J. Membrane Biol. 164, 59–69 (1998)

The Journal of
Membrane
Biology

Tight Junctions and the Experimental Modifications of Lipid Content

V. Calderón[1], A. Lázaro[2], R.G. Contreras[2], L. Shoshani[2], C. Flores-Maldonado[2], L. González-Mariscal[2], G. Zampighi[3], M. Cereijido[2]

[1]Department of Biochemistry and [2]Department of Physiology, Biophysics and Neuroscience of the Center for Research & Advanced Studies, Apartado Postal 14-740, México, D.F. 07000 México

[3]School of Medicine, University of California at Los Angeles, CA 90024, USA

Received: 7 November 1997/Revised: 20 March 1998

Abstract. Tight junctions (TJs) are cell-to-cell contacts made of strands, which appear as ridges on P faces and complementary furrows on E faces on freeze fracture replicas. Evidences and opinions on whether these strands are composed of either membrane-bound proteins or lipid micelles are somewhat varied. In the present work we alter the lipid composition of Madin-Darby canine kidney monolayers using a novel approach, while studying (i) their transepithelial electrical resistance, a parameter that depends on the degree of sealing of the TJs; (ii) the apical-to-basolateral flux of 4 kD fluorescent dextran (J_{DEX}), that reflects the permeability of the intercellular spaces; (iii) the ability of TJs to restrict apical-to-basolateral diffusion of membrane lipids; and (iv) the pattern of distribution of endogenous and transfected occludin, the sole membrane protein presently known to form part of the TJs. We show that changing the total composition of phospholipids, sphingolipids, cholesterol and the content of fatty acids, does not alter TER nor the structure of the strands. Interestingly, enrichment with linoleic acid increases the J_{DEX} by 631%. The fact that this increase is not reflected in a decrease of TER, suggests that junctional strands do not act as simple resistive elements but may contain mobile translocating mechanisms.

Key words: Epithelia — Cell/cell junctions — Tight junctions — Apical/basolateral polarity — Transepithelial electrical resistance — Lipid composition — Occludin — Paracellular

Correspondence to: M. Cereijido

Introduction

Tight junctions control the diffusion of ions and small molecules through the intercellular space (paracellular ''gate'' function), and of proteins and lipids between apical and lateral-basal surface of epithelial cells (the molecular ''fence'' function) (Cereijido, 1991; Mandel, Bacallao & Zampighi, 1993). Both functions depend on the establishment of intimate plasma membrane contacts between adjacent cells, which extend as a belt of strands around the entire cell's perimeter. The chemical composition of the strands is currently a topic of intense investigation. Tight junction strands are thought to be composed of either protein (Chalcroft & Bullivant, 1970; Staehelin, 1974; Cereijido et al., 1978*b;* Polak-Charcon, Shoham & Ben-Shaul, 1978; Griepp et al., 1983) or of lipids (Kachar & Reese, 1982; Pinto da Silva & Kachar, 1982; Meyer, 1983; Kan, 1993). The ''protein'' model depicts the strands constructed of two protein macromolecules (one per cell) joined through their external domains. The ''lipid'' model in turn, depicts the strands as cylindrical micelles with the polar groups of the lipids directed inward, and the hydrophobic tails immersed in the lipid matrix of the plasma membrane of both contacting cells.

Until recently, the ''protein'' model was supported by a body of indirect evidence, such as the inhibition of junction formation by treating MDCK monolayers with inhibitors of protein synthesis (Cereijido et al., 1978*a*,*b;* Cereijido, Meza & Martínez-Palomo, 1981), and the identification of proteins (ZO-1, ZO-2, p130, cingulin, BG9.1), that in spite of having no membrane spanning domains are closely associated to the TJ (Stevenson et al., 1986; Citi et al., 1988; Chapman & Eddy, 1989; Gumbiner, Lowenkopf & Apatira, 1989, 1991; Balda et

al., 1993). The scope has since changed with the identification of occludin, a 64 KD protein that localizes at the TJ, and contains four membrane-spanning domains and two extracellular loops (Furuse et al., 1993). This would suggest that occludin is a component of the strands, or that is a regulatory component of the TJ. However, transfection of occludin tagged with an HA epitope, and whose COOH domain has been deleted, into MDCK cells, increases both the transepithelial electrical resistance of the monolayer (TER) and the paracellular permeability to FITC-dextran, and impairs the ability of the TJ to prevent diffusion of the lipid probe BODIPY-sphingomyelin from the apical to the lateral domain, but fails to modify the number and pattern of the strands observed in freeze fracture replicas (Balda et al., 1996). Furthermore, transfection of this COOH-truncated occludin produces a segmentation of the pattern of occludin observed by immunofluorescence, without a concomitant segmentation of the strands observed in freeze fracture replicas (Balda et al., 1996). Taken together, this segmentation in the immunofluorescence pattern, absence of segmentation in freeze fracture replicas, as well as expression of much higher TER and paracellular permeability, suggests that, if strands are the elements responsible for the blockade of transit through the extracellular space, they should have additional molecular components besides of occludin.

The ''lipid'' model is based on the detailed interpretation of P (protoplasmic) and E (external) faces generated during freeze-fracture, in control or lipid modified cells (Lynch et al., 1991, 1993; Stankevich et al., 1996); by the fact that the lipid-soluble probe dipicrylamine can be transferred with the aid of an applied voltage from the cell membrane of a previously loaded cell to the membrane of a neighboring one (Turin et al., 1991); by the recovery of large bleached areas with lipids diffusing through the TJ (Grebenkämper & Galla, 1994); and by the cytochemical localization of phospholipids with gold complexed phospholipase A2 (Kan, 1993). However, several studies have failed to support or discard this model (Dragsten, Blumenthal & Handler, 1981; Spiegel et al., 1985; van Meer & Simons, 1986; Van Meer, Gumbiner & Simons, 1986; Nichols, Borgman & Young, 1986; Schneeberger et al., 1988).

The possibility exists that strands would not be made exclusively of proteins or lipids, or that lipids would participate indirectly as a source of a second messenger such as diacylglycerol (Balda et al., 1991; Balda, 1992). In the present work we take advantage of new experimental approaches to reinvestigate the point. We have altered systematically the percentage composition of alkyl chains, polar groups, sphingomyelin and cholesterol of Madin-Darby canine kidney (MDCK) monolayers using a method introduced by Schneeberger et al. (1988), and a second one using enzymes that modify the lipid while remaining extracellular. This procedure insures that the modification occurs at the outer leaflet of the plasma membrane and, if strands are composed by lipids, of these structures as well. We found that these procedures do not alter the ''gate'' function of tight junctions (as measured from their transepithelial electrical resistance) nor their ''fence'' function (as tested by the diffusion of BODIPY-sphingomyelin), nor modify the pattern of distribution of endogenous and transfected occludin, taken here as sort of ''molecular probe'' of the TJ. However, we find that the apical-to-basolateral flux of dextran (J_{DEX}), increases by 630% in linoleic-enriched MDCK cells. This apparent contradiction between the information afforded by TER and J_{DEX} is used to discuss the nature of the barrier offered by the TJ.

