EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

Purinoreceptor P2X7 Regulation of Ca^{2+} Mobilization and Cytoskeletal Rearrangement Is Required for Corneal Reepithelialization after Injury

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The process of wound healing involves a complex network of signaling pathways working to promote rapid cell migration and wound closure. Activation of purinergic receptors by secreted nucleotides plays a major role in calcium mobilization and the subsequent calcium-dependent signaling that is essential for proper healing. The role of the purinergic receptor P2X7 in wound healing is still relatively unknown. We demonstrate that P2X7 expression increases at the leading edge of corneal epithelium after injury in an organ culture model, and that this change occurs despite an overall decrease in P2X7 expression throughout the epithelium. Inhibition of P2X7 prevents this change in localization after injury and impairs wound healing. In cell culture, P2X7 inhibition attenuates the amplitude and duration of injury-induced calcium mobilization in cells at the leading edge. Immunofluorescence analysis of scratch-wounded cells reveals that P2X7 inhibition results in an overall decrease in the number of focal adhesions along with a concentration of focal adhesions at the wound margin. Live cell imaging of green fluorescent protein–labeled actin and talin shows that P2X7 inhibition alters actin cytoskeletal rearrangements and focal adhesion dynamics after injury. Together, these data demonstrate that P2X7 plays a critical role in mediating calcium signaling and coordinating cytoskeletal rearrangement at the leading edge, both of which processes are early signaling events necessary for proper epithelial wound healing. (*Am J Pathol* 2016, 186: 285–296; <http://dx.doi.org/10.1016/j.ajpath.2015.10.006>)

The process of epithelial wound healing in the cornea is essential for maintaining the health of the tissue and preventing pathologies that can result in pain and chronic erosion. The early response after injury is critical for initiating the signaling pathways required for proper wound healing. This early response includes the release of nucleotides, Ca^{2+} wave propagation from the wound site, and cytoskeletal rearrangements that promote migration to reestablish the epithelial barrier.

Immediately after injury, there is a release of nucleotides into the extracellular milieu.¹ Purinergic receptors bind these nucleotides and mediate downstream signaling. The P2 class of purinergic receptors is subdivided into P2Y receptors, which are G-protein–coupled receptors that cause an increase in intracellular Ca^{2+} via inositol 1,4,5 triphosphate–mediated signaling, and P2X receptors, which are trimeric ion channels that gate Ca^{2+} and other ions from the extracellular environment.²

Nucleotide release and P2 receptor signaling are required to stimulate wound-induced Ca^{2+} transients. This has been demonstrated with apyrase, an ectonucleotidase, which abolishes the Ca^{2+} response to injury.³ Although down-regulation of specific P2 receptors decreases Ca^{2+} mobilization in corneal epithelial cells,⁴ activation of P2 purinergic receptors by nucleotides causes increased phosphorylation of adaptor proteins and focal adhesion kinases.^{5,6} These changes mediate signaling events that regulate migration, demonstrating the critical role of nucleotide signaling in wound repair.

Aberrant purinoreceptor signaling is often associated with disease.⁷ Extensive research has been performed on the role

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of P2Y receptors in wound healing; however, P2X receptors are not as well characterized. Although the P2X7 receptor has been predominantly studied as a cell death receptor, recent studies have suggested a role in the cellular response to injury.^{8–11} P2X7 is necessary for proper epithelial cell adhesion to the basement membrane as well as the overall integrity of the corneal stroma.¹² However, the mechanism by which P2X7 mediates cell migration is poorly understood.

The cornea is an avascular, transparent tissue that is oxygenated via diffusion. It is also highly innervated by sensory nerves. Disruption of cell-cell junctions or the misalignment of collagen fibrils can result in corneal haze. Although the cornea is a tissue with unique features, the response of the epithelium to injury, including the release of nucleotides and the mobilization of Ca^{2+} , is similar to that of other epithelia.¹³ Thus, the cornea offers an attractive model for the study of epithelial wound healing.

Our goal was to determine the role of P2X7 in mediating both the initial Ca^{2+} mobilization and the downstream events of wound closure. We observed that P2X7 expression increases at the leading edge after injury, despite an overall decrease in P2X7 further back from the wound. Inhibition of P2X7 impairs wound closure and prevents the injury-induced change in P2X7 localization. *In vitro*, we demonstrated that inhibition of P2X7 attenuates both the amplitude and duration of Ca^{2+} mobilization in cells within close proximity to the wound. P2X7 inhibition also decreases the number and alters the localization of focal adhesions. Live cell imaging revealed that P2X7 inhibition alters actin cytoskeletal dynamics, impairs focal adhesion turnover, and leads to an increased frequency of both focal adhesion splitting and merging events. Together, our results demonstrate that P2X7 is a vital component in regulating the early response to epithelial injury, with a particular role in coordinating Ca^{2+} mobilization and cytoskeletal rearrangement at the leading edge.

Materials and Methods

Antibodies

Anti-P2X7 polyclonal rabbit antibodies directed against the extracellular domain of rat P2X7 (catalog number APR-008) were purchased from Alomone Labs (Jerusalem, Israel). Anti- β -actin monoclonal mouse antibodies (clone AC-15, catalog number A5441), anti-vinculin monoclonal mouse antibodies (clone hVIN-1, catalog number V9264), anti-talin monoclonal mouse antibodies (clone 8d4, catalog number T3287), and anti-p15^{INK} polyclonal rabbit antibodies (CDKN2B; catalog number SAB4500078) were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor-conjugated whole IgG secondary antibodies against mouse or rabbit were purchased from Invitrogen (Carlsbad, CA).

