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Cellular Injury Induces Activation of MAPK via P2Y Receptors

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Abstract Wound healing is a complex process that involves cell communication, migration, proliferation, and changes in gene expression. One of the first events after injury is the rapid release of Ca^{2+} that propagates as a wave to neighboring cells (Klepeis et al. [2001]: J. Cell. Sci. 114:4185–4195). Our goal was to examine the signaling events induced by cellular injury and identify extracellular molecules that induce the activation of extracellular signal responsive kinase (ERK) (p42/44). In this study we demonstrated that injury induced ERK1/2 activation occurred within 2 min and was negligible by 15 min. Treatment of unwounded cells with wound media caused activation of ERK that could be inhibited by apyrase III. Stimulation with epidermal growth factor (EGF) did not mimic the injury response and it was not detected in the wound media. To identify the active component, size fractionation was performed and factor(s) less than 3 kDa that induced the release of Ca^{2+} and activation of ERK1/2 were identified. Activity was not altered by heat denaturation, incubation with proteinase K but it was lost by treatment with apyrase. Adenosine triphosphate (ATP), uridine triphosphate (UTP), adenosine diphosphate (ADP), and uridine diphosphate (UDP) promoted activation by 2 min with similar profiles as that generated by injury. Preincubation with phospholipase C inhibitor, U73122, inhibited activation that was induced by injury and/or nucleotides. Lack of activation by alpha-beta-methylATP (α, β-MeATP) and beta-gamma-methylATP (β, γ-MeATP) to purinergic (P)2X receptors further indicated that activation occurs via P2Y and not P2X purinergic receptors. These results indicate that injury-induced activation of ERK1/2 is mediated by a P2Y signaling pathway.
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Key words: wound repair; ERK activation; cellular communication; purinergic receptors; injury

Cell communication can occur in several ways. Molecules can move through physical junctions or bind to transmembrane proteins such as integrins and cadherins, where clustering of receptors is initiated and signaling cascades are induced. In addition, the release of soluble factors such as growth factors and nucleotides bind to transmembrane receptors and elicit a signaling cascade [Clark, 1996 review]. Klepeis et al. [2001] demonstrated that while EGF induced propagation of Ca^{2+} waves and enhanced the injury induced Ca^{2+} mobilization, the tyrosine kinase inhibitor, AG1478, inhibited the EGF induced wave not the injury induced wave. These all indicate that cellular communication following injury must involve multiple signaling events.

Purines and pyrimidines are regulators of cellular functions such as cell growth, differentiation, and cell death. Changes in regulation often result in a number of pathologies from neurodegenerative disease to ischemic events and cancer [Abbracchio et al., 1996]. While ATP was historically thought to play a role as an intracellular energy source, it has also been shown to act as an extracellular signaling molecule that mediates cell communication. When corneal epithelial cells were injured, ATP was

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released and Ca^{2+} waves propagated in the presence of gap junction inhibitors and tyrphostin [Klepeis et al., 2001]. In other epithelial cells, mechanical stimulation of bronchial cells induces the release of ATP and a non-regenerative Ca^{2+} wave [Arcuino et al., 2002]. In astrocytes, ATP has been shown to move through hemichannels. Furthermore, the hemichannel activator quinine caused the release of ATP and Ca^{2+} waves which was inhibited by the connexin channel inhibitor, flufenamic acid [Stout et al., 2002]. Burnstock [1972] proposed that ATP was a neurotransmitter and in 1978 demonstrated that P1 and P2 receptors mediated the physiological effects of adenosine and ATP [Burnstock, 1972, 1978]. The P2 receptors are classified into two families, P2X and P2Y. The P2X receptors are ligand-gated channels that are permeable to cations including Ca^{2+} and recently an ATP gated channel at the nucleus (P2X7) was hypothesized to play a role in mediating cellular activity suggesting that it is coupled directly to changes in gene expression [Atkinson et al., 2002]. The P2Y receptors are metabotropic G-protein coupled receptors that possess seven transmembrane hydrophobic domains with short extracellular amino and intracellular carboxyl terminals. P2Y receptors have been linked to activation of the phosphoinositide pathway [reviews by Abbracchio and Burnstock, 1998; Ralevic and Burnstock, 1998; Gao et al., 1999].

ATP and UTP induce alterations in tyrosine phosphorylation of a myriad of proteins ranging from adhesion proteins to members of mitogen-activated protein (MAP) kinase family (p42 extracellular signal responsive kinase (ERK)) [Soltoff et al., 1998]. This evidence suggests that the P2Y receptors may play a role in signal transduction events. These receptors are linked via a heterotrimeric G-protein to phospholipase C, and promote the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) resulting in the release of Ca^{2+} [Tu et al., 2000]. IP_3 acts as an intracellular second messenger by binding to the specialized tetrameric receptor that spans the endoplasmic reticular membrane triggering the release of Ca^{2+} from the endoplasmic reticulum [Clapham, 1995]. In our model, Klepeis et al. [2001] demonstrated in corneal epithelial cells that propagation of a Ca^{2+} wave moves over acellular regions and can be inhibited with apyrase and not tyrphostin indicating that injury activates the purinergic receptor signaling pathway.

The cornea is an excellent tissue to study the response to injury. Epithelial cells are superficial, avascular, and devoid of direct lymphatic supply [review by Trinkaus-Randall, 2000]. Therefore, these cells have the ability to elicit an injury response without significant, direct influence of other tissues. The basal epithelial cells are innervated by a branch of the trigeminal ganglion that terminate as free nerve terminals and are known to influence wound repair. Understanding the short and long term elements underlying the response to injury in cornea should establish a prototype for injury responses in other tissues.

Our goal was to evaluate if the purinergic signaling pathway mediated the regulation of ERK in response to mechanical injury. We demonstrated that components secreted immediately after injury activate p42/44, are less than 3 kDa, can be inhibited with apyrase but are not inhibited by proteinase K. These results indicate that nucleotides mediate the immediate response to injury. Furthermore the use of specific agonists suggest that the P2Y family and not the P2X family of purinergic receptors play a critical role in regulating the signal activation in response to injury.

