J. Membrane Biol. 104, 45–55 (1988)

The Journal of
Membrane Biology

Regeneration of Resistance and Ion Transport in Rabbit Corneal Epithelium after Induced Surface Cell Exfoliation

J. Mario Wolosin
Departments of Ophthalmology and Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029

Summary. Exposure of the rabbit corneal surface to a 20-μM digitonin-0.9% NaCl solution leads to permeabilization of the most superficial cells of the stratified epithelium. The devitalized cells exfoliate spontaneously from the corneal surface. Detergent exposure limited to 4–8 min leads to permeabilization and rapid exfoliation of a monolayer of surface cells. Consistent with the presence of the epithelial paracellular permeability barrier in this cell layer, their permeabilization results in complete loss of transepithelial resistance (R_t). Within minutes after detergent removal an initial recovery of R_t can be noticed indicating generation of a new paracellular permeability barrier by the viable subsurface cells. This recovery proceeds rapidly and R_t reaches within 70 min a maximum equal to > 90% of the preexfoliation values (= 2.43 k$\Omega \cdot$ cm^2, $n = 22$). The R_t recovery is fully blocked in a reversible manner by 10 μM dihydrocytochalasin B. The recovery is not affected by inhibition of protein synthesis with 5 μM cycloheximide. When the ocular surface is treated again with digitonin the permeabilization and exfoliation of a monolayer of cells and loss of R_t are repeated. After the second detergent exposure an initial recovery of R_t occurs as before within minutes. However, the pace of R_t recovery is much slower; 4–5 hr are required to reach a stable maximal R_t values amounting to about 73% of initial control. This recovery can be fully blocked by 5 μM cycloheximide indicating that protein synthesis is required for generation of tight junctions by the second subcellular layer. With only a fraction of R_t recovered, short-circuit currents amounting to, at least, 50% of control values and attributable in part to cell-to-tear movement of Cl^- through the apical surface can be measured. This suggests that apical-type Cl^- channels are either present in the apically facing membrane of subsurface cells or that they are rapidly inserted in it from preexisting intracellular pools immediately following the devitalization of the surface cells by digitonin.

Key Words stratified epithelium · corneal epithelium · tight junction generation · cell polarization

Introduction

The genesis of occluding junctions and cellular polarity in epithelia has attracted increasing interest during the last few years. The use of immortalized cell lines has allowed the circumvention of the limitations imposed by the permanent character of the cells in monolayered simple epithelia thereby facilitating the study of assembly of tight junctions following their transient disassembly or digestion of its protein (Cereijido et al., 1978; Griepp et al., 1983; Cohen et al., 1985; Gonzalez-Mariscal, Chavez de Ramirez & Cereijido, 1985; Gumbiner & Simmons, 1986).

Unlike many ion-transporting monolayered epithelia, transporting stratified epithelia undergo constant cell loss and renewal (Hanna & O'Brien, 1960; Thoft & Friend, 1983; Budtz, 1985). As a result of this, the biogenesis of occluding junctions and membrane polarity in these tissues is a constant activity whose existence evokes a number of specific and intriguing questions. The corneal epithelium of vertebrates represents, in this context, a viable experimental model. This highly organized, five-layer-thick, stratified squamous epithelium is devoid of a *substratum corneum* and exhibits rapid cellular turnover. Cell kinetic studies in several mammalian species have indicated that in vivo the cell population at the surface is replaced daily and that the whole epithelium is renewed weekly (Hanna & O'Brien, 1960; Elgjo, 1969).

The stratified cells communicate in a syncytial arrangement (Klyce, 1972). A paracellular permeability barrier is located between the cells at the surface of the epithelium (Klyce, 1972; Burstein & Klyce, 1977). The nature of this barrier has not been investigated in detail but it is believed to result from tight junctions present between surface cells (Hogan, Alvarado & Weddel, 1970; Maurice, 1984). This is consistent with the observation that bathing in Ca^{2+}-free solution abolishes within minutes the transepithelial resistance (Candia, Montoreano & Podos, 1977). The apical membrane of the surface cell is endowed with channels which allow the movement of specific ions between the tear solution and the epithelium; coupling to transport processes

at the basolateral membrane results in vectorial ion transport, which can be expressed as a short-circuit current (I_{sc}). In the rabbit, a tear-to-stroma I_{sc} in the basal state is due to Cl^- movement from stroma to tear and, to a lesser extent, to Na^+ transport in the opposite direction (Klyce, 1975; Kylce & Crosson, 1985).

The barrier and transport activities of the corneal epithelium appear to be carried out efficiently in spite of the high rate of daily cellular attribution suggesting a high coordination of the process of replacement of surface cells (Pfister, 1973; Hazlett et al., 1980). This replacement process raises interesting questions with regard to a number of biological phenomena including: a) the signal(s) that triggers underlying cells to undergo final maturation into surface cells, b) the readiness of underlying cells to proceed through this maturation, and c) the cellular events involved in the maturation process and their temporal sequence. Previous studies which have addressed these questions have been hampered by the asynchrony of the surface cell replacement. To facilitate the study of the subjects outlined above, a method to induce the rapid exfoliation of full monolayers of corneal epithelial cells, based on controlled cellular permeabilization with digitonin, was developed. The capacity of the underlying cells to generate a permeability barrier across the epithelium was then assessed. The results provide significant clues on the progression of cellular maturity and polarity in this epithelium.

Materials and Methods

Adult albino rabbits (5 kg) were sacrificed by air embolism. Following aseptic enucleation the corneal surface was washed extensively with saline (0.9% NaCl) and either subjected to further treatment in the intact eye or dissected with a 1-mm scleral rim. Extreme care was taken to preserve the integrity of the endothelial surface both during and after the dissection.

Digitonin Treatment in the Intact Eye

The freshly enucleated eyes were placed with the corneal side down on top of a scintillation vial filled with 20 μM digitonin dissolved in saline. The vial diameter was slightly larger than the corneal diameter. The solution was stirred constantly with a magnetic stirrer. At the end of the treatment the corneal surface was washed with saline for 30 sec and stained for cell viability by dripping a 0.02% Trypan blue-saline solution from a Pasteur pipette for 1 min followed by a 1-min wash of excess dye by immersion in saline.

Incubations

Dissected corneas were transferred to 6 cm petri dishes containing 15 ml of a 1 : 1 mix of N-2-hydroxy-ethylpiperazine-N'-2 ethane sulfonic acid (HEPES)-buffered Dulbecco's modified Eagle medium and Ham F12, pH = 7.4 (D/F 12) and incubated at 36°C at 60 rev/min (circular motion) in a New Brunswick incubator shaker in an air atmosphere for times ranging from 5 min to 72 hr. Visual examination of transparency indicated that corneal deturgence was preserved during the 72-hr period. The corneas were then placed epithelium down on a glass coverslip and photographed in an Olympus microscope under regular illumination.

