Experimental Modulation of Occluding Junctions in a Cultured Transporting Epithelium

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ABSTRACT The experimental opening and resealing of occluding junctions in monolayers of cultured MDCK cells (epithelioid of renal origin) was explored by measuring changes in the electrical resistance across the monolayer and by freeze-fracture electron microscopy. As in natural epithelia, the function of occluding junctions as permeability barriers specifically depends on extracellular Ca^{++} concentration and fails if this ion is replaced by Mg^{++} or Ba^{++}. The removal of Ca^{++} and the addition of EGTA to the bathing medium opened the junctions and reduced the transepithelial resistance. Resealing was achieved within 10–15 min by restoring Ca^{++}. Quantitative freeze-fracture electron microscopy showed that junctional opening, caused by lack of Ca^{++}, was accompanied by simplification of the pattern of the membrane strands of the occluding junction without disassembly or displacement of the junctional components. Resealing of the cellular contacts involved the gradual return to a normal junctional pattern estimated as the average number of strands constituting the junction. The occluding junctions were also opened by the addition of the ionophore A23187, suggesting that the sealing of the contacts requires high Ca^{++} on the extracellular side and low Ca^{++} concentration of the cytoplasmic compartment. The opening process could be blocked by low temperature (7.5°C). Resealing did not depend on serum factors and did not require protein synthesis; therefore, it seems to be caused by reassembly of preexisting membrane junctional components. The restoration of the junctions occurred simultaneously with the establishment of ion-selective channels; the Na^+/Cl^- and the cation/cation selectivity were recovered with the same time-course as the electrical resistance. The role of the cytoskeleton in the process of junctional reassembly is reported in the companion article (Meza et al., 1980, *J. Cell Biol.*, 87: 746–754.).

The epithelioid cells of the established MDCK (Madin, Darby, Canine, Kidney) cell line (17, 20) form in culture monolayers that retain several physiological properties of mammalian kidney tubular cells. This led several authors to plate MDCK cells on permeable supports and use the preparation as a cultured epithelium of the simple cuboidal type constituted by a homogeneous population of cells (3–5, 29, 34). The cells in the monolayer are structurally and functionally polarized (6) and establish occluding junctions (or tight junctions) in ~15–20 h, so that the monolayer constitutes, by this time, an effective permeability barrier (4). The occluding junctions of MDCK cells do not form, as in most natural epithelia, a homogeneous belt with a uniform structure and resistance throughout the perimeter of adjoining cells but have, instead, "tight" regions intermixed with "leaky" ones (7).

As in the case of leaky epithelia, the monolayer has the ability to discriminate Na^+ from Cl^- with a 9:1 permeability ratio, and the monovalent cations of the series IA follow Eisenman's 6th pattern (corresponding to negative sites with a medium force field).

The occluding junctions of MDCK monolayers can be opened by removing Ca^{++} and adding EGTA to the bathing medium. Restoration of Ca^{++} reseals the junction (5). Junctional resealing, which is completed in 2–3 h, offers the opportunity to study the factors that participate in the reassembly process of the cellular contacts by monitoring changes in transmural electrical resistance and by modifications of the freeze-fracture electron microscopy pattern of the junctions.

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Meldolesi et al. (25) reported the slow (1–2 h) disassembly of occluding junctions of guinea pig pancreatic lobules incubated without Ca^{++} ions. This system has given valuable morphological information on the processes of disintegration and reconstitution of the occluding junctions. Taking advantage of the possibility of measuring transepithelial resistance in MDCK monolayers, we have used this cultured epithelium to determine the structural modifications that parallel the functional opening and resealing of these junctions.

In the present article, we report that the time-course of resealing of occluding junctions in MDCK monolayers depends on the duration of the period in which they are left open (2–60 min). A gradual simplification of the pattern of junctional strands is seen by freeze-fracture electron microscopy during this period, but there is no disassembly or displacement of the junctional components. Resealing seems to be achieved with preexisting junctional components. We analyze the role of Ca^{++}, both on resting monolayers and those opened and resealed, and explore several experimental conditions that influence resealing. The participation of microfilaments and microtubules is described in the companion paper (28). It was found that the cytoskeleton plays an important role in the process of resealing of the occluding junctions. Preliminary observations were presented elsewhere (3, 23).

MATERIALS AND METHODS

Cell Culture

MDCK (Madin, Darby, Canine, Kidney) cells constitute an established line obtained in 1958 by Madin and Darby (20) from the kidney of a normal cocker spaniel female dog. Cells in the 100–110th passages were grown at 36.5°C in roller bottles with an air-5% CO_2 atmosphere and 100 ml of complete Eagle's minimal essential medium (CMEM) with Earle's salts (Grand Island Biological Co. [GIBCO F-11] Grand Island, N. Y.), 100 U/ml of penicillin, 100 μl/ml of streptomycin, and 10% calf serum (GIBCO 617). Cells were harvested with trypsin-EDTA (GIBCO 540) and plated on 30-mm petri dishes (Lux Scientific Corp., Thousand Oaks, Calif.). When grown on a nonpermeable support (i.e., a petri dish), the cells form monolayers with blisters attributed to unidirectional water transport (17). When plated on disks of a Nylon cloth (HC-103 Nitex, Tetko Inc., Elsmford, N. Y.) coated with collagen extracted from rat tails, the monolayer can be used to study membrane phenomena as described below. Once sterilized under UV light, each disk was placed into a 16-mm well of a multichamber dish (Limbro Chemical Co., New Haven, Conn.), and a suspension of MDCK cells in 1.0 ml of CMEM was added to a density of 4×10^5 cells/cm^2. After incubation for 90 min at 36.5°C in an air-5% CO_2 atmosphere with constant humidity (V.I.P. CO_2 incubator 417, Lab Line Instruments, Inc., New Brunswick, N. J.) to allow for attachment of the cells, disks were transferred to another multichamber dish containing fresh CMEM without cells. A continuous monolayer is thus formed which starts to develop a measurable electrical resistance across in ~5 h, and reaches a steady value in ~20–24 h (3). The disk with the monolayer is translucent and permeable, and the state of the monolayer can be visualized with an inverted microscope. All monolayers used in the present study were confluent for 2 d or more.

Electrical Tests

Disks were mounted as a flat sheet between two Lucite chambers. The exposed area was 0.2 cm^2. A current pulse of 20 μA was delivered via two Ag/AgCl electrodes placed 2 cm from the disk, and the voltage change produced was measured with a second set of Ag/AgCl electrodes placed 1.0 mm from the disk. With those data, the electrical resistance was calculated. The contribution of a collagen-coated nylon disk without cells was subtracted from all calculated values of resistance.

