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OCCLUDING JUNCTIONS AND PARACELLULAR PATHWAYS STUDIED IN MONOLAYERS OF MDCK CELLS

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SUMMARY

MDCK cells (epithelioid, derived from the kidney of a normal dog) cultured in monolayers on a permeable support, exhibit properties of natural transporting epithelia. Comparisons of the electrical resistance across the plasma membrane of MDCK cells (as studied with microelectrodes) and the resistance across the whole monolayer, (mounted as a flat sheet between two chambers) indicate that most of the current flows through an extracellular pathway. Scanning of the electrical field over the apical surface shows that this pathway is located at the intercellular space. Yet conductance is not evenly distributed along the intercellular space as in leaky epithelia, but is restricted to sites scattered irregularly along the intercellular space. Studies of freeze fracture electron microscopy indicate that the number of strands of the junctions is also distributed irregularly, varying from 1 to 10 in a few nanometers. This suggests that regions with few strands would correspond to spots with high conductance and *vice versa*. However, in this preparation the sealing property of the junction bears little relationship to its structure. Thus by changing the temperature from 37 to 3 °C and back, the electrical resistance increases reversibly by 306 %, while the number and arrangement of the strands show no significant modification. The resistance of the monolayer varies also with the age of the cells, suggesting that sealing and ion-permeating components of the junction may be dynamic entities that are not permanently installed, but can be accommodated to the requirements of the tissue.

INTRODUCTION

The observation that the high conductance of epithelia like those of the proximal tubule (Kühn & Reale, 1975; Pricam, Humbert, Perrelet & Orci, 1974), the small intestine (Clarkson, 1967; Staehelin, Mukherjee & Williams, 1969), the gall bladder (Diamond, Barry & Wright, 1971; Moreno & Diamond, 1975; Reuss & Finn, 1977) and the choroid plexus (Wright & Diamond, 1968) is due to the existence of a predominant paracellular route, focused attention on the occluding junction that, from mere 'terminal bars' or blocking elements, came to be regarded as the main structural feature in the determination of the permeation rate. Furthermore, the observation that its properties change in response to physiological requirements

Key words: MDCK, paracellular, occluding junctions.

(Murphy, Swift, Mukherjee & Rogers, 1982*a*; Murphy *et al.* 1982*b*; Pitelka, Hammamoto, Duafala & Nemanic, 1973), foetal maturation (Tice, Carter & Cahill, 1977), pathological conditions (Pickett, Pitelka, Hammamoto & Misfeldt, 1975) and experimental challenge (Bentzel *et al.* 1980; Duffey, Hainau, Ho & Bentzel, 1981; Elias & Friend, 1976; Erlij & Martínez-Palomo, 1972; Humbert *et al.* 1976; Kachar & Pinto da Silva, 1981; Robenek, Themann & Ott, 1979; Urakabe, Handler & Orloff, 1970; Wade, Revel & Di Scala, 1973) led to the suspicion that the resistance offered by occluding junctions can be modulated so as to *regulate* the flux of substances through the paracellular route. The demonstration that cultured epithelial cells form occluding junctions *in vitro*, afforded new sources of information on occluding junctions (Cereijido *et al.* 1978*a*; Cereijido, Rotunno, Robbins & Sabatini, 1978*b*; Misfeldt, Hammamoto & Pitelka, 1976). The present article summarizes our observations with monolayers of MDCK cells, an epithelioid line derived from the kidney of a normal dog in 1958 by Madin & Darby.

GENERAL PROPERTIES OF MONOLAYERS OF MDCK CELLS

Monolayers are formed by plating cells at high densities (approximately 10^6 cells cm^{-2}) on a collagen-coated nylon cloth disc to saturate the area available for attachment. After 90 min the disc with the monolayer attached is transferred to fresh medium without cells. An electrical resistance develops within 4–6 h after plating and achieves some 100–400 Ωcm^2 after 12–16 h. Mature monolayers are morphological and functionally polarized. They contain junctional complexes composed of desmosomes and occluding junctions with properties similar to those of 'leaky' epithelia. These monolayers show: (1) a linear conductance/concentration relationship; (2) an asymmetric instantaneous current/voltage relationship; (3) a 9:1 Na^+/Cl^- discrimination; (4) a decrease in this discrimination when pH is lowered from 7·4 to 3·8 (suggesting that cation-specific channels, which exclude Cl^-, contain acidic groups dissociated at neutral pH) and (5) a characteristic pattern of ionic selectivity (Eisenman's series VI), which suggest that the negatively-charged sites are highly hydrated and of medium field strength (Cereijido, 1978*a*,*b*).

