Formation and Disassembly of Adherens and Tight Junctions in the Corneal Endothelium: Regulation by Actomyosin Contraction

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PURPOSE. To determine the role of actin cytoskeleton in the disassembly and reformation of adherens junctions (AJs) and tight junctions (TJs) in bovine corneal endothelial monolayers.

METHODS. Disassembly and reformation of AJs and TJs were induced by extracellular Ca^{2+} depletion and subsequent add-back of Ca^{2+} , respectively. Resultant changes in the transendothelial electrical resistance (TER), an indicator of integrity of TJs, were measured based on electrical cell-substrate impedance. Phosphorylated myosin light chain (ppMLC), a biochemical measure of actomyosin contraction, and activation of its upstream regulatory molecule RhoA-GTP were assessed by Western blot analysis.

RESULTS. Extracellular Ca^{2+} depletion led to activation of RhoA, increase in ppMLC, decrease in TER, contraction of the perijunctional actomyosin ring (PAMR), and redistribution of zonula occludens-1 (ZO-1) and cadherins. These effects were reversed on Ca^{2+} add-back. Pretreatment with Y-27632 and blebbistatin (as inhibitors of actomyosin contraction) reduced the rate of decline in TER, opposed the contraction of the PAMR, and blocked the redistribution of ZO-1 and cadherins. Both drugs reduced the recovery in TER and opposed the normal redistribution of ZO-1 and cadherins on Ca^{2+} add-back. Cytochalasin D, which led to dissolution of the PAMR, also reduced the recovery of TER on Ca^{2+} add-back.

CONCLUSIONS. The (Ca^{2+} depletion)-induced disassembly of AJs accelerates the breakdown of TJs through a concomitant increase in the actomyosin contraction of the PAMR. However, these data on reassembly show that a contractile tone of the PAMR is essential for assembly of the apical junctional complex. (*Invest Ophthalmol Vis Sci.* 2010;51:2139–2148) DOI:10.1167/iov.09-4421

The transparency of the cornea requires deturgescence of its connective tissue, the stroma. The cellular monolayer at the posterior surface of the cornea, the endothelium, is thought to be solely responsible for the maintenance of stromal deturgescence.¹ This essential physiological role of the endothelium is dependent on its barrier function and its fluid pump activity.^{2–5} The barrier function confers resistance to facile influx of water into the stroma from the aqueous humor secondary to the imbibition property of the glycosaminoglycans

in the tissue.^{6,7} The fluid pump activity, on the other hand, drives fluid out of the stroma into the aqueous humor, and it is based on the mechanism of active ion transport.^{2,5} Given this putative “pump-leak” phenomenon associated with the endothelium,⁷ a rigorous understanding of the mechanisms underlying the dynamic regulation of the barrier function becomes important for developing pharmacologic strategies against corneal edema. In this context, two significant challenges to maintaining the barrier integrity of corneal endothelium other than that associated with aging should be recognized. The first challenge involves loss of barrier integrity in response to cell signaling provoked by inflammatory stress,⁸ whereas the second threat entails endothelial cell loss and consequent exposure of the stroma to the aqueous humor.

As a characteristic among the epithelia, the corneal endothelium exhibits a thick band of actin cytoskeleton proximal to the apical junctional complex (AJC),⁹ which has been referred to as the perijunctional actomyosin ring (PAMR).^{9,10} This pool of actin cytoskeleton manifests structural associations with the adherens junctions (AJs) and tight junctions (TJs) through linker proteins such as zonula occludens-1 (ZO-1).^{11,12} Such interactions enable cell signaling, especially those involving the Rho family of small GTPases, to dynamically regulate the integrity of AJs and TJs through the PAMR.^{13–16} In fact, emerging evidence suggests that an enhanced tone of the PAMR (i.e., increased actomyosin contraction) is detrimental to the barrier integrity of cellular monolayers.^{15,17,18} It is plausible that when the PAMR undergoes excessive actomyosin contraction, the resultant centripetal forces reduce the cell-cell tether and consequently break down the barrier integrity.^{10,14,18,19}

A number of studies, especially with vascular endothelium, have demonstrated that actomyosin contraction is regulated by the small GTPase RhoA through its effector, Rho kinase.^{20–24} This kinase phosphorylates the regulatory subunit of myosin light chain phosphatase (i.e., MYPT1; 130 kDa)^{25,26} and thereby inhibits the dephosphorylation of myosin light chain (MLC). A consequent increase in the phosphorylation of MLC elicits myosin II ATPase-mediated actomyosin contraction.^{17,27,28} It has been demonstrated that thrombin-induced MLC phosphorylation along the locus of PAMR results in a breakdown of the barrier integrity in corneal endothelium.¹⁵ Similar effects have been noted with respect to other agents, some of which are relevant in response to inflammatory stress.^{15,28,29}

In contrast to the indirect influence of enhanced actomyosin contraction of the PAMR, cell loss presents a direct threat to barrier property of the corneal endothelium. Loss of corneal endothelial cells occurs constantly during aging but is reported to be pronounced during Fuch's dystrophy and in response to iatrogenic injury (e.g., phacoemulsification).³⁰ In transplanted corneas after keratoplasty, cell loss is known to be both acute and chronic.³¹ When endothelial cell density, which is typically 2500 cells/mm² in healthy adults, reduces to <700 cells/mm², the monolayer cannot sustain stromal hydration control,

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and corneal edema becomes inevitable.³ When the endothelium sustains loss of cells or is challenged by inflammatory stress,³² it is crucial to know the factors likely to impact the reassembly of cell-cell junctions, which is essential for resumption of the normal physiological activity of the monolayer.