Nucleotides and P2X7 Inhibitors

ATP, UTP, and ADP were purchased from Sigma-Aldrich. 2,3-O-(4-benzoylbenzoyl)-ATP (BzATP), a P2X7 agonist, was purchased from Sigma-Aldrich. Oxidized ATP (oxATP), an irreversible inhibitor of P2X7, was purchased from Sigma-Aldrich. oxATP is thought to act at the ATP binding site of P2X7.¹⁴ Although BzATP can act as an agonist for P2X1 and P2X3, this is not a concern because these receptors are not expressed in corneal epithelial cells.¹⁵ A438079 hydrochloride, a competitive inhibitor of P2X7, was purchased from Tocris Biosciences (Minneapolis, MN).

Organ Culture

Dissected corneas were cultured as described previously.^{6,16} Briefly, Sprague-Dawley juvenile rats (Charles River Labs, Wilmington, MA) were euthanized, and heads were kept on ice until corneal dissection. A 3-mm-diameter region in the center of the cornea was demarcated with a trephine, and the epithelium was removed from the region. Eyes were removed and placed in ice-cold Dulbecco's modified Eagle's medium (DMEM) containing 20 mmol/L HEPES (pH 7.5). Corneas were dissected, leaving 1- to 2-mm scleral tissue adjacent to the cornea. The lens and iris were removed, leaving a corneal cup. DMEM with 0.75% agar was pipetted into the posterior surface of the cornea to form a dome to maintain corneal curvature. The corneas were cultured anterior side up, with enough DMEM to cover the scleral ring. The corneal epithelium was exposed to air, and the epithelium was wetted with DMEM periodically to prevent drying.

Cell Culture

Human corneal limbal epithelial (HCLE) cells were a kind gift from Dr. Ilene K. Gipson (Harvard Medical School, Boston, MA).¹⁷ Cells were cultured in keratinocyte serum-free media supplemented with 25 $\mu\text{g/mL}$ bovine pituitary extract, 0.02 nmol/L epithelial growth factor, 0.03 mmol/L CaCl_2 , 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 0.5% amphotericin B (Fungizone; Invitrogen). Cells were passaged when 80% to 90% confluent, were plated for experiments at 80 to 100 cells/ mm^2 , and were grown to confluence. Where indicated, growth supplements were removed for 16 to 24 hours before stimulation or injury.

Organ Culture Wound Healing

To determine the rate of wound closure, corneas were stained with 1% methylene blue in phosphate-buffered saline (PBS; pH 7.4) at indicated times after injury. The dye was added for approximately 30 seconds and washed several times with PBS to remove excess stain. The exposed basement membrane retained the blue stain, allowing for

quantification of the wound area over time. Corneas were imaged with a Nikon 990 digital camera (Nikon, Melville, NY) attached to a Zeiss dissection microscope (Zeiss, Thornwood, NY). Wound sizes were quantified using FIJI/ImageJ version 1.49 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>).

Quantitative Real-Time PCR

RNA was isolated from rat organ cultured corneas by scraping two distinct cell populations (migrating and peripheral) into ice-cold PBS, spinning the cells, and resuspending them in RNA lysis buffer. Cells were homogenized, and mRNA was isolated using a spin column kit (Fisher Scientific, Pittsburgh, PA). Normalized concentrations were reverse transcribed, as previously described.⁶ Quantitative real-time PCR was performed using verified TaqMan probes (Life Technologies, Grand Island, NY) for target or 18S on an ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA). Relative expression was determined using the $\Delta\Delta C_t$ method, using 18S to normalize.

siRNA Transfection

HCLE cells were transfected as described previously.^{4,15} Briefly, complete medium was replaced with keratinocyte serum free media lacking antibiotics and supplements. The siRNA sequences directed against P2X₇ were as follows: 5'-GGAUCCAGAGCAUGAAUUAUU-3' (sense) and 5'-UAAUUCAUGCUCUGGAUCCUU-3' (antisense; Dharmacon product number J-003728-07; Dharmacon, Lafayette, CO). To identify transfected cells, the Silencer®Cy3 RNA labeling kit was used to label siRNA according to the manufacturer's directions (Ambion Life Technologies, Carlsbad, CA). A final concentration of 200 nmol/L siRNA against P2X7 or an equal concentration of nontargeting siRNA (Dharmacon product number D-001210-02) was transfected using Lipofectamine 2000 (Invitrogen). The transfection medium was replaced with complete keratinocyte serum free media 6 hours after transfection, and the cells were cultured for 4 days before experimentation. Knockdown of RNA and protein was routinely determined by real-time PCR and Western blot analysis before experimentation.

Western Blot Analysis

The two distinct populations of corneal epithelial cells were isolated from organ cultures, as described above. Cell pellets were resuspended in ice-cold radioimmunoprecipitation assay buffer (10 mmol/L Tris-Cl, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonylfluoride, and 1 mmol/L sodium orthovanadate, pH 7.5) and homogenized. For cell culture, HCLE cells were washed with cold PBS and lysed in radioimmunoprecipitation assay buffer. Protein concentration was determined using the BCA assay (Bio-Rad, Hercules, CA). Protein samples were boiled in Laemmli sample buffer for 10

minutes, resolved using SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for indicated proteins. Band quantifications were performed using FIJI/ImageJ.

Immunofluorescence and Confocal Microscopy

Cells or organ cultures were fixed in freshly prepared 4% paraformaldehyde in PBS for 20 minutes at room temperature (cells) or overnight at 4°C (organ cultures). Immunofluorescence staining was performed as described previously.^{5,6} Briefly, cells or tissues were permeabilized with 0.1% v/v Triton X-100 in PBS for 2 to 5 minutes and blocked with 4% bovine serum albumin in PBS for 1 hour. Cells or tissues were incubated in primary antibody solutions overnight at 4°C. Alexa Fluor–conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:100 in blocking solution for 1 hour at room temperature. Rhodamine-conjugated phalloidin (1:50; Invitrogen) was used to visualize F-actin. Cells or tissues were mounted using VectaSHIELD with DAPI (Vector Labs, Burlingame, CA). Images were taken on a Zeiss LSM 700 (Zeiss) confocal microscope with indicated objectives and settings. Images were analyzed using FIJI/ImageJ.

