STUDIES ON THE CORNEA

IX. Physiologic and Morphologic Effects of Cytochalasin B on Endothelium of Rabbit Corneas Perfused In Vitro

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INTRODUCTION

Previous reports from our laboratories have separately shown that the reversibility of the effects of Ca^{2+} depletion on rabbit corneal endothelium, including the morphologic restoration of the endothelial junctional complexes, depends on the morphologic restoration of a network of microfilaments in the apical cytoplasm (7, 9); and that cytochalasin B reduces both transendothelial potential difference and fluid transport measured in vitro (4, 5). The similarity of some of the morphologic effects of Ca^{2+} depletion in the rabbit corneal endothelium, namely, disruption of the apical microfilaments, cell shape change and loss of junctional integrity, (7–9), and the effects of cytochalasin B in morphogenesis, namely, loss of microfilament integrity and cell shape change (1, 17), led us to examine the effects of this agent on the rabbit cornea perfused in vitro according to the method of Dickstein and Maurice (2).

MATERIALS AND METHODS

Six sets of paired or triplicate perfusions were carried out at cytochalasin B concentrations of 1, 2, 5, 10, and 40 μg/ml in modified Dickstein-Maurice medium (MDMM) (4).[1] In addition, paired corneas were perfused with 0.25% and 0.5% DMSO (the solubilizing agent for the cytochalasin B at both the concentration used and twice the concentration used) and with a Ca^{2+}-free medium.

All corneas were observed directly through the specular microscope during perfusion and corneal thickness was measured every 7.5 or 15 min (2). Some endothelia were photographed directly through the specular microscope (2) during perfusion. At the end of the experimental period (ranging from 52.5 to 142.5 min) corneas were perfused with 2% OsO_4 in Sorensen's phosphate buffer at pH 7.4 or with phosphate-buffered 2% glutaraldehyde and processed for transmission (7, 8) and scanning electron microscopy, respectively.

All specimens for scanning were processed whole, dehydrated with a graded series of ethanol solutions and infiltrated with a graded series of ethanol-amyl acetate solutions before quatering and critical point drying from liquid CO_2 in a Denton critical point drying apparatus (Denton Vacuum Inc., Cherry Hill, N. J.) without exposure to air after fixation. Transmission specimens were examined with an RCA-EMU 3-G electron microscope and scanning specimens coated with gold-palladium were examined with a JEOLCO JSM-U3 scanning electron microscope at an accelerating voltage of 25 kv.

RESULTS

Neither the DMSO control perfusions at either concentration nor the 1 μg/ml concentration of cytochalasin B produce significant corneal swelling

[1] Modified D-M medium (MDMM): 110 mM NaCl, 3.8 mM $KHCO_3$, 39 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 1.0 mM KH_2PO_4; 1.7 mM $CaCl_2$, 6.9 mM glucose, 5.0 mM adenosine, 0.24 mM glutathione; pH 7.4 adjusted by bubbling with pure CO_2 before use.