Specific ion conductance for Na^+ and Cl^- was calculated from the values of the total conductance and the ratio of the sodium (P_{Na}) and chloride (P_{Cl}) permeabilities. This ratio was obtained through the Goldman-Hodgkin-Katz equation using the dilution potential measured across membranes mounted between a 2:1 concentration gradient of NaCl:

$$\Delta\Psi = -RT/F \ln \frac{(Cl)_i + \beta (Na)_o}{(Cl)_o + \beta (Na)_i} \quad (1)$$

where the subscripts o and i refer to the outer and inner bathing solutions, respectively, and β is the ratio P_{Na}/P_{Cl}. The solutions used on one side contained: 150 mM NaCl, 0.25 mM $CaCl_2$, and 1.5 mM Tris-HCl buffer at pH 7.4, and on the other side a similar solution with 75 mM NaCl replaced by sucrose. All measurements were corrected for potentials (E) between the electrodes because of the different chloride activities of the solutions (a_{Cl}) as follows:

$$E = \frac{RT}{F} \ln \frac{a'_{Cl}}{a''_{Cl}}. \quad (2)$$

Freeze-Fracture

Confluent monolayers of MDCK cells grown on petri dishes were fixed for 15 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, at room temperature. After fixation, the monolayers were washed twice with 0.1 M cacodylate buffer and were gradually impregnated with increasing concentrations of glycerol in cacodylate buffer for 30 min, up to a concentration of 20% glycerol, where they were left for 30 min and then removed from the substrate with a rubber policeman.

Fixed, glycerol-impregnated MDCK monolayers were rapidly frozen in the liquid phase of partially solidified Freon 22, cooled by liquid nitrogen, and stored in liquid nitrogen for 1–3 d. Freeze-fracture was carried out at −120°C in a Balzers 300 apparatus (Balzers Company, Liechtenstein) equipped with a turbomolecular pump.

Replicas were produced by evaporation from a carbon-platinum source. The specimens were shadowed at 2×10^{-6} mm Hg within 2–4 s of fracturing. After cleaning in sodium hypochlorite, replicas were washed with distilled water, mounted on Formvar-coated grids, and observed with a Zeiss EM 10 (Zeiss Company, Oberkochen, Germany) electron microscope.

After studying the replicas, we photographed the membrane regions where large fracture faces of occluding junctions were present. Original negatives were obtained at a final magnification of × 10,000, magnified three times, and the positive electron micrographs were used to make tracings of the junctional strands on transparent sheets as they appear under different experimental conditions. The total length of the junctional strands in a given replica, seen as ridges on P-fracture faces or as grooves on E faces, was measured with a map measurer (opisometer), and the value obtained was divided between the total linear length of the occluding junction studied in each replica.

RESULTS

Effect of Removal of Extracellular Calcium

The electrical resistance across MDCK monolayers that had been in confluence for 2–5 d was relatively stable with the normal concentration of Ca^{++} in the bathing solution (Fig. 1). However, when Ca^{++} was removed from the bathing solution (open circle at zero calcium), there was a sharp drop of the resistance. The addition of 2.4 mM EGTA to the bathing medium (filled circle) decreased the electrical resistance to values close to that of the free-solution. The specificity of EGTA action and the fact that a fraction of a millimole of Ca^{++} sufficed to confer 80% of the maximal resistance recorded suggested that the junctional sites bridged by Ca^{++} have a high affinity for this ion. This is further stressed by the finding that two other divalent cations tested failed to maintain the control levels of electrical resistance. Thus, Mg^{++} restored the electrical resistance to only 22.0 ± 2.8 Ωcm^2 (10) and Ba^{++} to 21.0 ± 1.3 (11). The findings described in Fig. 1 are comparable to those reported by Sedar and Forte (38) and Forte and Nauss (9) in the gastric mucosa.

The drop in the electrical resistance produced in MDCK monolayers by the removal of Ca^{++} and the addition of 2.4 mM EGTA (Fig. 2) was described by Cereijido et al. (6) and attributed to the opening of the occluding junctions. Unpublished observations by Meza et al. using a voltage scanning method devised by Cereijido et al. (7), have localized the decrease in resistance to the level of the intercellular space. Although a decrease also in the transcellular resistance may not be disregarded, the resistance of this pathway in epithelial

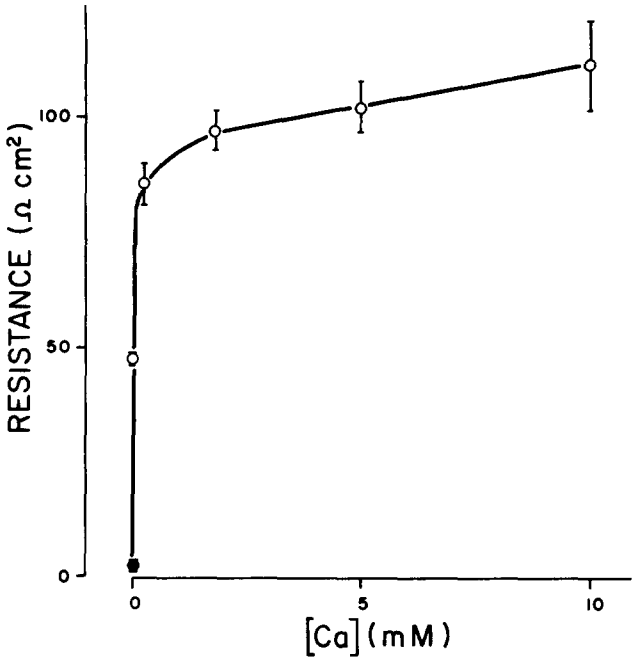

FIGURE 1 Dependence of the electrical resistance across MDCK monolayers on the concentration of Ca^{++} in the bathing medium. The medium contained (meq): 120 NaCl, 4 KCl, Tris Cl, and $CaCl^2$ and were adjusted to sum 31.5 meq when the concentration of Ca^{++} was varied. Disks were incubated at 37°C in petri dishes containing media with different concentrations of Ca^{++} for 45 and 90 min. They were then withdrawn and mounted in a Lucite chamber with the same media to measure the electrical resistance. No difference was observed in the values at 45 and 90 min and, therefore, the values at a given concentration of Ca^{++} were averaged together. In this and following figures, each point represents the mean ± SE. The electrical resistance of the empty disks bathed with each solution was subtracted from that of the disk with the monolayer. There are two points plotted at zero Ca^{++}. The one represented by a filled circle has no calcium but has 2.5 mM EGTA added. In this and following figures, values are given as mean ± SE. Number of experiments, n is 5–12. When standard error is not given, the value is an average of two to four determinations.

cells is so high (40, 44) that it is unlikely that this factor makes a significant contribution to the observed phenomenon. As shown in Fig. 2, this decrease consists of a rapid phase followed by a further reduction with much slower kinetics. Whereas the sharp drop may be attributed to the opening of the junction, the slower decrease may be caused by widening of the intercellular spaces (12). The value of the electrical resistance remaining after the first sharp drop varied from one group of disks to another. The reason for this variation was not obvious. Therefore, each experiment in this article included a control group.

