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Tight Junction Formation in Cultured Epithelial Cells (MDCK)

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Summary. Synthesis and assembly of tight junctions are studied in monolayers of MDCK cells plated at a density sufficient for confluence, allowed to attach for 1 hr, and transferred to fresh media without cells containing or not Ca^{2+}. 20 hr later, while monolayers with Ca^{2+} have fully developed junctions that confer an electrical resistance across of 346 ± 51 Ω cm², those without Ca^{2+} have a negligible resistance. If at this time Ca^{2+} is added, junctions assemble and seal with a fast kinetics, that can be followed through the development of electrical resistance, penetration of ruthenium red, and electron microscopy. Drugs that impair synthesis, maturation and transport of proteins (cycloheximide, tunicamycin, monensin) indicate that protein components are synthesized early upon plating, do not seem to require N-glycosylation, and are stored in the Golgi compartment. Upon addition of Ca^{2+} they are transferred to the membrane with the participation of microfilaments but not of microtubules. These components seem to insert directly in the position they occupy in the strands, and the cell circles its perimeter with one strand as early as 15 min, even if in some segments it only consists of a row of particles. New strands develop in association with previous ones, and the pattern completes in 4 to 6 hr. Ca^{2+} is required for the maintenance of the assembly and also for the sealing with neighboring cells. These processes cannot occur below 25°C. Serum is not required. Polarized distribution of intramembrane particles (IMP) in apical and basolateral regions follows the same time course as junction formation, in spite of the fence constituted by those strands that are already assembled. This suggests that IMP do not redistribute by lateral displacements in the plane of the membrane, but by removal and insertion in the apical and basolateral domains.

Key Words epithelial monolayers · MDCK cells · tight junctions · calcium · biosynthesis of junctions · junctional assembly · apical/basolateral polarization

Introduction

The cells from transporting epithelia have two basic features: 1) they form tight junctions that block diffusion from the lumen to the interstitial side, and 2) they polarize their plasmalemma into apical and basolateral domains which are morphological, chemical and physiologically different [5]. Studies of the processes of junction formation and polarization are facilitated by the fact that when cells of epithelioid lines (that have been previously harvested with trypsin-EDTA) are plated at confluence, they form junctions and polarize in a few hours under conditions amenable for experimental control (*see* [2]). Cereijido et al. [5] have shown that cycloheximide or puromycin, if added before the sixth hour of plating, prevent junction formation between MDCK cells in a reversible way, but that if these inhibitors are added later, they fail to block the development of electrical resistance across the monolayer, indicating that by this time the components of the junction are already synthesized. However, the fact that the development of resistance takes a total of 12 to 15 hr to reach a maximum, shows that although by the sixth hour the components of the junction are synthesized, they are not yet assembled into sealed junctions. This observation was confirmed by Hoi Sang et al. [13] and by Griepp et al. [10]. It is well known that sealing in natural epithelia requires Ca^{2+} [3, 20, 40]. In MDCK cells, Cereijido et al. [4] have shown that if this ion is removed from the bathing media one hour after plating, the cells remain attached, but do not form tight junctions, as demonstrated by the absence of strands in freeze-fracture replicas, by the penetration of ruthenium red, through the intercellular spaces, and by the absence of electrical resistance across the monolayer. If 20 hr later Ca^{2+} is restored, resistance develops and reaches a maximum with fast kinetics (4 to 6 hr).

When cells that have been harvested with trypsin-EDTA are plated at a density sufficient for confluence, they have to recover from this treatment, attach, spread, and adjust their borders to each other before junctions can be established. In monolayers plated and left 20 hr without Ca^{2+} instead, these processes are essentially completed and, therefore, the time resolution of the process of

junction formation and sealing is improved. Furthermore, Griepp et al. [10] have demonstrated that when harvested MDCK cells are maintained in a Ca-free spinner medium for 24 hr and then plated, occluding junctions form even in the presence of inhibitors of protein synthesis, indicating that attachment is not required to trigger the synthesis of junctional components. Therefore, the procedure of plating the MDCK cells, removing Ca^{2+} for 20 hr, and then restoring this ion, offers an opportunity to study where the components are stored, how they are assembled and sealed under a variety of experimental conditions. This is precisely the purpose of the present article. We follow the genesis of tight junctions by freeze-fracture electron microscopy, penetration of ruthenium red and development of electrical resistance across the monolayer. The freeze-fracture replicas are also used to evaluate the density and distribution of intramembrane particles (IMP) in E and P faces of apical and basolateral membranes. Furthermore, we assess the effect of temperature, serum and drugs that impair microtubules and microfilaments, and that block different steps of the synthesis of glycoproteins.

A preliminary communication of these results was made at the XXVII Meeting of the Mexican Society of Physiological Sciences (Morelia, Mexico, July 16, 1984).

Materials and Methods

Cell Culture

Starter MDCK cultures were obtained from the American Type Culture Collection (MDCK, CCL-34) [19]. In most experiments cells were between 60-80th passage. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3250, Cambridge, Mass.) with an air-5% CO_2 atmosphere (VIP CO_2 incubator 417, Lab Line Instruments, Inc., New Brunswick, N.Y.) and 20 ml of Dulbecco's modified Eagle's basal medium DMEM (Grand Island Biological Co. (GIBCO) 430-1600, Grand Island, N.Y.) with 100 U/ml of penicillin, 100 μg/ml of streptomycin (GIBCO 600-5145), 0.08 U/ml of insulin (Eli Lilly, Mexico D.F.), and 10% calf serum (GIBCO 200-6170); this complete medium is referred to in the following text as CDMEM. Cells were harvested with trypsin-EDTA (GIBCO 610-5305) and plated either on disks of a nylon cloth (diameter 1.8 cm) coated with rat tail collagen as described previously [3] and contained in a 24 well Falcon dish (Falcon, Labware, Oxnard, Calif.), or on tissue culture dishes (Lux 5221, Miles Laboratories, Naperville, Ill.). One hour after plating at a density sufficient for confluence (10^6 cells/ml) the newly formed monolayers were washed twice with Ca-free PBS, and transferred from CDMEM to Ca-free MEM (GIBCO 410-1300) for 20 hr. Monolayers were then incubated for different periods of time in fresh DMEM, or exposed for 5 hr to drug treatment.

Drug Treatment of Monolayers

Cytochalasin B (Sigma Chemical Co., St Louis, Mo.) was prepared as a 5 mg/ml stock in dimethylsulfoxide (DMSO) and added to DMEM to a final concentration of 5 μg/ml. DMSO final concentration in medium (0.1%) had no effect in control experiments. Colchicine (Sigma Chemical Co., St. Louis, Mo.) was added directly to DMEM to a final concentration of 2×10^{-5} M. Tunicamycin (Sigma Chemical Co., St. Louis, Mo.) was directly prepared in DMEM at a concentration of 1 μg/ml. Monensin (Calbiochem-Behring Corp., La Jolla, Calif.) was prepared as a 1.5×10^{-2} M stock in absolute ethanol. It was diluted directly in DMEM at a final concentration of 1 μM. The final concentration of ethanol in the medium had no effect in control experiments.

Electrical Measurements

The degree of sealing of the tight junction was evaluated by measuring the electrical resistance across the monolayer. Disks were mounted between two Lucite® chambers of 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; 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 solution was subtracted, and all values reported correspond exclusively to the monolayer. A given disk was used only for a single determination and discarded to avoid leaks due to edge damage.

Freeze Fracture

Freeze-fracture replicas were obtained from monolayers fixed with 2.5% glutaraldehyde for 30 min, and gradually infiltrated with glycerol up to 20% concentration, in which 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 at −120°C, and 2×10^{-6} mm Hg using a Balzers apparatus (FL 9496 Balzers, Leichenstein) equipped with a turbomolecular pump. 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 are shown with the shadowing direction from bottom to top.

