Occluding Junctions and Cytoskeletal Components in a Cultured Transporting Epithelium

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ABSTRACT MDCK cells form uninterrupted monolayers and make occluding junctions similar to those of natural epithelia. This article explores the relationship between these junctions and the cytoskeleton by combining studies on the distribution of microfilaments and microtubules with the effect of drugs, such as colchicine and cytochalasin B, on the degree of tightness of the occluding junctions. To study the degree of tightness, monolayers were prepared by plating MDCK cells on nylon disks coated with collagen. Disks were mounted as flat sheets between two Lucite chambers, and the sealing capacity of the junctions was evaluated by measuring the electrical resistance across the monolayers. Equivalent monolayers on coverslips were used to study the distribution of microtubules and microfilaments by indirect immunofluorescence staining with antibodies against tubulin and actin. This was done both on complete cells and on cytoskeleton preparations in which the cell membranes had been solubilized before fixation. Staining with antiactin shows a reticular pattern of very fine filaments that spread radially toward the periphery where they form a continuous cortical ring underlying the plasma membrane. Staining with antitubulin depicts fibers that extend radially to form a network that occupies the cytoplasm up to the edges of the cell. Colchicine causes a profound disruption of microtubules but only a 27% decrease in the electrical resistance of the resting monolayers. Cytochalasin B, when present for prolonged periods, disrupts the cytoplasmic microfilaments and abolishes the electrical resistance. The cortical ring of filaments remains in place but appears fragmented with time. We find that removal of extracellular Ca^{++}, which causes the tight junctions to open, also causes the microfilaments and microtubules to retract toward the center of the cells. The process of junction opening and fiber retraction is reversed by the restoration of Ca^{++}. Colchicine has no effect on either the opening or reversal processes, but cytochalasin B inhibits the resealing of the junctions by disorganizing the filaments in the ring and at the apical border of the cells. These cytochalasin B effects are fully reversible. The correlation among cell shape, cytoskeletal patterns, and electrical resistance in the EGTA-opened and resealed monolayers suggests that microfilaments, through their association with plasma membrane components, play a role in positioning the junctional strands and influence the degree of sealing of the occluding junctions.

MDCK cells plated on a permeable support form a monolayer with the characteristics of a transporting epithelium (8, 9, 10, 26). In the cultured monolayer, the lateral borders in the luminal region of adjacent cells are joined by occluding junctions (tight junctions or zonulae occludentes). We have previously shown that these junctions are both structurally and functionally heterogeneous, in that "leaky" regions alternate with "tight" portions over the length of a given junction (11).

The occluding junctions of MDCK cells can be opened and resealed by removing and restoring Ca^{++} with little initial effect on the structure of the junctions, which appear in freeze-fracture replicas as linear arrays of smooth ridges and particles (23). This reversible modification in the permeability of the occluding junctions occurs even in the presence of inhibitors of protein synthesis, indicating that resealing does not require the *de novo* synthesis of the membrane junctional components but

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rather junctional reassembly (23).

The mechanism that the cell uses to organize or reassemble the components of the junctional complex is poorly understood but probably involves the coupling of junctional elements to the cytoskeleton. There is increasing evidence in other systems for the participation of cytoskeletal elements in the lateral displacement of certain membrane proteins or receptors along the surface of the plasma membrane, or their positioning in a particular region (1, 15, 18, 29). The cytoskeleton has also been considered to play a role in changes in epithelial permeability elicited by various conditions (7, 14, 35). In addition, ultrastructural evidence indicates that microfilaments and microtubules show a close association with cellular junctions (3, 12, 13, 17, 37), although the specific relationship with the occluding junctions remains unsettled. Finally, we have recently demonstrated by immunofluorescence that in MDCK monolayers actin microfilaments form a complete ring at the lateral borders of the cells, where occluding junctions are located (10), suggesting that these filaments may play a role in positioning the junctional components. Therefore, we have studied the role of both microtubules and microfilaments in the maintenance of the permeability of the occluding junction in MDCK cells and their participation in the experimental assembly and reassembly of junctions. The transepithelial permeability of the MDCK cells was monitored by measuring the electrical resistance across the monolayer mounted as a flat sheet between two chambers. This allowed us to follow the time-course of the change in junctional permeability induced by the removal and addition of Ca^{++}. The intracellular distribution of microtubules and microfilaments was followed in equivalent preparations with actin and tubulin monospecific antibodies. Finally, the effect of microtubule and microfilament disruption on the reconstitution of the occluding junctions was tested with colchicine and cytochalasin B. Preliminary reports of this work have been presented (22, 25).

MATERIALS AND METHODS

Cell Cultures

A detailed description of the procedures used to cultivate MDCK cells and to test the electrical resistance of the cellular monolayers is given in the companion article (23).

Purification of Tubulin and Actin

Tubulin was isolated and partially purified from bovine brain by two polymerization cycles (33). The soluble fraction after cold depolymerization of microtubules was fractioned in DEAE-cellulose (6). The purity of the fraction eluted at 500 mM NaCl was verified by SDS-urea polyacrylamide gel electrophoresis (32). Alfa and β-tubulin monomers were the only bands visible in the polyacrylamide gels even when these were overloaded. The pure tubulin fraction was then reduced and carboxymethylated (31), and the modified protein, mixed with Freund's adjuvant at concentrations of 1–2 mg/ml was injected into rabbits. Actin was purified from rabbit skeletal muscle, following the procedure of Spudich and Watt (34). The G-actin obtained was verified for purity in SDS-urea gel electrophoresis in which only one band was visible. For injection into rabbits, actin (2 mg/ml) was denatured with 1% SDS, boiled for 1 min, and then mixed with an equal volume in Freund's adjuvant.

