The Myosin II ATPase Inhibitor Blebbistatin Prevents Thrombin-Induced Inhibition of Intercellular Calcium Wave Propagation in Corneal Endothelial Cells

Raf Ponsaerts,1,2 Catheleyne D’bondt,1,2 Geert Bullynck,1 Sangly P. Srinivas,3 Johan Vereecke,1 and Bernard Himpens4

PURPOSE. Thrombin inhibits intercellular Ca2+ wave propagation in bovine corneal endothelial cells (BCECs) through a mechanism dependent on myosin light chain (MLC) phosphorylation. In this study, blebbistatin, a selective myosin II ATPase inhibitor, was used to investigate whether the effect of thrombin is mediated by enhanced actomyosin contractility.

METHODS. BCECs were exposed to thrombin (2 U/mL) for 5 minutes. MLC phosphorylation was assayed by immunocytochemistry. Ca2+ waves were visualized by confocal microscopy with Fluo-4AM. Fluorescence recovery after photobleaching (FRAP) was used to investigate intercellular communication (IC) via gap junctions. ATP release was measured by luciferin-luciferase assay. Lucifer yellow (LY) uptake was used to investigate hemichannel activity, and Fura-2 was used to assay thrombin- and ATP-mediated Ca2+ responses.

RESULTS. Pretreatment with blebbistatin (5 μM for 20 minutes) or its nitro derivative prevented the thrombin-induced inhibition of the Ca2+ wave. Neither photo-inactivated blebbistatin nor the inactive enantiomers prevented the thrombin effect. Blebbistatin also prevented thrombin-induced inhibition of LY uptake, ATP release and FRAP, indicating that it prevented the thrombin effect on paracrine and gap junctional IC. In the absence of thrombin, blebbistatin had no significant effect on paracrine or gap junctional IC. The drug had no influence on MLC phosphorylation or on [Ca2+]i waves in corneal endothelial cells consists of gap junctional IC (GJIC) and hemichannel-mediated paracrine IC. (Invest Ophthalmol Vis Sci. 2008;49:4816–4827) DOI:10.1167/ iovs.07-1533

CONCLUSIONS. Blebbistatin prevents the inhibitory effects of thrombin on intercellular Ca2+ wave propagation. The findings demonstrate that myosin II–mediated actomyosin contractility plays a central role in thrombin-induced inhibition of gap junctional IC and of hemichannel-mediated paracrine IC. (Invest Ophthalmol Vis Sci. 2008;49:4816–4827) DOI:10.1167/ iovs.07-1533

The corneal endothelium is the interface between the corneal stroma and aqueous humor. Its main function is hydration control of the stroma, which is essential for corneal transparency. 1 In humans, the corneal endothelial cells are nonregenerative, and hence their loss is a concern during aging and senescence, hypoxia, inflammation, storage, and iatrogenic trauma. 2–4 However, the monolayer is known to be resilient, 5,6 sustaining hydration control so long as the endothelial density is 500 to 1000 cells/mm². A direct consequence of continuous cell loss in the absence of regeneration is that denuded space on Descemet’s membrane is filled by cell spreading and migration. This phenomenon inevitably leads to loss of cell shape (polymorphism) concomitant with an increase in cell size (polymegathism). These morphologic adaptations imply dynamical changes in the actin cytoskeleton, which is known to possess structural and functional interactions with connexins, directly or indirectly through actin binding proteins (e.g., ZO-1). 7–12

As in other epithelial and endothelial monolayers, intercellular communication (IC) is likely to contribute toward the resilience of the corneal endothelium by promoting a synchronized response against external stress or stimuli. 13 In this context, we have previously explored propagation of intercellular Ca2+ waves as a model of IC in corneal endothelial cells. 15–17 In this paradigm, we stimulated a single cell in a cultured monolayer by a mechanical stimulus and followed the increase in intracellular Ca2+ ([Ca2+]i) in the mechanically stimulated cell and in the neighboring cells over time. As we have reported, the propagation of such [Ca2+]i waves in corneal endothelial cells consists of gap junctional IC (GJIC) and paracrine IC (PIC). 15–17 As in many other cell types, 12,18 GJIC is mediated by gap junctional channels, which accrete in the proximity of adherens junctions to form gap junctions. 1 Each gap junctional channel is formed by docking of two hemichannels contributed by two adjacent cells. 14,19,20 The PIC component of the Ca2+ wave, on the other hand, involves release of ATP with subsequent activation of the P2Y purinergic receptors evoking autocrine and paracrine responses. 15–18 We also have provided evidence that ATP release involved in PIC is mediated by connexin (Cx) hemichannels. 17 Direct evidence for ATP efflux through Cx43 hemichannels has recently been obtained. 21

In two recent studies, 22–23 we investigated the effect of thrombin on IC, to study how PIC and GJIC are influenced by the actin cytoskeleton. This problem is of interest, not only because the continuous loss of endothelial cells modulates the cortical actin cytoskeleton, a determinant of cell shape, but also because many proinflammatory mediators influence the actin cytoskeleton. While the latter is well known in vascular endothelial cells, 24–25 recent studies have demonstrated that thrombin 26 and histamine 27 induce actomyosin contraction also in corneal endothelial cells, leading to significant changes in the organization of the cortical actin cytoskeleton. These changes in the cytoskeleton have been implicated in a loss of barrier integrity in corneal endothelial monolayers. Specifically, thrombin, which can be generated in the cornea, 26 and