Other laboratories, with the exception of Dr Simmon's group (Simmons, 1981), have reported similar findings (Rabito, Tchao, Valentich & Leighton, 1978). Simmons and co-workers have instead a batch of MDCK cells that, under the incubating and plating conditions they use (e.g. a naked Millipore instead of collagen) obtain monolayers with 4000 Ωcm^2 or more. It will be of great interest to discover the factor that determines whether an epithelium presents the characteristic of a 'tight' or a 'leaky' membrane. In this respect, Valentich, Tchao & Leighton (1979) have found that usual batches of MDCK cells contain more than one subclass, and that different culture conditions might favour the predominance of one or the other, a fact that may later be reflected in the electrical properties of the monolayer.

ELECTRICAL PROPERTIES OF MDCK CELLS

Permeation through epithelia may follow two main routes: transcellular and paracellular. To investigate whether the relatively high conductance of monolayers of

DCK cells is due to ionic transfer through their plasma membrane, we have developed a procedure using microelectrodes (Stefani & Cereijido, 1983). The values of the electrical potential were around −50 mV, and the electrical resistance was $61{\cdot}6 \pm 6{\cdot}3\ M\Omega\ cell^{-1}$. The electrical capacity was $45{\cdot}1 \pm 2{\cdot}9$ pF, somewhat higher

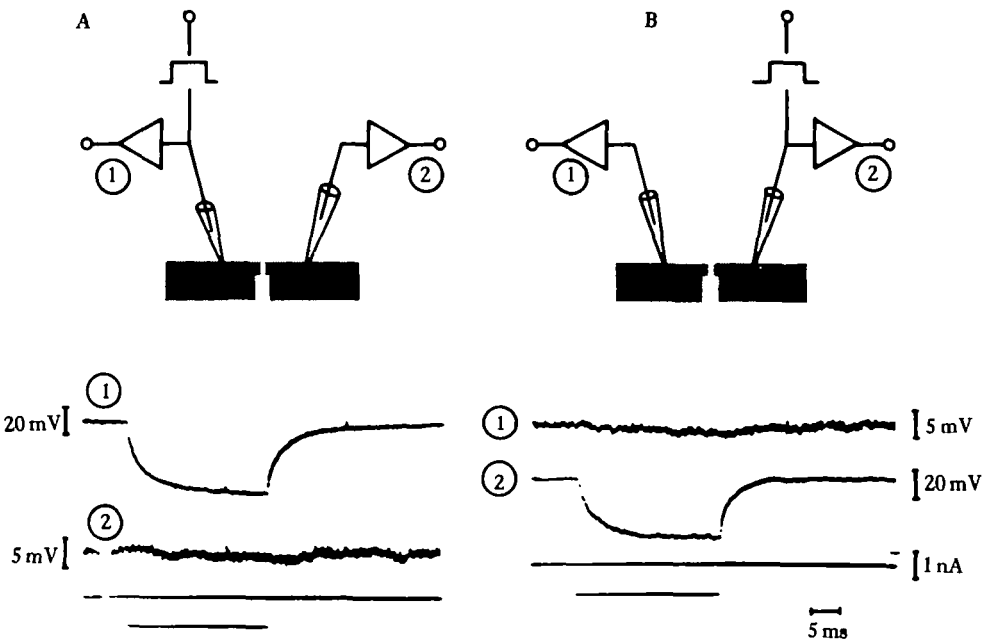

Fig. 1. Impalement of two adjacent MDCK cells. The upper schemes illustrate the experimental arrangement. Records 1 and 2 were obtained with microelectrodes 1 and 2, respectively. The resistance of the microelectrodes was around 90 MΩ. Notice the large gain in records A2 and B1. Cells were of passage 73 and were plated 4 days before. (From Stefani & Cereijido, 1983.)