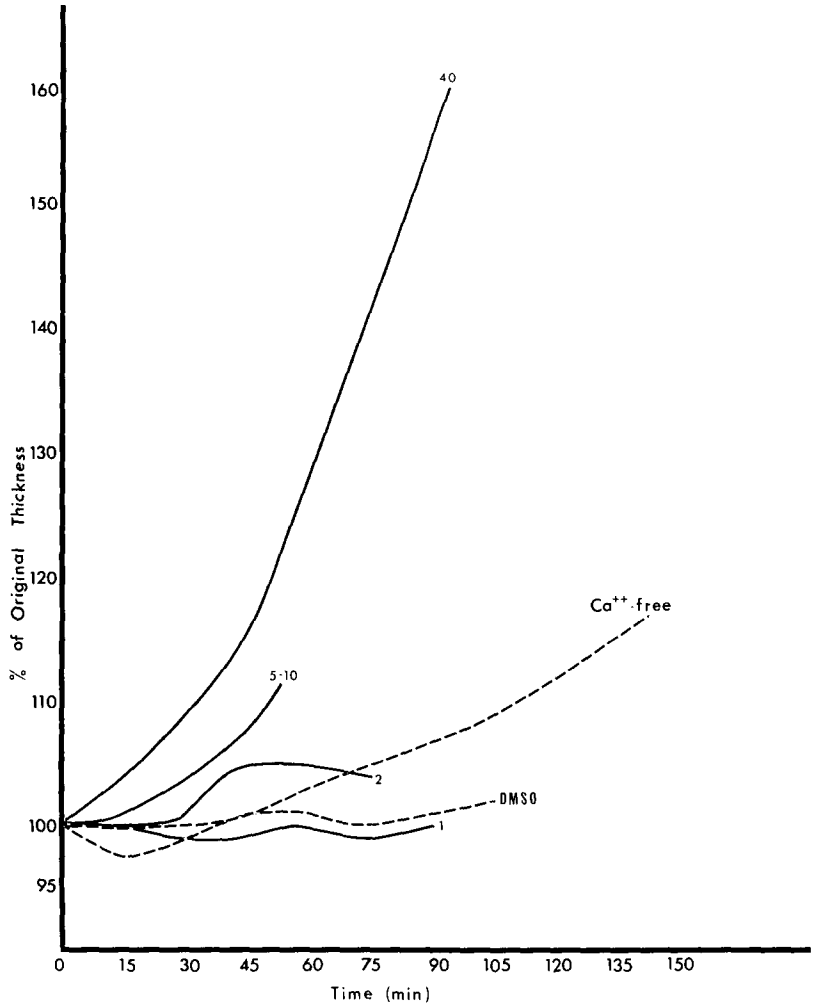

FIGURE 1 Thickness versus time curves for the several sets of corneas perfused with varying concentrations of cytochalasin B (1, 2, 5–10, and 40 μg/ml), with DMSO and with a Ca^{2+} free medium. Each curve is an average for 2–5 corneas. The rate of change of corneal thickness has been shown to be the most reliable measurement of transendothelial fluid movement (8, 9, 14).

(Fig. 1), but even this low concentration of cytochalasin B produces focal disruption of the apical microfilament network in the endothelial cells and apical cytoplasmic bulging (Fig. 2). In the DMSO controls, however, neither transmission nor scanning electron microscopy reveals any significant morphologic alteration of the cell surface (Figs. 3 *a* and 3 *b*). Above 2 μg/ml there is a progressive increase in both the degree and rate of corneal swelling (Fig. 1) accompanied by progressive change in endothelial cell shape (Figs. 4 *a*–4 *b*, 5 *a*–5 *b*). The apparent movement of endothelial cells observed through the specular microscope during perfusion with the higher concentrations of cytochalasin B is reflected in the fixed preparations by the extensive change in cell shape, as well as by the shift from a single layer of cells with a smooth continuous surface to a situation in which highly attenuated portions of endothelial cytoplasm alternate with zones in which endothelial cells (or, at least, their nuclear zones) overlap one another (Fig. 5 *a*). Even in the most extreme situation observed (40 μg/ml perfusion with swelling to more than 160% of normal thickness at a rate of more than 200 μm/h) endothelial junctions retain their morphologic integrity (Fig. 5 *b*) despite the attenuation of the cytoplasm in places to less than 0.5 μm (less than 10% of normal) (Figs. 5 *a*–*b*).

The microfilaments of the apical exoplasm of the corneal endothelial cells first become disrupted