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histamine may lead to the onset of centripetal forces in the perijunctional actomyosin ring (PAMR; i.e., dense band of cortical actin), which is found at the apical junctional complex. 26,27,29–31 This force is opposed by intercellular tethering forces, which are essential to establish interactions of the transmembrane proteins that make up the tight junctions. 26,31–35 Results of our recent work also imply that thrombin has effects on GJIC and PIC through actomyosin contractility. 22,23 Specifically, activation of PAR-1 receptors in bovine corneal endothelial cells (BCECs) by thrombin leads to significant inhibition of Ca2+ wave propagation as well as to inhibition of uptake of LY and of ATP release through connexin 43.22,23 Studies with pharmacologic agents have provided further evidence that the effect of thrombin is mediated by an increase in MLCK phosphorylation involving activation of MLCK, Rho kinase, and PKC. 22 Since MLCK phosphorylation results in enhanced contractility of the actin cytoskeleton, 36,37 in this study we investigated whether (~)-blebbistatin (a selective inhibitor of myosin II ATPase activity38–41) affects the thrombin-induced inhibition of IC in BCECs. We found that blebbistatin pretreatment prevented not only thrombin-induced inhibition of gap junctions but also of hemichannels.

Materials and Methods

Cell Culture

Primary cultures of BCECs were established as previously described. 15,17–22,23,26,27,30–35 First- and second-passage cells were seeded into two-chambered glass slides (15 580; Nunc, Roskilde, Denmark) at a density of 165,000 cells per chamber (4.2 cm2) and were allowed to reach confluence for 3 to 4 days.

Mechanical Stimulation for Inducing Ca2+ Waves

The mechanical stimulation consisted of an acute deformation of a cell by a brief touch of the cell membrane with a glass micropipette (tip diameter, <1 μm) coupled to a piezoelectric crystal (Nano-Positioner P-280; PI Polytech, Karlsruhe, Germany), as previously described. 15,17–22,23

Measurement of Ca2+ Wave Propagation

The Ca2+ wave propagation was assayed by imaging [Ca2+]i with the Ca2+ -sensitive fluorescent dye Fluo-4. The dye was excited at 488 nm, and its emission was collected at 530 nm. Spatial changes in [Ca2+]i, after point mechanical stimulation were measured with a confocal microscope (LSM510; Carl Zeiss Meditec, Inc., Jena, Germany) with a 40× objective. The neighboring cells (NB cells) immediately surrounding the mechanically stimulated (MS) cell were defined as neighboring cell layer 1 (NB1). Cells immediately surrounding the NB1 cells are defined as neighboring cell layer 2 (NB2), and so on. Normalized fluorescence of a cell was measured at different times after mechanical stimulation, by averaging the fluorescence over the entire area of the cell and dividing this value by the fluorescence before mechanical stimulation. Intercellular propagation of the Ca2+ wave was characterized by maximum normalized fluorescence (NF) of cells in different NB layers, as well as by the total surface area of responsive cells (active area, AA) with NF > 1.1.

Measurement of [Ca2+]i

The effect of blebbistatin on thrombin and ATP-induced Ca2+ transients were analyzed with a multimode benchtop microplate reader (FlexStation 3; Molecular Devices, Sunnyvale CA). Cells (8000 per well) were seeded onto 96-well plates (Cytowell 96F Fluorcarbon Black, 146520; Nunc) and allowed to reach confluence over 2 to 3 days. The cells were then loaded with Fura-2AM (at a final concentration of 1.25 μg/mL) for 30 minutes at room temperature. Ratiometric [Ca2+]i measurement was obtained by acquisition at 510 nm to excitation at 340 and 380 nm, respectively. ATP dose–response curves were fitted using the Michaelis-Menten model using the DRC-package (version 1.2.0) for R programming (version 2.5.1; R Foundation for Statistical Computing, Vienna, Austria).

Lucifer Yellow Uptake Assay

Cells were incubated in a Ca2+-rich PBS containing the drug of interest for 20 minutes. Then cells were exposed to PBS containing 2 mM EGTA and 2.5% Lucifer yellow (LY) for 5 minutes in the continued presence of the drug. In experiments with thrombin, cells were exposed to thrombin for 1 minute before exposure to LY. Dye uptake was recorded using the confocal microscope (LSM510; Carl Zeiss Meditec, Inc.) by excitation at 488 nm with emission recorded at 530 nm, as described previously.15,22,23

Measurement of ATP Release

ATP release, after mechanical stimulation on the confocal microscope, was followed using luciferin-luciferase bioluminescence. 15,22,23 Samples (100 µL) were taken from the 500-µL bathing solution covering the cells and transferred to a custom-built photocounting setup. 15,22,23

Fluorescence Recovery after Photobleaching

Cells were loaded with 6-carboxyfluorescein diacetate (10 µM) in Dulbecco’s PBS (Invitrogen-Gibco, Karlsruhe, Germany) for 5 minutes at room temperature, and fluorescence recovery after photobleaching (FRAP) was measured with the confocal laser scanning fluorescence microscope (LSM510; Carl Zeiss Meditec, Inc.) as previously described. 15,17–22,23 A single cell was bleached by exposure to laser light at 488 nm. The recovery of fluorescence in the bleached cells was measured every 10 seconds over a period of 5 minutes by excitation at 488 nm with emission recorded at 570 nm. The decrease in fluorescence in a region widely distant from the bleached cells was also measured as a reference for correction for bleaching due to the excitation light used for fluorescence detection during fluorescence recovery. The fluorescence in neighboring cells after bleaching a single cell was 0.984 ± 0.002 (n = 42) of the value before bleaching. Fluorescence recovery in the bleached cell at 5 minutes was compared with that of the prebleach scan, and the percentage recovery was calculated.