Characterization of Antisera

Antibodies were induced after several (five to eight) subcutaneous injections of the modified antigens. The titer of the immune sera was tested by double immunoprecipitation with ^{125}I-labeled purified tubulin or actin. The antisera were mixed with serial dilutions of the radioactive-labeled antigen for 2 h and the complex precipitated was measured in a gamma-ray counter. In these tests, the antibodies against tubulin precipitated 87% of the iodinated tubulin and those against actin precipitated 92% of the iodinated actin. A control using nonimmune serum was used in every titration. The antisera reacted specifically with tubulin or actin when tested in competition assays with a number of other proteins.

Monospecific antibodies were prepared by adsorption of the immune IgGs from each serum on Sepharose-4B columns to which purified tubulin or actin had been cross-linked (21). The bound antibodies were eluted with 0.3 N acetic acid, then buffered to neutral pH, concentrated, and stored at −20°C.

Drug Treatment of Monolayers

Colchicine solutions (Sigma Chemical Co., St. Louis, Mo.) were freshly prepared as a 10 mg/ml stock in H_2O and added directly to the culture medium to a final concentration of 2×10^{-5} M. Cytochalasin B (Aldrich Chemicals, Milwaukee, Wis.) was prepared as a 5 mg/ml stock in dimethylsulfoxide (DMSO) and added to the medium to a final concentration of 5 μg/ml. DMSO final concentration was 0.5% and had no effect on control experiments.

Immunofluorescence

Cells were grown to confluence on glass coverslips in complete Eagle's minimal essential medium (CMEM). The monolayers were treated as described below to visualize microtubules or microfilaments and, according to the treatment, they are designated complete cells and cytoskeletons. Complete cells: coverslips were rinsed with phosphate buffer saline (PBS), fixed for 20 min at room temperature with 3.7% formaldehyde in PBS, rinsed, immersed in cold acetone at −20°C for 5 min, and then rinsed again with PBS. The coverslips were then washed with 0.05 M NH_4Cl in PBS for 10 min to block free aldehyde groups and treated with 0.1% Triton X-100 for 2 min. The coverslips were then washed thoroughly with PBS and treated with the appropriate antibodies. Cytoskeletons: in these preparations, the cells were lyzed after the elements of the cytoskeleton were stabilized, but before fixation. The microtubules were well preserved in a stabilization buffer containing 0.1 M PIPES, 1 mM EGTA, 2.5 mM GTP, and 4% polyethylene glycol, pH 6.9, as described by Osborn and Weber (30). After the cells were rinsed with this buffer, they were lysed with 0.5% Triton X-100 in the same buffer for 3–5 min at 37°C. The coverslips were rinsed extensively with PBS, fixed with methanol for 10 min at −20°C, postfixed with 3% formaldehyde for 30 min, and then rinsed again. Antibodies were then applied, as in the complete cells. Microfilaments were preserved in the lysed cells by treatment with a 25 mM Tris, 5 mM glucose, 0.5 mM MgCl, 137 mM NaCl, 5 mM KCl, 0.025 mM $CaCl_2$, 0.5% Triton X-100, pH 7.4, buffer for 5–10 min as reported by Brown et al. (5). After lysis, the coverslips were rinsed thoroughly with PBS and the cells were fixed as described for microtublues. The staining with the corresponding monospecific antibody (100–500 μg/ml) was performed by incubating for 45 min at 37°C in a humid chamber, rinsing well with PBS, then overlaying with fluorescein-conjugated goat anti-rabbit IgGs (Bionetics Laboratory Products, Litton Bionetics Inc., Kingston, Md.) 1:10 dilution, and incubating for 45 min at 37°C. After rinsing thoroughly with PBS, coverslips were mounted with a 9:1 glycerol-PBS mixture and viewed in a Zeiss 18 standard microscope equipped with epifluorescence optics. All photographs were taken with a ×63 Planapochromat objective with Kodak Tri X film.

RESULTS

Immunofluorescent Pattern of the Cytoskeleton in Confluent MDCK Monolayers

Confluent cultures of MDCK cells resembled a simple cuboidal epithelium which on surface view appeared as a mosaic of small, polyhedral cells and which in cross-section appeared to be a single row of closely apposed cells. The pattern of cytoplasmic microtubules and microfilaments visualized by immunofluorescence with antitubulin and antiactin was studied both in complete cells and in cytoskeletons. Complete cells, i.e., cells fixed before the interaction with the antibodies (Fig. 1 *a* and *b*), showed a diffuse fluorescence that hindered the visualization of the fibrillar elements of the cytoskeleton, as previously reported by others in fibroblastic and in certain epithelial cells (2, 36). On the other hand, in cytoskeletons, i.e., cells lysed before fixation (Fig. 1 *c* and *d*), a clear pattern of the fiber network was obtained because of the reduced background from the soluble components.

In these cytoskeletal preparations, the microtubular pattern

appeared as a delicate network of radially oriented filamentous structures. At the periphery of the cell, the tubules followed the contour of the membrane and filled the cellular processes (Fig. 1 *c*). Actin microfilaments were present as an extremely fine mesh concentrated mostly in the perinuclear area, then extending toward the plasma membrane where a strongly fluorescent ring delineated the cellular borders (Fig. 1 *d*). The specificity of the staining with antitubulin was revealed in dividing MDCK cells, in which the mitotic spindle gave an intense fluorescence (Fig. 1 *a*, *inset*). On the other hand, the spindle fibers were not stained with actin antibodies.