Materials and Methods

Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34). Upon arrival, cells were cloned and all experiments reported here were performed in cells of Clone 7, chosen because of its intense blistering activity when plated on impermeable supports. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3250) in-5% CO_2 atmosphere (Forma Scientific CO_2 incubator, Steri-Cult 200). We used Dulbecco's modified Eagle's medium (GIBCO 430-1600), with 100 U/ml penicillin, 100 μg/ml of streptomycin (GIBCO 600-5145), 0.8 U/ml insulin (Eli Lilly) and 10% fetal calf serum (GIBCO 200-6170). This medium will be referred to as CDMEM. Cells were harvested with trypsin-EDTA and plated on disks of Millipore paper with pores of 0.45 μm diameter. Cells used for the experiments reported here were usually of the 60–80 passage. The MDCK cells transfected with the chicken occludin were kindly provided by M.S. Balda and K. Matter from Geneve, Switzerland (Balda et al., 1996).

Measurement of the Transepithelial Resistance (TER)

Filters with monolayers were mounted between two Lucite chambers with an exposed area of 0.69 cm^2. Current was delivered via $Ag/AgCl_2$ electrodes placed at 2 cm from the monolayer and the voltage deflection measured with electrodes placed at 1 mm from the membrane. To avoid leaks due to edge damage, each monolayer was used for a single measurement. Values of *TER* were corrected by subtracting the contribution of the filter and the bathing solution and were normalized with the formula:

$$TER = ter(M/m)$$

where *ter* is the resistance measured in the experimental monolayer, *m* the mean value in the monolayers under control conditions measured the same day, and *M* the mean value of all control monolayers. After measuring TER, lipid analysis was performed in the same monolayers.

Lipid Modification

Long Protocol

A sparse culture of MDCK cells was grown in the presence of ^{32}P-orthophosphate and one of the different bases (Serine 2 mM, ethanol-

amine 2 mM, or inositol 1 mM), lipids (sphingomyelin 15 mM, oleic acid 1 mM or linoleic acid 1 mM). 2–3 days later, when the culture had reached confluence, it was trypsined and cells were plated in Millipore filters and cultured in media containing the same base, lipid or inhibitor. One day later disks were mounted between two Lucite chambers, transepithelial electrical resistance (TER) was measured, and the same monolayers were extracted with chloroform-methanol (2:1v/v) and subjected to thin layer chromatography to analyze their lipidic composition.

To modify cholesterol content, Acyl-anthrolone or β-Hydroxi-Cuomarin were added to cells on Millipore filters two hours after plating at confluence. Cholesterol and TER were measured one day later.

Short Protocol

A sparse culture of MDCK cells was grown in the presence of ^{32}P-orthophosphate. Two days later, when the culture had acquired confluence, it was trypsined and cells were plated on Millipore filters. One day later monolayers were incubated for 2 hr with phospholipase D (1 Unit/ml media) and the different bases (10 mM: serine ethanolamine, and inositol) or sphingomielinase (25u/ml). TER and phospholipids were then measured as above.

Lipid Composition

Phospholipids

Cells were grown in a medium containing 5 μCi/ml of phosphate (^{32}P). Lipids were extracted by suspending MDCK monolayers in 5.0 ml methanol:chloroform (1:2 v/v). Phospholipids were identified in two-dimensional thin layer chromatography on glass plates (20 × 20 cm) of silica gel G-60. The first dimension was developed with solvents containing chloroform/methanol/water (65/25/4 v/v/v). The spots corresponding to different phospholipids were scrapped off the plates and the radioactivity measured in a scintillation counter (Cerbón and Calderón, 1991).

Fatty Acids

Lipid extracts were treated with 0.5 M sodium methoxide and the methyl esters analyzed in a 25 mm NB-351 silica capillary column (Perkin Elmer) by gas chromatography.

Cholesterol

Lipid extract in organic phase was dried under N_2 choloesterol was converted to cholestenone with cholesterol oxidase and assayed spectrophotometrically as described by Lange and Ramos (1983).

Electron Microscopy

Thin Sectioning

Monolayers were fixed by immersion in 3% glutaraldehyde, 4% paraformaldehyde in 0.2 M Na^+cacodylate buffer pH 7.3 for 2 hr at room temperature. The filters were washed in a solution of 0.2 M Na^+cacodylate and 4% sucrose, pH 7.3 three times at room temperature. The filters were post-fixed in 2% osmium tetroxide in 0.2 M Na^+cacodylate buffer for 90 min at room temperature. The filters were washed in 0.1 M Na + acetate buffer pH 5.0 and left overnight in a 0.5% uranyl acetate in 0.1 M Na^+acetate buffer. The filters were dehydrated in ethanol/water mixtures (50-75-95-100%), infiltrated in mixtures of Epon/ethanol and embedded in Epon 812. Sections were collected on carbon/Formvar coated grids, double stained with uranyl acetate and lead solutions and studied in a Zeiss EM10C electron microscope.

Freeze-Fracture

Monolayers fixed as described for thin sectioning were infiltrated with solutions containing 20% glycerol in 0.2 M Na^+ cacodylate buffer pH 7 for 1 hr at room temperature. The monolayers were scrapped from the filters and deposited on Balzers specimen holders. After removing the excess solution, the holders were frozen by immersion in liquid propane cooled in a liquid nitrogen bath. Fracture was performed in a Balzers 400K freeze-fracture-etch unit with a liquid nitrogen cooled knife. The fractured surfaces were shadowed with platinum at 45°C and carbon at 90°C. The replicas were cleaned with 4% Na hypochlorite and collected on single-hole copper grids coated with formvar and carbon.

Immunofluorescence

Glass coverslips containing MDCK monolayers cultured under the several experimental conditions described above, were rinsed twice with PBS, fixed and permeabilized with −20°C methanol for 20 sec, washed with PBS, incubated with 3% foetal bovine serum in PBS for 30 min, and treated with rabbit polyclonal antibody against human (Zymed 71-1500 San Francisco, CA) or chicken (a generous gift of M.S. Balda and K. Matter) occludin, for 1.0 h at 37°C. Monolayers were then rinsed 3 times for 5 min each, with PBS, incubated with a FITC-labeled goat-anti-rabbit antibody (Zymed) for 30 min, rinsed as above, mounted in FluoroGuard (Biorad 170-3140) and examined with a confocal microscope (MRC-600, Bio-Rad). Lateral views were generated by scanning the monolayers from apical to basolateral side along a 20 μm, using steps of 0.2 μm.