Visualization of MLC Phosphorylation by Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at 37°C and permeabilized using 0.5% Triton X-100 in PBS for 10 minutes at room temperature. After blocking with 10% goat serum and 3% BSA, the cells were incubated with antibody either against Ser19 phosphorylated MLC (MLC (Ser19) antibody, P7481; Invitrogen, Karlsruhe, Germany). Immunofluorescence images were acquired by confocal microscope (LSM510 Carl Zeiss Meditec, Inc.), with a 100× oil-immersion objective (1.3 NA). The images were analyzed for pixel intensities with the microscope software (LSM510, ver. 3.2; Carl Zeiss Meditec, Inc.).

RT-PCR Assay for Expression of Myosin II Isoforms

Total RNA was extracted from the BCECs (SV Total RNA Isolation System kit; Z1300; Promega, Mannheim, Germany). First-strand cDNA synthesis was performed (SuperScript II Reverse Transcriptase for RT-PCR, 11904-018; Invitrogen-Gibco) with random hexamers. To am-
plify cDNA for myosin II isoforms, PCR was performed as previously described,\textsuperscript{22} using the gene-specific primers listed in Table 1. Amplified cDNA was visualized with ethidium bromide after performing DNA gel electrophoresis (2% agarose) in the presence of a 50-bp DNA ladder (G4521; Promega).

### Western Blot Analysis for Expression of Myosin II Isoforms

Cells grown on 100-mm Petri dishes were lysed with an ice-cold RIPA lysis buffer containing 1% Triton X-100 and a protease inhibitor cocktail (11-873-580; Roche Applied Science, Mannheim Germany). After a preclearing centrifugation step (14,000 rpm for 15 minutes at 4°C), the whole lysate was subjected to SDS-PAGE (10 μg protein/lane; EA03752BOX; Invitrogen-Gibco). After blotting, membranes were blocked with TBST (pH 7.4, containing 0.1% Tween 20) containing 5% nonfat dry milk for 1 hour and were subsequently incubated with the primary antibodies (1:1000 dilution) for 1 hour: Polyclonal antibodies (ab24762; Abcam PLC, Cambridge, UK, and ab24761; Abcam PLC), directed against nonmuscle myosin IIA (predicted size: 226 kDa) and myosin IIB (predicted size: 200 kDa), respectively, were used. Blots

### Figure 1. Typical findings of the effects of blebbistatin on the reduction of the Ca\textsuperscript{2+} wave propagation induced by thrombin. Representative pseudocolored fluorescence images showing Ca\textsuperscript{2+} transients at different times after mechanical stimulation of a single cell in a confluent monolayer of BCECs in different conditions. For each condition, the first image shows the fluorescence intensities before stimulation, and the arrow in the second image identifies the mechanically stimulated (MS) cell. Right: line graphs show the time course of the normalized fluorescence value (NF) in the MS cell and of the average value of NF in the cells in each of the neighboring cell layers 1 to 5 (NB1–NB5). (A) Control conditions: The Ca\textsuperscript{2+} wave propagated to five neighboring cell layers. The total area of cells reached by the wave (active area) was 88,300 μm\textsuperscript{2}. (B) Effect of thrombin: thrombin (2 U/mL for 5 minutes) reduced the spread of the wave to three layers and the active area to 17,900 μm\textsuperscript{2}. (C) Effect of thrombin after pretreatment with (−)-blebbistatin (5 μM for 20 minutes): The Ca\textsuperscript{2+} wave propagated to four neighboring cell layers. The active area was 83,500 μm\textsuperscript{2}, similar to the value in control conditions, indicating that (−)-blebbistatin inhibited the effect of thrombin.

### Table 1. Gene-Specific Primer Pairs for RT-PCR of Bovine Myosin II Isoforms

<table>
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<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Size (bp)</th>
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<tr>
<td>MYH9</td>
<td>XM_612582</td>
<td>Forward</td>
<td>TTTCGTCGAGAAGGTGATGCAAGGA</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCTTATTCCAGCTTGCCAGGTAGGT</td>
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<tr>
<td>MYH10</td>
<td>NM_174834</td>
<td>Forward</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>TGGGACGTTGACTTCTGCTCCT</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGTCAGTGTCTCTTCTGTC</td>
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</table>
were washed with TBST three times (15 minutes each) and then were visualized using the alkaline phosphatase–conjugated secondary antibody and ECF detection kit (GE Healthcare, Buckinghamshire UK) on the Storm 840 instrument (Molecular Dynamics, Sunnyvale, CA).

**Chemicals**

Fluo-4 AM (F14217), 6-carboxyfluorescein diacetate (C1362), Dulbecco’s PBS (14190-091) Dulbecco’s modified Eagle’s medium (DMEM, 11960-044), L-glutamine (Glutamax, 35050-038), antibiotic-antimycotic mixture (15240-096), and EBSS (Earle’s balanced salt solution, 14155-048), trypsin (25300-054) were obtained from Invitrogen-Gibco. Fetal bovine serum (F-7524), a luciferin-luciferase bioluminescence assay kit (FL-AAM), ATP, thrombin (T-4648), LY (L-0259), and (-H11001)-blebbistatin (B0560; a 1-phenyl-1–2-pyrrolidine derivative) were obtained from Sigma-Aldrich (Deisenhofen, Germany). The (-H11001)-enantiomer of blebbistatin was obtained from Calbiochem (203392; Merck Biosciences, Darmstadt, Germany). The (-H11001) and (-H11002) enantiomers of nitroblebbistatin were kind gifts of Nicholas J. Westwood (University of Saint Andrews, UK). Gap27 peptide was synthesized at the Laboratory of Biochemistry, KULeuven, as described previously.17

**Data Analysis**

T-tests with Bonferroni correction were used to compare mean values for different treatments, and unpaired tests were used to compare results of experiments with a single treatment and a single control (Prism 4.0 for Windows; GraphPad Software Inc., San Diego, CA). *P* < 0.05 is considered significant. All data are expressed as the mean ± SEM. *N* indicates the number of independent experiments. In experiments involving mechanical stimulations, *N* indicates the total number of mechanically stimulated cells. In MLC phosphorylation experiments, *n* indicates the total number of images analyzed.

