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Effect of Temperature on the Occluding Junctions of Monolayers of Epithelioid Cells (MDCK)

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Summary. In previous works it was demonstrated that the monolayer of MDCK cells behaves as a leaky epithelium where the electrical resistance across reflects the sealing capacity of the occluding junction. In the present work we study whether this sealing capacity can be modified by temperature and whether this is accompanied by changes in the structure of the occluding junction. Monolayers were prepared on disks of nylon cloth coated with collagen and mounted as a flat sheet between two Lucite chambers. The changes in resistance elicited by temperature were large (306% between 3 and 37°C), fast (less than 2 sec), and reversible. An Arrhenius plot of conductance versus the inverse of temperature shows a broken curve (between 22 and 31°C), and the activation energies calculated (3.2 and 4.0 kcal · mol^{-1}) fall within the expected values for processes of simple diffusion. The morphology of the occluding junction was evaluated in freeze-fracture replicas by counting the number of strands and the width of the band occupied by the junction every 133 nm. In spite of the change by 306% of the electrical resistance and the phase transition, we were unable to detect any appreciable modification of the morphology of the occluding junction. Since the freeze-fracture replicas also show a density of intramembrane particles (IMP) different in the apical from that in the basolateral regions of the plasma membrane, as well as differences between face *E* and face *P*, we also investigated whether this is modified by temperature. Cold increases the population of IMP, but does not affect their polarization with the incubation time it takes to elicit changes in electrical resistance.

Key Words epithelial monolayers · MDCK cells · occluding junctions · intramembrane particles · electrical resistance · temperature

Introduction

The epithelioid line of MDCK cells, which was derived from the kidney of a normal dog by Madin and Darby (1958), can be cultured as monolayers on permeable supports. These preparations exhibit many properties of transporting epithelia (Misfeldt, Hamamoto & Pitelka, 1976; Cereijido et al., 1978*a*,*b*). One of these similarities is the ability to form occluding junctions (for a recent review *see* Cereijido, Meza & Martínez-Palomo, 1981). The present study is on the effect of temperature on the degree of sealing of these occluding junctions (as measured by the transepithelial electrical resistance) and on their structure (as studied in freeze-fracture replicas).

There were several reasons for performing these studies: (1) While leaky epithelia, like that of *Necturus* gallbladder, have the electrical conductance, the width of the band occupied by the occluding junction, and the number and arrangement of the strands homogeneously distributed along the intercellular space between two cells (Cereijido, Chávez de Ramírez & Stefani, 1982), the monolayer of MDCK cells has a conductance heterogeneously distributed and a chaotic pattern of strands that varies in number from one to ten in a few nanometers (Cereijido, Stefani & Martínez-Palomo, 1980). There seems to be a correlation between distribution of strands and conductance in the two preparations. However, the question of whether the degree of tightness of a junction is related to the number of its strands is still obscure (*see* Discussion). Part of the controversy is originated by the fact that one generally compares different epithelia from different animals and even from different species. We expected that, by using changes of temperature in the same set of monolayers of MDCK cells, we would be able to change the electrical resistance or the structure of the occluding junction, or both, and this would permit us to establish whether there is any relationship between them. (2) When a suspension of MDCK cells obtained by trypsinization of previous monolayers is plated at confluence, it forms new occluding junctions in a few hours through a process that requires protein synthesis (Cereijido et al., 1978*b*). However, this does not necessarily mean that the junctions themselves are constituted by proteins. In fact, recent studies sug-

gest that strands may be composed by lipids arranged in cylindrical micelles, of the type that would appear in X-ray crystalography as Hexagonal Phase II (Pinto da Silva & Kachar, 1982; Kachar & Reese, 1982). These liquid-crystalline phases, at least those formed by mixtures of pure lipids (Verkleij et al., 1980; Van Venetie & Verkleij, 1981) or lipids extracted from biological systems, are highly sensitive to temperature. Thus, depending on the lipid species, liquid-crystalline phases switch from *hexagonal* to *rectangular* to *complex-hexagonal* to *cubic* to *lamillar* to *gel*, etc. (Luzzati & Husson, 1962). Therefore, one would expect that, if the strands of an occluding junction are cylindrical lipidic micelles in Hexagonal Phase II, they would suffer drastic alterations when temperature is changed by several decades (3 to 37°C). (3) The strands of the occluding junctions usually appear in freeze-fracture replicas as ridges on the *P* face and grooves on the *E* face. Yet in many occluding junctions, in particular those of fast growing or developing tissues (Humbert, Montesano, Perrelet & Orci), 1976; Tice, Carter & Cahill, 1977) and in junctions that are being resealed with Ca^{2+} after a treatment with EGTA (Meldolesi et al., 1978; Hoi Sang, Saier & Ellisman, 1979), ridges are fragmented and seem to be constituted not by continuous filaments, but by rows of particles. It was even suggested that the strands of the occluding junctions are in fact formed by alignment and fusion of particles that were previously scattered in the plane of the membrane and that were cross linked by glutaraldehyde during fixation (Montesano, Friend, Perrelet & Orci, 1975; Van Deurs & Luft, 1979). However, this interpretation has been challenged (Kachar & Reese, 1982; Pinto da Silva & Kachar, 1982). Therefore, it seemed interesting to study whether the changes that temperature may introduce on the structure, or on the sealing capacity of the occluding junctions, would be accompanied by modifications in the density of intramembrane particles (IMP). A direct relationship between lipidic particles, which correspond to inverted lipid micelles, and the Hexagonal II Phase has been demonstrated in model systems (Verkleij et al., 1980; Van Venetie & Verkleij, 1981; Borovjagin, Vergara & McIntosh, 1982; Hope, Walker & Cullis, 1983).

