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Modulation of Tight Junction Morphology and Permeability by an Epithelial Factor

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Abstract. We report evidence of a factor secreted at the apical side of epithelial monolayers which modulates tight junction structure and permeability. This activity was detected within 4–7 days of conditioning of the apical medium by MDCK, A6 or Caco-2 epithelial cell lines cultured on permeable membranes in bipartite chambers. Apical conditioned medium (ACM), applied to the basolateral surface of a confluent monolayer, increased the transepithelial electrical resistance (TER), progressively reaching values 12–22% higher than the baseline within 5–10 min. After 40–60 min, the TER returned slowly to the basal value. This phenomenon was not observed either when using preheated ACM or the ACM filtrate obtained through a 30,000 MW cutoff membrane. The ACM maintained its activity even when applied to cell lines from different organs and species, as demonstrated when ACM from MDCK monolayers promoted an increase of 22% in the TER of Caco-2 cells. The increase of TER induced by the ACM treatment is accompanied by a change in the distribution of the number of tight junction strands, from an initial pattern, dominated mostly by junctions with one or two strands, to a new pattern after treatment dominated by junctions with two or three strands. Our results suggest the existence of a mechanism in epithelial cells that could signal leakage of apically secreted components to the basolateral side, thereby modulating the junction structure and permeability.

Key words: Tight junction — Intercellular junctions — Transepithelial permeability — Transepithelial electrical resistance (TER) — MDCK, A6, Caco-2 epithelial cell cultures

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Introduction

Tight junctions provide a selectively permeable occlusion along the paracellular pathway across epithelial cell layers. These structures limit the diffusion of molecules that are selectively concentrated on each side of the cell layer by secretory processes or transepithelial transport. The tight junctions are dynamic structures that change their permeability according to physiological and pathological conditions as well as under stimulation by pharmacological substances.

The basic component of tight junctions are structures formed as a result of the specialized linear interactions between membranes of adjacent cells that function as the actual intercellular permeability barriers. These regions of linear interactions are best visualized in freeze-fracture replicas separating the apical and the basolateral domain of the plasma membrane where they appear as strands. These strands vary in number and length and sometimes form elaborate networks.

The efficiency and selectivity of the tight junction barrier can be evaluated by measuring the transepithelial electrical resistance (TER) (Diamond, 1977; González-Mariscal, 1991). The sealing capacity of tight junctions in natural epithelia as well as in cultured monolayers depends not only on the length and number of tight junction strands (Claude & Goodenough, 1973; Claude, 1978) but also on several other factors. The existence of channels (Claude, 1978; González-Mariscal, Chávez de Ramirez & Cereijido, 1984); the compartmentation afforded by the frequent anastomoses between junctional strands (Cereijido, González-Mariscal, & Contreras, 1989); the biochemical state of junctional components (e.g., phosphorylation) (Stevenson et al., 1988, 1989); as well as the control provided by the relationship between the tight junction and the cytoskeleton (Montesano et al., 1975, 1976; Saxon et al., 1978; Bentzel et al., 1980; Elias et al., 1980; Meza et

al., 1980, 1982; Hecht et al., 1988; Madara et al., 1988), among other factors can determine TER. Once TER is stabilized, the permeability can increase as a result of chelation of extracellular Ca^{2+} (Sedar & Forte, 1964; Cereijido et al., 1978; Martinez-Palomo et al., 1980), low pH of the apical medium (Fischbarg & Whittembury, 1978), rise in intracellular Ca^{2+} concentration (Cereijido, Meza & Martinez-Palomo, 1981), the action of hormones such as insulin (Mc Roberts et al., 1990), some toxins (Hecht et al., 1988; Fasano et al., 1991) and several drugs (Meza et al., 1980; Duffey et al., 1981; Ojakian, 1981; Griepp et al., 1983). In contrast, some agents such as barium (Kottra & Frömter, 1990), plant cytokinins (Bentzel et al., 1980) and low temperature (González-Mariscal et al., 1984) have been reported to increase TER.