**RESULTS**

**Effect of Blebbistatin on Thrombin-Induced Inhibition of the Ca²⁺ Wave**

Figure 1 shows typical findings of intercellular Ca²⁺ wave propagation evoked by mechanical stimulation of a single cell, and its modulation by thrombin without and with blebbistatin pretreatment. Figure 1A illustrates the Ca²⁺ wave propagation evoked by mechanical stimulation of a single cell, and its modulation by thrombin without and with blebbistatin pretreatment. Figure 1B shows the effect of thrombin on the reduction in the active area of the Ca²⁺ wave induced by thrombin. (A) Effect of pretreatment with (-)-blebbistatin (5 µM for 20 minutes) on the reduction in active area (AA) by thrombin (2 U/mL for 5 minutes; *N* = 120). (B) Effect of pretreatment with the inactive isoform (+)blebbistatin (5 µM for 20 minutes) on the reduction in AA by thrombin (2 U/mL for 5 minutes; *N* = 48). *P* < 0.001 for comparison between AA in the presence versus absence of thrombin in each condition. *P* < 0.001 for comparison between AA in the presence versus absence of (-)-blebbistatin in the control condition.

**TABLE 2.** Mean Active Area Average for the Experiments with (-)-Blebbistatin over Each of the Experimental Days

<table>
<thead>
<tr>
<th>Animal/Eye/Passage Number</th>
<th>Active Area (µm²) Exp. Day</th>
<th>Control Mean</th>
<th>Control SD</th>
<th>Thrombin Mean</th>
<th>Thrombin SD</th>
<th>(-)-Blebbistatin Mean</th>
<th>(-)-Blebbistatin SD</th>
<th>(-)-Blebbistatin + Thrombin Mean</th>
<th>(-)-Blebbistatin + Thrombin SD</th>
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<tr>
<td>A1/E1/P1</td>
<td>1</td>
<td>32.189</td>
<td>12.185</td>
<td>13.808</td>
<td>8.585</td>
<td>30.802</td>
<td>11.143</td>
<td>17.942</td>
<td>9.388</td>
</tr>
<tr>
<td>A2/E1/P1</td>
<td>2</td>
<td>30.841</td>
<td>11.215</td>
<td>11.963</td>
<td>5.110</td>
<td>35.581</td>
<td>10.122</td>
<td>41.146</td>
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<td>49.391</td>
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<td>6.428</td>
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<td>73.529</td>
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<td>18.898</td>
<td>13.672</td>
<td>5.662</td>
<td>65.188</td>
<td>18253</td>
<td>59.530</td>
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</table>

* Average number of experiments per day per condition is eight.
in untreated cells (control condition). Figure 1B shows that the spread of the Ca\(^{2+}\) wave is reduced after exposure to thrombin (2 U/mL for 5 minutes). This inhibition is not apparent when cells were pretreated with (-)-blebbistatin (5 \(\mu\)M for 20 minutes) (Fig. 1C). In control conditions, the Ca\(^{2+}\) transients are observed up to five cell layers away from the mechanically stimulated cell (Fig. 1A). The line graph (at the right side of the images in Fig. 1) represents the time course of the Ca\(^{2+}\) transients (represented as normalized fluorescence [NF] values) in the mechanically stimulated cell and in the neighboring cell layers one to five (NB1, NB2, NB3, NB4, and NB5). Clearly, the peak normalized fluorescence is reduced in cells distant from the mechanically stimulated cell. Cells that are subjected to mechanical stimulation after incubation with thrombin also showed a Ca\(^{2+}\) wave, but the spread of the wave is reduced, and a marked Ca\(^{2+}\) increase is found only up to three cell layers (Fig. 1B), as previously described. When cells were pretreated with (-)-blebbistatin, thrombin failed to limit the spread of the wave. As shown in Figure 1C, the average value of the peak normalized fluorescence in NB5 is similar to the value found under control conditions.

Figure 2 summarizes the active area, a measure of spread of the Ca\(^{2+}\) wave, from 15 independent experiments (performed on different days on different preparations), in which we measured the effect of (-)-blebbistatin on the thrombin-induced inhibition of IC. The figure also includes data from six experiments with the inactive enantiomer (+)-blebbistatin. In each of these experiments, the Ca\(^{2+}\) wave propagation assay was repeated eight times in each condition, with stimulation of cells in separate areas of the monolayer. The active area of cells treated with thrombin (2 U/mL, 5 minutes) was significantly lower in the absence than in the presence of (-)-blebbistatin (5 \(\mu\)M, 20 minutes). No significant differences in the active area were found for Ca\(^{2+}\) waves in cells in control condition compared to cells treated with thrombin plus (-)-blebbistatin or (-)-blebbistatin alone (Fig. 2A). The inactive enantiomer (+)-blebbistatin did not have any significant influence on the active area in control conditions, as well as in the presence of thrombin (Fig. 2B). Table 2 shows the mean and SD values as well as number of replicates for each of 15 days of experiments in which we repeated control, thrombin, (-)-blebbistatin, and (-)-blebbistatin plus thrombin as a control. The variability among the absolute values of the active area appears to be multifactorial and may be due to differences in the breed and age of animals and small differences in culture time or passage number of the cells. However, as shown in Table 2, this variability apparently does not influence the effects of thrombin on IC, or the inhibition of the effect of thrombin by (-)-blebbistatin.