The primary aim of this study was to elucidate the role of actin cytoskeleton in the dynamic regulation of the integrity of AJs and TJs in corneal endothelial monolayers. Specifically, our goal was to investigate the influence of actin cytoskeleton on the dynamics of disassembly and reassembly of AJs and TJs on extracellular Ca^{2+} depletion and Ca^{2+} add-back, respectively. To underscore the impact of actin cytoskeleton during disassembly and reassembly, we chose pharmacologic agents to selectively modulate actin polymerization and actomyosin contraction. To follow the temporal course of the integrity of AJs and TJs during Ca^{2+} switch, we measured transendothelial electrical resistance (TER) and examined AJ and TJ markers by immunofluorescence at the AJC. Our results reconfirm the importance of increased actomyosin contraction in the breakdown of barrier integrity. Furthermore, our observations show that a significant reduction in actomyosin contraction prevents the reassembly of AJs and TJs. Thus, taken together, our findings contribute to further understanding of the barrier function of the corneal endothelium during health and disease.

MATERIALS AND METHODS

Drugs and Chemicals

Cell culture supplies were from Gibco (Carlsbad, CA) and Sigma-Aldrich (St. Louis, MO). ZO-1 and pan-cadherin antibodies were from Zymed Laboratories (San Francisco, CA) and Sigma-Aldrich, respectively. Phosphospecific MLC (Thr18 and Ser19; denoted as ppMLC) antibody was purchased from Cell Signaling Technology (Danvers, MA). Texas-red conjugated phalloidin and Alexa-488 conjugated goat anti-mouse antibodies were from Molecular Probes (Eugene, OR). RhoA activation assay kit was from Cytoskeleton, Inc. (Denver, CO). Gold electrodes (8W10E+) for measuring TER were from Applied Biophysics (Troy, NY). All other drugs and chemicals were from Sigma.

Cell Culture

Bovine corneal endothelial cells (BCECs) were harvested and cultured as described earlier.^{15,29,33} First- and second-passage cells were used in all experiments. To corroborate experiments with cultured BCECs, limited experiments were carried out with endothelium isolated from rabbit eyes (Pel-Freeze Biologicals; Rogers, AR). For immunostaining, rabbit eyeballs were cut at the equator, and the endothelial layer was exposed to the Ca^{2+} -free medium for 30 minutes. Similarly, to study the reassembly, Ca^{2+} -free medium was replaced with the Ca^{2+} -rich medium after 30 minutes and allowed to reassemble for 3 hours. At the end of 30 minutes or 3 hours, endothelium was isolated from the rabbit cornea and fixed immediately using paraformaldehyde (4%). The subsequent steps in the staining procedure are given below.

Ca^{2+} Switch Protocol

To induce dissociation of the AJs, cells were exposed to Ca^{2+} -free DMEM containing 2 mM EGTA for 30 minutes. Next, to induce the reassembly of AJs, Ca^{2+} -free DMEM was replaced with DMEM containing 1.8 mM Ca^{2+} (Ca^{2+} add-back). This approach was used to measure the changes in the TER and to study the remodeling of AJC by immunofluorescence. All experiments were carried out at 37°C in a humidified atmosphere containing 5% CO_2 .

Measurement of TER

Cells were seeded at a density of 5×10^5 cells/mL on gold electrodes ($250 \mu\text{m}^2$) and placed in the incubator at 37°C. To measure TER, a small AC current was applied across the electrode, and the impedance

for current flow was measured by a lock-in amplifier at ~ 0.1 Hz (ECIS model 1600R; Applied Biophysics). Initial experiments revealed that impedance at 4 kHz would provide the most sensitive changes in the measurement of TER for BCECs. Cell-substrate impedance was monitored continuously after cells were seeded. When the resistive component remained stable for at least 2 hours, cells were subjected to Ca^{2+} switch protocol, as described. On average, TER reached ~ 1700 to 2000Ω (total area of the electrode, $250 \mu\text{m}^2$) after 20 hours. For analysis, the TER values were normalized to the values obtained before Ca^{2+} depletion. TER was monitored for 30 minutes before the start of the experiments to ensure a steady baseline measurement. During the reformation of AJs and TJs after Ca^{2+} add-back, the normalized TER was averaged across trials and is represented as mean $\pm$ SEM.

Immunofluorescence

Double immunostaining was performed after cells were fixed with 4% paraformaldehyde at different time points during the Ca^{2+} switch. The cells were permeabilized with 0.2% Triton X-100 for 5 minutes, followed by blocking in 1% BSA and 5% goat serum for 1 hour at room temperature. The cells were incubated in 1:25 of ZO-1 and 1:1000 of pan-cadherins primary antibodies for 1 hour. After washing and incubation with the secondary antibody for 1 hour, cells were stained for F-actin using phalloidin. Coverslips were mounted on slides with antifade reagent. For the disassembly experiments, cells were fixed 30 minutes after Ca^{2+} depletion, and for the assembly experiments cells were fixed 3 hours after Ca^{2+} add-back in the presence or absence of the inhibitors. Freshly isolated rabbit endothelial cells were placed on microslides and processed similarly to cultured cells. Confocal images of the actin cytoskeleton and junctional protein ZO-1 and cadherins were taken with a fluorescence microscope (SP5; Leica, Wetzlar, Germany) to record the changes occurring during Ca^{2+} switch.