Materials and Methods

Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34) (Madin & Darby, 1958). In most experiments cells were between 60-80th passage. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3150, Cambridge, Mass.) with an air-5% CO_2 atmosphere (VIP CO_2 incubator 417, Lab Line Instruments, New Brunswick, N.Y.) and 20 ml of Complete Dulbecco's Minimal Essential Medium (CMEM) with Earle's salts (Grand Island Biological Co. (GIBCO) 430-1600, Grand Island, N.Y.), 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% calf serum (GIBCO 617). Cells were harvested with trypsin-EDTA (GIBCO 540) and plated on disks of a nylon cloth (diameter 1.3 cm), coated with rat tail collagen as described by Cereijido et al. (1978*a*) and contained in a 24-well Falcon dish (Falcon Labware, Oxnard, Calif.). Plating was made at confluence (10^6 cells/ml; 1.0 ml/well). After 90 min the cell suspension was removed, replaced by fresh CMEM, and those cells forming a confluent monolayer on the disks remained attached. They were kept at 36.5°C in an air-5% CO_2 incubator.

Electrical Measurements

The degree of sealing of the occluding junction was evaluated by measuring the electrical resistance across the disks. These were mounted between two Lucite chambers, 1.0 ml on each side, with an exposed area of 0.69 cm^2. Current was delivered via Ag/AgCl electrodes placed at 2 cm from the monolayer, and the voltage deflection elicited was measured with a second set of electrodes placed at 1.0 mm from the membrane. The contribution of the collagen support and the bathing solutions was subtracted, and all values reported corresponded exclusively to the monolayer. This contribution is 24.3 ± 0.36 Ωcm^2 (16 disks) at 37°C and 33.2 ± 0.5 Ωcm^2 (16) at 3°C, i.e., is small and varies very little compared to the variations reported below for the monolayer. A given disk was used only for a single determination to avoid edge damages, then discarded.

Electrical resistance was studied as a function of temperature, from 3 to 37°C. Several batches of monolayers were used, prepared on different days during several months. In each case, the whole range of temperature was tested so that minor differences in the preparation and handling of the monolayers would not distort the shape of the resistance/temperature curve. Most experiments were performed in the cold room, the warm room, and the general laboratory, so that monolayers, media, chambers, and instruments were at the temperature described below. Yet this was later found to be unnecessary, as the new value of resistance achieved by a change in temperature is attained in less than 2 sec, and therefore it immediately reflects only the temperature of the CMEM being injected in the chambers.

Morphological Studies

Freeze-fracture replicas were obtained from monolayers fixed with 2.5% glutaraldehyde for 30 min, and gradually infiltrated with glycerol up to 20% concentration, where they were left for 1 hr. The monolayers were then detached from the substrate and the isolated monolayers frozen in the liquid phase of Freon 22 cooled with liquid nitrogen. Freeze-fracture was carried out using a 300 Balzers apparatus (FL 9496 Balzers, Liechtenstein) equipped with a turbomolecular pump at −120°C, and a vacuum of 2×10^{-6} mm Hg. After evaporation of platinum and carbon, replicas were recovered in sodium hypochlorite, washed in distilled water, and mounted on Formvar-coated 100-mesh grids. Observations were carried out with a Zeiss EM10 electron microscope (Oberkochen, Germany). All micrographs of replicas

