Wounding Sheets of Epithelial Cells Activates the Epidermal Growth Factor Receptor through Distinct Short- and Long-Range Mechanisms

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Wounding epithelia induces activation of the epidermal growth factor receptor (EGFR), which is absolutely required for induction of motility. ATP is released from cells after wounding; it binds to purinergic receptors on the cell surface, and the EGFR is subsequently activated. Exogenous ATP activates phospholipase D, and we show here that ATP activates the EGFR through the phospholipase D2 isoform. The EGFR is activated in cells far (>0.3 cm) from wounds, which is mediated by diffusion of extracellular ATP because activation at a distance from wounds is abrogated by eliminating ATP in the medium with apyrase. In sharp contrast, activation of the EGFR near wounds is not sensitive to apyrase. Time-lapse microscopy revealed that cells exhibit increased motilities near edges of wounds; this increase in motility is not sensitive to apyrase, and apyrase does not detectably inhibit healing of wounds in epithelial sheets. This novel ATP/PLD2-independent pathway activates the EGFR by a transactivation process through ligand release, and it involves signaling by a member of the Src family of kinases. We conclude that wounding activates two distinct signaling pathways that induce EGFR activation and promote healing of wounds in epithelial cells. One pathway signals at a distance from wounds through release of ATP, and another pathway acts locally and is independent on ATP signaling.

INTRODUCTION

When an epithelium is wounded, cells at the edge undergo profound phenotypic changes and subsequently acquire an ability to migrate rapidly. Epithelial healing has been studied extensively using genetic, biochemical, and cell biological approaches, and it is now recognized to be a complex phenomenon involving interaction of the cells with extracellular matrix, with underlying stromal cells, with growth factors, and with various inflammatory cells. The initial response of epithelial cells is typically a rapid initiation of cell migration followed later by replenishment of lost cells by cell division (Jacinto *et al.*, 2001; Fini and Stramer, 2005; Jane *et al.*, 2005; Netto *et al.*, 2005; de Giorgi *et al.*, 2007; Jang *et al.*, 2007; Raja *et al.*, 2007).

Activation of the epidermal growth factor receptor (EGFR) is absolutely required for induction of motility in many epithelia including the corneal epithelium and the epidermis after wounding (Hansen *et al.*, 1997; Zieske *et al.*, 2000; Block *et al.*, 2004; Repertinger *et al.*, 2004; Xu *et al.*, 2004). Activation occurs as a result of proteolytic release of ligands of the EGFR, which resembles the well-characterized triple membrane-passing signaling pathway that causes trans-

activation of the EGFR after stimulation of numerous G-protein coupled receptors (Fischer *et al.*, 2003; Ohtsu *et al.*, 2006).

Recently, a few upstream signals that activate the EGFR after wounding sheets of epithelial cells have been identified. We have reported that phospholipase D (PLD), which catalyzes hydrolysis of phosphatidylcholine to the second-messenger phosphatidic acid (PA) (Exton, 2002; McDermott *et al.*, 2004; Jenkins and Frohman, 2005; Cazzoli *et al.*, 2006), is activated rapidly after wounding sheets of corneal epithelial cells, which in turn activates the EGFR (Mazie *et al.*, 2006). Extracellular ATP provides a second signal that has been identified to be upstream of EGFR activation. ATP is released after wounding sheets of epithelial cells and results in transactivation of the EGFR through the G-protein-coupled P2Y class of purinergic receptors (Klepeis *et al.*, 2004; Yang *et al.*, 2004; Boucher *et al.*, 2007; Yin *et al.*, 2007).

The EGFR transactivation processes induced by exogenously added ATP and PA are similar, and ATP is known to induce activation of PLD in many systems (el-Moatassim and Dubyak, 1992; Gargett *et al.*, 1996; Sun *et al.*, 1999; Kusner and Adams, 2000; Perez-Andres *et al.*, 2002; Pochet *et al.*, 2003; Le Stunff *et al.*, 2004). This leads to the hypothesis that ATP signals through PLD to activate the EGFR. Because ATP is freely diffusible, and the major driving forces for epithelial migration after wounding appear to be derived mainly from the first few rows of cells from the wound edge (Fenteany *et al.*, 2000; Farooqui and Fenteany, 2005), we were interested in analyzing the spatial aspects of EGFR activation in wounded epithelial cell sheets.