Detergent Extraction, MLC Phosphorylation, and Western Blot Analysis

After Ca^{2+} depletion for 30 minutes, the soluble fraction was collected by incubating cells in 200 μL of 0.5% Triton X-100 in PBS for 5 minutes on ice. To the supernatant, 40 μL of 5 $\times$ Laemmli sample buffer was added immediately. The insoluble fraction was resuspended in 240 μL of 5 $\times$ sample buffer. For each fraction, 40 μL sample was loaded on an 8% SDS-PAGE gel. For analysis of MLC phosphorylation (ppMLC), cells were rinsed in PBS and solubilized in 300 μL of 2 $\times$ Laemmli sample buffer. After protein estimation using the Lowry method, samples were boiled for 5 minutes, and equal protein was loaded in a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% fat-free milk for 1 hour, and incubated with the anti-phospho-MLC (ppMLC) or pan-cadherin antibody. Membranes were incubated with appropriate secondary antibodies for 1 hour, and blots were washed and developed using an enhanced chemiluminescence kit (Pierce, Rockford, IL).

RhoA Activation Assay

Confluent serum-starved cells were Ca^{2+} depleted for 30 minutes. These cells were lysed on ice using a lysis buffer (50 mM Tris, pH 7.5, 10 mM MgCl_2 , 0.5 M NaCl, 0.1% Triton X-100, and 0.1% SDS) containing protease inhibitor cocktail (Sigma). The lysate was centrifuged for 5 minutes at 12,000g at 4°C. The supernatant was incubated with rho assay reagent (Rhotekin-RBD; Cytoskeleton Inc., Denver, CO) beads (20–30 μg) for 1 hour at 4°C. Beads were washed with ice-cold Tris-buffer containing 10 mM MgCl_2 and 150 mM NaCl and were spun at 5000 rpm. The immunoprecipitated complex was resuspended in a 2 $\times$ SDS sample buffer, boiled at 95°C for 5 minutes, and subjected to 15% SDS-PAGE, followed by Western blot analysis. The separated proteins were immunoblotted with antibody against RhoA.

Statistical Analysis

For analysis of TER results, normalized values from individual experiments were pooled and expressed as mean $\pm$ SE. $P < 0.001$ was

considered statistically significant for TER measurements. For Western blot analysis of ppMLC and detergent extraction, data were compared by one-way analysis of variance with Bonferroni's posttest analysis using a statistical analysis software (Prism version 5.0; GraphPad Software, Inc., San Diego, CA) and results are shown in bar graphs with mean $\pm$ SEM. For RhoA activation assay, a *t*-test was conducted. $P < 0.05$ was considered statistically significant. For all results, *n* denotes number of independent experiments.

RESULTS

Changes in TER and Remodeling of the Apical Junctions during Ca^{2+} Switch

Figure 1 shows a summary of the dynamics of TER during the Ca^{2+} switch maneuver. Extracellular Ca^{2+} removal led to a rapid decrease in TER (90% $\pm$ 1% of the decrease in <10 seconds; $n = 3$), but its subsequent replacement (i.e., 1.8 mM Ca^{2+} add-back) returned TER to baseline in ~ 3 hours. Replacement with <1.8 mM Ca^{2+} led to a partial recovery (50% at 0.5 mM compared with 1.8 mM Ca^{2+} ; also shown in Fig. 1; $n = 3$). As expected, there is no recovery of TER in the absence of Ca^{2+} add-back. These changes in TER are indicative of the breakdown of TJs in response to Ca^{2+} removal and reformation on Ca^{2+} add-back, as expected.

To corroborate these findings of TER, we next examined the AJC by immunocytochemistry. As shown by confocal images in Figure 2, a contiguous distribution of ZO-1 (a marker of TJs) at intercellular borders was found to be fragmented and withdrawn from the cell-cell border on extracellular Ca^{2+} removal (Fig. 2A vs. 2D). Concomitantly, the PAMR compacted into a contractile ring (Fig. 2B vs. 2E). Consistent with the loss of TER and the withdrawal of ZO-1, the formation of intercellular gaps became evident, as shown by arrows in Figure 2D. Confocal z-scans showed colocalization of ZO-1 and PAMR in both untreated and Ca^{2+} -depleted cells (Figs. 2C, 2F). However, as shown in Figure 3, unlike ZO-1, the staining for cadherins decreased at the cell-cell borders and increased in the cytoplasm after Ca^{2+} removal (Fig. 3A vs. 3D). Although cadherins colocalize with the PAMR in untreated cells, they appear to be independent of PAMR after Ca^{2+} depletion (Figs. 3C, 3F). As shown in Figures 3G and 3H, differential extraction assay

FIGURE 1. Effects of Ca^{2+} switch on TER. Cells grown on the electrodes for ~ 24 hours and showing a steady state in TER were exposed to Ca^{2+} -free medium. This results in a precipitous decline in TER in <1 minute. Subsequent Ca^{2+} add-back induced a complete recovery in ~ 3 hours. Exposure to <1.8 mM Ca^{2+} reduced the rate and extent of recovery. TER, measured by lock-in amplifier, was sampled at ~ 0.1 Hz. Graph shows mean $\pm$ SEM of three independent experiments.

FIGURE 2. Effects of Ca^{2+} depletion on the PAMR and distribution of ZO-1. Confocal images of PAMR and ZO-1. (A–C) Untreated cells. At the focal plane of ZO-1, F-actin formed a characteristic dense band of PAMR (B). However, above and below the focal plane of ZO-1, F-actin was sparse (data not shown). Note that ZO-1 of neighboring cells share a common locus at the cell-cell borders indicating that cells are in complete apposition (A). (D–F) Similar images taken after 30 minutes of Ca^{2+} depletion. Notice the compaction of PAMR into a contractile ring (E) and the retraction of ZO-1 from cell-cell junctions (D) into loci of contractile PAMR. Formation of intercellular gaps (D, arrows).

shows that a decrease in the insoluble fraction of cadherins occurs concomitantly with an increase in the soluble fraction after Ca^{2+} depletion. This confirms that cadherins are endocytosed on Ca^{2+} depletion.