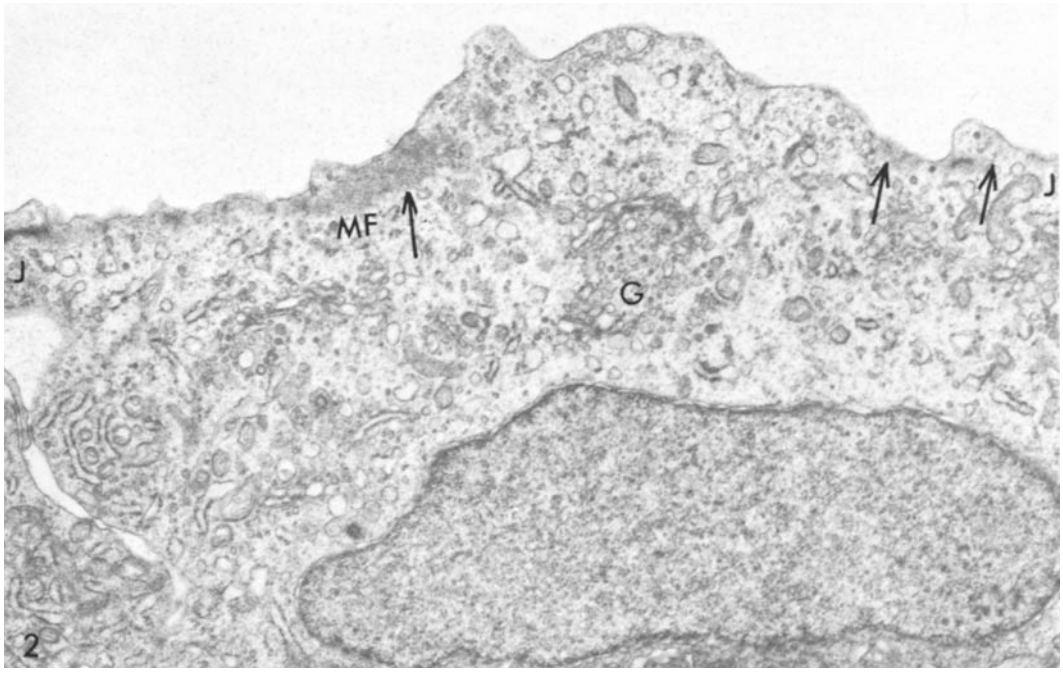

FIGURE 2 Electron micrograph of a portion of a cornea perfused with 1 μg/ml cytochalasin B. Although there was no swelling of this cornea, the endothelial cells show focal disruption of the microfilament network (*MF*), seen here primarily as cross sections of the long filaments which normally would subtend the entire apical plasma membrane (7, 9). The apical cytoplasm evaginates in the zones in which the microfilaments are absent. Arrows delimit the discontinuities in the microfilament network. *J*, junctional zone; *D*, Descemet's membrane; G, Golgi complex. × 11,500.

near the middle of the cell, above the nucleus (Fig. 2), and a cytoplasmic bulge forms at the site. Scanning electron micrographs of corneal endothelium fixed during perfusion with cytochalasin B at 5 or 10 μg/ml show that not all cells are equally affected; those which are, are distributed in a serpentine pattern in the endothelium (Fig. 4 *a*). The number of cells affected increases both with length of time of perfusion at a given concentration of cytochalasin B and with increased concentration of the agent.

Perfusion with the simple Ca^{2+}-free MDMM leads to the same rapid corneal swelling and separation of endothelial junctions as previously reported after perfusion with Ca^{2+}-free Kresge Eye Institute medium (7–9, 15). The early phase of this separation is better seen with scanning electron microscopy (Fig. 6) than with transmission electron microscopy (7–9).

Preliminary experiments suggest that, as with other agents or insults which affect the corneal endothelial barrier, the effects of cytochalasin B may be reversed or attenuated in this tissue as they are in other tissues (17) if the cornea is returned to a normal medium before it has swollen to more than 150–200% of normal thickness (Fig. 7).

DISCUSSION

The present study demonstrates that the apical microfilament network of the rabbit corneal endothelium (7, 9) is similar to that described in other cells (1, 17) in that it has a function in maintaining normal cell shape, and it can be disrupted by cytochalasin B. The corneal endothelium may be unique, however, in that these effects are easily demonstrable in a mature tissue in its normal location and can be monitored continuously by direct microscopic observation of the endothelium during perfusion and by measurement of several physiologic parameters normally used to evaluate the functional barrier characteristics of this cell layer (2, 4, 7, 14, 15).

The relationship between the effects of Ca^{2+} depletion (7–9) and cytochalasin B on the micro-

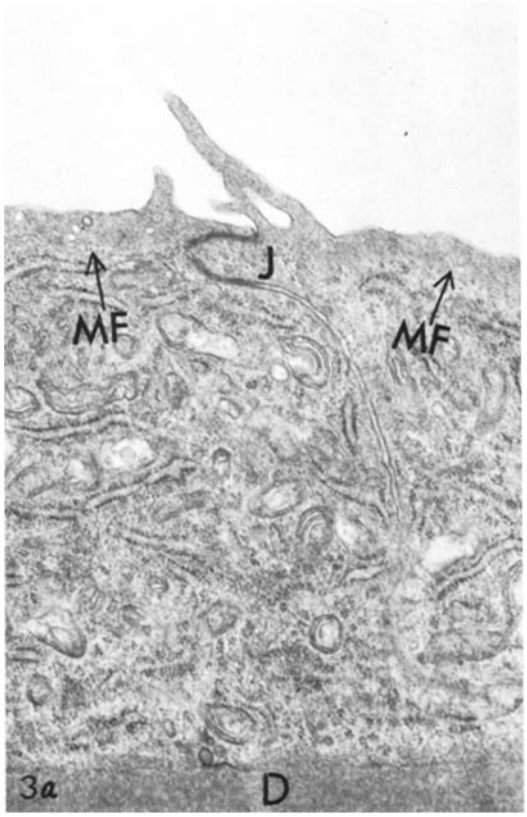

FIGURE 3 a Junctional zone and adjacent cytoplasm of a cornea perfused with DMSO. The apical surface of the cell is smooth and the dense zone immediately subtending the apical plasma membrane (which indicates the microfilament network (*MF*) in this glutaraldehyde fixed cornea) is regular and continuous. *J*, junctional zone; *D*, Descemet's membrane. × 26,400.