MATERIALS AND METHODS

Materials

Antibodies against phospholipase D2 PLD2 were the generous gift of Dr. Sylvain G. Bourgoin (Université Laval, Québec, Canada). Antibodies against

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Abbreviations used: AR, amphiregulin; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase; HCLE, human corneal-limbal epithelial; KSFM, keratinocyte serum-free medium; PA, phosphatidic acid; PLD, phospholipase D; RB2, reactive blue 2.

PLD1, EGFR, EGFR phosphorylated on tyr-1173, ERK1, and ERK1/2 phosphorylated on thr-202 and tyr-204 were from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing antibodies against AR, HB-EGF, and TGF α were from R&D Systems (Minneapolis, MN). Antibodies against Src-family kinases phosphorylated on tyr-416 and to the corresponding nonphosphorylated peptide were from Cell Signaling Technology (Beverly, MA). ATP, grade VII apyrase from potato, and reactive blue 2 were from Sigma (St. Louis, MO). Tyrphostin AG 1478, PP2, and Src kinase inhibitor 1 were from EMD Biosciences (San Diego, CA). Phosphatidic acid (1,2-dioctanoyl-*sn*-glycero-3-phosphate) was from Avanti Polar Lipids (Alabaster, AL). Cell culture reagents were from Mediatech (Herndon, VA), and other reagents and supplies were from Thermo Fisher Scientific (Pittsburgh, PA), unless noted.

Cell Culture and siRNA Transfection

Human corneal limbal epithelial (HCLE) cells (Gipson *et al.*, 2003) were cultured in human keratinocyte serum-free medium (KSFM, Invitrogen, Carlsbad, CA) supplemented with 0.3 mM CaCl₂, 25 μ g/ml bovine pituitary extract, and 0.2 ng/ml epidermal growth factor (EGF). Before stimulations, cells were cultured for 4–5 h in the same medium without pituitary extract and EGF. Small interfering RNA (siRNA) oligonucleotides encoding the sense and antisense target sequences were synthesized by Applied Biosystems (Foster City, CA). The PLD1 siRNA was GUUAAGAGGAAAUUCAAGC, and the PLD2 siRNAa and siRNAb were UGGGGCAGGUACUUUGCU and AGUCUUGAUGAGGUCUGCUC, respectively. BLAST searches (Altschul *et al.*, 1990) with siRNA sequences revealed significant sequence homologies with only the targeted mRNAs. Unless otherwise noted, 50 nM siRNA was transfected into subconfluent cells at the time of seeding using the siPORT NeoFX lipid-based reverse transfection reagent (Applied Biosystems). Two days after transfections, cells were reseeded to generate confluent cultures and were used the following day. For every experiment reported here, expression of the relevant PLD isoforms was monitored by Western blot and found to be similar to that shown in Figure 2, A and B.

Wounding Models

For analysis of signaling in cells near wounds, HCLE cells were grown to confluence around agarose droplets, forming lanes one to five cells wide, and the cell sheets were acutely wounded by removal of the droplets, as described previously (Block *et al.*, 2004). Protocols for Western blotting and the assay for PLD activity were described previously (Mazie *et al.*, 2006).

For analysis of signaling in cells far from wounds, HCLE cells were grown to confluence around a single agarose strip, and the cell sheets were wounded by the removal of the strip. Reactions were stopped 10 min later by plunging the bottom of the dish into ice water and replacing the medium with ice-cold phosphate-buffered saline (171 mM NaCl, 10.1 mM Na₂HPO₄, 3.35 mM KCl, 1.84 mM KH₂PO₄, pH 7.2). Cells proximal to the wound were scraped using a polyethylene cell lifter (Corning Costar, Acton, MA) trimmed to 7 mm, effectively removing $\sim$ 3 mm of cells from each side of the wound, and the remaining cells were lysed directly in 1% SDS.