The existence of cellular mechanisms controlling tight junction permeability and structure has been previously suggested (Griepp et al., 1983; Tao-Cheng, Nagy & Brightman, 1987). During the establishment of cell confluence in tissue culture, TER reaches a peak before assuming steady-state values. Also, after disruption of established monolayers, the resistance initially rebounds to higher than steady-state values (Griepp et al., 1983). Identification of endogenous factors that can influence the sealing capacity of tight junctions is fundamental to the understanding of the physiology and pathology of permeability barriers in various epithelia and can be important to the development of new therapeutic strategies.

We have used epithelial cell lines cultured to confluence on permeable filters, in bipartite chambers, to examine the possible release of endogenous factors that would affect tight junction structure and permeability. The confluent epithelial cell monolayers, grown on bipartite chambers, allow the separation of apical and basolateral media which in turn become conditioned by the polarized secretory activity of the cells. Both the apical and the basolateral conditioned media were tested for their ability to affect TER. We have found that the apical conditioned medium (ACM), when placed in contact with the basolateral side of the confluent epithelial monolayers, produces an increase of TER. We have examined the effects of ACMs on the permeability and structure of tight junctions in three different epithelial cell lines. Our results suggest the existence of a factor that is secreted to the apical side of epithelial monolayers that is capable of modulating the tight junction structure and permeability.

Materials and Methods

Cell Culture

Cells were obtained from American Type Culture Collection (Rockville, MD). MDCK (CCL 34) cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Irvine Scientific, Santa Ana, CA), 2 mM glutamine (Irvine Scientific), 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY) and 1% antibiotic-antimicotic solution (Biofluids, Rockville, MD). Caco-2 cells (HTB 37) were grown in Eagle's Minimum Essential Medium (EMEM) with nonessential amino acids and 20% fetal bovine serum (GIBCO-BRL). Both cell lines were grown at 37°C in an air-5% CO_2 atmosphere. A6 cells (CCL 102) were grown at room temperature with CL2-Amphibian medium (NIH-Media Section, Bethesda, MD), 2 mM glutamine, 10% fetal bovine serum and 1% antibiotic-antimicotic solution in an air-2% CO_2 atmosphere. Cells at confluence were harvested with 0.25% trypsin-1 mM EDTA (GIBCO-BRL) and plated on 4.7 cm^2 diameter and 0.4 μm pore size Transwell cell culture inserts (Costar, Cambridge, MA). A6 and MDCK cells were plated on polycarbonate filter and Caco-2 cells on collagen-treated filters. Confluent monolayers of MDCK cells reached a stable TER averaging 419 $\pm$ 9 $\Omega \cdot cm^2$ four days after seeding. A6 and Caco-2 reached stable TERs of 502 $\pm$ 17 $\Omega \cdot cm^2$ and 623 $\pm$ 17 $\Omega \cdot cm^2$, respectively, seven days after seeding.

MDCK cells were also cultured in an isotonic hormonally defined serum-free medium previously described for this cell type (Taub et al., 1979). These cells generated a steady-state TER of 425 $\pm$ 18 $\Omega \cdot cm^2$.

Electrical Measurements

The degree of sealing of tight junctions was evaluated by measuring the electrical resistance across the cell monolayers using an epithelial voltohmmeter (World Precision Instruments, New Haven, CT) operated at a constant current of 20 μA. The electrical resistance of the filter and the media was subtracted from the measured values and expressed for the area of the filter (4.7 cm^2).

Apical Conditioned Medium (ACM)

Epithelial cell monolayers plated on filter inserts with steady-state TER conditioned the apical medium by the secretory activity of epithelial cells. This medium, conditioned for 4–7 days, was used in the experiments described below.

Experiments

The ACM from donor cultures was applied to the basolateral side of receptor epithelial cell monolayers of either the same cell line (homologous combination) or a different cell line (heterologous combination) (Fig.1). The TER of the receptor and control cultures was monitored at 5 min intervals. As a control, we used a culture where the basolateral medium was replaced by fresh medium. Since changes in pH (Fromm et al., 1990) or temperature (González-Mariscal et al., 1984) would modify the value of TER, both ACM and fresh media were buffered to the same pH and the chamber plates were left at room temperature until the TER became stable. All experiments were performed on a minimum of 16 monolayers (8 experimental and 8 control).

