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P2Y Receptors Play a Critical Role in Epithelial Cell Communication and Migration

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Abstract Cellular injury induces a complex series of events that involves Ca^{2+} signaling, cell communication, and migration. One of the first responses following mechanical injury is the propagation of a Ca^{2+} wave (Klepeis et al. [2001] J Cell Sci 114(Pt 23):4185–4195). The wave is generated by the extracellular release of ATP, which also induces phosphorylation of ERK (Yang et al. [2004] J Cell Biochem 91(5):938–950). ATP and other nucleotides, which bind to and activate specific purinergic receptors were used to mimic injury. Our goal was to determine which of the P2Y purinergic receptors are expressed and stimulated in corneal epithelial cells and which signaling pathways are activated leading to changes in cell migration, an event critical for wound closure. In this study, we demonstrated that the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors were present in corneal epithelial cells. A potency profile was determined by Ca^{2+} imaging for nucleotide agonists as follows: ATP $\geq$ UTP $>$ ADP $\geq$ UDP. In contrast, negligible responses were seen for β,γ-meATP, a general P2X receptor agonist and adenosine, a P1 receptor agonist. Homologous desensitization of the Ca^{2+} response was observed for the four nucleotides. However, P2Y receptor internalization and degradation was not detected following stimulation with ATP, which is in contrast to EGFR internalization observed in response to EGF. ATP induced cell migration was comparable to that of EGF and was maximal at 1 μM. Cells exposed to ATP, UTP, ADP, and UDP demonstrated a rapid twofold increase in phosphorylation of paxillin at Y^{31} and Y^{118}, however, there was no activation elicited by β,γ-meATP or adenosine. Additional studies demonstrated that wound closure was inhibited by reactive blue 2. These results indicate that P2Y receptors play a critical role in the injury repair process. J. Cell. Biochem. 93: 1115–1133, 2004.

Key words: Ca^{2+} signaling; corneal epithelium; wound repair; P2Y receptors; migration

Epithelial cells form a protective barrier for all tissue surfaces and linings. When this barrier is compromised during injury, the cells must communicate with each other to signal that an injury event has occurred. It is known that epithelial cells respond to a variety of extracellular stimuli upon trauma, including growth factors, cytokines, and matrix components. These factors may be released by epithelial cells or other nearby cell types, such as fibroblasts, immune cells, or vascular cells, which function in an autocrine or paracrine manner. Binding of these factors to epithelial cells alters cell function, leading to stimulation of cell migration, proliferation and, ultimately, wound closure.

More recently, investigators have shown that nucleotides, including ATP and UTP, are released from cells and play a role as extracellular signaling molecules by regulating biological processes such as secretion, proliferation, and differentiation [Burnstock, 1997; Abbracchio and Burnstock, 1998; Neary et al., 1999]. Nucleotides can be released from cells by ligand

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binding, excitatory or mechanical stimulation, or by exocytotic release. Although the mechanism of active ATP release from epithelial cells is not well established [Schwiebert and Zsembery, 2003], it is known that injuring cells results in passive release of ATP. Previously, we showed that ATP functions as an intercellular messenger in corneal epithelial cells immediately following mechanical injury of a monolayer of cells [Klepeis et al., 2001]. This is consistent with studies performed using other cell types, including articular chondrocytes [Grandolfo et al., 1998], glial cells [Charles et al., 1991], pancreatic islet cells [Cao et al., 1997; Bertuzzi et al., 1999], osteoblastic cells [Jorgensen et al., 1997], and a variety of epithelial cells, including those originating from lens [Churchill et al., 1996], airway [Hansen et al., 1993], liver [Frame and de Feijter, 1997], and mammary [Enomoto et al., 1994] tissue. Furthermore, mechanical stimulation of bronchial epithelial cells has also been shown to induce release of ATP and propagation of a non-regenerative Ca^{2+} wave [Arcuino et al., 2002].

Extracellular nucleotides stimulate cells via activation of P2 purinergic receptors on the cell surface. This group of receptors is divided into two families, P2X and P2Y receptors, based on structural and physiological differences. ATP and other nucleotides can induce large Ca^{2+} oscillations, and these increases in cytosolic Ca^{2+} generated by activation of P2X or P2Y receptors occur via distinct mechanisms [Ralevic and Burnstock, 1998]. P2X receptors, which play an important role in excitable cell types, are ligand-gated ion channels that allow Ca^{2+} influx from the extracellular space following nucleotide binding. P2Y receptors are G-protein coupled receptors (GPCRs) that stimulate a release of intracellular Ca^{2+} stores through phospholipase C (PLC)-mediated PIP_2 hydrolysis and activation of the IP_3 pathway. These receptors produce a biphasic Ca^{2+} response in which the initial depletion of intracellular Ca^{2+} stores leads to a sustained Ca^{2+} release due to the opening of specific store-operated voltage-independent Ca^{2+} channels in the plasma membrane that allow Ca^{2+} entry from the extracellular space [Lewis, 1999].

P2X and P2Y receptors are expressed in a number of cell types. Agonist potency profiles have been established for each receptor based on Ca^{2+} signaling intensities, and they show that while a single receptor can bind to different nucleotides and vice versa, the receptors display agonist selectivities [Ralevic and Burnstock, 1998]. There are at least eight subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) [Communi et al., 2001; Nicholas, 2001; Sak and Webb, 2002; Abbracchio et al., 2003], and specific receptors have been categorized as having a preference for pyrimidines versus purines and/or dinucleotides versus trinucleotides. $P2Y_1$, $P2Y_6$, and $P2Y_{12}$ receptors are preferentially activated by nucleoside diphosphates, while $P2Y_2$ and $P2Y_4$ receptors are mainly activated by nucleoside triphosphates. ADP is the strongest ligand for $P2Y_1$ and $P2Y_{12}$ receptors while UDP is the strongest ligand for $P2Y_6$ receptors. ATP is a strong agonist for $P2Y_2$ but not $P2Y_4$ receptors, which preferentially binds pyrimidine triphosphates, such as UTP. In addition, $P2Y_1$, $P2Y_{11}$, and $P2Y_{12}$ receptors are activated by purine and not pyrimidine nucleotides [Ralevic and Burnstock, 1998; Sak and Webb, 2002; Lazarowski et al., 2003].

The cornea is an excellent model tissue for studying the response to injury and nucleotide signaling. It is avascular and innervated by a branch of the trigeminal ganglion that is known to influence wound repair in vivo. Understanding the response to nucleotides in epithelial cells will allow us to determine the role of purinergic receptors in initial events after injury through wound closure. We hypothesize that injury initiates localized signaling events through the activation of P2Y receptors by nucleotides. Previously, we had demonstrated that mechanical injury of a monolayer of epithelial cells results in propagation of an intercellular Ca^{2+} wave in cells surrounding the injury site [Klepeis et al., 2001]. The wave is generated by extracellular release of ATP, which also induces phosphorylation of ERK 1/2 [Yang et al., 2004]. Although the Ca^{2+} response to ATP has been reported in a number of epithelial cell types, the role of P2Y receptor subtypes in cell migration is not well understood.

The aim of this study was to characterize the expression of P2Y receptors and evaluate their role in migration and wound repair in an epithelial cell line and primary epithelial cells. A number of P2Y receptor subtypes, including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$, were detected by RT-PCR and were shown to participate in functional Ca^{2+} signaling. Furthermore, nucleotides induced cell migration and

the phosphorylation of paxillin, a migration-associated signaling molecule. We demonstrated for the first time in corneal epithelial cells that P2Y receptors play a role in chemotaxis and directed migration, which are critical components of wound repair.

MATERIALS AND METHODS

Antibodies and Reagents

The fluorescent Ca^{2+} indicator dye fluo-3/AM and pluronic acid were from Molecular Probes, Inc. (Eugene, OR). The purinergic receptor inhibitor reactive blue 2 (RB2) and the nucleotidase apyrase (grade III) were from Sigma-Aldrich, Inc. (St. Louis, MO), as were all of the nucleotides (adenosine 5′-triphosphate (ATP), uridine 5′-triphosphate (UTP), adenosine 5′-diphosphate (ADP), and uridine 5′-diphosphate (UDP)), synthetic agonists (adenosine 5′-[γ-thio]triphosphate (ATP-γ-S), β,γ-methyleneadenosine 5′-triphosphate (β,γ-meATP), and adenosine 5′-[β-thio]diphosphate (ADP-β-S)) and adenosine. Human recombinant epidermal growth factor (EGF) was purchased from Invitrogen Corporation (Carlsbad, CA). The EGF receptor tyrosine kinase inhibitor tyrphostin AG1478 was obtained from EMD Biosciences, Inc. (San Diego, CA).