The immunofluorescent pattern of the cytoskeleton of confluent MDCK cells differed from that reported for cultures of fibroblastic and subconfluent epithelial cells in which microtubules constitute a well-defined reticulum, and microfilaments form cables or "stress" fibers (2, 19, 20). To establish that the absence of these patterns in confluent MDCK cells was not artifactual, we stained cultured fibroblastic cells. As shown in Fig. 1 *e*, spreading 3T3 cells treated with nonionic detergent and stained with antitubulin, under the same conditions as confluent MDCK cells, show the usual striking arrangement of microtubules. When stained with antiactin, actin cables are also readily apparent in these fibroblastic cells (Fig. 1 *f*). These findings illustrate the difference in the cytoplasmic disposition of cytoskeletal components of spreading fibroblasts and MDCK confluent cells. In subconfluent cultures of MDCK cells, we have observed stress actin fibers and a better defined microtubule network.

In this work, we have concentrated on the changes in the cytoplasmic distribution of cytoskeletal components. Quantitative modifications, if present, could be explored by techniques other than immunofluorescence.

Effect of Cytochalasin B and Colchicine on Sealed Monolayers

The establishment of occluding junctions in confluent monolayers of MDCK cells conferred a transepithelial electrical

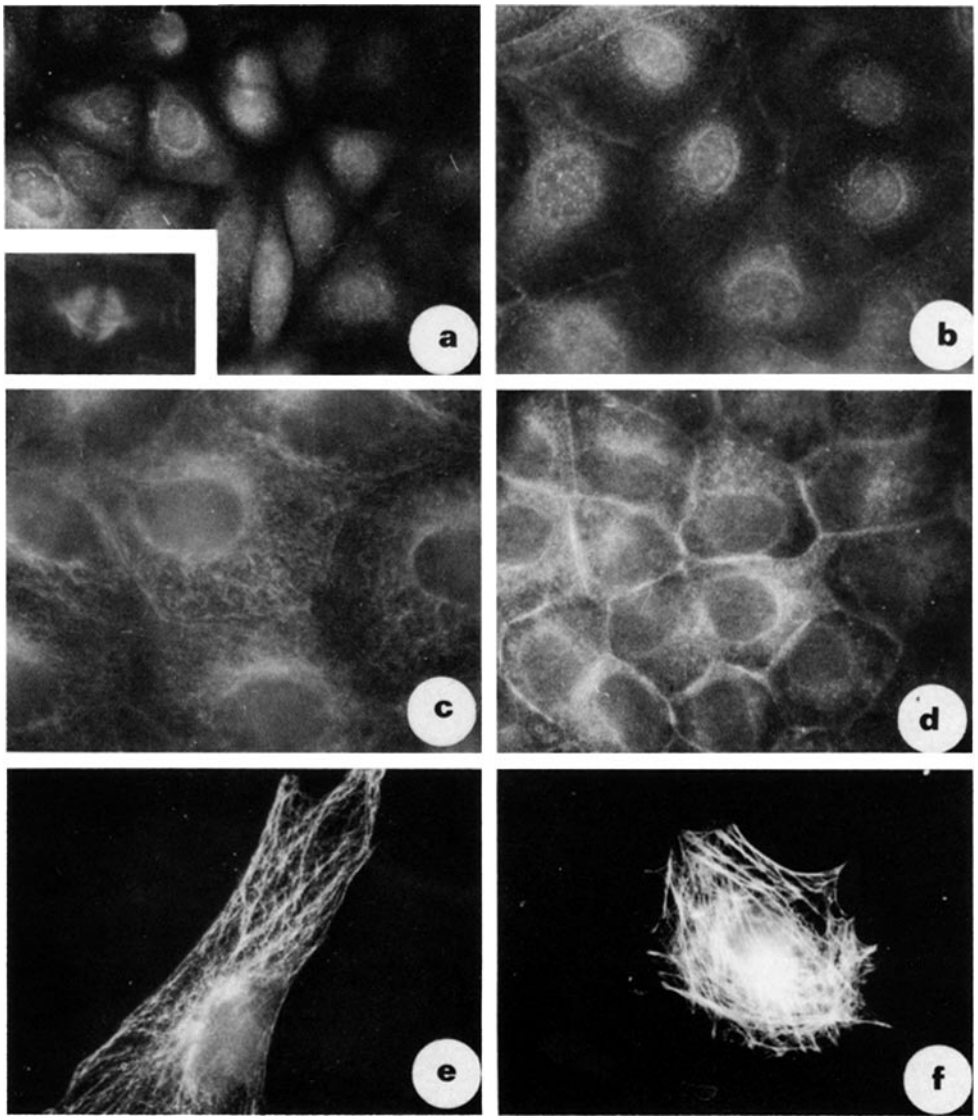

FIGURE 1 Indirect immunofluorescence staining with antibodies against tubulin and actin. (*a*) Complete MDCK cells stained with antitubulin. *Inset* shows a spindle of an MDCK cell in mitosis. (*b*) Complete MDCK cells stained with antiactin. (*c*) Cytoskeletons obtained after lysis in PEG and GTP to preserve microtubules and stained with antitubulin. (*d*) Cytoskeletons obtained in buffer to preserve microfilaments and stained with antiactin. (*e*) 3T3 fibroblast stained with antitubulin. (*f*) 3T3 fibroblast stained with antiactin. × 600.

resistance of 124 ± 7 Ω·cm² (point at zero time in Fig. 2). As shown in Fig. 2, cytochalasin B, at a concentration of 5 μg/ml, induced a slow and gradual drop in the resistance, starting 20 min after its addition. By 2 h the resistance was only 25% of the control value. Colchicine produced a rapid decrease in the resistance, to ~73% of the normal value within 30 min, but subsequently the resistance remained steady for >2 h.