To assess modifications in the pattern of tight junction strands, we counted the number of strands intercepting lines drawn perpendicular to the main axis of the junction, as well as the distance between the upper and lowermost strand. The density of IMP is obtained by counting the IMP present in small circles drawn in photographs of different membrane areas. All these procedures have been previously described in detail [9].

Transmission Electron Microscopy (TEM)

Monolayers were fixed with 2.5% glutaraldehyde after different times of incubation in CDMEM. After washing in 0.1 M cacodylate buffer, cells were post-fixed in 1% OSO_4-cacodylate buffer with ruthenium red (0.5 mg/ml), then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut with a diamond

knife, then examined and photographed in a Zeiss EM10 electron microscope.

Results

The ion-selective electrode used to measure the Ca^{2+} concentration in the present study gives a linear response down to 70 μM. Below this concentration the voltage/concentration curve bends and becomes constant. This is not necessarily due to a true Ca content, but to the fact that culture media have high concentrations of Na^+, K^+, H^+ and other ions that the electrode senses when the Ca concentration becomes very low. In any case, 70 μM is the maximal possible Ca^{2+} content in the solution termed "Ca-free." Figure 1 shows that confluent monolayers incubated until the 20th hour in this solution exhibit no transepithelial electrical resistance. Addition of Ca^{2+} at this time elicits an increase in resistance that reaches a maximum of 291 Ω cm^2 in about 4.5 hr, confirming the observation of Cereijido et al. [4]. It may be noticed that the electrical resistance at the 24th hour after restoration of Ca^{2+} (44 hr after plating) is lower than the level reached between the 4th through 6th hour. This effect has also been observed in newly plated monolayers bathed with Ca-containing media, and was attributed to a different time course between junctional sealing and installation of channels in the tight junction [3, 5].

Monolayers incubated without Ca^{2+} are easily recognized by the absence of blisters, and also because when they are collected for electron microscopy they do not come off as a sheet, but as a cloud of cells. In TEM they show no junctional complexes and are freely permeable to ruthenium red (Fig. 2*A*). 1.5 hr after restoration of Ca^{2+}, developing occluding and adhering junctions can be observed, but they fail to stop penetration of the extracellular marker (Fig. 2*B*). At the 4.5th hour the punctuated aspect of the tight junction in TEM is frequently observed in association with desmosomes (Fig. 2*C*) and the interspaces beyond these complexes appear to contain no ruthenium red (Fig. 2*C*). By this time the morphological changes observed by TEM upon addition of Ca^{2+} are completed, as samples taken at the 24th hour convey no new information (Fig. 2*D*).

To provide a quantitative support to the illustrations shown in Fig. 2, we scored the interspaces as penetrated or not by the extracellular marker (Fig. 3). Before addition of Ca^{2+} all interspaces were penetrated (100%), but none was at the 24th hour (0%). However, an intermediate percentage does not mean that interspaces were partially penetrated, because in a given monolayer one may find sealed and

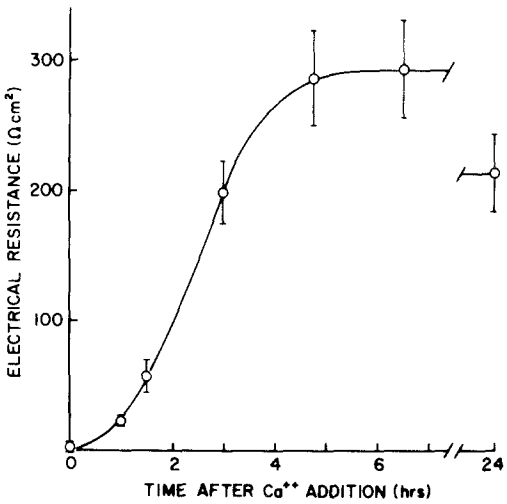

Fig. 1. Development of electrical resistance in monolayers of MDCK cells after Ca^{2+} restoration. Cells were plated at a density sufficient for confluence on collagen-coated nylon disks. One hr after plating disks were tranferred to Ca-free MEM, where they remained for 20 hr. At time marked zero in this figure (20 hr after plating) the monolayers were transferred from Ca-free MEM to CDMEM. Resistance was measured by mounting the monolayer between two Lucite® chambers with CDMEM, passing 100 μA cm^{-2} of current, and recording the voltage deflection. A monolayer was used for a single determination and discarded. The contribution of the collagen support and solutions to the total resistance was subtracted. Results are expressed as mean ± standard error

unsealed junctions. In this respect the curves of Figs. 1 and 3 reflect the macroscopic status of the monolayer, and not necessarily the degree of sealing of individual junctions.

Freeze-fracture replicas of monolayers maintained without Ca^{2+} do not show the image of tight junctions. Occasionally one may observe a knot of strands (Fig. 4*A*) or a short segment (Fig. 4*B*), usually in an abnormal position, such as in between the microvilli of the apical surface. Restoration of Ca^{2+} produces a quick (less than 15 min) development of strands. They appear in replicas as single, long strands, usually interrupted and with occasional loops that part and return to the main strand (Fig. 4*C*). This is reflected in the morphological analysis of the junction, performed as explained by Gonzalez Mariscal et al. [9], indicating that at this time almost 70% of the junctions are constituted by a single strand, and the rest contains almost exclusively two strands (Fig. 5, upper line). Replicas obtained at 1 hr with Ca^{2+} have more strands (Fig. 4*D*) (*see also* histogram in the upper line of Fig. 5), which are also discontinuous, and present loose ends oriented toward the basolateral region. Replicas at the 1.5th hour show junctions with a maxi-