Calcium Imaging

Calcium mobilization was performed as described previously.^{6,18} Briefly, cells were cultured to confluence onto fetal bovine serum–coated glass coverslips (for perfusion) or glass bottom dishes (for wounding). Growth supplements were removed 16 to 24 hours before experimentation. Cells were preloaded with 5 μ mol/L Fluo-3 AM fluorescent dye (Invitrogen) in a final concentration of 1% (v/v) dimethyl sulfoxide and 0.02% (w/v) pluronic acid in HEPES-buffered saline (pH 7.6), for 30 minutes at 37°C and 5% CO₂.^{6,15,18} For perfusion, coverslips were mounted onto an imaging chamber (Warner Instruments, Holliston, MA), and HEPES-buffered saline was perfused at a rate that did not induce fluxes. Images were collected every 786 milliseconds on a Zeiss LSM 510 confocal microscope.^{6,15,18,19} Approximately 50 frames were captured to establish baseline fluorescence, then stimuli and washes were performed at indicated times. For wounding, glass pipettes were pulled to a fine tip and positioned over cells with a micromanipulator (Sutter Instruments, Novato, CA), as also described previously.^{6,20} Baseline and post-wound frames were captured as above, at indicated times. Analysis was performed using FIJI/ImageJ.

Live Cell Imaging of Actin and Talin

Cells were plated onto fetal bovine serum–coated glass bottom dishes, transduced with CellLight Actin-Green Fluorescent Protein or Talin-Green Fluorescent Protein, BacMam 2.0 (Molecular Probes, Eugene, OR), and grown to confluence. Growth supplements were removed 16 to 24

hours before experimentation, and vitamin C (1 mg/mL) was added 1 hour before imaging to minimize phototoxicity. After scratch wounding, cells were imaged on a Zeiss LSM 510 confocal microscope equipped with an environmental chamber (37°C, 5% CO₂). The confocal pinhole was set to 2.79 Airy units. Time-lapse images were obtained at 10-minute intervals for 4 hours, with the initial images taken 30 minutes after injury. Twelve regions were imaged in parallel for each dish. Autofocusing was performed before each time point, and the detector gain and amplifier offset were automatically adjusted by the Zeiss LSM 510 imaging software version 4.2 for optimal brightness and contrast at each location. Four independent experiments were performed for each probe. Images were analyzed in FIJI/ImageJ. Automated focal adhesion tracking was performed using the TrackMate plugin for FIJI/ImageJ.

Statistical Analysis

Values are means $\pm$ SEM of at least three independent experiments. Statistical significance was determined by unpaired, one-tailed *t*-test or two-way analysis of variance with appropriate post hoc tests using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

Results

P2X7 Regulation in Intact and Wounded Corneas

To understand the role of P2X7 in migration, we first examined how P2X7 expression and localization changes after corneal epithelial injury. Epithelial abrasions were

performed on rat corneas before being placed in organ culture. Methylene blue–stained regions were measured to determine the rate of wound closure (Figure 1A). To determine the levels of P2X7 at baseline and after corneal injury, mRNA and protein were collected and measured. The epithelial cells were separated into two populations, as indicated (Figure 1A). Cells that crossed the initial wound margin were designated migrating, and the distal epithelial cells were designated peripheral. P2X7 mRNA decreased 4 hours after injury in both cell populations and remained low for the duration of the time course (Figure 1B). Similarly, P2X7 protein levels decreased 12 hours after injury, by approximately 90% in migrating cells and approximately 80% in peripheral cells (Figure 1C). To confirm the cell populations, the cell cycle inhibitor p15 was analyzed. The increase in p15 after injury confirmed the migrating phenotype of the cells (Figure 1C). Both P2X7 and p15 were normalized to β -actin, and data are presented as ratios after densitometric analysis.

Immunohistochemical analysis of P2X7 in the intact and wounded corneal epithelium demonstrated a striking change in localization that occurred after injury. In the unwounded epithelium, P2X7 was localized predominantly in the basal and the flat apical cells (Figure 1D). After injury, P2X7 was prominent at the leading edge of the migrating epithelium but down-regulated behind the leading edge. However, when the wound reepithelialized, P2X7 localization returned to the pattern detected in the unwounded epithelium (Figure 1D). To assess the change in intensity at the wound edge compared with areas back from the wound, the wounded or unwounded epithelium was divided into two regions of interest, each 150 μ m in length. The fluorescence

Figure 1 P2X7 localization and expression after corneal epithelial injury. **A:** Healing curve of organ-cultured rat corneal epithelium over time after wounding. At indicated times, organ-cultured corneas were stained with 1% methylene blue and imaged (inset). Wound size was measured using FIJI/ImageJ. The migrating and peripheral epithelial cell populations (for mRNA and protein isolation) are indicated (inset, dashed circle). **B:** Real-time PCR was performed on mRNA collected from the migrating (closed diamonds) and peripheral (open diamonds) cell populations at each time point. The data are graphed as relative expression of P2X7/18S. Data are representative of at least seven eyes per time point. **C:** Protein expression. Cell lysates were collected from the migrating and peripheral cell populations, resolved by SDS-PAGE (10%), and immunoblotted. Data are representative of at least six rats per time point. Densitometry was performed using FIJI/ImageJ, and data are expressed as P2X7/ β -actin or p15/ β -actin. **D:** Representative images show change in P2X7 localization as a result of injury. Radial sections were cut and stained for P2X7. The leading edges of the wounded epithelia are lined up for presentation and compared with unwounded and reepithelialized. The leading edge is indicated by an asterisk. Images were taken on a Zeiss LSM 700 confocal microscope (numerical aperture, 0.8). *N* = 3 per time point (A). Scale bar = 50 μ m (D). E, epithelium; S, stroma in the cornea.