The addition of cytochalasin B to confluent monolayers of MDCK cells mantained in normal medium containing calcium,

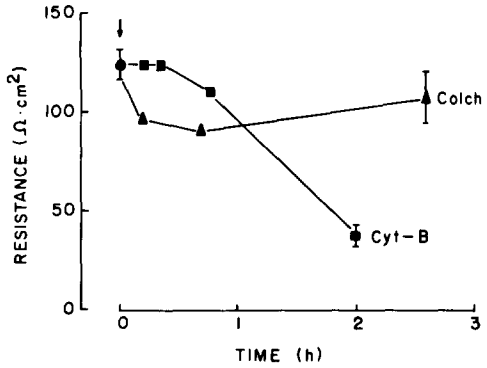

FIGURE 2 Effect of cytochalasin B and colchicine on the electrical resistance of MDCK monolayers plated at confluence 2 d before on disks of nylon cloth coated with collagen. Disks were mounted as flat sheets between two Lucite chambers (exposed area, 0.2 cm²) and the resistance was measured by passing pulses of 20 μA. The resistance of the support and the bathing medium was subtracted from the total resistance. Measurements were performed in CMEM. Results are expressed as mean ± SE. When a symbol has no standard error, it represents an average of two to four individual disks. Drugs were added at the time marked by the arrow and remained present throughout. Triangles, colchicine; squares, cytochalasin B.

for periods up to 60 min, did not significantly alter the pattern of actin microfilaments (Fig. 3*a*). At this time, the peripheral ring of microfilaments delineating the cellular borders was still present but its fluorescence was no longer homogeneous, suggesting that the ring was beginning to be fragmented and the filaments condensed. After 2 h in the presence of cytochalasin B and when the electrical resistance had dropped considerably, a pronounced disorganization in the microfibrillar network was already evident. At the end of 3 h, the network consisted of coarse filaments or bundles of filaments aggregated around the nucleus (Fig. 3*b*). However, even after 3 h in cytochalasin B, the shape of the cells was not changed and the peripheral ring of microfilaments, although fragmented and patchy, was still in place. Colchicine treatment of the monolayers for 30 min in normal medium resulted in a diminution of the fluorescence of microtubules although microtubules remained clearly visible at the cellular margins (Fig. 3*c*). At longer times, even these peripheral microtubules were depolymerized (Fig. 3*d*). After treatment of cells with colchicine, the shape of the cells in the sealed monolayer was maintained.

Changes in the Cytoskeletal Components During Opening and Resealing of Occluding Junctions

As previously described (9, 10, 23), the removal of Ca^{++} and the addition of EGTA to the medium produced a drastic drop in the electrical resistance. This lowering of the resistance is caused by the opening of the occluding junctions (23). Restoration of Ca^{++} produced a complete recovery of the electrical resistance.

The opening of the occluding junctions induced by Ca^{++} depletion produced little change in the pattern of the micro-

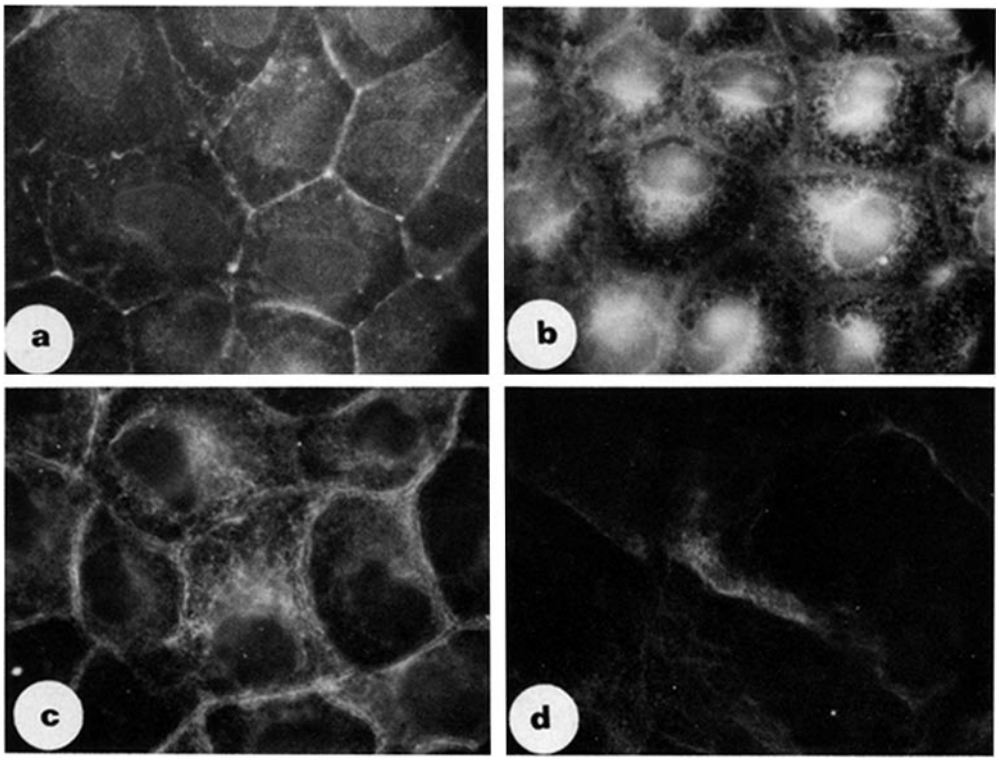

FIGURE 3 Effect of drugs on the cytoskeleton distribution in resting monolayers. Actin-microfilaments, as seen in cytoskeletons, of MDCK cells after being in the presence of cytochalasin B for 60 min (*a*) and for 180 min (*b*); microtubule-fibers in cytoskeletons of MDCK cells after being in the presence of colchicine for 30 min (*c*); and for 60 min (*d*). × 600.