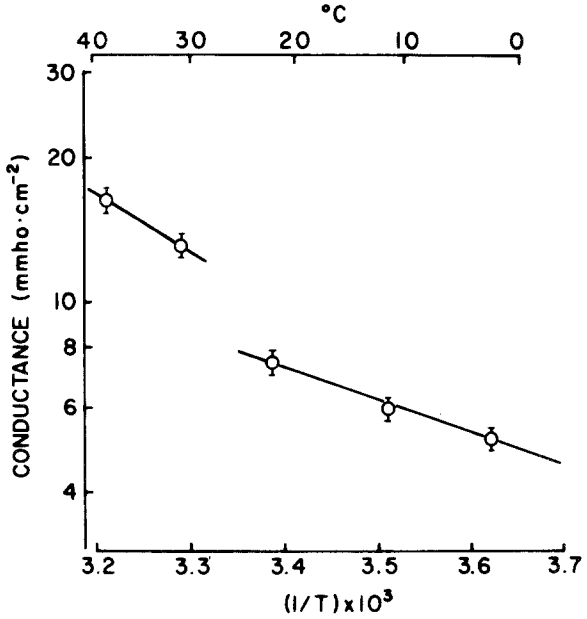

Fig. 1. Effect of temperature on the electrical conductance across monolayers of MDCK cells (passage 60 to 80°) 24 hr after plating at confluence on nylon disks coated with collagen. Conductance was calculated with the voltage deflection elicited by a current of 100 μA cm^{-2}. The contribution of the support, solutions, and electrodes was subtracted. Results are expressed as mean ± SE. Each point is an average of 5 to 28 individual monolayers. The slopes of the two portions of the curve (fitted by eye) correspond to 4.0 and 3.2 kcal mole^{-1}

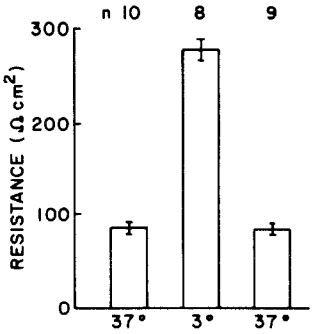

Fig. 2. Reversibility of the effect of temperature on the electrical resistance across the monolayers of MDCK cells. A population of monolayers was incubated at 37°C, and 10 of them were taken for electrical measurements. Disks were discarded after measurements. The rest was switched to CMEM at 3°C, and 8 more measurements were performed on disks that were also discarded. The remaining 9 disks, that have been at 37 and then at 3°C, were incubated now in CMEM at 3°C, and their resistance was measured as described above

are shown with the shadow direction from bottom to top. The procedures followed in order to assess modifications in the pattern of the strands of the occluding junctions, as well as in the density of the IMP, are described in Results.

Results are expressed a means ± SE, followed by the number of observations between parentheses.

Results

The electrical conductance across monolayers of MDCK cells is markedly decreased by cold (Fig. 1). Between 3°C (5.19 ± 0.24 mmho · cm^{-2}) and 37°C (16.03 ± 0.49) conductance changes by 306%. This effect of temperature on membrane permeability was previously observed in other membranes (Dreyer, Müller, Peper & Stertz, 1976; Fischbach & Las, 1978; Jähnig & Bramhall, 1982). Even when we injected the bathing solution at a new temperature as quickly as possible with a couple of large syringes (50 ml) into the small chambers (1 ml) at the two sides of the disk, the operation took a couple of seconds, and we failed to record a transient. If one takes into account that, besides the time taken by the operation, part of the delay is due to the diffusion of heat through unstirred layers, the change in conductance appears as a very fast phenomenon. The change in conductance is also completely reversible (Fig. 2).

The Arrhenius plot of the values of conductance shows (Fig. 1) a discontinuity and a change of slope between 22 and 31°C. This change of slope is usually taken as evidence of phase transition in the structure operating the permeation of ions (Dreyer et al., 1976; Anderson, Cull-Candy & Miledi, 1977; Schwarz, 1979). The two slopes can be used to calculate the energy of activation for the permeation of ions with the equation

$$\ln C = \frac{E^*}{RT} + \ln A \qquad (1)$$

where C is the electrical conductance, E^* is the activation energy, R is the gas constant, T the absolute temperature, and A the Arrhenius constant. Equation (1) gives an activation energy of 4.0 kcal/mole for the branch between 31 and 37°C, and 3.2 for the one between 3 to 22°C. Activation energies in this range correspond to mechanisms of diffusion that do not involve enzyme-like mechanisms (pumps, carriers, etc.) (Kniffki, Siemen & Vogel, 1981; Leech & Stanfield, 1981). This is in keeping with the view that ion translocation through monolayers of MDCK cells proceeds through a paracellular permeation route (Cereijido et al., 1980), and permeation through this route in leaky epithelia obeys diffusion laws (Barry & Diamond, 1971; Barry, Diamond & Wright, 1971; Wright, Barry & Diamond, 1971; Moreno & Diamond, 1975).