Figure 2 Inactivation of P2X7 alters wound healing. **A:** Oxidized ATP (oxATP) delays rate of wound healing. Debridement wounds (3 mm in diameter) were made, and corneas were cultured in the presence or absence of 10 $\mu\text{mol/L}$ oxATP for 12 hours. The remaining wound area was stained with methylene blue and imaged. Wound size was quantified using FIJI/ImageJ. Percentage wound closure at 12 hours is shown (one-tailed *t*-test). **B:** Representative images show oxATP inhibits injury-induced change in P2X7 localization. Rat corneas incubated with oxATP demonstrated that P2X7 remained along the basal cell membranes compared with control wounded (asterisk indicates leading edge). Unwounded control is shown for comparison. Images were collected on a Zeiss LSM 700 confocal microscope (numerical aperture, 0.8). Data are given as means $\pm$ SEM (**A**). $N = 6$ or more independent experiments (**A**). $*P < 0.05$. Scale bar = 50 μm (**B**). E, epithelium; S, stroma in the cornea.

intensity of the region of interest at the leading edge of the wounded tissue was consistently twofold greater than the adjacent region of interest. In contrast, in the unwounded epithelium, the fluorescence intensity values were constant over the entire length of the tissue. These data indicate that, although P2X7 expression increases at the leading edge after injury, it decreases back from the leading edge. We hypothesize that this change in localization may be a critical factor in orchestrating the onset of events for proper migration.

Effects of P2X7 Inhibition on Corneal Epithelial Wound Closure

To test the role of P2X7 in corneal wound repair, corneas were cultured in the presence or absence of oxATP, an irreversible P2X7 inhibitor.^{14,21} Wound sizes were

measured 12 hours after injury. Treatment with oxATP resulted in a significantly larger wound area (Figure 2A), supporting previous findings that P2X7 promotes wound healing.^{12,15} When the corneas were incubated with oxATP, P2X7 remained present along the basolateral membrane after injury (Figure 2B), a pattern similar to that seen in control unwounded tissue (Figures 1D and 2B). Similar results were obtained using A438079, a reversible P2X7 inhibitor (data not shown).

Role of P2X7 in Calcium Mobilization

To examine signaling events mediated by P2X7, we used an established corneal epithelial cell line (HCLE), which has been shown to exhibit Ca^{2+} mobilization in a manner similar to primary corneal epithelial cells.^{4,17} Previously, we demonstrated that P2X7 stimulation with BzATP, a P2X7 agonist, increases the migration rate in both a modified Boyden chamber and scratch-wound assay model and induces a prototypical mobilization of Ca^{2+} in HCLE cells.¹⁵

To further determine the role of P2X7 in Ca^{2+} mobilization, we examined the effects of P2X7 antagonism on BzATP-stimulated Ca^{2+} influxes. The BzATP-induced Ca^{2+} response was decreased by oxATP and A438079 compared with controls (Figure 3, A–C). These results were confirmed with siRNA experiments where the lack of fluorescence in response to BzATP was established by determining cells that were transfected as the siRNA was conjugated to Cy3 (Figure 3D). To confirm that BzATP did not activate P2Y11 (a G-protein–coupled purinoreceptor expressed in HCLE cells), cells were incubated in a Ca^{2+} -free medium and stimulated with BzATP. Only a minimal response was detected, indicating that P2Y11 was not activated (Figure 3C). To further determine specificity, cells were also stimulated with either UTP or ADP, agonists for P2Y2 and P2Y4 or P2Y1, respectively, three other G-protein–coupled purinoreceptors that are expressed in HCLE cells.^{4,18} Treatment with oxATP, A438079, or siRNA to P2X7 did not significantly inhibit the UTP- or ADP-stimulated Ca^{2+} response (Figure 3, C and D).

We then determined the effect of P2X7 inhibition on wound-induced Ca^{2+} mobilization. Because oxATP abrogated the P2X7-mediated Ca^{2+} response, confluent HCLE cells were injured in the presence or absence of oxATP and the intracellular Ca^{2+} wave was recorded (Figure 4A and Supplemental Movie S1). Inhibition of P2X7 significantly altered the overall relative fluorescence intensity wave in cells both immediately adjacent to and more distal to the wound ($P < 0.05$ by two-way analysis of variance with Sidak post hoc shown in end brackets) (Figure 4, B and C). In cells immediately adjacent to the wound, the intensity of the maximal fluorescence peak was significantly decreased by P2X7 inhibition ($P < 0.05$ by one-tailed *t*-test) (Figure 4B). In cells further from the wound, there was no significant difference in maximal fluorescence peak

Figure 3 P2X7 mediates calcium dynamics. Cells were washed in HEPES-buffered saline, incubated with Fluo-3AM, and washed to remove excess dye. Basal fluorescence levels were collected for 50 frames before agonist stimulation. **A:** Representative images using a pseudocolor intensity scale show the response of cells to 2,3-O-(4-benzoylbenzoyl)-ATP (BzATP) stimulation after preincubation with P2X7 inhibitors. **B:** Relative fluorescence intensity before and after stimulation in the presence or absence of inhibitors. **C:** Oxidized ATP (oxATP) and A438079 attenuate the BzATP-mediated, but not UTP- or ADP-mediated, calcium mobilization. In addition, oxATP only affects the BzATP-mediated calcium mobilization in the presence of Ca²⁺. Maximum fluorescence peak relative to control is graphed. **D:** Cells transfected with Cy3-labeled siRNA toward P2X7 or control were loaded with Fluo-3AM and stimulated with BzATP, UTP, and ADP. The Cy3-labeled cells are shown in black and white tiles, and the representative pseudocolored images show the response of cells to BzATP, UTP, and ADP using the color intensity scale shown in **A**. Arrows indicate transfected cells. Images were collected with a Zeiss LSM 510 confocal microscope (numerical aperture, 0.8) and analyzed using FIJI/ImageJ. Data are given as means $\pm$ SEM (one-tailed *t*-test; **C**). *N* = 4 or more independent experiments (**C**). ***P < 0.005. Scale bar = 50 μ m (**A** and **D**). Unstim., unstimulated.

intensity. There was, however, a significant difference in fluorescence intensity immediately after the initial peak, with oxATP-treated cells exhibiting a faster return to baseline (*P* < 0.05 by Sidak post hoc test) (Figure 4C). Finally, P2X7 inhibition did not alter either the rate of Ca²⁺ mobilization or the distance of propagation away from the wound

(Figure 4D). We speculate that the attenuated Ca²⁺ peak in cells immediately adjacent to the wound and the attenuated return to baseline in cells further back from the wound observed in oxATP-treated cultures may be detrimental to wound healing by attenuating downstream signaling events.