Ionophore Effect

These above-mentioned findings are comparable to those found in occluding junctions of the gastric mucosa by Sedar and Forte (38) and in communicating junctions by Loewenstein (18), who succeeded in altering the function of both kinds of junctions under a variety of conditions and proposed that these modifications depend on the increase of Ca^{++} on the cytoplasmic side. One of the lines of evidence supporting this hypothesis included the effect of Ca^{++}-transporting ionophores (11, 36, 37). Fig. 3 shows that the Ca^{++}-ionophore A23187 added to CMEM-bathed MDCK monolayers produced, at the end of 1 h, the abolition of the resistance across MDCK monolayers bathed with normal concentrations of Ca^{++} (squares). The other two groups depicted in Fig. 3 refer to the removal of Ca^{++} from the bathing solution (filled circles) and to the addition of the vehicle used for the ionophore (ethanol), respectively.

Effect of Calcium Removal on the Morphology of the Junctions

Previous studies of freeze-fracture replicas of the occluding junctions of MDCK cells (5, 6, 29) have shown that, as in natural epithelial, the occluding junctions consist of several strands appearing as ridges on the P face and complementary grooves on the E face. However, in normal MDCK cells, many junctional strands tend to appear fragmented on P faces and varying numbers of intramembrane particles may be found in E-face furrows. Treatment with zero extracellular Ca^{++} and EGTA produced no dramatic change in the overall pattern of distribution of strands (Fig. 4) during the 15 min (Table I)

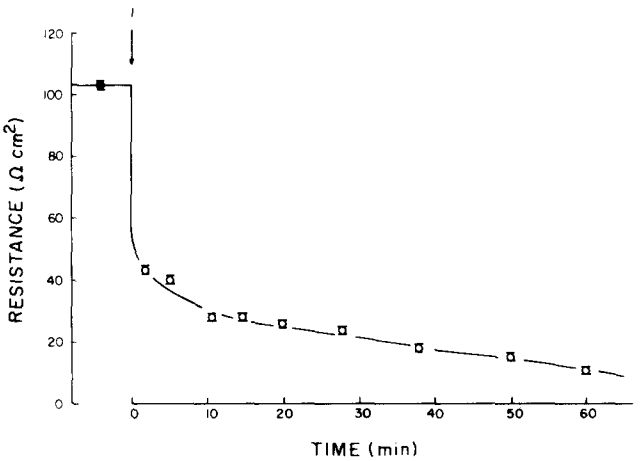

FIGURE 2 Effect of calcium removal. At arrow *1*, the disks are transferred to Ca^{++}-free MEM with 2.5 mM EGTA. The electrical resistance first drops sharply and then continues to decrease at a slower rate.

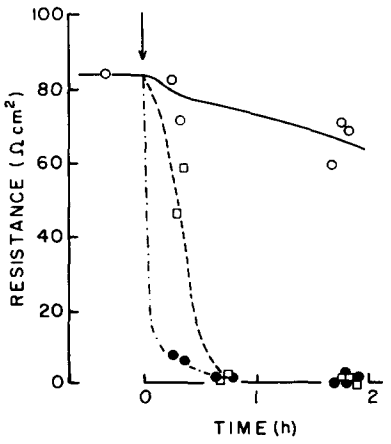

FIGURE 3 Effect of the Ca^{++}-transporting ionophore A23187. At zero time, the disks were divided into three groups. The first, represented by filled circles, was transferred to Ca-free MEM with EGTA and served as control. The second, represented by open circles, was transferred to CMEM containing 25 μl/ml of ethanol, which was the solvent used for A23187. The third group of disks, represented by open squares, was bathed in Ca^{++}-containing CMEM with 1.4 mM Ca^{++} and A23187 (5×10^{-5} M).

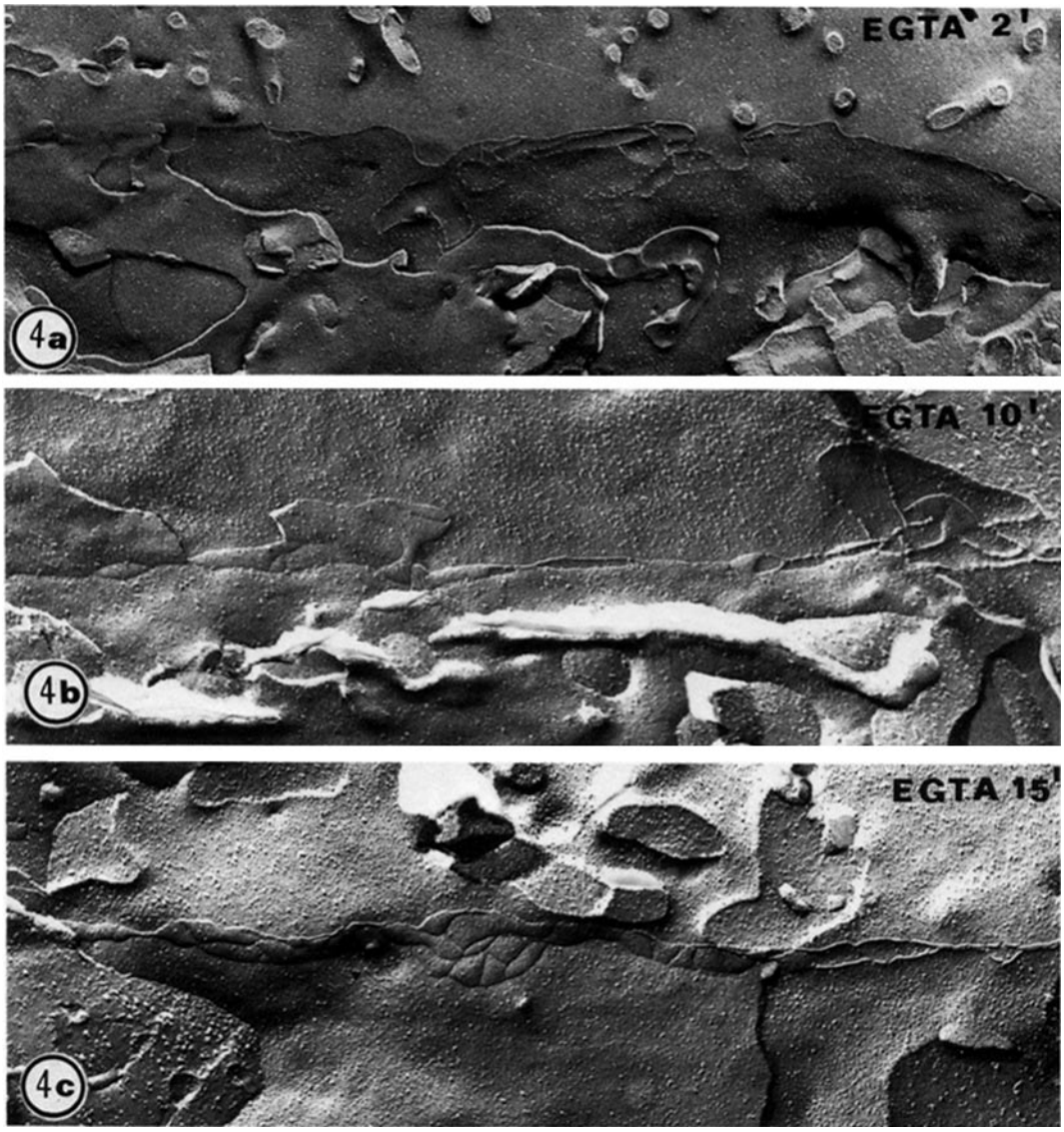

FIGURE 4 Freeze-fracture replicas of MDCK cells in confluent monolayers. The tight junctions are seen on both P and E faces of the plasma membrane just below the region of microvilli. The junctional strands vary in number (see Table I). Fig. *a*, MDCK cells treated for 2 min without Ca^{++} and with EGTA; *b*, 10-min treatment without Ca^{++} and with EGTA; *c*, 15-min treatment without Ca^{++} and with EGTA. Fig. *a*, × 23,000; *b*, × 40,000; *c*, × 46,000.