Diffusion of BODIPY-Sphingomyelin

To label cells with the fluorescent lipid, sphingomyelin/BSA complexes (5nmol/ml) were prepared in P buffer containing (mM): 145 NaCl, 10 HEPES (pH 7.4), 1.0 Na-piruvate, 10 glucose, 3 $CaCl_2$, using BODIPY-FL-sphingomyelin (Molecular Probes, Eugene, OR) and defatted BSA (Pagano & Martín, 1994). For confocal microscopy, filter-grown MDCK cells were labeled with BODIPY-sphingomyelin-BSA complexes for 10 min on ice. Then, the cells were washed with P buffer and incubated for 1 hr on ice or directly prepared for microscopy. To analyze the distribution of the fluorescent lipid, the filters were cut from the frame and mounted in P buffer on a glass slide that had in each corner a drop of dried nail polish. The samples were covered with a rectangular coverslip so that it was resting on the drops of nail polish. The samples were then analyzed by confocal microscopy as described above. In wild-type MDCK cells, polar lipid staining was stable for at least 20 min and lateral appearance of BODIPY-sphingomyelin was preceded by internalization. All pictures shown, however, were generated within the first 5 min of analysis (Balda et al., 1996).

Paracellular Flux

We measured paracellular flux of FITC-dextran (J_{DEX}) to 10 μg/ml in P buffer. The experiment was started by transferring the filter culture

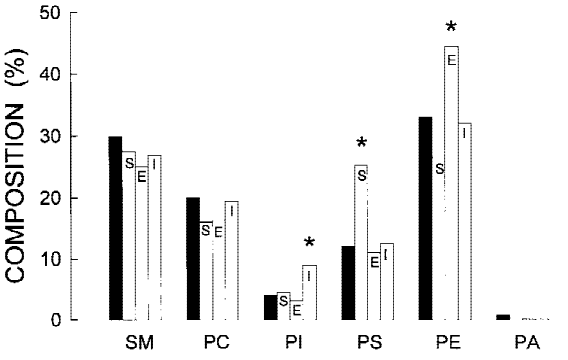

Fig. 1. Modification of the phospholipid composition of MDCK cells using the ''long'' protocol (3 days). MDCK monolayers were incubated with 10 mM of either serine, ethanolamine or inositol until they achieved confluence. Monolayers were separated into six groups labeled SM, PC, PI, PS, PE and PA. Each group included control (black columns) monolayers and experimental monolayers incubated with serine, ethanolamine and inositol, (empty columns labeled S, E, I, respectively). The content of sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidic acid (PA) was measured in the 24 monolayers by thin layer chromatography. The standard error was less than 10%. Asterisks indicate a significance better than 0.01.

to a 12-well dish containing 500 μl per well P buffer. Then, 250 μl tracer solution was added to the apical side and the monolayers were incubated at 37°C. After 3 hr, the media were collected, and FITC-dextran was measured with a fluorometer (excitation 492 nm; emission 520 nm).

Results are expressed as mean ± SE (number of observations).

ABBREVIATIONS

SM	sphingomyelin
PS	phosphatidylserine
PC	phosphatidylcholine
PI	phosphatidylinositol
PE	phosphatidylethanolamine
PA	phosphatidic acid
TER	transepithelial electrical resistance
FF	freeze fracture

Results

MODIFICATION OF THE POLAR HEAD GROUP OF PHOSPHOLIPIDS

The head group composition of phospholipids of MDCK cell monolayers was changed by incubating the monolayers for three days with 2.0 mM of either serine, ethanolamine or inositol and cultured until they achieved confluence (''long'' protocol). The content of SM, PC, PI, PS, PE and PA of the experimental groups was measured by thin layer chromatography and ^{32}P content. Figure 1 shows the percentage of SM, PC, PI, PS, PE and PA in monolayers that were left as control (black columns) or exposed to 2.0 mM of the bases serine, ethanolamine and inositol, respectively (empty columns). It may be noticed that the addition of a given base to the culture media affected only the percentage composition of the phospholipid containing such base. For example, of the four monolayers included in the experimental group labeled PS in Fig. 1, only the monolayer that contained serine in the medium increased the percentage content of PS ($P < 0.01$).

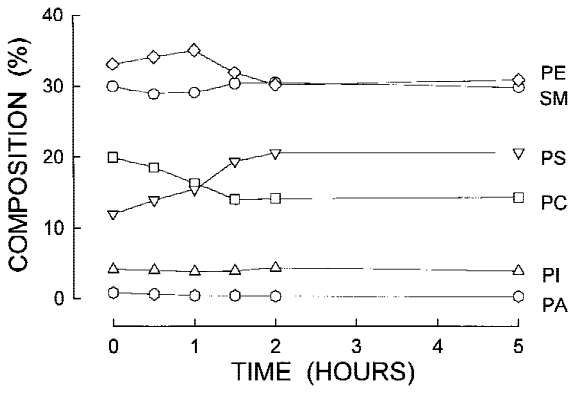

Fig. 2. Time course of the effect of 1.0 U/ml of phospholipase D on experimental monolayers incubated with 10 mM serine using the short protocol. The PC content decreased from about 20% to about 12% of the total phospholipid of the cells. The content of PS to almost double the original value (from about 10% to 20% of total phospholipid content).

When monolayers that were already confluent were incubated with 10 mM of serine in the presence of 1.0 U/ml of phospholipase D (PLD), the enzyme exchanged the head group of phosphatidylcholine (PC) for the base added to the culture medium. Figure 2 shows that this treatment achieves a steady composition of lipids in about 2 hr. Accordingly this incubation length was adopted as a ''short'' protocol. The fact that PLD works from the extracellular space, insures that it acts primarily on the lipids of the outer leaflet of the plasma membrane. Furthermore, if junctional strands are in fact composed by lipids, this treatment should modify their properties. Figure 3 shows that this short protocol achieves essentially the same pattern and amount of change in lipid composition observed with the long one. This validates the long approach used here, that was developed by Schneeberger et al. (1988), because our results demonstrate that an enzyme that starts working on lipids exposed to the bathing solution, produces an overall change that achieves a steady state in 2 hr as with the long protocol. It also reflects the fast dynamic of exchange between different lipid reservoirs in the cell.