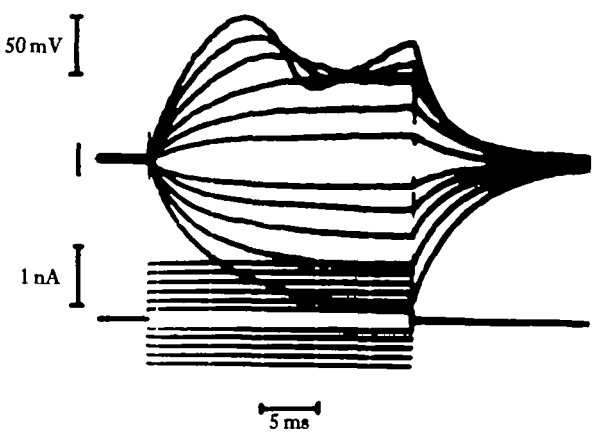

Fig. 2. Time-dependent resistance obtained during large positive pulses in the presence of 10^{-4} M-amiloride. Notice that even before reaching the time-dependent responses, the voltage deflections produced by the pulses of current were asymetric. (From Stefani & Cereijido, 1983.)

than expected for a cell the size of an MDCK (diameter 14 μm, height 5 μm), bu[redacted] keeping with the high degree of infolding of its plasma membrane. The high capacity cannot be attributed to intercellular coupling, as no evidence of this type of connection was found in 20 pairs of neighbouring cells (Fig. 1). The current/voltage relationship shows a marked asymmetry, and in some cells the voltage becomes time-dependent for large, depolarizing current pulses (Fig. 2).

For current to flow through a transcellular pathway it must twice traverse the plasma membrane of an MDCK cell. Theoretical considerations indicate that the minimal resistance of the route through a single cell will be 250 MΩ. A typical monolayer contains some 2–5 $\times$ 10^5 of such cells per square centimetre. These resistive elements in parallel would confer a minimal resistance of 500–1300 Ωcm^2, i.e., higher than the resistance actually offered by the monolayer (some 100–400 Ωcm^2). This indicates that part of the current does not flow through the cytoplasm of the cells. So in the next section we analyse whether it is a paracellular route, as in natural leaky epithelia (Frömter & Diamond, 1972), or merely a consequence of incomplete growth or faulty sealing of the monolayer.

THE SEARCH FOR A PARACELLULAR ROUTE

Square pulses of current of 20–50 $\mu A\,cm^{-2}$ and duration of 5 ms were passed first in the positive and then in the negative direction, while the voltage changes on the apical surface of the monolayer were scanned with a microelectrode. The amplitude of the signal measured by the microelectrode is proportional to the electrical conductance and permits the detection of the points where current flows through the monolayer (Cereijido, Stefani & Martínez-Palomo, 1980). This study indicated that: (1) in monolayers of MDCK cells current flows through a paracellular route, but (2) conductance may have large variations along the perimeter of a given cell, and the intercellular space is best represented by an actually 'tight' junction studded with conducting points.

The observed heterogeneity of the electrical field has a structural correlate. Thus junctional strands appear in freeze-fracture replicas as varying abruptly in number from 1 to 10 within a few nanometers. We wondered whether natural leaky epithelia would also exhibit heterogeneously distributed conductance, like the monolayers of MDCK cells, or would be evenly distributed. For this purpose we applied our procedure to the *Necturus* gallbladder (Cereijido, Stefani & Chávez de Ramírez, 1982). We found that in this preparation both conductance and the number of strands were homogeneously distributed along the interspace, except at the intersections between three cells (where conductance decreases, and freeze-fracture replicas showed an increase in the number of strands and a widening of the band occupied by the occluding junction). Therefore, the monolayer of MDCK cells exhibits characteristics of both 'leaky' and 'tight' epithelia. In this respect it might be worthwhile remembering the observations of Simmons (1981) and of Valentich *et al.* (1979) mentioned above, which indicated that culturing conditions can determine the resistance of the monolayer, probably through a shift in the proportion between sealing and conductive elements.

THE RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION OF THE OCCLUDING JUNCTION

Studies performed in natural epithelia have shown that under certain conditions junctional strands vary their arrangement and become loose, suggesting a correlation between arrangement and permeability (see, Bentzel *et al.* 1980; Meldolesi *et al.* 1978; Claude, 1978; Claude & Goodenough, 1973; Wade & Karnovsky, 1974). Also, the results described in the previous section indicate that an epithelium with irregularly distributed strands would have an irregularly distributed conductance and *vice versa*. Yet there is a great deal of controversy as to whether the number of strands has anything to do with the resistance of the junction (see Cereijido *et al.* 1980, 1982). Part of the problem comes from the fact that comparisons are made between different organs or even different animal species. We could avoid this problem by studying the structure of the occluding junction (as observed in freeze-fracture replicas) and the electrical resistance of the monolayer.