Electrophysiology

Corneas were mounted in Ussing-type chambers following a previously published method (Candia, 1972). A 0.5 cm^2 corneal area was exposed to the half-chamber. Both tissue sides were bathed with D/F 12. The tear side hemichamber contained 7 ml and the endothelial side hemichamber contained 10 ml of solution. The difference in volumes was used to generate a hydrostatic pressure difference of 3 cm H_2O which maintained the natural curvature of the cornea and minimized tissue undulations due to solution movement. Solutions in each half-chamber were continuously bubbled with air and kept at 36°C by heating loops immersed in the fluid. The transepithelial potential difference (PD_t) was monitored through agar-NaCl-filled polyethylene bridges that were connected to calomel half-cells. The tips of the PD_t bridges were placed at less than 2 mm from the tissue surfaces. External current pulses (I_e = 13.4 $\mu A/cm^2$) of 0.5-sec duration were passed through the cornea via agar/NaCl-filled bridges placed at 3 cm from the tissue surfaces. Current pulses were controlled automatically by an electronic timer allowing selection of pulse intervals between 5 sec and 2.4 min. Transepithelial resistance (R_t) was determined from the deflection (Δ) in PD_t generated by the current pulse as $\Delta PD_t/I_e$. The I_{sc} was determined as PD_t/R_t. The values obtained this way were compared to those measured directly with an automatic voltage-clamp circuit (Schoen & Candia, 1978) included as an alternative measuring mode in our apparatus. Shifting from open-circuit to voltage-clamp mode yielded instantaneous I_{sc}'s identical to those calculated as PD_t/R_t. Buffered saline solution (BSS) consisted of (in mM), 136 NaCl, 5.0 KCl, 1.4 $CaCl_2$, 0.85 $MgSO_4$, 5.5 glucose, 25 hemi Na HEPES, pH 7.4. In KCl-BSS all NaCl was replaced by KCl.

Determination of Recovery of Electrical Parameters After Digitonin Treatment of Ocular Surface

After control PD_t and R_t were determined, the flow of heating water through the loops was stopped. When the hemichamber solutions reached room temperature (RT) the tear solution was exchanged to saline containing 20 μM digitonin. After incubation for 2 to 16 min, two replacements with pure saline were performed over a 30-sec period. Afterwards, D/F 12 was reintroduced and the flow of heating water through the loops was restarted. The minimal dilution of detergent was estimated, by the dilution of a Dextran Blue marker, to be 2,500-fold. With a temperature microprobe it was determined that the chamber temperature increased to 36°C within 3 min.

Management of Aseptic Conditions

To prevent solution contamination during the experiments, the corneal hemichambers and all accessories brought in contact

with the cornea were immersed in 70% alcohol and left to dry in a sterile laminar flow hood, where corneal mounting was performed. Agar bridges were made in 500 mM NaCl stored in a large volume of 500 mM NaCl containing 0.05 volumes of a 10% iodine-povidone solution and washed with sterile saline prior to insertion in the chambers. The assembled chamber was transferred from the hood and immediately connected to a source of house air which was passed upstream through a 0.20-μm Millipore filter and a sterile 1% Na dicromate solution kept at 37°C. All solutions were sterilized prior to use, saline by autoclaving and others by passage through 0.2 μm Millipore filters.

Dihydrocytochalasin B, cycloheximide, epinephrine maleate, ouabain and powdered D/F 12 mix were purchased from Sigma. Digitonin was obtained from Sigma and from Calbiochem and amphotericin B from Squibb. All other chemicals used were of the highest purity available. All aqueous solutions were prepared in water purified by a Millipore system to a specific conductance larger than 10 MΩ.

Results

Digitonin Permeabilization of Cells at the Ocular Surface

Trypan blue staining efficiently revealed the presence of nonviable cells at the ocular surface. Under regular microscope illumination, cells at the corneal surface could not be distinguished. This is probably due to their extreme flatness and tight border aposition. Viability of the great majority of the cells was demonstrated by the lack of Trypan blue staining. After exposure to 20 μM digitonin, cells became permeabilized and were stained with Trypan blue (Fig. 1). A 2-min exposure permeabilized only a fraction of the cells. Exposures of between 4 to 8 min at room temperature led to uniform permeabilization of a single surface monolayer of mosaic-like cells with a fairly homogenous 25-μm frontal diameter and 12 μm nuclei. After a few minutes of incubation at 36°C the damaged layer could be exfoliated as a fragile sheet by minimal prodding with a smooth-surface glass rod or by a jet stream from a 20-gauge needle syringe. In the absence of overt physical intrusion, the cell monolayer separated spontaneously from the corneal surface upon a 30-min incubation at 36°C. The cohesion of the exfoliated monolayer was weak and the cells dispersed easily. A 16-min digitonin exposure, on the other hand, led to the permeabilization of more than one cellular layer judging from the crowded appearance of overlapping nuclear figures. In this case, a sheet of permeabilized cells with some internal cohesion could be released from the cornea. The surface underneath the exfoliated cell layers excluded Trypan blue and no further cellular sloughing was observed upon incubation in organ culture for 72 hr.

When a second 6-min digitonin treatment was made after removing the first exfoliated monolayer, a new monolayer of Trypan blue stainable cells was generated (Fig. 2). This new permeabilized monolayer was similar in frontal profile to the first layer, although the cells were smaller and showed a less-structured organization. The diameter of their nuclei was 20% smaller than those of the surface cell layer. After removal of this permeabilized layer, the corneal surface was comprised of nearly rounded cells. Unlike the case of the surface and first subsurface cells, these cells could be easily visualized in the whole cornea, in their viable state, under regular illumination.