Table 1 summarizes measurement of the transepithelial resistance (TER) under the two protocols. No statistical difference in the measurements of TER was observed in the four experimental groups. Therefore

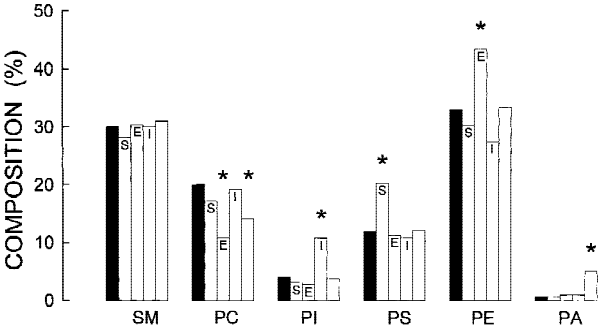

Fig. 3. Modification of the phospholipid composition of MDCK cells using the ''short'' protocol (2 hr). Confluent monolayers were incubated without phospholipase D (black columns) or in the presence of the enzyme (empty columns). The experimental monolayers were incubated with 10 mM of either serine, ethanolamine or inositol or nothing, respectively. The content of SM, PC, PI, PS, PE and PA was determined by thin layer chromatography in the 30 monolayers and expressed as percentage composition of the total lipid. Asterisks indicate a significance better than 0.01.

Table 1. Effect of changes in phospholipid head group composition on transepithelial electrical resistance

Protocol	PLD	Serine	TER ($\Omega \cdot cm^2$)
Long			500 ± 21 (146)
Long		2.0 mM	567 ± 26 (9)
Short	1 U/ml		483 ± 18 (28)
Short	1 U/ml	10 mM	476 ± 28 (12)

Monolayers were plated on nitrocellulose filters at confluence. ''Long'' refers to incubation with or without serine for 3 days, while ''short'' refers to incubation for 2 hr in the presence of phospholipase D. The transepithelial electrical resistance was measured as indicated in Materials and Methods.

changes in the composition of the phospholipid polar head group did not alter the ''gate'' function of tight junctions.

Figure 4 shows a typical tight junction pattern obtained by freeze fracture (FF) in control MDCK cells. We were unable to modify this pattern by incubating the monolayers under the several experimental conditions discussed in this work. As an example, Fig. 5 illustrates the case of monolayers treated with ethanolamine plus phospholipase D (*A*), sphingomyelinase (*B*), and serine plus phospholipase D (*C*).

Modification of Sphingomyelin, Fatty Acid Content and Cholesterol

Incubation in 15 mM sphingosine using the long protocol increases the SM content of MDCK monolayers by 20% (Fig. 6; group SM, second column). As a counterpart of

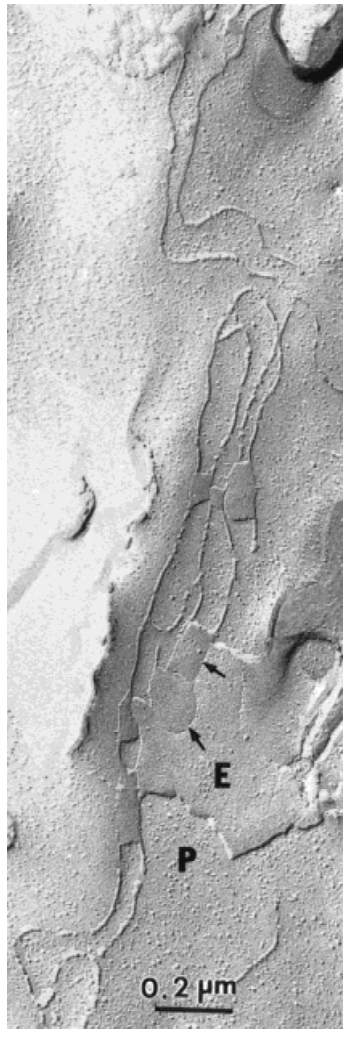

Fig. 4. Organization of a tight junction from control MDCK epithelium. In the P face (P), the junction appears composed of parallel strands made up of intramembrane particles that extend throughout the entire perimeter of the cell. In the E face (E), the junctional strands were represented by furrows (arrows) that were complementary of the strands of particles seen in the P face. The apical surface of the cell is to the left. Magnification: ×50,000.

this experiment, treatment of MDCK monolayers for 2 hr with 25 U/ml sphingomyelinase, an enzyme that hydrolyses SM into ceramide and phosphorylcholine, decreased the content of SM by 18% (Fig. 6, 3rd column). Table 2 shows that changes in the content of SM do not alter the values of TER measured in the monolayers.

The fatty acid chains of phospholipids were modified by incubating cells with 10 mM oleic (18:1) or linoleic (18:2) acids. Figure 7 illustrates the change in the distribution of chain length and saturation of the alkyl chain of treated monolayers (empty column) with respect to control monolayers (black columns). Table 3 shows that these modifications did not alter the value of TER measured in the monolayers.

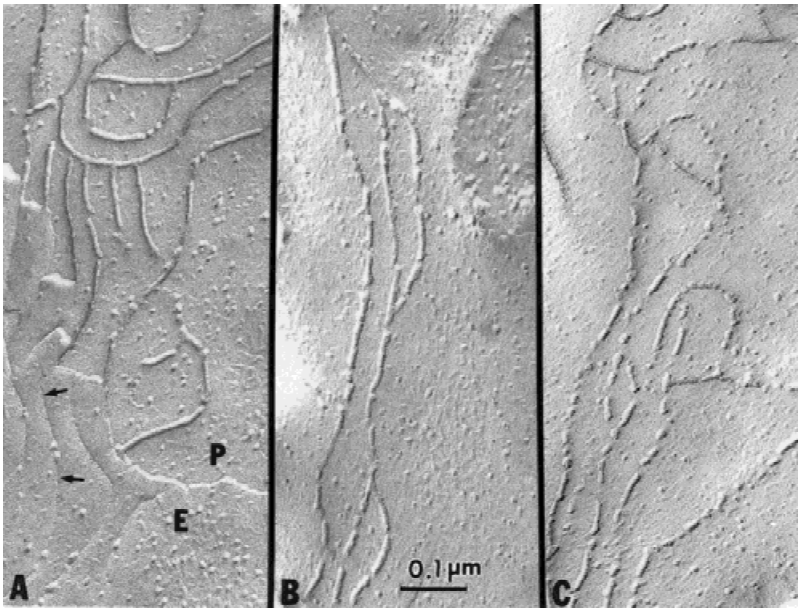

Fig. 5. Organization of tight junctions from monolayers to which the lipid composition had been altered using the ''short'' protocol (*see* Materials and Methods). Panel *A* shows a monolayer that was incubated in 10 mM ethanolamine and treated with phospholipase D. The step separating the P and E fracture faces corresponds to the place where the fracture plane passed from one cell into the partner cell forming the junction. The arrows in the E face indicate to the furrows formed by the tight junctional strands. Panel *B* shows a tight junction from a monolayer treated with 25 U/ml of sphingomyelinase. Panel *C* shows a tight junction from a monolayer incubated with 10-mM serine and treated with phospholipase D. The principal conclusion of this experiment was that the size of the particles in the strands remained unaltered after changing the lipid composition of the different monolayers. Magnification: ×100,000.