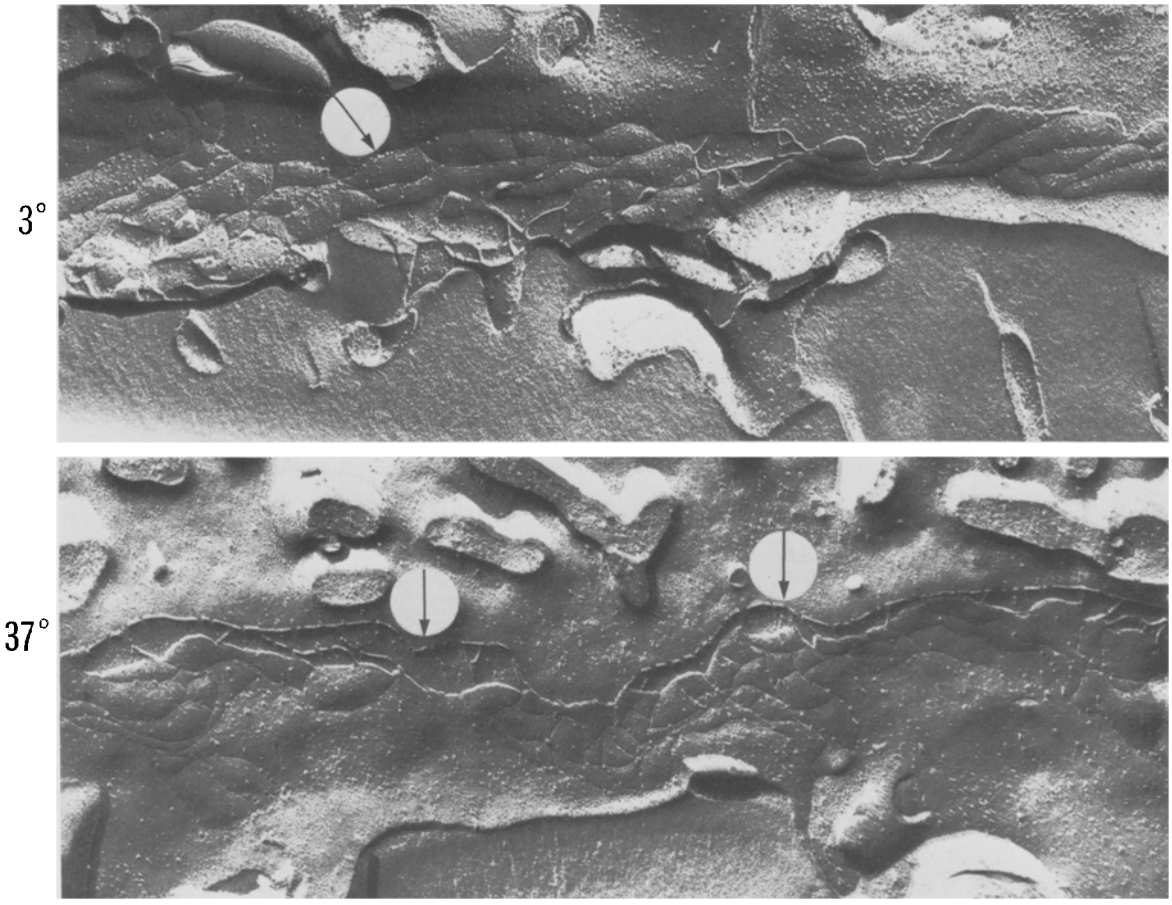

Fig. 3. Freeze-fracture replicas of MDCK cells in confluent monolayers incubated and fixed at 3 and 37°C. Strands appear as grooves on *E* faces and ridges on *P* faces. Note the irregularity of the pattern; the irregulatory in the number of strands, varying from 2 (*left arrow* at 37°C) to 6 (*right arrow* at 37°C); some strands are not continuous but appear as a row of particles (*arrow* at 3°C); ×48,000

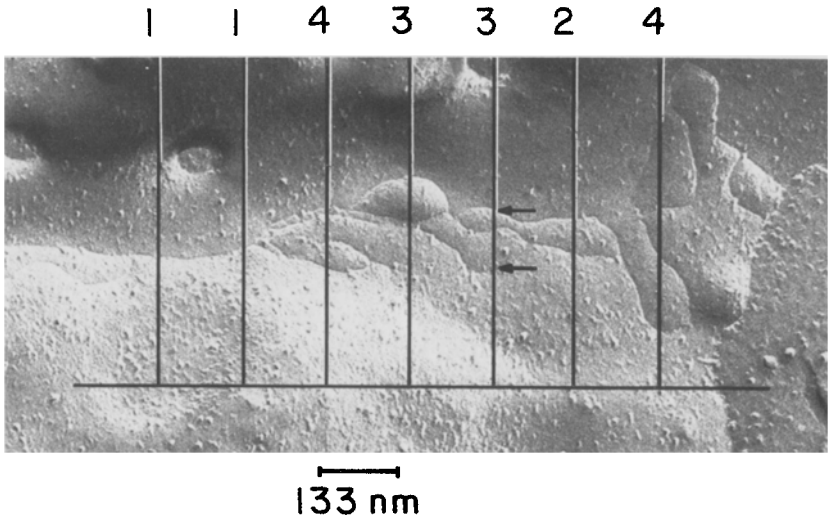

Fig. 4. Method to evaluate the number of strands and the width of the occluding junction. A line is drawn parallel to the main axis of the junction, and perpendicular lines to this line are drawn every 133 nm. The number of strands that intersect each of these lines is counted as on top of this figure. The distance between the upper and innermost strands (arrows) is taken as junctional width. A single strand is scored as zero width. ×83,000

