Journal of Cellular Biochemistry 18:407–421 (1982)
Cellular Recognition 687–701

Occluding Junctions in MDCK Cells: Modulation of Transepithelial Permeability by the Cytoskeleton

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In MDCK cell monolayers the opening and resealing of occluding junctions can be induced by removal and restoration of calcium to the external medium. The overall changes in permeability of the occluding junctions in the monolayer can be monitored by the drop and recovery of the total transepithelial electrical resistance. We have investigated the effects of cytochalasin B (CB) on this process. When CB is added to sealed monolayers there is a gradual drop in the electrical resistance across the monolayer. This drop is accompanied by a slow disorganization of the microfilament pattern of these cells, including a disturbance of a ring of cortical microfilaments that is normally associated with the junctions. Cells in open monolayers treated with CB will not reseal and have an altered filament distribution. These cells do not have a continuous cortical ring.

We have used a voltage scanning technique that uses a microelectrode to measure the resistance at selected points along the junction which surrounds a single cell. In untreated, closed monolayers, the junction is heterogeneous with alternating points of high and low conductance. In closed monolayers treated with CB, although there are low conductance points, we have observed an increased frequency of high conductance points that correlates with the change in the overall conductance. The frequency of high conductance points along the junction and the overall conductance both increase with time of exposure to CB.

In an effort to understand the molecular basis for the permeability changes induced by EGTA and CB, we have looked for differences in the protein components of the cell membranes of open, closed, and CB-treated MDCK monolayers. This was done by radioiodinating the surface membrane proteins under control and experimental conditions that bring about permeability changes. No significant differences in the labeled protein patterns were found under these conditions. These results suggest that the permeability changes involve only a structural rearrangement of membrane components. In addition we have observed that about 36% of the surface label remains bound to the insoluble cytoskeletons obtained from cells in control and experimental conditions that alter the permeability of the tight junctions. The iodinated proteins attached to the CS include polypeptides with M_r of $\geq$ 120K daltons as well as peptides with M_r = 56K, 50K, 36K, and 18K daltons.

Key words: MDCK cells, occluding junctions, permeability, cytoskeleton

Received August 28, 1981; accepted November 23, 1981.

0730-2312/82/1804-0407$04.50

Cultured MDCK cells form a transporting epithelial monolayer joined together by junctional complexes. These complexes include desmosomes and tight, or occluding junctions, which form a permeability barrier. These cultured monolayers develop a transepithelial resistance several hours after plating at high density [1]. Once established, this epithelial resistance can be reversibly raised and lowered by changing the Ca^{++} in the medium [2]. The removal of Ca^{++} results in the rapid opening of the occluding junctions as measured by a decrease in the transepithelial resistance. This decrease in resistance seems to be accompanied by a slight change in the distribution of the strands, seen using freeze fracture, that form the tight junction. Addition of Ca^{++} restores the resistance and the frequency of more complex patterns of junctional strands [3]. This Ca^{++}-dependent modification of the permeability of the occluding junction occurs in the presence of protein synthesis inhibitors indicating that the process does not require "de novo" synthesis of junctional proteins and suggests a rearrangement of preexisting components [3,4].

Using indirect immunofluorescence techniques we have followed the organization of microtubules and microfilaments during formation of the junctions and during opening and resealing. A detailed study of the cytoskeleton organization was first performed in well-spread MDCK cells. In these cells there is a radial distribution of microtubules around an organizing center. Actin filaments are present, arranged as stress fibers and thicker bundles of filaments underlying the plasma membrane [4,5]. In confluent monolayers the cells become taller and the superposition of optical planes impairs the clear visualization of individual fibers. However, we could observe that microtubules maintain their distribution; the actin stress fibers disappear, while cortical bundles form a continuous ring at the periphery of the cell. The actin staining also shows a diffuse filamentous network in both spreading and confluent cells [4,5]. We have shown that colchicine breaks down the microtubules in the MDCK cells but does not alter the microfilaments and does not affect the electrical resistance of the monolayers [6]. Cytochalasin B (CB), however, disorganizes the microfilament pattern and decreases the overall transepithelial resistance [6].

In subconfluent monolayers CB produces marked morphological changes similar to those described in other cell types by Goodman and Miranda [7] and termed arborization and formation of zeiotic blebs. The same changes are observed in confluent monolayers whose tight junctions have been opened with EGTA. However, confluent monolayers treated with CB but in the presence of Ca^{++} remain with their cells in apposition and do not arborize even after 2 hr of drug treatment. We now port the use of a voltage scanning technique to study in detail the effects of CB at selected points on a single junction. Our cytochalasin results suggest that microfilaments are necessary to establish and maintain the permeability barrier in the monolayer. The exact mechanism for formation of the occluding junction is unclear, but could involve direct microfilament-junctional component interactions. Therefore disruption of the microfilament network could alter the distribution of membrane, particularly junctional components. We report here on studies designed to look for differences in the iodination patterns of surface proteins in open, closed, and CB-treated monolayers and for changes in the interaction of these proteins with the cytoskeleton.

MATERIALS AND METHODS

Cell Culture

MDCK cells, passage 59, were obtained from Flow Laboratories (Irvine, CA) and grown in complete Eagle's minimal essential medium (CMEM) with Eagle's salts, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Tissue culture media and reagents were obtained from GIBCO (Grand Island, NY). Cells were harvested with trypsin-EDTA and plated at high density (2×10^6 cells/ml) on disks of nylon cloth coated with collagen or on Petri dishes. A confluent monolayer is formed that starts to develop a measureable electrical resistance in 5 hr and reaches a steady value in 20–24 hr [1,2]. In all the experiments described here we used monolayers that had a steady resistance for 24 hr or more. CB (Aldrich Chemical Co., Milwaukee, WI) was dissolved in DMSO. The final concentration of DMSO added to the cells was 0.1%. Similar concentration of DMSO by itself had no effect on the permeability of the junctions or structure of the cells.

Electrophysiological Measurements

Overall electrical resistance. Nylon disks with the confluent monolayers were mounted as a flat sheet between two Lucite chambers. The exposed area was of 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 measured with a second set of Ag/AgCl electrodes placed 1.0 mm from the disk. From the voltage change the transepithelial resistance can be calculated. The contribution of a collagen-coated nylon disk without cells was subtracted from all calculated values of resistance.

