Tri-nucleotide receptors play a critical role in epithelial cell wound repair

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Abstract

The cornea plays a major role in the refraction of light to the retina. Therefore, the integrity and transparency of the corneal epithelium are critical to vision. Following injury, a combination of rapid signal transduction events and long-term cell migration are essential for wound closure. We have demonstrated previously that injury resulted in the release of nucleotides that induce the propagation of a Ca^{2+} wave to neighboring cells. This suggests that nucleotides and their receptors are critical components of wound healing. Epidermal growth factor (EGF) and integrins also have been shown to play a role in injury. In this study, we demonstrate that pretreatment of cells with ATP and UTP inhibited the immediate wound response, while BzATP, ADP, and UDP did not affect this response. Tri-nucleotide pretreatment also reduced the EGF induced Ca^{2+} response. Additionally, lower EC_{50} concentrations of ATP and UTP triggered migration of cells that was enhanced further with EGF and was inhibited by the tripeptide, RGD. Results indicate that the desensitization induced by ATP and UTP was specific. While ADP and UDP cause a homologous desensitization of their own signal, they did not cause an inhibition of the wound response nor does BzATP. Neither Ca^{2+} wave propagation nor cell migration occurred in response to β,γ-MeATP. Together these results lead us to hypothesize that corneal epithelial wound repair is mediated by both $P2Y_2$ and $P2Y_4$ receptors.

Abbreviations: BAPTA – 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; DAG – diacylglycerol; EGF – epidermal growth factor; EGFR – epidermal growth factor receptors; GPCR – G-protein coupled receptors; HB-EGF – pro-heparin binding EGF; HCE-T – human corneal epithelial cells transformed; IP_3 – phosphoinositol tri-phosphate; MMP – matrix metalloproteases; PBS – phosphate buffered saline; PIP_2 – phosphatidylinositol 4,5-bisphosphate; PKC – Protein kinase C; PLC – phospholipase C; RGD – arginine-glycine-aspartic acid; RGE – arginine-glycine-glutamic acid

Introduction

Wound healing is a series of complex biological responses requiring cytoskeletal and extracellular matrix remodeling, signal cascades, and gene regulation. Initial and long-term signals after injury are communicated to the cells and promote wound healing. These signals and events must be understood to advance our knowledge of normal wound repair. Investigators have demonstrated that nucleotides are released from cells by mechanical stimulation, ligand binding, exocytotic release, or injury [1–5] Nucleotide stimulation can also lead to an increase in intracellular calcium. The increase in cytosolic calcium can be generated via activation of purinergic (P2Y or P2X) receptors through distinct mechanisms. In addition, purinergic receptors can promote cell migration, a critical component of wound repair. The exact role and identity of the various receptors and their importance in different systems has yet to be elucidated.

Nucleotides have been shown to accelerate wound closure [6]. Nucleotides can act as a chemotactic stimulus for cell migration in many cell types including corneal epithelial [7], arterial smooth muscle [8], microvascular endothelial [9], and immature dendritic cells [10]. P2 receptors also have been shown to play a role in repair after ischemia and renal tubular epithelial wound healing [11, 12]. Previously, we demonstrated that ATP plays a critical role in intercellular communication following mechanical injury. ATP induced cell migration, and Reactive Blue-2, a P2 antagonist, inhibited wound closure [7]. In addition, nucleotides induced calcium waves and phosphorylation of paxillin (pY^{118}) and ERK1/2 in a transient manner [7, 13]. Furthermore, neither occurred in the presence of β,γ-MeATP [7, 13]. Our goal is to determine how epithelial cells respond to nucleotides in order to understand the events that occur after injury.

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P2Y and P2X receptors are expressed in a number of cell types. As G-protein coupled receptors (GPCR), P2Y receptors are seven pass transmembrane receptors coupled to heterotrimeric G-proteins (review in [14]). P2Y receptors couple to $G\alpha_q$ and activate phospholipase C β (PLCβ), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to diacylglycerol (DAG) and phosphoinositol tri-phosphate (IP$_3$). IP$_3$ binds to Ca^{2+} channels on the endoplasmic reticulum and causes an increase in intracellular Ca^{2+}. This family of receptors consists of eight members: P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$. P2X receptors are ligand gated ion channels that open upon agonist binding allowing extracellular calcium to enter the cell. This family of receptors has seven members: P2X$_{1-7}$. Each receptor has a specific agonist potency profile for different nucleotides with some showing preference for a specific nucleotide and others preferentially responding to tri-nucleotides or adenine based nucleotides [15]. We have shown that corneal epithelial cells express a number of P2YR subtypes [7], and an intracellular Ca^{2+} increase is seen in response to both nucleotides and injury [5].

Epidermal growth factor receptor (EGFR) also plays an important role in injury response. The EGFR family has four receptor family members, ErbB1-4. These receptors can form both homo- and hetero-dimers when stimulated by the epidermal growth factor (EGF) or other ligands [16]. The EGFR becomes activated upon injury, and inhibition of ErbB phosphorylation limits proper wound closure *in vitro* [7]. EGF itself promotes cell migration and wound healing *in vivo* [17] and can up-regulate integrin expression [18]. Gross mechanical injury induces a Ca^{2+} wave that propagates out from the wound site. EGF has been shown to increase the intensity of the Ca^{2+} wave but is not required for propagation [5]. Recently, growth factor receptors and GPCRs have been demonstrated to participate in cross talk connecting signaling pathways through transactivation, which can occur via enhancement or downregulation of downstream signal cascades [19]. In addition, specific P2 receptors can associate with other signaling proteins. For example, P2Y$_2$ has an arginine–glycine–aspartic acid (RGD) domain on its first extracellular loop that can associate with integrins [20]. Currently, no other P2Y receptor has an identified RGD domain that interacts with integrins. In addition, this RGD domain promotes signaling and interactions with epidermal growth factor receptors (EGFR) [21].

Our goal is to determine if specific P2 receptors play a role in the injury response and wound healing in the cornea. Improper wound repair can alter refraction of light, one of the major roles of the cornea. Immediately after injury, corneal epithelial cells display an intracellular Ca^{2+} wave that propagates from the site of injury to neighboring cells [5]. This wave does not propagate via gap junctions but is mediated by the release of extracellular nucleotides activating P2Y receptors that lead to activation of intracellular signaling pathways such as ERK1/2 [13]. We hypothesize that injury induces an immediate localized event that stimulates later events such as release of growth factors and long-term signals.

In this paper, we provide evidence that specific subtypes of purinergic receptors regulate the injury response. Pretreatment of cells with ATP and UTP inhibited the propagation of the injury induced Ca^{2+} wave while BzATP, ADP, and UDP did not participate in the desensitization. In addition, pre-stimulation with tri-nucleotides, but not BzATP or di-nucleotides, resulted in a decrease in the intracellular Ca^{2+} release induced by EGF. ATP and UTP preferentially enhanced later cellular events, including migration. Cellular migration was enhanced further when cells were co-stimulated with EGF. In addition, the ATP mediated migration was reduced by the tri-peptide, RGD. The results indicate that immediate and long-term components of the wound response are mediated by tri-nucleotide receptors.

Materials and methods

Reagents

The nucleotides [adenosine 5$'$-triphosphate (ATP), uridine 5$'$-triphosphate (UTP), adenosine 5$'$-diphosphate (ADP), uridine 5$'$-diphosphate (UDP), β,γ-methyleneadenosine 5$'$-triphosphate (β,γ-MeATP), and 2$'$-3$'$-O-(4-Benzoylbenzoyl)adenosine 5$'$-triphosphate (BzATP)], adenosine, apyrase, and Reactive Blue 2 were purchased from Sigma Chemical Company (St. Louis, MO). Human recombinant epidermal growth factor (EGF) and penicillin-streptomycin were purchased from Invitrogen Corporation (Carlsbad, CA). The fluorescent Ca^{2+} indicator dye Fluo-3AM and pluronic acid were from Molecular Probes, Inc. (Eugene, OR). 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic (BAPTA) was purchased from Calbiochem (La Jolla, CA). RGD and RGE peptides were purchased from American Peptide (Sunnyvale, CA).