Figure 4 Inactivation of P2X7 alters the wound-induced calcium wave. **A:** Confluent human corneal limbal epithelial cells were preincubated with or without 100 $\mu\text{mol/L}$ oxidized ATP (oxATP) for 1 hour. After incubation with Fluo-3AM, basal fluorescence levels were collected for 50 frames before wounding (arrows indicate wound). Images were collected with a Zeiss LSM 510 confocal microscope (numerical aperture, 0.8). Representative images were pseudo-colored using a 16-color intensity scale to show the response of cells to injury. Brackets (B and C) indicate the cell populations analyzed. **B:** Relative fluorescence intensity of Ca^{2+} wave measured in cells adjacent to the wound (as indicated by brackets in A). Bar graph indicates the maximal peak fluorescence immediately after stimulation (arrow). **C:** Relative fluorescence intensity of Ca^{2+} wave measured in cells at a distance from the wound (as indicated by brackets in A). Analysis was performed using FIJI/ImageJ. **D:** Distance of furthest Ca^{2+} response from wound margin over time. **B–D** were analyzed by two-way analysis of variance with Sidak post hoc test, and bar graph of maximal fluorescence in **B** was analyzed by one-tailed *t*-test. Data are given as means $\pm$ SEM (**D**). *N* = 5 or more independent experiments (**B–D**). *P < 0.05. Scale bar = 50 μm (**A**).

Effect of P2X7 Inhibition on Cell Migration and Cytoskeletal Proteins

Wound-induced Ca^{2+} waves activate downstream signaling cascades that stimulate cytoskeletal rearrangements and promote migration. To determine whether cells *in vitro* exhibited a similar response to P2X7 inhibitors, as shown in organ culture (Figure 2), scratch-wound assays were performed in the presence or absence of oxATP (Figure 5A).

As demonstrated in organ culture, the oxATP-treated cells showed significantly delayed migration rates (two-way analysis of variance with Dunnett post hoc test: *P* < 0.05, *P* < 0.005) (Figure 5B). To determine whether oxATP inhibited BzATP-stimulated migration similar to siRNA knockdown,¹⁵ scratch-wound assays were performed with the addition of BzATP in the presence or absence of oxATP. OxATP significantly inhibited BzATP-stimulated migration (two-way analysis of variance with Sidak post hoc:

Figure 5 P2X7 mediates wound healing in cell culture. Confluent human corneal limbal epithelial cells were incubated in the presence or absence of oxidized ATP (oxATP) for 1 hour, scratch wounded, and imaged at indicated times after injury using an Olympus FSX-100 microscope. **A:** Images of control and oxATP-treated cells at 0 and 8 hours after injury are representative of four independent experiments. **B:** Quantification of wound size in the presence or absence of oxATP was performed using FIJI/ImageJ and graphed as percentage wound closure. **C:** Confluent cultures were wounded, and the media were replaced with fresh media containing 30 μmol/L 2,3-O-(4-benzoylbenzoyl)-ATP (BzATP) in the presence or absence of 100 μmol/L oxATP. Quantification of wound size was performed and graphed as percentage wound closure. Data are given as means ± SEM [two-way analysis of variance with Dunnett post hoc test (**B**) or Sidak post hoc test (**C**)]. $N = 4$ independent experiments (**B** and **C**). * $P < 0.05$, *** $P < 0.005$. Scale bar = 200 μm (**A**).

$P < 0.005$) (Figure 5C). Together, these data demonstrate that P2X7 mediates wound healing (Figures 2 and 5).

P2X7 signaling has been shown in lung epithelial cells to be upstream of focal adhesion formation.²² We therefore analyzed whether inhibition of P2X7 in corneal epithelial cells altered focal adhesions after injury. Cultures were incubated in the presence or absence of oxATP, and scratch-wounded cells were stained for talin or vinculin. Talin stabilizes integrin–extracellular matrix binding, and vinculin is an adaptor protein present in mature focal adhesions that plays a role in sensing and responding to mechanical tension in both cell–substrate and cell–cell interactions.^{23–25} Vinculin has been shown to be up-regulated in control wounded corneal epithelium.²⁶ Quantification of the entire field of view at low magnification revealed a net decrease in focal adhesions in the presence of oxATP (one-tailed *t*-test: $P < 0.05$, $P < 0.01$) (Figure 6A). Analysis of localization showed both talin- and vinculin-containing focal adhesions were at the leading edge of the wound margin and between cells in control cultures. In contrast, focal adhesions were prominent only at the leading edge in treated cultures (Figure 6, B and C). In addition, staining for F-actin revealed extensive filopodial projections in control cells compared with cells treated with oxATP; the latter also exhibited thicker actin bundles along the leading edge compared with control cells (Figure 6, B and C).

To gain further insight into these results, we analyzed actin cytoskeletal rearrangements and focal adhesion dynamics in live cells. Epithelial cells expressing actin–green fluorescent protein were scratch wounded in the presence or absence of oxATP and imaged for a period of 4 hours, with the initial images taken 30 minutes after injury (Figure 7A). Continuous membrane ruffling was observed in both cultures, but the oxATP-treated cultures displayed less searching or orienting behavior (Figure 7A and Supplemental Movie S2). In addition, although thick actin bundles formed after injury in both control and oxATP-treated cells, there was a trend for these bundles to remain perpendicular to the direction of migration in the treated cultures, indicating that there was minimal reorganization of actin filaments over time (Figure 7A and Supplemental Movie S2). Cell tracings color coded by time (Figure 7B) demonstrated decreased membrane ruffling in the treated cultures, which corroborated the impaired migration presented in Figure 5. Together, these data show that P2X7 is necessary for proper actin cytoskeletal rearrangements in response to injury.