Fig. 3 shows that by changing the temperature from 37 to 3 °C, the electrical resistance of the monolayer changes reversibly from 63 to 193 Ωcm^2 (L. González-Mariscal & M. Cereijido, unpublished observations). Yet when the monolayer is conventionally fixed with glutaraldehyde at either temperature, the pattern of the occluding junction, the statistical distribution of the number of strands (Fig. 4), and the width of the band occupied by the junction do not change appreciably.

Since the observations depicted in Figs 3 and 4 are part of a more extensive study whose results are not included in this review, their full implications will not be

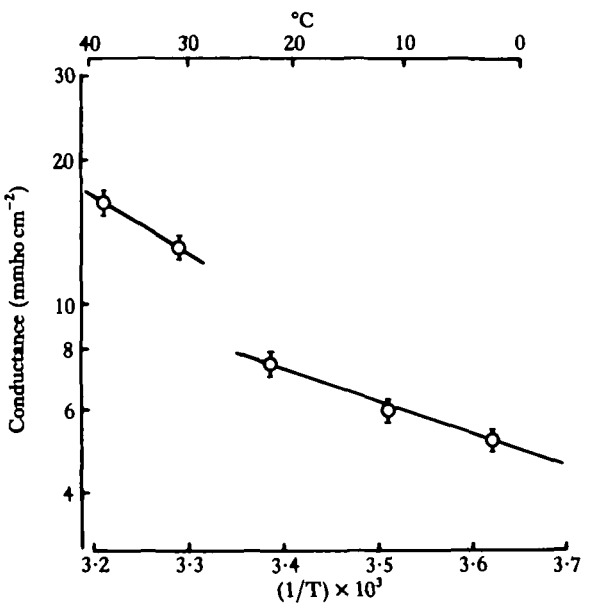

Fig. 3. Arrhenius plot of electrical conductance across monolayers of MDCK cells. Conductance was calculated with the voltage deflection elicited by a current pulse of a 100 $\mu A\ cm^{-2}$. (From L. González-Mariscal, B. Chávez de Ramírez & M. Cereijido, unpublished observations.)

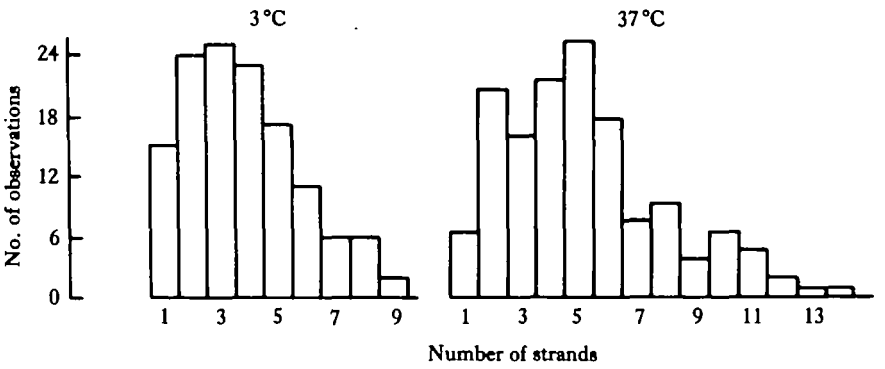

Fig. 4. Histograms of the number of strands, as counted in freeze-fracture replicas, in monolayers of MDCK cells incubated and fixed at 3 or 37 °C. (From L. González-Mariscal, B. Chávez Ramírez & M. Cereijido, unpublished observations.)

pursued here. Yet they suffice to suggest that since a change in the resistance of 306 % is not accompanied by detectable modifications in the arrangement of the strands, the irregularly distributed number of strands and heterogeneously distributed resistance described in previous sections might be coincidental. They also suggest that the resistive element of a junction might not necessarily be the strand itself. Substances contained in the strands, or between them, might play a more significant role.