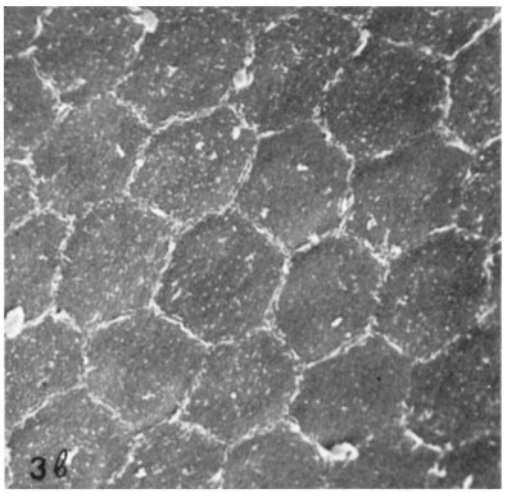

FIGURE 3 b Scanning electron micrograph of control cornea perfused with 0.5% DMSO. The normal hexagonal pattern of the cell margins of the flat endothelial cells is evident throughout. × 900.

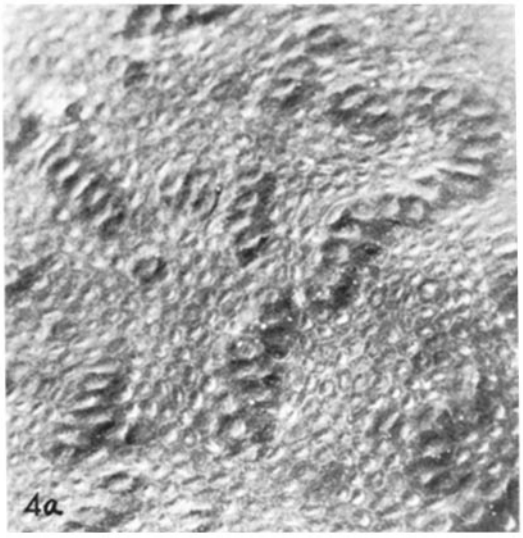

FIGURE 4 a Scanning electron micrograph of a cornea perfused with 5 μg/ml cytochalasin B. The early changes in the endothelium appear as serpentine patterns of cells with attenuated cytoplasm and supranuclear bulges. × 200.

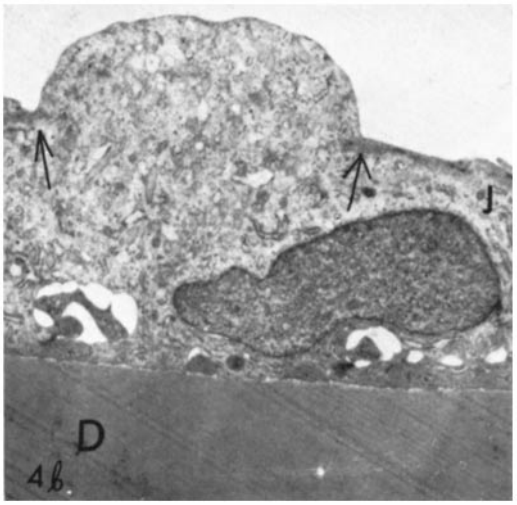

FIGURE 4 b Transmission electron micrograph of a cornea paired to that in Fig. 4 *a* showing the supranuclear bulge in that portion of the apical cytoplasm between the ends of the disrupted microfilament network (arrows), again seen primarily as cross sections not well resolved at this low magnification. *J*, junctional zone; *D*, Descemet's membrane. × 5,100.