Parallel experiments were performed using epithelial cells expressing talin–green fluorescent protein (Figure 7C and Supplemental Movie S3), and automated tracking of talin-containing focal adhesions was performed using the TrackMate plugin for FIJI/ImageJ. Focal adhesion trajectories color coded by lifespan revealed highly convoluted paths of focal adhesions in oxATP-treated cultures relative to control (Figure 7D). Quantification of focal adhesion turnover at the wound margin revealed a significant increase

Figure 6 Inactivation of P2X7 alters focal adhesion number and localization. Confluent human corneal limbal epithelial (HCLE) cells were preincubated with or without 100 $\mu\text{mol/L}$ oxidized ATP (oxATP) for 1 hour, scratch wounded, and incubated for 8 hours. Cells were stained for talin or vinculin and f-actin. **A**: Quantification of talin- and vinculin-containing focal adhesions using FIJI/ImageJ was performed by measuring particles of a thresholded area (one-tailed *t*-test). **B** and **C**: Representative images show talin (**B**) and vinculin (**C**) along with actin filament bundles in cells at wound edge. Images were taken on a Zeiss LSM 700 confocal microscope (numerical aperture, 1.4). *N* = 4 or more independent experiments (**A**). *P < 0.05, **P < 0.01. Scale bar = 20 μm (**B** and **C**).

in focal adhesion lifespan in oxATP-treated cultures (one-tailed *t*-test: *P* < 0.005) (Figure 7E). Furthermore, oxATP-treated cultures exhibited increased frequencies of both focal adhesion splitting and merging events relative to control cultures (one-tailed *t*-test: *P* < 0.01, *P* < 0.005) (Figure 7F). These results corroborate the trends presented visually in Figure 7D. Together, these data indicate that P2X7 activation promotes the turnover of focal adhesions at the leading edge. We speculate that the greater stability of focal adhesions observed in oxATP-treated cultures impairs cell motility during the wound response.

Discussion

Although the general importance of purinergic signaling to the early events of wound healing is established, the individual contributions of different P2 receptors to the overall purinergic wound response are not well understood. To determine whether the P2X7 receptor plays a specific and pivotal role in promoting cell migration after corneal epithelial injury, we used several approaches to analyze Ca^{2+} mobilization and cell motility in organ and cell cultures.

We report that a complex change in P2X7 expression occurs after corneal epithelial injury, which involves a marked increase in P2X7 expression at the leading edge despite an overall decrease in P2X7 mRNA and protein throughout the epithelium. P2X7 inhibition impairs migration and abrogates the P2X7 injury-induced change in localization. *In vitro*, we observed that P2X7 inhibition attenuates the intensity of the calcium peak in cells immediately adjacent to the wound, as well as the return to baseline in cells slightly further back. However, unlike other purinoreceptors, such as P2Y2, we found that P2X7 does not influence the distance or rate of Ca^{2+} wave propagation from the site of injury.^{3,4} Because the region of cells in which P2X7 modulates calcium dynamics correlates with the region of wounded epithelium in which P2X7 expression increases after injury, we speculate that localized activation of P2X7 acts in a feed-forward manner to up-regulate its expression at the leading edge.

Previous studies suggest that Ca^{2+} signaling is linked to the formation of focal adhesions via mechanosensory receptors.²⁷ Previously, our laboratory showed that the process of cell adhesion and phosphorylation of focal adhesion proteins was Ca^{2+} dependent.²⁸ These studies are supported by others demonstrating that P2X7-dependent Ca^{2+} signaling is necessary for focal adhesion formation and migration in astrocytes.²² On the basis of this evidence, we hypothesized that P2X7 activation and the subsequent change in P2X7 localization after injury promote rapid cytoskeletal rearrangements at the leading edge. Immunofluorescence studies demonstrated that focal adhesion proteins in cells that were scratch wounded in the presence or absence of oxATP were altered. Focal adhesion complexes

Figure 7 P2X7 mediates membrane ruffling and focal adhesion dynamics. Confluent human corneal limbal epithelial cells expressing actin–green fluorescent protein (GFP) or talin–GFP were scratch wounded in the presence or absence of oxidized ATP (oxATP). Cultures were monitored in an environmental chamber mounted on a Zeiss LSM 510 confocal microscope (numerical aperture, 1.4). Images were collected 30 minutes after injury for 4 hours. **A**: Representative cells expressing actin–GFP are shown at three time points after injury. **B**: Color-coded tracings of leading edges over a period of 4 hours. Note highly directional protrusions in control cultures. **C**: Representative cells expressing talin–GFP are shown at 4.5 hours after injury. **D**: Overlays of cells in **C** display focal adhesion trajectories color coded by lifespan. Automated focal adhesion tracking was performed using the TrackMate plugin for Fiji/ImageJ. **E**: Quantification of focal adhesion turnover at the wound margin (one-tailed *t*-test). **F**: Quantification of the relative frequency of focal adhesion splitting and merging events (one-tailed *t*-test). Data are representative of 12 distinct fields of view for each condition from four independent experiments for each probe. **P < 0.01, ***P < 0.005. Scale bar = 20 μ m (**A**, **C**, and **D**).

are essential for cell movement and migration because they link the actin cytoskeleton to the underlying matrix.^{29,30} It was recently shown that BzATP-induced activation of P2X7 enhances the activity of Fyn, a kinase that is upstream of

focal adhesion formation.³¹ Previously, Fyn was shown to be required for the regulation of the β 4 integrin and maintenance of hemidesmosomes, structures vital for epithelial integrity.³² Blocking the P2X7 activity with oxATP

inhibited the oligodendrocyte precursor cell migration induced by BzATP,³¹ which we demonstrated in our epithelial scratch-wound migration experiments.