Localization of conductive pathways by voltage scanning. When current crosses the monolayers through a narrow path, such as an intercellular space, the current density at the mouth of the opening is different than the density a few microns away. This difference can be measured by placing a microelectrode at the mouth of the opening or about 10 μ above the opening. Current pulses are generated across the monolayer and the sets of pulses from each location are accumulated in a computer and subtracted from each other. If a current is actually flowing through that point, there will be a net signal whose amplitude is proportional to the electrical conductance of the spot under the microelectrode. The measurement can be made at several points along a given junction to map the distribution of the conductive pathways (for further details, see [8]).

Immunofluorescence Staining

Monospecific antibodies against brain tubulin and muscle actin were prepared and purified as described previously [6]. Cytoskeletons were prepared by lysing the cells in the monolayer with 0.5% Triton X-100 for 5 min as described by Brown et al [9], then fixing with methanol and formaldehyde. Indirect immunofluorescence staining was done using a fluorescein-labeled goat antirabbit IgG serum obtained from Miles Laboratories, Elkhart, IN. Preparations were viewed in a Zeiss 18 standard microscope equipped with epifluorescence optics. All photographs were taken with a 63× Planapochromat objective.

Labeling and Electrophoresis of Surface Proteins

Cell monolayers grown on Petri dishes were labeled with ^{125}I following the technique described by Hubbard and Cohn [10] using 250 μCi/ml of ^{125}I (New England Nuclear, Boston, MA), 12.5 μg of lactoperoxidase (Sigma Chemical Co., St. Louis, MO), 0.003% H_2O_2, and 0.02 mM KI in a reaction mixture of 500 μl per 35 mm dish containing 8×10^5 cells. The dish was kept on ice and constantly rotated so that the cells were covered with the reaction mixture. Reaction was stopped adding an excess of cold 10^{-3} M KI. Monolayers were rinsed extensively with PBS containing 5 mM phenylmethylsulfonylfluoride (PMSF). Cells were scraped from the dish directly into 1% SDS with a rubber policeman and prepared for electrophoresis.

Cytoskeletons of radiolabeled cells were prepared the same way as those for immunofluorescence by solubilizing the labeled membranes with 0.5% Triton X-100 for 5 min. The solubilized fraction was centrifuged at 10,000g for 10 min and the supernatant was precipitated with an equal volume of 20% TCA, rinsed twice with acetone, and put into solution in the electrophoresis buffer. The cytoskeletons remaining attached to the dish were solubilized with 1% SDS, and prepared for electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Laemmli [11]. Equivalent numbers of counts were used for each protein fraction to facilitate comparison of the patterns. Gels were stained with Coomassie blue to visualize markers and then treated with dimethylsulfoxide and PPO for fluorography as described by Bonner and Laskey [12].

RESULTS

Effect of CB on the Cell Shape

Figure 1 shows the effect of CB in confluent and subconfluent monolayers as seen by phase microscopy. In the presence of normal concentration of Ca^{++} the cells in confluent monolayers do not become arborized when CB is added to the medium. A comparison of Figure 1A and 1B shows that under these conditions, cells remain in aposition and there are not marked structural alterations even after 90 min in the presence of the drug. In Figure 1B some cells have detached from their neighbors, leaving a free border where signs of blebbing begin to show. In Figure 1C and 1D, the effect of CB on subconfluent monolayers grown in the presence of normal concentration of Ca^{++} is shown. In this case the arborization of the cells and the formation of blebs is very clear. In Figure 1E and 1F the effect of CB on control monolayers and monolayers treated with EGTA, and therefore with the tight junctions open, is shown. These cells present signs of arborization as soon as 2 min after the addition of CB. The effect however is not as marked as in subconfluent cells. If Ca^{++} is added back to the medium, some cells recover their normal shape, even if CB is present. If CB is removed, the cells recover completely (figures not shown).

Effects of CB on Microfilaments and the Transepithelial Resistance

Figure 2A shows the effect of CB on the transepithelial resistance of closed MDCK cell monolayers. The average value for closed monolayers in the resting state is $124 \pm 7\ \Omega\ cm^2$. The addition of CB at 5 μg/ml with a final concentration of 0.1% DMSO produces a gradual drop in the resistance. Two hours after CB addition the resistance has fallen to about 25% of the control value. At the same time,