when a striking reduction in resistance was recorded. The only apparent morphological modification found was a reduction in the ratio between the total length of the strands and the length of the junctions. This indicates that the rapid phase of the resistance drop is caused by the removal of Ca^{++} bridges between junctional components at adjacent cells rather than to complete disarray of the junction itself. The disassembly and detachment of complete strands of the junctional belt formed by the occluding junctions, reported by Meldolesi et al. (25) in pancreatic acinar cells of the guinea pig devoid of Ca^{++} for several hours, were not observed at the short time intervals necessary to open the junctions in MDCK cells. In our laboratory, we have not observed the major changes in the occluding junction caused at short times by lack of Ca^{++} described in thin sections by Cereijido et al. (5). In Ca^{++}-depleted MDCK monolayers, the occluding junctions become freely permeable throughout the whole cellular perimeter to electron-dense tracers, such as ruthenium red (unpublished observations), but at short times (1–15 min) there is no other change observable in thin sections.

Effect of Prefixation

A further indication that the first rapid fall in resistance is caused by the removal of Ca^{++} salt links without simultaneous disarray of the junctional components was obtained in MDCK monolayers fixed with glutaraldehyde before opening. Fig. 5 shows the opening under control conditions (solid line) produced by the removal of Ca^{++}, as well as the evolution of the resistance of monolayers that were fixed with glutaraldehyde at arrow *1* (dashed line). At arrow *2*, the removal of Ca^{++} and the addition of EGTA reduced the electrical resistance in spite of the fact that the monolayers were previously fixed. However, this reduction in resistance was incomplete and consisted of a single rapid phase.

Effect of Calcium Restoration

The transference of the monolayers to CMEM containing Ca^{++} restored the electrical resistance with a time-course that depended on how long the junctions were left open (Fig. 6). This phenomenon is similar to the one reported by Johnson et

TABLE I

Ratio between Total Length of Junctional Strands and Total Length of Occluding Junctions, as Seen in Freeze-Fracture Replicas of MDCK Monolayers

Conditions	Total length of five junctions measured for each experimental condition	Strands/junction
	μm	
Control	28.6	2.4
EGTA, 2 min	50.0	1.7
EGTA, 10 min	20.0	1.9
EGTA, 15 min	9.7	1.8
Recovery, 50 min	44.0	2.0
Recovery, 165 min	50.5	2.2

EGTA, monolayers were placed in medium without Ca^{++} and EGTA. Recovery, after 15 min in medium without Ca^{++} and with EGTA, monolayers were incubated in normal medium.

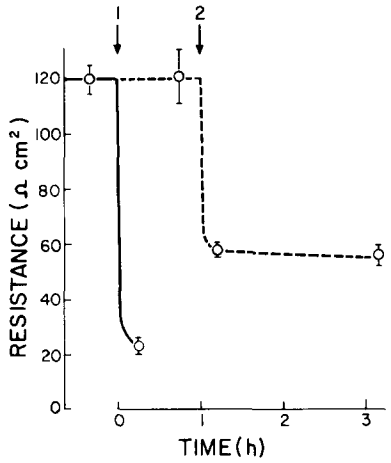

FIGURE 5 Influence of fixation on the electrical resistance. At arrow *1*, a group of disks was transferred to Ca^{++}-free MEM with EGTA, and their resistance dropped to 23 Ωcm^2; the rest were left in CMEM to which glutaraldehyde was added (final concentration 2%). At arrow *2*, this group was transferred to Ca^{++}-free MEM.

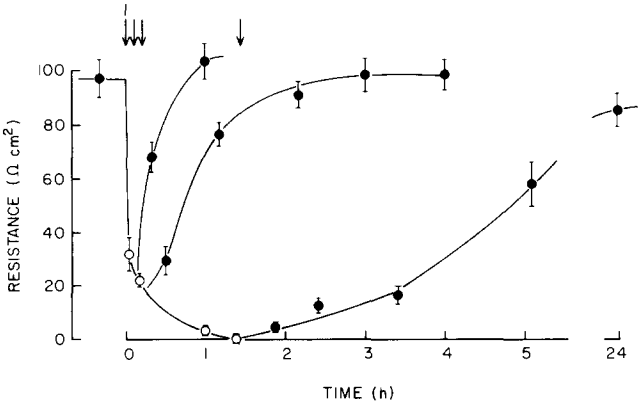

FIGURE 6 Recovery of the electrical resistance across MDCK monolayers. At arrow *1*, all disks were put into petri dishes containing Ca^{++}-free MEM with 2.5 mM EGTA. Ca^{++} was restored at the times marked by the arrows. Curves were drawn by eye.

al. (15) for the reformation of communicating junctions in Novikoff hepatoma cells that were previously dissociated with EDTA. Data in Fig. 7 show that the lack of Ca^{++} and not the presence of EGTA per se *is* the factor that keeps the junctions

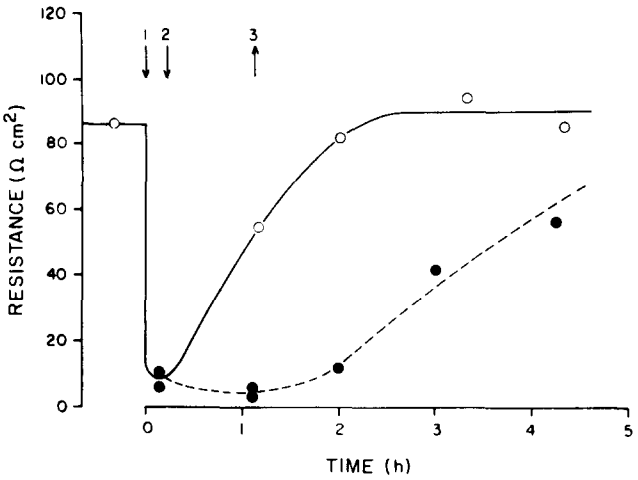

FIGURE 7 Presence of Ca^{++} vs. removal of EGTA. At arrow *1*, all disks were transferred to Ca-free medium containing 2.5 mM EGTA. At arrow *2*, disks were divided into two groups. The first, represented by open circles, was transferred to calcium containing (1.4 mM) CMEM. The second, represented by filled circles, was transferred to Ca^{++}-free MEM without EGTA. At arrow *3*, this group was bathed with Ca^{++}-containing CMEM.

