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The cytoskeleton of the cornea and its role in tight junction permeability

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Summary

The present article investigates the role of cell cytoskeleton on tight junction permeability in the cornea by combined methods based on microscopy and electrophysiology. Ultrastructural characterization of the cytoskeletal network of the cornea was performed using the S-1 myosin subfragment labeling technique and transmission electron microscopy. The effect of various cytoskeleton-active agents including cytochalasin B, phalloidin, colchicine, kinetin, and a calcium chelator, EDTA on the cytoskeleton and tight junction permeability was also studied using confocal fluorescence microscopy and electrical resistance measurements. Results from this study indicate that actin microfilaments, a major component of the cytoskeletal system, play a major role in controlling tight junction permeability of the cornea. Morphologically, these filaments are present throughout the entire cornea with heavy accumulation near the regions of tight junctions, superficial surfaces, and spot-desmosomes of the intercellular spaces. Treatment with EDTA and cytochalasin B results in a disruption of these filaments and subsequent reduction in electrical resistance. This effect is fully reversible upon removal of the compounds, except for prolonged exposure. Compounds that stabilize these filaments, i.e., phalloidin and kinetin, exhibit a reverse effect on the corneal resistance. Colchicine, a microtubule-selective agent, has no effect on the resistance. The correlation between cytoskeletal patterns and electrical resistance in the presence of drugs suggests that actin microfilaments are associated with the tight junction complex and play an important role in regulating the degree of sealing of the paracellular transport pathway.

Introduction

Tight junction integrity appears to be essential for directed transport to take place across an epithelium. In addition to being a region of cell-to-cell attachment, the tight junction restricts molecular diffusion, and probably, any induced bulk flow between intercellular spaces of the epithelia. Tight junction permeability has been shown to depend on a number of factors including: (a) degree of maturation of the epithelia (Pitelka et al., 1973; Humbert et al., 1976; Luciano et al., 1979); (b) response to physiological requirements (Tice et al., 1975; Humbert et al., 1976); (c) change in environmental conditions such as osmolarity and ionic strength (Urakabe et al., 1970; Wade et al., 1973); (d) presence of drugs, vitamins, and hormones (Pickett et al., 1975; Elias and Friend,

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1976). While it is unlikely that a single mechanism is responsible for such a variety of permeability changes, it is conceivable that a few agents play a central role in most of them. Two of these suspected common agents are the concentration of calcium and the cytoskeleton. The nature of these control mechanisms and their structural counterpart constitute the major thrust of this study.

The cytoskeleton is a complex protein network consisting of three major types of filamentous structures, classified according to their size, namely microfilaments, intermediate filaments, and microtubules. Under normal physiological conditions, microfilaments and microtubules, the smallest and largest cytoskeletal types, undergo rapid dynamic arrangements which give rise to a variety of functions. These include cell motility (Hitchcock, 1977; Korn, 1978; Lazarides and Revel, 1979), cell adhesion (Hynes and Destree, 1978; Singer, 1979), and proliferation (Schroeder, 1973; Sanger, 1975a,b; Herman and Pollard, 1979). In contrast, intermediate filaments are much more stable and rigid which helps provide mechanical support to cells. In epithelial tissues, intermediate filaments form tonofibrils that connect spot-desmosomes of the epithelial cells, providing tensile strength to the tissues (Darnell et al., 1986).

There is increasing evidence showing that the cytoskeleton participates in the regulation of epithelial permeability elicited by various conditions (Carasso et al., 1973; Taylor et al., 1973; Frederiksen and Leyssac, 1977). Ultrastructural evidence also indicates that microfilaments and microtubules show a close association with cellular junctions (Wessells and Evans, 1968; Craig and Pardo, 1970). Cereijido et al. (1980) demonstrated that actin microfilaments of the cultured epithelial cells form a complete ring at the lateral borders of the cells near the area of tight junctions. Disruption of these filaments, e.g. by treatment with specific cytoskeleton-active agents such as cytochalasin B and by removal of extracellular calcium, results in an increase in tight junction permeability (Meza et al., 1980; Griepp et al., 1983; Cohen et al., 1985; Gonzalez et al., 1985; Gumbiner and Simmons, 1986). The exact mechanism for regulation of the tight junction is unclear at present, but could involve specific interactions between microfilaments and certain tight junction components. Recently, a number of specific junctional proteins, such as ZO-1 (Stevenson et al., 1986) and Cingulin (Citi et al., 1988) have been identified in various epithelia, including colon, kidney, testis, artery, and brush border.

In the cornea, the nature of the paracellular permeability barrier has not been investigated in detail but it is believed to result from tight junctions presenting between surface cells (Hogan et al., 1980; Maurice, 1984). This is consistent with previous observations (Rojanasakul and Robinson, 1990b) that bathing in solutions containing calcium chelator or cytochalasin B drastically reduces transcorneal resistance. The existence and role of cytoskeleton on tight junction permeability is investigated in more detail in this study.

Experimental

Animals and materials

Male albino rabbits, New Zealand strain (New Franken, WI), weighing between 2.5 and 3.0 kg were used. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed. Lighting was maintained in the caging facilities 24 h a day to provide a constant experimental environment. FITC-labeled phalloidin, Na_2EDTA, cytochalasin B, phalloidin, colchicine, kinetin (6-furfurylaminopurine), and heavy meromyosin subfragment 1 (S-1), prepared from rabbit muscle myosin according to the method of Weeds and Taylor (1975), were obtained from Sigma (St. Louis, MO). All chemicals were used as received. Distilled deionized water was used in preparation of the buffer solutions.

Methods

S-1 labeling of actin filaments

The procedure for extraction and labeling of the specimens was similar to that described previously (Schliwa and Van Blerkom, 1981; Ryder et al., 1984) with slight modification. Briefly, the corneas were first extracted with 0.15% Triton X-100 in PHEM buffer containing 10 mM EGTA

for 30 min. They were washed twice in the same buffer and incubated in 1 mg/ml S-1 in PHEM/EGTA buffer supplemented with 10% glycerol for 1 h. Following another wash, the tissues were fixed with 4% glutaraldehyde and 0.5 tannic acid in buffer solution for 2 h. Finally, the tissues were post-fixed in 2% OsO_4 buffer solution for an additional 1 h.

Transmission electron microscopy

The S-1 treated samples were dehydrated in a graded series of ethanol and embedded in Durcupan embedding medium. The samples were then sectioned (Porter-Blum, MT 2-B Ultramicrotome), stained with uranyl acetate and lead acetate, and examined on a transmission electron microscope (Jeol 100 CX).