Figure 4 shows that the effects of Ca^{2+} depletion on the organization of the AJC are transient and that they are completely reversed on Ca^{2+} add-back. Specifically, after 3 hours of add-back, corresponding to the time at which TER recovered completely (Fig. 1), we note that the reorganization of the actin cytoskeleton and the reassembly of the apical junctions are comparable to those before Ca^{2+} removal (Figs. 4C, 4F, 4I). To show the remodeling of the PAMR and the AJC in intact endothelial monolayers, as in Figures 2 to 4, we examined the response to Ca^{2+} switch in rabbit corneas (Fig. 5). In these experiments, the endothelial surface was exposed to Ca^{2+} -free medium (30 minutes). Subsequently, patches of the endothelium were peeled onto glass slides for immunocytochemistry. As shown in Figure 5, the reorganization of the PAMR and the disposition of ZO-1 and cadherins in response to Ca^{2+} switch are similar to responses found with cultured endothelial cells (Figs. 2–4). Thus, our findings in Figures 1 to 5 indicate that the decrease in TER in response to Ca^{2+} removal is consistent with the breakdown of TJs and that its recovery occurs in parallel with the reformation of TJs.

Role of Actomyosin Contraction in the Disassembly of Apical Junctions

As noted earlier, the activity of the motor protein myosin II is central to the initiation of actomyosin contraction. Its ATPase activity is selectively inhibited by blebbistatin and, therefore, has been used to oppose actomyosin contraction.²⁷ In addition, Rho kinase induces actomyosin contraction by phosphorylating the regulatory subunit of myosin phosphatase (MYPT1) at its Thr696 and Thr853 residues.²⁵ These phosphorylations inhibit myosin light chain phosphatase leading to increased MLC phosphorylation and, consequently, enhanced actomyosin contraction. Thus, inhibition of Rho kinase is an alternative to reduce actomyosin contraction.

To examine the role of actomyosin contraction in the disassembly of the apical junctions, we exposed cells to blebbista-

FIGURE 3. Effects of Ca^{2+} depletion on the distribution of cadherins. (A–C) Confocal images of cadherins and F-actin in untreated cells. As can be observed, at the focal plane of the characteristic PAMR (B), cadherins exhibit a contiguous appearance at the cell-cell borders (A). (D–F) Images taken 30 minutes after Ca^{2+} depletion. In the absence of Ca^{2+} , most of the cadherins are internalized (D), and there is no observable colocalization with the compacted PAMR (F). (G) Differential extraction of the soluble (S) and insoluble (I) fractions of Ca^{2+} -depleted (CF) cells show that there is a decrease in the levels of cadherins in the insoluble fraction with a concomitant increase in the soluble fraction compared with untreated cells. This increase in the soluble fraction indicates endocytosis of the protein. (H) Shows densitometric analysis of the data shown in (G). Soluble fraction in Ca^{2+} -depleted cells increased by ~100% compared with the untreated cells (columns 1 and 2; $**P < 0.05$). Insoluble fraction of cadherins in Ca^{2+} -depleted cells decreased by ~60% compared with untreated cells (columns 3 and 4; $**P < 0.05$). Data are represented as the mean $\pm$ SE of three independent experiments.

tin (10 μM) or Y-27632 (5 μM) during Ca^{2+} depletion. Both drugs reduced the rate of decline in TER compared with control (Fig. 6). In the presence of blebbistatin, it took 20 ± 8 minutes ($n = 4$) for maximal reduction in TER compared with a >90% decrease in <1 minute in untreated cells. Similarly, in the presence of Y-27632, it took 12 ± 3 minutes ($n = 4$) for maximal reduction in the TER to occur. These results are also reflected in the distribution of ZO-1 and cadherins, as shown in Figure 7. When cells were pretreated with blebbistatin and Y-27632, compaction of the PAMR on Ca^{2+} depletion was less pronounced (Figs. 7C and 7D vs. 7B). It is also evident that both inhibitors opposed the endocytosis of cadherins (Figs. 7K and 7L vs. 7J) and the redistribution of ZO-1 (Figs. 7G and 7H vs. 7F) from cell-cell contacts after Ca^{2+} removal. These changes in the disposition of the key proteins at the AJC are consistent with the slower decline in the TER in the presence

FIGURE 4. Effects of Ca^{2+} add-back on the reorganization of PAMR and AJC. In untreated monolayers, cadherins and ZO-1 are continuous at the cell-cell borders (D, G), and those from the neighboring cells share a common locus. After 30 minutes of Ca^{2+} depletion, cadherins are internalized in contrast to ZO-1, which remains bound to the compacted PAMR (E, H). These effects are reverted within 3 hours of Ca^{2+} add-back. Note that the organization of the AJC, including the PAMR, is similar to those in untreated cells (C, F, I). (A, B) F-actin in untreated cells and on Ca^{2+} depletion. Images shown are representative of three independent experiments.

FIGURE 5. Ca^{2+} switch in the freshly isolated rabbit corneal endothelium. Under untreated conditions, distribution of ZO-1 and cadherins are contiguous at the cell-cell borders (D, G). Ca^{2+} depletion led to dislocation of ZO-1 and cadherins (E, H), concomitant with compaction of the PAMR (B vs. A). These effects reverted within 3 hours of Ca^{2+} add-back (C, F, I), similar to cultured BCECs, as shown in Figure 4.