open. At arrow *2*, one of the groups (open circles) was transferred to CMEM as in Fig. 6. The other one (filled circle, dashed line) was bathed in medium that contained no EGTA but to which Ca^{++} was not restored either. The resistance of this group remained low until Ca^{++} was added at arrow *3*. It may be noticed that, upon restoration of Ca^{++}, this second group of disks recovered its electrical resistance with a much slower kinetics. This difference in the kinetics of resealing was further demonstrated in the experiment described in Fig. 6 in which the junctions were left open for varying periods and Ca^{++} was restored at the times shown by the arrows. When Ca^{++} was added after >1 h, the resistance remained low for up to 5 h but recovered overnight. This suggests that when cells are treated with a Ca^{++}-free, EGTA-containing medium, recovery requires more than the mere reestablishment of Ca^{++} bridges.

A study of the resealing of the junction as a function of the concentration of Ca^{++} in the bathing solution indicated that maximal effect was achieved at very low concentrations (Fig. 8). The values reported in Fig. 8 refer to the resistance measured at 90 min, i.e., a time when the sealing was not yet completed (see Fig. 6). As in the case of Fig. 1, the steepness of the curve revealed a high selectivity for Ca^{++}.

When the occluding junctions had been opened for 15 min, the extent of noncontinuous, dotted junctional strands in freeze-fracture replicas studied with the electron microscope seems to increase, although this is difficult to demonstrate quantitatively. The ratio between the total length of the junctional strands and the total length of the occluding junctions decreased from 2.4 in control monolayers to 1.8 in cells treated for 15 min with zero Ca^{++} and EGTA (Fig. 4). 50 min after restoration of Ca^{++}, the ratio was 2.0 and 2.2 in cultures after 165 min of recovery in the presence of Ca^{++} (Fig. 9 and Table I). Therefore, the simplification of the freeze-fracture pattern of the junctions induced by the absence of Ca^{++} was reversed during the recovery period. This picture is in keeping with the suggestion made by Friend and Gilula (10) that these fragments of strands represent stages in the development of the junctions (24, 30, 41). The fact that many strands are prolonged by a row

of particles also agrees with the observation of Marin et al. (21) that regeneration of tracheal epithelium occluding junctions after mechanical injury proceeds through the development of small linear arrays of particles. Formation of occluding junctions in fetal liver seems to follow the same pattern (33). Even though differences in the degree of fragmentation of the occluding junctions may be induced by varying fixation conditions, the variations reported here were found in cells uniformly fixed with high (2.5%) concentrations of glutaraldehyde. Furthermore, the finding that glutaraldehyde fixation does not modify the transmural resistance should be taken into account. It should be emphasized that freeze-fracture reveals mostly the structure of the occluding junction components inside the plasma membrane but fails to reveal the surface aspect of the junctional elements which may, in principle, be visualized through the freeze-etching procedure.

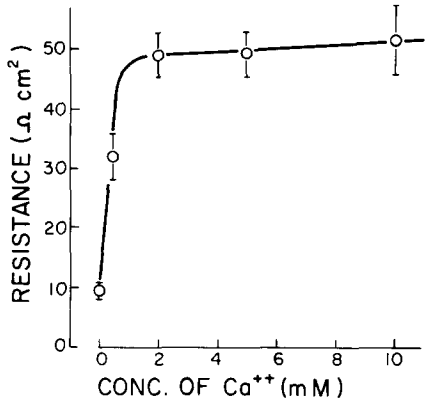

FIGURE 8 Recovery of the electrical resistance as a function of the concentration of Ca^{++} of the bathing solution. Once the junctions were opened with Ca-free MEM and EGTA, the disks were divided into several groups. To recover, each disk was placed in MEM with different concentrations of Ca^{++}. The electrical resistance was measured at 90 min, i.e., before the recovery was completed.

Ionic Discrimination

To study whether opening and resealing modifies the ion discriminating ability of the occluding junctions, we studied 2: 1 NaCl dilution potentials across monolayers at different degrees of resealing. Fig. 10 shows that when the junctions are opened by the removal of Ca^{++}, the paracellular permeation route becomes a water-filled pathway where ions move as in a free solution. As these free water pathways are resealed by Ca^{++}, the discriminating ability of the selective channels predominates and the ratio between the Na^{++} and the Cl^- permeability (P_{Na}/P_{Cl}) is proportional to the degree of recovery (as expressed by the electrical resistance). Essentially the same information was obtained for the cation/cation discrimination (Fig. 11). The left-hand side of Fig. 11 illustrates the electrical resistance of a group of disks (each one represented by a circle) in Li^+ Ringer's solution. The ascending branch of the curve is reproduced on the right-hand side together with those obtained in the same manner with different cations. There was no major change in the discrimination pattern, K^+ and Na^+ being the most permeable cations and Li^{++} the least one following the pattern of the resting monolayer (6). The experiments described in Figs. 10 and 11 suggest that the reassembly of the junction does not seem to involve a period of sealing followed by another of repopulation of ion-selective channels. A resealing followed by a reinstallation of channels would have given a recovery of the resistance to a level higher than control with ion-permeability ratios similar to that of free water. Only on a few occasions did the recovery of the electrical resistance exhibit an overshoot.

Effect of Low Temperature

Fig. 12 shows that cooling of the MDCK monolayers, by itself, did not open the occluding junctions (filled squares). But rather increased the resistance. The nature of this increase was not investigated. However, it is suspected that it is related to