For Western blot analysis and immunocytochemistry studies, rabbit anti-$P2Y_2$ receptor antibody was purchased from Alomone Laboratories, Inc. (Jerusalem, Israel). For Western blot analysis, the phosphotyrosine-specific antibodies rabbit anti-pY^{31}paxillin and rabbit anti-pY^{118}paxillin were obtained from BioSource International (Camarillo, CA). Mouse monoclonal antibodies specific for paxillin and RhoA were obtained from BD Biosciences (San Diego, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. The mouse monoclonal antibody that specifically recognizes the extracellular domain of the EGFR was from Santa Cruz Biotechnology, Inc. Rhodamine–phalloidin was obtained from Molecular Probes, Inc. and affinity purified fluorescently conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Culture

Studies were performed using both an epithelial cell line and primary epithelial cells isolated from fresh rabbit corneas (Pel-Freeze Biologicals, Rogers, AR). The human corneal epithelial cell line (HCE-T) was established by Araki-Sasaki et al. using a recombinant SV40-adenovirus vector and displays properties similar to normal corneal epithelial cells [Araki-Sasaki et al., 1995]. Corneas were removed from eyes and incubated in D-MEM containing 1.2 mg/ml Dispase II (Boehringer Mannheim Corporation, Indianapolis, IN) for 1 h at 37°C [Trinkaus-Randall and Gipson, 1984]. Epithelial sheets were teased off and seeded onto glass coverslips (No. 1) [Trinkaus-Randall et al., 1988]. All epithelial cells were cultured in serum-free keratinocyte medium (K-SFM) ($[Ca^{2+}] = 0.09$ mM) supplemented with 30 μg/ml bovine pituitary extract (BPE), 0.016 nM EGF, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Corporation).

Calcium Imaging

For the non-flow-through Ca^{2+} imaging experiments, epithelial cells were grown to confluency on 25-mm round glass (No. 1) coverslips and incubated for 18–24 h before experimentation in K-SFM lacking growth factors (BPE and EGF) [Klepeis et al., 2001]. For all experiments, cells were incubated in an HEPES-buffered saline solution containing 137 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 3 mM $CaCl_2 \cdot 2H_2O$, 25 mM glucose, and 10 mM HEPES [Cornell-Bell et al., 1990]. Briefly, cells were loaded with 5 μM fluo-3/AM for 30 min, rinsed, and placed in an open chamber (Molecular Probes, Inc.) containing HEPES solution and positioned on the stage of a Zeiss LSM 510 Axiovert confocal laser scanning microscope equipped with an Argon laser. All perturbations were made while continuously scanning the cells every 789 ms [Klepeis et al., 2001; Yang et al., 2004]. For each experiment, cells were initially scanned to establish a baseline fluorescence reading before the addition of an equal volume of growth factor and/or injury was made [Klepeis et al., 2001]. When the Ca^{2+} response to conditioned wound media was examined, media was collected within 30 s of injuring cells and then treated for 5 min with or without 30 U/ml apyrase (grade III) before addition to fluo-3/AM-loaded cells [Klepeis et al., 2001].

For the flow-through Ca^{2+} imaging experiments, confluent epithelial cells were loaded with fluo-3/AM and placed into an open chamber equipped with openings for up to six inlet

tubes (for six different solutions) and an outlet tube connected to a suction pump (Warner Instruments, CT). This allowed for continuous perfusion while imaging. For each experiment, cells were perfused with HEPES-buffered saline to establish a flow rate of approximately 1 ml/min (to ensure that the cells were not stimulated) and to establish a base line fluorescence reading. When agonist potency profiles or desensitization experiments were performed, cells were alternately perfused with HEPES-buffered saline and nucleotide in 100 s intervals.

Calcium Data Analysis

To evaluate Ca^{2+} dynamics, changes in average fluorescence of individual cells or an entire field of cells (460.6 × 460.6 μm) was plotted over time. The LSM 510 Imaging Software was used to determine average fluorescence of the region of interest for each 789-ms time-point. The data was transferred to KaleidaGraph to perform calculations. To calculate the percent change in average fluorescence with respect to the first time-point (F_0) reading, the following equation was applied to each 789-ms time-point (F):

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

and the results were plotted [Cornell-Bell et al., 1990]. Maximal percent increase in average fluorescence was determined for an experiment in order to comparison experiments.

Reverse Transcriptase-Polymerase Chain Reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to characterize the presence of specific P2Y receptors. Total cellular RNA was isolated from corneal epithelial cells using the Qiagen Kit (Qiagen, Inc., Valencia, CA). Five micrograms of total RNA was annealed with either oligo-$(dT)_{16}$ primer or random hexamers and the first strand cDNA synthesis was carried out with Superscript Reverse Transcriptase (Invitrogen Corporation). The products of the reactions were combined and served as a template for PCR amplification. The following primers were designed based on the P2 receptor cDNA sequences: $P2Y_1$ (GenBank accession no. Z49205) (80/81): forward primer: 5′-ATCGGCTTCCTGGGCAACA-3′, reverse primer: 5′-CCAAGGGGACACAGAACAT-3′, annealing temperature: 56°C, product length: 499 bp; $P2Y_2$ (GenBank accession no. NM_002564) (220/221): forward primer: 5′-GCCCCCGTGCTCTACTTTG-3′, reverse primer: 5′-CTGCTGCCCAACACATCTC-3′, annealing temperature: 60.1°C, product length: 572 bp; $P2Y_4$ (GenBank accession no. X91852) (202/203): forward primer: 5′-CTGCCCACCCTCATCTACT-3′, reverse primer: 5′-GCACTCATCCCCTTTTCTC-3′, annealing temperature: 57.8°C, product length: 876 bp; $P2Y_6$ (GenBank accession no. NM_004154) (206/207): forward primer: 5′-ACCTTGCTCTGGCTGACCT-3′, reverse primer: 5′-CAGGCACTGGGTTGTCACG-3′, annealing temperature: 58.8°C, product length: 281 bp; $P2Y_{11}$ (GenBank accession no. NM_002566): forward primer: 5′-GCCGTGGTCTTCTCTGTCC-3′, reverse primer: 5′-AGCCCAACCCCGCCAGCAC-3′, annealing temperature: 61°C, product length: 451 bp; $P2Y_{12}$ (GenBank accession no. NM_022788): forward primer: 5′-CAAACCCTCCAGAATCAAC-3′, reverse primer: 5′-TAGCCCCCAAGAGATTTTT-3′, annealing temperature: 52°C, product length: 466 bp. The DNA fragments were amplified by PCR according to the Perkin-Elmer protocol. Amplification was carried out for 36 cycles (1 min at 94°C, 1 min at the annealing temperature specific for each gene, and 1 min at 72°C) followed by 10 min at 72°C. The amplified PCR products were examined on 1 or 1.5% agarose gels. Samples were run alongside a marker (100 bp ladder) with prominent bands at 100, 600, 1,500, and 2,072 bp.

Western Blot Analysis

Cells were cultured to confluency in tissue culture dishes and incubated for 18–24 h before experimentation in K-SFM lacking growth factors. Cells were washed and incubated with specific agonists (prepared in K-SFM) for different amounts of time at 37°C, 5% CO_2. Cells were rinsed with cold HEPES/saline buffer (pH 7.4), placed on ice and lysed in 10 mM Tris-HCl (pH 7.4) containing 1% Triton X-100, 0.5% Nonidet P-40, , 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 μg/μl aprotinin, 1 μg/μl leupeptin, 1 μg/μl pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate (Na_3VO_4) and the samples were centrifuged for 10 min at 12,000 rpm. Total protein was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). SDS–PAGE was

performed and lanes were loaded with equivalent protein. Proteins were transferred to a PolyScreen polyvinylidene difluoride (PVDF) membrane (NEN Life Science Products, Inc., Boston, MA) by the semi-dry method. Nonspecific binding was blocked with bovine serum albumin (BSA) in wash buffer (8 mM Tris-HCl, 2 mM Tris-base, 154 mM NaCl). Membranes were probed with primary antibody of appropriate concentration in BSA, washed, and incubated with horseradish peroxidase-conjugated secondary antibody in BSA (BD Biosciences and Zymed Laboratories, Inc., South San Francisco, CA). The chemiluminescence enzymatic reaction was carried out according to the manufacturer's instructions (NEN Life Science Products, Inc.). Membranes were exposed and band analysis data was collected using a Kodak Image Station 440 CF. The Image Station software was used to quantify the results by calculating the net intensity of each band. Blots were stripped (62.5 mM Tris (pH 6.8), 2% sodium dodecyl sulfate, 100 mM β-mercaptoethanol), blocked and re-probed with the antibody of interest. Graphs were plotted using either normalized net intensity values or as fold change over control.

Immunocytochemistry

Cells were grown to confluency in 8-well glass chamber slides and were incubated for 18–24 h before experimentation in K-SFM lacking growth factors. After treating cells, they were rinsed with phosphate-buffered saline (PBS) and fixed for 15 min at RT with 4% paraformaldehyde (pH 7.2). Cells were rinsed and permeabilized with 0.1% Triton X-100 in PBS for 1 min at RT. After blocking with 3% BSA/PBS, cells were incubated overnight at 4°C with the antibody of interest (5 μg/ml) prepared in 1% BSA/PBS. Cells were rinsed with PBS, blocked in 3% BSA/PBS, and incubated with the appropriate fluorescently conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) (1:50 in 1% BSA/PBS). Negative controls were incubated without the primary antibody. Cells were rinsed and counterstained with rhodamine–phalloidin (1:50 in PBS) (Molecular Probes, Inc.) for 30 min at RT. Cells were rinsed, covered with Antifade (Molecular Probes, Inc.) and coverslipped, and imaged and analyzed on a Zeiss LSM 510 Axiovert confocal laser scanning microscope as described previously [Song et al., 2002, 2003].