To quantitate wound healing, HCLE cells were grown to confluence around a single agarose strip (Block *et al.*, 2004) and induced to differentiate into a stratified epithelium (Gipson *et al.*, 2003). Cells were transferred to Dulbecco's modified Eagle's medium:F-12 1:1 with 10% newborn calf serum (NCS), the agarose strip was removed, and healing was allowed to progress 14–18 h before fixation. Wound healing was monitored by measuring the widths of wounds, as described previously (Block *et al.*, 2004). Experiments with mitomycin C have previously demonstrated that wounds in HCLE cells heal as a result of cell migration (Mazie *et al.*, 2006).

Assays for AR and ATP Release

HCLE cells were incubated with KSFM without EGF and pituitary extract for 10 min. Conditioned medium was collected and centrifuged for 2 min at 5000 $\times$ g. The cell-free supernatants were aliquoted and stored at -20°C until further processing. AR was measured in the supernatants using the DuoSet ELISA (R&D Systems) according to manufacturer's protocol. ATP was measured in the cell-free tissue culture supernatants with the ATP Bioluminescent Assay Kit (Sigma). For normalization purposes, protein content of whole cell extracts was determined by the BCA protein assay (Pierce, Rockford, IL).

Immunofluorescence Analysis of Wounded Layers of HCLE Cells

HCLE cells were grown to confluence around a single agarose strip and were wounded by the removal of the strip. Reactions were stopped 10 min later by addition of 1/10 volume 37% formaldehyde. Cells were processed for immunofluorescence analysis as previously described (Block *et al.*, 2004). Seven contiguous aligned fields (a total of 5 mm from the wound edge) per sample were captured on a Nikon TE2000E automated microscope (Melville, NY) with a 10 $\times$ objective (NA 0.3) using the MetaMorph scanline utility (Universal Imaging, West Chester, PA). For quantitation of phospho-ERK signal intensities, images were acquired at identical exposures, and the average intensities of 250- or 62.5- μ m-wide regions were recorded for the entire 5-mm width of the image.

Figure 1. Wounding induces ATP release, leading to activation of PLD. (A) ATP contents in conditioned media were determined in HCLE cell cultures that were unwounded (NT), wounded, and incubated with medium for 10 min (W) or treated similarly in the presence of 5 U/ml apyrase (W+APY). ATP content was normalized to protein content of whole cell extracts. (B) PLD activities in HCLE cells that were unwounded (NT) or wounded (W) in the presence of 30 U/ml apyrase (APY) where indicated and incubated with medium for 5 min. The data points in A and B are the means of quadruplicates; error bars, SDs. Analysis of variance followed by Bonferroni's multiple comparison test showed that apyrase significantly reduced PLD activation by wounding ($p < 0.001$). The results are representative of at least three repetitions in this and the following figures.

Live Cell Microscopy

HCLE cells were stratified around agarose strips in a 12-well dish, and medium was changed to 1:1 DMEM:F-12 with 10% NCS 24 h before experiment. Cells received no treatment or 30 U/ml apyrase, and agarose strips were left in place or removed, such that $n = 3$ for each condition. Cell migration was monitored for the subsequent 24 h with a Live Automated Cell Imager (Schmidt *et al.*, 2008): cells were maintained in an environmentally controlled chamber (37°C and 5% CO₂) on a robotic stage and were visualized with an inverted Nikon Eclipse TE 2000 U microscope equipped with a 10 $\times$ objective and Photometric ES CoolSnap CCD camera (Woburn, MA). Time-lapse images were created, and cell velocities were calculated using MetaMorph by manually tracking individual cells. In each well, four cells from each of two regions at the wound edge and four cells from each of two regions 2 mm from the wound were analyzed. Cells were chosen arbitrarily, before viewing the time-lapse movie. The analysis was blinded to apyrase treatment, and in cells distal to the edge, the analysis was blinded to wounding. Because of errors in stage movement and manual tracking, the velocity of a fixed object was determined to be 0.1 μ m/min, which was subtracted from calculated cell velocities.