In the next series of experiments we investigated whether the 306% change in the degree of sealing of the occluding junction elicited by temperature is reflected in alterations of its image in freeze-fracture replicas. Monolayers were fixed at the lowest (3°C) and highest (37°C) temperatures that were electrically explored. The image of the junction always exhibits a major axis that, as shown before (Cereijido et al., 1978*b*; Martínez-Palomo, Meza, Beaty & Cereijido, 1980), corresponds to the separation of the apical and basolateral regions of the membrane (Fig. 3). Several authors have developed methods to perform a morphometric analysis of the occluding junctions (*see*, for instance, Lagarde, Elias, Wade & Boyer, 1981). In the present study we drew straight lines perpendicular to the axis of the occluding junctions every 133 nm and counted the number of strands intercepting such lines (Fig. 4). We also measured the width of the junctions as the distance between the lowest and the highest strands. The Table shows that these parameters are not significantly affected by temperature. Yet, since occluding junctions in monolayers of MDCK cells have a very irregular pattern, in Figs. 5 and 6 we compare the distributions of number of strands and junctional width at the two temperatures. The distributions do not show significant alterations that may account for the 306% change in resistance.

The constancy in the pattern of the occluding junction observed above, when the degree of sealing is modified by temperature, does not suggest that a net number of particles would be fused and added to the strands. Yet, the density or the polarized distribution of IMP may be altered by temperature for other reasons. We compared freeze-fracture replicas of the *E* and *P* faces from the apical and basolateral regions at the two temperatures. A description of these regions of MDCK cells was made in a previous article (Cereijido et al., 1980). In the present work we concentrated on the density of IMP on the different faces. Figure 7 illustrates the distribution of IMP in these faces. In order to make a valid comparison between IMP at 3 and 37°C we counted them in photographs at 110,000 × magnification. The errors introduced by microvilli, or regions where the plane of fracture abandons the lipid leaflets, were minimized by drawing small circles of 4.5 cm^2, representing a membrane area of 3.7×10^{-8} μm^2 (Fig. 8) and counting the IMP inside them. Figure 9 summarizes the results obtained at 3°C (white bars) and at 37°C (shaded bars). At the temperature close to that at which the cells are usually cultured (37°C), there are more IMP on *P* than on *E* faces, both in the apical and basolateral regions. At this temperature *E* face is not polarized into apical and basolateral domains (800 ± 51 *vs.* 811 ± 109 IMP/μm^2). In the *P* face instead, the density of IMP is much higher in the basolateral than in the apical region (1878 ± 81 *vs.* 1156 ± 74 IMP/μm^2).

Cold produces a significant increase in density of IMP in the *E* face of the apical membrane ($P < 0.001$) and on the *P* face of the basolateral membrane ($P < 0.001$). On the basis of these results it is not possible to assert whether this change in density is due to insertion of IMP from a pool or by formation of new IMP as result of a phase transition in the arrangement of molecules already present in the membrane (e.g., by micellization of the bilayer).

Taken together, the present results indicate that resistance can be increased by 306% without significant changes in the pattern of the occluding junction, that IMP do not decrease their density as a result of incorporation into the junctional strands, and that if junctional strands consist of cylindrical

Table. Occluding junctions of MDCK cells at 3 and 37°C

Junctional parameters	3°C	37°C
Number of strands	4.8 ± 0.2	5.2 ± 0.2
Junctional width (μm)[a]	0.266 ± 0.02	0.247 ± 0.01
Junctional density[b]	18.05	21.05
n	129	153

[a] Distance between uppermost and lowermost strand.
[b] Number of strands divided by junctional width.

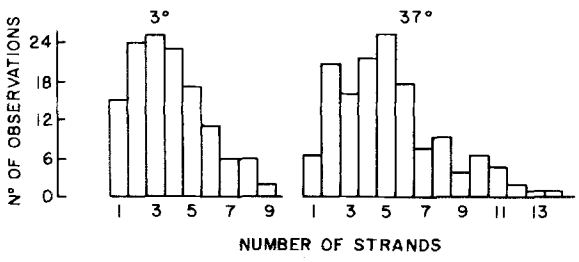

Fig. 5. Frequency distribution of the number of strands of occluding junctions, scored as described in Fig. 4, at 3 and 37°C