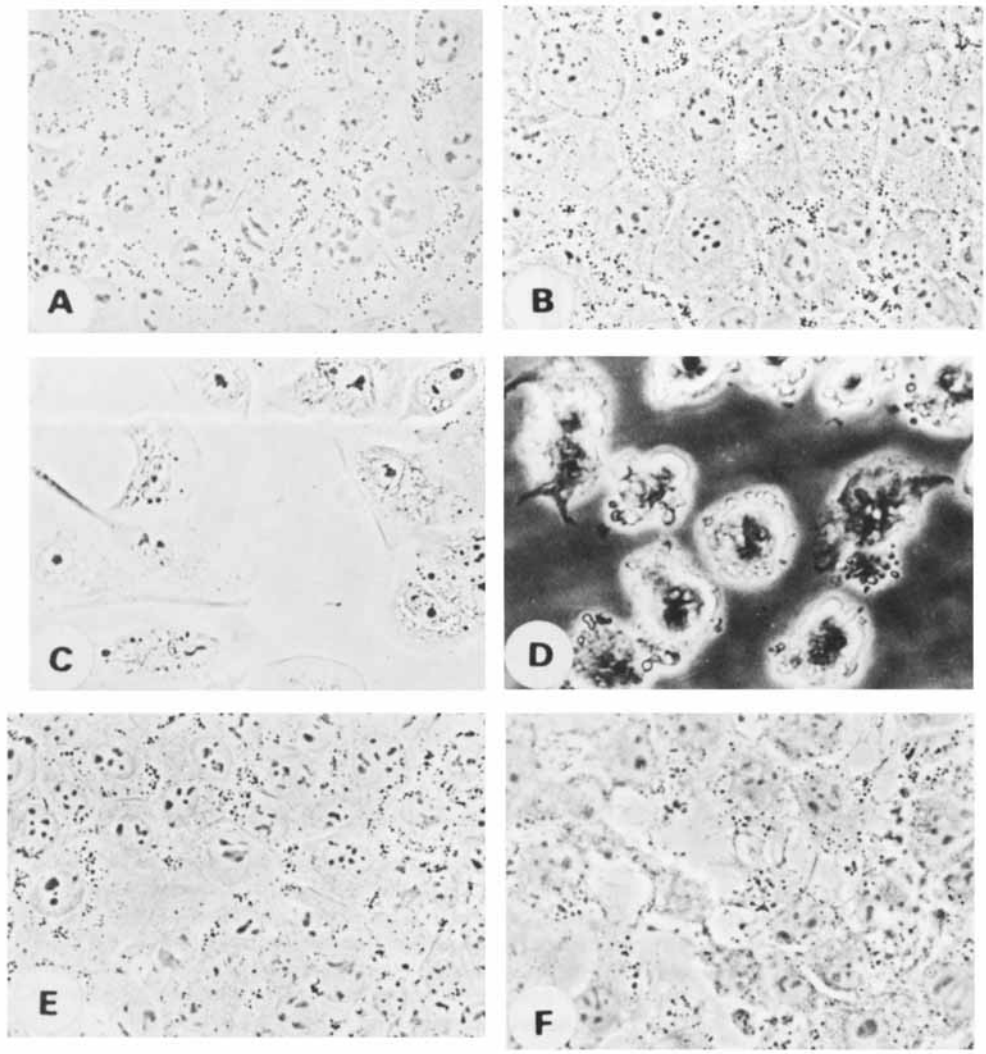

Fig. 1. Phase contrast microscopy of MDCK monolayers treated with cytochalasin B. (A) control monolayer plated at high density in CMEM and cultured for 28 hr. (B) A confluent monolayer treated with 5 μg/ml of CB for 90 min in the presence of normal concentrations of Ca^{++}. (C) Control monolayer plated at low density and cultured for 5 hr in CMEM. Many cells remain isolated and others begin to form groups of 2–3 cells. (D) A subconfluent monolayer treated with 5 μg/ml of CB for 15 min in the presence of normal concentrations of Ca^{++}. Note the marked effect on the drug on the shape of the cells (arborization and formation of blebs in the periphery). (E) Control monolayer, same conditions as in (A). (F) A confluent monolayer treated with 2mM EGTA in medium without Ca^{++} and 5 μg/ml of CB for 15 min. Note again the change in shape of the cells to the arborized type. × 800.

the drug produces an alteration in the microfilament network. By 30 min there is a slight patching of the fluorescence and discontinuity of the ring of actin filaments, which is visible near the membrane in the control cells (compare Fig. 2B and 2C). After 2 hr in CB, when the resistance is low, there is a more pronounced organization of the microfilaments in the ring and the central cytoplasm (compare Fig. 2B

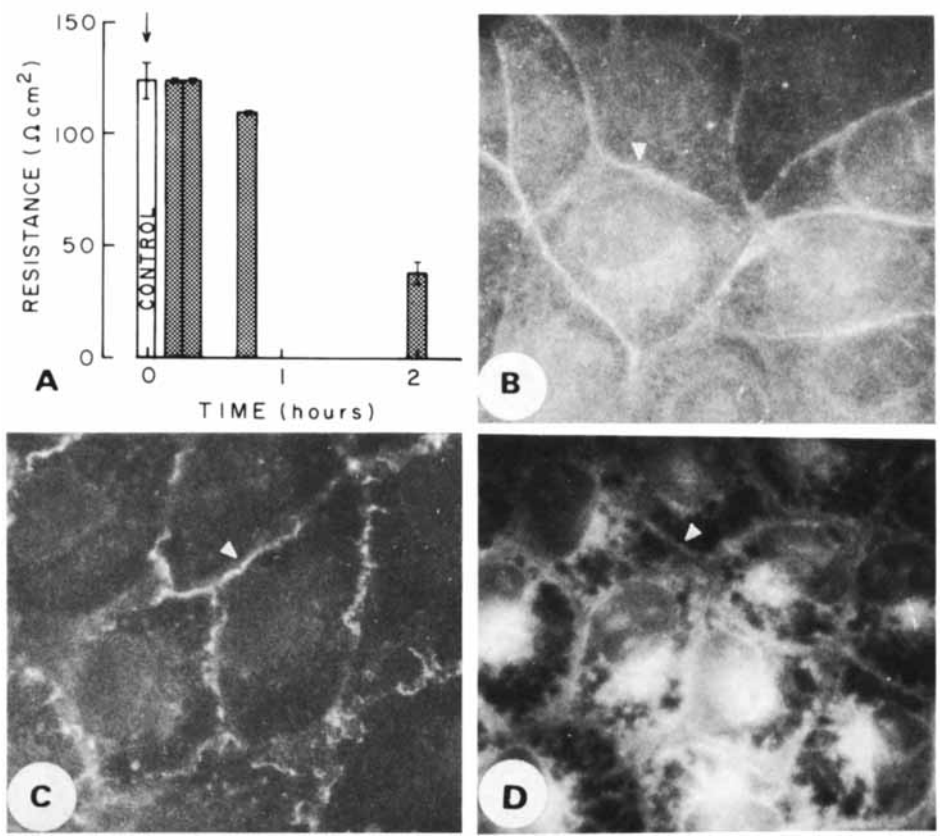

Fig. 2. Effect of CB on the overall electrical resistance and microfilament distribution of closed monolayers. (A) Resistance was measured as indicated in Methods. Results are expressed at the mean ± standard error. The number of observations in each point corresponds to 6–10 individual monolayers, each one withdrawn at the time of the measurement and discarded afterwards. CB (5 μg/ml) was added at the time marked by the arrow and was present throughout the experiment (shaded bars). (B) Actin distribution in a closed, untreated monolayer as visualized by immunofluorescence. (C) Actin distribution after 60 min in CB. (D) Actin distribution after 120 min in CB. × 2,400.

and 2D). Even after 2 hr in CB, when the monolayers are partially open, there is little indication of arborization and this occurs at the free edges of the monolayer or in places where a cell is lost.