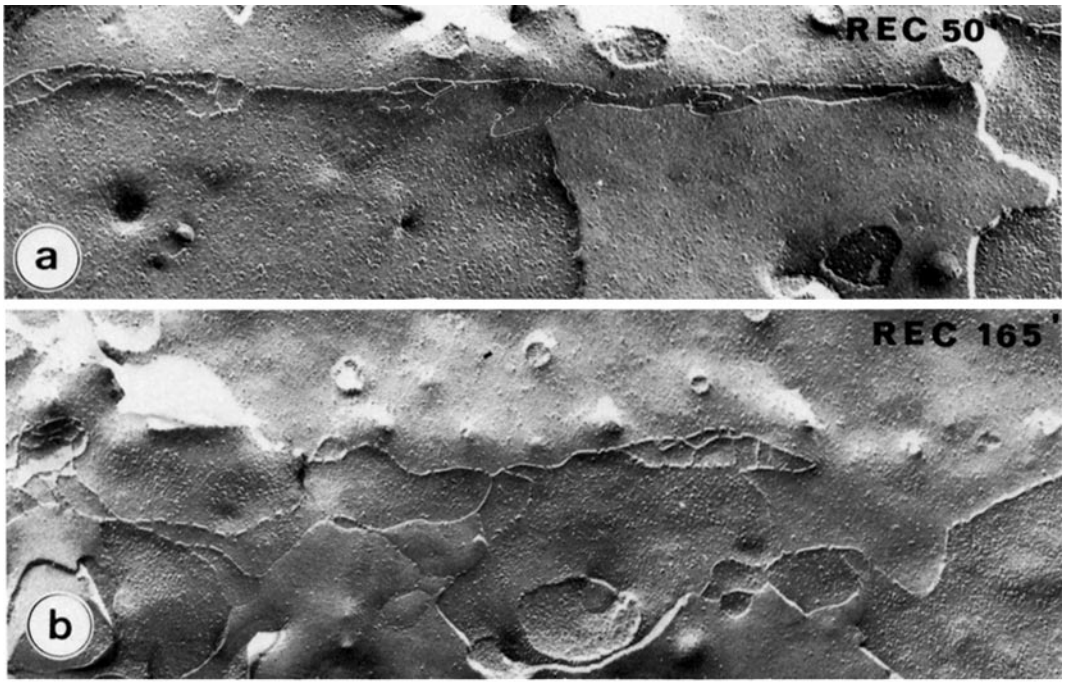

FIGURE 9 Freeze-fracture replica of MDCK monolayers that had been exposed to Ca-free media containing EGTA for 10–15 min, and then allowed to reseal in CMEM for 50 min (*a*) and 165 min (*b*). *a*, × 53,000; *b*, × 42,000.

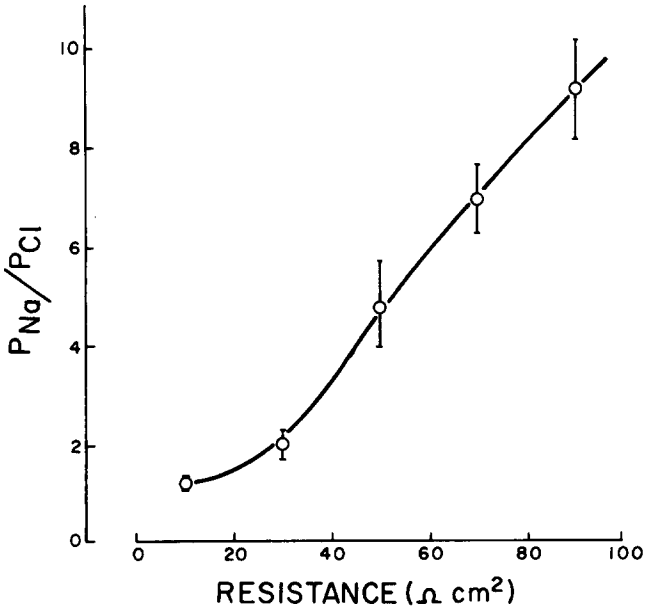

FIGURE 10 Cation/anion discrimination as a function of the electrical resistance during resealing. The ratio between the permeability to sodium (P_{Na}) and the permeability to chloride (P_{Cl}) was calculated with Eq. 1 on the basis of the dilution potential ($\Delta\Psi$) generated across the monolayers mounted between two different Ringer's solutions containing 150 and 75 mM NaCl. Osmolarity was kept constant with sucrose.

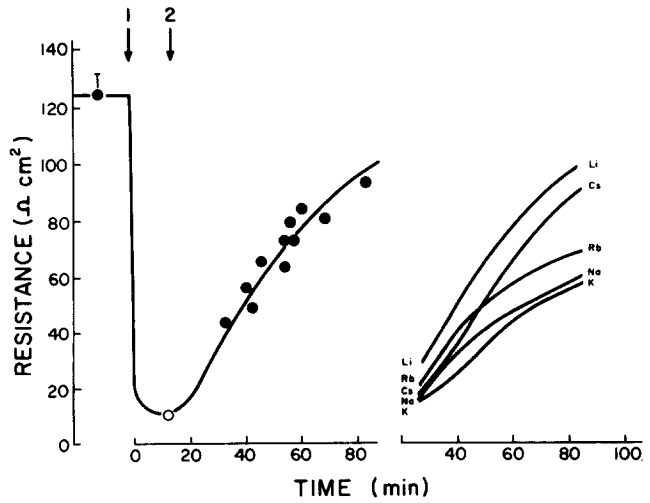

FIGURE 11 Cation/cation discrimination as a function of time. Monolayers were mounted on Ringer's solutions containing 150 mM of the chloride salt of the cations listed. In each case, the resistance of the empty disk bathed with the given solution was subtracted. The left-hand side shows the electrical resistance of the monolayer bathed in LiCl Ringer's. Arrow *1*, the opening of the junctions at the removal of Ca^{++}. Arrow *2*, the resealing elicited by the restitution of Ca^{++}. Only the first 80 min were studied because that was the period of maximal change. Each point corresponds to an individual disk. The curve was drawn by eye. The right-hand side shows the curves of the five alkali metal ions of series 1A obtained, as in the case of Li^+.

the swelling and collapse of the intercellular space caused by the arrest of the membrane pumps. Cold does not prevent the opening elicited by the removal of Ca^{++} (open squares). It comes as a surprise, though, that cold slows down the opening. It was expected that, if the initial drop were only caused by the removal of Ca^{++}, the effect of the cold would be very minor. This, together with the observation that the use of EGTA makes a clear difference (6), suggests that Ca^{++}-bridges are not overtly exposed and that their removal depends on the fluidity of the membrane. A collapse of the interspace, with the corresponding increase of its contribution to the transepithelial resistance, could also be taken into account. Fig. 13 describes the effect of low temperature on the process of resealing. Filled symbols are the controls described in detail in earlier figures: filled circles and squares correspond to restitution of Ca^{++} at arrows *2* and *4*, respectively, and the kinetics observed agree with the experiments of Figs. 6 and 8. The lowering of temperature seemed to protect the cells or delay changes, so that if the junctions were left open 1.5 h with the temperature low (open squares), upon restitution of both normal temperature (36.5°C) and normal Ca^{++} the resistance recovers with a faster kinetics. When Ca^{++} was restored, at arrow *2*, but temperature was

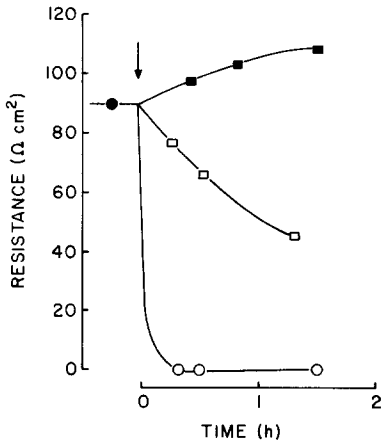

FIGURE 12 Effect of cold on the electrical resistance of MDCK monolayers. Open circles, disks incubated at 36.5°C. Ca^{++} was removed at arrow. Filled squares, disks incubated in CMEM with Ca^{++} refrigerated at 7.5°C from the arrow on. Open squares, disks incubated in CMEM at 7.5°C since 10 min before zero time and then switched to MEM without Ca^{++} at time marked by arrow. Each value corresponds to an average of two to four disks.