Cell culture

Primary rabbit corneal epithelial cells and an established human corneal cell line were used. Human epithelial cells transformed (HCE-Ts) were established by Araki-Sasaki et al. [22], using an SV-40 recombinant adenovirus vector. Primary corneal epithelial cells were isolated and cultured from freshly extracted whole rabbits eyes (Pel-Freeze Biologicals (Rogers, AR) [7].

Ca^{2+} imaging

HCE-T cells and primary cells were grown to confluency on 22 mm^2 coverslips and quiesced 18 to 24 h before experiments in unsupplemented serum-free keratinocyte medium (K-SFM). Cells were washed in a HEPES-buffered saline solution containing 137 mM NaCl, 5 mM KCl, 4 mM MgCl$_2$, 3 mM CaCl$_2$·2H$_2$O, 25 mM glucose and 10 mM HEPES [23]. Cells were incubated 20–30 min in 5 μM Fluo-3AM resuspended in HEPES buffer supplemented with 0.02% pluronic acid in DMSO [5, 7, 13]. Coverslips were mounted in a flow through perfusion

apparatus connected to inlet tubes with varying solutions and an outlet pump (Warner Instruments). The mounted coverslip was placed on the stage of a Zeiss Axiovert LSM 510 laser scanning confocal microscope, and cells were scanned with an Argon laser every 786 ms [7]. Cells were perfused with HEPES buffered saline at a rate that did not elicit a response, and baseline fluorescence was obtained. Cells were stimulated either by perfusing nucleotides or EGF prepared in HEPES buffered saline and/or by manually perturbing cells [5].

Ca^{2+} data analysis

Ca^{2+} data was recorded by LSM software as average fluorescence intensity (F) for a region of interest over time. The region was either a 460 μm × 460 μm field or single cells, over time. Raw data was entered into KaleidaGraph. Percent change in average fluorescence was calculated using the following formula

$$Percent\ change\ in\ average\ fluorescence = \frac{(F - F_0)}{F_0} \times 100$$

where F_0 is initial average fluorescence for the region of interest [5, 7]. Data was either graphed as average maximal fluorescence of at least three different experiments or as percent change in average fluorescence for time course. For the injury experiments, the area cleared by the wound was excluded from calculations.

Migration assay

HCE-T cells were quiesced 18 to 24 h in unsupplemented K-SFM. Cells were washed in phosphate buffered saline (PBS), trypsinized, and the reaction was stopped with filtered soybean trypsin inhibitor as described previously [7]. Cells were resuspended at a concentration of 125,000 cells/100 μl in binding buffer (unsupplemented K-SFM, 0.05% gelatin and 25 mM HEPES). Binding buffer, 600 μl, with or without added stimuli was placed in the wells of a 24 well plate. Costar Transwell inserts (6.5-mm diameter polycarbonate membranes, 8 μm pore size) were placed into the wells, and 100 μl of the cell suspension was added to the inserts. Experiments were conducted for 8 h at 37 °C.

To collect conditioned wound medium, cells were washed and incubated in binding buffer. Cells were scrape wounded as described previously [13]. The wound medium was collected immediately and used in migration assays. Unwounded medium was defined as binding buffer added to cells and collected without injury to cells. The conditioned wound medium was also treated with apyrase prior to the migration assays.

To determine migration, upper and lower chambers were aspirated and rinsed with PBS. Cells were fixed at room temperature for 10 min, rinsed with PBS, and non-migrated cells were removed. Migrated cells were stained with propidium iodide (5 μg/ml) (Molecular Probes, Inc., Eugene, OR) for 5 min and rinsed with PBS. Membranes were mounted onto a slide with SlowFade Antifade

(Molecular Probes, Inc., Eugene, OR). For each membrane (33.2 mm²), six random 10× fields (one field = 1.37 mm × 1.08 mm, or 1.48 mm²) were photographed. Cells were counted and averaged for each membrane and experiments were performed in triplicate.

Statistical analysis

Values were given as the mean ± SD. Statistical comparisons were made using Student's *t*-test with a stringency of $P < 0.01$ for significance. Data were fit using KaleidaGraph to calculate concentration for half the maximal response (EC_{50}) and maximal possible change in fluorescence for a given agonist (ΔF_{max}). A generalized single-site binding model was used as follows: $\Delta F = \frac{\Delta F_{max} \times [L]}{EC_{50} + [L]}$, where ΔF is the percent change in fluorescence and $[L]$ is the concentration of the agonist.

Results

Nucleotide dose response curves

Ca^{2+} dose response curves were determined for ATP, UTP, ADP, UDP, and BzATP over a concentration range of 10^{-7} to 10^{-3} M (Figure 1). The response to ATP and UTP was greater than that of ADP and UDP over the entire range. The BzATP response was similar to ADP and UDP at concentrations $\leq 10^{-4}$ but lower at higher concentrations. In addition, adenosine, an agonist for P1 receptors, and β, γ-MeATP, an agonist for P2X receptors, were evaluated. Neither of these agonists induced a change in intracellular Ca^{2+} (data not shown). The EC_{50} for ATP and UTP were found to be $(3.4 \pm 1.8) \times 10^{-6}$ M and $(3.4 \pm 2.0) \times 10^{-6}$ M respectively. The EC_{50} for ADP and UDP were higher than the tri-nucleotides, $(1.8 \pm 0.9) \times 10^{-4}$ M and $(1.83 \pm 0.12) \times 10^{-4}$ M. respectively. The EC_{50} for BzATP was $(8.6 \pm 5.9) \times 10^{-5}$. *R* values for line fit were 0.96 for ATP, UTP, and UDP, 0.93 for ADP, and 0.91 for BzATP.
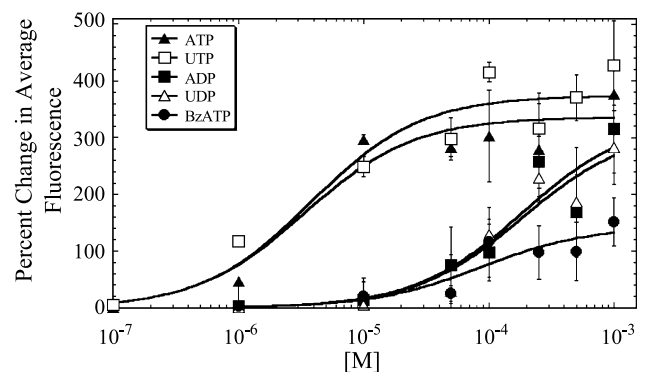

Figure 1. Nucleotide induced Ca^{2+} dose response curves. HCE-T cells were incubated in 5 μM Fluo-3AM for 30 min and imaged every 786 ms using a flow through apparatus on an LSM 510 confocal. Cells were stimulated with the indicated concentration of nucleotide for 60 s. Maximal percent change in average fluorescence of a 460 μm × 460 μm field was determined. Data was fit to a generalized single-site binding model. Graph represents a minimum of three experiments at each concentration tested.

To verify that P2 receptors caused the intracellular Ca^{2+} increase, cells were pre-incubated for 30 min in 100 μM Reactive Blue 2 prior to experimentation. Cells were placed in a flow through system and washed for at least 50 s in HEPES buffered saline. Cells were stimulated with 100 μM of the indicated nucleotide for 100 s and washed again with HEPES buffer. Non-pretreated cells were used as control. Reactive Blue 2 lowered the Ca^{2+} response to nucleotides. The ATP and UTP responses were reduced by one-fifth, and the ADP and UDP responses were reduced by half.