MATERIALS AND METHODS

Reagents

Antibodies against ERK1/2 and active MAPK (ERK1/2) were purchased from Promega (Madison, WI). The fluorescein isothiocyanate (FITC)-conjugated antibody to ERK1/2 [pTpY185/187] was from BioSource International (Camarillo, CA). Fluo-3AM, propidium iodide, and pluronic acid were purchased from Molecular Probes (Eugene, OR). The secondary antibody (horse radish peroxidase (HRP)-conjugated goat anti-rabbit used for immunoblot analysis was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the Protein Assay kit was purchased from Pierce (Rockford, IL). The Daiichi Silver Stain Kit was purchased from Owl Separation System (Portsmouth, NH) and ATP, UTP, ADP, UDP, adenosine, apyrase, α, β-MeATP, β, γ-MeATP, ATPγS, U0126, U73122 along with other routine chemicals were obtained either from Sigma-Aldrich (St. Louis, MO), Roche, Calbiochem (San Diego, CA), or from American Bioanalytical (Natick, MA).

Cell Culture

A human corneal epithelial cell line was developed using a recombinant SV40 adenovirus vector [Araki-Sasaki et al., 1995]. Cells seeded at a density of 10^4 cells/cm^2 were cultured in keratinocyte-SFM supplemented with 30 μg/ml bovine pituitary extract, 0.1 ng/ml EGF, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL/Life Technologies, Grand Island, NY) [Klepeis et al., 2001].

Injury Model

Cells were grown to confluency for biochemical and immunohistochemical experiments as described previously [Klepeis et al., 2001; Song et al., 2001]. To determine the injury response cells were washed with phosphate buffered saline (PBS, pH 7.2), and cell lysates collected at various times after injury. To collect conditioned wound media, cells were washed and incubated in HEPES buffered saline (137 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 3 mM $CaCl_2$, 25 mM glucose, and 10 mM HEPES, pH 7.2) [Cornell-Bell et al., 1990]. Cells were scrape wounded as described previously [Haq and Trinkaus-Randall, 1998] and the wound medium was collected immediately, and analyzed or added to unwounded cells for the duration of time indicated.

SDS PAGE Electrophoresis

Lysates were collected in a Tris/NaCl buffer containing 150 mM NaCl, 10 mM Tris, 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, 2 mM PMSF, and 2 mM Na_3VO_4. The lysates were sheared with a 25 G needle, centrifuged at 10,000 rpm to remove debris, and the supernatant was collected and protein concentration determined using the BCA assay (Pierce). Thirty micrograms of protein from each lysate were subjected to SDS–PAGE (12%) and transferred to polyscreen PVDF membrane (PerkinElmer, Boston, MA). Blots were blocked in a 10 mM Tris buffer (TBST: 10 mM Tris, 100 mM NaCl, 0.1% Tween-20 pH 7.4) containing 0.2% I-block (Applied Biosystems, Foster City, CA) and membranes were incubated with appropriate antibodies, washed and incubated with appropriate secondary antibodies, and rinsed again with TBST. Visualization was performed by enhanced chemiluminescence (PerkinElmer). Fractionated conditioned media was run on SDS–PAGE (4–20% gradient gels) (Bio-Rad, Boston, MA) and silver stained according to the manufacturers directions (Daiichi Silver Stain Kit, Owl Separation Systems, Portsmouth, NH).

Sizing Fractionation

The conditioned medium was fractionated using Centricon centrifugal filter devices with a 3 kDa nominal molecular weight limit (Millipore, Bedford, MA) according to manufacturers' directions. The conditioned media was centrifuged at 7,500*g* for 2 h. Retentate and filtrate were added separately to cells and the responses were evaluated using Ca^{2+} signal-signaling and MAPK activation. Aliquots of the control unfractionated conditioned medium were retained for comparison.

Confocal Microscopy

Live cell imaging experiments were performed using conditioned medium on the Zeiss LSM 510 Inverted Microscope equipped with one argon and two HeNe lasers [Klepeis et al., 2001]. Cells were loaded with Fluo-3 AM (4 μM) in the presence of 0.02% pluronic acid and 1% DMSO (Molecular Probes), and washed to remove excess probe according to Klepeis et al. [2001]. Baseline images were taken in the presence of HEPES and the response to the retentate, filtrate, intact conditioned medium, and control medium, and 50 and 10 μM ATP were analyzed. For each experiment, the response was continuously followed for a minimum of 200 s. Percent change in fluorescence was calculated over time as determined by Cornell-Bell and Finkbeiner [1991] and Klepeis et al. [2001].

Fig. 1. Injury activates MAP kinase. **A**: Confluent cells were cultured and at time points ranging from 0 to 60 min after scrape injury, lysates were collected (W). Equal amounts of protein were resolved in SDS–PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were also probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases (ERK 1/2). Parallel cultures were performed where cells were not injured and lysates were collected and analyzed (U). Densitometric analysis of the blots show relative activation. Blot is representative of five independent experiments. **B**: Confluent cells were cultured and injured at 5, 15, and 30 min and compared to uninjured controls. Cells were fixed and direct immunohistochemistry was performed with an anti-phospho-ERK/MAP kinase antibody conjugated to fluorescein and counter stained with propidium iodide. Arrows indicate the site of the scrape injury. Images are representative of three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