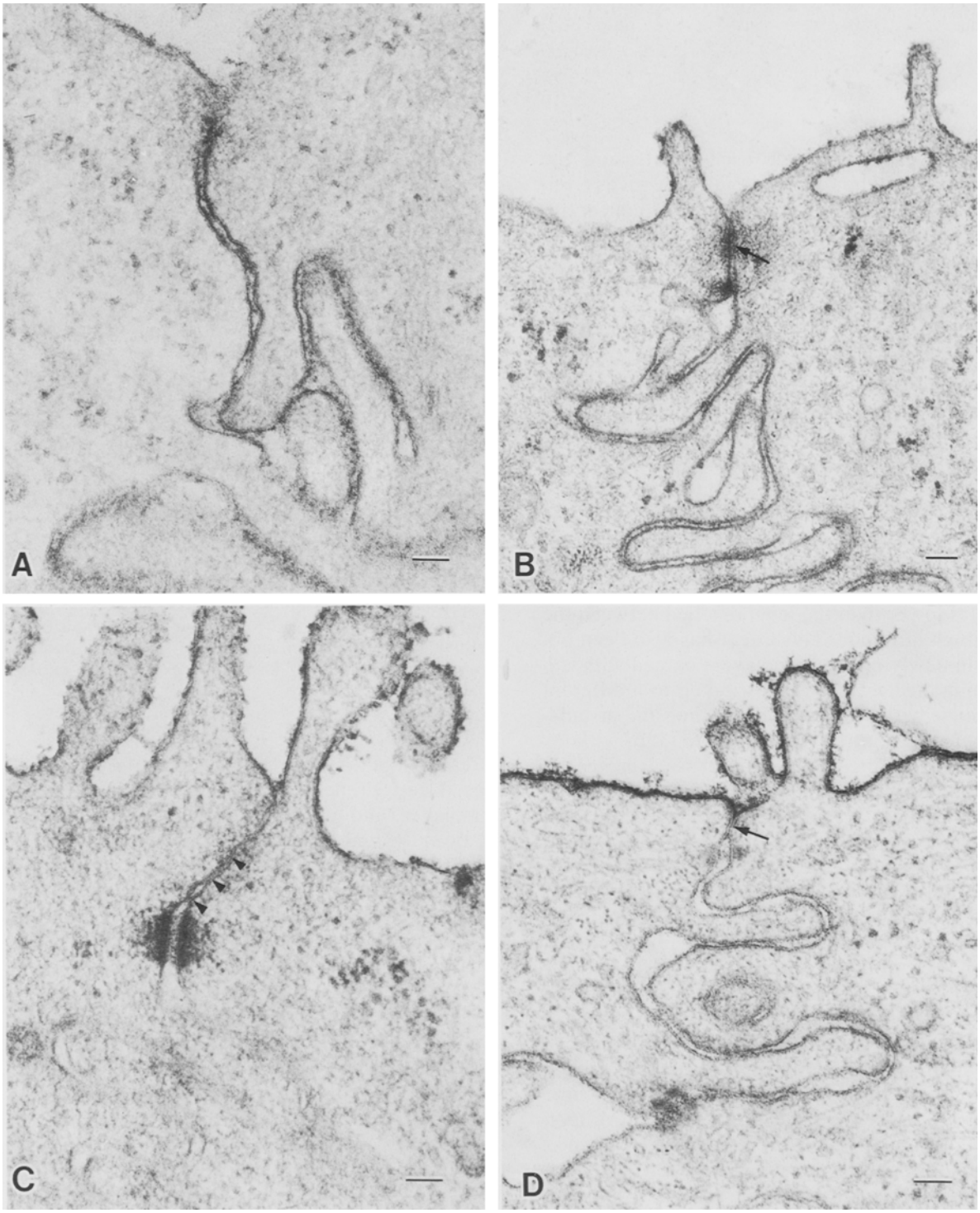

Fig. 2. MDCK monolayers treated with ruthenium red. Cells were plated in CDMEM and transferred 1 hr later to Ca-free MEM. After 20 hr in this medium they were placed in fresh CDMEM. *A*) Cells incubated for 20 hr in Ca-free MEM. Ruthenium red permeates freely though the intercellular space. 60,000×; bar = 100 nm. *B*) Cells incubated for 1.5 hr in fresh CDMEM. Ruthenium red marks the interspace. The arrow points to a developing occluding and adhering junction. 50,000×; bar = 100 nm. *C*) Cells incubated for 4.5 hr in CDMEM. Tight junction intersection points are clearly discernable (*arrowheads*). Ruthenium red does not pass beyond the tight junction. 60,000×; bar = 100 nm. *D*) Cells incubated for 24 hr in fresh CDMEM. The penetration of ruthenium red to the intercellular space is blocked by the tight junction (*arrow*). 60,000×; bar = 100 nm

mum of parallelism and a minimum of intercrossing (Fig. 4*E*). This parallelism is lost in replicas at the 4.5th hour (Fig. 4*F*). By this time the heterogeneity in the distribution of the strands resembles that of mature monolayers (Fig. 4*G*) [1, 3] except that at the 4.5th hour, loose ends are still present, and the degree of crossing is somewhat smaller.

Compared with those of natural epithelia [6], tight junctions between MDCK cells have a very irregular pattern and the number of their strands varies abruptly from 1 to 10 [3, 9]. Therefore, to evaluate the amount of junction produced we defined the following function:

$$\text{Amount of junction} = \sum_{n=1}^{\infty} n_i \%_i \qquad (1)$$

where n_i is the number of strands in a segment of tight junction, and $\%_i$ is the percentage of segments having that number of strands. For instance, if 70% of the segments would have only one strand ($n = 1$; $\% = 70$) and the rest would have two strands ($n = 2$; $\% = 30$), Eq. (1) would indicate that the "amount of junction" is equal to 130. In this way, the second row of Fig. 5 indicates that, after restoration of Ca^{2+}, the strands do not simply change their distribution, but that the amount of material incorporated to the junction increases steadily up to the 4.5th hour. The last row in this figure indicates that, as more substance is incorporated, the junction occupies a wider belt. The final values of the morphological parameters do not differ significantly from those found previously in our laboratory in cells that have always been incubated with Ca^{2+} (number of strands 4.42 ± 0.26 *vs.* 5.2 ± 0.2; width 0.189 ± 0.006 *vs.* 0.247 ± 0.01 μm) [9]. As an incidental observation, we have found some desmosomes trapped between the strands of these junctions, a feature that we never observe with older monolayers.

It may be noticed that, as expected, there is a close agreement between the development of the junction and the degree of sealing expressed by the electrical resistance across the monolayer (Fig. 1 and last row of Fig. 5). Accordingly, in the Table we use this parameter to evaluate the effect of a variety of experimental conditions on the development of the occluding junction. This Table is organized as follows: the first column indicates the condition in which the monolayers were incubated until the 20th hour (yet notice that the first of these 20 hr after plating corresponds to the time allowed for attachment in CDMEM). The second column in the Table reports the value of the electrical resistance at the 20th hour. The third column indicates the condition

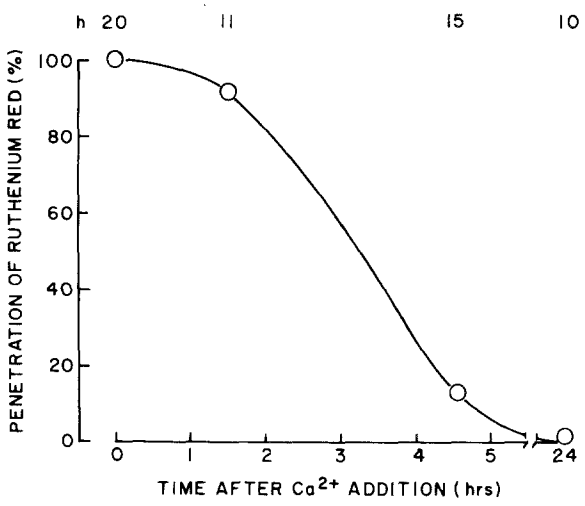

Fig. 3. Percentage of penetration of ruthenium red to the intercellular space of MDCK cells at different terms of incubation with Ca^{2+}. The upper line (n) in this and following figures indicates the number of observations

to which the monolayers were transferred at this time. Finally, the fourth reports the electrical resistance after 4 to 6 hr in such conditions. The first line corresponds to the basic control: monolayers that were incubated for 20 hr in the regular, serum-containing medium and, at this time, were transferred to the same but fresh medium. The different changes do not seem to affect appreciably the value of their electrical resistance (346 *vs.* 331 Ω cm^2). The second line of the Table reports the value found in a set of monolayers that, after the first hour of plating at confluence, have been transferred to medium without serum, and then this medium was renewed at the 20th hour for another 4 to 6 hr. The third line corresponds to monolayers transferred to Ca-free medium after plating. At the 20th hour these monolayers had a negligible resistance across (2.5 Ω cm^2), but their incubation in complete (serum and Ca^{2+}-containing) medium seals their junction as in control monolayers. If instead of transferring them to a complete medium, they are incubated with serum-free DMEM (fourth line) sealing occurs as in control monolayers. These lines indicate therefore that junction formation does not require serum.

If Ca^{2+} is added only for a brief period (15 to 60 min) strands form (Fig. 4) but then vanish (*not shown*) and, accordingly, at the 6th hour the monolayers present minimal electrical resistance (Table, lines 5 and 6).