THE FORMATION OF THE OCCLUDING JUNCTIONS

The monolayers of MDCK cells are obtained by trypsinization of monolayers growing in plastic bottles. This treatment destroys both the occluding junctions and the apical/basolateral polarity. On plating the cells the monolayer is formed in less than 30 min, yet it has no electrical resistance for another 4–6 h. At this time the electrical resistance starts to increase and reaches a maximum in some 12–16 h. If the synthesis of proteins is inhibited by cycloheximide or puromycin the cells cannot make the junctions (Fig. 5).

If the inhibitors are applied, instead, in a pulse (between 1·5 and 3·5 h after plating) they only delay the formation of the junction (Cereijido *et al.* 1978*a*). Inhibitors of the synthesis of RNA, like actinomycin, have no appreciable effect. This would indicate that the synthesis of proteins is required to make the occluding junction, and that the information (i.e. mRNA) is already available in the cytoplasm of the plated cells. Yet it does not necessarily prove that the junctions themselves are constituted by proteins.

CALCIUM AND THE OCCLUDING JUNCTION

If one removes Ca^{2+} from the bathing solution, and adds EGTA, the electrical resistance at first drops sharply and then at a slower rate (reaching zero in about 1·5 h) due to the opening of the occluding junctions. Restitution of Ca^{2+} re-seals the junction with a time course that depends on the time that they had remained in the op

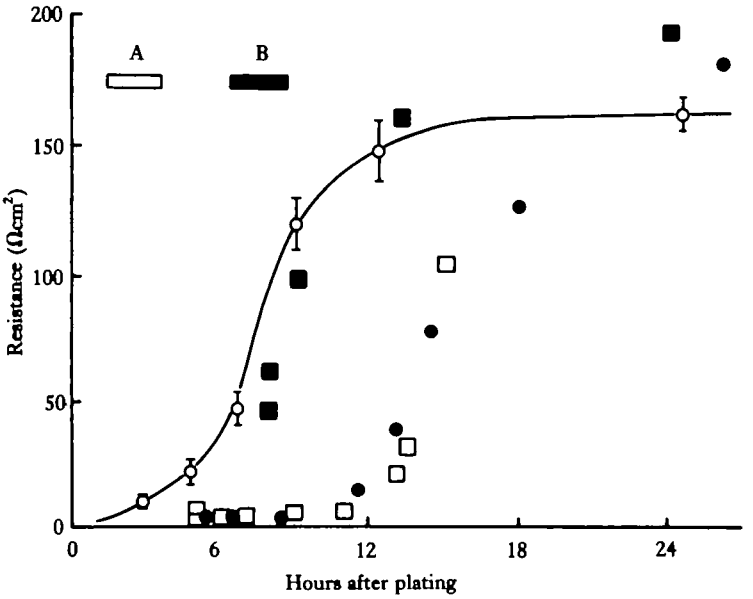

Fig. 5. Development of electrical resistance across monolayers of MDCK cells plated at confluence at time zero. Although the monolayer is completed in less than 0·5 h, electrical resistance develops slowly as junctions are synthesized and sealed (—○—). Cycloheximide (6 μg ml^{-1}), present during the period marked A, delays development of junctions (—□—). Puromycin (10^{-4} M) has the same effect (—●—). If cycloheximide is present instead during period marked B, it does not prevent the rise of electrical resistance (—■—). (From Cereijido, Meza & Martínez-Palomo, 1981.)

condition (Cereijido *et al.* 1978*b*; Martínez-Palomo, Meza, Beaty & Cereijido, 1980). Other divalent cations tested (Mg^{2+}, Ba^{2+}) do not re-seal the junction. Yet extracellular calcium does not seem to be a physiological regulator of the permeability of the junction, for complete removal, or unphysiologically high concentrations, are needed to alter transepithelial resistance. It is even conceivable that the changes in the concentration of Ca^{2+} in the bathing solution would modify its cellular concentration, and/or perturb organelles, and not necessarily act through a direct variation of cell-to-cell salt linkages.