Migration Assay

Epithelial cells grown to confluency were incubated for 18–24 h before experimentation in K-SFM lacking growth factors. Migration assays were performed using Costar Transwell inserts (6.5-mm diameter polycarbonate membranes, 8 μm pore size) as described in Song et al. [2002]. Binding buffer (0.05% gelatin and 25 mM HEPES in K-SFM) was used for both diluting growth factors and resuspending the cells. Chemotactic factor was added to each of the bottom chambers; and unsupplemented binding buffer was used as a negative control for migration. Trypsinized cells were resuspended in binding buffer and 125,000 cells were added to the top chambers. Migration was performed at 37°C for 8 h, and migrated cells were fixed with methanol for 10 min at RT. Nonmigrated cells were removed, and the remaining cells were stained with 5 μg/ml propidium iodide (Molecular Probes, Inc.). The polycarbonate membranes were washed, removed and mounted onto glass slides with SlowFade Antifade (Molecular Probes, Inc.). For each membrane (33.2 mm^2), the total number of cells were counted in each of 6 random 10× fields (one field = 1.37 × 1.08 mm, or 1.48 mm^2) and an average and standard deviation were calculated.

Scrape Wound Assay

Corneal epithelial cells were grown to confluency in 8-well glass chamber slides. Cells were pre-incubated for 30 min at 37°C (5% CO_2) in the presence or absence of inhibitor. A single linear wound 50 μm in width was made with a 25 G7/8 needle. Images of three separate areas along the wound were taken immediately after injury using a SPOT camera connected to a Nikon inverted light microscope. Experimental and control cells were incubated at 37°C (5% CO_2) until wound closure occurred in the control cells, and images were taken of the original imaged sites. The degree of wound closure was determined for each condition and calculated as percentage of control.

RESULTS

This is a novel study that characterizes P2Y receptors in corneal epithelial cells and evaluates their role in chemotaxis and directed cell migration. Previously, we demonstrated that a Ca^{2+} wave rapidly propagates to neighboring

cells in response to mechanical injury of epithelial cells [Klepeis et al., 2001]. The wave did not depend on gap junctions and propagated across an acellular space. Furthermore, apyrase, an enzyme that cleaves the terminal phosphate groups off of ATP and ADP, inhibited the Ca^{2+} wave. To determine the ability of wound media collected immediately after injury to induce a Ca^{2+} response, media was added to fluo-3/AM loaded cells and imaged using confocal laser scanning microscopy. Wound media caused an average peak percent increase in fluorescence (i.e., Ca^{2+} signaling) of 81% (±25%). In contrast, the Ca^{2+} response was abolished when wound media were treated for 5 min with 30 U/ml apyrase prior to addition to the fluo-3 loaded cells (Fig. 1). In addition, other studies have demonstrated that the active component was less than 3 kDa in size and was not inactivated by proteolysis or heat denaturation [Yang et al., 2004]. Together these indicate that mechanical injury causes the release of nucleotides into the extracellular space, resulting in a Ca^{2+} response.

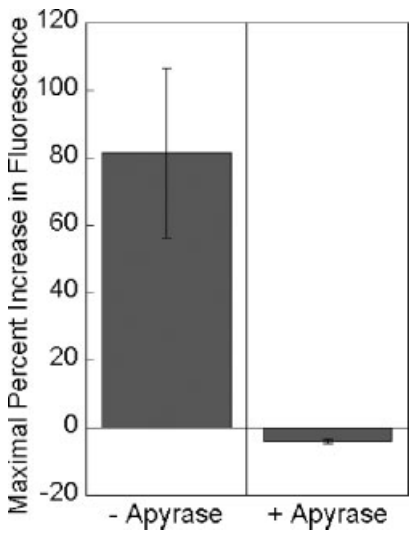


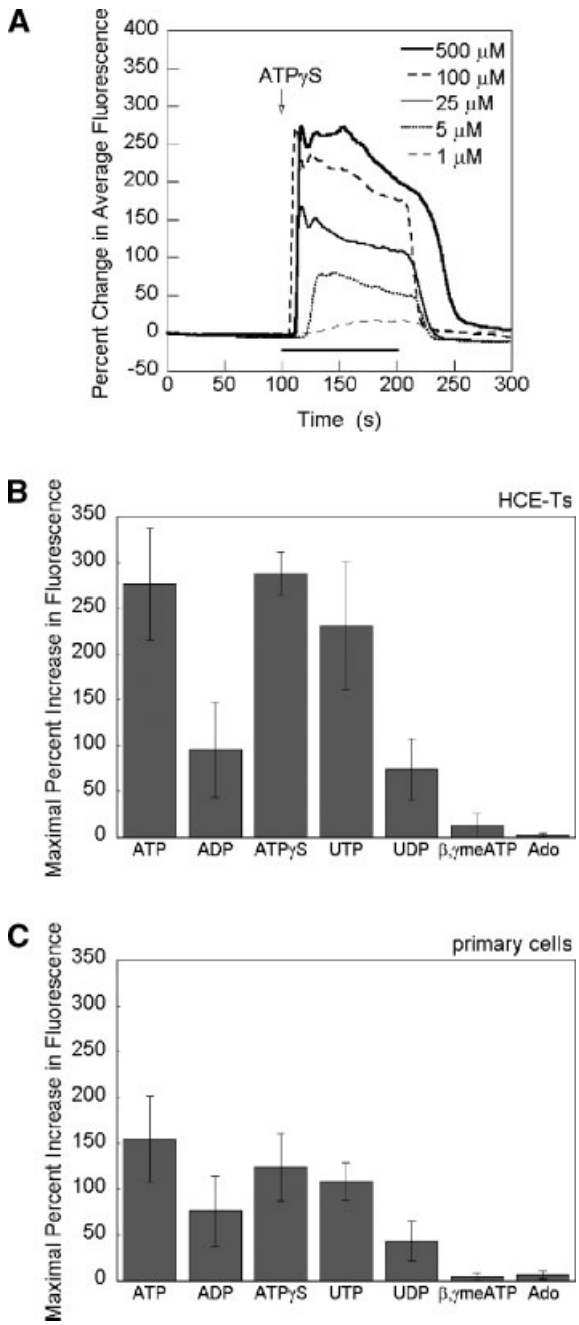
Fig. 1. Injury-induced Ca^{2+} response is inhibited with apyrase. Confluent cells were washed with HEPES-buffered saline and multiple parallel wounds (2.5-mm intervals) were made using a 25 G7/8 needle. Wound media was collected within seconds and added to fluo-3/AM-loaded cells and the Ca^{2+} response was imaged using confocal laser scanning microscopy. The maximal percentage increase in fluorescence elicited by the wound media was determined. Standard deviations are indicated by error bars. In a parallel set of experiments, the response was inhibited when the wound media was treated with 30 U/ml apyrase for 5 min before addition to the fluo-3/AM-loaded cells. Results represent an average of three separate experiments.

Mobilization of Intracellular Calcium by Different Purinergic Receptor Agonists

There are at least eight human subtypes of the P2Y receptor ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$), each with its own agonist potency profile for purines and pyrimidines. Using the accepted agonist selectivities, P2Y receptor subtype expression on the epithelial cell surface was evaluated by exposing fluo-3/AM-loaded cells to specific agonists. The experiments were conducted using a flow-through apparatus, with images collected every 789 ms. Cells were perfused for 100 s in HEPES-buffered saline alone to obtain background images, then switched to the agonist of choice for 100 s and perfused again with HEPES-buffered saline for 100 s to allow a return to background. A potency profile was determined for each agonist (1–500 μM), and a separate culture of cells was used for each agonist and each concentration. A typical dose response for ATP-γ-S in HCE-T cells is shown in Figure 2A. Data were plotted as percent change in fluorescence of an entire field of cells over time, and results for each concentration of ATP-γ-S (1, 5, 25, 100, and 500 μM) were superimposed onto one graph for comparison. Increasing concentrations of agonist caused stronger Ca^{2+} responses, and the response was maximal at 100 μM and plateaued at 500 μM. A very rapid response was observed for all but the lowest agonist concentration (1 μM). Analysis of individual or clusters of cells did not alter the shape of the resulting graph.

In Figure 2B, responses to a number of agonists (ATP, ADP, ATP-γ-S, UTP, UDP, β,γ-meATP, and adenosine) at 100 μM are shown. The results are plotted and compared as the maximal percentage increase in average fluorescence that was induced for the entire field of HCE-T cells (460.6×460.6 μm) (average of three separate experiments). A parallel set of experiments were performed and revealed similar responses for primary epithelial cells (Fig. 2C). While the potency profile for the primary cells is the same as for HCE-T cells, the response for the former is less intense. The strong responses to ATP-γ-S and ATP (288 and 276% average maximal increases in HCE-T cells and 124 and 154% average maximal increases in primary epithelial cells, respectively) suggests the presence of $P2Y_2$ and $P2Y_{11}$ receptors. A similarly strong response to UTP

(230% average maximal increase for HCE-T cells and 109% average maximal increase for primary epithelial cells) supports the hypothesis that both $P2Y_2$ and $P2Y_4$ receptors are present and activated (Fig. 2B,C). In addition, Figure 2B,C demonstrates that dinucleotides induce specific Ca^{2+} responses, indicating the potential presence of $P2Y_1$, $P2Y_6$, and $P2Y_{12}$ receptors. The Ca^{2+} responses to ADP and UDP are approximately 33% (for HCE-T cells) or 50% (for primary cells) of the ATP-γ-S or UTP responses (95 and 74% average maximal increases for HCE-T cells and 76 and 44% average maximal increases for primary cells, respectively). While primary cells displayed smaller responses, the trend is consistent with the cell line. Overall, the cells responded with a potency profile of ATP ≥ UTP > ADP ≥ UDP. Negligible responses were seen for β,γ-meATP, a general agonist for P2X receptors, and adenosine, a P1 receptor agonist. From these data we predict that P1 and P2X receptors do not play a role in the nucleotide induced Ca^{2+} response.