tubular network studied in complete cells. This conclusion is somewhat tentative because it is difficult to visualize individual tubules in these unlysed preparations. The fluorescent network appeared somewhat more intense around the nucleus and slightly retracted from the cellular margins, which in the phase microscope still appeared to be in close apposition. However, in cytoskeletal preparations there was a pronounced change in the microtubular web, which resulted in its thickening and retraction, leaving large empty spaces between cells devoid of fibers (Fig. 4 *a*). This apparent retraction of the fibers was even more evident in cytoskeletal preparations of monolayers stained with antiactin. The peripheral ring, which was normally found under the plasma membrane, seemed to contract, decrease its diameter, and shrink down around the nucleus. The impression with both antibodies was that the fiber networks were contracted but intact and that neighboring cells were separated by large intercellular gaps generated by the retraction (Fig. 4 *d*).

The retraction in the cytoskeletons was only apparent when the junctions in the monolayer were opened by Ca^{++} removal. Lysis before fixation of the cells with sealed junctions did not produce retraction.

Restitution of Ca^{++} to the monolayers within 10–15 min resulted in the recovery of the transepithelial resistance within 2–3 h (9, 10, 23). The resealing process was accompanied by a gradual return of the microtubular (Fig. 4 *b* and *c*) and microfilament (Fig. 4 *e* and *f*) networks to their normal position.

Effect of Cytochalasin B During Opening and Resealing of Occluding Junctions

The presence of cytochalasin B did not inhibit the drop in electrical resistance across MDCK monolayers produced by Ca^{++} removal (Fig. 5 arrow *2*). Cytochalasin had no effect on cell shape in the sealed monolayer (Fig. 3 *a* and *b*) but produced pronounced changes when applied to open monolayers (Fig. 6 *a*). The appearance of the cytochalasin B-treated cells in open monolayers is similar to that described for other types of cells by Godman and Miranda (16) as caused by a contracture of the cytoplasm and zeiotic blebbing. The microfilament pattern in the drug-contracted cells was distorted; the peripheral ring was retracted toward the center of the cell and appeared discontinuous. If the drug was left in the medium and Ca^{++} was restored, the occluding junctions failed to reseal (Fig. 5, filled squares). Under these conditions, the microfilament network remained retracted.