Role of nucleotides in injury response

We have demonstrated that epithelial cells respond to nucleotides in a saturable manner. However, these results did not identify which P2 receptors were involved in the injury response. To evaluate the wound response, cells
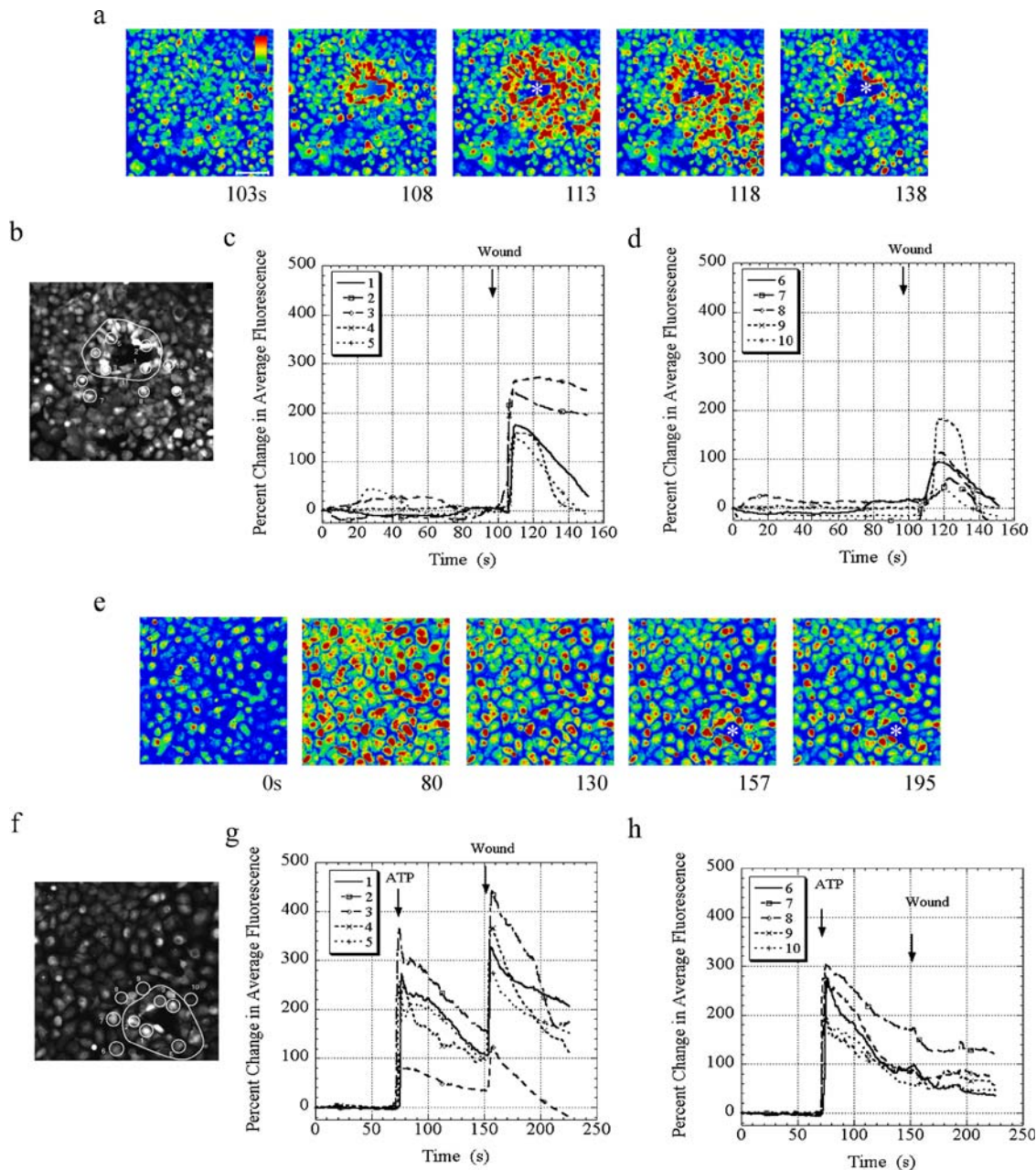

Figure 2. Pre-stimulation with ATP inhibits injury induced Ca^{2+} wave. Primary corneal epithelial cells were incubated in 5 μM Fluo-3AM for 30 min and imaged in a flow through apparatus on an LSM 510 confocal. Cells were washed in HEPES buffered saline with Ca^{2+} for at least 30 s, and stimulated by wounding or with ATP. Individual cells were analyzed for percent change in average fluorescence. Intensity scale is shown in (a) with *red* indicating highest Ca^{2+} levels and *blue* indicating lowest Ca^{2+} levels. The *horizontal white bar* in (a) represents 100 μm. a) Cells were washed in HEPES buffer containing Ca^{2+} and wounded. A series of images taken from a time course of a representative experiment of a wound (shown at *asterisk*) is presented. b) A single image taken after wounding is shown. Cells (#1–5) immediately adjacent to the wound (c) and cells away (#6–10) from the wound (d) were analyzed. e) Cells were washed in HEPES buffer containing Ca^{2+}, stimulated with 100 μM ATP (80 s), and wounded (157 s). A series of images taken from a time course of a representative experiment is presented (wound shown at *asterisk*). f) A single image taken after wounding is shown. Cells (#1–5) immediately adjacent to the wound (g) and cells away (#6–10) from the wound (h) were analyzed. Images are representative of at least 10 independent experiments.

were placed in a flow through apparatus. Background images were collected in HEPES buffered saline, and the cells perturbed manually (Figure 2a). Percent change in average fluorescence of individual cells adjacent to the wound (Figures 2b, c (#1–5)) are shown inside the large white circle and graphed (Figure 2c). These depict a rapid increase in fluorescence after injury that is followed by attenuation. The rate of attenuation of injured cells was variable. While these cells did respond they were not a part of the calcium wave that propagated from the site of injury [5]. We also examined cells not immediately adjacent to the wound, cells involved in the wave (Figures 2b, d). When individual cells that were not adjacent to the wound were evaluated [(Figures 2b, d (#6–10) (outside of *white circle*)], the percent response was lower on average and the response time was delayed (Figure 2d). The delay in response time could be accounted for by the time it took the Ca^{2+} wave to travel the distance to nonadjacent cells.

To evaluate what role ATP played in the initial injury response, cells were stimulated by ATP and wounded. To perform the experiment, cells were placed in the flow through apparatus, equilibrated with HEPES buffered saline, and stimulated with HEPES buffered saline con-

taining 100 μM ATP. The response to ATP was allowed to attenuate (approx. 80 s), and then the cells were wounded (Figure 2e). The cells were analyzed for average change in percent fluorescence for individual cells adjacent to the wound (Figures 2f, g) and those involved in wave propagation (Figures 2f, h). As predicted from Figure 2a and d, cells adjacent to the wound responded (Figures 2f, g). The Ca^{2+} wave was absent when it followed pretreatment with ATP (Figures 2f, h). In its place was a minor inflection at the time of injury that resembled the wound response induced in the absence of intracellular Ca^{2+} [5] (see Figure 5). These responses indicate that the injury response was inhibited by the desensitization of specific P2Rs by ATP.