Detection of P2Y Receptor mRNA and Protein Expression Using RT-PCR and Immunocytochemistry

It is difficult to make definitive conclusions about P2Y receptor expression based on Ca^{2+} studies alone because there are no specific agonists or antagonists for each of the different receptor subtypes, and because potency orders are influenced by the stability of the ligands in the presence of endogenous ectonucleotidases. Verification of P2Y receptor expression was performed by RT-PCR. Based on the nucleotide sequences for each of the P2Y receptors, specific primers were designed. We have detected transcripts for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors in HCET cells (Fig. 3). The $P2Y_{12}$ receptor was not detected, consistent with its specific expression in platelets. We have also identified expression of a similar composition of P2Y receptors in primary cells (data not shown).

Fig. 2. Comparison of Ca^{2+} responses for P2Y receptor agonists. **A**: Epithelial cells were loaded with fluo-3/AM and perfused with ATP-γ-S (1–500 μM) in HEPES-buffered saline. Data are presented as percentage change in average fluorescence of an entire field of confluent cells over time. A separate set of cells was used for each concentration. The strongest initial Ca^{2+} responses were equivalently induced at 100 and 500 μM, with a longer sustained response at 500 μM. The horizontal bar at the bottom of the graph indicates time of agonist exposure. Representative of three experiments. **B**: Fluo-3/AM loaded HCE-T cells were perfused with P2Y receptor agonists (ATP, ADP, ATP-γ-S, UTP, and UDP) a P2X agonist (β,γ-meATP) and a P1 agonist (adenosine (Ado)) at 100 μM. A separate set of cells was used for each agonist. Percent change in average fluorescence over time was calculated for each experiment and the maximal percentage increase was averaged. Standard deviations are indicated by error bars. ATP, ATP-γ-S, and UTP produced the strongest Ca^{2+} responses, while ADP and UDP produced lesser responses. A negligible response was detected upon exposure to β,γ-meATP or adenosine. **C**: Parallel experiments were performed in primary epithelial cells. Cells were perfused with the same purinergic receptor agonists (100 μM) as described in (B). Graphs represent a minimum of three experiments.

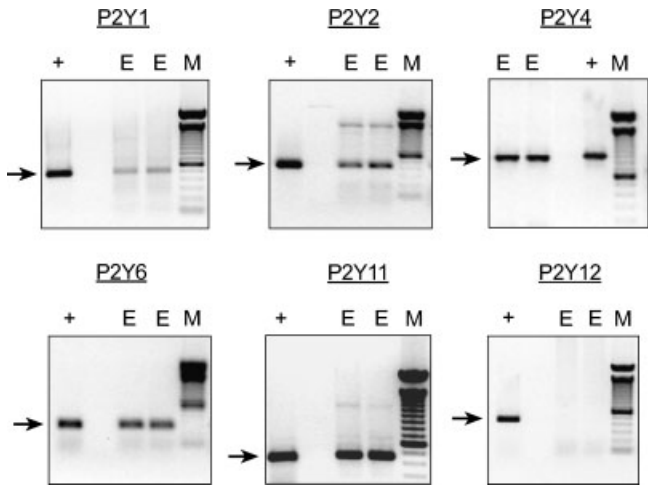


Fig. 3. P2Y receptor subtype expression in HCE-Ts. Total RNA from corneal epithelial cells (E) was reverse transcribed, and the cDNA was PCR-amplified. Aliquots of the PCR reaction were analyzed by agarose gel electrophoresis. Transcripts of the predicted sizes were obtained for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$. **Lane M** contains a 100 bp ladder with stronger bands at 100, 600, 1,500, and 2,072 bp. Plasmids containing receptors cloned from HUVEC (human umbilical vein endothelial cells) were used as positive controls (+) for $P2Y_1$ and $P2Y_4$. Plasmid containing the $P2Y_2$ receptor cloned from epithelial cells was used as a positive control (+) for $P2Y_2$. Positive controls (+) for $P2Y_6$ and $P2Y_{11}$ represent RNA isolated from a human U937 monocytic cell line, and the positive control (+) for $P2Y_{12}$ represents RNA isolated from human platelets. Gels are representative of three independent experiments.

Together, these results indicate that epithelial cells express a variety of functional P2Y receptors. In addition, we have detected P2Y receptor protein by immunocytochemistry (Fig. 5A).

P2Y Receptor Signaling Is Desensitized Upon Repeated Stimulation With Nucleotides and Is not Associated With Receptor Degradation

Some growth factor receptors, including ErbB1, i.e., the epidermal growth factor receptor (EGFR), undergo ligand-induced desensitization following stimulation with certain growth factors. This is hypothesized to be necessary for downregulation of certain signaling pathways as well as stimulation of others [Sorkin, 2001]. Many GPCRs, including P2Y receptors, also undergo desensitization following agonist stimulation [Ferguson, 2001]. Live-cell imaging of fluo-3/AM-loaded HCE-T cells was performed using a perfusion system to follow desensitization in response to P2Y receptor agonists. Cells were alternately perfused in 100-s intervals with HEPES-buffered saline solution or HEPES containing agonist, and the resulting data were plotted as percentage change in average fluorescence over time. As seen in Figure 4A, repeated stimulation with either ATP, UTP, ADP, or UDP (100 μM) in both primary epithelial and HCE-T cells caused homologous desensitization of the Ca^{2+} response, and by the third stimulation with agonist there was a 34, 35, 32, and 54% decrease in each nucleotide-induced response, respectively.

To further delineate the P2Y receptor subtypes expressed in epithelial cells, we conducted desensitization studies to determine whether stimulation with one nucleotide affected the Ca^{2+} response to another nucleotide. Heterologous-desensitization of the UTP-induced Ca^{2+} response was detected following repeated stimulation with ATP. This supports the functional presence of the $P2Y_2$ receptor subtype, which is able to bind either ligand with equal efficiency (Fig. 4B, top). In contrast, UDP, which does not share equal binding efficiency with UTP-specific receptors, did not diminish the UTP-induced Ca^{2+} response (Fig. 4B, bottom).

Since there was desensitization of the nucleotide induced Ca^{2+} response, experiments were performed to determine whether ligand binding led to P2Y receptor internalization and degradation. When cells were treated for various amounts of time with 100 μM ATP, there were no significant changes in receptor localization detectable by immunocytochemistry (Fig. 5A). Parallel experiments were performed for the EGFR and verified that receptors localized along the plasma membrane became internalized following EGF stimulation (Fig. 5A, arrows). As these data alone do not explain whether P2Y

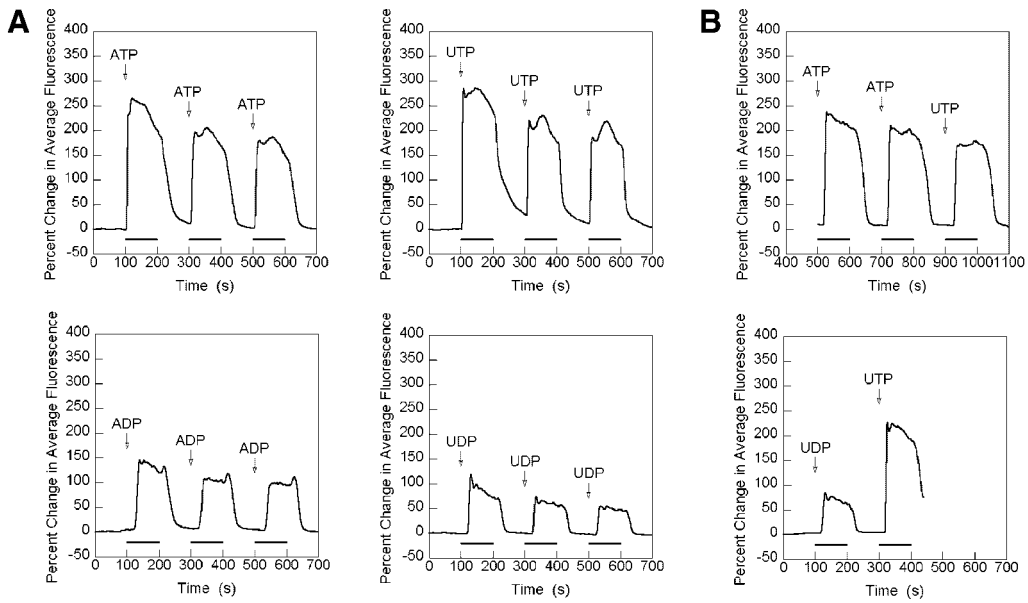


Fig. 4. Repeated nucleotide stimulation causes desensitization of the Ca^{2+} response. Cells loaded with fluo-3/AM were alternately perfused in 100-s intervals with HEPES-buffered saline solution or HEPES containing 100 μM of the indicated nucleotide. Percent change in average fluorescence of an entire field of cells was plotted over time. Horizontal bars at the bottom of each graph indicate time of agonist exposure. **A**: Homologous desensitization of the Ca^{2+} response following repeated stimulation with ATP, UTP, ADP, or UDP, respectively. **B**: Heterologous desensitization of the UTP-induced response following ATP exposure is demonstrated in the top graph. Lack of desensitization of the UTP-induced response following UDP exposure is demonstrated in the bottom graph. Results are representative of three separate experiments.

receptor internalization and degradation is responsible for desensitization of the Ca^{2+} response, additional experiments with lysates analyzed by Western blotting were performed (Fig. 5B,C, see below). It is also possible that recycling of the receptor occurred with the receptor remaining along the cell periphery at the time-points observed, however, the internalization and degradation typically seen following EGF binding to the EGFR (Fig. 5A) was not detected for the $P2Y_2$ receptor.