Confocal fluorescence microscopy

The MRC-500/Lasersharp fluoroscan confocal system (Biorad, Cambridge, MA) with an argon ion laser source, operating at 488 nm, was used. Details of the system have been previously described (Rojanasakul et al., 1990). Corneas, taken after specific treatments, were directly mounted in a non-fluorescing mounting medium (Polysciences Inc., Warrington, PA) and were examined microscopically without further tissue processing.

Electrical resistance measurements

Corneas were isolated and mounted in Ussing-type chambers following a previously published method (Rojanasakul and Robinson, 1990a). A 0.67 cm^2 corneal area was exposed to the half-cell. After the cornea was securely mounted, 7 ml of glutathione-bicarbonate Ringer's solution (GBR), pH 7.4, was first added to the receptor cell and an equal volume of the same buffer containing specific drugs was subsequently added to the donor cell. Drug concentrations used in this study were fixed at 0.1 mM. Solutions in each half-cell were continuously bubbled with 95% O_2 and 5% CO_2 and kept at 37°C by heating loops immersed in the external circulating fluid. In confocal microscopic studies, the corneas obtained at the end of perfusion studies were incubated with 0.01% FITC-labeled phalloidin in GBR buffer for 30 min prior to viewing.

Transcorneal electrical resistance was measured by the four-electrode (Ag/AgCl) system; two electrodes (positioned 1 cm from each side of the corneal surfaces) were used to record the potential difference and the other two (located 2 cm from each surface) were used to inject current pulses. To avoid problems associated with tissue polarization, sinusoidal currents (current density, 1–10 μA cm^{-2}; frequency, 1 Hz), generated from a function generator, were applied across the cornea and the voltage deflections resulting from the currents passing through it were recorded on a Keithley-197 microvoltmeter. To obtain the actual resistance of the cornea, resistance between the two sensing electrodes, determined in the absence of the cornea, was subtracted from that obtained in the presence of the cornea. Details of the experimental setup and determination of the resistance have been described previously (Rojanasakul and Robinson, 1990a).

Results and Discussion

Ultrastructural organization of actin microfilaments in the cornea

Transmission electron micrographs of Triton-extracted, S-1 labeled, cornea showing organization of actin filaments are presented in Fig. 1. These filaments can be observed throughout the entire epithelium with dense bundles accumulated near the apical surface, tight junctions, and spot-desmosomes of the intercellular spaces (Fig. 1a). Intracellular filaments appear to be denser in the basal cell layers as compared to the apical layers. These filaments form a random loose network with no specific directional organization. Higher magnification micrographs of the surface and wing cells showing detailed structural organization of these filaments are shown in Fig. 1b and c, respectively. A high magnification TEM of the apical surface of the control cornea (no extraction and S-1 decoration) is also shown in Fig. 1d. From these micrographs, it appears that the apical actin networks are associated with the plasma membranes and microvilli of the superficial cells. Previous studies in the intestinal epithelium have indicated that these filaments are present in bundles

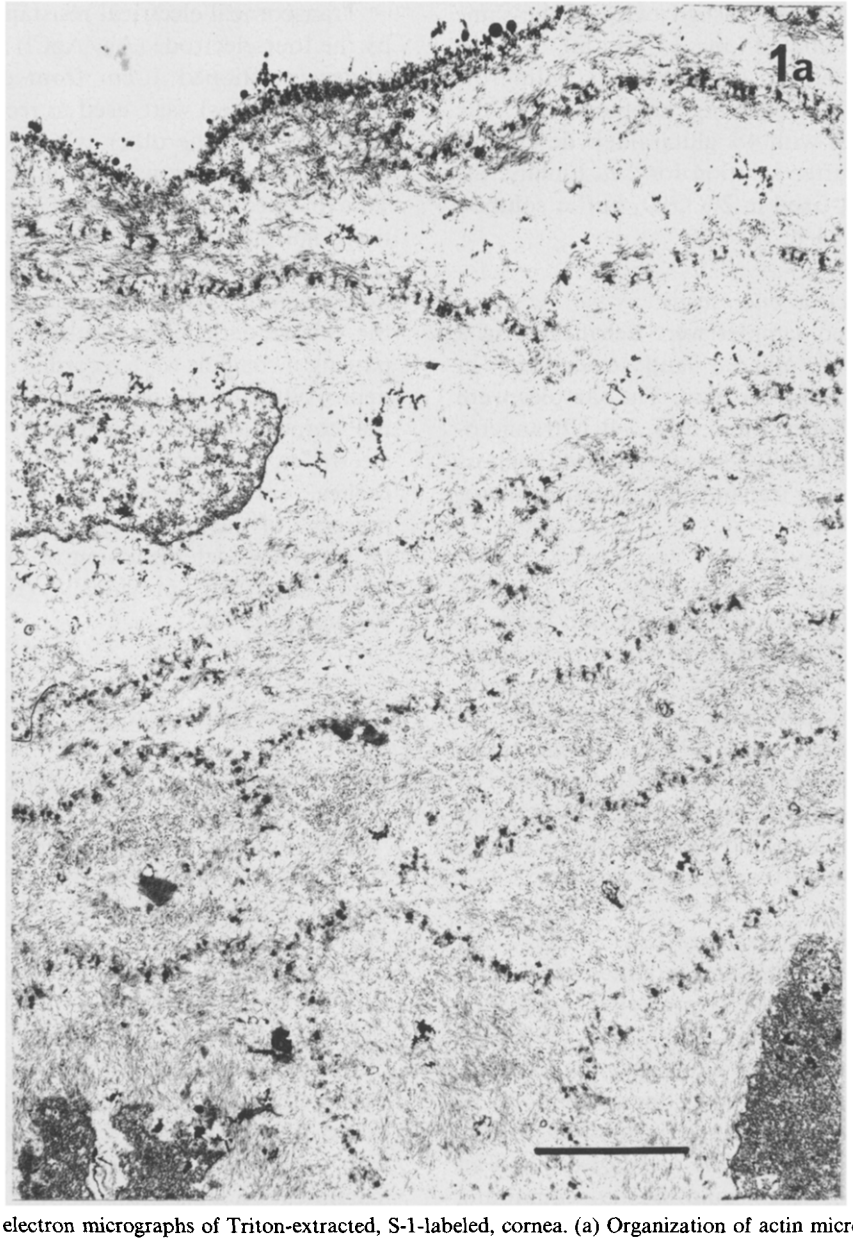

Fig. 1. Transmission electron micrographs of Triton-extracted, S-1-labeled, cornea. (a) Organization of actin microfilament networks in the epithelium. Note the heavy accumulation of these filaments near the apical surface, tight junctions, and spot-desmosomes of the intercellular spaces; bar = 5 μm. Higher magnification of (a) showing apical cytoskeletal networks at the apical surface (b) and the wing cells (c); bars = 1 μm. (d) Transmission electron micrograph of the control cornea (no extraction and S-1 labelling) showing tight junctions, intercellular spaces, and microvilli of the apical epithelial surface; bar = 1 μm. Note the accumulation of actin filaments at the apical and basal regions of the cell; bar = 5 μm.