To determine which nucleotides could desensitize the Ca^{2+} release in response to wounding, cells were pretreated with nucleotides and injured (Figures 3 and 4). Both ATP and UTP pre-stimulation inhibited the wound-induced wave (Figure 3), while ADP, UDP, and BzATP did not inhibit the injury induced Ca^{2+} wave (Figure 4). Together, these results indicate that both ATP and UTP desensitize receptors and inhibit the injury response at an equivalent molar concentration.
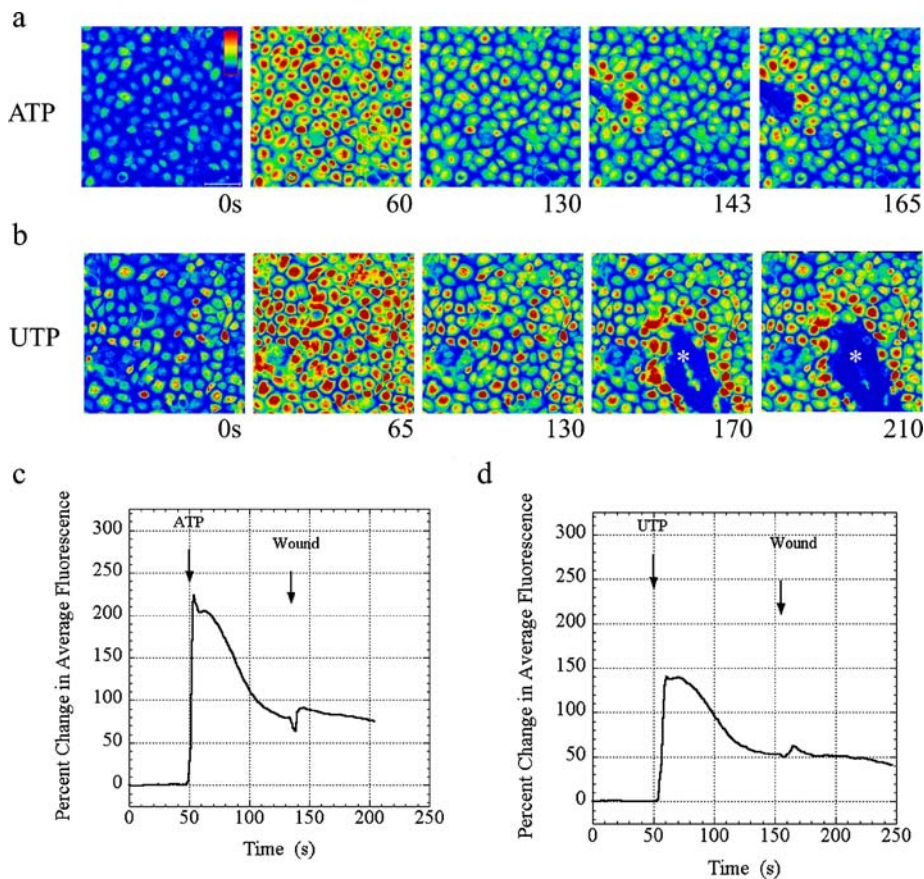

Figure 3. Tri-nucleotides attenuate the injury induced Ca^{2+} wave. Primary corneal epithelial cells were incubated in 5 μM Fluo-3AM for 30 min and imaged in a flow through apparatus on an LSM 510 confocal. Cells were washed in HEPES buffered saline with Ca^{2+} for at least 30 s and stimulated with the indicated nucleotide and wounded. Intensity scale is shown in (a) with *red* indicating highest Ca^{2+} levels and *blue* indicating lowest Ca^{2+} levels. The *horizontal white bar* in (a) represents 100 μm. a, b) Cells were washed in HEPES buffer with Ca^{2+}, stimulated with 100 μM of the indicated nucleotide, and wounded. A series of images taken from a time course of a representative experiment is presented (wound shown at *asterisk*). c, d) Percent change in average fluorescence for the whole field (460 μm × 460 μm) was calculated and graphed over the time course for each experiment. Images are representative of at least 10 independent experiments. Series of images are taken from Movie 1 (see online version of article at www.springeronline.com).
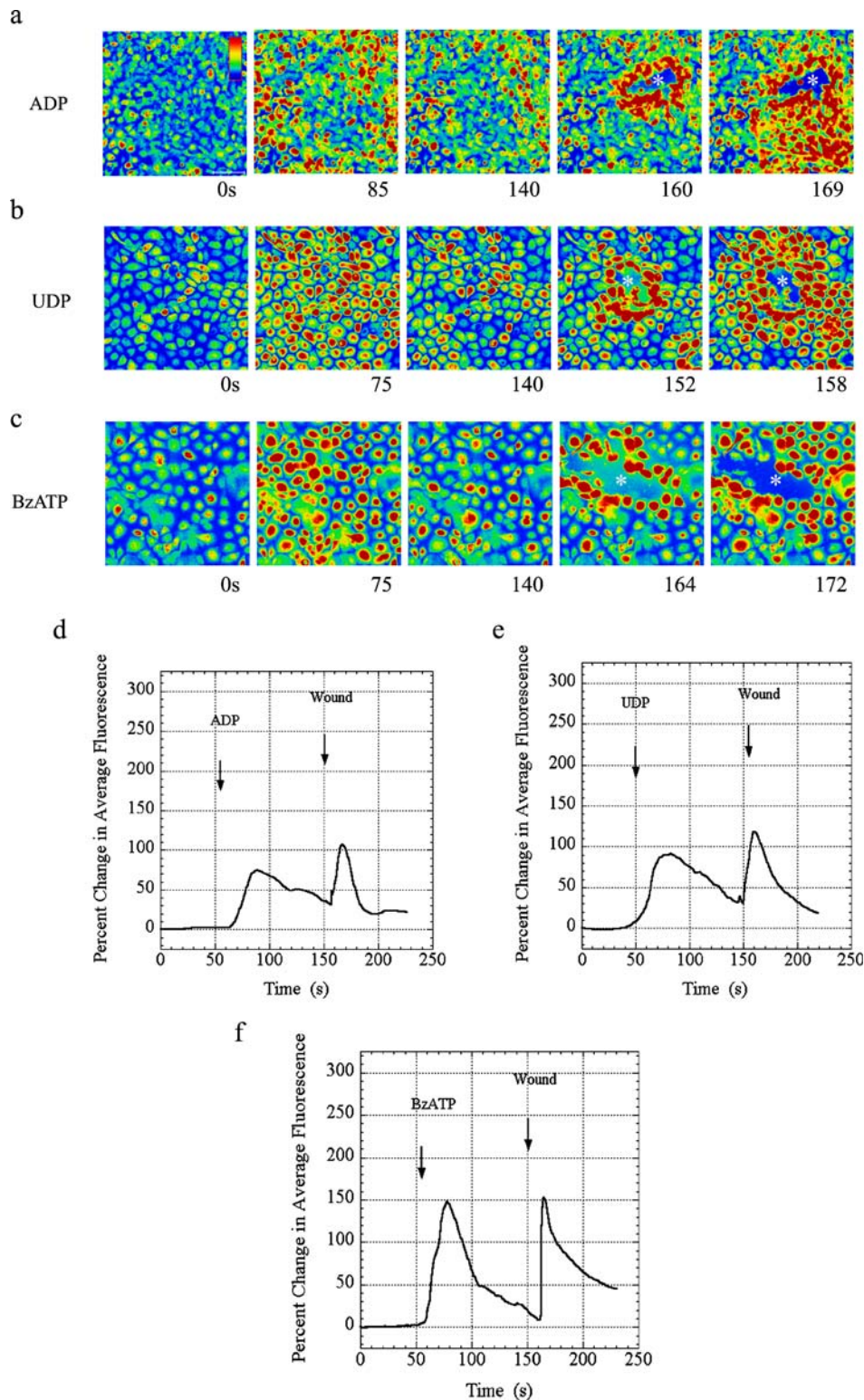
Figure 4.   Di-nucleotides and BzATP do not attenuate the injury induced Ca^{2+} wave. Primary corneal epithelial cells were incubated in 5 μM Fluo-3AM for 30 min and imaged in a flow through apparatus on an LSM 510 confocal. Cells were washed in HEPES buffered saline with Ca^{2+} for at least 30 s and stimulated with the indicated nucleotide and wounded. Intensity scale is shown in (a) with *red* indicating highest Ca^{2+} levels and blue indicating lowest Ca^{2+} levels. The *horizontal white bar* in (a) represents 100 μm. (a–c) Cells were washed in HEPES buffer with Ca^{2+}, stimulated with 100 μM of the indicated nucleotide, and wounded. A series of images taken from a time course of a representative experiment is presented (wound shown at *asterisk*). (d–f) Percent change in average fluorescence for the whole field (460 μm × 460 μm) was calculated and graphed over the time course for each experiment. Images are representative of at least 10 independent experiments. Series of images are taken from Movie 1 (see online version of article at www.springeronline.com).