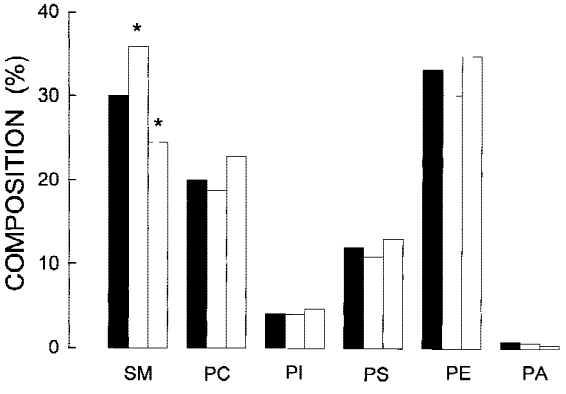

Fig. 6. Modification of the composition of sphingomyelin (SM) of MDCK cells with the long protocol. The composition of SM, PC, PI, PS, PE and PA was measured in control monolayers (black columns) and experimental monolayers incubated with 10 mM sphingosine (second empty column) or 10 U/ml of sphingomyelinase (third empty column). As expected the incubation with sphingosine increased the content of SM and the incubation with the enzyme decreased the content of the lipid only in the group labeled SM. Asterisks indicate a significance better than 0.01.

To change the cholesterol content, MDCK monolayers were treated with β-hydroxi-cuomarin or acylanthrolone. Table 4 shows that the value of TER remained unaltered after treating the monolayers with the drug.

Experimental Modification of Lipids Changes J_{DEX}

Usually, the electrical resistance through the monolayer (TER) is taken to reflect the sealing capacity of the TJ, an assumption supported by the fact that variations in TER

Table 2. Effect of changes in phospholipid tail group composition on transepithelial electrical resistance

	TER ($\Omega \cdot cm^2$)
Control	500 ± 21 (146)
Sphingosine	536 ± 30 (10)
Sphingomyelinase	440 ± 24 (10)

Monolayers, plated as in Table 1, were incubated with 15-mM sphingosine (long protocol) or 25 U/ml of sphingomyelinase (short protocol). Transepithelial electrical resistance was then measured as indicated.

are closely correlated to changes in the transepithelial flux of extracellular markers (González-Mariscal et al., 1990). However, Balda et al. (1996) found that transfection of some deletion constructs of occludin into MDCK cells, may vary the two parameters in the same direction, indicating that an increase of resistance can be accompanied by an increase in permeability. To investigate whether lipids may be involved in these phenomena, we measured J_{DEX} in two different conditions, one in which we enriched the polar heads with serine using the short protocol (Fig. 8, first two bars), and one in which we varied the degree of saturation of the hydrocarbon chain with the long protocol (Fig. 8, last two bars). It can be seen that while linoleic enrichment does not change TER (Table 3), it does produce a 631% increase in J_{DEX}.

Modifications of Lipids Do Not Impair the Retention of BODIPY-Sphingomyelin in the Apical Domain of the Cells

TJs are thought to prevent the diffusion of proteins and lipids in the outer leaflet of the plasma membrane be-

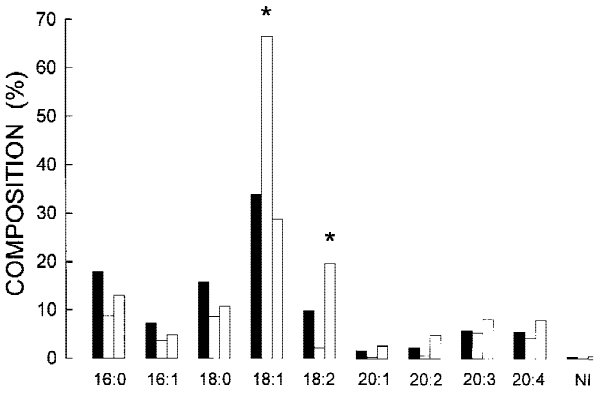

Fig. 7. Modification of the fatty acid composition on MDCK cells with the long protocol. The black columns indicate the control monolayers and empty columns the composition of experimental monolayer. The experimental monolayers were incubated with 10 mM oleic (second column) or linoleic (third empty column) acid. The percentage composition of the different fatty acids was measured by gas chromatography. As expected only the percentage composition of 18:1 (second column) and 18:2 (third column) fatty acids was modified in the experiment. Asterisks indicate a significance better than 0.01.

Table 3. Effect of fatty acid content on transepithelial electrical resistance

	TER ($\Omega \cdot cm^2$)
Control	500 ± 21 (146)
Oleic	457 ± 25 (10)
Linoleic	494 ± 28 (12)

Monolayers were cultured on millipore filters in media supplemented with oleic or linoleic acids with the long protocol as described methods (lipid modifications). Transepithelial electrical resistance was then measured as indicated.

Table 4. Effect of cholesterol content on transepithelial electrical resistance

		Cholesterol content (μg/mg protein)	TER ($\Omega \cdot cm^2$)
Control		0.32 ± 0.018	500 ± 21 (146)
β-Hydroxi-Cuomarin	50 μM	0.20 ± 0.045	465 ± 14 (20)
β-Hydroxi-Cuomarin	100 μM	0.18 ± 0.039	455 ± 14 (20)
Acyl-Anthrolone	20 μM	0.27 ± 0.032	526 ± 46 (12)
Acyl-Anthrolone	200 μM	0.22 ± 0.025	590 ± 58 (16)

Monolayers were cultured on Millipore filters in media supplemented with the inhibitors as described in methods (long protocol). Transepithelial electrical resistance was then measured as indicated, and cholesterol content was determined.