(-)-Blebbistatin is inactivated by exposure to UV light but also to radiation at 488 nm, the wavelength used for excitation of Fluo-4, and its inactivation products were reported to have cytotoxic effects. Therefore, we performed several control experiments to exclude that the results obtained with blebbistatin are confounded by nonspecific or toxic effects. We performed intercellular Ca\(^{2+}\) wave propagation experiments with photoactivated (-)-blebbistatin. The latter was obtained by illuminating (-)-blebbistatin with UV light for 30 minutes immediately before the experiment. Photoinactivation was confirmed by comparing the absorption spectrum before and after exposure to the UV light. The absorption spectrum of (-)-blebbistatin shows a pronounced peak between approximately 350 and 500 nm, with a maximum at 430 nm, which is greatly diminished after the solution is illuminated. The optical density (OD) measured at 430 nm of a 5 \(\mu\)M (-)-blebbistatin-containing solution was 0.0034 in our experiments. Photoinactivation reduced the amplitude of peak in the absorption spectrum of (-)-blebbistatin at 430 nm by 77.5% (corrected for baseline). As shown in Figure 3A, photoinactivated (-)-blebbistatin did not prevent the effect of thrombin (\(N = 83\)). In contemporaneous experiments, (-)-blebbistatin (not exposed to UV light) prevented the effect of thrombin (\(N = 32\)). These results show that the active area is not affected by possible toxicity of photoinactivated blebbistatin, since in the absence of thrombin, the active area of the Ca\(^{2+}\) wave propagation in the presence of inactivated (-)-blebbistatin was not significantly different (\(N = 46\), \(P = 0.13\)) from the value in the control condition (Fig. 3A).

We also measured Ca\(^{2+}\) wave propagation in the presence of (-)-nitroblebbistatin, a derivative that is much less sensitive to photoinactivation. The active area in the presence of (-)-nitroblebbistatin was slightly higher (9%) than that in the

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932946/ on 11/28/2018)
Inhibition of PIC

In our recent studies, thrombin was found to inhibit both PIC and GJIC. Therefore, we investigated whether the active area in the presence of thrombin was AA in the absence of thrombin. After inhibition of GJIC by thrombin, we obtained a significantly higher AA compared with thrombin in the absence of thrombin. 

We also used LY uptake and ATP release assays to examine PIC. In a previous study, we have shown uptake of LY (2.5% for 5 minutes) in a Ca^{2+}-free medium containing 2 mM EGTA. This approach is attributed to influx through hemichannels, as demonstrated in other cell types. The LY uptake was not blocked by thrombin, indicating that the proteinase inhibits the hemichannel-mediated PIC. Although BCECs treated with thrombin (2 U/mL, 5 minutes) did not show uptake of LY (Fig. 5), as demonstrated previously, the cells showed LY uptake in the presence of thrombin after pretreatment with (-)-blebbistatin (5 μM, 20 minutes; N = 5), but not in the presence of (+)-blebbistatin. This observation provides evidence that (-)-blebbistatin prevents the effect of thrombin on the hemichannel-mediated PIC pathway.

This issue was further examined by measuring ATP release in response to mechanical stimulation without and with (-)-blebbistatin. Thrombin significantly reduced the ATP release to 35% ± 7% (N = 5) of the value in control condition, whereas in the presence of thrombin plus (-)-blebbistatin the ATP release was 108% ± 9% (N = 4) of the value in control condition and was significantly larger than with thrombin in the active area in the presence of Gap27 alone or in the presence of the combination of Gap27 and (-)-blebbistatin.

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control condition (N = 25; P = 0.03). In the presence of (-)-nitroblebbistatin, the active area of the Ca^{2+} wave upon mechanical stimulation was significantly higher (by a factor of 5) than in the presence of thrombin alone (N = 25; P < 0.001) and not significantly different (P = 0.5) from the control condition (N = 40; Fig. 3B). Therefore, we can conclude that (-)-nitroblebbistatin also prevents the effect of thrombin on Ca^{2+} wave propagation. After preincubation of the cells with (+)-nitroblebbistatin, thrombin caused a significant reduction of the active area of the Ca^{2+} wave upon mechanical stimulation. The active area in the presence of (+)-nitroblebbistatin plus thrombin was not significantly different from the value with thrombin alone (Fig. 3B).

Effect of Blebbistatin on Thrombin-Induced Inhibition of PIC

Our previous studies have demonstrated that intercellular Ca^{2+} wave propagation in BCECs is mediated largely by PIC (consisting of ATP release through hemichannels) and to a smaller extent by GJIC. In our recent studies, thrombin was found to inhibit both PIC and GJIC. Therefore, we investigated whether (-)-blebbistatin affects the inhibition of PIC and GJIC by thrombin.

We first tested whether (-)-blebbistatin can prevent the effect of the protease on PIC in cells pretreated with Gap27, a connexin mimetic peptide that inhibits GJIC in BCECs without influencing PIC. In agreement with our findings published before, application of Gap27 reduced the propagation of the Ca^{2+} wave, and thrombin caused a further decrease of the active area in the presence of Gap27 by affecting PIC. As shown in Figure 4, (-)-blebbistatin pretreatment prevented the effect of thrombin even in the presence of Gap27. Thus, the active area in the presence of both Gap27 and thrombin was significantly increased by (-)-blebbistatin (Fig. 4). The active area in the presence of the combination Gap27, thrombin, and (-)-blebbistatin was not significantly different from
the absence of (−)-blebbistatin. Application of (+)-blebbistatin did not significantly affect the release of ATP. In the presence of thrombin and (−)-blebbistatin, ATP release relative to the control condition was 40% ± 9% (N = 3), which was not significantly different from the value in the presence of thrombin alone. These results indicate that inhibition of myosin II activity by (−)-blebbistatin prevents the effect of thrombin on ATP release after mechanical stimulation, whereas its inactive enantiomer (+)-blebbistatin has no effect.