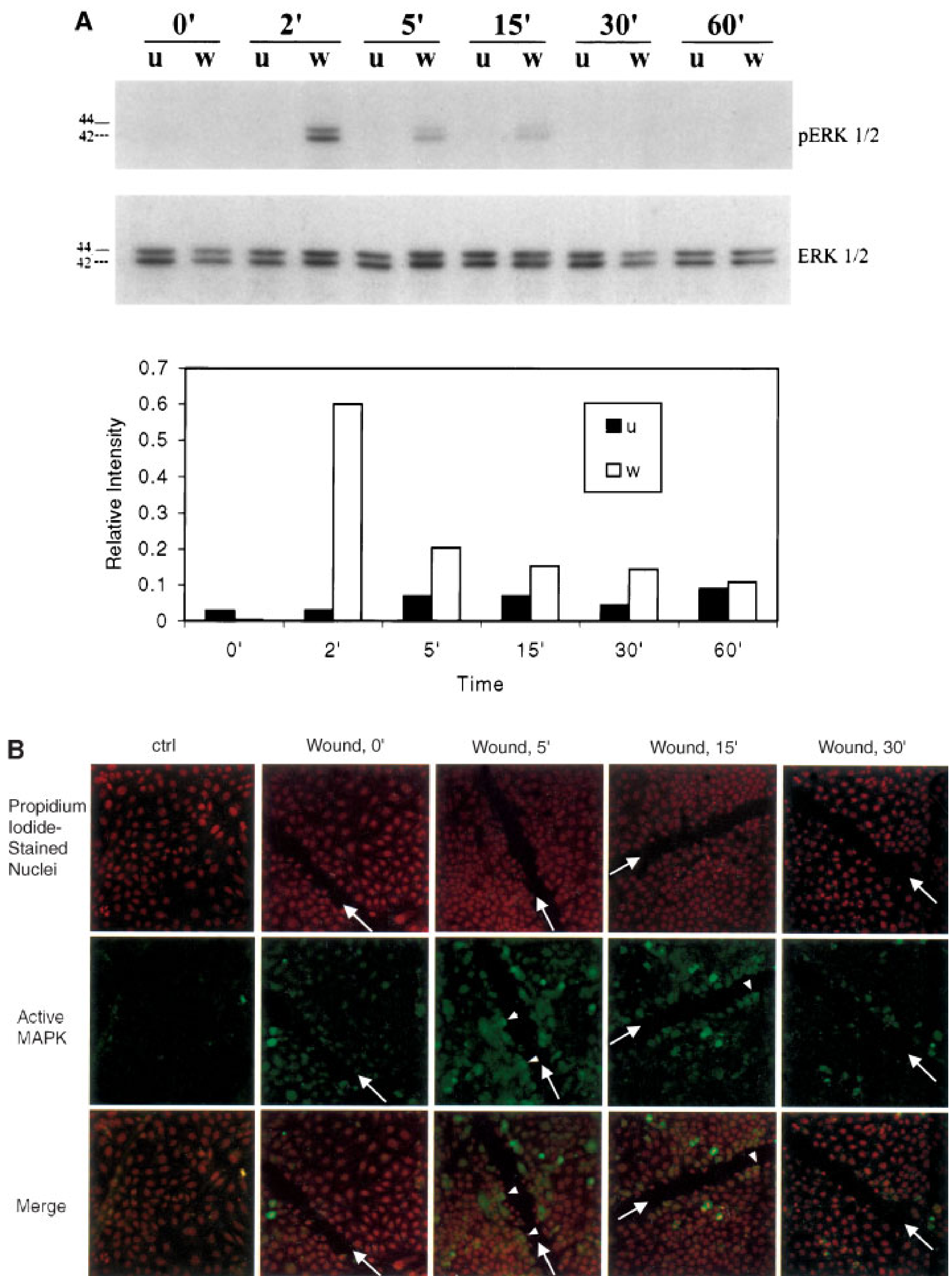

Fig. 1.

Direct immunohistochemistry was performed to localize activated ERK1/2 using confocal microscopy. The ERK1/2 antibody was directly conjugated to FITC (Biosource International). Briefly, cells were fixed in 1% freshly made paraformaldehyde for 15 min at 4°C, washed and permeabilized with 0.1% Triton-X, and blocked with the blocking buffer. Cells were incubated with the FITC-conjugated antibody to ERK1/2 [pTpY185/187] at 4°C overnight, washed and counterstained with propidium iodide (1:2,000) [Song et al., 2000, 2001; Klepeis et al., 2001].

RESULTS

Induction of ERK Activation With Injury or Wound Media

To determine if ERK1/2 is activated in response to injury, cells were wounded and the cell lysates were probed with anti-ERK and anti-phospho-ERK antibody. Unwounded cultures were evaluated for each experiment at each time point. Activation was normalized to total ERK and densitometric analysis was performed on all blots. Injury promoted rapid and transient phosphorylation of both ERK 1 (44–45 kDa) and ERK 2 (42 kDa) within 2 min (Fig. 1A). Densitometric analysis of the blots demonstrated that there was a 12-fold increase over control at 2 min that decreased significantly over 15 min. The blots represent the lysate of wounded and unwounded cells, and are an average of the entire culture (Fig. 1A). In addition, we demonstrated that the injury response could be inhibited when cells were incubated with the ERK inhibitor, U0126, immediately prior to the injury (data not shown). To localize the activation, a fluorescently conjugated antibody directed against the phospho- ERK 1/2 was used and activation was detected along the wound edge after injury and decreased with time (see arrowheads) (Fig. 1B). Activation was negligible in the unwounded cultures.

Previous experimental results demonstrated that cellular injury resulted in the propagation of a calcium wave to neighboring cells [Klepeis et al., 2001]. When wound media was collected and added to unwounded cells, activation of ERK 1 and 2 was detected within the same time course that was detected from injured cells (Figs. 1A and 2). Likewise, addition of control medium did not promote activation of ERK. Together these indicate

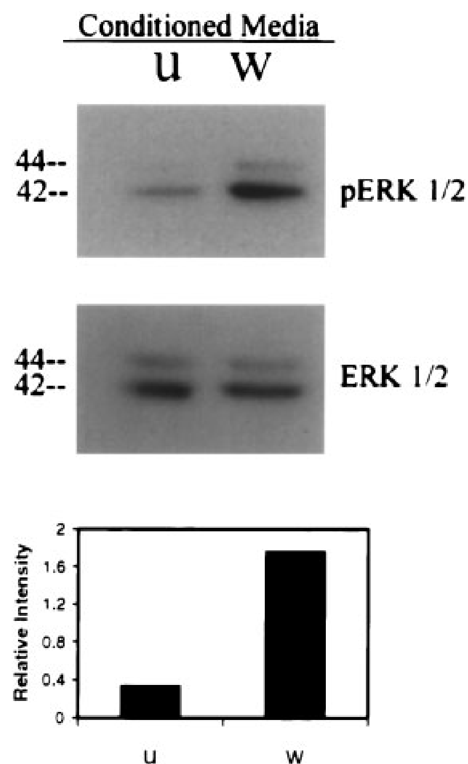

Fig. 2. Wound media activates MAP kinase. Confluent cells were cultured and a scrape injury was made and the medium was collected immediately and added to a second set of cultures (W). In a parallel experiment, media was collected from unwounded cultures and added to cultures and lysates were collected (U). Equivalent amounts of protein were resolved in SDS–PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases and densitometric analysis was performed. Blot is representative of four independent experiments.

that the active component is released with injury.