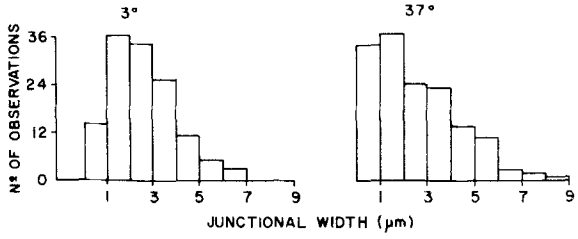

Fig. 6. Frequency distribution of the width of the band occupied by the occluding junction, measured at two different temperatures as described in Fig. 4

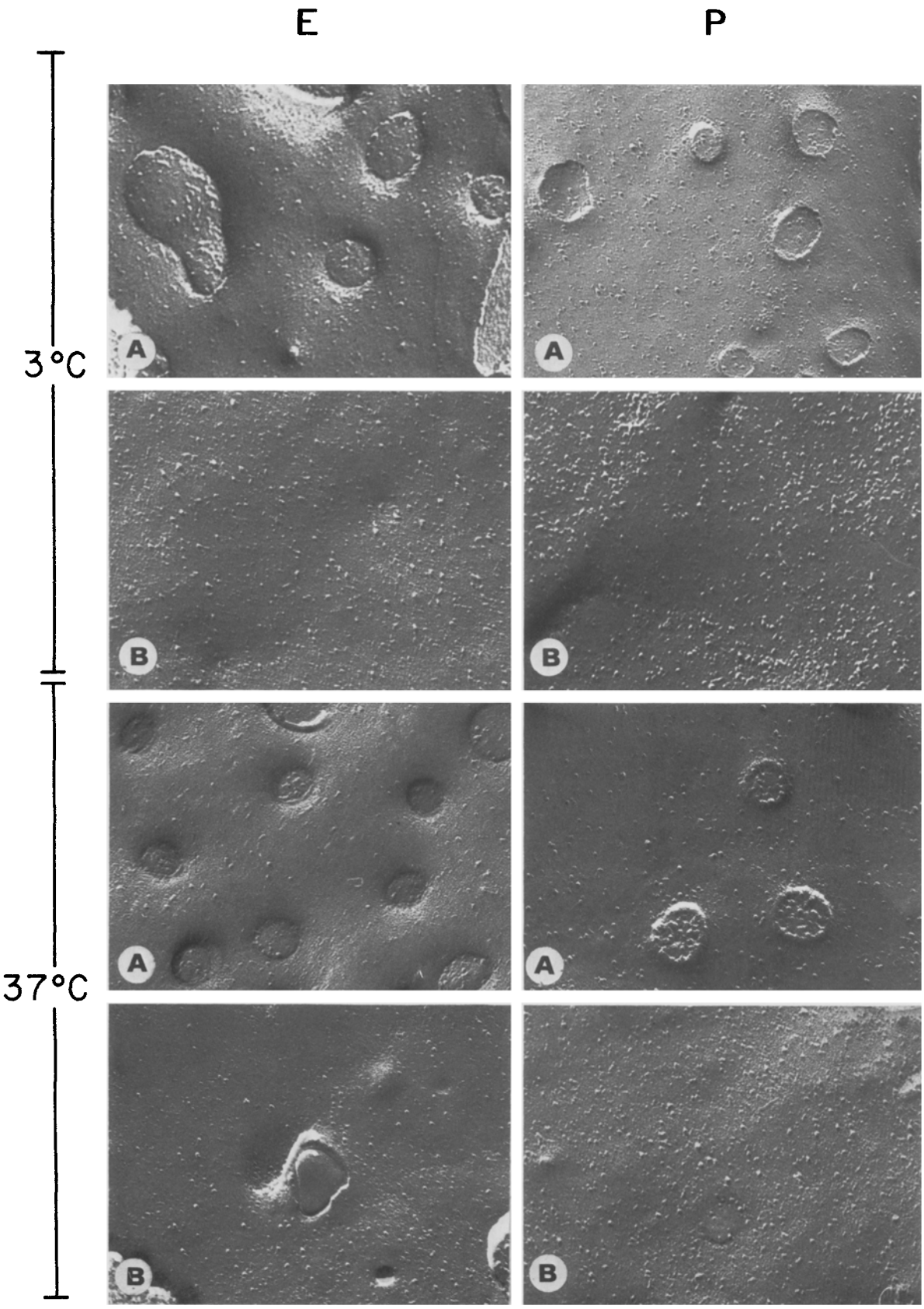

Fig. 7. Freeze-fracture replicas of *E* and *P* faces of the apical (*A*) and basolateral (*B*) regions of MDCK cells incubated and fixed at 3 and 37°C. Note the differences in density of IMP between the different regions and faces. ×48,000

micelles they are more stable to temperature changes than any other cylindrical micelles formed in other systems known to us and withstand a temperature span that provokes a clear phase transition between 22 and 31°C.