To further evaluate receptor internalization and degradation, additional studies were performed. HCE-T cells were treated over four different time periods (5 min, 1, 3, 8 h) with 100 μM ATP or unsupplemented media (negative control) and lysates were collected, normalized for total protein, and analyzed using a polyclonal antibody specific for the $P2Y_2$ receptor. Specificity of the antibody was verified by preabsorbing the antibody with antigenic peptide, as suggested by the manufacturer (Fig. 5B). The presence of multiple bands may be due to posttranslational modifications, such as glycosylation [Sage and Marcus, 2002] or dimerization of the receptors. Compared to the well-characterized EGFR internalization and degradation that occurs following EGF binding (Fig. 5A), there was only a negligible decrease in total $P2Y_2$ receptor protein even after 8 h of exposure to ATP (Fig. 5C). These results were consistent with the observation that desensitization of the ATP-induced Ca^{2+} response, even after multiple stimulations with nucleotide, was not complete.

Nucleotides Stimulate Motility of Epithelial Cells in a Dose-Dependent Manner

Investigators have shown that nucleotides can function as chemotactic agents for microglial cells [Honda et al., 2001] and smooth muscle cells [Chaulet et al., 2001]. Using the Transwell migration assay, we compared the role of nucleotides and EGF on epithelial cell migration. HCE-T cells were allowed to migrate for 8 h at 37°C in the presence or absence of chemotactic factors at different concentrations. For each condition, migrated cells were counted in six 10× fields (one field = 1.48 mm^2) and averaged (Fig. 6). ATP induced a similar degree of migration as EGF, a known chemotactic factor for corneal epithelial cells. Migration to each factor was dose-dependent and displayed a bell-shaped curve. Exposure to low agonist

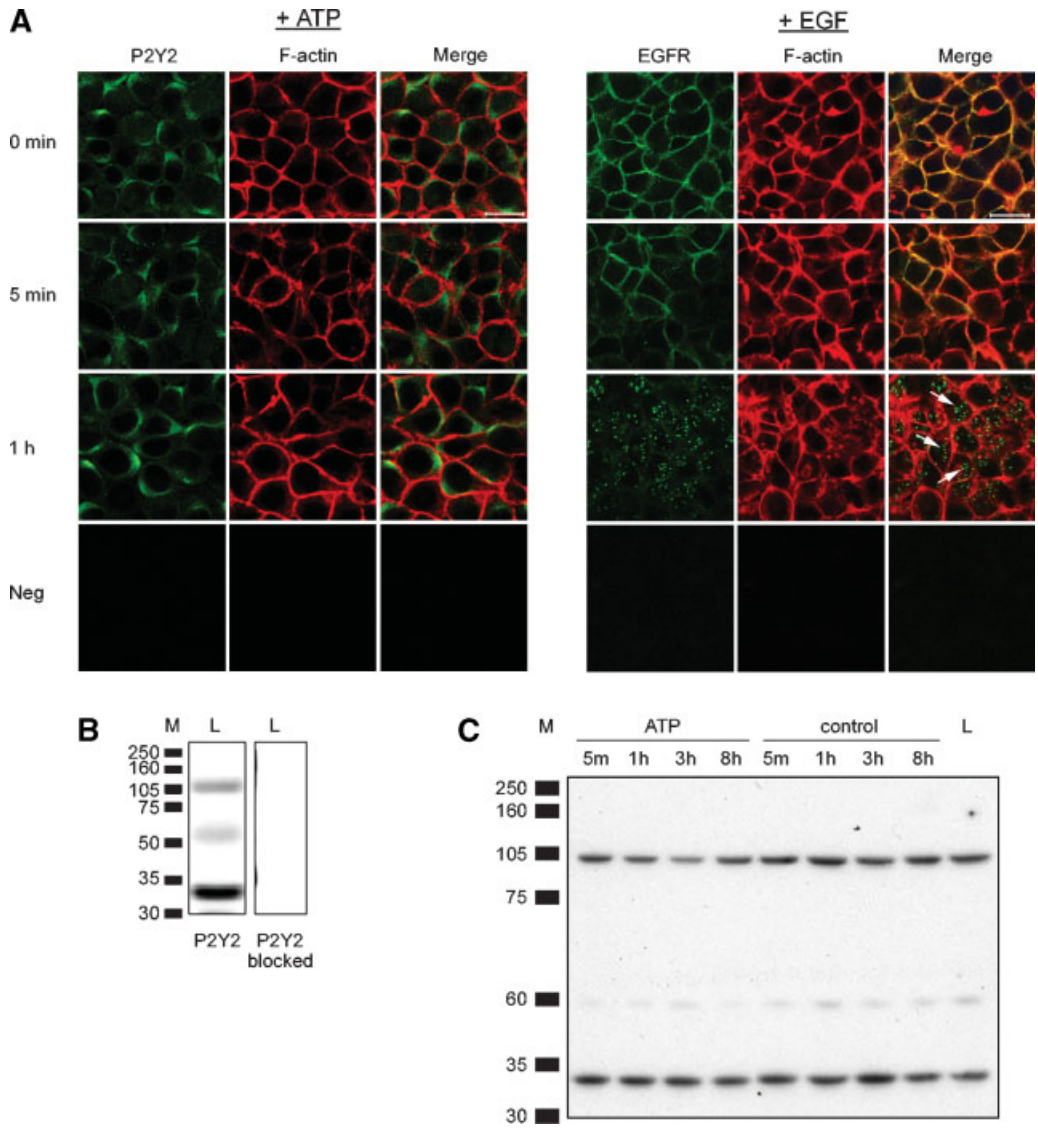


Fig. 5. Localization and expression of $P2Y_2$ receptor protein. **A**: Confluent cells were stimulated with ATP (100 μM), fixed, and probed with a rabbit polyclonal antibody specific for the intracellular C-terminus of the $P2Y_2$ receptor followed by FITC-conjugated IgG anti-rabbit. Cells were co-stained with rhodamine–phalloidin. Representative images show that $P2Y_2$ receptor subcellular localization does not change at either 5 min or 1 h. For the negative control, cells were probed only with FITC-conjugated IgG anti-rabbit. Parallel experiments were performed where EGF (4 nM) was added to cells and the EGFR was detected using a monoclonal antibody specific for the extracellular domain of the receptor and detected using FITC-conjugated IgG anti-mouse. Note internalization of the EGFR within 1 h after stimulation with EGF (arrows). Confocal micrographs represent single optical sections of 2 μm in the mid region of the cells. Scale bar equals 20 μm. The images are representative of three experiments. **B**: Cell lysates (equivalent protein concentrations) were run on a 12% SDS–PAGE, transferred and immunoblotted with a polyclonal antibody specific for the $P2Y_2$ receptor. All three bands were blocked following preabsorption of the antibody with antigenic peptide. **C**: Total $P2Y_2$ receptor protein was detected by Western blot analysis. HCE-T cells were exposed to 100 μM ATP for 5 min, 1, 3, or 8 h), lysates collected and run as equivalent protein. Lysates from untreated cells (L). No substantial change in total $P2Y_2$ receptor protein was detected with ATP. Gel is representative of two separate experiments.

concentrations (1 μM nucleotide or 0.16 nM EGF) was associated with the strongest motility (Fig. 6). However, high concentrations (100 μM nucleotide or 16 nM EGF) failed to induce strong migration. In Figure 6, ATP caused maximal motility at a concentration of 1 μM (317 cells, ±16), while EGF caused maximal motility at a concentration of 0.16 nM (277 cells, ±20). Similarly, other nucleotides, such as ATP-γ-S, a non-hydrolyzable form of ATP, and UTP, induced maximal migration at a concentration of 1 μM (data not shown).

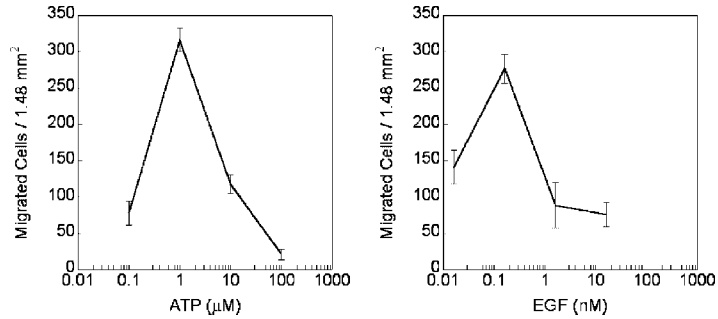


Fig. 6. Nucleotides induce migration in a dose-dependent manner. Cells were migrated for 8 h at 37°C to EGF or ATP. Migrated cells were fixed, stained with propidium iodide, and counted. For each condition, the number of cells that migrated in six 10× fields (one field = 1.48 mm^2) were averaged and standard deviations calculated. Migration was dose-dependent, with ATP causing maximal cell migration at 1 μM and EGF causing maximal cell migration at 0.16 nM. Results are representative of at least three separate experiments.

