lower than in cultured proximal tubular cells. It is possible that the observed differences in the fluid phase transcytosis between these two models reflect different functional specializations of epithelial cells along the nephron.

In summary, PTC grown as a monolayer on a collagen-coated semipermeable support represent a convenient model to study transfer of markers of fluid phase pinocytosis. This polarized epithelial monolayer expresses vectorial (luminal-to-basolateral) transcytosis of a marker of fluid phase pinocytosis, Lucifer Yellow CH. This process is blocked at low temperature, is inhibited by potassium depletion, and is stimulated twofold upon activation of protein kinase C.

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Note Added in Proof

The authors apologize for a miscalculation of Lucifer Yellow transfer. All the values for transfer should be divided by a factor of 250. We are grateful to Dr. K. Simons (Heidelberg) for directing our attention to this mistake.