Response to EGF

Our laboratory and others have shown that EGF receptors are upregulated from corneal injury by 15 min that lasts for over 30 min [Zieske et al., 2000; Song et al., 2001, 2003]. As the active component is present in the wound media, we hypothesized that growth factors would be present in the wound media and play a role in mediating the response. We found that 5–10 ng/ml of EGF elicited a response with similar magnitude to that detected in response to injury (Fig. 3A). However, the concentration of EGF necessary to induce activation is significantly greater than that found in vivo (0.1 ng/ml). A time course demonstrated that EGF promoted a rapid (2 min) and sustained

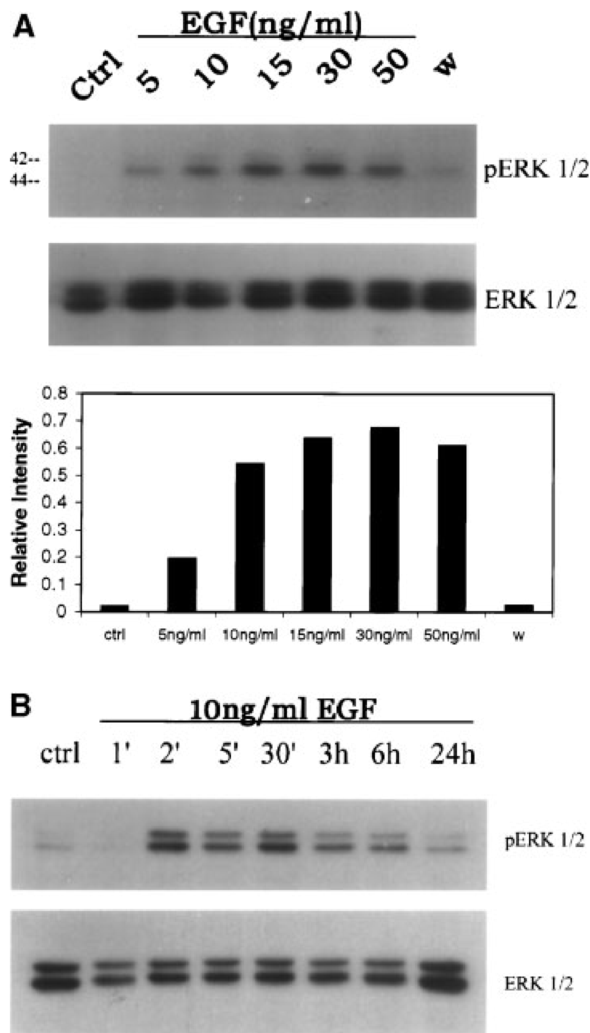

Fig. 3. Response to EGF. **A**: Confluent cells were cultured and varying concentrations of EGF were added to cell cultures and compared to control buffer (ctrl) and wounded (w). Lysates were collected and equal amounts of protein were resolved in SDS–PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were also probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases. Densitometric analysis of the blots shows relative activation. Blot is representative of three independent experiments. **B**: Confluent cells were cultured and incubated with 10 ng/ml EGF for periods of time ranging from 1 min to 24 h and compared to unwounded cultures (u). Lysates were collected and equal amounts of protein were resolved in SDS-PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were also probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases. Blot is representative of three independent experiments.

activation (over 6 h) of ERK, unlike the more rapid and transient injury induced activation (Fig. 3B). Furthermore, the addition of EGF to injured cells amplified the wound response (data not shown). However, while exogenous EGF promoted ERK 1/2 activation it was not detected in the wound media over a period from 2 min to 3 h by ELISA sandwich assay (Biosource International). Therefore, it is unlikely that EGF is a critical player in the initial ERK activation following injury.

Characterization of Wound Media

Several assays were performed to evaluate the active components(s) in the wound media. Cells were wounded and the wound medium was collected at 2 min and treated with proteinase K (10 μg/ml), heat denatured (100°C 10 min), or degraded with apyrase (30 U/ml), an exo-nuclease. The treated wound medium was added to cells and the activation of ERK was compared to control and untreated wound medium at 2, 5, and 15 min. When the wound media was incubated with apyrase (30 U/ml) activation of ERK 1 and 2 was inhibited. However, neither heat denaturation nor incubation with proteinase K inhibited the activation of ERK 1 or 2 (Fig. 4).

To further characterize the wound medium, size fractionation was performed. A number of preliminary experiments demonstrated that the active component was less than 50 kDa. Filters (3 kDa) were employed to distinguish between nucleotides (approximately 400–600 Da) and growth factors (most are greater than 3 kDa). Wound medium was collected and immediately fractionated. The high (retentate) and low (filtrate) molecular weight fractions of control and wounded cells were added back to cells to evaluate: (1) activation of ERK and (2) induction and propagation of a Ca^{2+} wave. Molecular mass(es) of the filtrate and retentate were verified using gradient gel electrophoresis followed by silver stain. Parallel control experiments were performed on fractionated medium collected from unwounded cells.