and form the core of the microvilli (Mooseker and Tilney, 1975; Matsudaira and Burgess, 1982). The apical actins have also been shown to participate in positioning and movement of surface protein receptors (Nicolson, 1976; Gabbiani et al., 1977; Hoch and Smith, 1978) as well as in maintaining

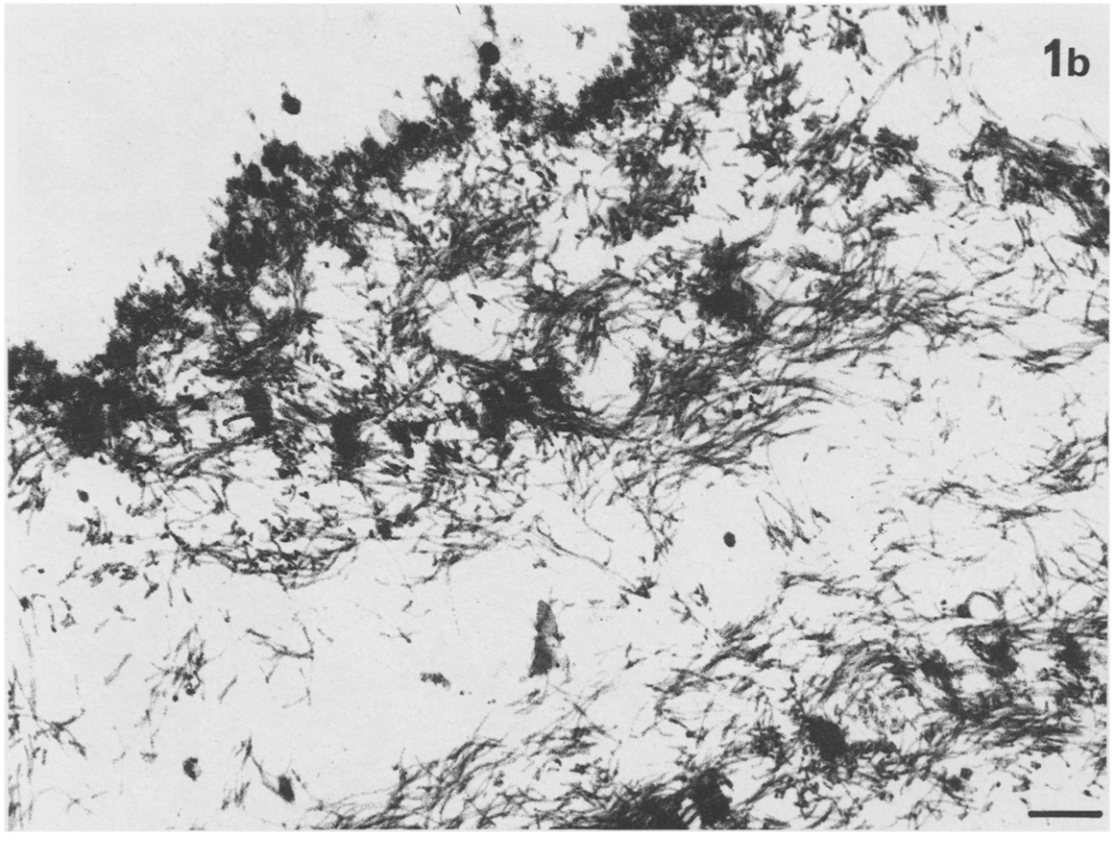

Fig. 1 (b).

the regular array and motility of the microvilli (Gipson and Anderson, 1977). The actin networks near the tight junctions and the intercellular spaces, on the other hand, appear to be involved in cell-to-cell attachment and present physical barrier to molecular transport in the paracellular pathway. Subsequent results from fluorescence microscopic and electrical resistance studies further support these findings.

Confocal fluorescence microscopy

Fluorescent phalloidin derivatives have been extensively used to identify, localize, and quantify F-actin in many different cell types (Haugland, 1989). Unlike actin antibody, the fluorescent phalloidin, due to its relatively small size (MW ≈ 1000), readily penetrates into living and fixed cells (Barak et al., 1980). Its extraordinary specificity to actin has facilitated many actin-related studies without the usual problems associated with the use of the antibody, i.e., non-specific interaction with myosin, tropomyosin, and DNase I (Haugland, 1989). Even more significantly, phalloidin-labeled actin filaments remain functional. Muscle fibers labeled with fluorescent phalloidin contract and move on solid phase myosin substrates in vitro (Haugland, 1989). These unique features of the compound along with the capability of the confocal system to optically section thick specimens make it possible to study live actin-related phenomena at varying depths in the tissue.

Confocal fluorescence images of the control cornea labeled with fluorescent phalloidin are shown in Fig. 2. On the epithelial surface (Fig. 2a), actin filaments form a continuous band at cell borders near the tight junctions. Bundles of these filaments are also present in the cells near the cell membrane surface. In deeper layers of the epi-