When the occluding junctions are opened, by chelation of extracellular Ca^{++} with 2.4 mM EGTA, there is an immediate drop in the electrical resistance (Fig. 3A, first arrow). The readdition of Ca^{++} (Fig. 3A, second arrow) restores the resistance (open columns). The presence of CB does not affect the drop in resistance upon removal of Ca^{++} (shaded columns) but does block the recovery of the resistance after Ca^{++} restoration. Addition of EGTA and CB results in a rapid change (2 min) in cell shape. The cells detach from their neighbors, arborize, and form zeiotic blebs. In these arborized cells, there is a marked movement of the ring toward the nucleus (compare Fig. 3b and 3C). The ring usually does not extend out into the cell processes and clearly is no longer tightly associated with the cell periphery, which ap-

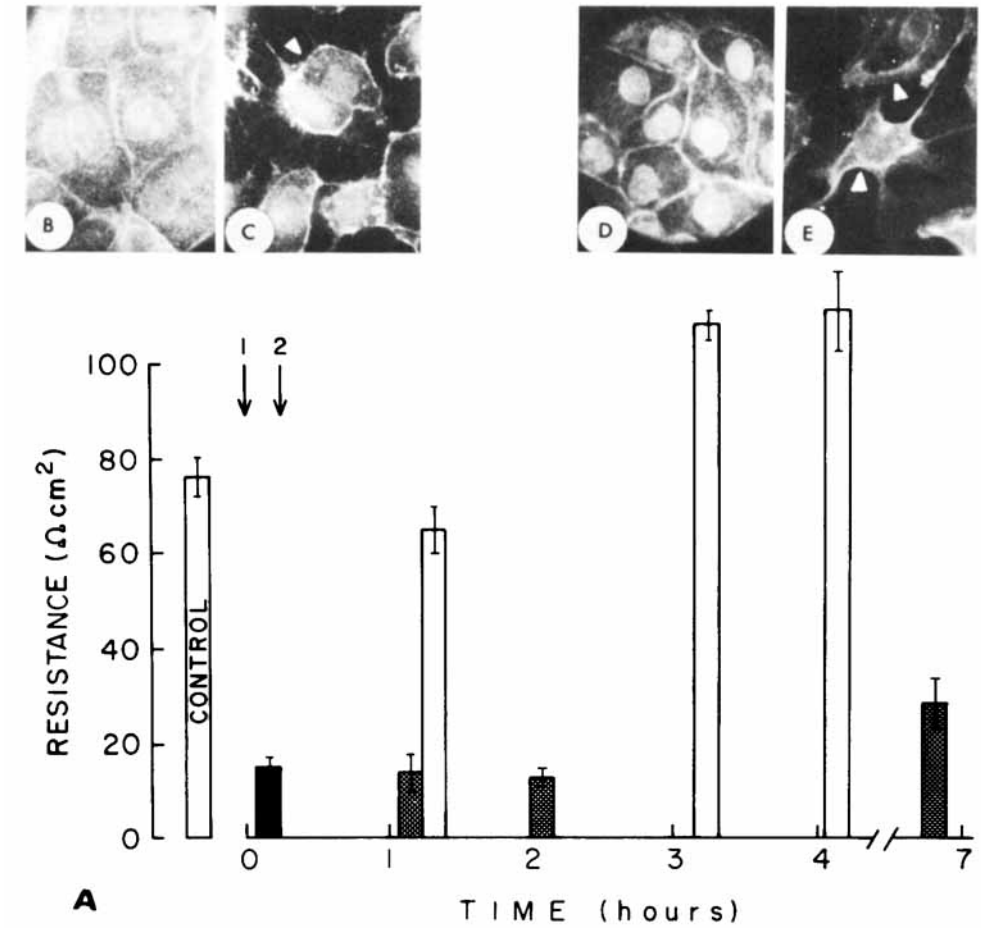

Fig. 3. Effect of CB on the opening and resealing of tight junctions and the microfilament distribution during this process. (A) Open bars refer to control monolayers and those recovered after restoration of CA^{++}. At arrow 1, Ca^{++} was removed and 2.4 mM EGTA was added. At arrow 2, Ca^{++} was restored and the disks divided into two groups. One group recovered in the absence of CB (open bars) and the other recovered in the presence of CB (shaded bars). The number of observations per point is similar to those of Figure 2. (B) Distribution of actin in untreated monolayers. (C) Actin pattern in monolayers treated with EGTA and CB. Arrow points to the retracted ring. A faint fluorescence corresponds to the cytoplasm outside the ring. (D) Actin patterns in monolayers after recovery in CMEM without CB for 180 min. (E) Altered shape and microfilament distribution in cells recovered for 90 min in CMEM with CB. Arrows point to the retracted ring. × 900.

pears as a clear zone outside the ring in Figure 3B and 3E. It is not clear if this is owing to an actual contraction of the ring. If Ca^{++} is added back to the medium and CB is removed, the resistance recovers at about the same rate as the controls without CB (see Fig. 3A and reference [6]). The morphology of these cells and the filament patterns gradually return to normal (Fig. 3D). The readdition of Ca^{++} in the presence of CB does not result in the recovery of the resistance, a normal cell shape, or a normal microfilament pattern (Fig. 3E). After prolonged treatment with CB

(3–4 hr), we can find some patches of confluent cells, but they retain the abnormal filament patterns.