To identify which fractions could induce propagation of a Ca^{2+} wave, the retentate and filtrate fractions from wounded and unwounded cultures were added to cultures that had been previously loaded with Fluo-3AM (4 μM) and live cell imaging was performed. The ability to generate release of Ca^{2+} and propagation of waves to neighboring cells was compared to that of HEPES (negative control, data not shown), media from unwounded cells (rose line) and ATP (10 and 50 μM) (positive control, red line). As demonstrated previously, the addition of ATP caused a rapid increase in fluorescence intensity in the entire field of cells [Klepeis et al.,

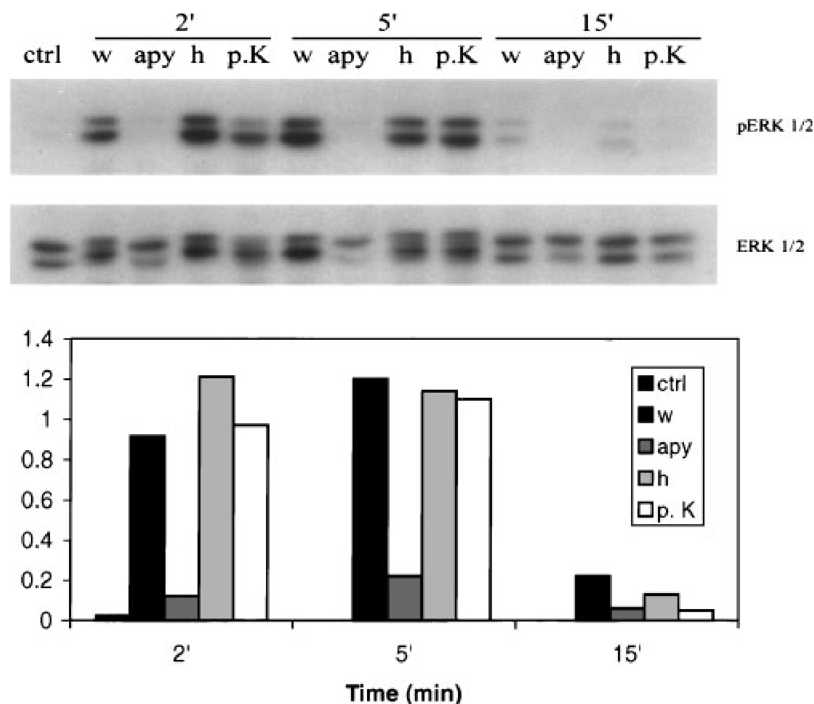

Fig. 4. Characterization of wound media. Wound media was collected after injury to cells and subjected to heat denaturation (h), proteinase K (PK) (10 μg/ml), or apyrase (apy) (30 U/ml). The treated wound media was added to cell cultures and lysates were collected at 2, 5, and 15 min. Treated wound media were compared to control (ctrl) and injured (w) cell lysates. Equal amounts of protein were resolved in SDS–PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were also probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases. Densitometric analysis of the blots show relative activation. Blot is representative of four independent experiments.

2001] (Fig. 5A). The data is expressed as % change in fluorescence of the entire 512×512 pixel field of cells over time. In all of the experiments, when a response was elicited, the entire field of cells responded. The addition of ATP resulted in an immediate increase in the release of Ca^{2+} that was followed later by oscillations. The addition of unfractionated wound media also caused an immediate increase (200% over HEPES baseline) (green line). Interestingly, only the addition of the filtrate fraction (low molecular mass fraction) (purple line) from wounded media elicited an immediate increase (120% over HEPES baseline) that attenuated rapidly with cell oscillations detected over the entire field. In contrast, the retentate media (higher molecular mass fraction) (blue line) did not elicit a release in Ca^{2+}. As predicted, neither the addition of media from unwounded cells (rose line) nor the filtrate fraction from these cultures induced a change in fluorescence intensity (Fig. 5A). All experiments were performed a minimum of three times from independent cultures.

To further evaluate the response to wound media, filtrate and retentate fractions were added to cells and activation of ERK1/2 was compared to control unwounded and wounded media. Neither the control nor the 0-time point promoted activation. After 2 and 5 min of incubation with the filtrate wound media fraction, activation was comparable to that of complete wound media (Fig. 5B). By 15 min, the filtrate wound media fraction only elicited minimal activation which was comparable to the complete wound media shown here and in Figure 1A. Minimal or no activation was induced by addition of the retentate fraction to cells at 2 min and activation remained less than 50% of the filtrate at 5 min. In addition, when the wound media was incubated with hexokinase there was a decrease but not complete loss of ERK activation. These all indicate that nucleotide(s) are released into the media.

Response to Nucleotides

The role of nucleotides on ERK1/2 activation was then evaluated from 0 to 60 min. Initial experiments were conducted in the presence or absence of ATP. We demonstrated that phosphorylation of ERK occurred by 2 min and that the response was negligible by 15 min, a time