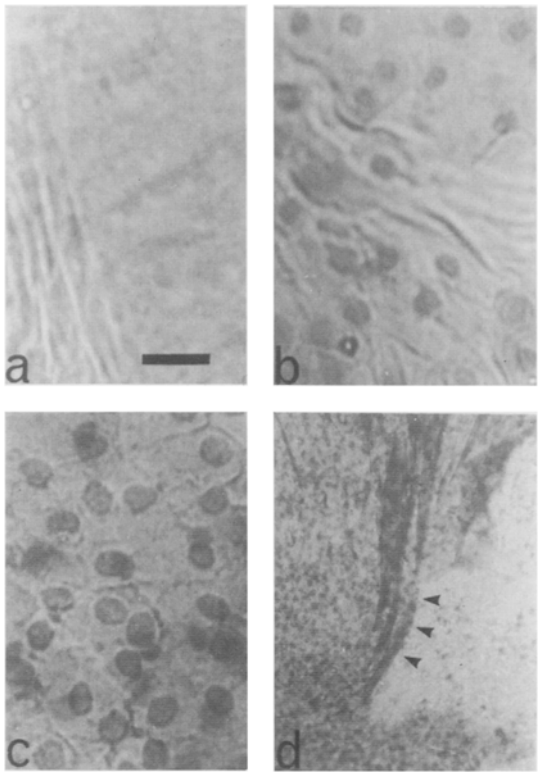

Fig. 1. Visualization of digitonin permeabilization of corneal epithelial cells by Trypan blue. (*a*) Control cornea. (*b*) 2-min digitonin. (*c*) 8-min digitonin. (*d*) 8-min digitonin followed by 30-min incubation at 36°C. The exfoliation of a sheet of permeabilized surface cells is shown (arrows). In (*a*) and (*b*) wrinkles result from placing entire corneas epithelial-side-down on top of coverslip. These wrinkles were useful for focusing on the corneal surface plane. Bar equals 25 μm (*a,b,c*) or 150 μm (*d*)

Recovery of Electric Properties After Induced Surface Cell Exfoliation

The studies to be described henceforth include relatively long (24 hr) incubations of corneas in Ussing-type chambers. Conclusions are drawn from both, time-dependent changes in R_t and in I_{sc} following experimental manipulations and comparison of

maximal R_t's and I_{sc}'s before and after these manipulations. Therefore in preliminary studies the effects of keeping corneas for 24 hr in the Ussing chamber were assessed. It was found that after an initial equilibration period of 60–90 min freshly dissected corneas maintained a nearly constant R_t and I_{sc} for a full day (*see* Fig. 4).

Digitonin Effect on PD_t, R_t and I_{sc}

In spite of the visible release of surface cells following digitonin treatment, it was noted that corneas incubated for 1 hr at 36°C and then mounted in Ussing chambers exhibited electrical properties not significantly different from control corneas in which digitonin permeabilization was omitted. The question then arose as to how the overt release of a layer of surface cells could be compatible with an apparent conservation of ion barrier and transport functions. To address it, the permeabilization protocol was applied to corneas premounted in Ussing chambers. Results of a typical experiment are presented in Fig. 3 which describes the continuous changes in PD_t, R_t and I_{sc} as sequential changes in chamber temperature and composition of the tear solutions were performed. The initial PD_t, R_t and I_{sc} of control corneas were 17.5 ± 3.9 mV, 2.43 ± 0.45 kΩ · cm^2 and 7.2 ± 1.8 $\mu A/cm^2$, respectively (mean ± SD for 22 experiments). Lowering the temperature increased R_t and decreased I_{sc} with little effect on PD_t. Upon introduction of the digitonin-saline solution on the tear side the electrical parameters changed in a biphasic fashion. During the first 40–80 sec, the R_t decreased while the PD_t increased, slightly indicating an increased I_{sc}. Afterwards, an accelerated R_t decrease led to a decrease in PD_t even though the I_{sc} continued to rise. At about 3 min, the resistance reached a value very near to that of the solution. This low R_t preempted any further accurate determination of the I_{sc} by our experimental setup.

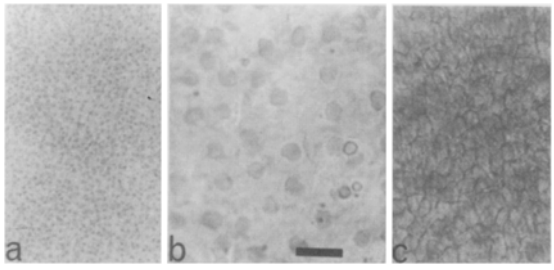

Fig. 2. The ocular surface after a second digitonin exposure. (*a*) and (*b*) Low and high magnifications, respectively, of the surface immediately after Trypan blue staining following a 5-min digitonin treatment. (*c*) The corneal surface after removal of the second layer of permeabilized cells. Bar equals 150 μm (*a*) or 25 μm (*b,c*)

Recovery of R_t

After digitonin removal and return to 36°C an initial recovery of resistance was seen within minutes. For digitonin exposures of between 2 and 8 min stable R_t values that remained essentially unchanged for several hours were always achieved in less than 1 hr. The half-time for R_t ($t_{1/2}$) recovery over this period (Table 1) was essentially independent of the length of exposure. Increasing the digitonin exposure to 16 min affected dramatically the recovery time; the $t_{1/2}$

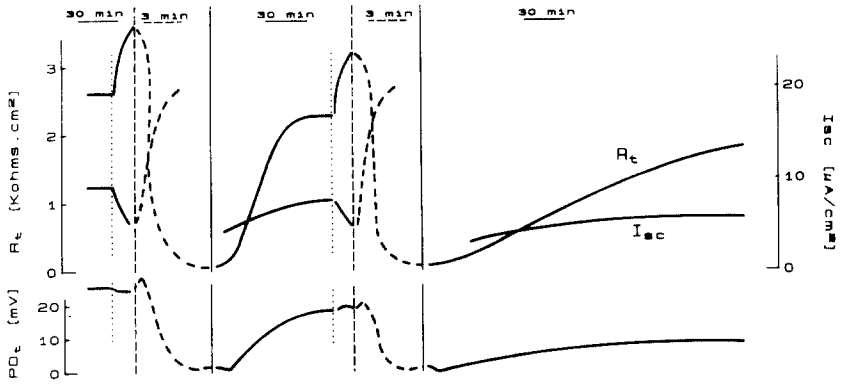

Fig. 3. Changes in PD_t, R_t and I_{sc} during and following successive exposures of the ocular surface to digitonin. The solid traces represent periods during which D/F 12 solution was present in the tear side solution. The dashed traces represent the periods of digitonin exposure. Between the pointed and dashed vertical lines heating was stopped and chamber temperature decreased from 36°C to room temperature. At the vertical solid lines D/F 12 was reintroduced in the tear side solution and chamber temperature was brought back to 36°C. Note expansion of time scale during the digitonin exposure period. The I_{sc} trace is interrupted because I_{sc} determination at low R_t was inaccurate

was extended more than threefold. Decreasing the temperature during the recovery period from 36 to 25°C increased the recovery time 2.5-fold. Between 2 and 8 min, the time of detergent exposure has little effect on the fractional recovery of the R_t which, measured 90 min after detergent removal, was about 90% of control (Table 1). For the 16-min exposure, the fractional R_t recovery was determined 4 hr after detergent removal, when a constant maximal R_t values was observed. Recovery in this case was lower than those for the shorter exposures. To test the physical effect of digitonin in the Ussing chamber, two corneas treated for 8 min were removed and stained with Trypan blue. One cornea, removed 5 min after the treatment, revealed a permeabilized cell monolayer. The cornea of the contralateral eye, removed 60 min later, after complete R_t recovery, revealed a surface free of stainable cells.