Voltage Scanning of CB-Treated Cell Junctions

We have previously described results obtained with the voltage scanning method using cells in closed monolayers [8]. These observations indicated that the electrical conductance along a given junction has a heterogeneous pattern of high and low conductance points. This heterogeneous pattern of conductance can be correlated with the heterogeneous appearance of the junctional strands in these cells as seen in freeze fracture replicas [8]. The pattern of conducting and nonconducting spots does not change with time, in the sense that the overall resistance of the monolayer stays constant. However, recordings made on the reputedly same spot may be observed to change in amplitude with time (up to 30% increase or decrease in an hour). Since the density of the field varies sharply with the distance from the junction and, as noted above, along the periphery of a given cell, a variation with time of 30% in the conductance could be due to the inapparent displacement of the microelectrode tip by as little as 1 μm. We now use this method to explore in detail

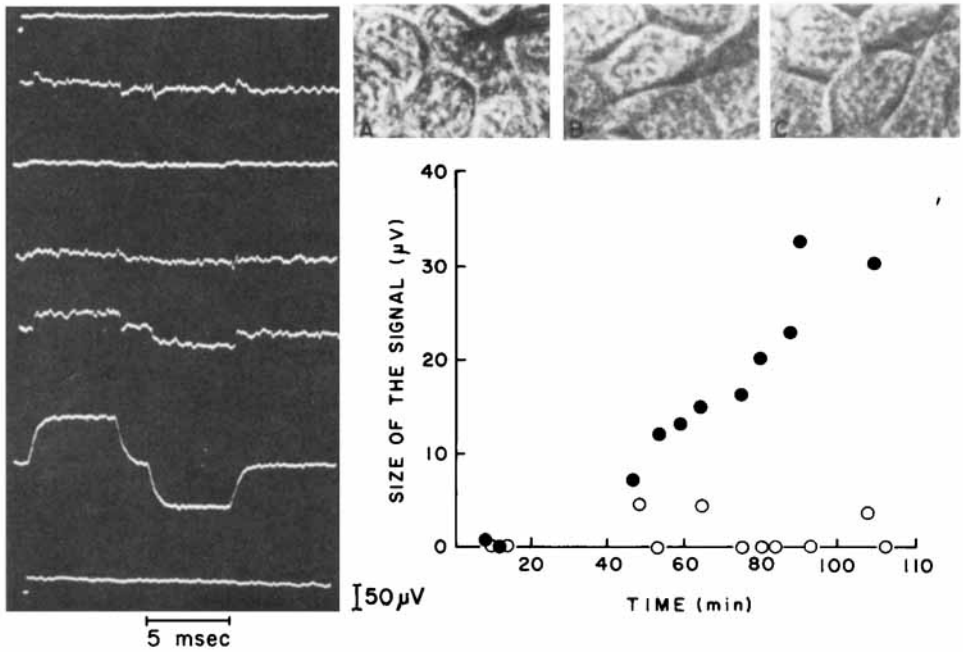

Fig. 4. Electric field scanning of an MDCK monolayer cultured on a permeable support. The tip of the microelectrode is on the center of a cell at (A), on a junction at (B), and at the intersection between three cells at (C). The left panel illustrates seven recordings. The top three recordings correspond to points (A), (B), and (C) in the resting monolayer. The next three recordings were made over a junction after 20, 52, and 100 min of CB treatment. A comparison of the sixth recording, over the junction with the final trace, over the cell body, shows that the center of the cell has no signal after 90 min of CB treatment, while the junction has a very high conductance. The graph summarizes the results. Filled circles correspond to recordings obtained on occluding junctions as a function of treatment with the drug. Open circles correspond to recordings on the center of the cell bodies.

the local effects of CB on the junction. We have described previously the effect of this drug on the overall transepithelial resistance [6].

Figure 4 shows the results of a typical experiment where we measured the resistance at points on the monolayer before and at various times after CB addition (Fig. 4A–4C). The left panel illustrates the individual electrical recordings. The graph summarizes the increase in amplitude of the signal as a function of time after CB addition. When the measurement is made over the junctions (full circles), there is an increase in conductance. Signals with the amplitude illustrated in the sixth recording of Figure 3 are never observed in control monolayers. The electrical resistance of the paracellular route consists of two basic components: 1) the occluding junction and 2) the intercellular space [13]. The contribution of the second component may be important in epithelia with low resistance (eg, the kidney proximal tubule with 5–10 Ω cm^2, see Moreno and Diamond [14]) or in circumstances where the osmolarity of the bathing solution is changed, and the intercellular space is experimentally collapsed [15,16]. However, in epithelia like the present preparation, with resistances beyond 80 Ω cm^2, volumetric expansion of the interspace is unlikely to make a detectable decrease of the resistance. The observed appearance of spots with high conductance in CB-treated monolayers suggest therefore that the occluding junction itself is being opened. If the measurement is made over the cell body (panel A and open circles) there is no change.

Figure 5 shows a schematic diagram of a region of a monolayer in which the conductance was monitored. A through D are points on the same junction where measurements were made. At point A, the conductance was measured at zero time

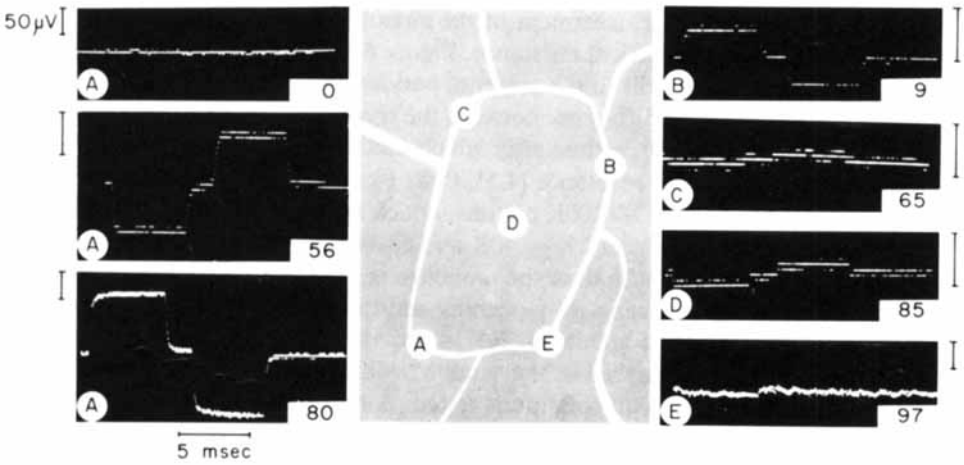

Fig. 5. Effect of CB on the permeability of the occluding junction of an MDCK cell monolayer. The center panel illustrates the silhouette of a cell and the approximate sites, marked by letters, where the microelectrode was placed. Recordings on the left show the increase of conductance at point (A) after 0, 56, and 80 min of treatment with CB. Recordings on the right show the amplitude at the points and times indicated. Vertical bars indicate 50 μV. Notice first the irregularity in the opening of the junction, eg, at 80 min point (A) is highly conductive and point (E) is not. Note also that the body of the cell has a negligible signal.

and at 56 and 80 min after CB addition. By 56 min, the junction was open at this point. Point B was partially open at 9 min, while points C and E are still closed at 65 and 90 min, respectively. Point D, corresponding to the cell body, has a barely detectable conductance even at a time when neighbor points, point A for example, are highly conductive. Repeated recordings on a given spot of a closed control monolayers, yield almost identical electrical signals.