Effect of Blebbistatin on Thrombin-Induced Inhibition of GJIC

Previously, we have demonstrated that GJIC also contributes to some extent to the Ca\(^{2+}\) wave propagation and that PIC can be inhibited by Gap26, a connexin mimetic peptide that inhibits hemichannels in BCECs.\(^{17}\) To determine whether (−)-blebbistatin can overcome the GJIC component of the Ca\(^{2+}\) wave, we tested its effect in the presence or absence of thrombin after inhibition of PIC by Gap26. As in our previous study,\(^{17}\) we

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932946/)
found a very pronounced reduction of active area by 82% in response to Gap26 (data not shown). In the presence of Gap26, we were also unable to demonstrate a further significant reduction of the active area by thrombin, or a significant effect of (−)-blebbistatin in the presence of thrombin, which may be due to the relatively small contribution of GJIC toward IC in BCECs.\textsuperscript{17}

Since our experiments with Gap26 failed to delineate significant effects of blebbistatin on GJIC, we performed FRAP experiments to investigate the influence of the drug on the effect of thrombin on GJIC more directly. We measured fluorescence recovery at 3 minutes after photobleaching 6-carboxyfluorescein in a single cell. As in our earlier study,\textsuperscript{22} thrombin led to a significant reduction in fluorescence recovery. The reduction was completely prevented by preincubation with (−)-blebbistatin. The drug had no effect on fluorescence recovery in the absence of thrombin. Furthermore, the inactive enantiomer (+)-blebbistatin did not significantly influence the fluorescence recovery in the presence or absence of thrombin (Table 3).

**Specificity of the Mechanism of Action of Blebbistatin on the Thrombin Effect**

To exclude that blebbistatin prevents the thrombin effect by signaling pathways other than myosin II ATPase, we investigated the influence of the drug on MLC phosphorylation in response to thrombin, as well as on the Ca\textsuperscript{2+} transients in response to thrombin and ATP.

**Thrombin-Induced MLC Phosphorylation in the Presence of Blebbistatin.** In BCECs, thrombin-induced cleavage of PAR-1 receptors induces activation of the RhoA – Rho kinase axis,\textsuperscript{49} resulting in enhanced phosphorylation of the regulatory light chain of myosin II (myosin light chain [MLC]).\textsuperscript{56,58} MLC is phosphorylated by MLCK at Thr18 and Ser19 residues and is dephosphorylated by MLCK phosphatase.\textsuperscript{56,57} MLC phosphorylation is a prerequisite for actomyosin contraction.\textsuperscript{57} To verify that the prevention of the effect of thrombin by (−)-blebbistatin is not due to decreased MLC phosphorylation, we determined the phosphorylation status under different conditions by quantitative immunofluorescence.

Figure 6 shows typical confocal images of immunostaining with a monoclonal antibody against diphosphorylated MLC (ppMLC) under various conditions. The images were obtained by focusing on the perijunctional actomyosin ring (PAMR), which was stained with Alexa 546 conjugated to phalloidin. The left column of the figure shows immunostaining of ppMLC (green) and F-actin (red), and the middle column highlights ppMLC alone. In untreated cells (control condition), basal MLC phosphorylation is evident, mainly along PAMR (Fig. 6A), indicating localization of contractile PAMR at the apical junctional complex. After treatment with thrombin, enhanced fluorescence can be observed, indicating higher levels of ppMLC (Fig. 6B). This is consistent with Rho-kinase-induced inactivation of myosin light chain phosphatase (MLCP) in response to thrombin-mediated PAR-1 receptor activation.\textsuperscript{22} No marked effect of (−)-blebbistatin on ppMLC was noted in control conditions (Fig. 6C). Furthermore, blebbistatin had no apparent effect on the enhanced MLC phosphorylation by thrombin (Fig. 6D).

Negative controls in the presence or absence of thrombin did not show any ppMLC staining (right column).

To make a quantitative comparison of the relative extents of phosphorylated MLC under different conditions, immunostained images were acquired by confocal fluorescence microscopy, using identical settings for excitation and for detection of fluorescence in each condition. The acquired images for ppMLC and pMLC were then quantified by representing them in the form of a fluorescence intensity distribution (FID). Figure 7A illustrates the analysis. For each image in a given condition, the FID of ppMLC fluorescence signals was constructed by summing the number of fluorescent pixels of a given intensity between 0 and the maximum of 255. Then, the number of fluorescent pixels of a given intensity (n(I)) was plotted as a function of pixel intensity (I) and the mean fluorescence intensity (MFI) was calculated as

$$MFI = \frac{\Sigma [n(I) \cdot I]}{\Sigma [n(I)]]}.$$  

Figure 7A shows a marked right shift of the peak of the FID for ppMLC in response to thrombin compared to untreated cells. (−)-Blebbistatin did not affect the FID in the presence or absence of thrombin. The average pixel intensities in the

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932946/)
example shown in the figure were 23.0 ± 0.9 in untreated cells, compared to 42.3 ± 1.1 in the presence of thrombin. (−)-Blebbistatin had no influence on MFI. The MFI was 24.5 ± 0.7 in the presence of (−)-blebbistatin and 40.5 ± 1.4 in the presence of the combination (−)-blebbistatin plus thrombin. The results from this typical experiment suggest enhanced ppMLC by thrombin and also absence of an effect of (−)-blebbistatin on ppMLC.