ATP Activates Signaling Molecules Important in Cell Motility

As nucleotides have been shown to play a role in motility, we hypothesized that signaling molecules, such as paxillin, would show changes in phosphorylation (Y^{31} and Y^{118}). Paxillin is an important component of focal adhesions and cell migration. In a dose-response study, ATP was added to mimic injury and induce a Ca^{2+} response by activation of P2Y receptors. Briefly, HCE-T cells were exposed to a range of concentrations of ATP (1, 5, 10, 25, 50, 100, 250 μM) or EGF (0.16, 0.8, 1.6, 4, 8, 12, 16 nM) for 5 min. Two controls were included in which cells were untreated (C) or treated with basal medium lacking agonist (0) for 5 min. Protein lysates were normalized for total protein, and resolved with reducing 7.5% SDS–PAGE and Western blot analysis. Maximal phosphorylation of Y^{118}paxillin correlated closely with maximal cell migration and inversely with agonist concentration (Fig. 7A). Densitometric analysis was performed on the phosphorylated protein for cells treated with various concentrations of ATP or EGF. The results for each agonist concentration were plotted as fold increase in phosphorylation with respect to control cells treated without agonist, as addition of unsupplemented basal medium stimulated paxillin phosphorylation. ATP and UTP induced greater than twofold phosphorylation of paxillin at Y^{118} over control at a concentration range of 1–25 μM, while EGF caused greater than twofold phosphorylation at a concentration range of 0.8–8 nM (Fig. 7B). Similar results were obtained for phosphorylation of paxillin at Y^{31} (data not shown).

To evaluate the change in pY^{118}paxillin over time, cells were stimulated with ATP (25 μM) or EGF (4 nM). Protein lysates were collected at the following times after ATP or EGF addition: 1, 5, 15, 30 min, 1, 3, and 8 h. A parallel control study was performed in which cells were treated with unsupplemented basal medium. Results were plotted as fold increase in paxillin phosphorylation with respect to control. Both ATP and EGF induced rapid (within 1 min) and transient phosphorylation of paxillin at Y^{118}, with maximal activation occurring 5 min following exposure (Fig. 8A). As expected, the temporal characteristics for phosphorylation of Y^{31} paxillin were similar, with activation occurring by 1 min and maximal by 5 min (Fig. 8B).

Since activation by ATP was maximal at 5 min, studies were performed to compare the response with that induced by other purinergic receptor agonists at this time-point. Cells were stimulated with either β,γ-meATP, ADP-β-S, ATP-γ-S, UTP, adenosine, ATP, ADP, or UDP at a concentration of 100 μM. Phosphorylation was induced by all of the P2Y receptor agonists with similar intensity. Minimal phosphorylation of Y^{118}paxillin was present under control conditions and when cells were stimulated with adenosine, a P1 receptor agonist, or β,γ-meATP, a P2X receptor agonist (Fig. 8C).

Additional experiments were performed to determine if nucleotides mediate cytoskeletal organization by evaluating localization of RhoA, a small GTPase. Stimulation of HCE-T cells did alter RhoA localization (Fig. 9). RhoA protein translocated to the cell periphery after 5 min of ATP exposure and was still detected there after 15 min, a staining pattern indicative of Rho GTPase activation (Fig. 9, arrows) [Kranenburg

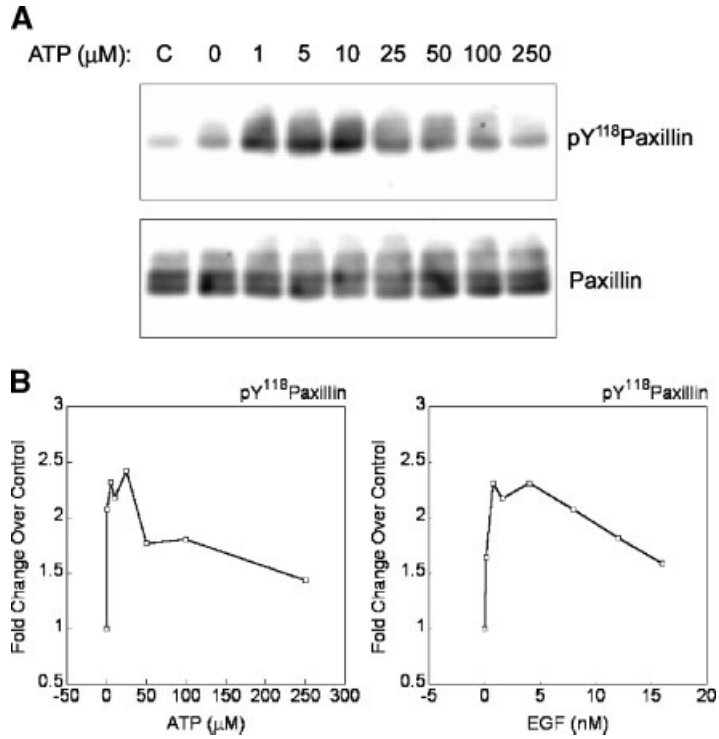


Fig. 7. Comparison of ATP- and EGF-stimulated paxillin phosphorylation. Cells were stimulated with ATP (1, 5, 10, 25, 50, 100, 250 μM) or EGF (0.16, 0.8, 1.6, 4, 8, 12, 16 nM) for 5 min. Protein lysates were collected and equal amounts of protein were subjected to SDS–PAGE. **A**: Representative blot shows ATP induced phosphorylation of paxillin at tyrosine 118 (pY^{118}paxillin). Controls included untreated cells (**lane C**) and addition of unsupplemented basal medium (**lane 0**). The blot was stripped and re-probed for total paxillin. **B**: Densitometric analysis of the blots. The data were plotted as fold change in phosphorylation of tyrosine 118 with respect to the control (lane C). ATP and EGF induced a greater than twofold increase in paxillin phosphorylation above control. Results are representative of three separate experiments.

et al., 1997]. Interestingly, at later time-points (3 h (data not shown) or 8 h) following stimulation, RhoA was localized as large clusters within the cytoplasm, i.e., not along the cell periphery (Fig. 9, arrowheads).

P2Y Receptors Play a Role in Wound Repair

We have shown that nucleotides play a role in chemotactic migration and that paxillin and Rho activity is mediated by ATP. Our goal was to establish the role of P2Y receptors in epithelial wound closure. Using a scrape wound model, we evaluated the effect of a general P2 receptor inhibitor, RB2 [Burnstock and Warland, 1987], on wound closure. Briefly, confluent cells were incubated overnight in medium lacking growth factors. Cells were pre-incubated for 30 min in the presence or absence of 100 μM RB2 or 10 μM tyrphostin AG1478, a specific inhibitor of EGF receptor tyrosine kinase activity. Images were taken immediately after injury and again after wound closure was achieved in injured non-treated control cells. Wound closure was inhibited in cells incubated with tyrphostin AG1478, as well as in cells treated with RB2 (Fig. 10). These results suggest that like EGF receptors, nucleotide receptors play a critical role in the ability of epithelial cells to repair a wound.

DISCUSSION

We previously established that mechanical injury results in release of a component into the extracellular space that induces propagation of a Ca^{2+} wave [Klepeis et al., 2001]. Yang et al. demonstrated that the active components released were nucleotides, and they caused ERK 1/2 activation via P2Y and not P2X receptors [Yang et al., 2004]. Our goal was to determine which of the P2Y receptors are expressed and stimulated in corneal epithelial cells and which signaling pathways are activated leading to changes in cell motility, an important component of wound