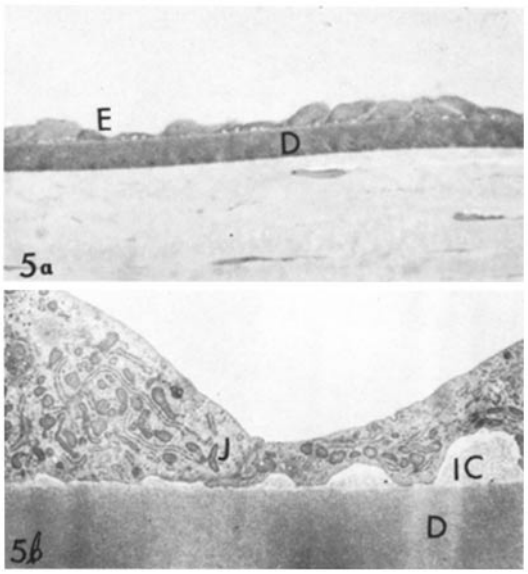

FIGURE 5 a Phase-contrast photomicrograph of 1-μm Epon section of a cornea perfused with 40 μg/ml cytochalasin B. The extreme attenuation of parts of endothelial cells (*E*) is evident along with the apparent clumping of perikarya of several cells, rotation of long axis of the nuclei, and distention of the intercellular spaces. *D*, Descemet's membrane. × 350.

FIGURE 5 b Electron micrograph of a portion of the same cornea showing the cobblestone shape of two endothelial cells, the distended intercellular space (*IC*) at the basal surface, the absence of an apical microfilament layer, and the preservation of the junction (*J*) between the two cells. Amorphous dense patches just under the apical plasma membrane at several sites probably represent remnants of the microfilament layer. *D*, Descemet's membrane. × 5800.

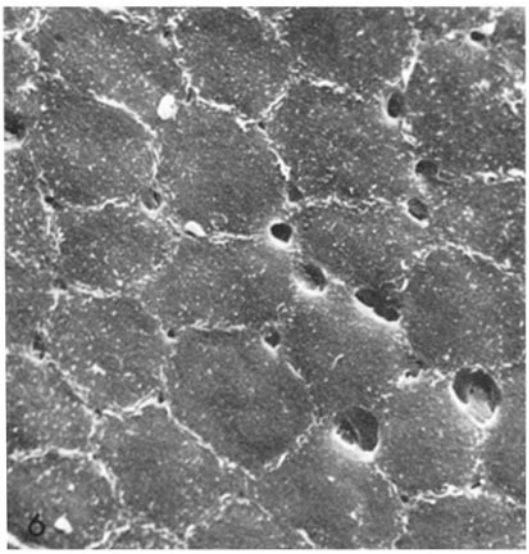

FIGURE 6 Scanning electron micrograph of control cornea perfused with a Ca^{2+}-free medium. This cornea was fixed at approximately 120% of normal thickness. The cells have begun to assume the cobbled appearance previously described (7–9) and separation of cells at their junctional zones is evident in several places, disturbing the normal hexagonal pattern. × 900.

filaments is still unclear. There have been suggestions from other laboratories that both the contraction of microfilaments and the effects of cytochalasin B may be calcium-dependent[2] (6). There is, as yet, no evidence that the microfilaments of the corneal endothelium are contractile as well as structural. Their presence appears essential to maintain the normal shape of the endothelial cells and the flat apical endothelial surface (7–9; Figs. 2, 4, 5); their presence also appears essential for the extension of the cytoplasmic flaps which participate in the restoration of normal structure and function during reversal of the effects of Ca^{2+} depletion (7–9). In this regard they resemble the microfilaments found in growing nerves more closely than those in differentiating glandular epithelium (17). It is, however, conceivable that a network of microfilaments under constant tension with attachment at the junctional zone could maintain a flat endothelial surface in much the same way that tensioning wires in prestressed concrete construction can maintain stability of flat concrete slabs over large spans (11).

We have previously shown that ^{45}Ca localizes in the junctional zone in the corneal endothelium recovering from the effects of perfusion with a Ca^{2+}-free medium (7). The lability of the endothelial junction and the incorporation of Ca^{2+} during repair suggests that loosely bound calcium may be a normal component of the junction. It may be significant, therefore, that the first recognizable morphologic effects of cytochalasin B on the corneal endothelium are found at the portion of the plasma membrane farthest from the junction (Fig. 2) where the concentration of loosely bound calcium would presumably be lowest. Attempts to modify the effects of cytochalasin B on the corneal endothelium by increasing the availability of free Ca^{2+} (increasing the Ca^{2+} concentration in the medium to two times and three times normal) have been unsuccessful. Conversely,

[2] Bernfield, M. R. 1972. Personal communication.