RESULTS

Wounding Activates PLD2 through ATP Signaling

Mechanically wounding sheets of epithelial cells results in robust release of ATP, presumably mostly as a result of leakage from damaged cells (Klepeis *et al.*, 2001; Yang *et al.*, 2004; Yin *et al.*, 2007). We have found that major cell damage is not required for healing of wounds in sheets of corneal epithelial cells by a more gentle procedure that is based on removal of agarose strips or droplets in the cell layer (Block *et al.*, 2004). To test whether ATP is released in this wounding assay, we used HCLE cells, which are human corneal limbal epithelial cells that have been immortalized by abrogation of p16^{INK4A}/Rb and p53 functions and overexpression of the catalytic subunit of the telomerase holoenzyme (Gipson *et al.*, 2003). As is seen in Figure 1A, wounding by this procedure also results in significant release of ATP. We have previously shown that wounding sheets of HCLE cells results in activation of PLD, and to determine whether this is a consequence of the released ATP, cell sheets were wounded in the presence of apyrase, which dephosphorylates extracellular ATP and ADP to AMP. Inclusion of 5 U/ml apyrase eliminated ATP effectively from the medium (Figure 1A), and apyrase reduced the PLD activity to basal levels after wounding (Figure 1B), which implies that activation of PLD is stimulated by the ATP that is released after wounding.

Figure 2. Wounding selectively activates the PLD2 isoform. (A) Western blots of whole cell extracts of HCLE cells untransfected (No siRNA) or transfected with siRNA against PLD1 or PLD2 cultured around agarose droplets. The Western blotting membrane was cut guided by molecular-

weight markers and immunoblotted with antibodies against PLD1, PLD2, or β -actin as a load control. PLD2 is 20 kDa smaller than PLD1. (B) Densitometry of immunoblots transfected with the indicated siRNAs. The values are means of six determinations; error bars, SDs. (C) PLD activities in HCLE cell sheets untransfected (NT) or transfected with siRNAs against PLD1 or PLD2 and wounded (W), as described in *Materials and Methods*. Data points are the means of triplicates; error bars, SDs. Analysis of variance followed by Bonferroni's multiple comparison test showed that PLD2 siRNAa significantly reduced PLD activation by wounding ($p < 0.001$).

No satisfactory chemical inhibitor of PLD activity currently exists, so we chose an siRNA-based approach to evaluate whether PLD is necessary for wound- and ATP-stimulated EGFR activation. Mammalian cells express two PLD isoforms, PLD1 and PLD2. SiRNA oligonucleotides targeted to PLD1 and PLD2 were transfected into HCLE cells, and whole cell extracts were prepared 3 d after transfection, and as illustrated by the immunoblots in Figure 2, A and B, the siRNA-mediated knockdowns of the PLD1 or PLD2 isoforms were efficient and specific. Quantitation of the intensities of the bands shows that expression of PLD1 was reduced by 70% and PLD2 by 80%. PLD activation after wounding was greatly diminished in cells transfected with one of the oligonucleotides (PLD2 siRNAa) but was unaffected in cells transfected with PLD1 siRNA, and knockdown of both isoforms had no greater inhibitory effect than knockdown of the PLD2 isoform alone (Figure 2C). The PLD2 siRNAa was also seen to reduce expression of PLD2 at lower concentrations, and a second PLD2 siRNA oligonucleotide (PLD2 siRNAb), which was made to control for off-target effects, also reduced the level of PLD2 expression (cf. Supplemental Figure S5). These results suggest that wound-induced PLD activation is predominantly determined by the PLD2 isoform. In the following experiments using siRNAs, PLD1 and 2 were verified to be down-regulated to levels similar to that shown in Figure 2.

ATP Induces EGFR Transactivation through PLD2 Signaling

Exogenously added ATP stimulates PLD activity in HCLE cells (Figure 3A), as it does in many other cell types (el-Moatassim and Dubyak, 1992; Gargett *et al.*, 1996; Sun *et al.*, 1999; Kusner and Adams, 2000; Perez-Andres *et al.*, 2002; Pochet *et al.*, 2003; Le Stunff *et al.*, 2004). Similarly to wound-stimulated PLD activity, ATP-stimulated PLD activity was blocked in HCLE cells transfected with PLD2 siRNAa but