The activity of the ACM was also tested at 2, 5, 7 and 10-fold concentrations. ACM collected from a large number of donor cultures was concentrated using a Centriprep concentrator with a 30,000 MW cutoff membrane (Amicon, Beverly, MA). Prior to concentration, the ACM was cleared by centrifugation at 100,000 $\times$ g, 4°C for 1 hr. In addition, to test whether the activity would resist heating, the concentrated ACM was exposed to 65°C for 10 min. The ACM filtrate

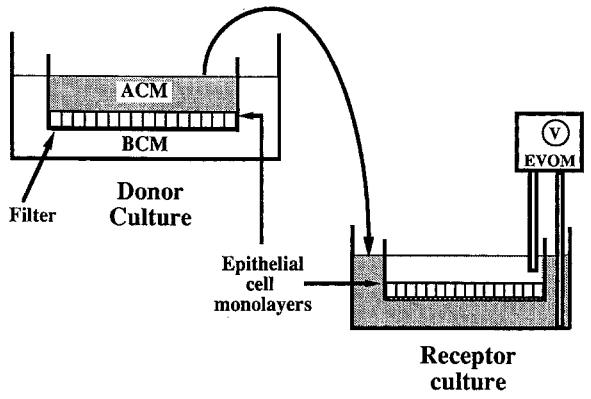

Fig. 1. Diagram of the experimental procedure. Epithelial cells were grown on permeable filters in bipartite tissue culture chambers. Medium conditioned by the cells for a period of four to seven days was collected from the apical compartment of mature donor culture monolayers and applied to the basolateral side of a receptor monolayer of either the same cell line (homologous combination) or a different cell line (heterologous combination). The TER of the receptor and control cultures was monitored using an Epithelial VoltOhmMeter (*EVOM*).

obtained through the 30,000 MW cutoff membrane was applied to the basolateral side of the control monolayers.

The experimental changes of TER were expressed as a percentage of increase in relation to the baseline values (Eq. 1).

$$\text{Net increase of TER (\%)} = \% \left[\frac{\Delta R}{R}\right]_t - \% \left[\frac{\Delta R}{R}\right]_c \quad (1)$$

where ΔR is the difference in resistance after replacing the basolateral medium by ACM, and R is the baseline value of TER at the time zero of the control (c) or treated (t) monolayers. Resistance changes are expressed as mean ± SEM.

SDS-PAGE

Proteins from serum-free ACM were separated on 0.75 mm thick, 10% polyacrylamide gels running at a constant 50 V using the discontinuous system of Laemmli (1970) and revealed by silver stain (Stratagene, La Jolla, CA). Molecular weight determinations were based on the apparent molecular weights of prestained high molecular weight standards (GIBCO-BRL).

Freeze-Fracture

The cell monolayers attached to the filter inserts were fixed by immersion in 2% glutaraldehyde in PBS for 2 hr, washed several times in PBS, removed from the surface of the filter, impregnated in 30% glycerol, mounted and then frozen for conventional freeze-fracture. Specimens were freeze-fractured at −110°C in a Balzers 301, shadowed with platinum-carbon and viewed in a JEOL 100CX electron microscope.

Morphometrical Analysis

Morphometric analysis of tight junction strands was performed according to a standard procedure (Bentzel et al., 1980; Lagarde et al., 1981; González-Mariscal et al., 1984). The micrographs of the freeze-fracture replicas were printed at 70,000× magnification. The test pattern consisted of one line placed parallel to the main axis of the junction and a series of perpendicular lines drawn at 5 mm intervals. The number of tight junction strands per cross-section was defined by the number of strands intersecting each perpendicular line crossing the junction. Tight junction depth was the distance between the most apical and basal strands intersecting each perpendicular line. Zero depth was assigned to a single strand junction. Results are expressed as the mean ± SEM. Differences between the data were assessed by the Student's unpaired t-test.

Results

Electrophysiological Changes

The ACM in a homologous combination showed an ability to trigger an increase of TER which was detected normally in less than 5 min (Fig. 2). The peak resistance change, resulting from the addition of ACM to the basolateral surfaces, ranged from 12 to 22% and was reached in 15–20 min. The TER slowly returned to basal values within 40–60 min. When basolateral conditioned medium was applied to the apical side of the monolayers, no effect on TER was observed (*data not shown*).