Discussion

The monolayer of MDCK cells behaves as a leaky epithelium where most of the current flows through the paracellular route (Misfeldt et al., 1976; Cereijido et al., 1978*a,b*; Rabito, Tchao, Valentich & Leighton, 1978; Stefani & Cereijido, 1983). Since this route is controlled by the occluding junction, the changes in electrical resistance observed in the present study reflect changes in the sealing capacity of this structure. There are many circumstances where the sealing capacity, the number of strands, or their arrangement were found to bear a correlation: (1) during foetal development (Humbert et al., 1976; Tice et al., 1977); (2) in different locations of a given tissue (Schiller & Taugner, 1982); (3) in a given tissue subject to different physiological requirements (Pitelka, Hamamoto, Duafala & Nemanic, 1973; Morgan & Wooding, 1982; Murphy et al., 1982*a,b*); (4) in a tissue under pressure or undergoing mechanical distortions (Hudspeth, 1975; Mutoh, 1981; Pitelka & Taggart, 1983); (5) in the presence of hyperosmolar solutions (Wade & Karnovsky, 1974); (6) under the effect of temperature (Kachar & Pinto da Silva, 1981; Tadvalkar & Pinto da Silva, 1983); (7) under the effect of drugs (Bentzel et al., 1980; Ojakian, 1981; Duffey, Hainau, Ho & Bentzel, 1981; Pitelka, Taggart & Hamamoto, 1983).

However, there are many circumstances where no correlation is found. Thus Martínez-Palomo and Erlij (1975) have observed that tissues like the frog urinary bladder and the rabbit intestinal mucosa have electrical resistances across of 125 and 12,000

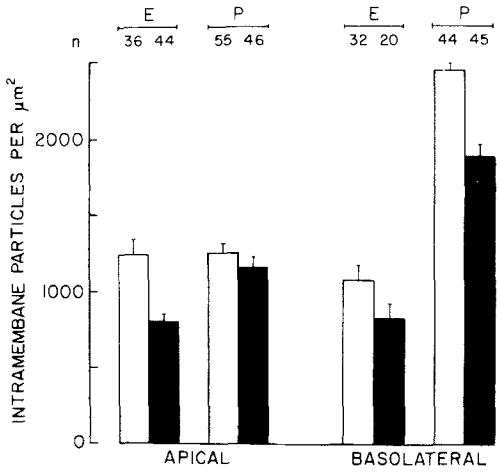

Fig. 9. Density of IMP in the membrane of MDCK cells incubated and fixed at 3°C (white bars) and 37°C (shaded bars). *Note.* the *P* face of the basolateral domain has a density of IMP higher than the *P* face of the apical at both temperature; *E* face does not seem to be polarized; cold trends to increase the density of the IMP

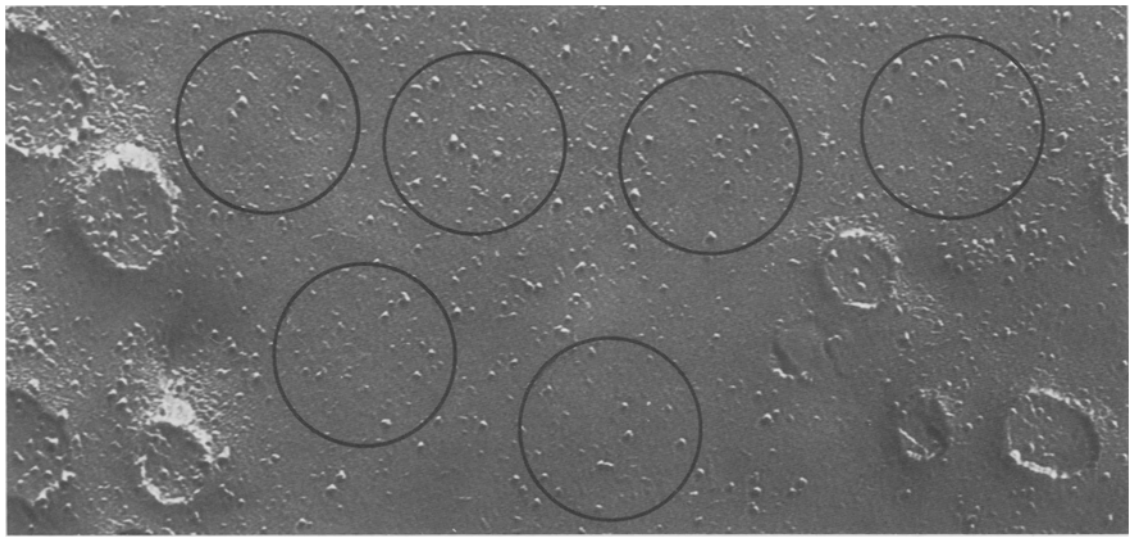

Fig. 8. Method to count the density of IMP in the different freeze-fracture replicas of MDCK cells. Circles of 4.52 cm^2, representing 0.037 μm^2 of membrane, are drawn on photographs of the replicas at a magnification of ×110,000. The circles are meant to avoid structures like microvilli, broken regions of the replicas, or sections where the plane of fracture abandons the plasma membrane, introducting considerable errors in the evaluation of the area