Figure 7B summarizes the results of measurements of the diphosphorylated form of MLC (ppMLC) obtained from four independent experiments, with 20 images taken for each condition. For each image, MFI was calculated, thus resulting in 20 MFI values per condition per experiment. For each condition, the average of the MFIs was taken and the values were normalized versus the control condition. The resulting normalized MFI values for each condition from four independent experiments (N = 4, n = 80) were then averaged, and the results are presented in bar graphs in the figure. The data show enhanced ppMLC by thrombin, in agreement with the findings obtained by Western blot analysis by Satpathy et al. Our experiments demonstrate that (−)-blebbistatin has no significant effect on ppMLC at the level of the PAMR, in the presence and absence of thrombin.

Our experiments with immunostaining for the monophosphorylated form of MLC (pMLC) showed that in addition to enhancing ppMLC, thrombin also enhanced the level of pMLC (data not shown), in agreement with the findings by Satpathy et al. (−)-Blebbistatin did not significantly affect the pMLC in the absence and in the presence of thrombin.

Expression of Myosin II Isoforms. Since blebbistatin is a selective inhibitor of myosin II ATPase, we verified the expression of myosin II in BCECs. RT-PCR showed positive bands at the expected band sizes of 116 and 263 bp for the transcripts of myosin IIA and IIB isoforms, respectively (Fig. 8A). The corresponding protein expression profile was confirmed by Western blot analysis, as shown in Figure 8B. It may be noted that the EAHY926 cells, a hybridoma of primary HUVEC cells, used as a positive control for myosin IIA, show expression of myosin IIA. Expression of myosin IIA and IIB was confirmed also by immunofluorescence, as shown in Figure 8C.

**Effects of Blebbistatin on the Ca\textsuperscript{2+} Transient in Response to Agonist Stimulation.** We wanted to exclude that the effects of (−)-blebbistatin are mediated by changes in the signal transduction cascade between thrombin receptor activation and the Ca\textsuperscript{2+} rise in BCECs. Therefore, we measured the Ca\textsuperscript{2+} rise in response to thrombin in the presence and absence of (−)-blebbistatin and (−)-nitroblebbistatin by ratiometric measurements, using Fura-2AM in a high-throughput assay using a microplate reader (FlexStation 3; Molecular Devices). Specifically, we assayed the ratio of fluorescence emission to excitation at 340 nm and 380 nm (F340/F380). The peak value of the Ca\textsuperscript{2+} rise evoked by thrombin (2 U/mL) was not significantly altered by the presence of (−)-blebbistatin or of (−)-nitroblebbistatin (Fig. 9A).

Since mechanical stimulation causes ATP release, resulting in autocrine and paracrine effects, we wanted to exclude that the inhibitory effects of (−)-blebbistatin are mediated by changes in response of the cells to ATP. Therefore, we measured the rise in Ca\textsuperscript{2+} in response to different concentrations of ATP in the presence and absence of (−)-blebbistatin and of (−)-nitroblebbistatin (Figs. 9B, 9C). Figure 9B shows the dose-dependent Ca\textsuperscript{2+} rise in BCECs provoked by different concentrations of ATP in control condition. Figure 9C shows that the EC\textsubscript{50} for ATP-induced Ca\textsuperscript{2+} rise in BCECs was not significantly altered by the addition of (−)-blebbistatin or (−)-nitroblebbistatin.

**Discussion.** The major finding of this study is that inhibition of the myosin II ATPase activity by (−)-blebbistatin prevented the thrombin-mediated inhibition of PIC and GJIC in BCECs. Hence, our experiments indicate that thrombin-induced contraction of the actin cytoskeleton inhibits IC, evoked by a point-mechanical stimulation, by acting on hemichannels and gap junctions. Our results, therefore, provide novel functional evidence for the regulation of hemichannels by contraction of the actomyosin cytoskeleton, in line with the recent findings that several ion channels are direct or indirect molecular targets for regulation by the actomyosin-based cytoskeleton.
Recently, we have shown that thrombin reduces the propagation of intercellular Ca\textsuperscript{2+} waves in BCECs by inhibiting both PIC and GJIC. Furthermore, the inhibitory effects of thrombin on IC could be attributed to an enhanced phosphorylation of MLC, leading to contraction of the actomyosin cytoskeleton.\textsuperscript{22-23} Here, we used (-)-blebbistatin, a selective inhibitor of myosin II ATPase, to investigate the role of the actomyosin contraction in the thrombin-dependent regulation of IC. Consistent with expression of myosin IIA and IIB, we have found that (-)-blebbistatin prevents the thrombin-induced inhibition of Ca\textsuperscript{2+} wave propagation and its GJIC and PIC components.

Several lines of evidence demonstrate that the effects of (-)-blebbistatin on IC were not confounded by toxicity of the photoinactivated compound, or effects not related to myosin ATPase activity.\textsuperscript{(1)} In our Ca\textsuperscript{2+} wave experiments, the cells were exposed to 488 nm excitation for a total of \(-2\) minutes at a (-)-blebbistatin concentration of 5 \(\mu\text{M}\). For 2 minutes of exposure to (-)-blebbistatin (5-20 \(\mu\text{M}\)), Kolega\textsuperscript{42} found no significant effect on the viability of bovine aortic endothelial cells.\textsuperscript{52} (2) Photoinactivated (-)-blebbistatin did not affect the Ca\textsuperscript{2+} wave propagation in the presence or absence of thrombin. (3) The nitro derivative of (-)-blebbistatin, which was shown to be stable to prolonged irradiation,\textsuperscript{45} had an effect similar to that of (-)-blebbistatin. (4) In the LY uptake experiments, LY is taken up before the cells are imaged for LY fluorescence, and thus (-)-blebbistatin is not exposed to excitation light during the dye uptake. (5) (-)-Blebbistatin did not significantly affect thrombin- or ATP-induced Ca\textsuperscript{2+} mobilization from the internal Ca\textsuperscript{2+} stores in BCECs. (6) (-)-Blebbistatin did not change MLC phosphorylation status in the presence of thrombin, implying that the drug has no significant effect on kinases and phosphatases upstream of MLC phosphorylation.