To evaluate the inflection detected in Figures 2e–h, cells were pretreated with BAPTA (100 μM) for 30 min and individual cells were analyzed (Figures 5a–d). Cells responded to the injury in a manner similar to the cultures that had been pretreated with ATP (Figures 3 and 4). These results indicate that the cellular response adjacent to the wound did not occur via P2Y receptor signaling since the Ca^{2+} increase came from extracellular stores
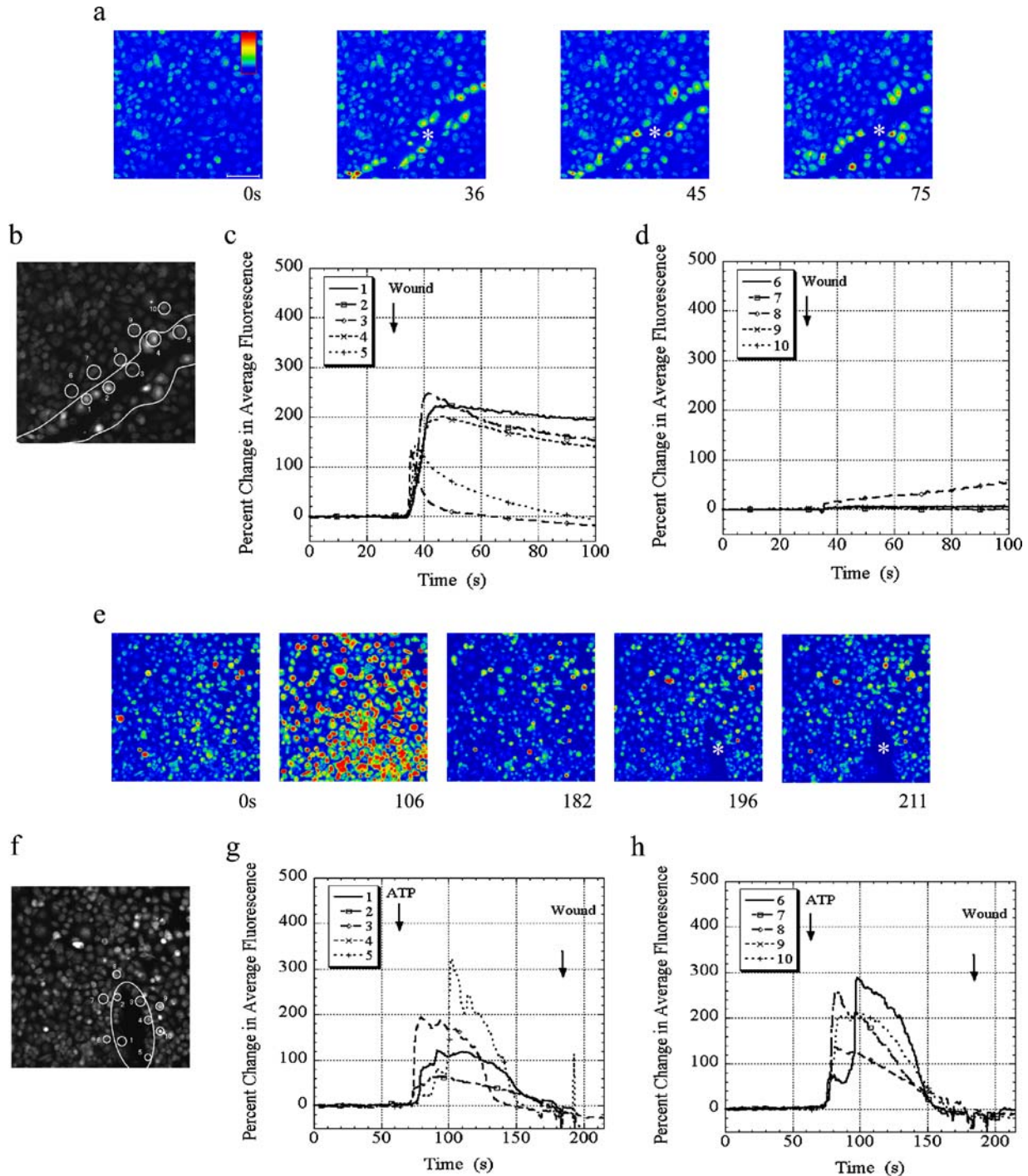

Figure 5. Ca^{2+} free media inhibits an injury response after ATP stimulation. Primary corneal epithelial cells were incubated in 5 μM Fluo-3AM for 30 min and imaged in a flow through apparatus on an LSM 510 confocal. Cells were washed in HEPES buffered saline for at least 30 s, and stimulated by wounding or ATP. Individual cells were analyzed for percent change in average fluorescence. Intensity scale is shown in (a) with *red* indicating highest Ca^{2+} levels and blue indicating lowest Ca^{2+} levels. The *horizontal white bar* in (a) represents 100 μm. a) Cells were pre incubated in BAPTA (100 μM) washed in HEPES buffer containing Ca^{2+} and wounded. A series of images taken from a time course of a wound (shown at *asterisk*) of a representative experiment is presented. b) A single image taken after wounding is shown. Cells (#1–5) immediately adjacent to the wound (c) and cells away (#6–10) from the wound (d) were analyzed. e) Cells were washed in Ca^{2+} free HEPES buffer containing EGTA, stimulated with 100 μM ATP, and wounded. A series of images taken from a representative time course is presented (wound shown at *asterisk*). f) A single image taken after wounding is shown. Cells (#1–5) immediately adjacent to the wound (g) and cells away (#6–10) from the wound (h) were analyzed. Images are representative of at least 10 independent experiments. The series of images are taken from Movie 2 (see online version of article at www.springeronline.com).

(Figures 5a–d). This agrees with previous results using thapsigargin [5]. To evaluate the requirement for extracellular Ca^{2+}, cultures were placed in the flow through apparatus in Ca^{2+} free HEPES buffer containing EGTA. These cells were stimulated with 100 μM ATP made in Ca^{2+} free HEPES buffered saline. Once the response had attenuated (approx. 100 s), the cells were wounded (Figure 5e), and individual cells were analyzed for average change in percent fluorescence in cells adjacent to the wound margin (Figures 5f, g) and those involved in wave propagation (Figures 5f, h; as in Figure 2). When the experiment was performed in Ca^{2+} free media, the inflection detected previously in the injury response was absent, completely eliminating any Ca^{2+} response to injury (Figures 5e–h).

Nucleotides affect EGF induced Ca^{2+} response

Cross-talk between the EGF and ATP signaling pathways occurs in many cell systems. To determine if this was a property unique to ATP, the response to EGF was evaluated after pre-stimulation with other nucleotides. HCE-Ts were perfused with HEPES buffered saline to establish baseline fluorescence, and the cells were stimulated with 100 μM ATP, UTP, ADP, UDP or BzATP for 100 s. Cells were washed with HEPES for 100 s and stimulated with 8 nM EGF for 100 s. Maximal percent change in fluorescence of the entire 460 μm × 460 μm field was calculated (Figure 6a). The results were compared with cells washed with HEPES buffer and stimulated with 8 nm EGF alone for 100 s (− pretreatment). Cells exhibited an attenuated Ca^{2+} response upon EGF stimulation when they were pretreated with either ATP or UTP. In contrast, when the cells were pre-stimulated with BzATP, ADP, or UDP, there was no detectable desensitization (Figure 6a). The reverse experiment was performed where cells were pre-stimulated with EGF and then by specific nucleotides (data not shown). Pretreatment with EGF did not desensitize the cells indicating that EGFR stimulation with EGF did not inhibit the response to nucleotides.