The next set of incubating conditions was used to obtain information on the degree of maturation of the junctional components. These monolayers started with the same incubating conditions (Ca-

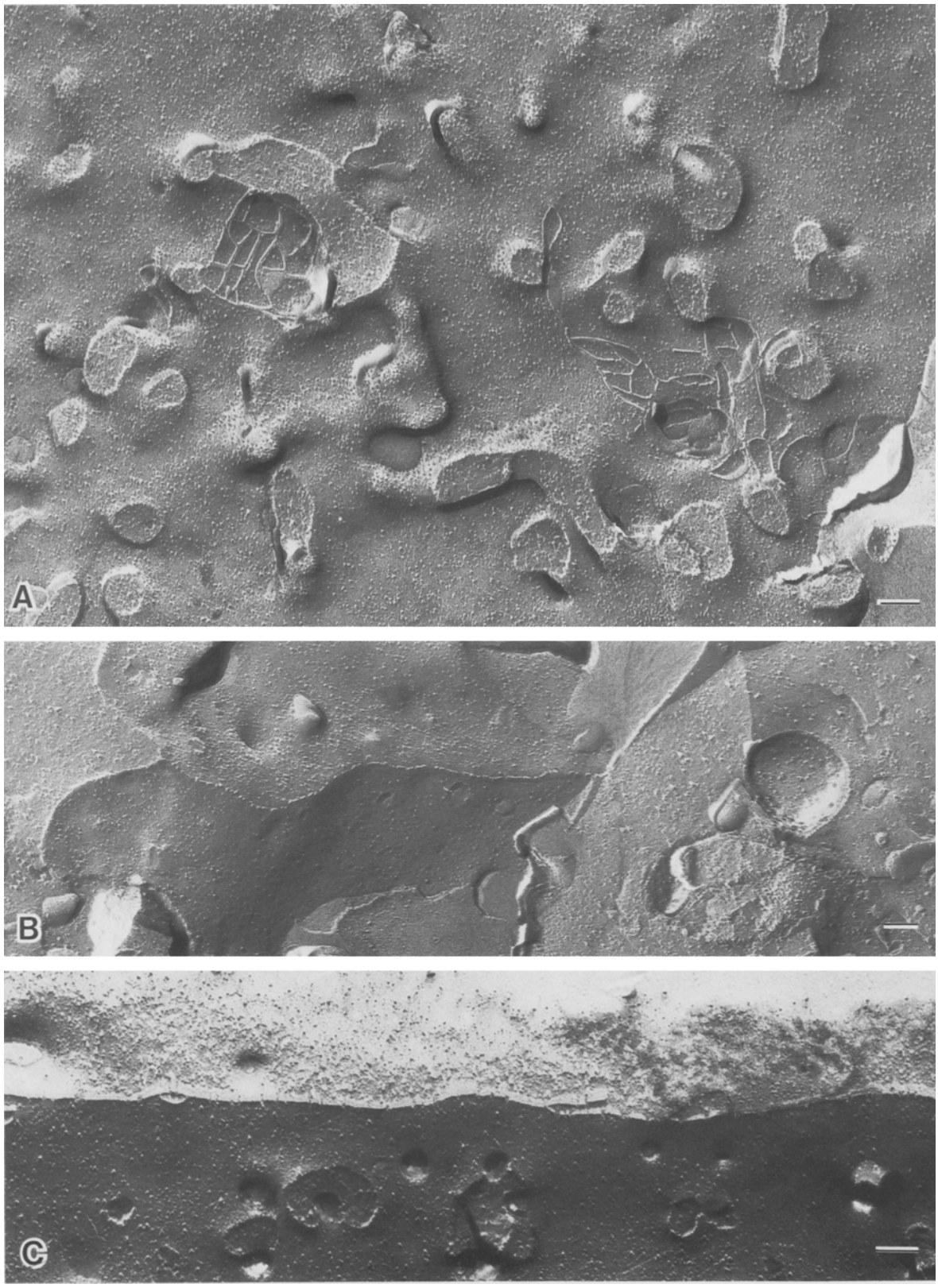

Fig. 4. Freeze-fracture replicas of MDCK cells placed for different periods of time in CDMEM after 20 hr of incubation in Ca-free MEM. *A*) Cells incubated for 20 hr in Ca-free MEM. Knots of strands can occasionally be observed between apical microvillosities. 65,000×; bar = 100 nm. *B*) Cells incubated for 20 hr in Ca-free MEM. The tight junction appears as a single rudimentary short segment. 65,000×; bar = 100 nm. *C*) Cells incubated for 15 min in fresh CDMEM: tight junctions appear as long single strands. 70,000×; bar = 100 nm

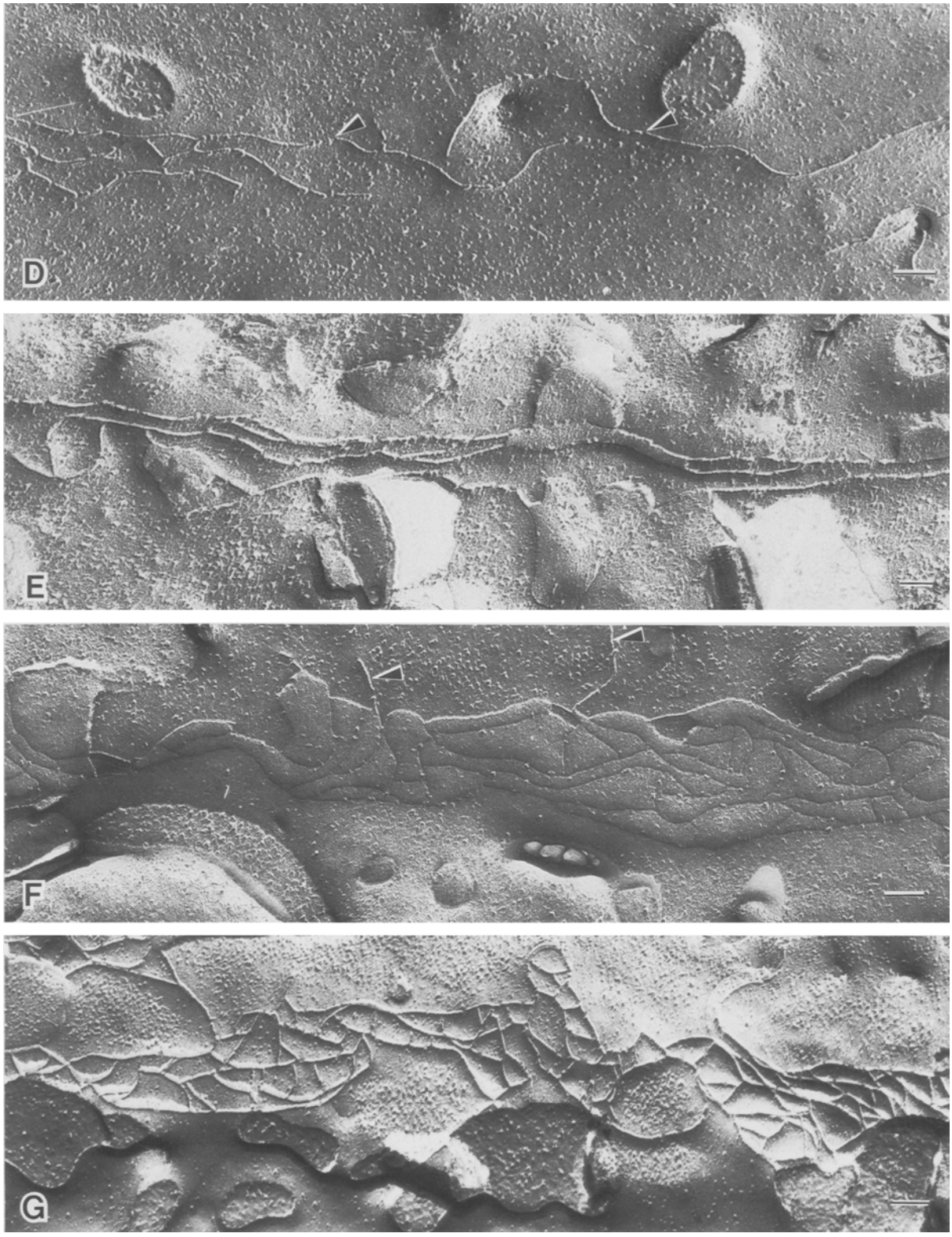

Fig. 4. *D*) 1 hr of incubation in CDMEM. Tight junction most often consists of one, two or three growing and frequently interrupted strands (*arrowhead*). 70,000×; bar = 100 nm. *E*) Cells incubated for 1.5 hr in CDMEM. Note that strands run parallel to each other. 68,000×; bar = 100 mn. *F*) 4.5 hr of incubation in CDMEM. The junction is wider and consists of 5 to 8 undulated strands; loose ends are often found growing from the tight junction strands to the basolateral domain (*arrowheads*). 68,000×; bar = 100 nm. *G*) Cells incubated for 24 hr in CDMEM. The junction consists of a fully developed and irregular network of strands. Two desmosomes are found above the junctional band, bordering the apical face (*arrowhead*). 68,000×; bar = 100 nm