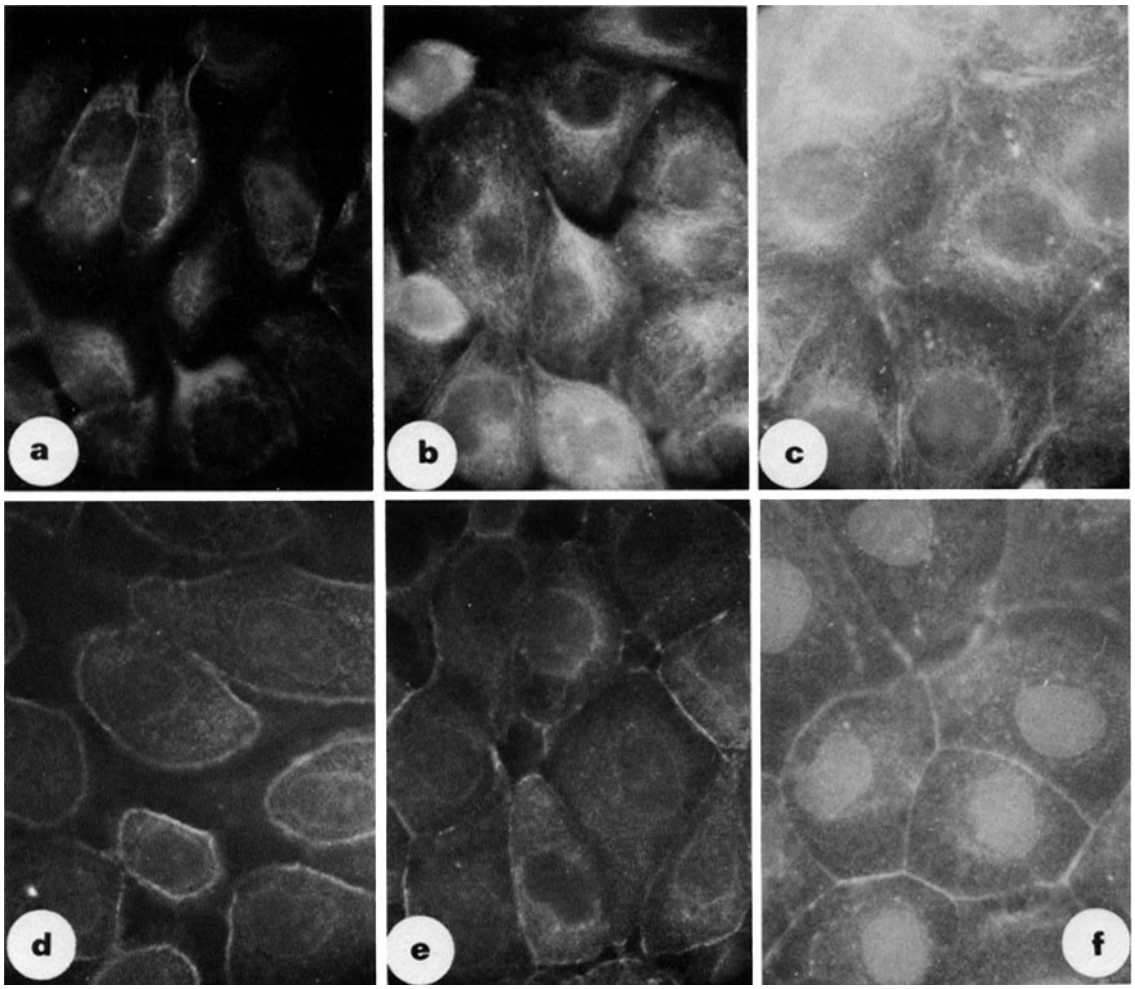

FIGURE 4 Effect of EGTA on the microtubule and microfilament patterns. (*a*) Microtubule fibers in cytoskeletons of cells treated with EGTA (15 min). (*b* and *c*) Microtubule fibers from MDCK cells which, after been treated with EGTA (15 min), were restituted in Ca^{++}-containing medium for 60 and 180 min, respectively. (*d*) Actin-filaments in cytoskeletons of cells treated with EGTA (15 min). (*e* and *f*) Actin fibers from cells which, after being treated with EGTA (15 min), were restituted with CMEM for 60 and 180 min, respectively.

After a few hours in cytochalasin B, some cells began to reorganize the basolateral region where desmosomes are located. These cells come into contact with their neighbors and, although their apical region is still disorganized, they show a partially reconstituted filament pattern (Fig. 6*b*). If, at the same time that Ca^{++} is restored cytochalasin B is also removed from the medium, the resistance of the monolayers recovers to the control levels (Fig. 5, crosses). At the same time, the microfilaments reorganize (Fig. 6*c*) and by the end of 3 h their normal distribution is reestablished (Fig. 6*d*). The microtubule network remained intact during cytochalasin B treatment of the sealed monolayer and during opening and resealing of the occluding junctions.

Effect of Colchicine During Opening and Resealing of Occluding Junctions

Treatment of MDCK monolayers with colchicine did not inhibit the drop in electrical resistance induced by Ca^{++} removal (Fig. 7, arrow *1*). In fact, the resistance decreased somewhat faster when colchicine was present (open triangles). As in the case of cytochalasin, colchicine affects the shape of the cells only when the monolayers are open and have a lower resistance. In these unsealed monolayers treated with colchicine, the cells became rounded and the microtubules were disorganized. Remnants of tubules were clearly visible at the cell periphery and in the region around the centrosome (Fig. 8*a*). If Ca^{++} was added back to the cultures but colchicine was left in the medium, the resistance recovered in spite of the fact that the microtubular pattern remained disorganized (Fig. 8*b*).

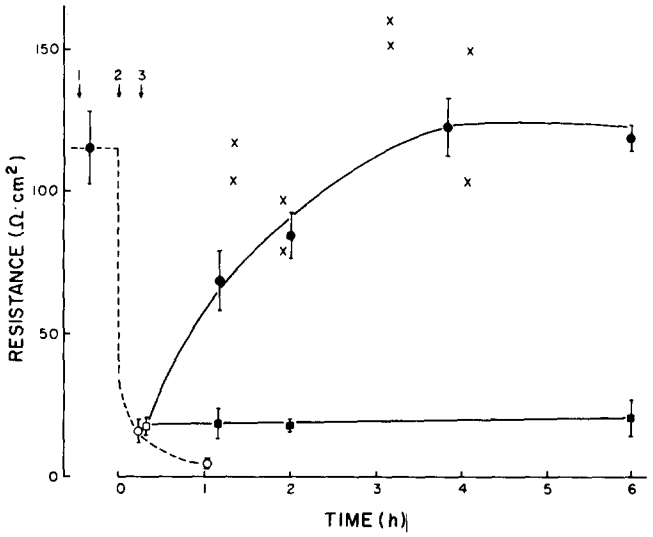

FIGURE 5 Effect of cytochalasin B on the opening and resealing of tight junctions. As in Fig. 3, open symbols refer to monolayers incubated in the absence of Ca^{++}. Circles refer to control monolayers. At arrow *1*, the drug was added to some monolayers and remained present throughout in all subsequent solutions. The rest of the monolayers were left as controls. At arrow *2*, all monolayers, treated and controls, were switched to media without calcium and with EGTA. At arrow *3*, calcium was restored to control monolayers which recovered their electrical resistance (●) in 2–4 h. Monolayers left in Ca-free MEM showed a further decrease in resistance (○). Restoration of Ca^{++} to monolayers treated with cytochalasin B failed to recover their resistance (■). If the drug was removed at the same time that Ca^{++} was restored, monolayers recovered their electrical resistance (×). Each cross represents an individual monolayer.

After 2–3 h in the presence of colchicine, the fluorescence disappeared almost completely and the electrical resistance started to fall (Fig. 7, filled triangles). Because the monolayers could reform their junctions in the presence of the drug and because a resistance value of ~90 $\Omega \cdot cm^2$ was achieved before showing a decline, the decrease in resistance observed after 3 h in the drug is presumably not an effect of colchicine on microtubules. When normal medium was restored to cells that were treated with colchicine during the opening of their junctions, the normal pattern of microtubules was reestablished by the end of 2 h (Fig. 8*c*).

DISCUSSION

The cytoplasmic microtubules of confluent monolayers of MDCK cells form a dense network of fibers that extend radially to the periphery of the cells. Colchicine treatment disrupts this pattern and leaves short remnants of microtubules in close contact with the plasma membrane at the cell periphery and in the perinuclear region near the centrosome. The electrical resistance of colchicine-treated monolayers is ~75% of untreated monolayers, suggesting that the occluding junctions continue to function as permeability barriers in the absence of microtubules and that tubules are not essential for junctional integrity.