The results shown in Figures 4 and 5 indicate that the increase in conductance elicited by CB is actually owing to opening of the occluding junction and not to leakage across or damage to the cell body.

Iodination of Surface Proteins

In order to obtain sufficient material for labeling, cells were grown on Petri dishes instead of on the nylon-coated disks used for the electrical measurements. Under the light microscope the cells in the Petri dishes have the same general appearance as those grown on the disks. The only difference observed was the presence of blisters owing to the accumulation of fluid under the monolayer. On either substrate, cells were plated at the same density and were treated simultaneously with the same concentration of CB for the same time. We assume that the behavior of cells on the solid substrate is identical to those grown on the disks and will refer to them as open, closed, or resealed monolayers. One should bear in mind, however, that the electrical measurements are only feasible on the permeable substrate.

Proteins on the surface of MDCK cells were labeled with ^{125}I using lactoperoxidase [10] and analyzed using one-dimensional SDS-gel electrophoresis layering with the same amount of cpm per lane. The efficiency of labeling was found to be lower for the CB-treated cells, although we could not detect cell loss during the treatment. Figure 6 shows the results for trypsinized cells and for cells in a closed monolayer 28 hr after plating. Parallel controls show that these monolayers have a normal electrical resistance. Furthermore, treatment of the monolayers with the iodination mixture did not alter their electrical resistance. Figure 6 also shows the protein patterns for opened and resealed cells in the presence and absence of CB. The results show that there is a qualitative difference between the trypsinized cells (A) and those plated for 2 hr or more (B), a time after which addition of cycloheximide does not inhibit the recovery of the resistance [4,5]. Cells from the monolayer display a group of proteins, M_r = 120–200K daltons, which are absent from the trypsinized cell surfaces. Other proteins at M_r = 40K and 58–60K are present in the trypsinized cells but are less prominent than in the monolayers. The control, closed monolayer pattern, is not markedly changed by opening and resealing even in the presence of CB (C to F). The bands of high M_r (120K $\leq M_r \leq$ 200K), that of 82K and those of 40K and 36K daltons as well as the small M_r which are also labeled in trypsinized cells, remain prominent in all conditions tested. A band corresponding to the molecular weight of BSA is found in all the preparations and could correspond to the BSA in serum that remains strongly attached to the cell surface even after prolonged washings. However we find a protein with this M_r in purified membrane preparations (unpublished results).

In order to study potential junctional component-cytoskeleton interactions, we have first iodinated the surface proteins, then lysed the cells with Triton X-100 to solubilize the membranes, and isolated a cytoskeletal fraction. A distribution of the label in the different fractions is included in Table I.

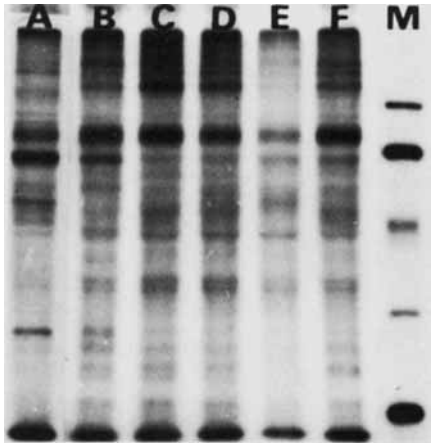

Fig. 6. Fluorograph of a gel of the surface proteins of MDCK cells labeled with ^{125}I. (A) Pattern from trypsinized cells. The cells were trypsinized, and resuspended in medium with serum, then washed several times with PBS by centrifugation, and labeled for 10 min at 2°C. The labeling conditions were the same for all the experiments. (B) Pattern from a confluent monolayer established for 28 hr. (C) Pattern from a confluent monolayer incubated in the absence of Ca^{++} for 60 min, then labeled. (D) Pattern from cells incubated in the absence of Ca^{++} with CB present for 60 min, then labeled. (E) Pattern from cells incubated as in (D), then allowed to recover for 3 hr in CMEM, and labeled. (F) Pattern from monolayers incubated in the presence of Ca^{++} and CB for 2 hr prior to labeling. Markers used were also iodinated and correspond to phosphorylase b (94K), BSA (67K), ovalbumin (43K), carbonic anhydrase (30K), and trypsin inhibitor (20K).

TABLE I. Binding of Surface Membrane Proteins Iodinated With I^{125} to the Cytoskeleton

	Cytoskeleton (percentage)[a]	Soluble fraction (percentage)[a]
Control	36.9 ± 5.1 (8)	63.1 ± 5.1 (8)
CB[b]	37.9 ± 7.8 (5)	62.0 ± 7.8 (5)
EGTA[c]	36.2 ± 5.8 (8)	63.7 ± 5.8 (8)
EGTA + CB[d]	42.8 ± 6.3 (8)	57.2 ± 6.3 (8)
Recovered from EGTA + CB[e]	35.2 ± 5.5 (8)	64.7 ± 5.5 (8)
Just detached[f]	46.4 ± 7.4 (6)	53.3 ± 7.3 (6)

[a]Values are expressed as percentage ± standard error (number of observations) of the sum (100%) of the CPM of I^{125} in the cytoskeleton and soluble fraction of Triton-treated MDCK monolayers.
[b]CB: 5μg/ml for 2 hr.
[c]EGTA: 2 mM for 60 min in Ca-free medium.
[d]EDTA + CB: treated as in (b) and (c), except that CB was present during 60 min instead of 2 hr.
[e]Recovered from condition (d) in normal medium for 3 hr.
[f]Cells detached from confluent monolayers with EDTA-trypsin.