Ninety minutes after the recovery of a stable electrical resistance, corneas which were treated originally for 2, 6, or 8 min with digitonin were exposed to the detergent again for 5 min. The time course of R_t (and I_{sc}) changes during the exposure was identical to those observed during the first period (Fig. 3). Permeabilization of a cell monolayer was again confirmed by Trypan blue staining. Initial recovery of resistance was observed a few minutes after the removal of digitonin, but in contrast, the process of R_t recovery was much slower than that observed after the first digitonin treatment. The half-times for R_t recovery, determined from the stable R_t values reached at between 4 and 5 hr, were similar to those observed after a single 16-min treatment.

Corneas kept for 72 hr in D/F 12 did not shed any noticeable amounts of cells into the incubating medium (released cells will be easily identified because of their typical squamous structure). When mounted in Ussing chambers, these corneas expressed electrical properties similar to those of freshly dissected tissues, although a certain increase in I_{sc} may have occurred. The R_t recovery times after each of two digitonin treatments were similar to those of the fresh corneas (Tables 1 and 2).

Recovery of I_{sc}

After R_t increased sufficiently above the solution resistance, it was possible to determine a I_{sc} (Fig. 3). It was surprising to find that a substantial I_{sc} was expressed from the point where measurements were begun and that the I_{sc} exhibited only small increases during the period of R_t increase. The control I_{sc} values and their fractional recovery after the digitonin treatments are presented in Table 1. The fractional recovery of I_{sc} at the time of complete resistance recovery was clearly smaller than unity, in particular after the removal of two cell layers. On a number of occasions when the recovered I_{sc} was a small fraction of the control, the corneas were left in the Ussing chamber for 16 hr. During this period, while the resistance remained essentially constant or decreased slightly, the I_{sc} increased gradually

Table 1. Effect of length of exposure to digitonin on the rapidity of recovery of R_t and on the magnitude of R_t and I_{sc}[a]

t (min)	n	$t_{1/2}$ (min)	Recovery			
			R_t		I_{sc}	
			%	Abs. Cont. (kΩ · cm²)	%	Abs. Cont. (μA/cm²)
2	4	32.0 ± 5.1	83 ± 10	(2.10 ± 0.81)	80 ± 9	(6.90 ± 0.10)
3	2	33.0 ± 4.2	98 + 3	(3.35 ± 1.90)	71 ± 1	(6.90 + 0.3)
4	4	35.3 ± 2.3	93 ± 29	(2.27 + 0.11)	68 ± 7	(10.2 ± 4.3)
6	4	33.6 ± 2.1	85 ± 13	(2.21 ± 0.44)	75 ± 19	(6.0 + 0.8)
8	4	38.8 ± 3.6	91 ± 24	(2.92 ± 0.96)	64 + 13	(7.2 ± 1.3)
16	4	113 + 22	67 ± 12	(2.22 ± 0.63)	53 ± 14	(6.3 + 1.8)
6-RT[b]	2	86 + 8.5	99 ± 3	(5.05 + 0.35)	81 + 5	(2.4 + 0.5)
6	3(O.C.)[c]	36.7 + 4.6	97 + 19	(2.26 + 0.11)	65 ± 24	(11.4 ± 4.2)

[a] Mean values (±SD). % recoveries were calculated as the ratio between the values after and before digitonin treatment times 100. The mean (±SD) R_t's and I_{sc}'s for each group are given in parentheses. t is the length of digitonin exposure, $t_{1/2}$ is the half-time for R_t recovery.

[b] 6-RT: The experiment was carried out at room temperature.

[c] O.C.: These experiments were carried out with corneas kept in organ culture for 72 hr.

Table 2. Half-times for R_t recovery and % recovery of R_t and I_{sc} after a second, sequential digitonin exposure

t (min)		n	t (min)	% Recovery	
1st	2nd			R_t	I_{sc}
2	5	2	109 ± 18[a]	77 ± 10	66 ± 21
6	5	2	128 ± 32	61 ± 16	65 ± 1
8	5	4	130 ± 21	77 ± 30	63 ± 23
6	5	3(O.C.)[b]	157 ± 14	89 ± 15	57 + 6

[a] Mean ± SD.

[b] O.C.: These experiments were carried out with corneas kept in organ culture for 72 hr.

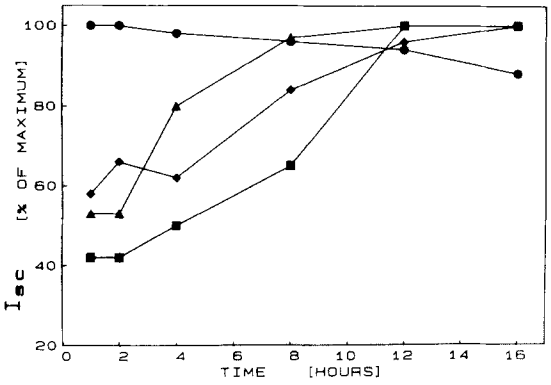

Fig. 4. Changes in I_{sc} as a function of time in control corneas and corneas treated with digitonin. For each one of the conditions studied two corneas were maintained for 16 hr in the Ussing chamber under open-circuit conditions. The I_{sc} was calculated at various times as the quotient between PD_t and R_t. Each point is the mean of the value for the two corneas in each condition; ●, control, no digitonin treatment, $t = 0$ represents time of tissue mounting on chamber; ▲, single 8-min digitonin exposure; ■, single 16-min digitonin exposure; ◆, two digitonin exposures (8-min followed by 5-min); $t = 0$ represents in each last three cases the end of last digitonin exposure

over several hours. These increases in I_{sc} and their contrast with the changes in untreated corneas are depicted in Fig. 4.