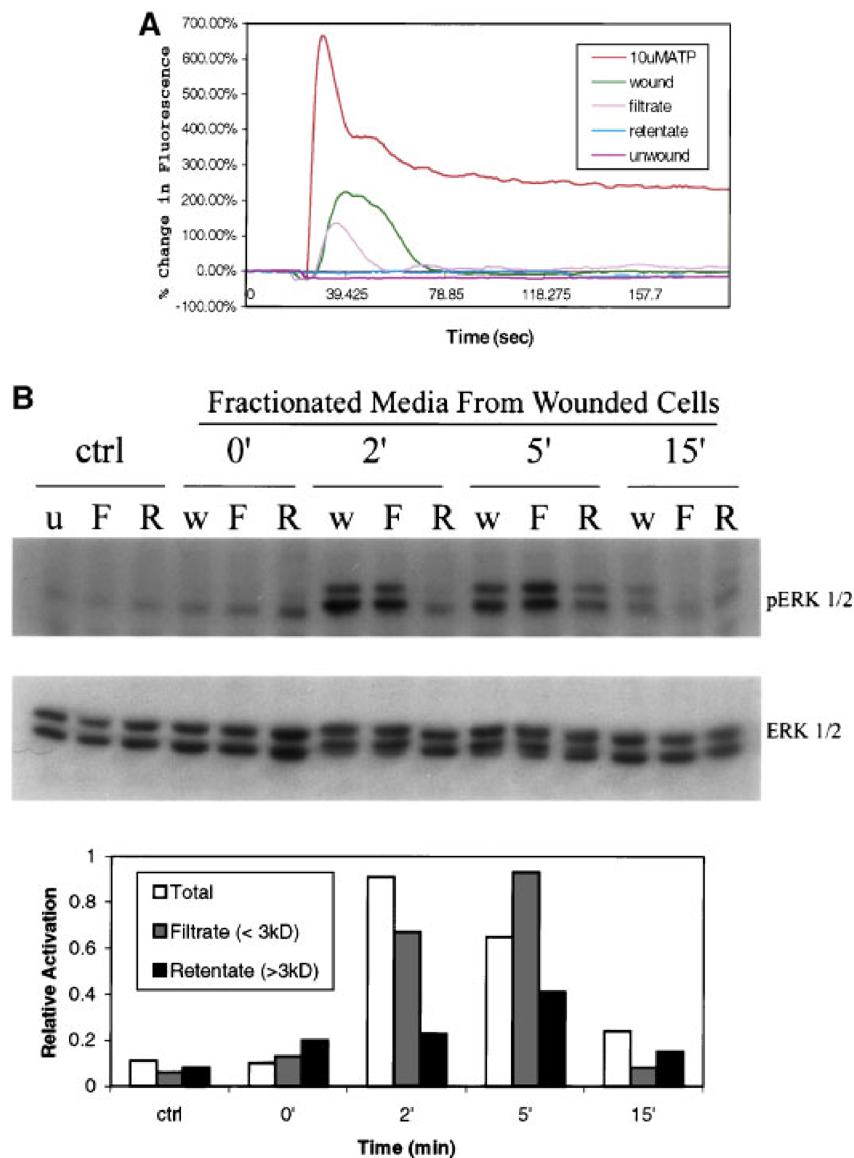

Fig. 5. Characterization of fractions of conditioned wound media. **A**: Evaluation of high and low molecular weight fraction activity using live cell imaging. Conditioned media from wounded or control cultures, filtrate and rentate fractions were added to fluo-3AM loaded cells and imaged using confocal microscopy. The data is presented as percent change in fluoresence ($(F-F_o)/F_o \times 100$). Wound media (green line), ATP (red line), and the filtrate (purple line) (a low molecular weight fraction (<3 kDa)) displayed a response. The unwounded media (rose line) and higher molecular weight fractions (retentate-blue line) did not induce a response. Graph is representative of three independent experiments. **B**: Activation of ERK with fractionated media. Conditioned media, filtrate, and rentate fractions from wounded and unwounded cultures were added to confluent cells. Lysates were collected and equal amounts of protein were resolved in SDS–PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases. Densitometric analysis shows relative activation. Data is representative of three experiments.

course that resembled the response to injury (Fig. 6A). Phosphorylation was not detected in the absence of ATP. Experiments were also conducted to evaluate the response to other nucleotides and we found that UTP was equipotent and equivalent in phosphorylation (Fig. 6B). In addition, the response to ATPγS was similar to that of ATP and UTP. When cells were exposed to ADP and UDP we found that the response was surprisingly similar to that of ATP and UTP (Fig. 6B). However, when experiments were performed in the presence of hexokinase, the response to UDP was lessened suggesting contamination of UDP with UTP. Incontrast, adenosine did not elicit any activation (data not shown), indicating that the P1