The ability of the ACM to promote an increase of TER was tested after preheating as well as filtration. ACM, preheated to 65°C for 10 min, lost its ability to increase TER (Fig. 2, left and center panels). The filtrate remaining, after the ACM was passed through a 30,000 MW cutoff membrane, was also unable to affect TER. For this reason, this filtrate was used as a control in experiments using concentrated ACM.

The capacity of the ACM to promote an increase of TER of the three cell lines could only be observed when the medium was conditioned for a minimum of 3–4 days. Peak TER changes of 9.6 ± 4.3 and 20.0 ± 3.8% were observed when Caco-2 monolayers were exposed to ACM conditioned for five and seven days, respectively. Higher increases of TER were obtained when the ACM was concentrated. As shown in Fig. 2, the maximum increase values were 21.5 ± 3.2% for the A6 monolayers, 30.7 ± 4.8% for Caco-2 and 23.6 ± 2.6% for MDCK. The increase of TER was proportional to the concentration of ACM. The effect of increasing concentrations of ACM on MDCK monolayers is shown in Fig. 3.

The action of ACM was also tested across different cell lines (heterologous combination). The concentrated apical medium, conditioned by MDCK monolayers for four days and then placed in contact with the basolateral membrane of Caco-2 cells, elicited an increase in TER, beginning 5 min after addition and reach-

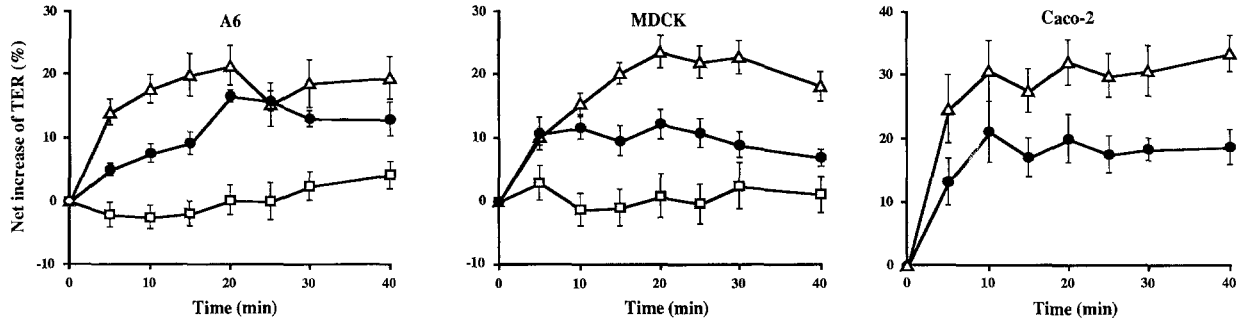

Fig. 2. TER changes in homologous combinations of donor and receptor cultures of A6 (left panel), MDCK cells (center panel) and Caco-2 cells (right panel) following replacement of the basolateral medium by ACM (●); 10-fold concentrated ACM (△); and heat-inactivated 10-fold concentrated ACM (□). Each data point represents the mean ± SEM obtained from 8–11 monolayers. Note the higher increases in TER using concentrated ACM as compared to nonconcentrated ACM and inactivation of ACM by preheating at 65°C for 10 min.

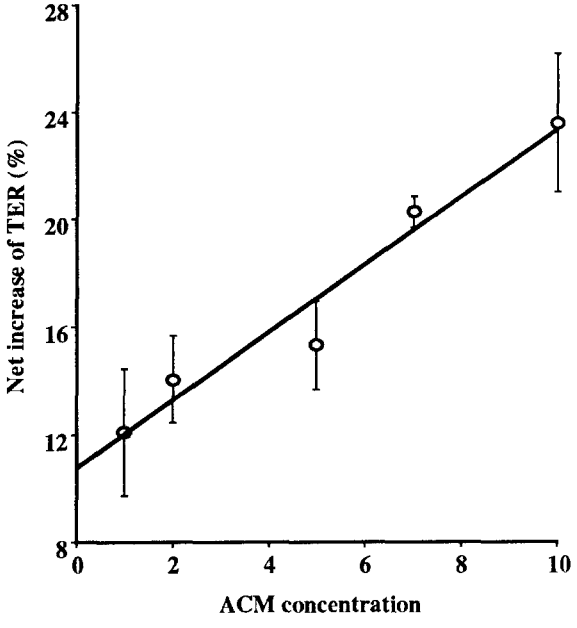

Fig. 3. Concentration dependence of the ACM activity. MDCK cells grown on polycarbonate filters were treated with homologous ACM concentrated 2, 5, 7 and 10-fold. The activity of ACM increases linearly with the concentration (correlation coefficient $r = 0.95$). Each data point represents the mean ± SEM obtained from 3 to 11 monolayers.