ohm/cm^2, respectively, yet the number of strands in their occluding junctions is very similar. Møllgård, Malinowska and Saunders (1976) found no correlation between tight junction morphology and permeability in the sheep choroid plexus during development. This opens the possibility that permeant structures in (e.g., pores) or between the strands, and not the strands themselves, would play a more significant role in the sealing capacity of the junction (*see* Claude, 1978). This view would agree with the present results, indicating that the occluding junction of the monolayer of MDCK cell may change its electrical resistance by 306% without a noticeable modification of its structure, as studied in freeze-fracture replicas.

Considering the wide range of temperature explored and the fact that the Arrhenius plot suffers in between a break in conductance of the kind usually identified with phase transitions, the structure of the occluding junction appears remarkably stable. Today little is known about the chemical nature of this structure. Cereijido et al. (1978*b*) have shown that occluding junctions do not form in newly plated MDCK cells if the protein synthesis is impaired with cycloheximide or puromycin (*see also* Cereijido, 1981). This has been confirmed by Hoi Sang, Saier and Ellisman (1980) and Griepp, Dolan, Robbins and Sabatini (1983). This shows that proteins play an important role in the formation of the junctions, yet it does not necessarily indicate that the strands are composed by proteins. Margolis, Neyfakh, Bergelson and Vasiliev (1982) have shown that solid liposomes attach to contact free lateral edges of epithelial cells, but that this attachment is prevented by trypsin, suggesting that this edge contains proteins. However, this does not prove that the proteins where liposomes attach are contained in the strands that one finds in freeze-fracture replicas.

Kachar and Reese (1982) have suggested that the strands are constituted by long lipidic micelles of the type that appear in X-ray spectroscopy as Hexagonal Phase II, i.e., inverted micelles with the polar head group toward a central, aqueous core, and with the hydrophobic chains outwards. These liquid crystalline structures are highly sensitive to temperature (*see* Introduction). In keeping with their view that strands are constituted by lipidic micelles with a long, cylindrical shape, Kachar and Pinto da Silva (1981) have observed a massive proliferation of strands in the occluding junction of the prostate gland when temperature is shifted from an unspecified temperature to 37°C (*see also* Tadvalkar and Pinto da Silva, 1983). However, in the present study the occluding junctions of the monolayer of MDCK cells do not exhibit such changes in spite of the fact that their sealing ability does change significantly. This raises the question of whether the strands of the occluding junctions have a universal structure with identical composition and permeability in the different preparations. It is even possible that the morphology of the strands were only indirectly related to permeability. Thus strands may contain channels whose states (open or closed) would depend on gates sensitive to temperature. A change in the ionic mobilities in the vicinity of the strands may not be discarded either as a plausible explanation to changes in conductance with temperature.

Some strands do not appear as continuous fibers, but as a row of discrete particles (Bullivant, 1982). Also, in regenerating tissues, clusters of particles that resemble gap junctions appear in regions of pre-existing occluding junctions (Yancey, Caster & Revel, 1979). Polak-Charcon, Shoham and Ben-Shaul (1978) and Suzuki and Nagano (1979) have shown that in some cases the formation of tight junctions is preceded by the appearance of a rather broad, elevated zone in the plasma membrane, where large areas of packed IMP are associated with segments of strands. A similar observation was made in monolayer of MDCK cells by Hoi Sang et al. (1979). Therefore, there seems to be a close association between junctions and IMP. There are also indications that both junctions (Cereijido et al., 1978*b*; 1981; Griepp et al., 1983) and IMP (Hong & Hubbell, 1972; Fischer & Stoeckenius, 1977) contain proteins. In spite of these similarities, we do not observe any interrelationship between junctions and ordinary IMP.

In this article we demonstrate that IMP are polarized in a double sense: face *P* has a density different from *E*, and the apical leaflets have densities different from the basolateral ones, and this agrees with previous observations in natural epithelia (De Camilli, Peluchetti & Meldolesi, 1974; Hoi Sang et al., 1979, 1980). We also find that the distribution of IMP is sensitive to temperature, but have no indication that the small changes observed would be associated with events in the occluding junction.

In summary, the sealing capacity of the occluding junctions of MDCK cells does not bear a direct relationship to the number and arrangement of its strands. If these strands were in fact constituted by long, cylindrical micelles, these micelles must be remarkably stable, as they keep their morphology unmodified from 3 to 37°C. The strands do not seem to be closely related to common IMP, in the sense that IMP may vary while the strands remain constant.

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