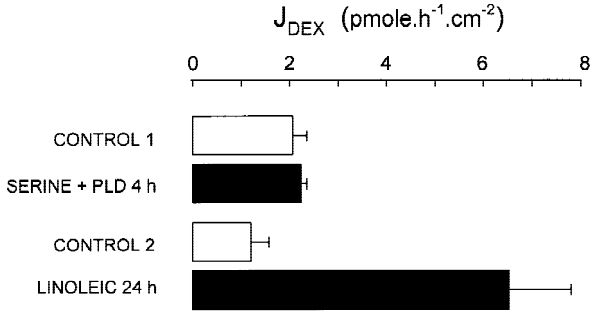

Fig. 8. Dextran flux across monolayers of MDCK cells. Monolayers were prepared on polycarbonate filters (Transwell, Costar Nucleopore, 24 mm in diameter). FITC-dextran of 4 kD was added to the apical side. Values of J_{DEX} in the first and second bars were obtained with the ''short'' protocol, and those in the third and fourth with the ''long'' one. The number of observations was 6 in every experimental conditions.

tween the apical and basolateral cell surface domains (Dragsten et al., 1981; van Meer et al., 1986; van Meer & Simons, 1986; Nichols et al., 1986; Spiegel et al., 1985). To investigate whether modification of the lipid composition affects this function of tight junctions (''fence'') we labeled the apical cell surface with BODIPY-sphingomyelin for 10 min at 4°C. Cells were then washed and left for an additional 60-min period on ice, followed by observation by confocal microscopy (Mandel et al., 1993; Balda et al., 1996). The z-sections obtained immediately after removing the probe or 1 hr later (Fig. 9) demonstrate that control MDCK cells effectively retain the fluorescent lipid in the apical plasma membrane. In monolayers treated with 2.0 mM EDTA (second line, left) and in a clone showing a negligible value of TER (second line, right) the fluorescent probe diffused through the tight junction, labeled the basolateral space, and appeared to penetrate the cells. To investigate the role of lipid composition, we used the protocol of adding serine for 3 days, adding this aminoacid for 2 hr in the presence of phospholipase D, and adding linoleic for 3 days. As observed in Fig. 9, none of the conditions tested impaired the ''fence'' role of the TJ. As an incidental observation, it was noticed that the probe tends to disappear with time from the central region of the apical membrane, and accumulate in the periphery, right above the tight junction (*see* for instance third line, right).

Experimental Modification of Lipids Does Not Change the Pattern of Distribution of Occludin

Occludin appears to be a membrane component of the TJ (Furuse et al., 1993; Balda et al., 1996). It is conceivable that if strands contain a sizable amount of lipids, the experimental modification of polar heads and hydrocarbon tails would affect the distribution of occludin. To test this point we used a policlonal antibody prepared against human occludin that would label endogenous occludin, and an antibody against the HA epitope that would label exogenous one (for details *see* Balda et al., 1996). Figure 10 shows that the modification of the lipid

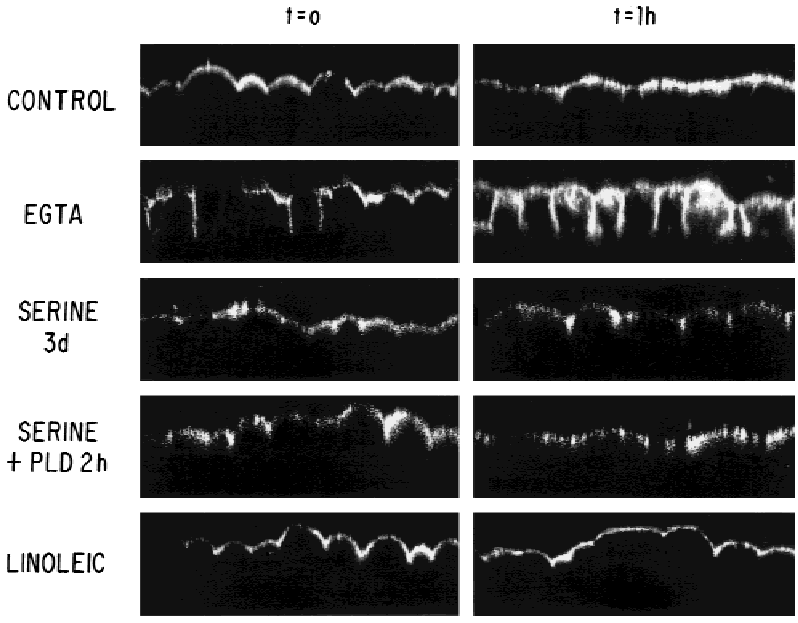

Fig. 9. Lateral diffusion of BODIPY-sphingomyelin in MDCK cells. The apical surface of filter-grown confluent monolayers was labeled for 10 min with BODIPY-sphingomyelin/BSA complexes on ice. Then, the cells were immediately mounted (t = 0) or left for an additional 60 min (t = 1 hr) on ice. The distribution of the fluorescent lipid was then analyzed by z-sectioning. In control monolayers the probe is effectively retained in the apical domain, whereas in cells treated with 2.8 mM EGTA (second line, left) or in a clone of MDCK cells with negligible value of TER (second line, right) it gains access to the lateral domain and appears later inside the cells as if, once in contact with this membrane domain, cells incorporate the lipid probe. In the rest of the samples the probe was effectively restricted to the apical region, despite of the modifications in lipid composition produced experimentally.

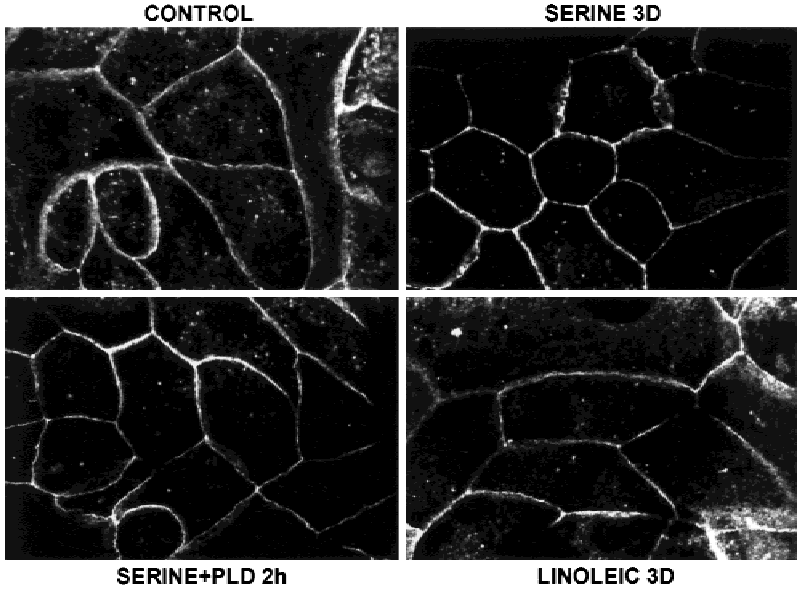

Fig. 10. Distribution of endogenous occludin in MDCK cells. Cells were plated and processed as indicated in Materials and Methods. An antibody against human occludin was used. A similar pattern is obtained with MDCK cells transfected with HA-tagged chicken occludin, using the antibody against HA.

composition of MDCK cell produces no detectable change in the distribution of occludin.