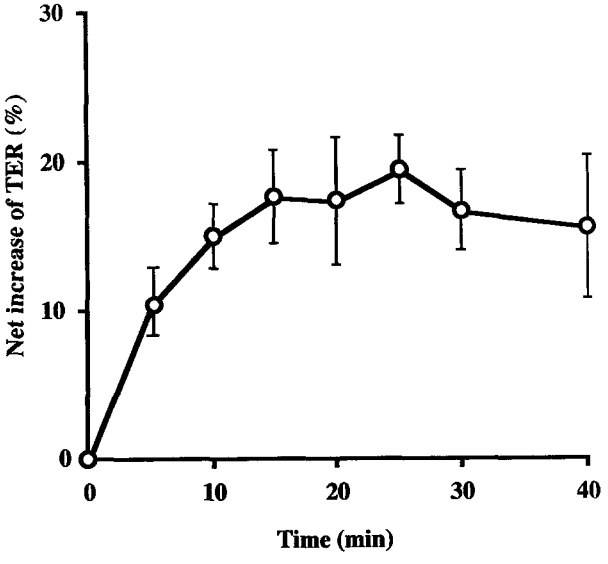

Fig. 4. Demonstration of the effect of ACM in a heterologous combination of donor and receptor cultures. At time 0, the basal medium of Caco-2 monolayers was replaced by 10-fold concentrated ACM obtained from MDCK monolayers (o). Each data point represents the average value obtained from 10 monolayers.

ing its maximum value (21.3 ± 4.0%) 25 min later (Fig. 4).

We also performed experiments to test whether the increases in TER observed depended on factors present in the serum that supplements the culture medium. MDCK monolayers exposed to a homologous serum-free ACM showed a pattern of TER increase similar to the one obtained using ACM-containing serum. The maximum value observed was 14.3 ± 2.0% at 20 min (Fig. 5). Subsequently, the TER returned slowly to baseline values. SDS-PAGE of the supernatant of MDCK serum-free ACM is shown in Fig. 6, where numerous bands can be observed. In contrast, the fresh medium showed only transferrin (76 kD), a supplement required for the culture.

Morphological Changes

The freeze-fracture replicas of A6 cells contained large expanses of membrane, clearly showing the organization of the tight junction strands (Fig. 7). The morphometric analysis showed that the average number of

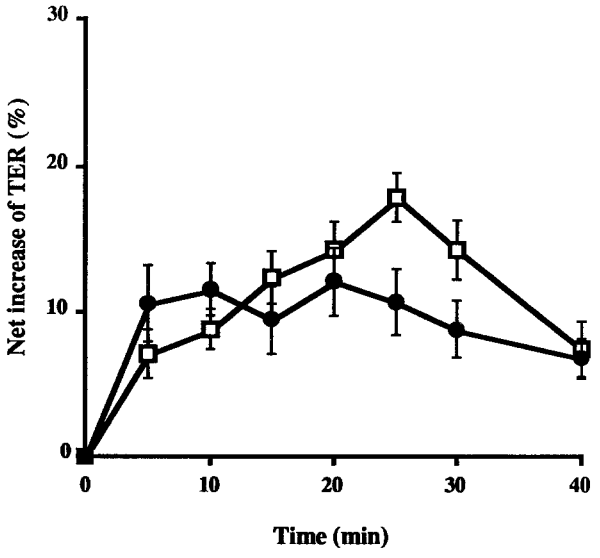

Fig. 5. TER changes in MDCK cell monolayers following incubation with homologous serum-free ACM (●) or serum-containing ACM (□). Each data point represents the average value for 10 monolayers.