As desensitization occurred after cells were pre-stimulated with ATP or UTP and wounded or stimulated with EGF, our goal was to determine if desensitization of the EGF receptor occurred in our wound model. Cells were placed in the flow through apparatus, perfused with HEPES buffered saline to establish baseline fluorescence, and injured. Once fluorescence returned to the base line, the cells were stimulated with EGF. The analysis was performed on individual cells that responded to both the injury and EGF (Figure 6b). Results were compared to individual cells stimulated with EGF alone. The Ca^{2+} response to EGF was significantly lowered after injury. The high standard deviation was due to the variable size of the wound.

Nucleotides and EGF induce cell migration

The long-term effects of P2 receptors on wound healing were examined using Transwell migration assays. Previ-
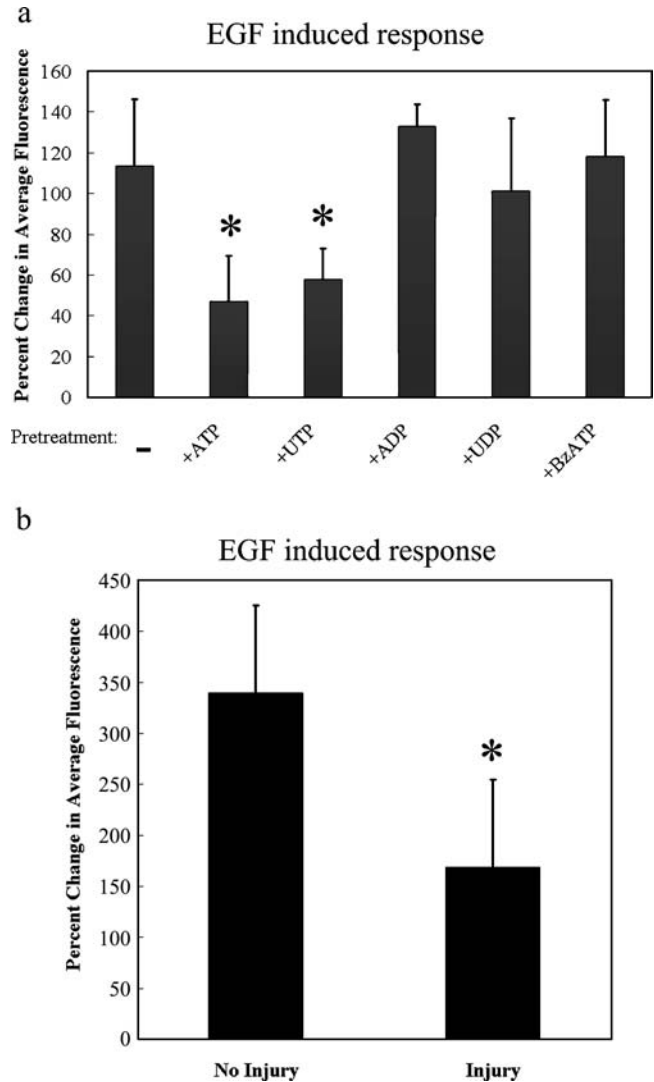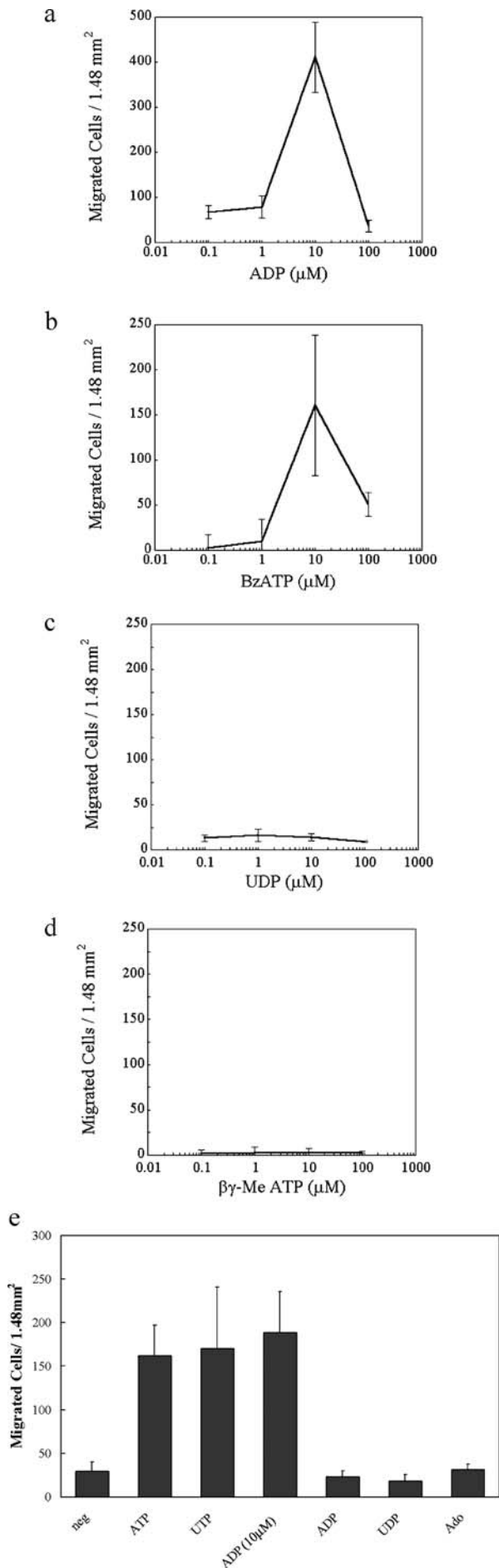

Figure 6. Injury and tri-nucleotides decrease subsequent EGF induced Ca^{2+} response. HCE-T cells were incubated in 5 μM Fluo-3AM for 30 min and imaged every 786 ms in a flow through apparatus on an LSM 510 confocal. a) Cells were stimulated with 100 μM of the indicated nucleotide for 100 s, washed in HEPES buffered saline for 100 s, and then stimulated with 8 nM EGF or stimulated with EGF without pretreatment. Maximal percent change in average fluorescence of a 460 μm × 460 μm field was determined. b) Cells were either wounded and stimulated with 8 nM EGF or stimulated with EGF without injury. Individual cells were selected for analysis. In the wound model, cells that responded to the wound were selected. (*t*-test, * $P < 0.0005$) Data represent a minimum of three independent experiments.

ously we showed that P2 receptors were necessary for wound repair in directed migration scratch assays [7]. Furthermore, we demonstrated that migration was optimal at 1 μM for ATP and 0.16 nM for EGF in Transwell assays [7]. In these experiments, migration to other nucleotides and adenosine were evaluated over a range of concentrations from 0.1 to 100 μM (Figure 7). Optimal migration for ADP and BzATP occurred at 10 μM (Figures 7a, b), while optimal migration for ATP and UTP occurred at 1 μM ([7] and data not shown). UDP and β,γ-MeATP did not induce migration at any concentration (Figures 7c, d). To assess preferential migration to any one particular nucle-

otide or adenosine, the nucleotides and adenosine were evaluated and compared. The cellular response was tested at 1 μM, the optimal concentration for ATP and UTP. In addition, ADP was tested at 10 μM, its optimal concentration. ATP, UTP, and ADP induced comparable migration at their optimal concentrations (162 ± 39, 170 ± 70, and 188 ± 47 cells/1.48 mm², respectively). However, ADP at 1 μM, UDP, adenosine, or binding buffer did not induce cell migration (23 ± 7, 18 ± 7, 31 ± 6 and 29 ± 11 cells/1.48 mm², respectively) (Figure 7e).