Table. Sealing of tight junctions between MDCK cells, as evaluated by the electrical resistance across the monolayer[a]

Conditions after plating	Electrical resistance, 20 hr (Ω cm^2)[b]	Conditions during incubation	Electrical resistance, 24–26 hr (Ω cm^2)
CDMEM	346 ± 51 (7)	CDMEM	331 ± 38 (20)
DMEM	275 ± 13 (8)	DMEM	238 ± 15 (10)
Ca-free MEM	2.5 ± 0.5 (49)	CDMEM	289 ± 18 (66)
		DMEM	359 ± 28 (9)
		15 min CDMEM → 4.45 hr Ca-free MEM	66 ± 31 (6)
		CDMEM + Cycloheximide	329 ± 18 (12)
		CDMEM + Tunicamycin	334 ± 48 (14)
		CDMEM + Monensin	252 ± 58 (10)
		CDMEM + Cytochalasin B	4 ± 1 (8)
		CDMEM + Colchicine	250 ± 35 (15)
		CDMEM 5°C	11 ± 1 (8)
		CDMEM 25°C	29 ± 3 (7)
CDMEM + Tunicamycin			365 ± 72 (5)
CDMEM + Monensin			194 ± 54 (5)

[a] *First column:* incubating conditions from plating until the 20th hour. *Second column:* electrical resistance at the 20th hour. *Third column:* incubating conditions from the 20th to the 24–26th hour. *Fourth column:* resistance at the 24–26th hour. *1st line:* After 20 hr in complete medium (with serum) monolayers have 346 Ω cm^2 and the change of medium at this time does not modify the resistance 331 Ω cm^2. *2nd line:* In the absence of serum resistance is somewhat lower. *3rd line:* In Ca-free medium resistance does not develop but, upon addition of CDMEM (with Ca^{2-}) at the 20th hour junctions are formed and sealed. *4th line:* If this medium does not have serum junctions also seal. *5th line:* A 15 min Ca^{2+} pulse at the 20th hour achieves only a minor degree of sealing. *Lines 6–10:* report the effect of several drugs. *Lines 11 and 12:* show the effect of temperature. *Line 13:* Tunicamycin present throughout does not impair sealing. *Last line:* monensin decreases the degree of sealing.

[b] Electrical resistance was calculated with the voltage deflection elicited by a current of 100 μA cm^{-2}. The contribution of the support and media was subtracted. DMEM = Dulbecco's modified Eagle's basal medium. CDMEM = DMEM + 10% calf serum + 1000 U/ml penicillin + 100 μg/ml streptomycin + 0.08 U/ml insulin. Cycloheximide = 6 μg/ml. Tunicamycin = 1 μg/ml. Monensin = 1×10^{-6} M. Colchicine = 2×10^{-5} M. Cytochalasin B = 5 μg/ml.

free medium), and at the 20th hour had a negligible electrical resistance across. The inhibition of the synthesis of proteins at this time has no effect on junction formation and sealing (329 Ω cm^2). This complements the information obtained by Cereijido et al. [2, 5], indicating that synthesis of proteins is not required after the 6th hour of plating, and that obtained by Griepp et al. [10], indicating that Ca^{2+} is not required before the cycloheximide-sensitive step. Tunicamycin inhibits the formation of N-linked oligosaccharides in glycoproteins by blocking the earliest steps in the transfer of sugars to the nascent polypeptide [16, 49]. This drug, added either along with Ca^{2+} at the 20th hour (Table, line 8), or 1 hr after plating and present throughout (penultimate line) has no effect in the degree of sealing (Table: 334 and 365 cm^2). But it does affect the junctional pattern (Fig. 6): strands occupy a much wider belt, as if they were pulled in the apical-basolateral axis. Monensin produces a marked slowing of the transit through the Golgi complex of newly synthesized secretory proteins, proteoglycans and plasma membrane glycoproteins [11, 42, 43, 45, 50]. This effect has been confirmed in MDCK cells [33]. The Table shows that this drug produces a significant decrease of the electrical resistance, which is more accentuated when the drug is added 1 hr after plating (last line), and tends to be low (not significantly) when monensin is added at the 20th hour (line 9).

In mature monolayers of MDCK cells the addition of cytochalasin B opens the tight junctions, prevents their resealing when they had been previously opened by EGTA, and disrupts the framework of actin filaments as observed with immunofluorescence antibodies [23, 24]. Meza et al. [24] have mapped the points where current flows

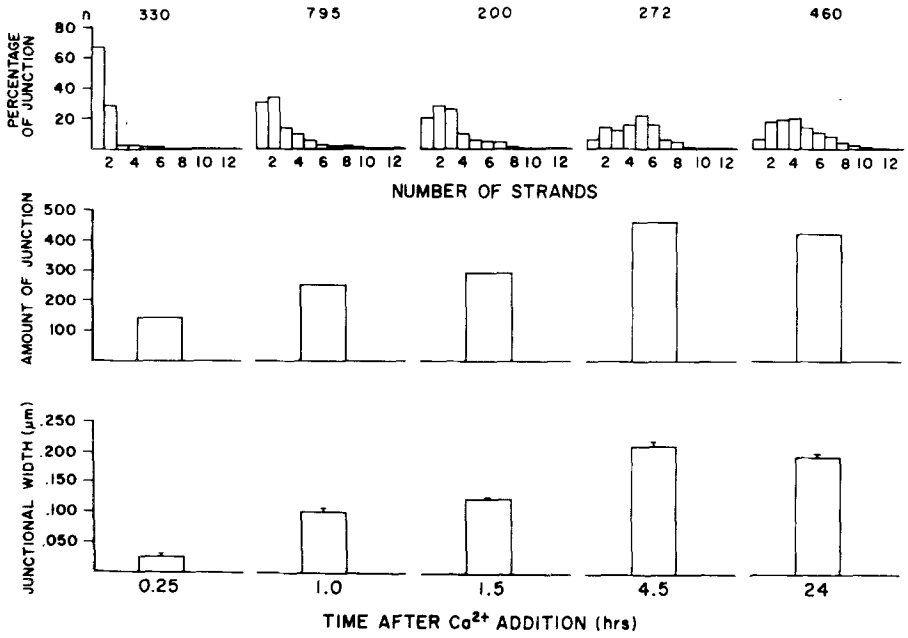

Fig. 5. Morphologic parameters of tight junctions as studied in freeze-fracture electron microscopy. Following 20 hr of incubation in Ca-free MEM monolayers were transferred for different periods of time to CDMEM. *Upper panel:* Frequency of one, two or more strands. Each bar represents the percentage of times that junctions with the indicated number of strands were found. *Middle scheme:* amount of junction at different times of incubation with Ca^{2+}, as calculated with Eq. (1). *Lower scheme:* junctional width