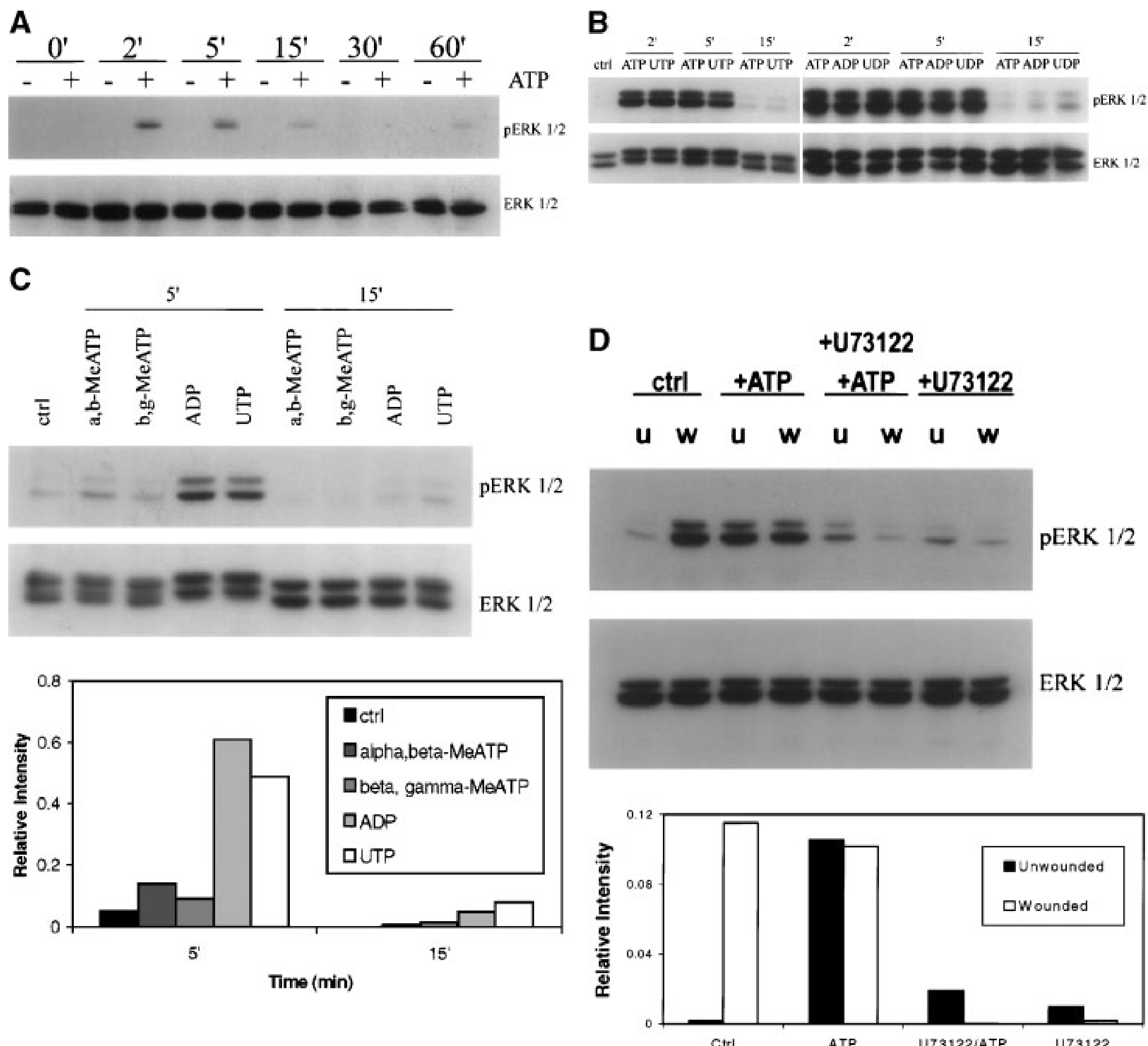

Fig. 6. Activation of epithelial cells by nucleotides. Confluent cells were cultured and incubated in the presence (+) or absence (−) of nucleotides for periods of time ranging from 0 to 60 min. **A**: Activation by 50 μM ATP. **B**: Comparison of activation by ATP, UTP, ADP, and UDP at three time points. **C**: Cells stimulated with α,β-MeATP (a,b-MeATP), β,γ-MeATP (b,g-MeATP), and responses compared to ADP, UTP, and ctrl. **D**: Activation by ATP, injury, and or both was inhibited by U73122. Lysates were collected and equal amounts of protein were resolved in SDS–PAGE and blotted with an anti-phospho-ERK/MAP kinase (MAPK) antibody. Membranes were also probed with an anti-MAP kinase antibody that recognizes both active and non-active forms of MAP kinases. Data is representative of three independent experiments.

receptor is not critical for activation. To further assess whether P2Y or P2X receptors were involved in the injury-induced signaling cascade, we evaluated the response to α,β-MeATP and β,γ-MeATP and compared the response to that of ADP and UTP at 5 and 15 min (Fig. 6C). α,β-MeATP and β,γ-MeATP are P2X receptor-specific agonists while ADP and UTP are P2Y receptor-specific. At both time points, there was no activation of ERK 1 and 2 by the synthetic agonists, while the nucleotides (ADP, UTP) induced a typical response. Phosphorylation of ERK was also detected in the total antibody with an increase in molecular mass (Fig. 6C). Furthermore, live cell imaging experiments have shown that while all the cells in a field of view respond to the four nucleotides, there is a repeatable potency response that is dose dependent (ATP > UTP > ADP > UDP). Together, these indicate the potential activation of purinergic receptors; P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11.

To determine the role of PLC on the injury induced response and nucleotide activation, cultures were preincubated with the phospholipase C inhibitor, U73122 and then either injured or stimulated with nucleotide and/or injured and

stimulated with nucleotide. We found that U73122 was capable of diminishing or inhibiting the activation of ERK by injury alone, by exogenous ATP or injury accompanied by addition of ATP (Fig. 6D). Together, these data indicate that injury to cells causes release of nucleotides that bind to purinergic receptors and activate a P2Y purinergic signaling pathway.

DISCUSSION

Wound healing is a complex process that includes cell signaling, gene expression, cell proliferation, cell adhesion, and migration (review by Clark, 1996). Our goal was to characterize the events that occur immediately after injury and identify the factors that activate the response. Previously, injury to cells has been shown to activate the EGFR while migration can be inhibited with tyrphostin AG1478 [Zieske et al., 2000; Song et al., 2001] or by transfection with ErbB1 anti-sense RNA [Song et al., 2003]. Previously, others have demonstrated that a number of growth factors are released in response to injury and affect the wound response [Schultz et al., 1991; Wilson et al., 1992; Bennett and Schultz, 1993]. The majority of these investigations were focused on events that occur after cells become migratory and are actively repairing the void caused by the injury. More recently, we demonstrated that calcium waves were generated within seconds after injury and these could be selectively enhanced by EGF [Klepeis et al., 2001]. However, it is still not well understood what factors generated the injury response.

Previously, investigators have shown that mechanical stimulation of cells causes an immediate transient elevation in $[Ca^{2+}]_i$ that spreads to neighboring cells [Charles et al., 1991; Hansen et al., 1993; Enomoto et al., 1994; Frame and de Feijter, 1997]. The response to physical injury induced a similar increase in $[Ca^{2+}]_i$ that spread to neighboring cells and decreased over time and distance (representative of a non-regenerative wave). Propagation of a Ca^{2+} wave may occur via gap junctions [Hansen et al., 1993; Boitano et al., 1994; Venance et al., 1997] or across acellular regions [Enomoto et al., 1994; Hassinger et al., 1996; Sammak et al., 1997; Guthrie et al., 1999; Klepeis et al., 2001]. The latter results suggest that injury induces the release of an extracellular messenger that diffuses from the injury independent of gap junctions. Our laboratory has demonstrated that the injury induced response was independent of gap junctions in epithelial cells that express connexins [Klepeis et al., 2001]. Further complicating the picture is evidence in connexin deficient glioma cells that Ca^{2+} signals have been shown to be received but not propagated [Cotrina et al., 2000]. In this study, we investigated the factors that are released when a localized injury to epithelial cells occurs causing the release of Ca^{2+} and propagation of a wave to neighboring cells and triggers the activation of ERK.

In mesangial cells ATP and UTP were demonstrated to activate both the MAPK and the p38-MAPK cascade [Huwiler and Pfeilschifter, 1994; Huwiler et al., 2000]. These nucleotides have recently been shown to activate the PKB/Akt pathway which is hypothesized to play a role in cell growth [Huwiler et al., 2002]. In monocytic cells, the depletion of extracellular or intracellular stores did not significantly alter the ability of growth factors to induce MAPK activation [Santiago-Perez et al., 2001]. In this study, we demonstrated that factors released from an injury triggered the activation of MAPK. This activity could be inhibited by apyrase but not by proteinase K, trypsin, or chymotrypsin. In other experiments, we demonstrated that the addition of wound media and/or nucleotides resulted in the release of Ca^{2+}. In addition, when epithelial cells were preincubated with thapsigargin which results in a rapid release of Ca^{2+} from IP_3 mediated stores, MAPK activation was enhanced. Additionally, the release of the active factor from injury seems to occur prior to that of growth factors such as EGF. Taken together, these results suggest that nucleotides play a major role in the early injury induced events.