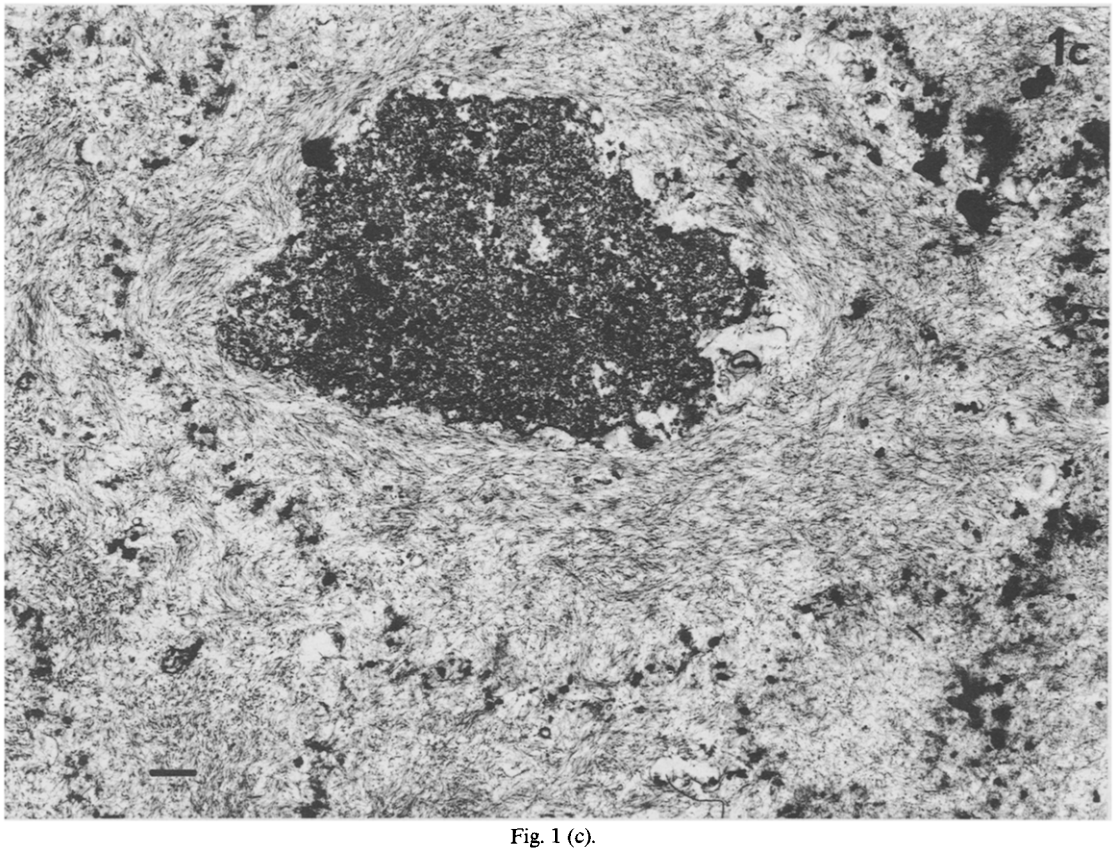

Fig. 1 (c).

thelium, localization of fluorescent phalloidin is observed mainly near the area of the intercellular spaces (Fig. 2b). Absence of cytoplasmic actin labeling may be attributed to limited penetration of the dye in the deeper layers of the epithelium. In the endothelium (Fig. 2c), the actin filaments form a striking complex network at the intercellular junctions with loosely distributed filaments observed inside the cells. This filamentous network is composed of two major bands running parallel along cell peripheries with numerous interconnecting strands joining these two bands and, presumably, the two cells together. The observation of intercellular actins in the endothelium is somewhat controversial since cell attachment in the epithelial tissues is believed to occur via some intermediate junctional proteins and not directly through actin bridges. Results to be discussed below on selective disruption of these strands by cytochalasin B further substantiate this finding.

Corneas treated with EDTA (Fig. 3) and cytochalasin B (Fig. 4) for 3 h, both at 0.1 mM concentration, show changes in organization of actin filaments. In the case of EDTA, while no dramatic change is observed on the epithelial surface (Fig. 3a), a complete loss of the interconnecting filaments and severe cell separation are evident on the endothelium (Fig. 3c). Unlike cytochalasin B, EDTA also causes loss of cell shape which is most dramatic in the deeper layers of the epithelium (Fig. 3b). Since it has been shown that the assembly of all three major cytoskeletal components requires calcium and some of these components, i.e., microtubules and intermediate filaments, are known to participate in preservation of cell shape (Lazarides, 1980; Clarkson et al., 1984)

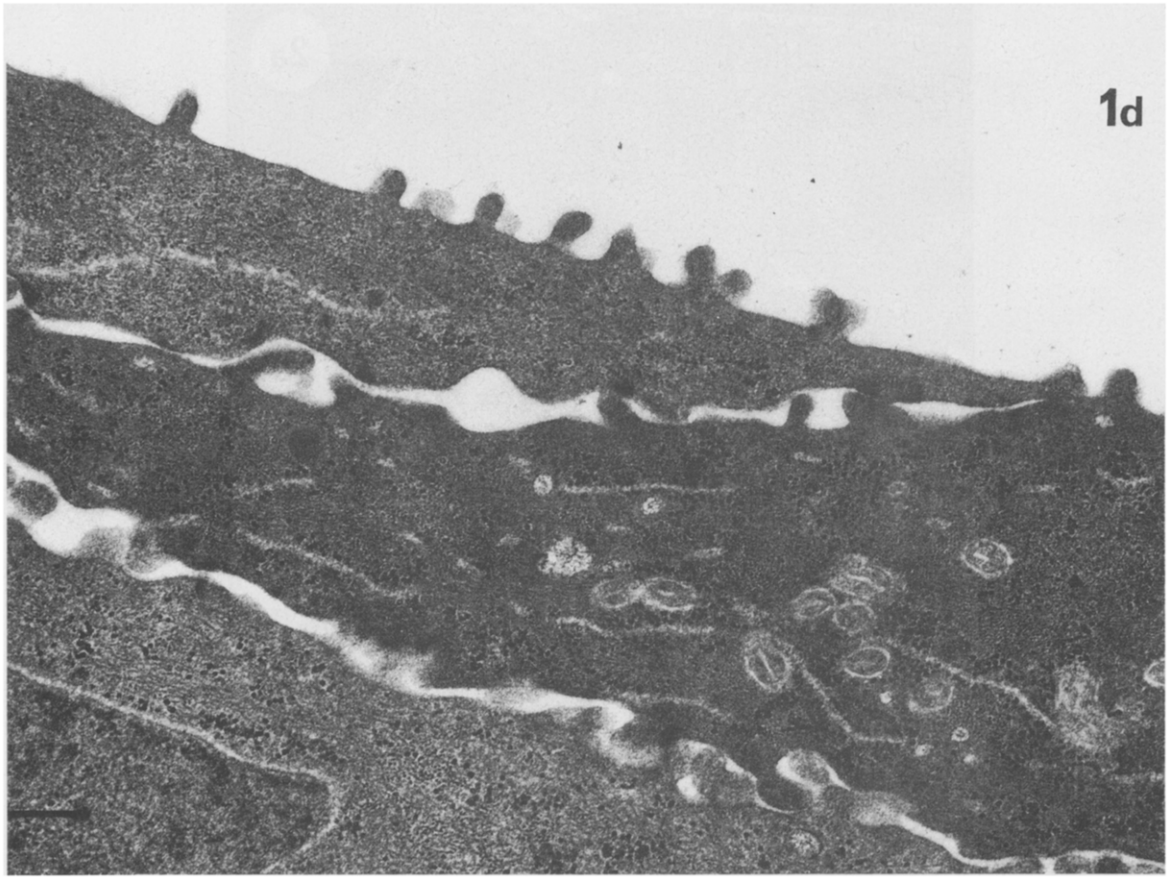

Fig. 1 (d).

it is conceivable that the loss of cell shape may result from EDTA effect on these filaments. In contrast to EDTA, cytochalasin B, a compound known to act specifically on actin filaments by interfering with their polymerization (Flanagan and Lin, 1980; Brown and Spudich, 1981), shows no effect on cell shape (Fig. 4b). Disruption and partial loss of the actins can be seen on both epithelial (Fig. 4a) and endothelial surfaces (Fig. 4c).