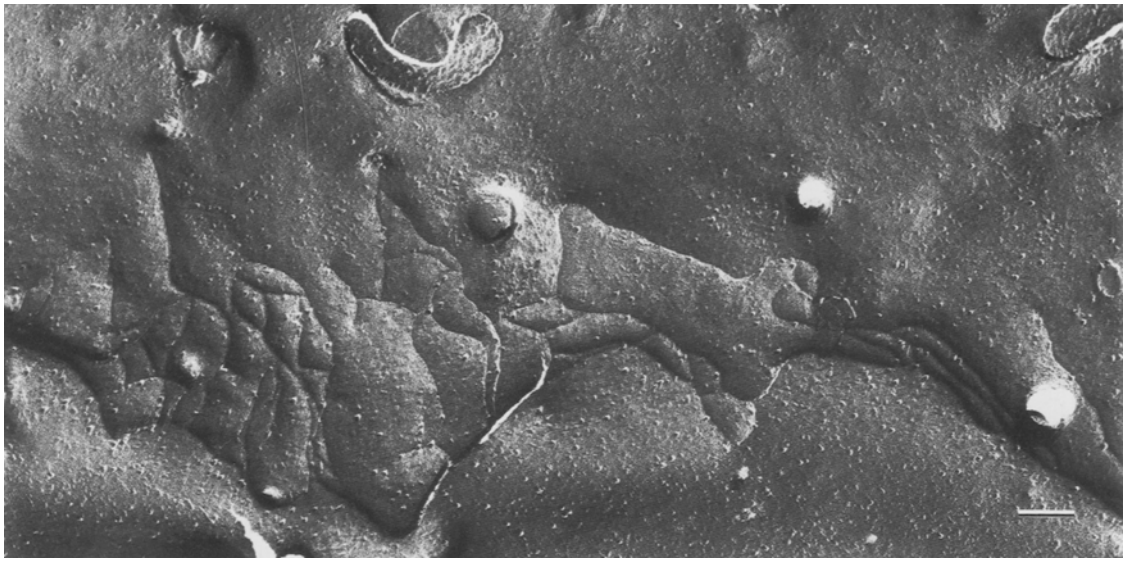

Fig. 6. Freeze-fracture replica of MDCK cells treated with tunicamycin (1 μg/ml) for 5 hr, after 20 hr of incubation in Ca-free MEM. Note that junctional strands occupy a much wider belt as if they were pulled in the apico/basolateral axis. 70,000×. bar = 100 nm

through the monolayer, and observed that the drop of electrical resistance elicited by cytochalasin B is due to the opening of tight junctions and not to the appearance of shunting pathways in the cell soma. However, the methods employed do not allow us to discriminate whether the change is due to a decrease in the sealing capacity of the strands, or because they are pulled apart by morphological changes of the cells. Cytochalasin B also impairs tight junction formation between newly plated MDCK cells [13]. Then it is not surprising that it prevents sealing by Ca^{2+} in the monolayers reported in the Table (line 10). However, it was not known whether the effect of cytochalasin is only due to the opening of sealed strands, inhibition of the first steps in the transfer of components between internal membrane compartments or prevention of their assembly. Freeze-fracture replicas of the

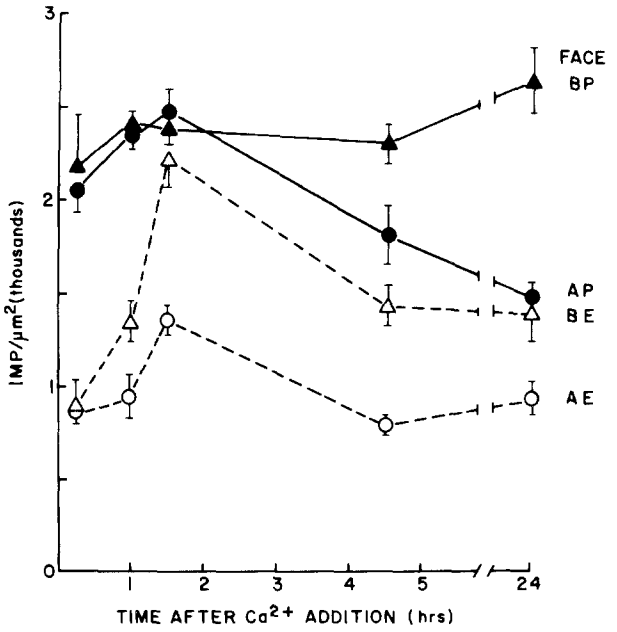

Fig. 7. Density distribution of IMP in plasma membrane faces of MDCK cells at different times of incubation with Ca^{2+}. Each point represents the average of 10 to 94 areas measured. (▲) basolateral P face; (●) apical P face; (△) basolateral E face; (○) apical E face

monolayers treated with cytochalasin B exhibit no strands (*not shown*), suggesting that microfilaments participate in the assembly of the already available components of the strands. Colchicine does not prevent assembly nor sealing of the junctions (Table, line 11) in agreement with previous information obtained in mature monolayers, indicating that they can be opened and resealed by removal and restoration of Ca^{2+} in the presence or absence of this drug [23, 24]. Meldolesi et al. [22] have shown that colchicine has no effect on the pattern of resting tight junctions of acinar pancreatic cells of guinea pig, but it does affect reassembly when they have been subject to removal and restoration of Ca^{2+}, and Rassat et al. [30], have found that this drug disorganizes the strands between rat hepatocytes. Colchicine, therefore, seems to have a distinct effect on tight junctions of different tissues.

A set of experiments on the effect of temperature (lines 12 and 13 in the Table) shows that electrical resistance does not develop below 25°C. When these monolayers are collected for electron microscopy they come off as a cloud of cells and their freeze-fracture replicas resemble those of monolayers maintained without Ca^{2+}. We have recently studied the effect of temperature on the pattern and degree of sealing of mature monolayers, and have observed that a change from 36 to 4°C produces a reversible 304% increase in the electrical resistance without a detectable modification of the junctional pattern [9]. This indicates that the variation of the electrical resistance is due to changes in the state of permeating elements (channels) in the strands, but not in the number nor in the arrangement of the strands themselves. The present results show that cold affects the assembly of the strands.

Several authors [9, 12, 20], have found that IMP are not distributed homogeneously in the plasma membrane of MDCK cells, but show a doubly polarized distribution in E and P faces of apical and basolateral regions. Furthermore, Hoi Sang et al. [13] have demonstrated that the polarized distribution of IMP in newly plated monolayer follows a similar time course as the formation of strands, and Cereijido et al. [4] have demonstrated that in monolayers left for 20 hr without Ca^{2+}, IMP have different densities in E than in P faces, but that they are not polarized in the apical and basolateral regions. The experiments in Fig. 7 were designed to study the fate of IMP when Ca^{2+} was restored. At zero time, before Ca^{2+} is added, the P leaflet (full symbols) has the same density of IMP in its basolateral (BP) than in its apical region (AP). The same lack of polarized distribution is observed in E leaflet (open symbols). The main effect of Ca^{2+} appears to be an addition of IMP to the basolateral region of the outer leaflet (BE) and a removal from the cytoplasmatic leaflet of the apical region (AP). Therefore, Ca^{2+} does not seem to act simply by setting a fence represented by the strands.

Discussion

Previous observations with MDCK cells have indicated that proteic components of the junction are synthesized between the 2nd and the 6th hour after trypsinization [2, 5]. Studies by Griepp et al. [10], have shown that attachment of the cells to a substrate is not a necessary condition for this synthesis, as it may be observed in cells maintained in suspension in a Ca-free spinner medium. In agreement with these observations, cycloheximide does not prevent junction formation in the present study, where cells have been attached for 20 hr before Ca^{2+} is added. Stevenson and Goodenough [41] obtained strong indications that the fundamental protein component of the tight junction in liver cells is constituted by a 37 K peptide. If tight junctions of mammalian origin have a universal basic composition, we may assume that this peptide is also part of the structure of the junctions of MDCK cells. Since tunicamycin does not prevent assembly nor sealing, even when it is added 1 hr after plating, this peptide

might not be glycosylated. In addition, Quaroni et al. [29], have shown that colchicine decreases the intracellular transport of glycoproteins to the apical surface of intestinal epithelial cells, and in the present work we have found no effect of colchicine on tight junction assembly. The inhibition caused by monensin suggests that the junctional peptide is stored in the Golgi complex, as it is known that this drug blocks the transit of secretory proteins, proteoglycans, and plasma membrane proteins from mid to trans Golgi complex [11, 42, 43, 45, 50]. Upon addition of Ca^{2+} this peptide is transferred to the plasma membrane and assembled.