THE CYTOSKELETON AND THE OCCLUDING JUNCTION

Cytochalasin B (a disruptor of microfilament organization) opens the occluding junctions of resting monolayers, and prevents their resealing if they had been opened by the removal of Ca^{2+} as described in the previous section. On the other hand, colchicine (an inhibitor of microtubule assembly), has no effect on these parameters (Meza *et al.* 1980). We have prepared specific rabbit antibodies against actin (the major component of microfilaments) and tubulin (the major component of the microtubules) and treated monolayers of MDCK cells with them. A second antibody (goat-anti-rabbit) which contained a fluorescent probe, was then used to localize microfilaments and microtubules. This indicated that microfilaments are in close association with the occluding junction, a position that would be in keeping with the effect of Cytochalasin B on electrical parameters. Microtubules instead, are associated

mainly with the perinuclear region and not with the junction. Moreover, these structures are affected by changes in the concentration of Ca^{2+}, of EGTA, and by the state (opened or closed) of the junction. Studies by Hoi Sang, Saier & Ellisman (1980), indicate that Cytochalasin B also interferes with junction formation in newly plated monolayers of MDCK cells. We have also tested Cytochalasin B under this circumstance. But in our hands, the formation of junctions takes many hours, and prolonged exposure to the drug induces drastic changes which do not permit us to confirm that the effect has been elicited specifically on the positioning of junctional elements. Changes in the structure and function of the occluding junction related to the cytoskeleton have also been observed in natural epithelia (see Bentzel *et al.* 1980).

The occluding junctions are closely associated with the cytoskeleton and the strands might consist of (or contain) proteins. Thus when the plasma membrane is dissolved with detergents some membrane proteins remain attached to the cytoskeleton. We have investigated the possibility that some of these proteins might be identified and related to the junctions (Meza, Sabanero, Stefani & Cereijido, 1982). To do this we radio-iodinated the surface membrane proteins using a lactoperoxidase technique in MDCK cells fixed under several of the conditions described in this review (lightly trypsinated, sealed, opened by removing Ca^{2+} and adding EGTA, with and without Cytochalasin B, etc.). We then solubilized the remainder of the cells (mainly the cytoskeleton with the membrane proteins that would be still attached) with 1 % SDS, and ran an electrophoretic analysis in polyacrylamide gels. No significant differences in pattern of labelled proteins were found under the conditions tested. However, one cannot eliminate the possibility that the junctions contained proteins but in too small a quantity to be detectable by the method of radio-iodination. Alternatively, the changes in permeability observed under different experimental conditions might be due to changes in the rearrangement or state of resistive elements, and not necessarily to their presence or absence.

THE OCCLUDING JUNCTION AS A FUNCTION OF TIME

Natural epithelia contain cells (and, therefore, intercellular junctions) of different types and in different states of the cellular cycle. Observations made on their occluding junctions are therefore likely to be made on an average of the properties of all of them. When the cells are trypsinized and plated they have to make junctions *de novo*. This introduces a degree of synchronization in the age of the junctions of the monolayer. Fig. 6 shows the evolution of the electrical resistance of the monolayers as a function of their age. Following an initial rapid increase, the electrical resistance decreases. This phenomenon may, of course, be associated with the number of cells in the monolayer: when the number of cells increases, there are more conductive elements in parallel. Moreover, the length of the intercellular space per unit area of monolayer also increases. *A priori* it would seem possible to study the electrical resistance as a function of the cell density, and extrapolate to the value of the conductance of the occluding junction, or to the value of the cell membrane. The actual experimental results (Fig. 7), show that in 3- to 5-day-old monolayers the electrical resistance falls with the cell density as expected. But in younger monolayers exactly the opposite trend occurs. This may be due to the fact that the sealing of the intercellular spaces, and the