The microfilaments of confluent MDCK cells appear to form a fine web spreading throughout the cytoplasm. Stress fibers are conspicuously absent from these cells but the filaments of the web appear concentrated at the periphery where they form a continuous ring beneath the plasma membrane. Cytochalasin B disorganizes the web and partially fragments the ring, but has no striking effect on the position of the ring as judged by immunofluorescence. The specificity of this drug is demonstrated by its exclusive modification of the immunofluorescent pattern of the microfilaments but not of the microtubular one. Besides its inhibitory action on microfilament assembly, cytochalasin B may have collateral effects (16). However, these effects are not primarily responsible for the drop in electrical resistance, as shown by Martínez-Palomo et al. (22) using a method devised by Cereijido et al. (11) to scan the points where the electric current is flowing through the monolayer. With this method, we found that the changes in resistance produced by cytochalasin B were caused by the opening of the paracellular permeation route. Therefore, even when this drug might have nonspecific effects, we shall ascribe the observed changes in resistance to modifications in the paracellular route. The structural changes in the microfilament web and ring, which parallel the slow decrease in the electrical resistance across the monolayer, suggest that filament integrity is required for the maintenance of the junctional permeability barrier.

We have examined the effect on the cytoskeletal fibers of opening the occluding junctions by the removal of calcium. In complete cells, the changes observed in microtubule and microfilament distribution are minimal, suggesting that opening of the junctions does not strongly affect the pattern of the cytoskeleton. In these preparations, the borders of the cells appear to be in close apposition when observed by phase-contrast microscopy. The distribution of both microtubules and microfilaments is strikingly different in the lysed cells or cytoskeletons with open junctions. The cytoskeletal fibers retract toward the nucleus, and the peripheral ring of actin microfilaments is displaced from its position under the plasma membrane, leaving what appears, with immunofluorescence, as empty cytoplasmic spaces. Lysis by itself does not cause the

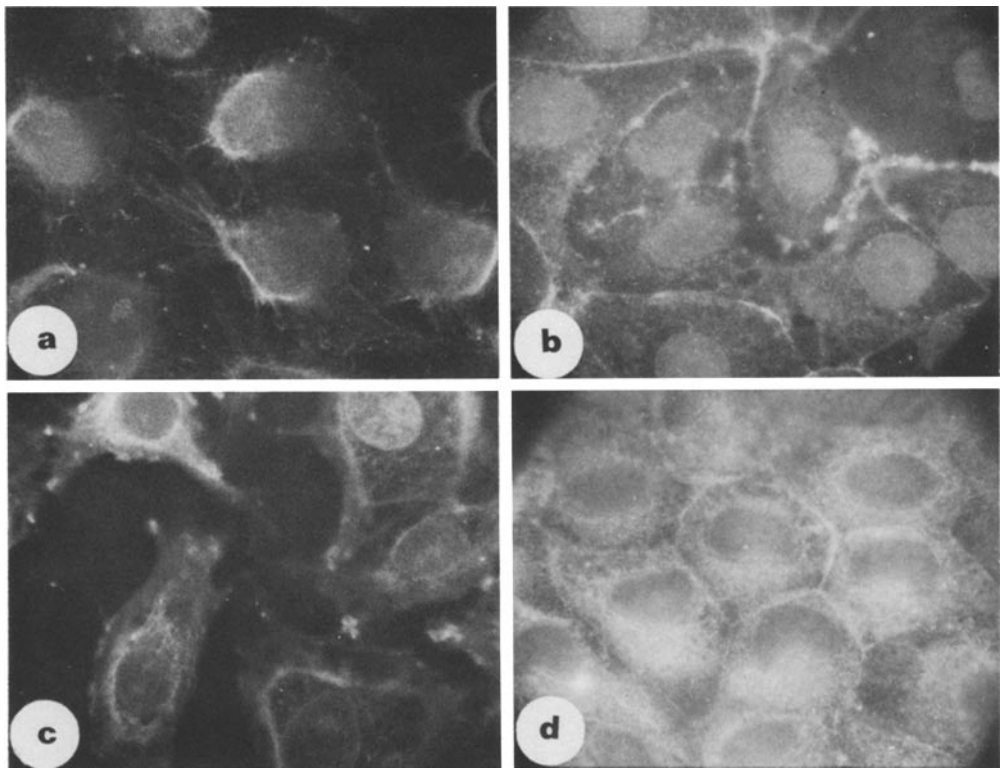

FIGURE 6 Effect of cytochalasin B on the microfilament pattern. (*a*) Pattern of microfilaments in cytoskeletons of cells subjected to cytochalasin B and EGTA for 15 min. (*b*) Microfilament distribution in cells that had been in the presence of cytochalasin B and normal Ca^{++} for 4 h. (*c*) Microfilament pattern as seen in the cytoskeleton preparations from cells treated as in *a* and then recovered in the presence of Ca^{++} for 90 min. (*d*) Actin fibers in cells that had recovered in normal medium (180 min) after being in the presence of cytochalasin B and EGTA. × 600.

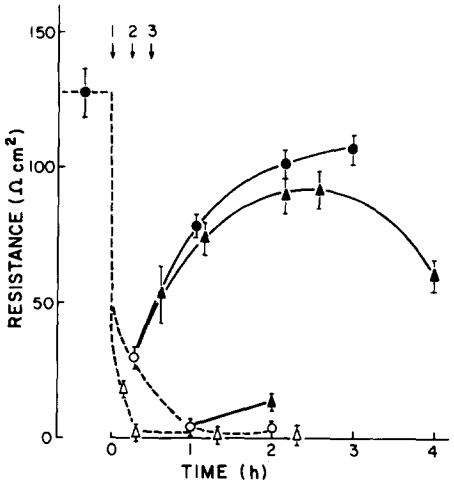

FIGURE 7 Effect of colchicine on the opening and resealing of tight junctions. Symbols are the same as those in Figs. 2 and 5. At arrow *1*, calcium was removed and 2.5 mM EGTA was added. This produced a sharp drop in the electrical resistance of both control (○) and colchicine-treated monolayers (△). Restoration of Ca^{++} at arrow *2* produced a recovery of the electrical resistance in control (●) and treated (▲) monolayers. Disks remaining in Ca-free media, either with or without the drug, failed to recover their resistance (open symbols). Restoration of Ca^{++} after a long treatment (20–30 min) with EGTA (arrow *3*) failed to recover the resistance with the same kinetics exhibited by those that had no calcium in the medium for 10–12 min. Colchicine was added at arrow *1* and remained present throughout.