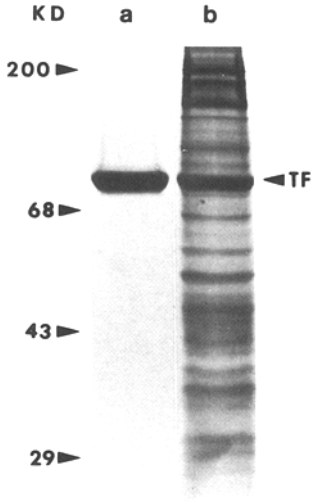

Fig. 6. SDS-PAGE of serum free medium before (*a*) and after six days conditioning by MDCK cells (*b*). Transferrin (*TF*) a 76 kD protein is the only supplement present in the fresh serum-free medium.

strands did not change with the ACM treatment (Table). However, the distribution of the number of strands (Fig. 8) changes from an initial pattern with a broad distribution (ranging from 1 to 11 strands), dominated mostly by junctions with 1 or 2 strands, to a narrower distribution (ranging from 1 to 9 strands), dominated by junctions with 2 or 3 strands. Furthermore, with the ACM treatment the tight junctions become more compact as the measured depth decreases by 19.4 % (Table).

To evaluate the relationship between the number of strands in the tight junction and the paracellular resistance, we modeled the tight junction as an electric circuit. Madara and Dharmsathaphorn (1985) demonstrated the advantage of using an electrical circuit analysis of the frequency distribution of the strands instead of simply the mean value of the number of strands. The electrical circuit analysis of the frequency distribution of the number of strands that occur in response to the treatment of the A6 cell monolayers with ACM is illustrated in Fig. 9. We analyzed our structural data as one would analyze an electrical circuit using the following relationship $(1/R_T) = (1/R_1)\ (F_1) + (1/R_2)\ (F_2) + \ldots (1/R_n)\ (F_n)$, where R_T is the total circuit resistance; $R_{1,2,\ldots n}$ are the resistances in series of individual resistors measuring an arbitrary value xΩ each; and F is the frequency of each specific type of resistor in the circuit. Taking into account the overall frequency distribution of the number of strands in the A6 cells (Fig. 8), the estimated TER is 0.020 xΩ for the control and 0.023 xΩ for the ACM-treated cells. This calculated 10% increase is very close to the increase of TER experimentally recorded following the ACM treatment.

Discussion

Apical medium conditioned by epithelial cells (ACM), when in contact with the basolateral side of confluent A6, Caco-2 or MDCK cell monolayers, is able to induce modifications in the morphology of the tight junctions and alteration in the tight junction permeability. A consistent increase of transepithelial electrical resistance (TER) was observed when the basal side of confluent cell monolayers was exposed to the ACM. This finding could be observed 5 min after contact with ACM, reaching a peak (12–22%) in 15–20 min and slowly returning to baseline values within 1 hr. The active factor present in ACM and required for this response must have a MW higher than 30 kD, since only the fraction obtained after filtration of ACM through a 30,000 cut-off membrane was inactive. Thermosensitivity is an additional characteristic of this ACM factor since it can be inactivated by preheating at 65°C for 10 min.

Serum-free ACM showed an equivalent ability to increase TER as ACM-containing serum, indicating that the active factor should be a product of the epithelial cells and not originated from the fetal bovine serum. In fact, upon examination by SDS-PAGE, the ACM showed several polypeptide bands, indicating a substantial secretory activity of the epithelial monolayers. One or more of the polypeptides revealed by SDS-