To better understand these results and further investigate the role of P2X7 in promoting cell migration at the leading edge, we examined actin cytoskeletal rearrangements and focal adhesion dynamics in live cells. A marked decrease in membrane ruffling and minimal extension of lamellipodia at the leading edge were observed in oxATP-treated cells. Although thick actin bundles that ran perpendicular to the direction of migration formed after injury in both groups, in control cultures, these structures tended to turn over in favor of shorter and thinner actin bundles that were oriented more obliquely relative to the direction of migration. This was in contrast to the less dynamic oxATP-treated cultures in which the structures often persisted throughout the time course. In the treated cultures, we observed an increased frequency of both focal adhesion splitting and merging events compared with control cells. Splitting and merging events were recently reported when focal adhesion disassembly was inhibited.³³ These findings may explain why less cell searching and slower motility were observed in oxATP-treated cultures.

Collectively, our results indicate that P2X7 plays a critical role in the overall migratory response to corneal epithelial injury through its modulation of Ca^{2+} mobilization to coordinate cytoskeletal rearrangement at the leading edge. Such a role for P2X7 would suggest a complementary relationship with the P2Y2 purinoreceptor. For instance, our laboratory previously showed that down-regulation of P2Y2 attenuates the distance of wound-induced Ca^{2+} wave propagation,⁴ whereas in the present study, we demonstrated that P2X7 modulates Ca^{2+} amplitude and duration in cells within close proximity to the wound without affecting the distance or rate of Ca^{2+} mobilization. Furthermore, although P2X7 expression is enhanced at the leading edge after injury, there is an overall decrease in P2X7 protein and mRNA throughout the epithelium; this contrasts with the previously reported overall increase in P2Y2 mRNA that occurs as cells begin to migrate.⁵ On the basis of these observations, we speculate that both P2X7 and P2Y2 are necessary for proper wound repair in the corneal epithelium and may play distinct roles in wound repair, with P2X7 coordinating the response in cells along the wound margin and P2Y2 coordinating the response in cells further back from the wound.

In recent years, there has been a focus on P2X7 as a marker for cancer cells,⁹ and studies have shown that P2X7 activation can increase cancer cell invasiveness^{8,10} and migration.^{34,35} Interestingly, oxATP has been used as an antitumorigenic agent.³⁶ In other pathologies, however, the relationship of P2X7 to migration and disease is less clear. For example, corneas from diabetic patients express higher P2X7 levels than controls, yet cell migration after injury is known to be impaired rather than increased.¹⁵ In addition, P2X7 plays critical roles in several ocular tissues, including

the retina and trabecular meshwork.^{37,38} Together, these observations suggest that misregulation of P2X7 may be an important cause of pathological migration and disease.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.10.006>.

References

1. Yin J, Xu K, Zhang J, Kumar A, Yu F-SX: Wound-induced ATP release and EGF receptor activation in epithelial cells. *J Cell Sci* 2007, 120:815–825
2. Abbracchio MP, Burnstock G: Purinergic signalling: pathophysiological roles. *Jpn J Pharmacol* 1998, 78:113–145
3. Klepeis VE, Cornell-Bell A, Trinkaus-Randall V: Growth factors but not gap junctions play a role in injury-induced Ca^{2+} waves in epithelial cells. *J Cell Sci* 2001, 114:4185–4195
4. Boucher I, Rich C, Lee A, Marcincin M, Trinkaus-Randall V: The P2Y2 receptor mediates the epithelial injury response and cell migration. *Am J Physiol Cell Physiol* 2010, 299:C411–C421
5. Kehasse A, Rich CB, Lee A, McComb ME, Costello CE, Trinkaus-Randall V: Epithelial wounds induce differential phosphorylation changes in response to purinergic and EGF receptor activation. *Am J Pathol* 2013, 183:1841–1852
6. Lee A, Derricks K, Minns M, Ji S, Chi C, Nugent MA, Trinkaus-Randall V: Hypoxia-induced changes in Ca^{2+} mobilization and protein phosphorylation implicated in impaired wound healing. *Am J Physiol Cell Physiol* 2014, 306:C972–C985
7. Burnstock G, Knight GE, Greig AVH: Purinergic signaling in healthy and diseased skin. *J Invest Dermatol* 2012, 132:526–546
8. Tafani M, Schito L, Pellegrini L, Villanova L, Marfe G, Anwar T, Rosa R, Indelicato M, Fini M, Pucci B, Russo MA: Hypoxia-increased RAGE and P2X7R expression regulates tumor cell invasion through phosphorylation of Erk1/2 and Akt and nuclear translocation of NF- κ B. *Carcinogenesis* 2011, 32:1167–1175
9. Di Virgilio F, Ferrari D, Adinolfi E: P2X(7): a growth-promoting receptor-implications for cancer. *Purinergic Signal* 2009, 5:251–256
10. Jelassi B, Chantôme A, Alcaraz-Pérez F, Baroja-Mazo A, Cayuela ML, Pelegrin P, Surprenant A, Roger S: P2X(7) receptor activation enhances SK3 channels- and cystine cathepsin-dependent cancer cells invasiveness. *Oncogene* 2011, 30:2108–2122
11. Adinolfi E, Raffaghello L, Giuliani AL, Cavazzini L, Capece M, Chiozzi P, Bianchi G, Kroemer G, Pistoia V, Di Virgilio F: Expression of the P2X7 receptor increases in vivo tumor growth. *Cancer Res* 2012, 72:2957–2969
12. Mayo C, Ren R, Rich C, Stepp MA, Trinkaus-Randall V: Regulation by P2X7: epithelial migration and stromal organization in the cornea. *Invest Ophthalmol Vis Sci* 2008, 49:4384–4391