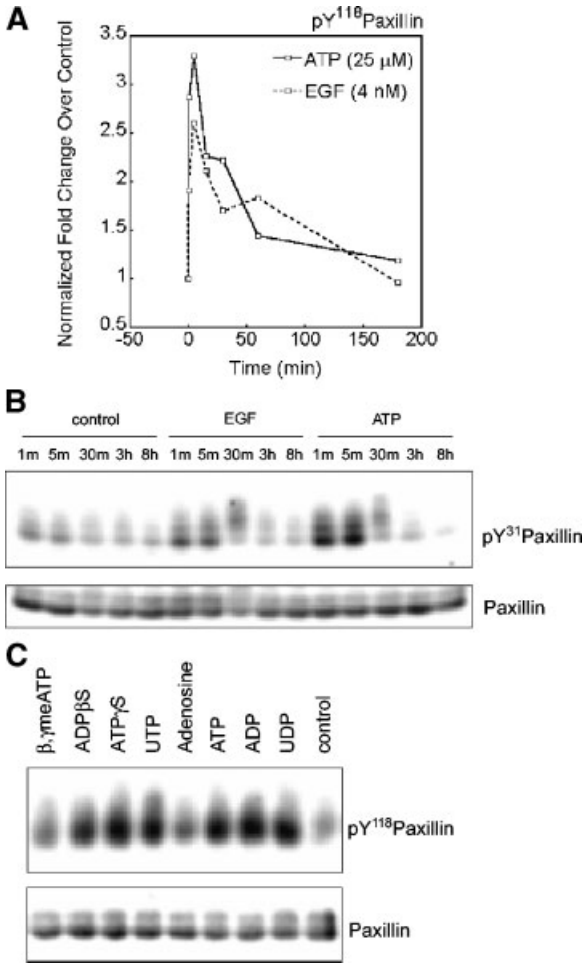


Fig. 8. Time-course of paxillin phosphorylation. Cells were stimulated for seven time periods (1, 5, 15, 30 min, 1, 3, 8 h) with EGF, ATP, or unsupplemented basal medium lacking agonist (control). Protein lysates were collected, loaded as equal protein, and paxillin phosphorylation was detected by Western blot analysis. All blots were stripped and re-probed for total paxillin so that values could be normalized. **A**: Densitometric analysis of paxillin phosphorylation at tyrosine 118 (pY^{118}paxillin). Data were plotted as fold change in phosphorylation compared to control untreated cells. ATP and EGF stimulated maximal phosphorylation within 1–5 min. **B**: Time-course of paxillin phosphorylation at Y^{31} (pY^{31}paxillin) following EGF or ATP stimulation. Control cells treated with unsupplemented basal medium are shown (control). The blot was stripped and re-probed for total paxillin. **C**: Response of cells to purinergic receptor agonists were compared at a concentration of 100 μM. Paxillin phosphorylation (pY^{118}paxillin) was detected. Control lysate (no agonist) was included as a negative control. All of the P2Y receptor agonists (ADP, ADP-β-S, ATP-γ-S, UTP, UDP, and ATP) induced activation. P1 (adenosine) or P2X (β,γ-meATP) receptor agonists did not induce activation over control. The stripped blot was re-probed for total paxillin.

repair. Our observations contribute greatly to the knowledge of chemotactic and directional motility, important underlying events for wound repair in cells that are avascular.

It was shown by RT-PCR (Fig. 3) that HCE-T cells express a variety of P2Y receptors, and Ca^{2+} imaging studies suggested cell surface expression of the receptors (Fig. 2), since ligand binding to a receptor expressed on the cell-surface is required for a response to occur. Furthermore, the Ca^{2+} imaging results for the corneal epithelial cell line corresponded with those for primary epithelial cells isolated from rabbit corneas [Kimura et al., 1999] (Fig. 2B,C). Our results are consistent with other studies demonstrating that a variety of P2Y receptor subtypes are co-expressed in epithelial cells [Dubyak, 2003]. The G_q-coupled P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) have been localized to most epithelial cell types [Communi et al., 1999; McAlroy et al., 2000; Insel et al., 2001; Choi et al., 2003], and some epithelia also express ionotropic P2X receptors [Slater et al., 2002; Shariatmadari et al., 2003; Zsembery et al., 2003]. While P2X receptors are present in HCE-T cells (data not shown), they are not players in Ca^{2+} signaling, since the P2X-specific agonist β,γ-meATP did not induce Ca^{2+} oscillations (Fig. 2B,C), paxillin activation (Fig. 8C) or ERK 1/2 activation [Yang et al., 2004]. The final degradation product of ATP, adenosine, a ligand for the P1 family of purinergic receptors, also did not stimulate these events.

As a complex mixture of cellular components are released upon physical damage to a cell, it is not surprising that a number of purinergic receptors are present on the cell surface. Total cellular levels of ATP have been shown to be 25- to 75-fold higher than UDP, ADP, or UTP in a number of cells [Werner et al., 1991]. Our results indicate that the $P2Y_2$ receptor is abundantly expressed in corneal epithelial cells. This is supported by Ca^{2+}-imaging studies using ADP and UDP, selective ligands for $P2Y_1$ and $P2Y_6$ receptors, respectively, which induced lower Ca^{2+} responses compared to ATP and UTP (Fig. 2B,C). However, this prediction assumes that $P2Y_1$ and $P2Y_6$ receptors activate PLC with equal efficiency as the $P2Y_2$ and $P2Y_4$ receptors. ATP is also rapidly hydrolyzed upon extracellular release by soluble and membrane bound ectonucleotidases to ADP, which preferentially binds $P2Y_1$ receptors. In fact, it has been shown that $P2Y_1$ receptors can mediate mechanically induced Ca^{2+} waves when $P2Y_2$ receptor expression is repressed [Gallagher and Salter, 2003]. There is also evidence for release of UTP, which was shown using a novel assay

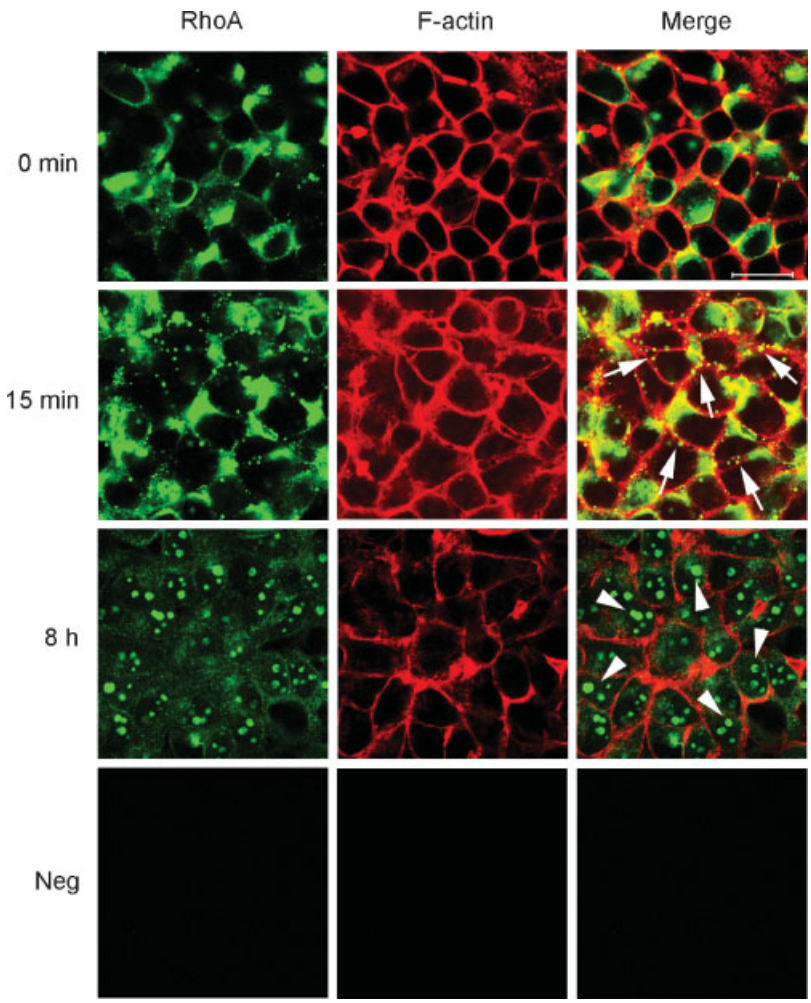


Fig. 9. Localization of RhoA following ATP stimulation. Confluent cells were treated with ATP (100 μM) and evaluated at 15 min and 8 h. Cells were fixed, permeabilized, blocked, and probed using a monoclonal antibody specific for the small GTPase RhoA that was then detected with FITC-conjugated IgG anti-mouse. Cells were double stained with rhodamine–phalloidin. RhoA is shown in untreated cells (0 min), and at 15 min and 8 h following ATP treatment. Arrows indicate increased localization of RhoA in small clusters along the cell periphery after 15 min. Intracellular localization was noted at the 8-h time-point (arrowheads). A negative control, in which no primary antibody or phalloidin was included, is shown (Neg). Confocal images represent single optical sections of 2 μm taken in the mid region of the cells. Scale bar equals 20 μm. Images are representative of three experiments.

based on the nucleotide specificity of UDP-glucose phosphorylase [Lazarowski et al., 1997].

Immunohistochemical staining and Ca^{2+} imaging studies in a variety of cell types have demonstrated that localization of P2Y receptors is specific not only to cell type but also cell region [Dubyak, 2003]. In general, $P2Y_4$ and $P2Y_6$ receptors are localized to apical plasma membranes, while $P2Y_1$ and $P2Y_{11}$ receptors are commonly found in the basolateral plasma membranes. $P2Y_2$ receptors have been found in both apical and basolateral membranes of epithelial cells [Sage and Marcus, 2002]. While our culture conditions did not allow us to evaluate cellular polarization, we demonstrated a subcellular localization for the $P2Y_2$ receptor (Fig. 5A). Organocultures that have developed polarized epithelium will be useful in future studies. While it is possible that receptor expression is altered in cell lines with passage number [Mateo et al., 1996; Okuda et al., 2003], physiologic and biochemical results obtained from the epithelial cell line and primary cells were consistent.