FIGURE 6. Influence of reduced actomyosin contraction on TER dynamics during disassembly. Cells were pretreated with Y-27632 (5 μ M) and blebbistatin (10 μ M) for 5 minutes and 20 minutes before Ca^{2+} depletion. In the continued presence of these inhibitors, extracellular Ca^{2+} was depleted. In untreated cells, addition of the Ca^{2+} -free medium led to a rapid decrease in TER (*closed circles*). However, the presence of Y-27632 (*squares*) and blebbistatin (*open circles*) reduced the rate of decline in TER and increased the time taken for maximum decline by 12 ± 3 minutes and 20 ± 8 minutes, respectively. Graph shown is a representative of four independent experiments.

of inhibitors of actomyosin contraction. Thus, the findings in Figures 6 and 7, taken together, suggest that the RhoA-Rho kinase-mediated actomyosin contraction is involved in the enhancement of the (Ca^{2+} depletion)-induced disassembly of the apical junctions and the consequent decline in TER.

Reorganization of the PAMR in Response to Ca^{2+} Depletion

Although the loss in TER (Fig. 1) can be explained based on the breakdown of the Ca^{2+} -dependent AJs, compaction of the

PAMR into a contractile ring after Ca^{2+} depletion suggests that the rate of decline could be attributed partly to the increased tone of the PAMR. As noted earlier, an increase in the contractility of the PAMR leads to a loss of barrier integrity secondary to inflammatory molecules such as histamine and thrombin.^{15,17} To confirm the apparent increase in the tone of PAMR and its influence on disassembly, we examined the phosphorylation of MLC using an antibody specific to the diphosphorylated form (ppMLC).

As shown in Figures 8A and 8B, after Ca^{2+} depletion, we observed a time-dependent increase in the phosphorylation of MLC with a maximum increase at 30 minutes. The locus of this increase in MLC phosphorylation is along the PAMR, as shown by immunofluorescence staining for ppMLC (Fig. 9E). Because RhoA is associated with outside-in signaling at the level of cadherin engagement,³⁴ we examined RhoA activity after Ca^{2+} depletion. Consistent with the increase in MLC phosphorylation, RhoA was upregulated, as shown in Figures 8C and 8D. Furthermore, treatment of cells with Y-27632 opposed MLC phosphorylation and compaction of the PAMR after Ca^{2+} depletion (Figs. 9C, 9F). These findings suggest that although the disengagement of cadherins and the consequent loss of AJs initiate the decrease in TER (Fig. 6), the concomitant increase in the contractility of the PAMR after Ca^{2+} depletion accentuates the rate of decline.

Role of Actomyosin Contraction in the Assembly of Apical Junctions

We first highlight that PAMR is essential for the reformation of AJs and TJs using cytochalasin D, which is a cell-permeable inhibitor of actin polymerization.³⁵ As shown in Figure 10A, exposure to cytochalasin D results in a dose-dependent decrease in the rate and extent of recovery of TER after Ca^{2+} add-back. Staining for cadherins and ZO-1 in the presence of the highest dose (0.125 μ g/mL) of the drug shows discontinuities at the cell-cell borders (Figs. 10E, 10G). In addition, as indicated by arrows in Figure 10C, PAMR is disrupted, and large intercellular gaps appear at 3 hours after recovery, indi-

FIGURE 7. Effects of reduced actomyosin contraction on the organization of AJC after Ca^{2+} depletion. Cells were pretreated with Y-27632 (5 μ M) and blebbistatin (10 μ M) followed by Ca^{2+} depletion in the presence of these drugs. Both inhibitors prevented contraction of the PAMR (C and D vs. B and A) and intercellular gaps. These inhibitors also opposed Ca^{2+} removal-induced redistribution of ZO-1 and cadherins from the cell-cell borders (E–H, I–L). Images are representative of three independent experiments.

FIGURE 8. Effects of Ca^{2+} depletion on actomyosin contraction. (A) MLC phosphorylation, as a biochemical measure of actomyosin contraction, was assessed at different time points after exposure to Ca^{2+} -free medium by Western blot analysis. Ca^{2+} depletion-induced MLC phosphorylation as shown by the increase in intensity of the diphosphorylated MLC (ppMLC). Treatment with Y-27632 (5 μM ; Y) prevented MLC phosphorylation completely. Thrombin was used as a positive control. Blots were probed for β -actin as an internal control for equal protein loading. (B) Bar graph of densitometric analysis of data shown in (A). There was a significant increase in MLC phosphorylation at 15 minutes with a maximum increase occurring after 30 minutes of Ca^{2+} depletion (** $P < 0.001$ vs. control). (C) Concomitant with the increase in MLC phosphorylation, there was a significant activation of RhoA after Ca^{2+} depletion (** $P < 0.005$). (D) Bar graph of densitometric analysis of data shown in (C). The error bars in both graphs represent SEM ($n = 3$).

cating compromised cell-cell junctions. At lower doses of the drug (0.062 $\mu\text{g}/\text{mL}$ and 0.031 $\mu\text{g}/\text{mL}$), the organization of the PAMR, cadherins, and ZO-1 were relatively continuous (data not shown). The immunofluorescence data correlate with the

FIGURE 9. Locus of increased actomyosin contraction after Ca^{2+} depletion. Diphosphorylated MLC (ppMLC) was imaged at the focal plane of PAMR (A–C) by immunofluorescence. Note the increase in the staining for ppMLC overlapping with the contractile PAMR after Ca^{2+} depletion (E vs. D), which is opposed by treatment with Y-27632 (5 μM ; F).