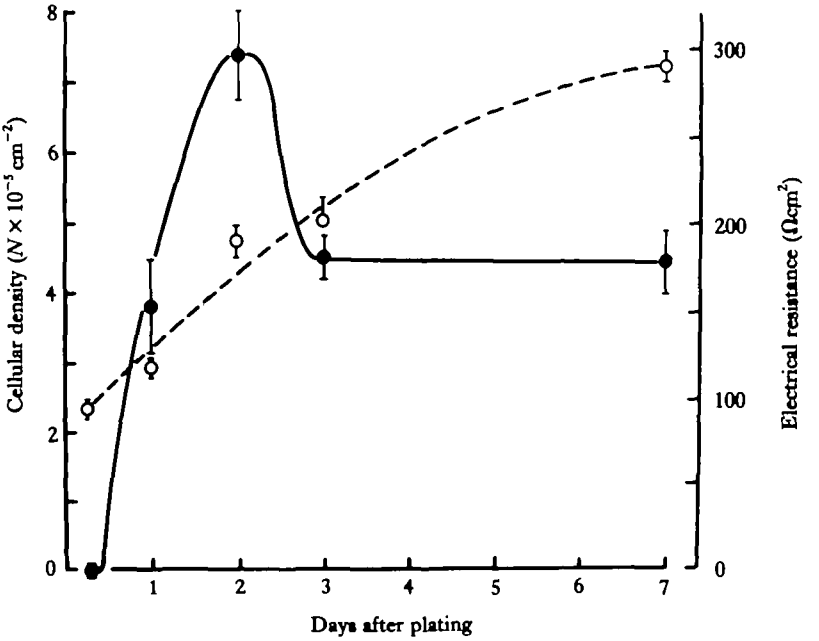

Fig. 6. Cellular density (—○—) and electrical resistance across monolayer (—●—) of MDCK cells as a function of time after plating. Cellular density was counted in the same disc used for the electrical measurements. (From L. Borboa, A. González-Robles & M. Cereijido, unpublished observations.)

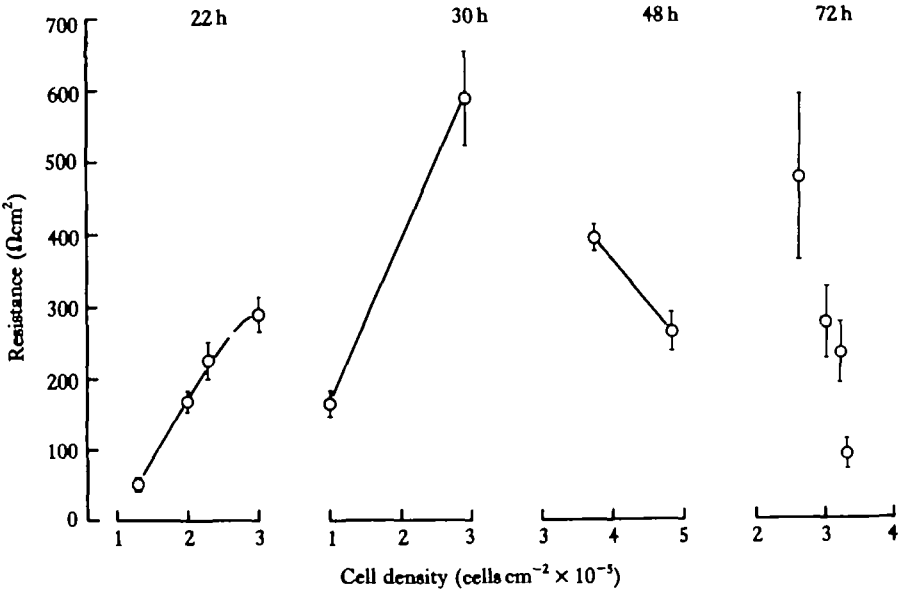

Fig. 7. Electrical resistance as a function of cellular density in monolayers which had been plated for 22, 30, 48 and 72 h. The cellular density was counted in the same disc used for the electrical measurements. (From L. Borboa, A. González-Robles & M. Cereijido, unpublished observations.)

instalation of conductive elements follow different time courses. Thus, we have observed that 24 h after plating (when the monolayer exhibits its maximal resistance) its ability to discriminate between Na^+ and Cl^- is still not developed (Cereijido *et al.* 1978*a*).

In summary, the epithelioid line of cells MDCK retains the ability to make occluding junctions *in vitro* under several experimental conditions. This permits us to study these structures from establishment to steady state. In order to make these junctions the cells resort to protein synthesis and link their strands with those of neighbouring cells, in a process that requires Ca^{2+} and the participation of the cytoskeleton. On the basis of the available information it is not possible to determine whether the number and arrangement of strands observed in freeze-fracture replicas are directly related to permeation or whether they are, instead, appurtenances that hold the cells together, or merely *contain* the molecules that operate the permeation of ions. Whatever these permeating elements are, they appear to be dynamic structures that do not remain constant throughout the life of the monolayer, an observation that may be of interest in natural epithelia where different types of cells, and cells of different ages, coexist at any given moment.

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