Electrophysiology

Transepithelial resistance is primarily a measure of ionic conductance through shunt or paracellular pathway, consisting of the tight junctions and intercellular spaces in series. Previous work in the cornea (Rojanasakul and Robinson, 1990a) have indicated that changes in the integrity of this pathway, i.e., junctional expansion, result in a parallel drop in the electrical resistance. Wolosin (1988) has shown that the transepithelial resistance of the cornea originates almost entirely from the tight junctions of the superficial epithelial layer. Despite its significance in controlling transport properties of the cornea, the nature of this barrier, especially with relevance to drug delivery, has not been fully investigated. In this section, the effect of various drugs affecting cytoskeleton and tight junction permeability was evaluated electrophysiologically by means of resistance measurements.

Effect of drugs on transcorneal electrical resistance

Drugs affecting organization of cell cytoskeleton such as EDTA and cytochalasin B have been previously shown to affect transepithelial permeability in a dose-dependent manner (Rojanasakul and Robinson, 1990b). To be consistent with pre-

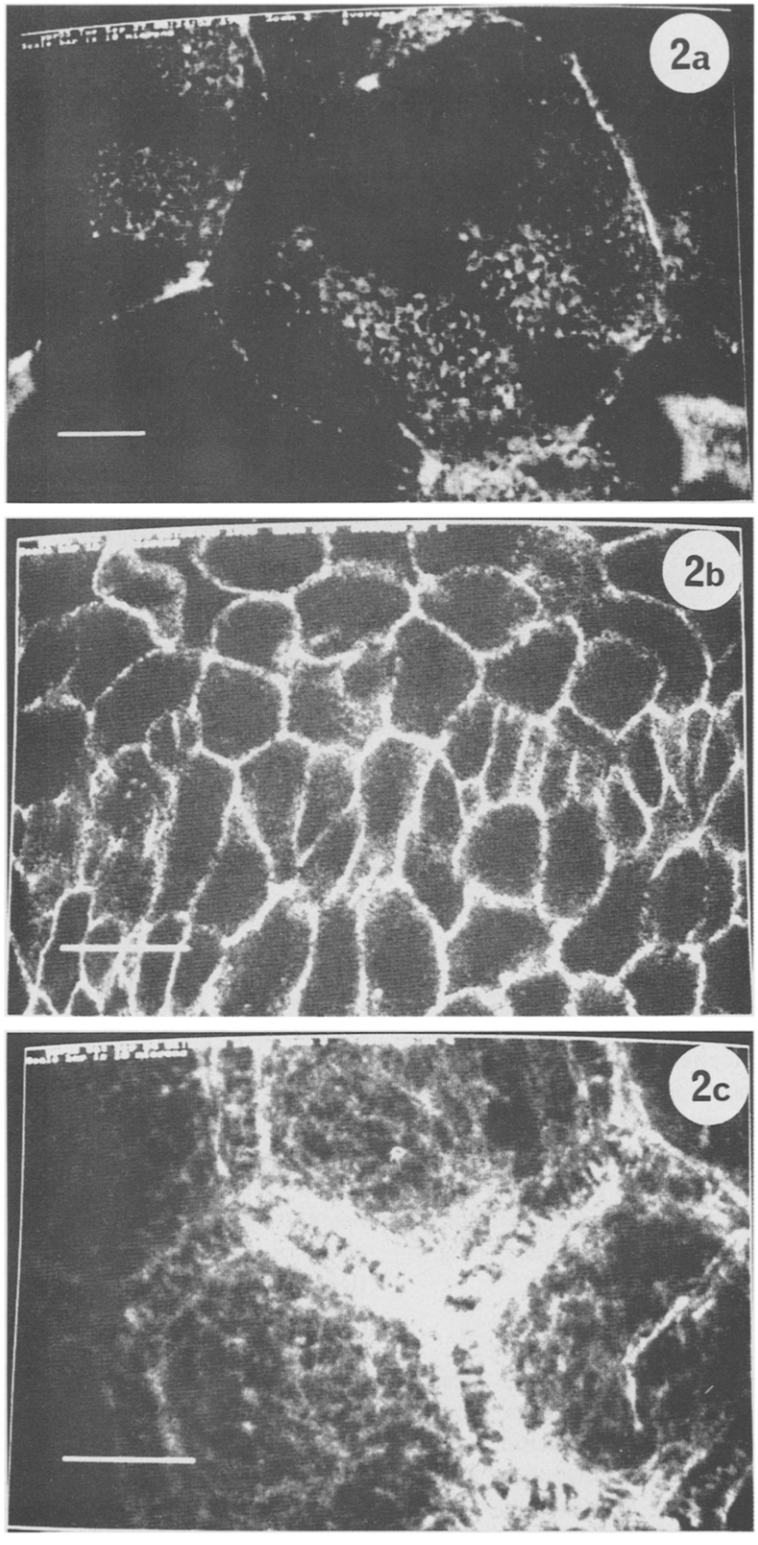

Fig. 2. Confocal fluorescence images of the phalloidin-labeled, control, cornea showing actin microfilament patterns. (a) Surface view of the epithelium. Note the distribution of cytoplasmic and tight junction-associated actins; bar = 10 μm. (b) Wing cells of the epithelium showing intense localization of actin filaments near the intercellular spaces; bar = 25 μm. (c) Endothelium showing complex actin networks in the intercellular junctions, bar = 10 μm.