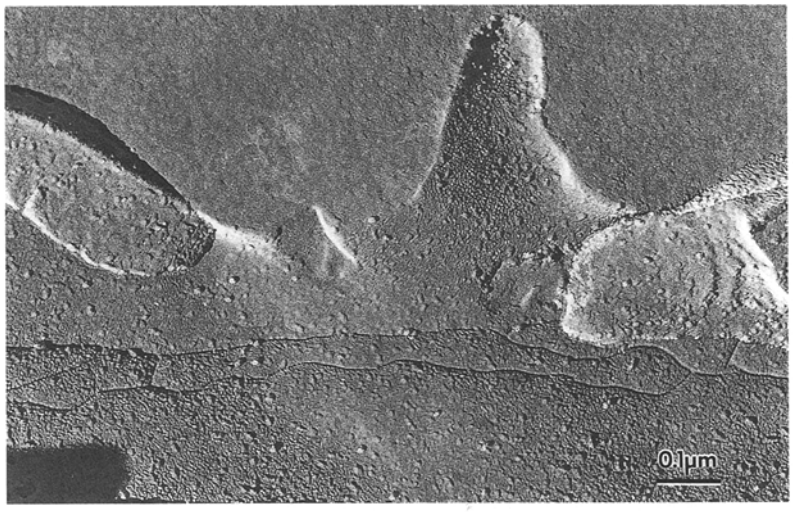

Fig. 7. Freeze-fracture image of a tight junction between A6 cells fixed after 15 min of incubation with homologous ACM. The micrograph illustrates the most frequent appearance of the network with two to three parallel strands in a relatively compact organization.

Table. Morphometrical analysis of tight junctions in A6 cells

Condition	n	Number of strands	Junctional depth (μm)
Control	1,422	3.09 ± 0.05	0.201 ± 0.006
10-fold concentrated ACM	1,110	2.92 ± 0.05	0.162 ± 0.005*

* Significantly different from control ($P < 0.001$).

PAGE could be responsible for the changes in the tight junction structure leading to the increase of TER.

The capacity of ACM to promote an increase of TER of the three cell lines could only be observed when the medium was conditioned for at least 3–4 days. ACM becomes more effective as the conditioning time increases. We have also shown that there is a linear correlation between the activity and the concentration of ACM. It is likely that the ACM activity depends on a factor secreted by the epithelial cells that are slowly accumulated in the apical medium.

A heterologous effect of ACM among different epithelial cell monolayers was also observed. Apical medium conditioned by canine renal cells (MDCK cell line) was able to increase the TER of human intestinal cells (Caco-2 cell line). Thus, the action of the ACM components on TER could represent a general tissue response seen in different types of epithelia, even with differences in organ origin and species. Although most of the epithelia have changing luminal contents, the luminal contents are often conditioned for long periods of time before they are completely replaced. In other cases, such as the bladder, the content is enriched in factors produced by the large number of nephrons in each kidney. Recently, Gallardo Montoya et al. (1992) have presented evidence for the existence of a factor in the urine of different animal species that is capable of increasing TER by 266% when applied to the basolateral side of MDCK monolayers. However, the increase of TER in response to the factor present in the urine, while more effective, was observed only after hours of exposure and the urine could not be inactivated by preheating treatments.

Tight junctions regulate the permeability of the paracellular permeation route, and this pathway accounts for up to 90% of the flux across some epithelia (Cereijido, 1991). Moreover, because the resistances of the plasma membrane are, in most cases, relatively high, the transepithelial electrical resistance is a measure of current flowing through the paracellular pathway and hence tight junction permeability (Frömter & Diamond, 1972; Diamond, 1977). Thus, the increase of TER observed is more likely to result from changes in the tight junction structure than from changes in permeability of the plasma membrane. Because the average number of strands did not change, it is improbable that the ACM treatment is promoting a rapid assembly (Kachar & Pinto da Silva, 1981) of new strands. However, a significant compaction of the network of tight junction strands and changes in the distribution of the number of strands were observed. These changes in the organization of the strands, from an initial pattern with a broad distribution (ranging from 1 to 11 strands), dominated mostly by junctions with 1 or 2 strands, to a narrower