Desensitization of GPCRs occurs after agonist stimulation to regulate activation of downstream signaling pathways and occurs through phosphorylation of residues in the carboxy-terminus [Ferguson, 2001]. We showed that

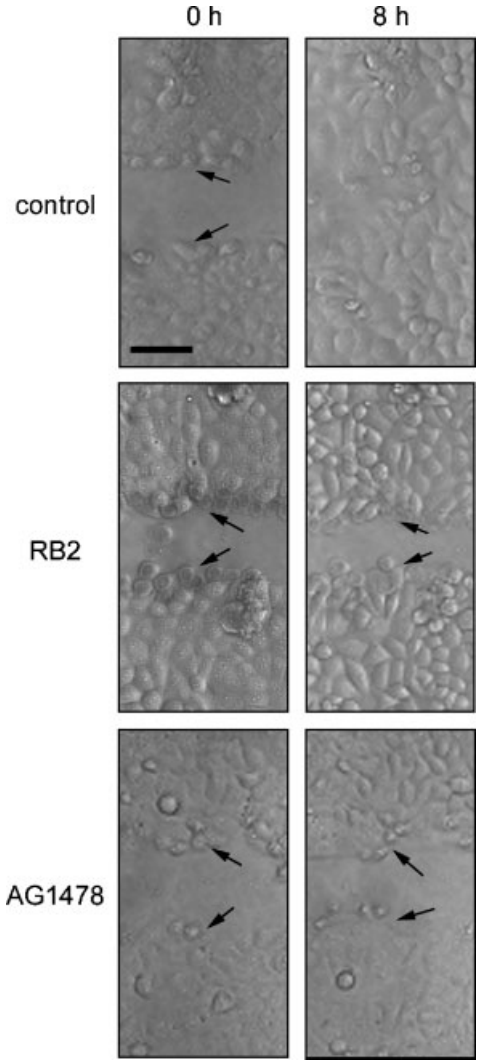


Fig. 10. Purinergic receptors are critical to wound repair. Confluent cells were incubated overnight in K-SFM lacking growth factors. Cells were treated for 30 min with basal medium containing either 100 μM RB2, a general P2 receptor inhibitor, or 10 μM tyrphostin AG1478, an EGFR inhibitor. Images were taken immediately after making a linear wound 50–75 μm in width. Images taken of the same region after wound closure was completed in control cells (8 h). Note lack of closure when cells were treated with either inhibitor. Wound edges are marked by arrows. Micrographs are representative of at least three separate experiments (scale bar equals 100 μM).

desensitization of P2Y receptor Ca^{2+} signaling occurred after repeated stimulation with nucleotide (Fig. 4). Our results also agreed with those of other investigators demonstrating that desensitization was not complete after consecutive stimulations with nucleotide. These groups demonstrated that the Ca^{2+} response attained a 40–55% lower steady-state level within 10 min of nucleotide exposure [Martin and Harden, 1989; Wilkinson et al., 1994]. We found a 50–60% decrease in the maximal Ca^{2+} response following a third stimulation with nucleotide. To demonstrate that desensitization of receptors occurred and not Ca^{2+} depletion, cells were stimulated with thapsigargin following stimulation with nucleotide. Full recovery of the Ca^{2+} response has been demonstrated within 45–60 min after removal of the nucleotides, suggesting that desensitization involves rapid and reversible modification of the receptor rather than degradation [Martin and Harden, 1989]. Interestingly, it is hypothesized that only long term exposure to nucleotide (hours) requires protein synthesis for recovery [Sromek and Harden, 1998]. We predict a similar mechanism for recovery in our long-term experiments.

Despite these observations, the exact mechanisms of P2Y receptor desensitization has not been established. In an extensive study conducted by Sromek et al., the $P2Y_2$ receptor was tagged with hemagglutinin A at its amino-terminus and expressed in human astrocytoma cells to follow receptor internalization. They were able to detect a decrease in cell surface immunofluorescence of the receptor after 15–30 min of agonist stimulation. Furthermore, they found that a decrease in IP_3 accumulation preceded loss of cell-surface reactivity of the receptor. Furthermore, by inhibiting receptor internalization with low temperature (4°C), they did not affect desensitization of the Ca^{2+} response [Sromek and Harden, 1998]. In other GPCRs, phosphorylation of the receptor led to uncoupling from G proteins and internalization. Exposure to phosphatases caused dephosphorylation and reappearance of receptors at the surface. Studies also indicated that a phosphorylation-dephosphorylation cycle regulated desensitization and resensitization of P2Y receptors, since phosphatase inhibitors block resensitization [Otero et al., 2000]. However, while dependence on protein kinase C was demonstrated by one group [Wilkinson et al., 1994], several others have presented conflicting findings [Galas and Harden, 1995; Otero et al., 2000], suggesting that other protein kinases may be involved.

The fundamental goal of our studies was to determine the role that P2Y receptors play in epithelial wound repair. In chemotactic assays, growth factors, such as EGF, are well-established chemotactic factors for corneal epithelial cells [Grant et al., 1992; Maldonado and Furcht, 1995; Zieske et al., 2000; Song et al., 2001]. In

addition, we demonstrated that ATP induced a similar degree of migration as EGF (Fig. 6). In fact, studies conducted in our laboratory have confirmed that ADP, UTP, and ATP-γ-S can stimulate migration (data not shown). In other systems, nucleotides, including ATP-γ-S, ATP, and ADP-β-S, have been shown to induce migration of neutrophils, while adenosine or P2X agonists did not [Verghese et al., 1996]. Furthermore, ADP, ATP, and UTP induced mast cell migration [McCloskey et al., 1999], UTP and UDP caused migration of smooth muscle cells [Pillois et al., 2002], and ATP and ADP stimulated migration of glial cells [Honda et al., 2001]. These indicate that chemotactic signals are cell-type specific. It is interesting to note that deletion of CD39, the main ectonucleotidase of endothelial cells and monocytes, has been shown to alter the ability of cells to migrate in response to nucleotides and is associated with downregulation of P2Y receptor signaling [Goepfert et al., 2001]. In fact, preliminary RT-PCR data demonstrated expression of CD39L4 in corneal epithelial cells (data not shown).

We demonstrated that epithelial cells were not motile when exposed to high concentrations of nucleotide or EGF. This is in contrast to studies performed in smooth muscle cells [Chaulet et al., 2001] and eosinophils [Dichmann et al., 2000], in which high concentrations of UTP and ATP, respectively, were required for maximal migration. The response we observed in epithelial cells was consistent with published reports of nucleotide-induced migration of dendritic cells [Idzko et al., 2002], and other reports of EGF-induced migration of epithelial cells [Grant et al., 1992; Song et al., 2003]. In contrast to migration and other long-term events, higher nucleotide concentrations induced the greatest Ca^{2+} responses. To explain this apparent discrepancy one should keep in mind the different time-courses of these responses. Ca^{2+} mobilization occurs within seconds after ligand binding, while downstream signaling events leading to chemotaxis take longer to occur (minutes to hours). Moreover, phosphorylation and desensitization of the receptor takes longer than activation of PLC but not as long as the events leading to chemotaxis. Therefore, high nucleotide concentrations may be inhibitory because most of the receptors would be phosphorylated and downregulated [Maghazachi, 2000]. While there are apparent differences in the Ca^{2+} and migration responses, PLC is hypothesized to be required for motility by establishing cell asymmetry. It is recruited to the area of the cell stimulated by the chemotactic factor, directing the cell toward the factor. In one model, inhibition of PLC activation decreased tumor cell invasiveness in prostate cancer [Kassis et al., 1999]. While Ca^{2+} mobilization may initiate the signaling process, it alone is not sufficient for motility [Wells et al., 1999].

A variety of intracellular signaling molecules are activated at the leading edge of migrating cells, resulting in changes in cell adhesion and cytoskeletal organization [Maghazachi, 2000], such as that of paxillin and RhoA. While paxillin is an important component of focal adhesions and is phosphorylated upon cell contact [Trinkaus-Randall et al., 2000] it has no tyrosine kinase activity and functions as a scaffold for downstream signaling molecules following phosphorylation [Turner, 2000]. Through the use of mutants, Nakamura demonstrated that tyrosines 31 and 118 played a role in migration [Nakamura et al., 2000]. We observed phosphorylation of tyrosines 31 and 118 of paxillin following stimulation with a variety of P2Y receptor agonists in a dose-dependent manner. Interestingly, concentrations needed to achieve maximal paxillin phosphorylation were similar to those required to induce maximal migration (Fig. 7). Furthermore, the phosphorylation of paxillin by ATP occurred prior to that by EGF (Fig. 8). The temporal sequence may be accounted for by the requirement for dimerization of the EGF receptor or slower diffusion of EGF (6,200 Da), which is larger than ATP (550 Da). Together these suggest that the ATP response does not function through indirect activation of the EGF receptor.

The signaling functions of nucleotides and P2Y receptors allow us to gain a better understanding of their potential physiological significance. Cowlen et al. have performed in situ hybridization for $P2Y_2$ on cryosections of ocular tissue, and found $P2Y_2$ receptor expression at the transcript level in a variety of cell types, including conjunctival epithelium, corneal epithelium, ciliary body epithelium, subcapsular epithelium of the lens, corneal endothelium, and various layers of the retina [Cowlen et al., 2003]. The overall localization indicates that these receptors are important endogenous regulators of ocular cell function. The demonstration that RB2, a general P2 inhibitor, inhibited

wound closure (Fig. 10) contributes to our understanding in the role of purinergic receptors in injury repair. While the current data indicate that a number of P2Y receptors are expressed and may be activated following injury, the cooperativity that exists among them and the cascade of signaling events that are shared or specific to receptor subtypes is not known. Our results suggest that the components of wound repair are tightly controlled by a number of receptors and suggest critical interactions between families of receptors, such as P2Y and EGF receptors. Additional studies exploring interactions between P2Y and EGF receptors, both well-established mediators of epithelial wound repair are being undertaken.

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