Figure 3. The EGFR is activated by ATP through PLD2 signaling. (A) PLD activities in confluent HCLE cells untreated (NT) or transfected with siRNA against PLD1, PLD2, or both and then treated for 5 min with 50 μ M ATP. Analysis of variance followed by Bonferroni's multiple comparison test showed that PLD2 siRNAa significantly reduced PLD activation by ATP ($p < 0.001$). (B) PLD activities in confluent HCLE cells untreated (NT) or treated for 5 min with 50 μ M ATP (ATP), 50 μ M ATP that had been preincubated for 15 min with 5 U/ml apyrase (ATP+APY), or 50 μ M ATP after 15 min pretreatment of cells with 10 μ M tyrphostin AG 1478 (ATP+AG). Data points in A and B are the means of triplicates; error bars, SDs. (C) Confluent HCLE cells were untreated (NT), treated with 50 μ M ATP for 10 min (ATP), transfected with siRNA against PLD2 (siPLD2a), or transfected with PLD2 siRNAa and treated with ATP (ATP+siPLD2a), and whole cell extracts were analyzed by Western blotting for EGFR phosphorylated on tyr-1173 (pEGFR). The membrane was stripped and reprobed with antibodies recognizing total EGFR (EGFR). (D) Densitometry of the experiments illustrated in C. The data points are means of four determinations; error bars, SDs. (E) Confluent HCLE cells untransfected or transfected with siRNA against PLD1 or PLD2 were untreated or treated with 50 μ M ATP for 10 min. Conditioned media were collected and assayed for AR concentration and normalized to protein content of whole cell extracts. Data points are the means of six determinations; error bars, SDs. Analysis of variance followed by Bonferroni's multiple comparison test showed that PLD2 siRNAa significantly reduced EGFR activation and AR release by ATP ($p < 0.001$).

not in cells transfected with PLD1 siRNA (Figure 3A). Stimulation with apyrase-treated ATP (Figure 3B) or with 50 μ M adenosine (not shown) did not activate PLD, indicating that activation is a direct effect of ATP. Addition of the EGFR kinase inhibitor tyrphostin AG 1478 did not inhibit activation of PLD by ATP (Figure 3B), although it completely inhibited EGFR activation by ATP in parallel incubations (not shown), indicating that the observed PLD activation is not a result of EGFR signaling.

To test the role of PLD signaling in ATP-stimulated EGFR activation, cells were transfected with siRNA oligonucleo-

Figure 4. EGFR activation in wound-distal cells is dependent on ATP signaling. (A) HCLE cells were treated with 30 U/ml apyrase (APY) as indicated. Wound-distal cells were analyzed as described in *Materials and Methods*. Membranes were cut and probed with antibodies against the phosphorylated forms of EGFR (pEGFR) and ERK1/2 (pERK1/2). The membranes were subsequently stripped and reprobed using antibodies detecting total levels of the kinases (EGFR, ERK1). (B) Western blots from three independent experiments, each performed in triplicate, were subjected to analysis by densitometry, and the ratio of the signal intensities of phosphorylated EGFR (pEGFR) and total EGFR (EGFR) compared with untreated controls was plotted. Data points represent means; error bars, SDs. Analysis of variance followed by Bonferroni's multiple comparison test showed that apyrase significantly reduced EGFR activation by wounding ($p < 0.001$).

tides. Activation was monitored by Western blotting with an antibody that recognizes the EGFR phosphorylated on tyrosine 1173. Both PLD2 siRNAa and siRNAb blocked EGFR activation, whereas PLD1 siRNA was inactive (Figure 3, C and D, Supplemental Figure S1). Transfection of PLD2 siRNA did not block activation of the EGFR by EGF (Supplemental Figure S2). This provides direct evidence that activation of the EGFR by ATP is mediated through PLD2 signaling.