Discussion

Efforts to select between the protein and lipid models of tight junctions have followed several approaches. One of the first, studied the diffusion of fluorescent lipid probes added to the outer leaflet of the apical membrane of MDCK cells. These probes were unable to diffuse into the basolateral membrane, presumably due to the barrier formed by tight junctions. Only lipid probes with the ability to flip-flop from the outer into the cytoplasmic leaflet were able to circumvent the tight junction (Dragsten et al., 1981). Although the results appear inconsistent with the lipid model, it was argued that the first type of lipid probes do not diffuse across strands made of lipids, for the same reason that they are not able to flip-flop between the two leaflets of the membrane, i.e., their hydrophilic heads are too large. In a second approach, different clones of MDCK cells, one of them expressing glycosphingolipid Forssman antigen (GFA), were cocultured at confluence (van Meer & Simons, 1986). It was expected that if junction strands were composed of lipids, the outer leaflet of neighboring cells would constitute a sort of lipidic continuum, and co-culture should

allow diffusion of GFA from a cell that expresses this molecule towards the neighbors that do not. However, this was not the case. Although this result appears inconsistent with the lipid model, it may not disprove the model because, as pointed out by Grebenkämper and Galla (1994), GFA may be too large to diffuse across the cylindrical lipid micelle. A third approach resorted to modifications of the length and degree of saturation of the phospholipids by supplementing cultured cells with fatty acids (Schneeberger et al., 1988). It was expected that the modification of the fatty acid chains of the lipids in the cell would also modify lipids composing the strands, and that such a modification would change in turn the functions of tight junctions. However, these lipid modifications did not alter the paracellular ''gate'' function of TJs in MDCK cell monolayers. A fourth approach consisted in photobleaching large areas of the cell membrane of MDCK cells that were previously loaded with the lipid fluorescent probe C_6-NBD-PC, and measure the fluorescence recovery or the ability of the probe to diffuse to neighboring cells. Using this method, Grebenkämper and Galla (1994) have found that the probes diffuse in the plane of the membrane of the same cell, as well as towards neighboring ones, provided temperature is kept above the melting point of the hydrophobic chains. So the available evidence is conflictive.

We have extended the approach of Schneeberger et al. (1988) by changing systematically the polar groups, the hydrophobic chain length, and the content of cholesterol and sphingomyelin, and study their influence on the value of TER and on the pattern of distribution of strands observed in freeze fracture replicas, but have also tested the ability of the TJs to prevent diffusion of lipids from the apical to the basolateral domain, the distribution of occludin, and the transepithelial flux of dextran. The fact that in some experiments these changes were elicited with enzymes that remain in the extracellular space, suggests that these changes are achieved primarily at the outer leaflet of the cell membrane. Furthermore, our observation that the short protocol using an extracellular enzyme achieves essentially the same modification of lipid composition than the longer one introduced by Schneeberger et al. (1988), supports the assumption of these workers that the modifications of lipids that are measure in an extract of the whole cell, reflects the composition of in the lipids of the plasma membrane. On the same basis, one expects that if junctional strands are actually constituted by long cylindrical micelles, their modification may somehow be reflected in the parameters studied.

Occludin is the sole TJ-associated protein known to belong to the membrane (Furuse et al., 1993) and whose experimental mutation modifies both the value of TER and the paracellular permeability (Balda et al., 1996). Transfected chicken occludin tagged with an HA epitope increases the value of TER and paracellular permeability to dextran by more than 200%, demonstrating that, contrary to the expectation, electrical resistance and junctional permeability not only can be dissociated, but can vary in opposite directions (Balda et al., 1996). When the COOH terminal is truncated, occludin remains at its typical position at the outermost end of the paracellular space, but instead of forming a continuous ring, it adopts a segmented pattern. Interestingly, this discontinuous pattern adopted by transfected occludin is induced in the endogenous occludin as well, but is not reflected in the structure of the strands observed in freeze fracture replicas (Balda et al., 1996). In the present work we observe that changes in lipid composition do not modify the distribution of occludin.

In natural epithelia TER ranges from 10 $\Omega \cdot cm^2$ (kidney proximal tubule) to several thousands (colon mucosa); a range almost exclusively due to variations in the diffusion through tight junctions (Reuss, 1991). Although chemical analysis showed that the two independent experimental protocols used in the present work (the ''long'' and the ''short'' ones) did change the head group, the degree of saturation of fatty acids and the concentration of lipids such as PS and SM, we failed to detect any significant modification in the value of TER, nor alterations in the freeze fracture pattern, results that are inconsistent with the idea that tight junction strands are *exclusively* composed of lipid micelles. However, the same holds for proteins because, as mentioned above, the expression of mutated forms of occludin, that change permeability and/or electrical resistance by several hundreds percent, fails to produce any detectable modification of the FF pattern in MDCK cells (Balda et al., 1996). Taken together, these facts suggest that the model of a paracellular route limited by strands that constitute a simple lump of matter blocking the diffusion of substances may be too simplistic. The very existence of a complex machinery of molecules (ZO-1, ZO-2, cingulin, etc.) closely associated to the TJ, with segments of amino acid whose sequence identify them with sites for the specific association with other molecular species or to undergo phosphorylation, and whose arrangement responds to cell-cell contacts, presence of hormones, Ca^{2+}, Cd^{2+}, and pharmacological agents, indicates that simple blocking-models are unsatisfactory. Actually, our finding that J_{DEX} may increase very significantly in the absence of any detectable modification of TER, is in itself a demonstration that substances cross the TJ by means of highly elaborated mechanisms.

Carriers offer a straightforward interpretation to the observed discrepancies between electrical resistance and permeability, in particular when they constitute what is known as an ''exchange diffusion'' mechanism, and translocate thousands of ions per second without an electrical manifestation. Since during their cyclic work carriers should undergo a series of reversible changes in configuration to deliver the ligand to the opposite side of

the membrane, they are particularly sensitive to the viscosity of the lipid matrix. Interestingly, when we enriched the membrane with lipids with double bonds in their hydrophobic chain (linoleic replacing oleic acid), J_{DEX} increased by a factor of 6, as if whatever the translocator may be, it was now operating in a more fluid environment. The TJ has always been regarded as a mere sealing element, a characteristic reflected in its very name. Consequently, the constellation of junction-associated molecules being discovered in these days, is thought to be involved in the regulation of the degree of tightness. In this respect, our results open the possibility that the TJ may not be a silent block of substance interposed in the diffusion pathway, but may contain carriers, and that some of these junction-associated molecules may conceivably operate the insertion, removal, or regulation of translocating mechanisms.

We thank the valuable discussion and advice of Drs. M.S. Balda and Karl Matter, the efficient secretarial work of Mrs. Elizabeth del Oso, and the economic support of the National Council of Science & Technology of México (CONACYT).