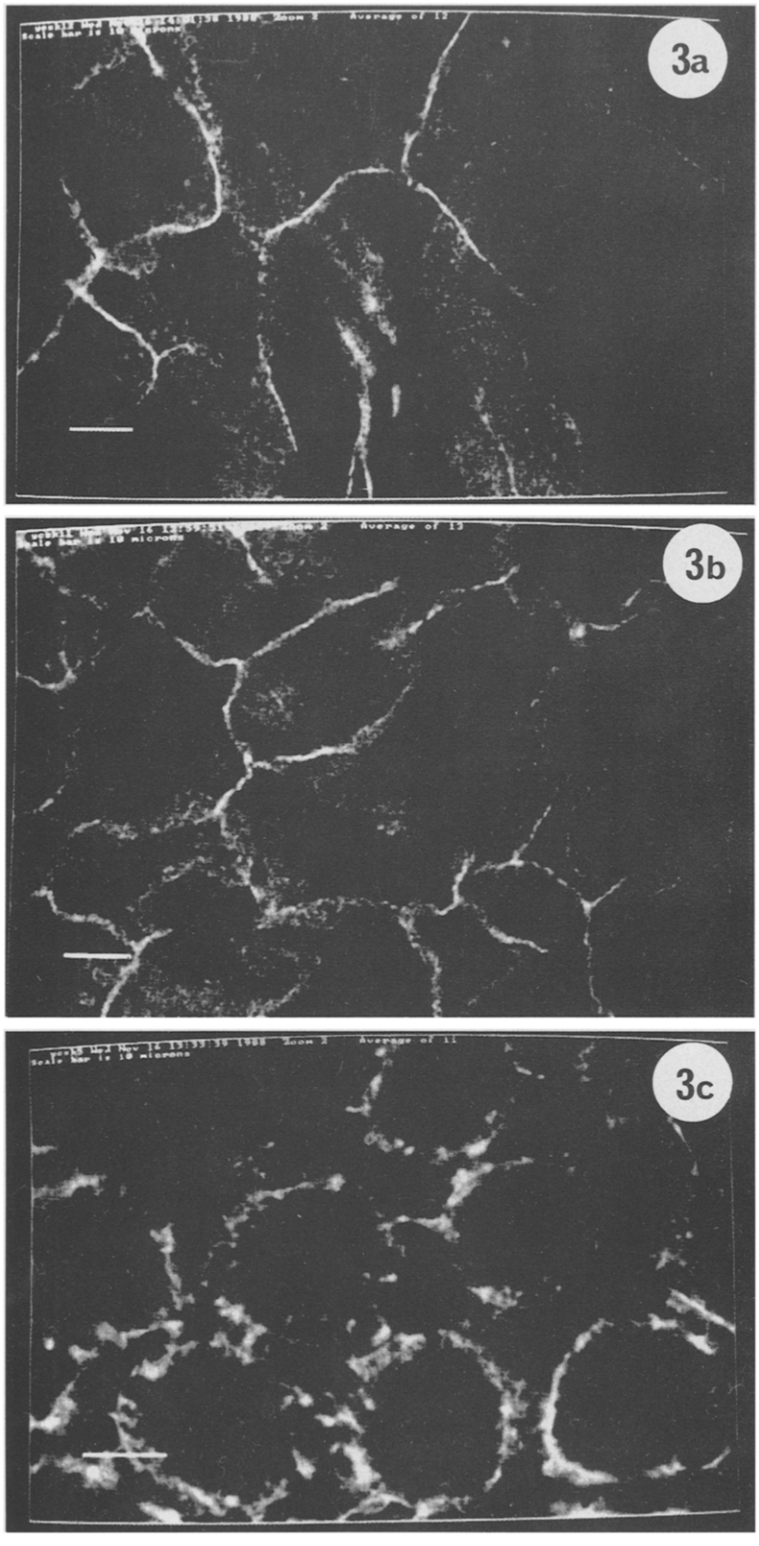

Fig. 3. Confocal fluorescence images of EDTA-treated cornea. (a) Superficial cells and (b) wing cells of the epithelium. Note the change in cell shape of the wing cells; bars = 10 μm. (c) Endothelial cells showing disruption of the interconnecting filamentous strands and cell separation; bar = 10 μm.