Extracellular ATP transactivates the EGFR through stimulation of proteases at the cell surface, which cleave precursors of ligands for the receptor (Boucher *et al.*, 2007; Yin *et al.*, 2007). To establish a measure of the transactivation process, we assayed one such ligand, amphiregulin (AR), and found that wounding, addition of ATP, and stimulation with a water-soluble analog of PA, 1,2-octanoyl-*sn*-glycero-3-phos-

phate, all increased AR release (Supplemental Figure S3). Furthermore, neutralization experiments indicated that AR contributes to the activation of the EGFR in response to these treatments (Supplemental Figure S4). Because ATP activates the EGFR through PLD2, reduction of expression of PLD2 was expected to decrease secretion of AR in response to ATP. Reducing the level of PLD2 resulted in reduction of basal levels of secretion of AR and a larger reduction of the response to ATP stimulation, whereas interfering with the cellular levels of PLD1 had no effect (Figure 3E). The reduction of PLD2 by the two siRNA oligonucleotides and AR release correlate closely, suggesting that the reduction of AR release is the result of knockdown of PLD2 rather than of off-target effects (Supplemental Figure S5).

ATP Functions as a Long-Range Messenger that Activates the EGFR

Because ATP is freely diffusible, we examined whether extracellular ATP mediates EGFR activation in cells at a distance from wound edges (for details of the procedure, see *Materials and Methods*). Inclusion of apyrase in the medium had no detectable effect on basal levels of EGFR activity, but it abolished wound-induced stimulation (Figure 4). Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are downstream targets of the EGFR that are important for wound healing (Rubinfeld and Seger, 2005; Katz *et al.*, 2007; McKay and Morrison, 2007), and they responded similarly. As a control, we noted that treatments with apyrase did not reduce the activation of EGFR and ERK1/2 kinases after addition of EGF (Supplemental Figure S2).

To visualize the extent of EGFR signaling induced by extracellular ATP directly, we performed immunofluorescence microscopy after removal of a single agarose strip. We were not able to identify an antibody to the activated EGFR that generated a satisfactory read-out, but phospho-ERK1/2 antibodies produced good signals. ERK1/2 were activated at least up to 0.5 cm from the wound edge (Figure 5). The specificity of the immunofluorescence signals was verified by the fact that UO126, which inhibits ERK1/2 activation, blocked the signals, and the EGFR-dependence of the signals was verified by the observation that they were quenched by AG 1478, which inhibits the EGFR kinase. Inclusion of

Figure 5. Activation of ERK1/2 is dependent on signaling by ATP in cells far from, but not near wounds in HCLE cells. Sheets of HCLE cells were cultured around an agarose strip and were left unwounded (No Wound), wounded (Wound), or wounded in the presence of 30 U/ml apyrase (Wound + APY), fixed 10 min later, and stained with antibodies recognizing the activated forms of ERK1/2. Images were acquired and analyzed as described in *Materials and Methods*. Results of quantitative analysis are shown below the immunofluorescence images, in the same scale. Images of cells wounded in the presence of 10 μ M UO126 (wound + UO) or 1 μ M tyrphostin AG 1478 (wound + AG) were also analyzed.

Figure 6. The EGFR is activated in wound-proximal cells independently of signaling by ATP and PLD2. (A) HCLE cells were wounded by removal of agarose droplets with or without 30 U/ml apyrase (APY). Membranes were probed with antibodies that recognize the phosphorylated forms of EGFR (pEGFR) and ERK1/2 (pERK1/2) and after stripping were reprobed with antibodies that recognize total EGFR and ERK1. (B) HCLE cells were untreated or transfected with PLD siRNAs as indicated, and whole cell extracts were prepared for immunoblotting after wounding. Membranes were cut and immunoblotted with antibodies against the EGFR phosphorylated on tyr-1173 (pEGFR), PLD2, or β -Actin. The membrane containing EGFR was stripped and reprobed with antibody that recognizes total epidermal growth factor receptor (EGFR).

apyrase in the medium resulted in quenching of the signals at a distance from the wounds, whereas ERK1/2 activation was still clearly detected within 250 μ m of the wound edge. This result indicates that the EGFR is activated by a mechanism that is independent of extracellular ATP near wound edges.