References

Balda, M.S. 1992. Intracellular signals in the assembly and sealing of tight junctions. *In:* Tight Junctions. M. Cereijido editor. pp. 121–137. CRC Press, Boca Raton, Florida

Balda, M.S., González-Mariscal, L., Contreras, R.G., Cereijido, M. 1991. Participation of a G protein modulation system in the assembly and sealing of tight junctions. *J. Membrane Biol.* **122:**192–202

Balda, M.S., Gonzalez-Mariscal, L., Matter, K., Cereijido, M., Anderson, J.M. 1993. Assembly of the tight junction: the role of diacylglycerol. *J. Cell Biol.* **123:**293–302

Balda, M.S., Whitney, J.A., Flores, C., González, S., Cereijido, M., Matter, K. 1996. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J. Cell Biol.* **134:**1031–1049

Cerbón, J., Calderón, V. 1991. Changes of the compositional asymmetry of phospholipids associated to the increment in the membrane surface potential. *Biochim. Biophys. Acta* **1067:**139–144

Cereijido, M. 1991. Introduction: Evolution of ideas on the tight junction. *In:* Tight Junctions. M. Cereijido editor. pp. 1–13. CRC Press, Boca Raton, Florida

Cereijido, M., Meza, I., Martínez-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*b*. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell. Biol.* **77:**853–880

Cereijido, M., Rotunno, C.A., Robbins, E.S., Sabatini, D.D. 1978*a*. Polarized epithelial membranes produced *in vitro*. *In:* Membrane Transport Processes. J.F. Hoffman, editor. pp. 433–461. Raven Press, New York

Chalcroft, J.P., Bullivant, S. 1970. An interpretation of liver cell membrane and junction structure based on observations of freeze-fracture. *J. Cell Biol.* **47:**49–60

Chapman, L.M., Eddy, E.M. 1989. A protein associated with the mouse and rat hepatocyte junctional complex. *Cell Tissue Res.* **257:**333–341

Citi, S., Sabanay, H., Jakes R., Geiger, B., Kendrich-Jones, J. 1988. Cingulin, a new peripheral component of tight junctions. *Nature* **333:**272–275

Dragsten, P.R., Blumenthal, R., Handler, J.S. 1981. Membrane asymmetry in epithelia: is the tight junction a barrier to the diffusion in plasma membrane? *Nature* **294:**718–722

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita S., Tsukita, S. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol.* **123:**1777–1788

González-Mariscal, L., Contreras, R.G., Bolívar, J.J., Ponce, A., Chávez de Ramirez, B., Cereijido, M. 1990. Role of calcium in tight junction formation between epithelial cells. *Am. J. Physiol.* **259:**C978–C986

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

Grebenkämper, K., Galla, H.J. 1994. Translational diffusion measurements of a fluorescent phospholipid between MDCK-I cells support the lipid model of the tight junctions. *Chem. Phys. Lipids* **71:**133–143

Gumbiner, B., Lowenkopf, T., Apatira, D. 1991. Identification of a 160 KD polypeptide that binds to the tight junction protein zo-1. *Proc. Natl. Acad. Sci. USA* **88:**3460–3464

Kachar, B., Reese, T. 1982. Evidence for the lipidic nature of tight junction strands. *Nature* **296:**464–466

Kan, F.W.K. 1993. Cytochemical evidence for the presence of phospholipids in epithelial tight junction strands. *J. Hystochem. Cytochem.* **41:**649–656

Lange, Y., Ramos, B.V. 1983. Analysis of the distribution of cholesterol in the intact cell. *J. Biol. Chem.* **258:**15130–15134

Lynch, R.D., Tkachuk, L., Shastry, R., Rabito, C.A., Schneeberger, E.E. 1991. Alterations in Ca^{2+}-induced development of transepithelial electrical resistance (TER) by MDCK cells monolayers depleted of cholesterol (CH). *J. Cell Biol.* **115:**480*a*. (*Abstr*.)

Lynch, R.D., Tkachuk, L.J., Ji, X., Rabito, C.A., Schneeberger, E.E. 1993. Depleting cell cholesterol alters calcium-induced assembly of tight junctions by monolayers of MDCK cells. *European J. Cell Biol.* **60:**21–30

Mandel, L.J., Bacallao, R., Zampighi, G. 1993. Uncoupling of the molecular ''fence'' and paracellular ''gate'' functions in epithelial tight junctions. *Nature* **361:**552–555

Meyer, H.W. 1983. Tight junction strands ere lipidic cylinders. *Naturwissenshaften* **70:**251–252

Nichols, G.E., Borgman, C.A., Young, W.W. Jr. 1986. On tight junction structure: Forssman glycolipid does not flow between MDCK cells in an intact epithelial monolayer. *Biochem. Biophys. Res. Commun.* **138:**1163–1169

Pinto da Silva, P., Kachar, B. 1982. On tight junctions structure. *Cell* **28:**441–450

Polak-Charcon, S., Shoham, J.J., Ben-Shaul, Y. 1978. Junction formation in trypsinized cells of human adenocarcinoma cell line. *Exptl. Cell Res.* **116:**1–13

Reuss, L. 1991. Tight junction permeability to ions and water. *In:* Tight Junctions. M. Cereijido, editor. pp. 49–66. CRC Press, Boca Raton, FL

Schneeberger, E.E., Lynch, R.D., Kelly, C.A., Rabito, C.A. 1988.

Modulation of tight junction formation in clone 4 MDCK cells by fatty acid supplementation. *Am. J. Physiol.* **254:**C432–C440

Spiegel, S., Lumenthal, R., Fishman, P.H., Handler, J.S. 1985. Gangliosides to not move from apical to basolateral plasma membrane in cultured epithelial cells. *Biochim. Biophys. Acta* **821:**310–318

Staehelin, L.A. 1974. Structure and function of intercellular junctions. *Int. Rev. Cytol.* **39:**191–283

Stankevich, M.C., Francis, S.A., Vu, Q.U., Schneeberger, E.E., Lynch, R.D. 1996. Alterations in cell cholesterol content modulate $Ca^{(2+)}$-induced tight junction assembly by MDCK cells. *Lipids* **31:**817–828

Stevenson, B.R., Siliciano, J.D., Mooseker, M.S., Goodenough, D.A. 1986. Identification of zo-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* **103:**755–766

Turin, L., Béhé, P., Plonsky, I., Dunina-Barkovskaya, A. 1991. Hydrophobic ion transfer between membranes of adjacent hepatocytes: A possible probe of tight junction structure. *Proc. Natl. Acad. Sci. USA* **88:**9365–9369

Van Meer, G., Gumbiner, B., Simons, K. 1986. The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. *Nature* **322:**639–641

Van Meer, G., Simons, R. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **5:**1455–1464