FIGURE 10. Effect of actin polymerization on TER recovery and AJC reassembly. (A) Cells were pretreated with the indicated concentrations (0.125 $\mu\text{g}/\text{mL}$, 0.062 $\mu\text{g}/\text{mL}$, 0.031 $\mu\text{g}/\text{mL}$) of cytochalasin D for 10 minutes in Ca^{2+} -free medium and allowed to reassemble after Ca^{2+} add-back in the presence of the drug. In control cells, subjected to Ca^{2+} switch, TER reached near baseline values after Ca^{2+} add-back. Compared with control, there was a dose-dependent decrease in the rate and extent of recovery of TER in cells treated with cytochalasin D. Data are expressed as mean $\pm$ SEM of three independent experiments. Immunofluorescence data show complete reassembly of AJC after Ca^{2+} add-back in control cells (B, D, F), which appear similar to their organization before Ca^{2+} depletion (Figs. 4A, D, G). Treatment with 0.125 $\mu\text{g}/\text{mL}$ of cytochalasin D led to a complete disruption of the PAMR (C, arrows). Staining for both cadherins and ZO-1 was discontinuous with numerous intercellular gaps (E and G), indicating incomplete cell-cell adhesion and reformation of TJs.

dose-dependent changes in the TER. These results, taken together, suggest that actin polymerization is crucial for the assembly and function of the AJC.

We next examined the role of actomyosin contraction in the assembly of AJs and TJs, as shown in Figure 11A. Exposure to either Y-27632 (5 μM ; 5 minutes) or blebbistatin (10 μM ; 20 minutes) before Ca^{2+} add-back reduced the rate of recovery of TER. Accordingly, organization of the PAMR was incomplete in the presence of blebbistatin or Y-27632 (Figs. 12B, 12C). With Y-27632, consistent with the marginal recovery of TER, only part of the ZO-1 underwent redistribution and formed a con-

FIGURE 11. Effect of Rho kinase and myosin II ATPase inhibition on the TER dynamics during reformation of AJC. (A) Cells were pre-treated with Y-27632 (5 μ M) for 5 minutes or blebbistatin (10 μ M) for 20 minutes during Ca²⁺ depletion. This was followed by Ca²⁺ add-back in the continued presence of the drugs. In untreated cells, TER reached the initial baseline levels after Ca²⁺ add-back. The presence of Y-27632 or blebbistatin significantly reduced the rate and extent of recovery in TER. Data are expressed as mean $\pm$ SEM of three independent experiments. (B) The Ca²⁺ switch maneuver was performed in the presence of the drugs, and TER was allowed to recover. After 3 hours of recovery, the medium containing the drugs was removed and replaced with fresh medium (without drugs). TER reached near baseline values within 6 to 7 hours, confirming that the observed changes in TER during reassembly were not due to drug toxicity. Graph is representative of three similar experiments.

tiguous band at the cell-cell borders. However, a small pool of ZO-1 remained retracted from the cell-cell border, implying incomplete reformation of TJs (Fig. 12F; indicated by arrows). In contrast to the response obtained with Y-27632, blebbistatin treatment does not show any apparent effects on the assembly of ZO-1 along the cell periphery, but its distribution is discontinuous (Fig. 12E; indicated by arrowheads). Under both treatment conditions, however, redistribution of cadherins is incomplete and exhibited diffuse and tortuous staining along the cell border (Figs. 12H, 12I). Taken together, Figures 10 to 12 suggest that both polymerization and actomyosin contraction of the PAMR are critical for the reformation of AJs and TJs.

To determine whether cytotoxicity of the drugs played a role in these observations, we also examined the reversibility of their effect on TER recovery. As shown in Figure 11B, the recovery of TER is near complete in 5 hours ($n = 3$) after removal of the drugs from the bathing medium. Thus, both Y-27632 and blebbistatin, at the concentrations used in this study, are nontoxic to BCECs.

DISCUSSION

In this study, for the first time, we have addressed the role of actomyosin contraction in the assembly and disassembly of AJC in the corneal endothelium. The findings of this study show that excessive contraction of the PAMR breaks down barrier integrity but that, at the same time, significant loss of its contractile tone is not permissive for reassembly of the AJC. In addition, our findings highlight the vital role of the RhoA-Rho kinase axis in a dynamic regulation of barrier integrity summarized in Figure 13.

In our recent studies, we characterized the breakdown of barrier integrity of the corneal endothelium in response to thrombin,¹⁵ histamine,¹⁷ and nocodazole.³⁶ These agents induced contraction of the PAMR, as indicated by an increase in the phosphorylation of MLC along the locus of AJC. Such an increase led to breakdown of the TJs, which could be inhibited by agents that opposed MLC phosphorylation.^{29,37} In contrast, the effect of contraction of the PAMR on the reassembly of AJs and TJs had not been investigated. To simulate the reassembly of the AJC, we chose the Ca²⁺ switch protocol, which is an established technique used to study remodeling of the AJC in tight epithelia such as the MDCK cells³⁸ and as shown recently with corneal endothelial cells.³⁹ Given a priori knowledge that the breakdown and reassembly can be rapid,^{13,40,41} we followed their dynamics by TER. Because the corneal endothelial monolayers are leaky, we chose a lock-in amplifier (ECIS; Applied Biophysics) to measure TER because the approach is known for high temporal resolution and high sensitivity and

FIGURE 12. Effects of reduced actomyosin contraction on the reassembly of AJC. (A, D, G) Images of the AJC in untreated cells 3 hours after Ca²⁺ add-back, which were similar to those before Ca²⁺ removal. However, in cells treated with blebbistatin and Y-27632, the reorganization of the PAMR is incomplete (B, C). In blebbistatin-treated cells, most of the ZO-1 reassembled at the cell-cell borders, but its appearance is discontinuous (E, arrowheads). In the presence of Y-27632, although ZO-1 is assembled at the cell borders, there is a pool of ZO-1 that remains retracted from the cell-cell borders (F, arrows), indicating incomplete reassembly of TJs. In the presence of both drugs, cadherins appear diffuse and are not contiguous at the cell-cell borders (H, I), indicating incomplete reassembly of the AJs. Images are representative of three separate experiments.