Figure 7 shows the patterns for the cytoskeleton and the Triton-soluble fraction of control monolayers labeled with ^{35}S-methionine and also shows fluorographs of the cytoskeletons and soluble fractions from the series of opening and resealing experiments shown in Figure 6. The ^{35}S-met labeled pattern show that actin and intermediate filament proteins are prominent components in the cytoskeletons of

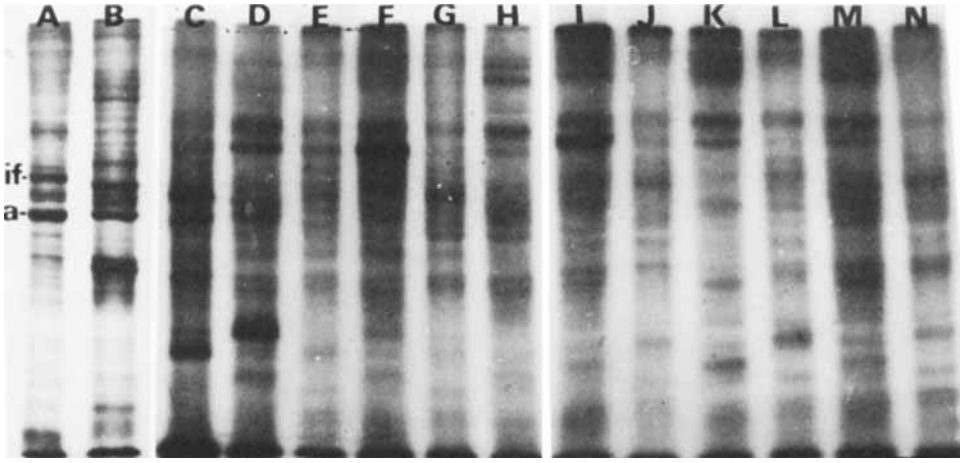

Fig. 7. Surface proteins associated with the cytoskeleton and soluble fraction of Triton-treated MDCK cells. Lanes (A) and (B) show the cytoskeleton and soluble fraction of a monolayer labeled with ^{35}S-methionine for 18 hr (25 μCi/ml of CMEM) as comparison to the surface labeled proteins. The two more prominent bands in the cytoskeleton correspond to actin and intermediate filament proteins. (C) and (D) show the cytoskeleton and soluble fraction of trypsinized cells. (E) and (F) cytoskeleton and soluble fraction of a confluent monolayer in culture for 24 hr. (G) and (H) cytoskeleton and soluble fraction of confluent monolayers treated with 2 mM EGTA for 60 min. (I) and (J) show the soluble fraction and cytoskeleton of a monolayer treated with CB for 120 min. (K) and (L) soluble and cytoskeleton of a monolayer treated with EGTA and CB for 60 min and then recovered in normal medium for 3 hr. (M) and (N) soluble fraction and cytoskeleton of a monolayer treated with CB (5 μg/ml) and EGTA for 60 min.

MDCK cells and show actin also present in the soluble fraction (A and B). Cytoskeletons of other cell types, prepared by Triton solubilization, show the same prominent protein components [9,17]. In trypsinized cells (C and D) and in control closed monolayers, a large number of the iodinated surface proteins remain attached to the cytoskeleton (E and F). Actin, a cytoplasmic component, is never labeled. Triton extraction releases proteins from the cells that have M_r = 120 and 200K daltons, 68–90K daltons and part of the prominent bands at 25K, 30K, 43K and 56K daltons. The majority of the 40K and 56K protein and all of the high molecular weight proteins remain associated with the cytoskeleton. Two prominent low molecular weight peptides at M_r = 36 and 18K daltons are also associated with the cytoskelton. Opening and resealing with EGTA has no significant effect on the protein patterns or their distribution with the cytoskeletons after Triton extraction (G, H and K, L). The disorganization of the actin cytoskeleton with CB also does not produce any changes in the patterns or distribution of the surface proteins (I, J, M, N). When the monolayers are treated with EGTA before labeling, and are presumably open, a better labeling of some bands is obtained as if label had a better access to these proteins. However the pattern remains qualitatively the same and the percent of protein in the cytoskeleton and soluble fractions remains very similar (see Table I).

DISCUSSION

We have previously shown that there is a ring of microfilaments associated with the junctional region in closed monolayers of MDCK cells [4,5]. In closed monolayers, CB disrupts this ring and the loose microfilament network in the central cytoplasm but does not cause arborization of the cells. The disruption of the actin microfilament pattern is closely associated with the opening of the occluding junctions, measured by a decrease in the transepithelial resistance, which is also caused by CB. Arborization produced by CB is only observed in subconfluent MDCK monolayers and in monolayers treated with EGTA that have no electrical resistance. The monolayers opened by Ca^{++} removal will not reseal after restoration of Ca^{++} if CB remains present. In these cells, the ring does not reorganize although some cells recover the normal shape. If CB is removed the cells recover the electrical resistance and the normal pattern of actin microfilaments and normal shape. We have proposed than an organization of actin filaments is required for the establishment and maintenance of the tight junction [6]. Several authors [18–20] have described actin filament networks in the apical region of other epithelia. Louvard et al [21] have also described the presence of actin filaments in close association with the tight junctions of MDCK cells. The precise nature of this association is not well understood but may involve direct interactions between junctional components and actin filaments or through linking molecules like spectrin [22].

The effect of CB on the shape of the cells is clearly evident in cells that do not touch their neighbors. In confluent monolayers where cells are in aposition the effect is not as strong unless the occluding junctions are opened. We could think that CB directly affects occluding junction-cytoskeleton components and that this causes the opening of the junction. However, we can not discard the posssibility that in the presence of CB, the cells in closed monolayers tend to change shape and that the occluding junction can not hold by itself the cell-to-cell attachment and therefore the junction would open.