Cell signaling via extracellular nucleotides and nucleosides has been demonstrated in both excitable and non-excitable cells [Neary et al., 1999] where they were shown to mediate both proliferative and cytoskeletal alterations on astrocytes, PC12 cells, airway cells, and endothelial cells [Murrin and Boarder, 1992; Wolkoff et al., 1995; Neary et al., 1996, 1999; Pelleg and Shulman, 2002]. In some cell types, extracellular ATP plays a role in mitogenicity, while at other times it has been demonstrated to be antiproliferative [Rapaport et al., 1983; Schulze-Lohoff et al., 1992]. The P2Y receptors have been found on a large variety of cell types ranging from freshly isolated cells to cell lines

[Dubyak, 1999] and they are involved in a number of signaling pathways. It has been demonstrated that the purinergic signaling pathway is involved in the response of astrocytes to injury where the release of purines following injury such as hypoxia may contribute to common neurological disorders such as stroke, degenerative, and demyelinative disorders [Norenberg et al., 1994]. In addition, vascular function has been shown to be mediated by the P2 family of receptors [Boarder and Hourani, 1998] and there is preliminary evidence that the addition of nucleotides generates oscillatory behavior in specific human endothelial cell populations. Furthermore, the release of di- and tri-nucleotides released in response to injury is currently thought to play a role in airway disorders [Pelleg and Schulman, 2002]. In this study, we demonstrated that injury elicits a localized and transient response. We hypothesize that the injury response that is generated in epithelial and other cells may be a mechanism that cells employ to promote localized cell–cell communication.

We have demonstrated that injury induced a signaling pathway distinct from that induced by EGF. In addition, we have shown that the EGF response can be inhibited by tyrphostin while the injury response is not [Klepeis et al., 2001]. EGF was not detected in the wound media immediately after injury and its activation of MAPK has a longer duration than that seen with wound media. However, the addition of EGF to wounded cells does enhance the injury response. These results correlate with those obtained from PC12 cells where NGF and ATP enhance ERK activation [D'Ambrosi et al., 2001].

When the wound media was fractionated we found that the active component had a low molecular mass (<3 kDa). Furthermore, it possessed a time course of activation similar to that of the complete wound media, in contrast to the higher molecular weight fractions. Treatment of this fraction with apyrase inhibited the activation of ERK, while incubation with proteinase K or heat denaturation did not alter the activity. In addition, the low molecular weight fraction from wounded cells induced a brief non-regenerative Ca^{2+} wave while the retentate did not induce a wave further supporting our hypothesis that nucleotides were the primary factors that communicated information to other cells immediately after injury. This is further supported by the recent evidence that diadenosine polyphosphates are normal constituents of tear fluid and are hypothesized to stimulate tear secretion via interaction with P2Y receptors in vivo [Pintor et al., 2002]. In addition, mechanical stimulation of bronchial epithelial cells has been shown to cause the release of ATP and generate short non-regenerative Ca^{2+} bursts [Arcuino et al., 2002]. Extracellular nucleotides have also been localized to the surface of living cells that had been stressed [Wang et al., 2000]. The cells that released ATP have been demonstrated (in at least one cell line) to be viable as calcein was taken up and retained [Arcuino et al., 2002]. The ATP that is released thus either diffuses into the adjacent extracellular medium or is hydrolyzed by ecto-ATPases. Taken together, these indicate that release of ATP is one method that cells can employ to rapidly and transiently communicate to cells in a localized region.

The addition of ATP, UTP, ADP, UDP to cells confirmed that nucleotides were capable of promoting a rapid and transient activation of ERK similar to that of injury. However, the addition of the nucleoside, adenosine, did not elicit a response indicating that the release of nucleotides with injury acts via a P2 receptor and not hydrolysis to adenosine and subsequent binding to adenosine receptors. This is in contrast to the signaling pathways found in mast cells [Gao et al., 1999]. In addition, agonists for P2X receptors did not activate ERK. These results indicate that the injury response is mediated via a P2Y receptor signaling pathway. Experiments demonstrating that ATP and UTP elicited activation indicate the presence of P2Y2, P2Y4, or P2Y11 receptors. This is further supported by preliminary experiments in the presence of reactive blue, an antagonist to P2Y11, where inhibition of the ATP response suggests the presence of the P2Y11 receptor. While response to both ADP and UDP indicate the presence of P2Y1 and P2Y6 receptors, respectively, it was not known whether contaminants of UTP could explain the response to UDP. Incubation with hexokinase did decrease the response but did not inhibit the activation of MAPK or propagation of a Ca^{2+} wave indicating that the P2Y6 receptor is expressed. Furthermore, incubation of the wound media with hexokinase also resulted in a decrease, verifying the pharmacological induced response to UDP. These all indicate that while ATP can

be detected in the wound media using a luminometer, a number of nucleotides may also be present. In fact it has been demonstrated that cells possess cytosolic levels of ATP ranging from 3 to 5 mM while the concentration of UTP and UDP is several fold less [Werner et al., 1991; Warny et al., 2001].

Regardless of which P2Y receptors are activated and which nucleotides are involved, all the P2YR's are known to activate PLC-β. Purinergic signaling has been hypothesized to elicit an intracellular signaling cascade that is initiated with the production of IP_3 and diacylglycerol by the action of PLC resulting in the release of Ca^{2+} from intracellular stores [North and Barnard, 1997]. Our results demonstrating that the PLC inhibitor, U73122, inhibited activation of the injury response in the presence or absence of ATP are consistent with this. Further studies will include evaluating the role of phospholipase C subfamilies in the injury induced signaling cascade and explore the interaction between specific subtypes of purinergic and EGF receptors.

We propose that a simple yet elegant mechanism exists in cells where a localized injury causes the release of a nucleotide that increases cytosolic Ca^{2+} resulting in a non-regenerative wave in a localized region. In addition, the redundancy of purinergic receptors demonstrates that these biological events are tightly controlled and play a critical role in maintaining the balance in injury and disease.

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