FIGURE 13. An overview of disassembly and reassembly of AJC in the corneal endothelium during the Ca^{2+} switch maneuver. In disassembly, we showed that extracellular Ca^{2+} depletion resulted in disengagement of cadherins (i.e., breakdown of AJs). This triggered outside-in signaling involving the activation of RhoA which, through its effector Rho kinase, increased MLC phosphorylation and actomyosin contraction. Our results with Y-27632 (Rho kinase inhibitor) and blebbistatin (myosin II ATPase inhibitor) showed that these inhibitors delayed the rate of decline in TER after Ca^{2+} depletion, confirming that increased actomyosin contraction of the PAMR, accelerated the breakdown of TJs. In assembly, the add-back of Ca^{2+} promoted cadherin ligation and clustering through mechanisms involving filopodia/lamellipodia formation. Actin polymerization was important for the support of these nascent structures because disruption of actin by cytochalasin D prevented the reassembly of AJs and TJs. In addition, the lack of recovery in the presence of Y-27632 and blebbistatin confirmed that RhoA-Rho kinase-induced actomyosin contraction is crucial for the reassembly of TJs. Results with these inhibitors show that the tone of the

PAMR is crucial for stabilizing the intercellular junctions at the cell-cell borders to form a functional barrier. These results show that actomyosin contraction plays a critical role during disassembly and reformation of AJC and that this contraction is mediated in part by the RhoA-Rho kinase pathway.

affords real-time measurement.⁴² Given that only TJs occlude the paracellular space and thereby limit ionic flux, TER measurements reflect the assembly of TJs and not of AJs. However, given that TJ assembly follows cell-cell adhesion,⁴¹ it is clear that TER dynamics indirectly reflect on AJ assembly as well.

Dynamics of TER and AJC Remodeling during Ca^{2+} Switch

Previous studies in the corneal endothelium have shown that the depletion of extracellular Ca^{2+} results in a complete separation of the AJC with a resultant increase in stromal swelling.^{43,44} In this study, (Figs. 1–5) we characterized the dynamics of TER and AJC remodeling during the Ca^{2+} switch maneuver. As expected, the depletion of extracellular Ca^{2+} resulted in a precipitous decline in TER (Fig. 1), consistent with the breakdown of TJs after the disengagement of cadherins at AJs in the absence of external Ca^{2+} . That the TER decline actually reflects the breakdown of TJs is evident from the staining of ZO-1 (Fig. 2A vs. 2D), a putative marker of the TJ complex.¹² This cytoplasmic protein is contiguous at the cell-cell border when the barrier integrity is intact (Fig. 2A). On disassembly, ZO-1 separates from cell boundaries, indicating the formation of intercellular gaps (Fig. 2D, arrows). Despite this separation, ZO-1 remains localized with the PAMR, which now manifests as a contractile ring (Fig. 2E). The breakdown of AJs themselves is evident in Figure 3D, which, in the presence of extracellular Ca^{2+} , is continuous at the cell-cell borders (Fig. 3A). The endocytosis of cadherins is reflected not only in the immunofluorescence data but also by Western blot analyses (Figs. 3G, 3H), which show an increase in the soluble fraction of the protein indicating its extraction from the membrane on Ca^{2+} depletion.

In contrast with Ca^{2+} depletion, Ca^{2+} add-back enabled the reassembly of AJs and TJs. Although the specific sequence of assembly is not apparent in the TER dynamics, it is unambiguous from immunofluorescence data that engagement of cadherins is complete at the end of 3 hours, by which time TER returns to the baseline value before Ca^{2+} depletion (Fig. 1). At this time point, TJ assembly is also complete, as demonstrated by realignment of ZO-1 at the loci of cell-cell borders. These results not only establish the baseline characteristics of TER dynamics during Ca^{2+} switch, they also clearly show the high temporal resolution and sensitivity of the lock-in amplifier (ECIS; Applied Biophysics)-based TER measurements. Although previous studies in corneal endothelium have used this instrument for studying its barrier property,^{36,45} immunofluorescence imaging of the AJC in Figure 4 provides corroborating evidence that the cell-substrate impedance measurements can be used to extract information on TER.

Disassembly of the Apical Junctions after Ca^{2+} Depletion

The TER measurements in Figures 1 and 6, in combination with immunofluorescence data in Figures 4, 5, and 7, suggest that the disassembly of the TJs in response to Ca^{2+} depletion is promoted by actomyosin contraction of the PAMR in addition to the loss of cell-cell adhesion by the disengagement of cadherins. This is apparent in several of our experimental findings. First, as noted in Figures 2 and 3 and as discussed above, the PAMR assumes a contractile form, as shown by the dramatic change in its appearance from a hexagonal pattern to one of contractile ring. Second, the rate of decrease in TER on Ca^{2+} depletion is significantly reduced by Y-27632 and blebbistatin, inhibitors of actomyosin contraction (Fig. 6). Finally, increased

MLC phosphorylation (Figs. 8A, 8B) along the locus of the PAMR (Fig. 9E) and RhoA activation (Figs. 8C, 8D) confirm that the PAMR undergoes actomyosin contraction in response to disassembly of the AJs. The latter is consistent with earlier reports that engagement of cadherins inhibits RhoA.³⁴ Thus, although loss of AJs by itself would prevent cell-cell adhesion and thereby eventually cause the breakdown of TJs, the concomitant induction of actomyosin contraction accelerates the process of disassembly.