The possibility that surface proteins may be displaced or rearranged during opening and resealing of the tight junctions was investigated by labeling surface proteins and following the peptide patterns in different experimental conditions that affect the permeability of the junction. Changes of the membrane components by treatment of liver cells with phalloidin, a drug that disaggregates actin filaments have been reported [23]. We have used CB, a drug that not only alters the integrity of the microfilament network but also alters the permeability of the tight junctions, to investigate the distribution of surface membrane proteins. The results presented here (Figs. 6 and 7) show that there is no marked difference in the surface iodination patterns of MDCK cells in open or closed monolayers in the presence or absence of CB. We expected to see a different set of bands of surface proteins labeled with ^{125}Iodine when the tight junctions were opened either with EGTA alone or with EGTA and CB. Under these conditions we should have been labeling also the basolaterial components of the membrane. We find only a quantitative increase in the amount of label for some of the proteins when the tight junctions are open, as if opening will permit a better access to the label, but we can not detect different proteins. The labeling of the apical versus the basolateral surfaces in these monolayers is being studied in our laboratory and some possible causes are explored in another paper [5]. We do see a diminution in the number of proteins labeled

following trypsinization. These components reappear at the surface within 2 hr after plating even in the presence of cycloheximide, which we have shown previously [4,5] inhibits the development of electrical resistance in newly formed monolayers. We studied the association of the iodinated surface proteins with the cytoskeleton by lysing the cells with Triton X-100 to isolate a soluble and a cytoskeletal fraction. Similar cytoskeletal preparations have been obtained from other cells in culture [9]. Association of membrane proteins to the insoluble cytoskeleton has been reported [24]. In Table I we can see that about 36% of label remains attached to the insoluble cytoskeletons. There are five major groups of surface-iodinated proteins that are retained in the cytoskeletal fraction under our lysis conditions. These include a group of proteins with high M_rs at 120–200K daltons and four prominent components with M_rs of 90K, 56K, 30K, and about 18K daltons. Some of the 90K and 56K proteins and two molecules with M_r of about 220K daltons are released into the Triton-soluble fraction. The distribution of the polypeptides between the soluble and cytoskeletal phases is not markedly changed by any of the opening and resealing conditions (Table I). The results show that 1) treatment of monolayers with EGTA and CB does not change the pattern of labeling but does make some proteins more accessible to lactoperoxidase catalyzed iodination (Fig. 6C and 6D). 2) Surface proteins are normally associated with the cytoskeletal fractions and disorganization of actin filaments does not markedly change the partitioning of these surface proteins between the soluble and cytoskeletal phases (Fig. 7 and Table I). These results are compatible with the idea that surface membrane components are redistributed on the membrane when monolayers are opened and resealed with EGTA or CB rather than internalized. The disorganization of the actin filament network does not produce a drastic change in the distribution of the membrane components visualized with the iodination technique. We have begun to investigate the nature of the redistribution of the membrane components. Two extremes would be that the junctional components are disassociated by both EGTA and CB and then are freely diffusible in the cell membrane. Alternatively, Ca^{++} removal may directly disassociate the occluding junction, while CB may cause clustering or patching of the junctional strands. The first case can be argued against because CB does not appear to disassociate the cells unless Ca^{++} is also removed. The clustering of junctional components by CB should lead to regions of high conductance and some regions of low conductance. The voltage scanning results are consistent with the idea that CB clusters the junctional components, since we can record the opening of selected regions but also find the retention of low conductance regions even after 1 to 2 hr in CB. This idea is also supported by the patching of actin in CB-treated cells stained with antiactin.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Bryan for his critical review of the manuscript and thank A. Lazaro for technical assistance. This work was supported by grants 220 and 1508 from the Consejo Nacional de Ciencia y Technologia (CONACYT) of Mexico and grant AM 26-481 from the US Public Health Service. IM was a fellow of the Guggenheim Foundation.

REFERENCES

1. Cereijido M, Rotunno CA, Robbins ES, Sabatini DD: In Hoffman JF (ed): "Membrane Transport Processes." New York: Raven, 1978, pp 433–461.
2. Cereijido M, Robbins ES, Dolan WJ, Rotunno CA, Sabatini DD: J Cell Biol 77:853, 1978.
3. Martínez-Palomo A, Meza I, Beaty G, Cereijido M: J Cell Biol 87:736, 1980.
4. Cereijido M, Ehrenfeld J, Fernández-Castelo S, Meza I: Proc NY Acad Sci in press, 1980.
5. Cereijido M, Meza I, Martínez-Palomo A: Am J Physiol 240:C96, 1981.
6. Meza I, Ibarra G, Sabanero M, Martínez-Palomo A, Cereijido M: J Cell Biol 87:746, 1980.
7. Goodman GC, Miranda AF: In Tanenbaum SW (ed): "Cytochalasins Biochemical and Cell Biological Aspects." Amsterdam: North Holland, 1978, pp 277–429.
8. Cereijido M, Stefani E, Martíez-Palomo A: J Memb Biol 53:19, 1980.
9. Brown S, Levinson W, Spudich JA: J Supramol Struct 5:119, 1976.
10. Hubbard AL, Cohn AZ: J Cell Biol 64:438, 1975.
11. Laemmli UK: Nature (London) 227:680, 1970.
12. Bonner WM, Laskey RA: Eur J Biochem 46:83, 1974.
13. Boulpaep EL: Am J Physiol 222:517, 1972.
14. Moreno JH, Diamond JM: J Membr Biol 21:197, 1975.
15. Rawlins FA, González E, Pérez-González M, Wittembury G: Pfluegers Arch 253:287, 1975.
16. Reuss L, Rinn AL: Am J Physiol 232:C99, 1977.
17. Osborn M, Weber K: Exp Cell Res 106:339, 1977.
18. Craig SW, Pardo JV: J Cell Biol 80:203, 1979.
19. Mooseker MS, Tilney LG: J Cell Biol 67:725, 1975.
20. Bentzel CJ, Hainau B, Edelman A, Agnastopoulos T, Benedetti EL: Nature (London) 264:666, 1976.
21. Louvard D: Proc Natl Acad Sci USA 77:4132, 1980.
22. Bennett V, Stenbuck PJ: J Biol Chem 254:2533, 1979.
23. Tuchweber B, Vonk RJ, Yousef IM: Can J Biochem 59:165, 1980.
24. Ben-Zev A, Duerr A, Solomon F, Penman S: Cell 17:859, 1979.