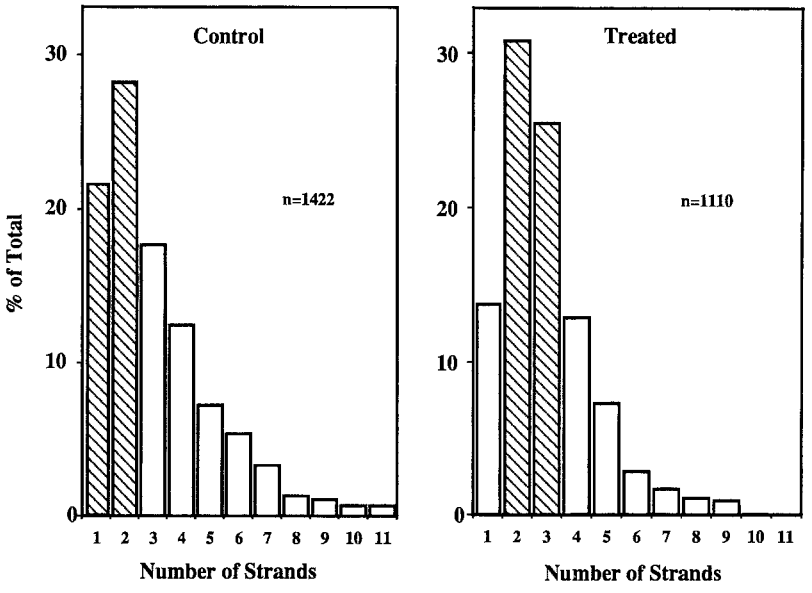

Fig. 8. Histograms showing the frequency distribution of the number of tight junction strands in A6 cells. In the control, the most frequent number of tight junction strands is one or two. After exposure to ACM, the most frequent number of tight junction strands changes to two or three.

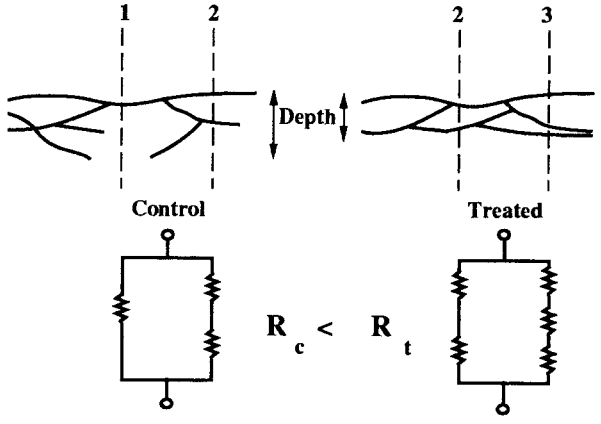

Fig. 9. Schematic representation of the structural and electrical changes of the tight junction in response to the treatment of A6 cell monolayers with ACM. The ACM treatment produces a compaction of the network of strands and a change in the distribution of the number of strands, from an initial pattern, dominated mostly by junctions with one or two strands, to a new pattern after treatment, dominated by junctions with two or three strands. The upper part of the figure illustrates the observed changes in the number and compaction of the network of strands. The lower part of the figure illustrates the equivalent electrical circuit composed of unitary resistors corresponding to individual strands at two representative lines across the junction. The higher number of strands arranged in series correlates with a higher summating resistance ($Rt > Rc$).

distribution (ranging from 1 to 9 strands) dominated by junctions with 2 or 3 strands, forms a more effective system of barriers resulting in the increase of TER (Fig. 9).

The increase of TER is only observed when the ACM is placed in contact with the basolateral side of epithelial cell monolayers. The basolateral conditioned medium showed no effect when applied to the apical side of the cell monolayers. Therefore, some type of factor must be selectively secreted to the apical side of epithelial cells and be effective only on the basolateral membrane. We suggest that the increase of TER is mediated by receptors localized on the basolateral plasma membrane. Receptors located in the basolateral side of epithelial cells have been previously proposed to mediate the decrease of TER in response to insulin (Mc Roberts et al., 1990). Tight junctions are known to be structurally and functionally associated with cytoskeletal elements (Montesano et al., 1975, 1976; Saxon et al., 1978; Bentzel et al., 1980; Elias et al., 1980; Meza et al., 1980, 1982; Hecht et al., 1988; Madara et al., 1988). Thus, it is possible that rearrangements of the strands with the compaction of the tight junction network depends on a concerted rearrangement of cytoskeletal elements. In fact, alterations in the cytoskeletal components at the apical border of epithelial cells have been associated with increases in TER values (Bentzel et al., 1980).

In conclusion, our study presents evidence for the existence of a factor secreted to the apical side of epithelial monolayers capable of changing the morphology of tight junction and increasing the TER when in contact with the basolateral side of the epithelium. Leakage of this endogenous factor from the apical to the basolateral side of the epithelial cells could be involved in a feedback mechanism controlling junctional permeability. This mechanism could be activated during physiological or pathological conditions to restore the steady-state permeability of the paracellular pathway. Identification of this putative factor could lead to new knowledge on the physiology and pathology of perme-

ability barriers in epithelia and could also lead to the development of new therapeutic strategies for the delivery of substances across tight junction barriers.

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