Effect of Dihydrocytochalasin B

Addition of 10 μM dihydrocytochalasin B to the tear side chamber had a small stimulatory effect on PD_t (*not shown*) with a simultaneous R_t decrease (Fig. 5). This effect subsided within a few minutes and afterwards a stable resistance was maintained for at least 2 hr. In contrast, this microfilament disrupting agent inhibited completely the R_t recovery following exfoliation of the layer of surface cells. Its removal from the chamber brought a slow recovery of

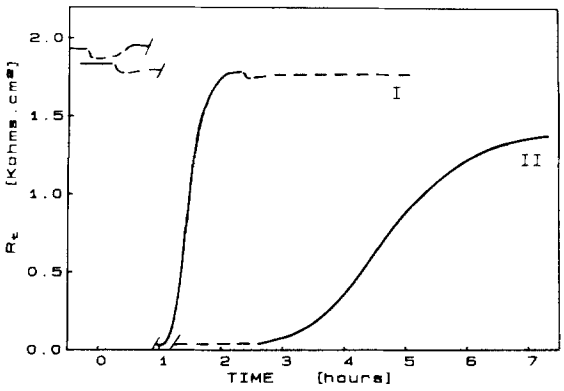

Fig. 5. Effect of dihydrochalasin B on recovery of R_t. Corneas from a single rabbit were studied simultaneously for the effect of 10 μm dihydrocytochalasin B added to the tear side solution at different times through the desquamation and R_t recovery periods. The drug was added in an aliquot of dimethylsulfoxide (1 μl/ml of solution) and was removed by changing the solution three times as done for digitonin. Its presence in the tear side hemichamber is indicated by the dashed traces. The interruption in the traces represents the period of digitonin treatment (6 min) which is not shown

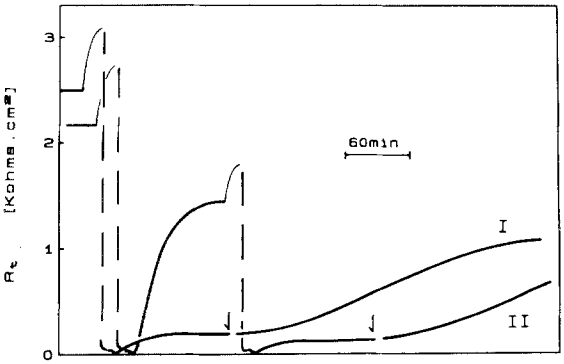

Fig. 6. Effect of cycloheximide on recovery of R_t. The two corneas were subjected to the digitonin exposure as described in Materials and Methods and Fig. 3. Thick solid traces represent periods of incubation at 36°C in D/F 12. Thin traces represent the time during which heating was arrested and temperature decreased to room temperature (RT). The dashed traces represent the digitonin exposure periods (at RT). One cornea (I) was treated one time for 16 min, the other (II) was treated twice, first for 6 min then for 5 min. Cycloheximide (5, μM) was present in the tear side solution from the start of the trace until the point indicated by the arrows, where it was washed out by three successive solution replacements

R_t over several hours to 88 ± 6% ($n = 2$) of controls. The I_{sc} of the recovered barrier remained nearly constant throughout the recovery period and was equal to 92 ± 9% of the control value. After full R_t recovery dihydrocytochalasin B had no significant effect on R_t.

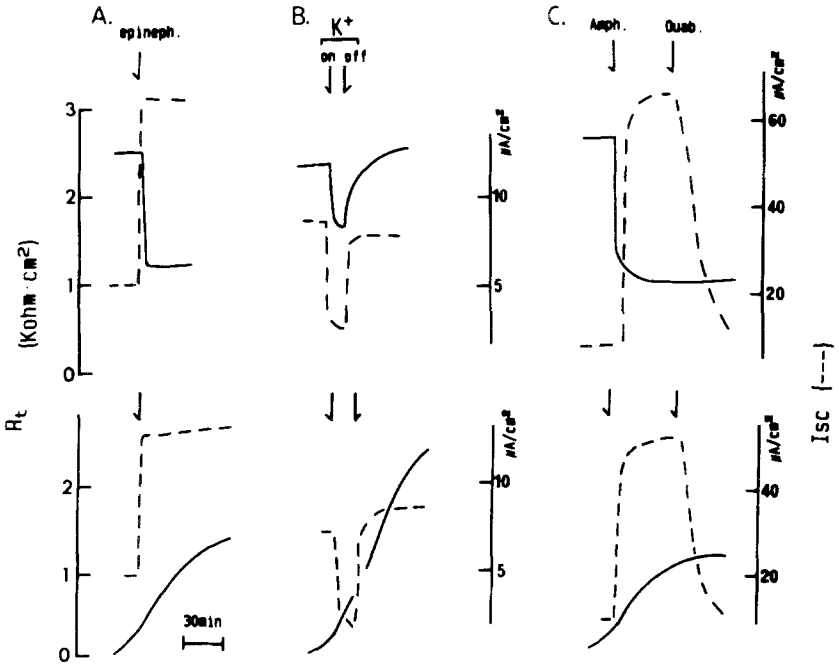

Fig. 7. Effect of epinephrine, K^+ and amphotericin B on the I_{sc} and the R_t in untreated and digitonin-treated corneas. Upper traces: untreated corneas; lower traces: corneas treated for 6 min with digitonin as described in Fig. 3. The R_t traces shown start from the point where D/F 12 was reintroduced in the tear hemichamber and heating was restarted. (*A*) Effect of addition of 10^{-5} M epinephrine (epineph.) to the tear side hemichamber. (*B*) Effect of replacing 30% of total volume in both tear and stromal hemichambers by KCl-BSS (K^+ on) to raise $[K^+]$ to 47 mM. At K^+ off, fresh D/F 12 was reintroduced in both hemichambers. (*C*) Effects of 2×10^{-5} M amphotericin B (amph.) and 10^{-3} M ouabain (ouab.) added to the tear and stromal hemichambers, respectively

Effects of Cycloheximide

The contribution of protein synthesis to regeneration of the permeability barrier after induced desquamation was tested with cycloheximide (Cereijido, Meza & Martinez-Palomo, 1981). This agent had no effect on the electrical parameters of the cornea kept in control condition or the rapidity of R_t recovery after surface cell exfoliation (Fig. 6). The fractional R_t recovery (53 ± 7.5% mean ± SE, $n = 4$) was significantly smaller than in its absence. In contrast to its minimal effects following single-layer exfoliation, cycloheximide completely inhibited the recovery of electrical resistance when two cell layers were removed either by a single 16-min digitonin exposure or by sequential removal of two layers. Removal of the agent elicited a gradual R_t increase but recoveries at 16 hr were only partial. This inhibitory action was also observed with two corneas which were maintained in organ culture in D/F 12 for 72 hr prior to measurement of R_t recovery in the Ussing chamber.