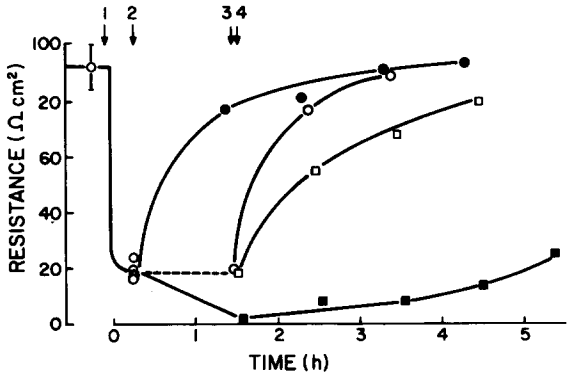

FIGURE 13 Effect of cold on the opening and resealing of the junctions. At arrow *1*, all disks, which were at 36.5°C, were transferred to Ca-free medium with EGTA. At arrow *2*, disks are divided into four groups: filled circles, disks were transferred to CMEM; filled squares, disks left in Ca-free medium until CMEM was restored at arrow *4*; open circles, disks were transferred to CMEM like those represented by filled circles, but at 7.5°C until arrow *3* (at that moment, they were incubated at 36.5°C), and open squares, disks left in Ca-free medium like those represented by filled squares, but at 7.5°C until arrow *3*. At that moment, they were transferred to 36.5°C.

lowered to 7.5°C (open circles), the junctions did not reseal. When the temperature of this group was brought back to 36.5°C (arrow *3*), the junctions resealed with fast kinetics. So the presence of Ca^{++}, by itself, does not suffice to reseal the junctions.

Effect of Serum and Inhibition of Protein Synthesis

Many phenomena related to the attachment of cells to the substratum and to one another depend on the presence of serum components in the bathing media. The process of resealing, however, does not seem to depend on factors contained in the serum. As described in Fig. 14, resealing proceeds in MEM (open circles) as in CMEM (filled circles). Furthermore, it also does not seem to depend on the synthesis of new proteins. As shown in Fig. 15, opening and resealing was not affected by cycloheximide, indicating that the components of the resealed strands were the same as when they were opened, or else the lack of action of the cycloheximide may reflect the existence of a large pool of components. The results shown in Fig. 15 agree with the information available on natural epithelia (14, 25). Protein components of plasma membranes are in a state of constant turnover and renewal and many have half-lives of ~85 h (2, 43). If the lack of effect of cycloheximide on junction formation in MDCK cells is actually caused by the presence of a pool of components, this pool would also have a long life. This interpretation is supported by the results described in Fig. 16 in which a large group of disks were left in CMEM with cycloheximide and then withdrawn periodically to perform an opening and resealing experiment. For up to 12 h in cycloheximide, the monolayers were still able to reseal their junctions. This experiment was not prolonged beyond 12 h as any observable change after such a long exposure could be caused by indirect effects. The last opening and resealing procedure did not reduce the electrical resistance >30–40%, perhaps because of swelling of the cells. These observations differ from those of Cereijido et al. (5) performed with MDCK cells harvested with trypsin from a roller bottle and plated at confluence on disks. Treatment with cycloheximide or puromycin under those conditions prevented the development of occluding junctions.

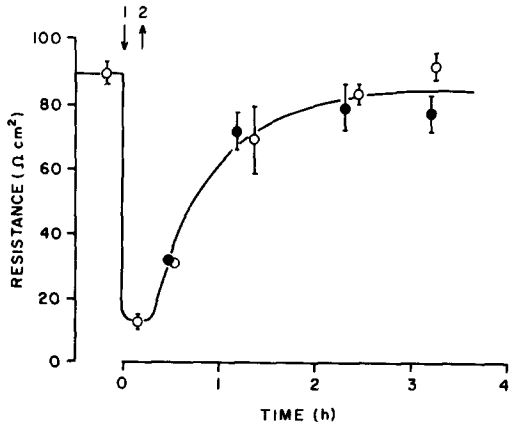

FIGURE 14 CMEM vs. MEM: Ca^{++} was removed at arrow *1*. At arrow *2*, disks were divided into two groups. The group represented by filled circles were bathed in CMEM. Open circles represent monolayers recovering in MEM. Circles without standard error marks represent means of two values.

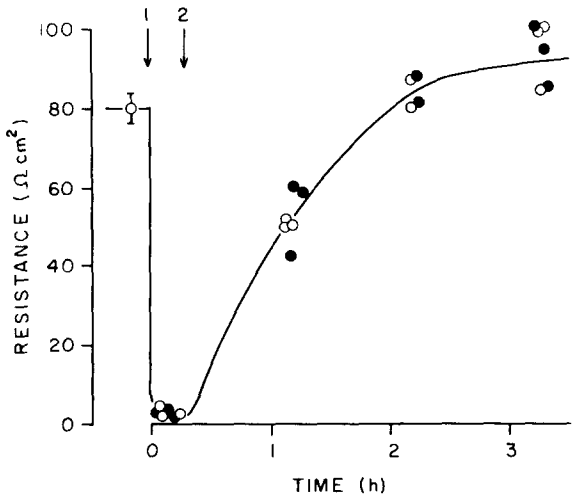

FIGURE 15 Effect of cycloheximide. A group of disks were incubated with CMEM with 10^{-} M cycloheximide 10 min before the removal of Ca^{++} at arrow *1* (filled circles). Open circles represent control monolayers. Ca^{++} was restored at arrow *2*. Each circle represents an individual monolayer.

Effect of Trypsin

One of the characteristics that made this series of experiments possible was the adherence of the cells to the collagen substrate in spite of their detachment from their neighbors when placed in Ca^{++}-free solutions. This suggests that the sites involved in cell-to-cell attachment are different from those participating in the attachment of the cells to the substratum, as suggested by data illustrated in Table II. The monolayers were treated with trypsin in the presence of Ca^{++}. Trypsin, at the concentrations used, did not open the junctions in a period of 125 min. It is interesting that when the concentration was raised to 10 mg/ml, the cells detached from the substrate but not from their neighbors and the monolayers rolled upon themselves in the

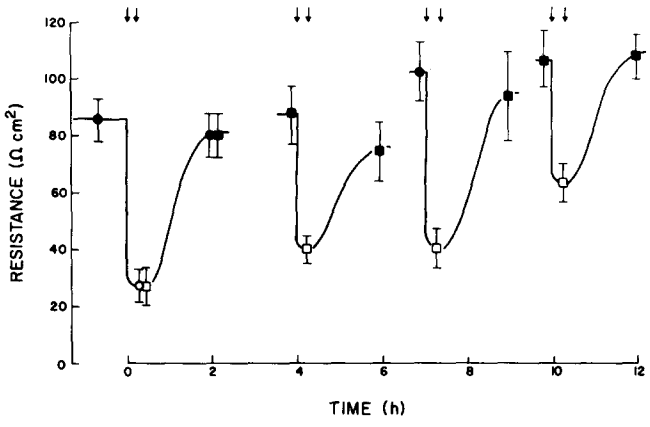

FIGURE 16 Effect of cycloheximide. In this figure, open symbols correspond to disks bathed in Ca-free MEM and square symbols to membranes treated with cycloheximide. Each pair of arrows correspond to the removal and restoration of Ca^{++}, respectively. 10 min before zero time, the membranes represented by squares were treated with the drug that remained present throughout. The opening and resealing procedure was performed at 0, 4, 7, and 10 h. Notice that after resistance measurement, disks were discarded and the different openings and resealing represented in this figure were not repeated on the same monolayers. Each one represents a procedure performed on disks that were incubated with the drug for progressively longer periods.