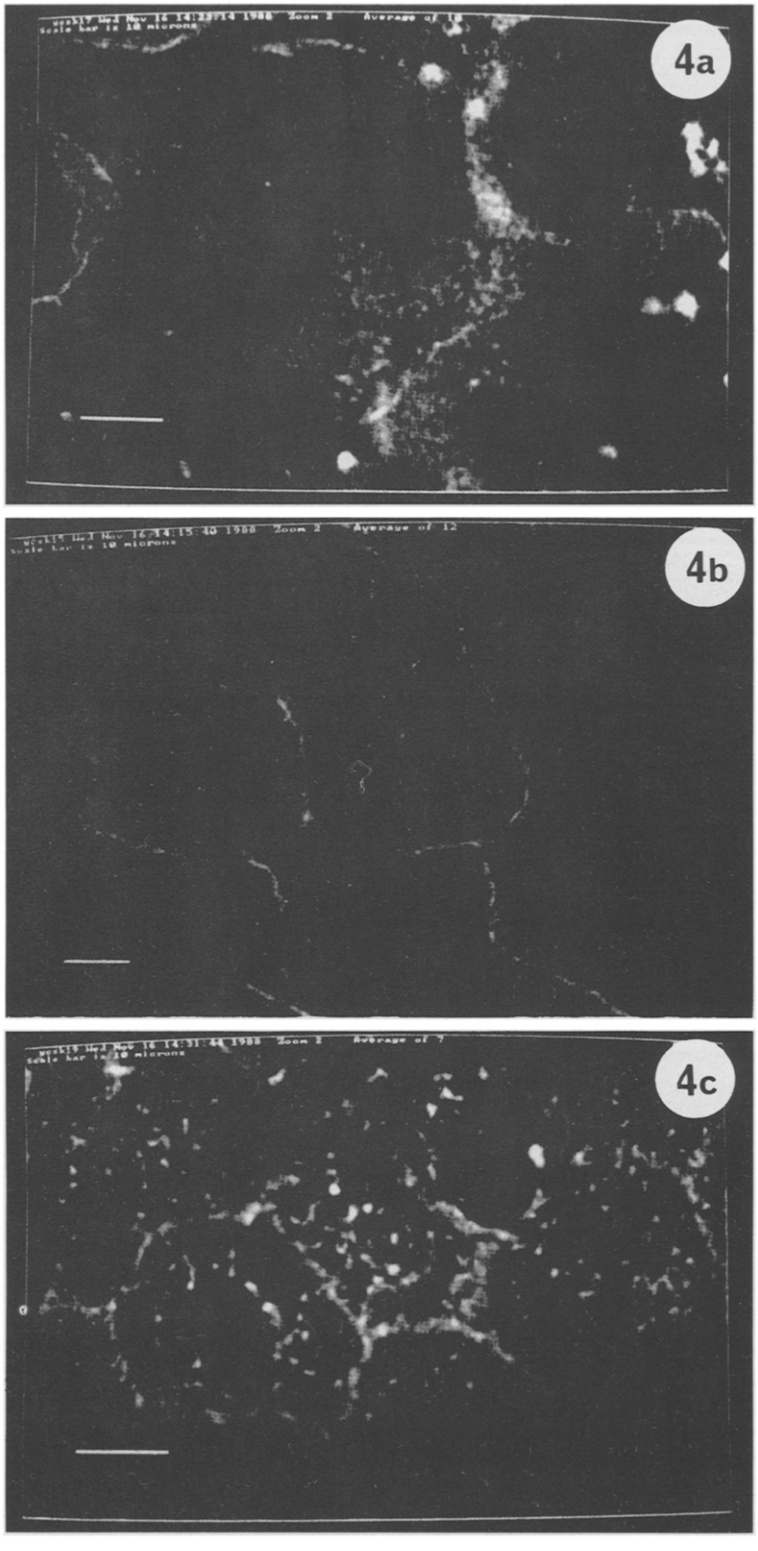

Fig. 4. Confocal fluorescence images of cytochalasin B-treated cornea showing actin microfilament patterns. (a) Superficial cells of the epithelium. Note the partial loss of tight junction-associated actins; bar = 10 μm. (b) Wing cells of the epithelium. Note the preservation of cell shape of these cells; bar = 10 μm. (c) Endothelial cells. Note the partial loss of the junctional actin bands and the preservation of cell-to-cell adhesion; bar = 10 μm.

vious microscopic studies and for comparison purpose, the concentrations of the drugs used in the study were fixed at 0.1 mM. At this concentration, EDTA and cytochalasin B decrease the corneal electrical resistance (Fig. 5) while other cytoskeleton-active drugs, namely phalloidin, colchicine, and kinetin, have no or reverse effects on corneal resistance (Fig. 6). Fig. 5 shows time profiles of the transcorneal electrical resistance of the control, EDTA, and cytochalasin B-treated corneas. Interestingly, the resistance of the control changes with time. This change was previously shown to result from changes in membrane potential and expansion of the paracellular pathway of the cornea during in vitro perfusion (Rojanasakul and Robinson, 1990a). Reduction in resistance caused by these drugs is observed through the entire period of the study with EDTA being more effective. Both drugs are believed to promote corneal permeability primarily by affecting tight junction integrity, i.e., by dissociating actin microfilaments from certain junctional proteins. The fact that EDTA causes disruption of actin networks and increases junctional permeability also suggests that maintenance of tight junction integrity by calcium may be operating through the same cytoskeletal mechanism, although the direct effect of the compound on specific junctional components can not

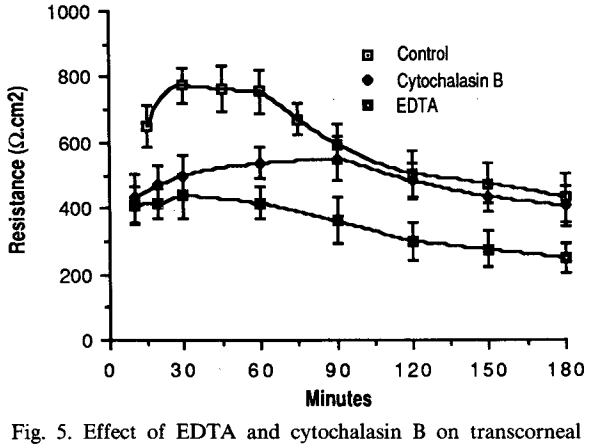

Fig. 5. Effect of EDTA and cytochalasin B on transcorneal electrical resistance. A sine wave with current density of 1–10 μA cm^{-2} and frequency of 1 Hz was applied across the cornea and the resistance was determined. Studies were conducted in GBR buffer, at 37°C. Drug concentrations were fixed at 0.1 mM. Bars indicate 1 SE; $n = 6$.

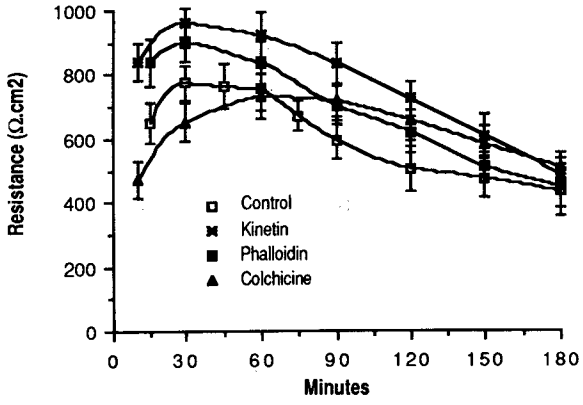

Fig. 6. Effect of phalloidin, colchicine, and kinetin on transcorneal electrical resistance. Studies were conducted in GBR buffer, at 37°C. Drug concentrations were fixed at 0.1 mM. Bars indicate 1 SE; $n = 6$.

be discarded. In addition to affecting cytoskeleton, EDTA, like many surfactants, has been shown to cause a direct deteriorating effect on the plasma membrane of the corneal superficial cells (Rojanasakul and Robinson, 1990b). The effect was not observed in cytochalasin B which may explain why EDTA causes a more drastic reduction in corneal resistance than cytochalasin B.

Phalloidin is another actin-specific agent which, unlike cytochalasin B, exhibits an opposite effect on polymerization of actin filaments. This compound stabilizes actin filaments against a variety of depolymerizing or even denaturing stimuli, decreases the critical concentration for assembly, and accelerates the rate of polymerization (Faulstich et al., 1977; Estes et al., 1981; Coluccio and Tilney, 1984). Corneas treated with phalloidin show no decrease in electrical resistance, instead, the reverse is observed (Fig. 6). A slight increase in resistance caused by phalloidin may be attributed to its stabilization effect on actin filaments which, presumably, undergo gradual disintegration during in vitro perfusion, as suggested by a gradual drop in resistance and concomitant expansion of the paracellular pathway (Rojanasakul and Robinson, 1990a). This result also explains a detection of 'intact' actin filaments by the fluorescent phalloidin in previous microscopic studies. Like phalloidin, kinetin, a microfilament-active drug which is best known for its capacity to

stimulate the growth of plant cells (Fernandez-Pol et al., 1977; Bentzel and Hainau, 1980), increases transcorneal resistance with the magnitude slightly higher than that observed in phalloidin (Fig. 6). The effect of kinetin on transepithelial resistance has been previously shown to result from an increased disorder of the tight junctional microfilament meshwork (Bentzel and Hainau, 1980).

While it is clear that microfilaments play a significant role in regulating tight junction permeability, little is known about the role of other cytoskeletal components, i.e., intermediate filaments and microtubules, on this permeability. Studies on the role of the intermediate filaments have proved difficult since no drug is known to act specifically on these filaments, although recent work by Graham et al. (1986) has suggested that 2,5-hexanedione may do so. Functionally, intermediate filaments have been proposed to serve as mechanical integrators in the cytoplasmic space and hence, having a structural role in maintaining cell shape and internal organization (Lazarides, 1980). Preliminary immunohistological studies conducted in this laboratory have indicated that these filaments are present quite uniformly inside the cells with no accumulation observed in the tight junctional and intercellular areas. No evidence, to date, has indicated the involvement of these filaments in tight junction permeability. To test whether microtubules participate in tight junction permeability, the cornea was treated with a known microtubule-disruptive drug, colchicine, and its resistive response was measured. Results of this study indicate minimal effect of this compound on the corneal electrical resistance (Fig. 6).