Effect of KCl, Epinephrine and Amphotericin B on the I_{sc} and the R_t in Control Conditions and During the R_t Recovery Phase

I_{sc}'s amounting to a significant fraction of the control values were measured in the digitonin-treated tissues as soon as the recovering R_t reached a value allowing reliable I_{sc} determinations. This suggested that the cellular machinery responsible for active ion transport was not affected by the previous digitonin exposure. To provide further confirmation of this hypothesis, the electrical response to three physiological perturbances of tissues in the initial phases of R_t recovery ($R_t < 0.4$ kΩ · cm²) were compared again to that of untreated controls (Fig. 7).

The relative contributions of apical Cl^- channels to the I_{sc}'s in the two cases was compared by studying the effect of epinephrine, an agent that increases the I_{sc} by increasing specifically the apical Cl^- conductance (Klyce, Neufeld & Zadunaisky, 1973; Klyce & Wong, 1977; Candia & Neufeld, 1978). The secretagogue increased the control I_{sc} by 320 ± 28% (mean ± SE, $n = 4$) and the I_{sc} of corneas in the post-digitonin stage ($R_t < 0.4$ kΩ · cm²) by 310 ± 12% ($n = 4$). The R_t of the controls decreased by 50 ± 8%. In contrast, there was no decrease in the recovering R_t during the period of I_{sc} increase (<1 min).

Active transport depends on a negative cell potential. This potential promotes the movement of Cl^- from cell to tear in spite of a concentration gradient in the opposite direction (Klyce, 1975; Klyce & Crosson, 1985). The main contributor to the potential is the K^+ permselectivity of the basolateral membrane. Thus, an increase in $[K^+]$ in the medium should decrease the I_{sc}. Raising $[K^+]$ to 47 mM reduced within 10 min the control and post-digitonin

I_{sc}'s by 75 ± 7% and 84 ± 5% (n = 3), respectively. These decreases were reverted rapidly by return to D/F 12. When BSS was used instead of KCl-BSS the I_{sc} remained essentially unchanged.

The third comparison is based on the fact that amphotericin B, added to the tear side, induces channels for monovalent ions in the apical membrane. This new pathway in combination with the electrogenic basolateral Na^+ pump results in tear-to-stroma net cation movement and thus, in enhancement of the I_{sc} (Burstein & Klyce, 1977). Addition of 2×10^{-5} M of the antibiotic to the tear side solution increased within 10 min the control I_{sc} from 7.9 ± 1.3 to 64 ± 4 $\mu A/cm^2$ (mean ± SE, n = 3) and simultaneously decreased R_t from 2.1 ± 0.3 to 0.9 ± 0.1 $k\Omega \cdot cm^2$. Added in the post-digitonin stage amphotericin B increased the I_{sc} from 4.6 ± 2.2 to 52 ± 4 $\mu A/cm^2$ (n = 4). During the period of I_{sc} increase and after its completion the R_t continue to rise reaching a final value of 1.1 ± 0.1 $k\Omega \cdot cm^2$. In both control and post-digitonin stages addition of 1 mM ouabain to the stromal hemichamber eliminated most of the I_{sc} without affecting the R_t.

Discussion

Digitonin permeabilizes cholesterol-containing membranes to the extent that cytosolic components of high molecular weight are released. Yet, permeabilization can be limited to the outermost exposed membrane. Apical or basolateral membranes or the whole plasma membrane have been permeabilized without affecting the respective basal or apical counterparts or intracellular organelles (Malinowska et al., 1981; Bonventre & Cheung, 1986; Morita et al., 1985; Dawson, 1987).

Application of digitonin at the ocular surface led to a loss of viability of the most exposed cells leaving inner aspects of the epithelium unscathed. Even though the cells are anchored to each other and to underlying cells by numerous spot desmosomes (Hazlett et al., 1980), the devitalized surface cells detached properly from the viable underlying strata. Hudspeth (1975) has observed a similar rapid detachment of single, mechanically permeabilized cells in gallbladder epithelium.

The electrical changes observed in the Ussing chamber provide some insight into the molecular events involved. The initial R_t decrease with concomitant I_{sc} increase is probably due to an initial permeabilization of the apical membrane, much like the effect of amphotericin B. The ensuing R_t loss may result from the permeabilization of the basolateral membrane or the disassembly of the paracellular permeability barrier.

The subsequent recovery of R_t, afforded the study of the mechanisms of generation of paracellular permeability barrier in corneal epithelium by the emerging, new surface cells. By the criteria of high transepithelial resistances ($R_t > 2$ $k\Omega \cdot cm^2$) the corneal epithelium of the rabbit is a tight epithelium. Yet, due to the low permeability of its apical membrane the cellular resistance (R_c) is higher than the paracellular (R_p) counterpart (Klyce, 1972). Since $R_t^{-1} = R_p^{-1} + R_c^{-1}$, providing R_c remains high, R_t identifies with R_p during most of the recovery period. Only when R_p becomes high will R_c influence the R_t value. However, it could be argued that small amounts of detergent seeping beyond the permeabilized cells decrease the resistance of the cellular membranes and that, as a result, the experimental R_t recovery observed reflects, in part, increases in the resistance of these membranes following detergent desorption. Since the normal R_c is 5–20 $k\Omega \cdot cm^2$ (Klyce, 1972), attributing the low R_t (~0.3 $k\Omega \cdot cm^2$) to R_c implies ascribing to the cellular membranes nonspecific ion permeabilities severalfold higher than that resulting from their specific ion pathways. Such a hypothesis is not compatible with the experimental evidence. The comparative studies of the effects of epinephrine and elevated medium K^+ suggested that soon after detergent removal both apical and basolateral membranes exhibit Cl^- and K^+ permselectivities not distinct from those in untreated tissues. Moreover, the amphotericin B effect demonstrated that the apical membrane of the new surface cell, which should be the most liable to detergent damage, retained its very limited Na^+ permeability. If a significant membrane permeability increase of the cells remaining viable does occur, its reversion after desorption must be much faster than the observed time course of R_t recovery. Thus, the increases in R_t are likely to primarily reflect increases in R_p.

Following devitalization of the surface cell monolayer, recovery of R_t was noted within minutes after detergent removal suggesting that the great majority of the cells participate in the formation of the new paracellular permeability barrier. R is a magnitude directly proportional to the length of the resistance pathway. Since the length of the zonula occludens is a fraction of a μm, while the length of the whole paracellular pathway is in the order of 100 μm, even a small percent of nonparticipating cells will suffice to diminish the surface resistance below that of the rest of the paracellular pathway. Therefore the observed time course of resistance increase is likely to represent the average time course of increased resistance through the intercellular space between two neighboring cells rather than a random distribution of tight junction

formation events within an asynchronous cellular population.