retraction, as lysis of monolayers with closed junctions produces few changes in the cytoskeleton pattern. The suggestion here is that the cytoskeletal network, most probably the microfilament web, loses its connections with the membrane when the junctions are opened and actually contracts when the cells are lysed. If the junctions are intact and closed, the force of the contraction is distributed over the whole monolayer and only a minimal local change is evident. In the cells with open junctions, however, contraction of the ring and/or web would cause the fiber network of an individual cell to retract because the junctional attachments are broken.

This interpretation implies that at least one element of the cytoskeleton is directly associated with the junction. The effects of drugs demonstrate that microtubules are probably not involved because tubule depolymerization does not abolish junctional resistance or prevent the calcium-dependent resealing of the junction measured by electrical resistance. Cytochalasin B, on the other hand, greatly lowers the electrical resistance and completely prevents resealing of the junctions, demonstrating that microfilament integrity and the normal morphology of the cells are essential for the maintenance and assembly of tight junctions.

Attachment of actin filaments to the plasma membrane has been visualized by electron microscopy and demonstrated in isolated membrane fractions (27). The attachment of cytoskeletal components to membrane particles (4, 18) has been recently demonstrated. In contrast to our results, Meldolesi et al. (24) found no interference of cytochalasin B in the reassembly of occluding junctions of guinea pig pancreatic acinar cells.

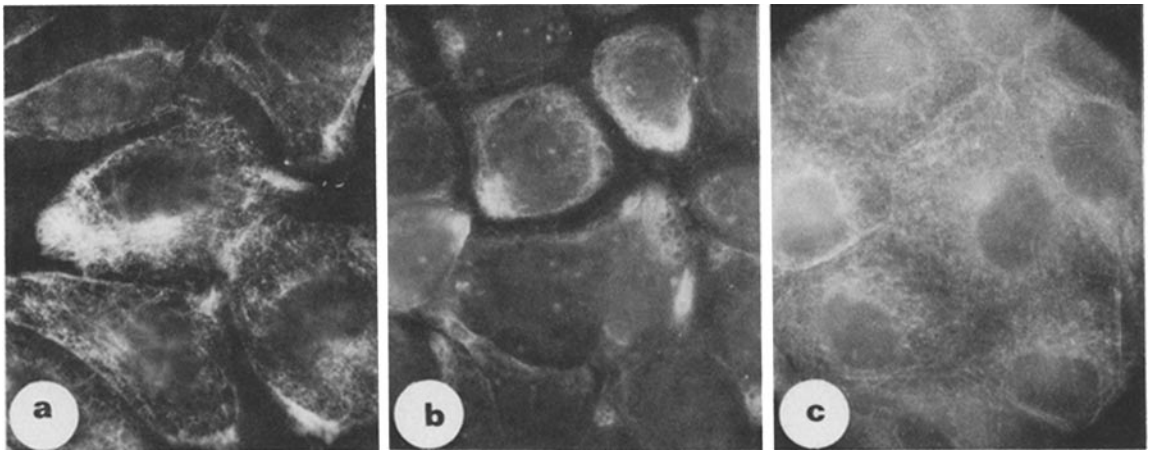

FIGURE 8 Effect of colchicine on the microtubular pattern. (*a*) Cytoskeletons prepared from cells treated with EGTA and colchicine (15 min). (*b*) Cytoskeletons showing the microtubular pattern after Ca^{++} has been restored to the medium but colchicine was still present (45 min). (*c*) Pattern of microtubules in cytoskeletons of cells to which Ca^{++} has been restored and colchicine removed. They had been in normal medium for 120 min. × 600.

However, this does not necessarily involve a contradiction because those authors treated the acinar cells without Ca^{++} and with EGTA for periods much longer than the ones we report here. As shown in the companion paper (23), when the junctions are left open for periods >15 min, the resealing has very different kinetics. Also, as mentioned above, we have evidence that cytochalasin B interferes with the sealing of the junctions at particular points (22). Moreover, it has been reported that cells treated with cytochalasin B for long periods may become insensitive to its action (28).

In summary, the present findings suggest that the association of microfilaments and the plasma membrane in the region of the occluding junctions results in a concerted action that regulates the function of the junction. This conclusion is based on the following observations: (*a*), the normal position of the peripheral ring of actin in the region where adjacent cells contact their neighbors; (*b*), the effect of cytochalasin B on the electrical resistance of resting monolayers; and (*c*), the blockage of the resealing of the junctions by cytochalasin B. The information thus obtained from our study of the distribution patterns of cytoskeletal elements and the occluding junctions of MDCK cells agrees with reports on the association of microfilaments with components of the plasma membrane (1, 15, 18, 27, 37) and suggests that microfilaments are involved in the redistribution of the surface components that constitute the occluding junctions. However, we still do not know what arranges the components of the junction in interwoven strands, why they are located at the apico-basolateral borders and, more pertinent to our system, why the strands of the occluding junctions in these cells are irregularly arranged so that the paracellular conductive pathway around the cells is heterogeneous (12).

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