Reversibility of drug effect on corneal permeability

Previous work by Hudspeth (1975) and Bentzel et al. (1980) has indicated that the tight junction is a dynamic structure capable of rapid assembly and disassembly in response to various stimuli. Upon removal of compounds affecting tight junction integrity, such as EDTA and cytochalasin B, membrane permeability has been shown to reverse (Cereijido et al., 1980; Martinez et al., 1980; Meza et al., 1980). The reversibility and specificity of these compounds on tight junction integrity have attracted a great deal of interest from researchers due to the potential benefit of these compounds in drug delivery, i.e., they may be used to promote absorption of macromolecular drugs. Selective and transient modification of membrane permeability by these compounds may allow a safe and effective means of drug delivery.

As previously demonstrated, among many compounds affecting cytoskeleton, only EDTA and cytochalasin B were found to be effective in reducing the corneal electrical resistance, and thus, they were further evaluated for their reversibility. Results of these studies are shown in Figs 7 and 8, respectively. In both cases, replacement of these compounds after 30 min treatment with drug-free GBR buffer results in a total restoration of the electrical resistance, a process that is completed within 30 min after solution replacement. Prolonged exposure of the corneas to either EDTA or cytochalasin B, i.e., more than 1 h, results in only partial or sometimes no recovery of the electrical resistance. This observation is consistent with previous findings in cultured epithelia by Johnson et al. (1984) and Martinez-Palomo et al. (1980). In EDTA-treated corneas, recovery of the electrical resistance is extended for a longer period of time as compared to that observed in cytochalasin B-treated or even the control corneas. Long-term study of the reversibility effect of these two compounds is difficult due to the natural deterioration

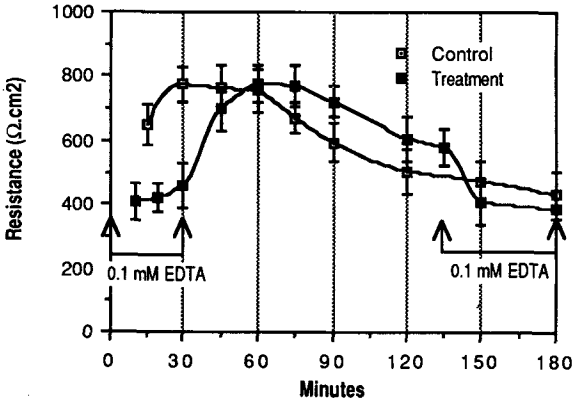

Fig. 7. Effect of EDTA on the opening and resealing of corneal tight junctions. Studies were conducted at a fixed drug concentration of 0.1 mM in GBR buffer, at 37°C. The arrows indicate the time of addition and removal of EDTA from the media. Bars = 1 SE; $n = 6$.

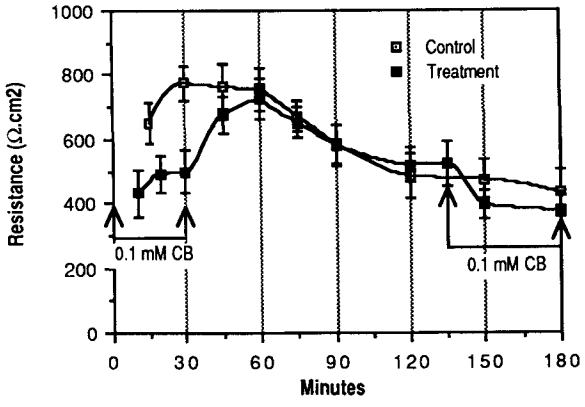

Fig. 8. Effect of cytochalasin B on the opening and resealing of corneal tight junctions. Studies were conducted at a fixed drug concentration of 0.1 mM in GBR buffer, at 37°C. The arrows indicate the time of addition and removal of cytochalasin B from the media. Bars indicate 1 SE; $n = 6$.

of the elecrical resistance and, presumably, the integrity of the cytoskeleton during in vitro perfusion. Resubstitution of the two compounds in the bathing solutions after approx. 100 min following the first solution substitution results in a drop in the resistance similar to that observed in the early period of treatment. In a separate experiment, when a calcium-free GBR solution was used instead of EDTA-GBR solution, a similar reversibility effect was observed (Fig. 9), indicating that the lack of calcium and not the presence of EDTA is

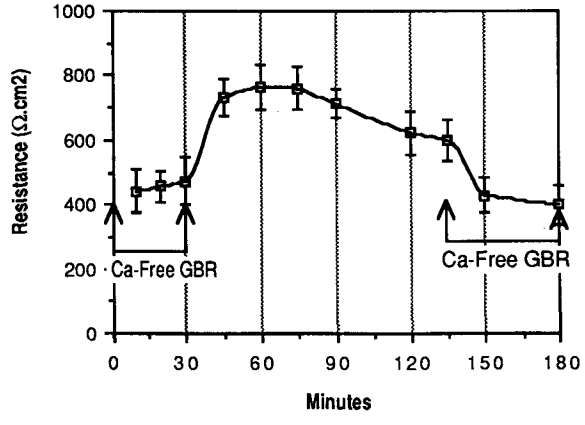

Fig. 9. Effect of calcium on the opening and resealing of corneal tight junction. The arrows indicate the time of addition and removal of calcium-free GBR buffer. Bars = 1 SE; $n = 6$.

the determining factor for maintenance of tight junction integrity.

Conclusions

The present work demonstrates the relationship between the tight junction permeability and organization of cytoskeleton in the cornea. Ultrastructural characterization of the cytoskeleton of S-1 decorated corneas indicates accumulation of actin microfilaments near tight junctions, spot-desmosomes of the intercellular spaces, and at the apical surface of the epithelium. These filaments appear to be the major cytoskeletal components influencing tight junction permeability. Drugs that disrupt organization of actin network such as EDTA and cytochalasin B also cause an increase in corneal permeability, while those stabilizing it, i.e., phalloidin and kinetin, have the reverse effect. Colchicine, a microtubule-active drug, has no effect on corneal permeability. The effect of EDTA and cytochalasin B is fully reversible upon their removal, but only with limited exposure time. These compounds may be of potential use in promoting absorption of drugs, especially those permeating the membrane paracellularly.

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