We evaluated the role of factors released into the wound media on cell motility and compared these effects to stimulation with nucleotides and EGF. Wound media was collected immediately after injury and added to migration assay chambers. Luciferase assays performed on wound media demonstrated presence of ATP [5]. Cell migration was 3.7-fold greater when cells were stimulated with wound media compared to wound media treated with apyrase or unwounded control media (Figure 8a). Previously we established that epithelial cells migrate to ATP or EGF in a dose dependent manner and optimal migration was determined to be 1 μM and 0.16 nM respectively [24]. To evaluate the combined effect of ATP and EGF, cells were co-stimulated with the optimal concentrations of each and compared. In a representative experiment an average of 369 (±34) cells migrated/1.48 mm² in response to 0.16 nM EGF while an average of 314 (±31) cells migrated/1.48 mm² in response to 1 μM ATP (Figure 8b). When cells were co-stimulated with 1 μM ATP and 0.16 nM EGF, migration increased more than threefold with an average of 1043 (±66) cells/1.48 mm² (Figure 8b). Co-stimulation experiments were performed with UTP, ADP and UDP at their respective optimal concentrations (Figures 7 and 8c). Cells exposed to ADP or UDP (1 μM) and co-stimulated with EGF (527 ± 61 and 637 ± 51 cells/1.48 mm², respectively) showed migration similar to that of EGF alone (457 ± 115 cells/1.48 mm²). When cells were co-stimulated with UTP and EGF, migration was enhanced in an additive manner (801 ± 133 cells/1.48 mm²). In addition when cells were exposed to 10 μM ADP and EGF, migration was greater than either alone (753 ± 173 cells/ 1.48 mm²).

To understand how nucleotides regulate cell migration, we asked whether integrins mediated the response. Integrins are known to play an important role in cell migration, and the integrin binding tri-peptide, RGD, that is found in the first extracellular loop of the P2Y$_2$ receptor, has been shown to interact with integrins [20]. In addition, expression of α$_6$β$_4$ in corneal epithelial cells is up-regulated by

Figure 7. Cell migration is nucleotide specific. Transwell migrations were performed for 8 h at 37 °C with the indicated nucleotide. HCE-T cells were stained with propidium iodide, counted in six randomly chosen fields (1.46 mm²), and averaged. Dose response curves for a) ADP, b) BzATP, c) UDP, and d) β,γ-MeATP are shown. Experiments were performed at least three times and representative curves were chosen. e) Cells were stimulated with binding buffer (neg), 1 μM ATP, UTP, ADP, UDP, or Adenosine (Ado), or 10 μM ADP. Experiments were performed in triplicate.

EGFR activation [18]. To evaluate the role of integrin receptors in nucleotide mediated migration, RGD peptide was added to the cell suspension prior to its addition to the Transwell chamber. RGE peptide was used to control for non-specific binding. Cells were migrated to binding buffer
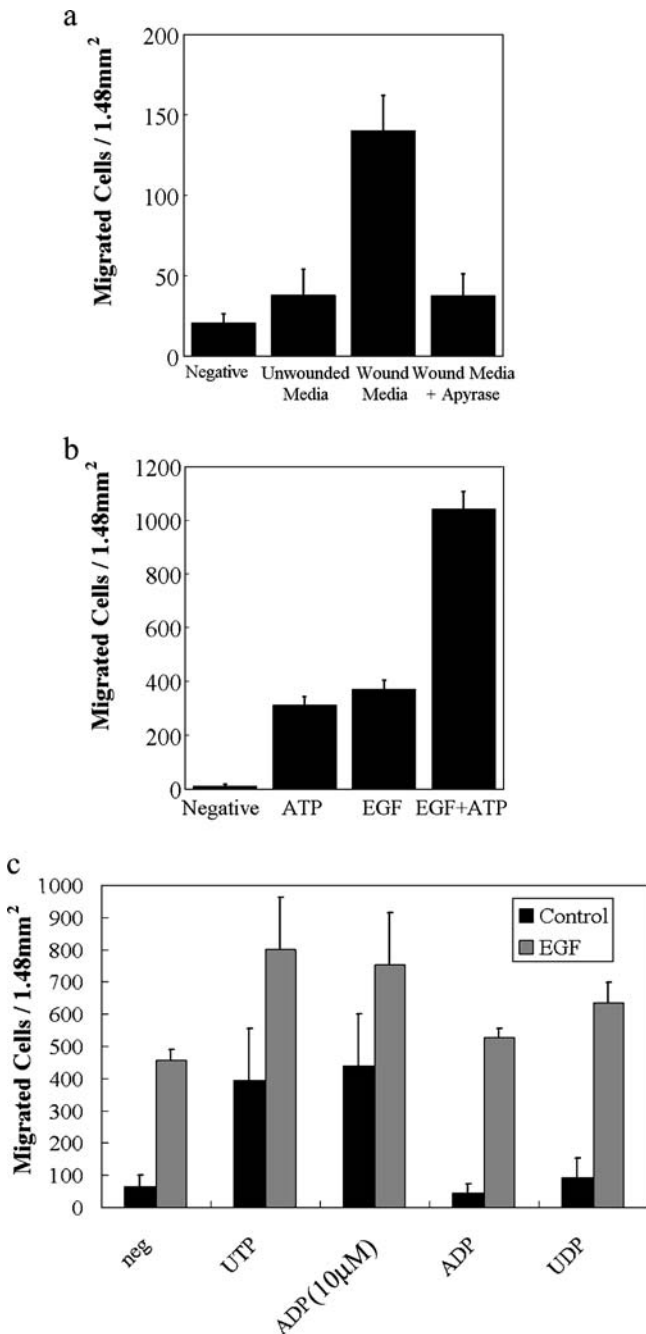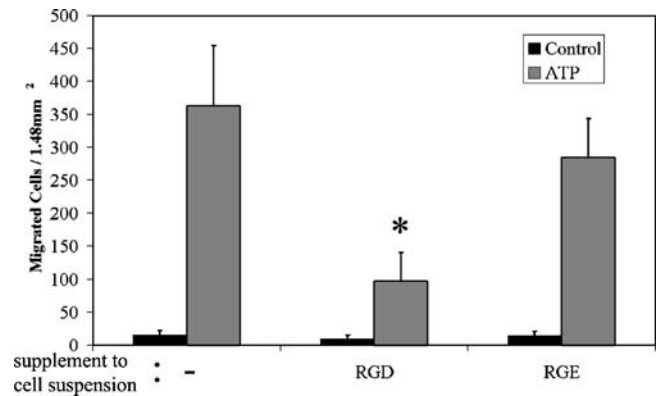

Figure 9. ATP and integrins mediate cell migration. Transwell migrations were performed for 8 h at 37 °C with the indicated nucleotides. HCE-T cells were stained with propidium iodide, counted in six randomly chosen fields (1.46 mm^2) and averaged. RGD or RGE peptide, 1 μM, was added to the cell suspension. Cells were stimulated with binding buffer (control) or 1 μM ATP. (*t*-test, *$P < 0.0005$) Experiments were performed in triplicate.

or 1 μM ATP. When either the RGD or RGE peptide was added to the cells migrating to binding buffer, there was no detectable difference over control (Figure 9). Cells stimulated with ATP migrated 362 (±92) per field. Interestingly, cell migration was reduced almost fourfold when RGD was present (96 ± 43 cells/1.48 mm^2) (Figure 9). The control peptide did not decrease migration significantly from control (284 ± 59). Since RGD peptide can inhibit migration, it is likely integrins play a role in ATP induced cell migration. The nucleotide receptors, EGFR, and integrins mediate calcium signaling and cell migration. Thus, interaction between these protein families may be critical in regulating the cellular signals after injury.