The involvement of actin microfilaments in the regeneration of a transepithelial resistance was demonstrated by the inhibitory action of dihydrocytochalasin B. Microfilaments play a large number of roles in cellular function, the inhibition may be related to transportation of a junctional component(s) along the plane of the membrane or to their role in intracellular translocation and membrane insertion (Meza et al., 1980). When two cell layers were removed, R_t recovery was blocked by cycloheximide suggesting that the generation of tight junctions was limited at this stage by the unavailability of some essential protein(s) component in at least some of the cells. The lower maximal R_t's attained by the deeper epithelial layer may be due to the fact that the cells are smaller and therefore, the total junctional length per unit of area is larger (Rabito, 1986).

The constant, rapid cell turnover in vivo implies that a more or less defined amount of time has elapsed since a cell located at a given position in the basal-to-surface stratification axis left its basal position. In rat and mice the cellular transport through the stratum has been estimated to be between four and six days (Hanna & O'Brien, 1960). Thus, the question arises as to whether the dependence of R_t recovery on protein synthesis after removal of two cell layers results from the cell location, or whether it reflects the "younger" character of the cells. To address this question, corneas were kept in organ culture under conditions (serum-free medium) favoring establishment of a quiescent state. Hence, preexisting suprabasal cells were allowed to age an additional 72 hr. Yet, the cycloheximide effects persisted suggesting that synthesis of proteins needed for tight junction generation is a spatially rather than a temporally controlled phenomena.

In addition to the remarkable rapidity of R_t recovery after surface cell removal, the other surprising finding was the relatively high I_{sc} values attained soon after detergent removal and the little further change in I_{sc} during the R_t recovery period. This behavior is of particular significance for our understanding of the development of membrane polarity in this stratified transporting epithelium. Just prior to digitonin exposure, the emerging apical surface was part of what is customarily viewed as the basolateral surface of the epithelial syncytium. That surface is assumed to contain basolateral transport systems such as the Na^+,K^+-ATPase (Tervo & Palkama, 1975) and K^+ channels. Yet, Cl^- ion channels appear to be already present at the membrane emerging as the new apical surface. Thus, polarization of transport activities may occur in the cells in parallel to its morphological change as it progresses spatially from a basal to a surface location. Patch-clamp studies of basal cells indicate that K^+ channels are abundant on its basal surface but absent at the apically facing pole, which appear to be devoid of an ion pathway (Keys et al., 1986). This observation and our results are consistent with the studies demonstrating a polarized insertion of membrane components in MDCK cells in the absence of tight junctions (Vega-Salas et al., 1987). If maturation of the cells include the gradual accumulation of Cl^- channels in the apically facing surface of Cl^- channels in the apically facing surface of intraepithelial cells, those channels should be abundant and easily detectable on the basolateral epithelial membrane of the syncytium. However, no significant Cl^- conductance on the basolateral membrane could be detected in tissues in which the apical surface was permeabilized to monovalent ions by amphotericin B (*unpublished observations*). The possibility that apical channels and tight junctional proteins are accumulated on the apical pole of the subsurface cells during maturation, and become inserted rapidly on the membrane only after a signal triggers the expression of tight junctions, should then be considered. By the time a paracellular resistance barrier develops, the new surface cells will already contain the specialized channels that confer to the epithelium its vectorial ion transport capacity.

The I_{sc} values observed at the time of maximal R_t recovery were always smaller than the control values. As a result of exfoliation, the total amount of basolateral membrane diminishes. Also, the number of total available ion channels in the exposed immature cells may be smaller than in the cells reaching the surface through a longer maturation time span. Thus, the gradual increases in I_{sc} observed between the 2nd and 16th hours after surface desquamation could reflect gradual increases in the density of ion channels at the apical membrane and/or an increased basolateral uptake activity.

The patterns of R_t recovery in corneal epithelium could be compared with those observed in other systems which have been used to study regeneration of paracellular barriers. In tryptinized Madin-Darby canine kidney (MDCK) cells reseeded at high density, an R_t increase occurs over several hours after a time lag reflecting the need for *de novo* synthesis of protein components and the asynchronous nature of the population (Cereijido et al., 1981; Griepp et al., 1983). Cell cycle synchronization and presynthesis of components shortens the time needed to reach maximal resistance (Gonzalez-Mariscal et al., 1985; Rabito, 1986), but even in such cases the R_t rise in cultured cells is slower than seen in the corneal epithelium. The rapidity of R_t recovery after devitalization of a single-cell layer

approached that observed in MDCK cells following brief tight junction disassembly in Ca^{2+}-free medium (Martinez-Palomo et al., 1980; Gumbiner & Simons, 1986). R_t recovery in that case reflects, primarily, the resealing of the prepositioned apposing components of the two cells (Pitelka, Taggart & Hamamoto, 1983). Thus, membrane insertion, physical aggregation and spatial positioning of tight junction components in the first subsurface cell layer of the corneal epithelium must be relatively fast processes.

The digitonin-induced exfoliation and the recovery of epithelial resistance and ion transport can also be compared with the moulting process in the Na^+-absorbing stratified frog skin (Nielsen, 1969; Vouté et al., 1969). During moulting the outermost layer of the *stratum granulosum* becomes cornified, while the second layer of the *stratum granulosum* replaces it functionally (Nielsen, 1972). Moulting occurs 7 hr after its induction by aldosterone. This long period of time may reflect the involvement of a multiplicity of changes, as cells modify radically their function, and is consistent with the essentially periodic, hormonally synchronized nature of moulting. In the cornea, in contrast, individual cells need to respond not only to age-dependent cell desquamation but also to unpredictable, environmentally determined death of individual surface cells. Thus it makes telelogical sense that the arrangement of cellular maturation will provide for a first subsurface cell whose maturation stage is arrested at a point such that replacement of a dying surface cell can be accomplished rapidly on demand and independently of systemic hormonal control.

This work was supported by an unrestricted grant from Research to Prevent Blindness. The author is the recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship.