TABLE II

Effect of Trypsin on the Monolayer of MDCK Cells

	Concentration	Time	Electric resistance
	mg/ml	*min*	$\Omega \cdot cm^2$
Control			80.2 ± 6.1 (5)
EGTA 1		10–20	20.6 ± 3.3 (5)
Trypsin	0.1	15–35	81.0 ± 4.6 (4)
Trypsin	0.1	60–97	82.6 ± 5.7 (5)
Trypsin	1.0	15–47	81.2 ± 5.9 (5)
Trypsin	1.0	75–78	76.7 ± 2.3 (3)
Trypsin	10	15	detachment

Disks incubated in MEM without serum.

form of continuous sheets. Because the monolayers were mounted on permeable disks where trypsin could penetrate from both the apical and basolateral sides, it seems unlikely (but not neglectable) that the cells could remain attached to one another because of the lack of penetration of the enzyme into the junctional region.

DISCUSSION

The importance of Ca^{++} in various phenomena involving membrane interactions, such as cell attachment, transcellular permeability, and secretion, has been recognized for a long time (8, 13, 26, 35). In particular, the participation of Ca^{++} in the establishment of several kinds of cell junctions has been the subject of thorough analysis (9, 11, 15, 19, 36–38). The long-term effect of Ca^{++} depletion in occluding junctions of pancreatic epithelial cells has been explored by Meldolesi et al. (25), who found progressive loosening and disarrangement of the junctional strands (visualized by freeze-fracture electron microscopy) with subsequent fragmentation and displacement over the plasma membrane. Restitution of Ca^{++} to pancreatic lobules resulted in the rapid reassembly of the occluding junctions. These observations have given us valuable information concerning the structural basis of occluding-junction disassembly and reassembly, and have also provided evidence on the participation of these junctions as barriers for maintaining the surface heterogeneity of membrane particles, that differentiates the lumenal from the basolateral plasma membrane regions (25). On the other hand, our experimental model has facilitated our following the early effects of Ca^{++} absence both on the structure of occluding junctions and on the function of the junction permeability barriers.

We have found that the occluding junctions of MDCK monolayers are specifically sealed by Ca^{++} present in the extracellular compartment and that they become permeable when the intracellular Ca^{++} concentration rises through the action of ionophores. Ca^{++} seems not only to govern the sealing, but also to stabilize the structure of the occluding junction. Because it is necessary to add EGTA to enhance the junctional opening and because this process may be markedly retarded by cold, it may be anticipated that the role of Ca^{++} is not restricted to the formation of simple bridges between two well-exposed negative sites placed on the junctional components in register between neighboring cell membranes. Katchalsky (16) suggested that negative sites on a protein molecule may form stereospecific regions and permit Ca^{++} to establish bonds far stronger than the ordinary electrostatic type. Ca^{++} may also play a role in the assembly of particles of the junction into strands and in the anchorage of these to other cellular components. Because all these possibilities are little understood at present, our discussion rests on the simplifying assumption that the only action of EGTA is to remove Ca^{++} from the junctions, but the possibility of side effects should not be forgotten.

The electrical resistance of the paracellular permeation route has been attributed to the sealing capacity of the junction and to the resistance of the intercellular space (40, 44). The rapid phase of junctional opening in MDCK cells placed in Ca^{++}-free media might be caused by the removal of Ca^{++} bridges without a significant change in the interspace. It might take some time for the junctional elements in register on opposite plasma membranes to separate upon Ca^{++} removal. In fact, control and treated monolayers are not distinguishable by phase-contrast microscopy nor by freeze-fracture electron microscopy. There is no disappearance or gross disarray of the junctions, and the only evident change is the simplification of the junctional pattern. The exposure for longer times widens the intercellular space (6) and abolishes the electrical resistance.

Once Ca^{++} is removed, the occluding junctions become functionally open, but for up to 15 min the overall disposition of membrane junctional components remains *in situ*, although there is a tendency for the strands to become fragmented on P faces with a higher number of intramembrane particles on E furrows. Martínez-Palomo and Erlij (22) have previously reported that in natural epithelia the osmotic opening of occluding junctions may occur in the absence of gross distortion of the freeze-fracture image of the junctional strands. We have now extended this observation to occluding junctions of MDCK cells rendered permeable through the absence of extracellular Ca^{++}. In the latter system, a reduction in the ratio between the number of junctional strands and the total length of the junction was found. The relative importance of this reduction and of the increased fragmentation of junctional strands in terms of the lowering of the resistance remains to be established. The study of long-term (i.e., 1 h) effects of Ca^{++} absence on the morphology of occluding junctions is in progress in our laboratory. It remains clear, however, that drastic reduction in transepithelial resistance does not require complete disassembly of occluding junctions.

Cereijido et al. (5) have observed that in MDCK cells isolated by treatment with EDTA and trypsin, the formation of the occluding junctions does depend on the synthesis of proteins. However, in the present work, trypsin by itself failed to open the junctions and when monolayers were treated with this enzyme, they rolled up like sheets detaching from the substrate, but the cells remained attached to one another. The sites seem to become exposed to trypsin only after they have released Ca^{++}.

The view that resealing of occluding junctions in MDCK cells is achieved with preexisting elements is supported by the observation that, as soon as they are positioned and bridged, they have the same ion-discriminating ability as the monolayer at rest (i.e., before opening). This suggests that the whole process may depend on the fixation of the strands in the position they have at the apical/basolateral boundary where the occluding junctions govern the permeability of the paracellular pathway and seem to act as a fence that preserves the polarization of the plasma membrane. Quantitative modulation of communicating and occluding junctions has been described in various tissues after treatment with proteolytic enzymes (1, 27, 32, 39). Observations derived from these studies tend to support the notion that the precursors of the cellular junctions may exist, at least partially, in a masked or immature state.

Because it has been shown that microtubules and microfilaments are responsible for the movement and topographical distribution of membrane components in certain cells (31, 42), in the companion paper we have explored the role of the cytoskeleton in the opening and resealing of the occluding junctions in MDCK monolayers (28).

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