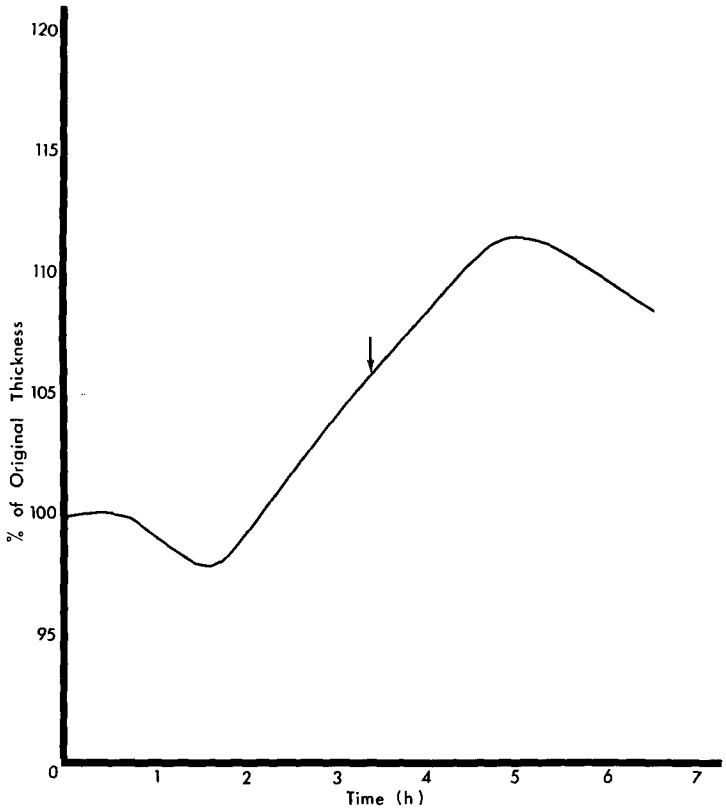

FIGURE 7 Thickness versus time curve for a pair of corneas perfused first with 10 μg/ml cytochalasin B and then switched to MDMM (at arrow). The corneas continue to swell during the mixing of the media in the chamber but, eventually, the effects of cytochalasin B are reversed and the cornea thins. Preliminary transmission electron microscope examination of these corneas shows a smooth apical surface and complete microfilament network.

corneas swollen with 10 μg/ml cytochalasin B can be made to swell more and more rapidly by switching to Ca^{2+}-free MDMM. Also of interest is the fact that the initial pattern of affected cells in the endothelium resembles that seen in many epithelia when electrical coupling at junctions is investigated (12, 13).

In the Ca^{2+}-free perfusions it has been relatively easy to demonstrate that the progressive disruption of the endothelial junction is responsible for an increase in the passive permeability of the corneal endothelium to nontransported nonelectrolytes (7–9, 15) paralleling, and probably responsible for the corneal swelling. Fischbarg (4, 5) has shown that cytochalasin B reduces net transendothelial fluid transport in a corneal preparation lacking epithelium studied in vitro (see also, 2, 14) and has suggested that the effect is principally on the barrier function of the endothelial cells (5). The precise relationship between the change in cell shape caused by perfusion with cytochalasin B (Figs. 2, 4, 5) and corneal swelling (Fig. 1) is not clear as it is in the Ca^{2+}-free condition (References 7–9; Figs. 1 and 6). It is possible that membrane deformation leads to disruption of a fluid transport system which normally functions to maintain a precise level of corneal hydration (2, 14, 15) or, more simply, it may be that the extreme attenuation of portions of endothelial cells, shortening of intercellular channels, and possible stretching of portions of the apical plasma membrane along with a change in the conformation of the exoplasmic microfilament network makes an already relatively permeable barrier (5, 7–9, 14) even more leaky. Studies of passive permeability to nontransported nonelectrolytes must be carried out on cytochalasin B perfused corneas in order to enable one more precisely to compare these with Ca^{2+} depleted corneas.

The present study has clearly demonstrated,

however, that cytochalasin B, even at low concentrations, produces progressive corneal swelling and significant changes in the morphology of the endothelial cells, particularly the progressive disruption of the microfilament network. While much work remains to be done to elucidate the possible relationships between the similar effects of Ca^{2+}-free perfusion and cytochalasin B on the corneal endothelium, and to investigate whether cytochalasin B may exert its effect through actions on transport or utilization of metabolic substrates (3, 10, 16), we believe that the corneal endothelium provides a nearly ideal system in which to study these problems because of the ease of manipulation, the considerable body of data on the effects of other agents on this tissue, the possibility in the present perfusion system for continuous visual monitoring of the development of the morphophysiologic changes in the living system and the relative ease of preparation for both transmission and scanning electron microscopy.

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