References

Bonventre, J.V., Cheung, J.Y. 1986. Cytosolic free calcium concentration in cultured renal epithelial cells. *Am. J. Physiol.* **250:**F329–F338

Budtz, P.E. 1985. Epidermal tissue homeostasis. I. Cell pool size, cell birth rate and cell loss by moulting in the intact toad, *Bufo bufo*. *Cell Tissue Kinetics* **18:**521–532

Burstein, N.L., Klyce, S.D. 1977. Electrophysiological and morphological effects of ophthalmic preparations on rabbit corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* **16:**899–911

Candia, O.A. 1972. Ouabain and sodium effects on chloride fluxes across the isolated bullfrog cornea. *Am. J. Physiol.* **233:**1053–1057

Candia, O.A., Montoreano, R., Podos, S.M. 1977. Effect of the ionophore A23187 on chloride transport across isolated frog cornea. *Am. J. Physiol.* **233:**F94–F101

Candia, O.A., Neufeld, A.H. 1978. Topical epinephrine causes a decrease in density of β-adrenergic receptors and catecholamine-stimulated chloride transport in the rabbit cornea. *Biochim. Biophys. Acta* **543:**403–408

Cereijido, M., Meza, I., Martinez-Palomo, A. 1981. Occluding junctions in cultured epithelial monolayers. *Am. J. Physiol.* **240:**C96–C102

Cereijido, M., Robbins, E.S., Dolan, W.J., Rotunno, C.A., Sabatini, D.D. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **77:**853–880

Cohen, E., Talmon, A., Faff, O., Bacher, A., Ben Shaul, Y. 1985. Formation of tight junctions in epithelial cells. *Exp. Cell Res.* **156:**103–116

Dawson, D.C. 1987. Properties of epithelial K^+ channels. *In:* Current Topics in Membranes and Transport. G. Giebish, editor. Vol. 28, pp. 41–71. Academic, Orlando, Florida

Elgjo, K. 1969. Cell renewal of the normal mouse cornea. *Acta Pathol. Microbiol. Scand.* **76:**25–30

Gonzales-Mariscal, L., Chavez de Ramirez, B., Cereijido, M. 1985. Tight junction formation in cultured epithelial cells (MDCK). *J. Membrane Biol.* **86:**113–125

Griepp, E.B., Dolan, W.J., Robbins, E.S., Sabatini, D.D. 1983. Participation of plasma membrane proteins in the formation of tight junctions by cultured epithelial cells. *J. Cell Biol.* **96:**693–702

Gumbiner, B., Simons, K. 1986. A functional assay for proteins involved in establishing an epithelial occluding barrier: Identification of a uromorulin-like polypeptide. *J. Cell Biol.* **102:**457–468

Hanna, C., O'Brien, J.E. 1960. Cell production and migration in the epidermal layer of the cornea. *Arch. Ophthalmol.* **64:**536

Hazlett, L.D., Spann, B., Wells, P., Beak, R.S. 1980. Desquamation of the corneal epithelium in the immature mouse: A scanning and transmission microscopy study. *Exp. Eye Res.* **31:**21–30

Hogan, M.J., Alvarado, J.A., Weddell, J.E. 1971. Histology of the Human Eye. W.B. Saunders, Philadelphia

Hudspeth, A.J. 1975. Establishment of tight junctions between epithelial cells. *Proc. Natl. Acad. Sci. USA* **72:**2711–2713

Keys, C., Nelson, S., Deutsch, W., Rae, J. 1986. Patch clamp recordings from mammalian corneal epithelium. *Invest. Ophthalmol. Vis. Sci. Suppl.* **27:**350

Klyce, S.D. 1972. Electrical profiles in the corneal epithelium. *J. Physiol. (London)* **226:**407–429

Klyce, S.D. 1975. Transport of Na^+, Cl^- and water by the rabbit corneal epithelium at resting potential. *Am. J. Physiol.* **228:**1446–1452

Klyce, S.D., Crosson, C.D. 1985. Transport processes across the rabbit corneal epithelium: A review. *Curr. Eye Res.* **4:**323–331

Klyce, S.D., Neufeld, A.H., Zadunaisky, J.A. 1973. The activation of chloride transport by epinephrine and db-cyclic AMP in the cornea of the rabbit. *Invest. Ophthalmol. Vis. Sci.* **13:**127–139

Klyce, S.D., Wong, R.K.S. 1977. Site and mode of adrenalin action on chloride transport across the rabbit corneal epithelium. *J. Physiol. (London)* **266:**777–799

Malinowska, D.H., Koelz, H.R., Hersey, S.J., Sachs, G. 1981. Properties of the gastric proton pump in unstimulated permeable gastric glands. *Proc. Natl. Acad. Sci. USA* **79:**5908–5912

Martinez-Palomo, A., Meza, I., Beaty, G., Cereijido, M. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* **87:**736–745

Maurice, D.M. 1984. *In:* The Eye, 3rd edition. H. Davson, editor. Vol. 1-b, pp. 1–158. Academic, London—New York

Meza, I., Ibarra, G., Sabanero, M., Martinez-Palomo, A., Cereijido, N. 1980. Occluding junctions and cytoskeletal components in a cultured transporting epithelium. *J. Cell Biol.* **87:**746

Morita, K., Levine, M., Heldman, E., Pollard, H.B. 1985. Ascorbic acid and catecholamine release from digitonin treated chromafin cells. *J. Biol. Chem.* **260:**15112–15116

Nielsen, R. 1969. The effect of aldosterone *in vitro* on the active sodium transport and moulting of the frog skin. *Acta Physiol. Scand.* **17:**85–92

Nielsen, R. 1972. The effect of polyene antibiotics on the aldosterone induced changes in sodium transport across isolated frog skin. *J. Steroid Biochem.* **3:**121–128

Pfister, R.F. 1973. The normal surface of corneal epithelium: A scanning electron microscopic study. *Invest. Ophthalmol. Vis. Sci.* **12:**654–668

Pitelka, D.R., Taggart, B.N., Hamamoto, S.T. 1983. Effects of extracellular calcium depletion on membrane topography and occluding junctions of mammary epithelial cells in culture. *J. Cell Biol.* **96:**613–624

Rabito, C.A. 1986. Reassembly of the occluding junctions in a renal cell line with characteristics of proximal tubular cells. *Am. J. Physiol.* **251:**F978–F987

Schoen, H.F., Candia, O.A. 1978. An inexpensive high output voltage clamp for epithelial membranes. *Am. J. Physiol.* **235:**C69–C72

Tervo, T., Palkama, A. 1975. Electron microscopic localization of adenine triphosphatase ($Na^+ + K^+$-ATPase) activity in the rat cornea. *Exp. Eye Res.* **21:**269–279

Thoft, R.A., Friend, J. 1983. The X, Y, Z, hypothesis of corneal epithelial maintenance. *Invest. Ophthalmol. Vis. Sci.* **1:**1442–1443

Vega-Salas, D.E., Salas, P.J.I., Gundersen, D., Rodriguez-Boulan, E. 1987. Formation of the apical pole of epithelial cells (Madin-Darby canine kidney): Polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions. *J. Cell Biol.* **104:**905–916

Voûte, C.L., Dirix, R., Nielsen, R., Ussing, H.H. 1969. The effect of aldosterone on the isolated frog skin epithelium (*R. Temporaria*). *Exp. Cell Res.* **57:**448–450

Received 16 October 1987; revised 1 March 1988