The finding that (-)-blebbistatin completely prevents the effect of thrombin on the Ca\textsuperscript{2+} wave, implies that the GJIC as well as the PIC components of IC are inhibited by (-)-blebbistatin. FRAP experiments confirmed that (-)-blebbistatin can prevent the thrombin effect on GJIC, while LY uptake and ATP release experiments demonstrated that it prevents the effects of the protease on PIC, which we have shown is due to connexin hemichannels. Our findings provide evidence that actomyosin contraction not only inhibits gap junctions, but also hemichannels.

**Mechanisms of Thrombin Action on GJIC and PIC**

The inhibition of GJIC by actomyosin contraction is likely to be due to loss of intercellular tethering forces, which are essential for stable docking of hemichannels from neighboring cells. This effect is similar to that of thrombin on tight and adherens junctions, which break down in response to increased contraction of the perijunctional actomyosin ring.\textsuperscript{56-58} However, the marked inhibition of hemichannels is unexpected. Although an exact mechanism underlying the inhibition of hemichannels cannot be well delineated based on our current findings, it is likely to involve direct or indirect association of the actomyosin cytoskeleton with hemichannels as has been shown for several other ion channels.\textsuperscript{50-53} We can, however, rule out certain possibilities based on the well-known selectivity of blebbistatin to inhibit myosin II ATPase.\textsuperscript{38-41,54}

**Direct Phosphorylation of Connexins.** As a serine protease, thrombin activates PAR-1 receptors, which are ex-
pressed in BCECa \(^{22,26}\) and brings about activation of both PKC and Rho kinase. \(^{26,30-31}\) PKC is known to cause phosphorylation of several connexin isoforms, including Cx43, and this phosphorylation is well known to modulate assembly, trafficking, and gating of the gap junction channels. \(^{14}\) Therefore, thrombin may cause enhanced phosphorylation of hemichannels. It cannot be ruled out that also other molecules downstream of actomyosin contraction are phosphorylated. However, our findings on the effects of blebbistatin (Figs. 4, 5) exclude the possibility that changes in the phosphorylation of connexins through a transduction cascade not involving myosin ATPase activity are responsible for the effects of the protease.

**Influence on Direct Phosphorylation of Proteins That May Interact with Hemichannels.** Many proteins, such as actin-binding proteins and putative scaffold proteins, link the actin cytoskeleton to membrane proteins, including ion channels and transporters. It is reported that organization of actin cytoskeleton near the membrane could affect the activity of ion channels by affecting their retention at the plasma membrane, trafficking to the plasma membrane, and gating of channel activity through scaffolding proteins (e.g., NHERF coupling to CFTR via PDZ domain) and adapter proteins (e.g., polycystin-2 associates with α-actinin; reviewed in \(^{51}\)). It has been reported that certain ion channels (e.g., ENaC, \(^{51}\) CLC-2 \(^{55}\)) can directly interact with F-actin, causing altered gating of the channel. As an example of scaffolding proteins that could be influenced by thrombin, ERM proteins (i.e., ezrin, radixin, and moesin) provide regulated linkage of actin to plasma membrane proteins. \(^{56,57}\) Activation of ERMs by serine/threonine phosphorylation and/or interaction with PIP2 is found in response to activation of RhoA. \(^{35}\) Thus, although ERMs could eventually be activated through stimulation of PAR-1 receptors by thrombin, the specificity of blebbistatin makes it unlikely that the inhibitory effect of thrombin on PIC and GJIC is due to a direct effect on ERMs.

**Direct Actin Remodeling.** Although thrombin-induced activation of Rho kinase is known to influence actin remodeling (i.e., actin polymerization) through LimI kinase, \(^{37}\) the selectivity of blebbistatin also excludes that the effect of thrombin on hemichannels is due to such an effect. Specifically, the action of blebbistatin on myosin II alone precludes that any potential actin remodeling regulated by processes upstream of actomyosin contraction takes part in the effect of thrombin.

**Potential Mechanisms Underlying the Thrombin Effect**

Although our data exclude mechanisms independent of actomyosin contractility, they do not allow us to pinpoint a specific mechanism downstream of actomyosin contractility involved in the thrombin effect. However, several possibilities can be suggested on the basis of findings in the literature. First, analogous to mechanosensitive channels that respond to extracellular mechanical stimuli (e.g., shear stress), the action of thrombin could be due to direct inactivation of the hemichannels involved in PIC secondary to tractional forces in the submembranous actin cytoskeleton. \(^{58}\) It has been shown that certain hemichannels (Cx46) are mechanosensitive and implicated in mechanotransduction during accommodation of the intraocular lens. \(^{59}\) Another possibility is that the gating of hemichannels is affected by changes in localization of proteins such as ZO-1 in response to increased contractility of the PAMR. ZO-1, through its PDZ-domain, also can link connexins (e.g., Cx43) to the actin cytoskeleton. \(^{60-62}\) Another possible mechanism could be associated with trafficking or membrane insertion of hemichannels as has been suggested to explain the involvement of actin cytoskeleton in the regulation of epithelial ion channels. \(^{51}\)

In summary, we have demonstrated for the first time that enhanced actomyosin contraction can inhibit GJIC and hemichannel-mediated PIC. Since a large number of proinflammatory mediators affect the actin cytoskeleton and contractility, our findings suggest that hemichannel-mediated effects can contribute to the response of the corneal endothelium during inflammation.

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**References**