Discussion

The results of these studies demonstrate that P2 receptors play a role in the initial cellular responses that occur after injury and the long-term signals that are necessary for wound closure in corneal epithelial cells. We have demonstrated that nucleotides are the active component released with injury and are responsible for the intercellular Ca^{2+} wave that is propagated in neighboring cells [5, 13]. We have shown that ATP, UTP, ADP, UDP and BzATP promote an intracellular Ca^{2+} increase, β,γMe-ATP does not. Our goal was to demonstrate that nucleotides play a prominent role in the cellular response to injury, in migration, and in the cross-talk with EGF.

Recently involvement of P2 receptors in wound healing has begun to be demonstrated [2]. Desensitization experiments demonstrate the role of nucleotides in the injury response. We have shown pretreatment of cells with ATP and UTP inhibits the response to a subsequent wound (Figure 3). Several explanations are plausible: 1) Specific receptors desensitize via receptor internalization or signal transduction down regulation [25] or 2) When cells are exposed to a solution of a tri-nucleotide, ATP and/or UTP

Figure 8. Wound media, nucleotides, and EGF mediate cell migration. Transwell migrations were performed for 8 h at 37 °C with the indicated nucleotides and/or EGF. HCE-T cells were stained with propidium iodide, counted in six randomly chosen fields (1.46 mm^2) and averaged. a) Migration of cells stimulated with wound media were compared to those stimulated with unwounded control media, wound media treated with apyrase and binding buffer (negative). b) A representative experiment is shown where cells were stimulated with optimal concentration of ATP (1 μM) and EGF (0.16 nM) and compared to co-stimulation with ATP and EGF and negative binding buffer. c) Cells were simulated with binding buffer (neg), 1 μM UTP, ADP, or UDP, or 10 μM ADP +/− 0.16 nm EGF.

receptors have bound agonist thus saturating the receptors. We predict that the amount of nucleotide used to desensitize the wound approximates the concentration needed for the maximal Ca^{2+} response (Figure 1). In either case, the result is that nucleotides that are released would not be able to induce any further effect upon the receptors. In addition, injury, ATP and UTP, induce a decrease in the EGF induced Ca^{2+} response (Figure 6). Since nucleotides are released with injury [13], we hypothesize that desensitization of the EGF receptor occurs via nucleotides. In contrast, while ADP and UDP cause homologous desensitization of their own Ca^{2+} signal [7], ADP, UDP, and BzATP do not affect either the EGF or wound response even when the receptors are saturated (Figures 4 and 6). Therefore, we hypothesize that $P2Y_1$, $P2Y_6$, and $P2X_7$ receptors do not play a role in the Ca^{2+} wave that occurs after injury.

We have demonstrated that the source of the calcium for the injury induced wave is intracellular ([5] and Figure 5). Pretreatment with either thapsigargin or BAPTA inhibits the Ca^{2+} wave but not oscillations of cells at the wound margin. In addition, pretreatment with either ATP or UTP inhibits only the wave but not the Ca^{2+} increase in the cells adjacent to the wound. Cells adjacent to the wound edge utilize extracellular calcium (Figure 5), but the method of extracellular calcium entry is unknown. One possibility is that the plasma membranes of the injured cells are damaged, and Ca^{2+} freely enters. The response could also be mediated via stress receptors or P2X receptors, both of which are ion channels that permit entry of extracellular calcium [26]. While these cells are not involved in the propagation of the wave, we cannot rule out the possibility that they play a role in wound healing, and that is an area of current exploration.

Activation of intracellular signaling pathways by P2 receptors is emerging as intricate patterns of cell specific processes. Involvement of $\alpha_V\beta_3$ integrins has been shown to be essential for both ATP induced intracellular Ca^{2+} increase and ERK1/2 activation in 1321N1 $P2Y_2$ transfected cells [20]. We have shown in our system that integrins play a role in the response to nucleotides (Figure 9). As integrins are known to play a role in cell migration and wound healing, their coordinated role in nucleotide signaling is of great interest. Purinergic signaling is also known to activate downstream signaling events using a number of receptors. We have shown that ATP, UTP, ADP, and UDP promote activation of ERK1/2, but α,β-MeATP and β,γ-MeATP do not [13]. $P2X_2$ and $P2Y_1$ receptors have been shown to play a role in activating ERK1/2 in stretch induced injury of astrocytes in Ca^{2+} dependent manner [27]. In contrast in embryonic kidney cells, $P2X_7$ activates ERK1/2 in a Ca^{2+} independent manner [28]. Additionally PKC and PKD both have been shown to play role in $P2X_7$ induced ERK1/2 activation [29]. In pro-monocytic cells, activation of ERK1/2 by $P2Y_2$ has been shown to involve c-Src but not PKC [30].

Recently, the P2YRs have been hypothesized to play a role in cross-talk with the EGFR [7, 13, 21, 31, 32]. There are a number of potential pathways, several of which may occur. One current hypothesis is cross-talk occurs via the triple membrane pass system where GPCR activated matrix metalloproteases (MMPs) cleave pro-heparin binding (HB)-EGF from the membrane and HB-EGF can then activate the EGFR [33, 34]. Another potential method for cross-talk is via Src. While Src is known to play a role in the phosphorylation of EGFR, its involvement in corneal epithelial injury has not been determined. GPCR stimulation has been found to lead to phosphorylation of the EGFR in a Src and/or Pyk2 dependent manner in many cell types [21, 31, 35, 36]. Future experiments will be performed to test the hypothesis that Src and Pyk2 play an active role in nucleotide-mediated activation of the EGFR in corneal epithelial cells. Inhibition of the EGFR activation with tyrphostin has been shown to inhibit ATP induced cell migration in epithelial cells [7]. These indicate that cross talk between GPCR and the EGFR occur over the entire time course of wound response and repair.

ATP and UTP are the major players in Ca^{2+} waves after injury and the cross talk with the EGFR (Figures 3 and 6). However, this does not preclude that other nucleotides may mediate other facets of the wound response. While ADP and BzATP do not play a role in the injury induced Ca^{2+} wave, they can stimulate migration, albeit at a higher concentration than ATP or UTP (Figure 7). Though UDP does not stimulate migration, both ADP and UDP have the ability to activate downstream signals. We have shown that ERK1/2 and paxillin become phosphorylated upon stimulation with both tri- and di-nucleotides [7, 13]. Additionally, BzATP has been shown to activate downstream signals such as ERK1/2 in other cell systems [28, 29]. The signals propagated by di-nucleotides may be artifacts of saturating the system with larger than physiological concentrations [37]. Another possibility is that the signal is real and that after injury the receptors are available at a density that is unable to elicit a detectable downstream cascade. This may explain why ADP can induce migration at 10 μM. However, this does not take into account that high concentrations of ADP are still not able to inhibit the injury induced Ca^{2+} wave (data not shown). Proper signaling relies on optimal concentrations of nucleotides. At high concentrations where maximal Ca^{2+} release upon nucleotide exposure occurs (>100 μM), epithelial cells do not migrate toward the stimuli. The desensitization of the calcium response that occurs at 100 μM may explain why cell migration is minimal. In contrast, at lower concentrations where the maximal migration occurs, little desensitization occurs. This bell shape curve is not atypical and has been demonstrated to occur with nucleotide induced migration in epithelial and dendritic cells [7, 10].

It is likely that different signals are needed for the initial communication of an injury and the long-term need for wound healing, such as cell migration and changes in protein expression. The receptor specificity and the numerous signaling pathways provide an intricate network allowing for differential regulation of short versus long term signals. Our study provides evidence that $P2Y_2$ and possibly $P2Y_4$ play a role in the initial signals involved in the injury response and these receptors and other P2 receptors play a

role in long term cell migration. Future studies will involve understanding the critical interactions between signaling pathways that play a role in wound healing.

Acknowledgements

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