Meza et al. [23, 24], have observed that anti-actin antibodies distribute in a framework that circles MDCK cells in intimate relationship with the tight junction, and that the use of cytochalasin B impairs junctional sealing in a reversible way. When this drug is added 6 hr after plating it prevents junction formation [13]. This, together with our present observation that the drug also inhibits junction formation when its components are already synthesized, suggests that the cytoskeleton of microfilaments may be involved in the transference of the protein of the junction to its position in the cell surface, as well as in the maintenance of the sealed state of the strands. Rodriguez-Boulan and coworkers have demonstrated that, in MDCK cells infected with different types of viruses, budding occurs polarizedly due to the insertion of the enveloped protein in either the apical or the basolateral regions [31, 32, 34, 35], and it was observed that microfilaments are not involved in the polarized insertion of viral proteins [17, 37]. Therefore, it is conceivable that microfilaments might participate in the addressing of some elements (e.g. junctional proteins), but not of others (e.g. those of the viral envelope). This view is supported by the observations of Cereijido et al. [4], that in MDCK cells whose interspaces are left open because of the lack of Ca^{2+}, as in the present work, certain elements in the plasma membrane are polarized (e.g. microvilli, viral proteins) while others are not (e.g. Na,K-ATPase, IMP). It should be emphasized that these interpretations of the effect of the different drugs tested rest on the assumption that they only modify the synthesis, maturation and assembly of proteins. We may not discard the possibility that these drugs would indirectly modify the degree of sealing by introducing changes in the cell's shape. Thus, any secondary effect that would create tensions or impair the exact matching of the strands in neighboring cells would act to decrease the electrical resistance.

Taub et al. [46, 47], have introduced a totally defined media to culture MDCK cells, and have demonstrated that its ability to replace serum is due mainly to its content of hormones, ferritin and selenium. Our observation that serum is not required to form and seal the junctions indicates therefore, that these elements do not participate in these cellular events.

Formation of tight junctions in some tissues is preceded by an aspect of honeycomb in the plasma membrane [14, 18, 25, 28, 38, 39, 44, 48]. We fail to observe such structure in our preparation. We observe instead that, 15 min after the addition of Ca^{2+}, a straight, single strand is already present, even when in some segments it only consists of a string of single particles. The fact that these particles, in spite of not yet being linked to their future neighbors in the strands, are already in position, strongly suggests that the precise location occupied by a tight junction in the surface of the cell is due to a carefully directed insertion of the components, perhaps by a membrane recycling mechanism similar to those reviewed by Farquhar [7]. It seems as if the highest "priority" of the cell were to circle its perimeter with a strand, using whatever junctional material becomes available. Complication of the pattern is observed at later times, as more material is added ("amount of junction," Fig. 5).

The whole process is dependent on temperature: it does not proceed below 25°C. This is in keeping with the known inhibition that cold produces on the transfer to the membrane of newly synthesized proteins, such as viral hemagglutinin [21], and acetylcholinesterase [36].

Ca^{2+} is also required to seal the strands of one cell with those of a neighboring one [20]. Several authors have shown in some natural epithelia, that if Ca^{2+} is absent for many hours strands disassemble [8, 22]. There are at least three reasons to suspect that the sealing role of Ca^{2+} might not be a simple salt link between registered strands in register on opposite cells: 1) the time course of opening and resealing by removal and restoration of Ca^{2+} in mature monolayers is much slower than one would expect from the diffusion of this ion from the bulk solution to the junctional belt or vice versa; 2) in mammary epithelial cells the removal of Ca^{2+} seems to open the junctions through the development of a centripetal tension, and not necessarily through a direct effect on the junction itself [27], and 3) Stevenson and Goodenough [41], have used Ca-chelating agents to fractionate the cellular membrane of mouse hepatocytes and obtained one which was rich in tight junctions. In spite of the Ca-free solutions and chelating agents used, junctions were present in paired vesicles resulting from fractions of two neighboring cells. Apparently, these vesicles were devoid of the cytoplasmic compo-

nents where Ca^{2+} displacements would have elicited the opening of the junction and the disassembly of the strands.

Our experimental protocols were focused on conditions and drugs that convey information on proteic components, but do not exclude the possibility that a quantitatively important component of the junction were made of lipids, as proposed by Kachar and Reese [15], and Pinto da Silva and Kachar [26]. Lipids, though, do not seem to play a central role in the degree of sealing nor in paracellular permeation (*see* [9]).

The observations that the polarized distribution of IMP [20], develops simultaneously with the establishment of tight junctions in newly plated MDCK cells [13], as well as in the preparation used in the present work, might stress the common misconception that junctions act as fences that restrain apical and basolateral components from mixing by diffusing in the plane of the membrane, and are "*therefore*" responsible for their polarized distribution. Apart from the fact that some elements may present a polarized distribution even in the absence of junctions [4], when a fence is installed in an MDCK cell whose membrane components became randomized during their harvesting with trypsin-EDTA, it cannot promote, but *impair* sorting of membrane elements through lateral diffusion in the plane of the membrane. Furthermore, it may be observed (Fig. 4) that a strand is already present by 15 to 90 min, but polarization of IMP takes a much longer time (Fig. 7), including periods where the mature pattern of the tight junction is essentially completed. This suggests that polarization of IMP is also achieved by insertion and removal, probably by a directed membrane recycling mechanism, and not by lateral displacements of membrane components.

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References

1. Cereijido, M., Ehrenfeld, J., Meza, I., Martinez-Palomo, A. 1980. Structural and functional membrane polarity in cultured monolayers of MDCK cells. *J. Membrane Biol.* **52:**147–159
2. Cereijido, M., Meza, I., Martinez-Palomo, A. 1981. Occluding junctions in cultured epithelial monolayers. *Am. J. Physiol.* **240:**C96–C102
3. Cereijido, M., Robbins, E.S., Dolan, W.J., Rotunno, C.A., Sabatini, D.D. 1978*a*. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **77:**853–880
4. Cereijido, M., Rodriguez-Boulan, E., Borboa, L., Gonzalez-Robles, A., Beaty, G. 1983. The relationship between occluding junctions and polarity in epithelioid cells (MDCK). *J. Cell Biol.* **97:**80a
5. Cereijido, M., Rotunno, C.A., Robbins, E.S., Sabatini, D.D. 1978*b*. Polarized epithelial membranes produced *in vitro*. *In:* Membrane Transport Processes. J.F. Hoffman, editor. Vol. 1, pp. 433–461. Raven, New York
6. Cereijido, M., Stefani, E., Chávez de Ramírez, B. 1982. Occluding junctions of the *Necturus* gallbladder. *J. Membrane Biol.* **70:**15–25
7. Farquhar, M.G. 1983. Multiple pathways of exocytosis, endocytosis, and membrane recycling: Validation of a Golgi route. *Fed. Proc.* **42:**2407–2413
8. Galli, P., Brenna, A., De Camili, P., Meldolesi, J. 1976. Extracellular calcium and the organization of tight junctions in pancreatic acinar cells. *Exp. Cell Res.* **99:**178–183
9. Gonzalez-Mariscal, L., Chávez de Ramírez, B., Cereijido, M. 1984. The effect of temperature on the occluding junctions of monolayers of epithelioid cells (MDCK). *J. Membrane Biol.* **79:**175–184
10. Griepp, E.B., Dolan, W.J., Robbins, E.S., Sabatini, D.D. 1983. Participation of plasma membrane proteins in the formation of tight junctions by cultured epithelial cells. *J. Cell Biol.* **96:**693–702
11. Griffith, G., Quinn, P., Warren, G. 1983. Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from medial to trans Golgi cisternae in baby hamster kidney cells infected with semliki forest virus. *J. Cell Biol.* **96:**835–850
12. Hoi Sang, U., Saier, M.H., Jr., Ellisman, M.H. 1979. Tight junction formation is closely linked to the polar redistribution of intramembranous particles in aggregating MDCK epithelia. *Exp. Cell Res.* **122:**384–392
13. Hoi Sang, U., Saier, M.H., Ellisman, M.H. 1980. Tight junction formation in the establishment of intramembranous particle polarity in aggregating MDCK cells. *Exp. Cell Res.* **128:**223–235
14. Humbert, F., Montesano, R., Perrelet, A., Orci, L. 1976. Junctions in developing human and rat kidney: A freeze-fracture study. *J. Ultrastruct. Res.* **56:**202–214
15. Kachar, B., Reese, T. 1982. Evidence for the lipidic nature of tight junction strands. *Nature (London)* **296:**64–66
16. Lennarz, W. 1975. Lipid linked sugars in glycoprotein synthesis. *Science* **188:**986–991
17. Lopez-Vancell, R., Beaty, G., Cereijido, M. 1982. El estado de las permeabilidades pasiva y activa al sodio y potasio de las membranas de celulas MDCK durante la gemacion asimetrica de virus. XXV Congreso Nacional de Ciencias Fisiologicas. Guadalajara, Jal. Julio 21 de 1982
18. Luciano, L., Thiele, G., Reale, E. 1979. Development of follicles and of occluding junctions between the follicular cells of the thyroid gland. *J. Ultrastruct. Res.* **66:**164–173
19. Madin, S.H., Darby, N.B. 1958. As catalogued in: American Type Culture Collection. *Catalog of Strains.* **2:**574–576
20. Martinez-Palomo, A., Meza, I., Beaty, G., Cereijido, M. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelia. *J. Cell Biol.* **87:**736–745
21. Matlin, R.S., Simmons, K. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosilation. *Cell* **34:**233–243
22. Meldolesi, J., Castiglioni, G., Parma, R., Nassivera, N., De Camilli, P. 1978. Ca^{++}-dependent disassembly and reassem-