13. Cordeiro JV, Jacinto A: The role of transcription-independent damage signals in the initiation of epithelial wound healing. *Nat Rev Mol Cell Biol* 2013, 14:249–262
14. Michel AD, Xing M, Thompson KM, Jones CA, Humphrey PPA: Decavanadate, a P2X receptor antagonist, and its use to study ligand interactions with P2X7 receptors. *Eur J Pharmacol* 2006, 534:19–29
15. Mankus C, Rich C, Minns M, Trinkaus-Randall V: Corneal epithelium expresses a variant of P2X(7) receptor in health and disease. *PLoS One* 2011, 6:e28541
16. Gordon MK, DeSantis A, Deshmukh M, Lacey CJ, Hahn RA, Beloni J, Anumolu SS, Schlager JJ, Gallo MA, Gerecke DR, Heindel ND, Svoboda KKH, Babin MC, Sinko PJ: Doxycycline hydrogels as a potential therapy for ocular vesicant injury. *J Ocul Pharmacol Ther* 2010, 26:407–419
17. Gipson IK, Spurr-Michaud S, Argüeso P, Tisdale A, Ng TF, Russo CL: Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci* 2003, 44:2496–2506
18. Weinger I, Klepeis VE, Trinkaus-Randall V: Tri-nucleotide receptors play a critical role in epithelial cell wound repair. *Purinergic Signal* 2005, 1:281–292
19. Klepeis VE, Weinger I, Kaczmarek E, Trinkaus-Randall V: P2Y receptors play a critical role in epithelial cell communication and migration. *J Cell Biochem* 2004, 93:1115–1133
20. Oswald DJ, Lee A, Trinidad M, Chi C, Ren R, Rich CB, Trinkaus-Randall V: Communication between corneal epithelial cells and trigeminal neurons is facilitated by purinergic (P2) and glutamatergic receptors. *PLoS One* 2012, 7:e44574
21. Murgia M, Hanau S, Pizzo P, Ripa M, Di Virgilio F: Oxidized ATP: an irreversible inhibitor of the macrophage purinergic P2Z receptor. *J Biol Chem* 1993, 268:8199–8203
22. Henríquez M, Herrera-Molina R, Valdivia A, Alvarez A, Kong M, Muñoz N, Eisner V, Jaimovich E, Schneider P, Quest AFG, Leyton L: ATP release due to Thy-1-integrin binding induces P2X7-mediated calcium entry required for focal adhesion formation. *J Cell Sci* 2011, 124:1581–1588
23. Calderwood DA, Ginsberg MH: Talin forges the links between integrins and actin. *Nat Cell Biol* 2003, 5:694–697
24. Wehrle-Haller B: Assembly and disassembly of cell matrix adhesions. *Curr Opin Cell Biol* 2012, 24:569–581
25. Bays JL, Peng X, Tolbert CE, Guilluy C, Angell AE, Pan Y, Superfine R, Burridge K, DeMali KA: Vinculin phosphorylation differentially regulates mechanotransduction at cell–cell and cell–matrix adhesions. *J Cell Biol* 2014, 205:251–263
26. Zieske JD, Bukusoglu G, Gipson IK: Enhancement of vinculin synthesis by migrating stratified squamous epithelium. *J Cell Biol* 1989, 109:571–576
27. Kobayashi T, Sokabe M: Sensing substrate rigidity by mechano-sensitive ion channels with stress fibers and focal adhesions. *Curr Opin Cell Biol* 2010, 22:669–676
28. Trinkaus-Randall V, Kewalramani R, Payne J, Cornell-Bell A: Calcium signaling induced by adhesion mediates protein tyrosine phosphorylation and is independent of pHi. *J Cell Physiol* 2000, 184:385–399
29. Mitra SK, Hanson DA, Schlaepfer DD: Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* 2005, 6:56–68
30. Jockusch BM, Bubeck P, Giehl K, Kroemker M, Moschner J, Rothkegel M, Rüdiger M, Schlüter K, Stanke G, Winkler J: The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* 1995, 11:379–416
31. Feng J-F, Gao X-F, Pu Y-Y, Burnstock G, Xiang Z, He C: P2X7 receptors and Fyn kinase mediate ATP-induced oligodendrocyte progenitor cell migration. *Purinergic Signal* 2015, 11:361–369
32. Mariotti A, Kedeshian PA, Dans M, Curatola AM, Gagnoux-Palacios L, Giancotti FG: EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J Cell Biol* 2001, 155:447–458
33. Stehbens SJ, Wittmann T: Analysis of focal adhesion turnover: a quantitative live-cell imaging example. *Methods Cell Biol* 2014, 123:335–346
34. Takai E, Tsukimoto M, Harada H, Sawada K, Moriyama Y, Kojima S: Autocrine regulation of TGF-β1-induced cell migration by exocytosis of ATP and activation of P2 receptors in human lung cancer cells. *J Cell Sci* 2012, 125:5051–5060
35. Takai E, Tsukimoto M, Harada H, Kojima S: Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells. *Purinergic Signal* 2014, 10:487–497
36. Seki S, Tsukimoto M, Suzuki A, Hattori F, Takai E, Ohshima Y, Kojima S: Anti-angiogenic effect of P2X7 receptor antagonist oxidized ATP as a mechanism of anti-tumor growth. *Pharmaceut Anal Acta* 2012, 3:190
37. Sanderson J, Dartt DA, Trinkaus-Randall V, Pintor J, Civan MM, Delamere NA, Fletcher EL, Salt TE, Grosche A, Mitchell CH: Purines in the eye: recent evidence for the physiological and pathological role of purines in the RPE, retinal neurons, astrocytes, Müller cells, lens, trabecular meshwork, cornea and lacrimal gland. *Exp Eye Res* 2014, 127:270–279
38. Lu W, Hu H, Sévigny J, Gabelt BT, Kaufman PL, Johnson EC, Morrison JC, Zode GS, Sheffield VC, Zhang X, Laties AM, Mitchell CH: Rat, mouse, and primate models of chronic glaucoma show sustained elevation of extracellular ATP and altered purinergic signaling in the posterior eye. *Invest Ophthalmol Vis Sci* 2015, 56:3075–3083