Role of Actin Cytoskeleton in the Assembly of Apical Junctions

Although loss of barrier integrity in response to Ca^{2+} depletion has been examined in the corneal endothelium,^{43,44,46} this is the first study that has sought to delineate a role for actin cytoskeleton in the reassembly of AJs and TJs. In a recent study, Mandell et al.^{39,47} demonstrated the importance of the transmembrane protein JAM-A in the formation of TJs. It was shown that in the presence of an antibody against JAM-A, the recovery in TER after Ca^{2+} depletion was significantly reduced. Similarly, corneal swelling induced by transient Ca^{2+} depletion was enhanced in the presence of JAM-A antibody. However, the importance of actin cytoskeleton in the establishment of AJC is not addressed in these studies. Our data in Figures 10 to 12 demonstrate that both actin remodeling and actomyosin contraction play a role in the assembly of AJC.

As shown in Figure 10A, treatment with cytochalasin D elicited a dose-dependent decrease in the recovery of TER. At the highest concentration of the drug, immunofluorescence of AJC revealed a sparse distribution of cadherins and ZO-1 at the cell-cell border (Figs. 10E, 10G). This is consistent with the presence of intercellular gaps (i.e., incomplete cell-cell adhesion) and a remarkable decrease in the extent of recovery of TER (Fig. 10A). These actions of cytochalasin D suggest that either the PAMR is important for the onset of reassembly or that the drug inhibited actin polymerization thought to be essential at the zones of initial cell-cell contacts.⁴⁸ There is evidence in keratinocytes, for example, that Ca^{2+} add-back induces the formation of filopodia-like structures, which form nascent cell-cell contacts and accordingly exhibit an accumulation of cadherins.⁴⁸ Moreover, these nascent structures also manifest radial actin filaments penetrating into their leading edges.⁴⁸ Therefore, it is possible that in addition to dissolution of the PAMR, cytochalasin D prevented the localized activity of actin polymerization at the nascent contacts.

We have further examined the influence of actin cytoskeleton using blebbistatin and Y-27632, agents well known to influence actomyosin contraction.²⁷ As shown in Figure 12, the status of AJC recovery at 3 hours after Ca^{2+} add-back with and without blebbistatin treatment is markedly different. Exposure to the drug disrupted the PAMR (Fig. 12B) and blocked recovery in TER (Fig. 11A). However, exocytosis of cadherins that was internalized on Ca^{2+} depletion was largely unaffected (Fig. 12H). In contrast to the disposition of cadherins, ZO-1 redistribution remained discontinuous (Fig. 12E; indicated by arrowheads) and is reflected in the lack of recovery of TER. Given the selectivity of blebbistatin toward myosin II ATPase, its major effect at the AJC is brought about by the inhibition of actomyosin contraction of the PAMR^{27,49} and presumably of the actin bundles of the filopodia. Without a normal tone of the actin bundles in the filopodia, progression of the adhesion zipper along cell-cell borders is unlikely to show completion. The apparently normal organization of ZO-1 at the cell-cell borders in the absence of TER recovery, therefore, may indicate its association with the F-actin at the AJs. Consistent with this claim, Rajasekaran et al.⁵⁰ demonstrated that in MDCK cells, cadherin engagement after Ca^{2+} add-back prompts re-

cruitment of ZO-1 to the AJs. During maturation of the AJs, however, ZO-1 was found to segregate toward TJs. Thus, we conclude that although blebbistatin does not inhibit the initial formation of AJs, it inhibits the completion of TJ assembly. In other words, in the presence of blebbistatin, the apparent contiguous assembly of ZO-1 is not indicative of stable TJs.

In contrast to blebbistatin, Y-27632 is a selective inhibitor of Rho kinase, but it also eventually inhibits actomyosin contraction. As shown in Figure 11A, exposure to Y-27632 impeded the assembly of TJs. However, we observed subtle differences in the recovery of TER and AJC remodeling compared with blebbistatin. First, unlike blebbistatin, TER shows a modest recovery with Y-27632 at 3 hours after Ca^{2+} add-back. It is noteworthy that the recovery in TER manifested after a lag of approximately 1 hour. Second, unlike the effects of blebbistatin, PAMR and cadherins showed increased colocalization (Figs. 12C, 12D). These differences between the effects of blebbistatin and Y-27632 are possibly due to additional targets of Rho kinase. Inhibition of Rho kinase also downregulates the LIM kinase-cofilin axis, which promotes actin polymerization.⁵¹ In other words, Rho kinase can show some effects similar to those of cytochalasin D. This may underlie the apparent lag in the recovery of TER with Y-27632. Taken together, our findings with cytochalasin D, blebbistatin, and Y-27632 suggest spatiotemporal organization in the reassembly of AJs and TJs involving actin remodeling and actomyosin contraction at the level of PAMR and of the actin filaments along the nascent filopodia-like structures.

In conclusion, this study has used the paradigm of Ca^{2+} switch to examine the dynamics of disassembly and reassembly of the AJC and its regulation by actomyosin contraction (Fig. 13). Our results highlight that intact PAMR with significant tone is essential for reformation of the AJC.

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