bly of occluding junctions in guinea pig pancreatic acinar cells. Effect of drugs. *J. Cell Biol.* **79:**156–172
23. Meza, I., Ibarra, G., Sabanero, M., Martinez-Palomo, A., Cereijido, M. 1980. Occluding junctions and cytoskeletal components in a cultured transporting epithelium. *J. Cell Biol.* **87:**746–754
24. Meza, I., Sabanero, M., Stefani, E., Cereijido, M. 1982. Occluding junctions in MDCK cells: Modulation of transepithelial permeability by the cytoskeleton. *J. Cell. Biochem.* **18:**407–421
25. Montesano, R., Friend, D.S., Perrelet, A., Orci, L. 1975. *In vivo* assembly of tight junctions in fetal rat liver. *J. Cell Biol.* **67:**310–319
26. Pinto da Silva, P., Kachar, B. 1982. On tight junction structure. *Cell* **28:**441–450
27. Pitelka, D.R., Taggart, B.N., Hamamoto, S.T. 1983. Effects of extracellular calcium depletion on membrane topography and occluding junctions of mammary epithelial cells in culture. *J. Cell Biol.* **96:**613–624
28. Porvaznir, M., Johnson, R.G., Sheridan, J.D. 1979. Tight junction development between cultured hepatoma cells: Possible stages in assembly and enhancement with dexamethasone. *J. Supramol. Struct.* **10:**13–30
29. Quaroni, A., Kirsch, K., Weisen, M.M. 1979. Synthesis of membrane glycoproteins in rat mold intestinal villus cells. Effect of colchicine on the redistribution of L-[1,5,6-3H] fucose-labelled membrane glycoproteins among Golgi, lateral, basal and microvillus membranes. *Biochem. J.* **182:**213–221
30. Rassat, J., Robenek, H., Themann, H. 1982. Alterations of tight and gap junctions in mouse hepatocytes following administration of colchicine. *Cell Tissue Res.* **223:**187–200
31. Rodriguez-Boulan, E. 1983. Membrane biogenesis, enveloped RNA viruses, and epithelial polarity. *In:* Modern Cell Biology. Vol. 1, pp. 119–170. Alan R. Liss, New York
32. Rodriguez-Boulan, E., Paskiet, K., Sabatini, D.D. 1981. Asymmetric budding of enveloped viruses from isolated epithelial cells attached to a collagen substrate. *J. Cell Biol.* **91:**121a
33. Rodriguez-Boulan, E., Paskiet, K., Salas, P., Bard, E. 1984. Intracellular transport of influenza virus hemaglutinin to the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* **98:**308–319
34. Rodriguez-Boulan, E., Pendergast, M. 1980. Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. *Cell* **20:**45–54
35. Rodriguez-Boulan, E., Sabatini, D.D. 1978. Asymmetric budding of viruses in epithelial monolayers: A model system for study of epithelial polarity. *Proc. Natl. Acad. Sci. USA* **75:**5071–5075
36. Rotundo, R.L., Fambrough, D.M. 1980. Secretion of acetylcholinesterase: Relation to acetylcholine receptor metabolism. *Cell* **22:**595–602
37. Salas, P.J.I., Vega-Salas, D.E., Rodriguez-Boulan, E. 1985. Roll of cytoskeleton in epithelial cell polarity. Effect of cytochalasin D and colchicine on the polarized budding of enveloped virus from Madin-Darby canine kidney (MDCK) cells. *J. Cell Biol. (in press)*
38. Sasaki, T., Higashi, S., Tachikawa, T., Yoshiki, S. 1982. Formation of tight junctions in differentiating and secretory ameloblasts of rat molar tooth germs. *Arch. Oral Biol.* **27:**1059–1068
39. Schneeberger, E.E., Walters, D.V., Olver, R.E. 1978. Development of intercellular junctions in the pulmonary epithelium of the foetal lamb. *J. Cell Sci.* **32:**307–324
40. Sedar, A.W., Forte, J.G. 1964. Effects of calcium depletion on the junctional complex between oxyntic cells of gastric glands. *J. Cell Biol.* **22:**173–188
41. Stevenson, B.R., Goodenough, D.A. 1984. Zonulae occludentes in junctional complex-enriched fractions from mouse liver: Preliminary morphological and biochemical characterization. *J. Cell Biol.* **98:**1209–1221
42. Strous, G., Lodish, H.F. 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. *Cell* **22:**709–717
43. Strous, G., Willemsen, R., Kerkhof, P. van, Slot, J.W., Genze, H.J., Lodish, H.F. 1983. Vesicular stomatitis virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. *J. Cell Biol.* **97:**1815–1822
44. Suzuki, F., Nagano, T. 1979. Morphogenesis of tight junctions in the peritoneal mesothelium of the mouse embryo. *Cell Tissue Res.* **198:**247–260
45. Tartakoff, A.M. 1983. Perturbation of vesicular traffic wih the carboxylic ionophore monensin. *Cell* **32:**1026–1028
46. Taub, M., Chuman, L., Jr., Saiers, M.H., Sato, G. 1979. Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone supplemented, serum-free medium. *Proc. Natl. Acad. Sci. USA* **76:**3338–3342
47. Taub, M., Sato, G. 1980. Growth of functional primary cultures of kidney epithelial cells in defined medium. *J. Cell. Physiol.* **105:**369–378
48. Tice, L.W., Carter, R.L., Cahill, M.C. 1977. Tracer and freeze fracture observations on developing tight junctions in fetal rat thyroid. *Tissue Cell* **9:**395–417
49. Tkacz, J.S., Lampen, J.O. 1975. Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf-liver microsomes. *Biochem. Biophys. Res. Commun.* **65:**248–257
50. Uchida, N., Smilowitz, H., Ledger, P.W., Tanzer, M.L. 1980. Kinetic studies of the intracellular transport of Procollagen and Fibronectin in human fibroblasts. Effects of the monovalent ionophore monensin. *J. Biol. Chem.* **255:**8638–8644

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