Characterization of a Mechanism of EGFR Activation that is Independent on Signaling by Extracellular ATP and PLD2

In our standard wounding model, cells are grown as strips that are one to five cells wide, and extracts are therefore largely from cells near the wounds. When HCLE cells were wounded in the presence of apyrase in this model, no reduction in EGFR activation was observed (Figure 6A), in sharp contrast to the reduction seen in cells further from the wound (Figure 4). We also found that reduction of PLD2 expression with siRNAa did not inhibit activation of the EGFR by wounding, indicating that PLD2 does not play a role in activation of the EGFR in wound-proximal cells (Figure 6B). This provides biochemical evidence that neither extracellular ATP nor PLD2 is necessary for activation of the EGFR in cells close to wounds.

To address the mechanism of activation of the EGF receptor in the ATP/PLD2-independent pathway, we first noted that AR is released independently of extracellular ATP signaling in our standard model (Figure 7A). Inclusion of the general protease inhibitor GM 6001 inhibited activation of the EGFR (Figure 7B), although it did not inhibit activation by exogenous ligand (Block *et al.*, 2004), which is consistent with the notion that EGFR activation is the result of a proteolytic event. Also, the LA1 antibody, which blocks activation of the EGFR by ligands, and neutralizing anti-AR antibodies inhibited EGFR activation. Taken together these data support that the wound-proximal pathway activates the EGFR through a transactivation mechanism.

Inhibitor studies have suggested that the Src family of kinases (SFKs) acts upstream of EGFR activation after wounding (Xu *et al.*, 2006). We first analyzed the possible role of the SFKs in the ATP/PLD2-dependent pathway by testing the effects of two structurally dissimilar SFK inhibitors, PP2 and Src Kinase Inhibitor I (SKI I), which are known to have distinct nonspecific effects (Bain *et al.*, 2007). Incubating with either inhibitor blocked ATP-induced release of AR and activation of the EGFR (Supplemental Figure S6). To examine the role of SFKs in the ATP/PLD2-independent

Figure 7. Transactivation of the EGFR in the ATP/PLD2-independent pathway. (A) HCLE cells were untreated (NT) or wounded (W) by removal of agarose droplets with or without 30 U/ml apyrase (APY). Conditioned media were collected and assayed for AR concentration, and the results were normalized to protein content of whole cell extracts. Data points are the means of four determinations; error bars, SDs. (B) HCLE cells were untreated (NT) or wounded by the removal of agarose droplets in the presence of 30 U/ml apyrase and, where indicated, 50 μ M GM 6001, a combination of 10 μ g/ml nonimmune mouse IgG and 20 μ g/ml nonimmune goat IgG (NIS), 10 μ g/ml anti-EGFR antibody (LA1), or 20 μ g/ml neutralizing anti-AR antibody (α AR). Ten minutes after wounding, whole cell extracts were prepared and subjected to Western blotting for active (pEGFR) and total (EGFR) levels of EGFR. (C) Densitometry analysis of Western blots as shown in B. Data points are the means of four determinations; error bars, SDs. Analysis of variance followed by Bonferroni's multiple comparison test showed that GM, LA1, and α AR significantly reduced EGFR activation by wounding in the presence of apyrase ($p < 0.001$).

Figure 8. The EGFR is activated through SFK signaling in the ATP/PLD2-independent pathway. (A) Sheets of HCLE cells were not treated (NT) or wounded by the removal of agarose droplets in the presence of 30 U/ml apyrase where indicated. Ten minutes after wounding, whole cell extracts were prepared and subjected to immunoblotting for active (pSrc) and inactive (non-pSrc) levels of SFKs. (B) Densitometry of immunoblots shown in A. Analysis of variance followed by Bonferroni's multiple comparison test showed that apyrase did not reduce Src family kinase activation significantly after wounding. (C) HCLE cells were not treated (NT) or incubated with 30 U/ml apyrase and 1 μ M SKI I or 10 μ M PP2, as indicated, and were wounded 30 min later by the removal of agarose droplets. The concentrations of AR released in the culture medium were determined after 60-min incubation and normalized to protein contents of the cells on the plates. Analysis of variance followed by Bonferroni's multiple comparison test showed that either Src inhibitor reduced AR release significantly by addition of ATP ($p < 0.001$). (D) Cells were treated as in C, and whole cell extracts were prepared 10 min after wounding and subjected to Western blotting for active (pEGFR) and total (EGFR) levels of EGFR. (E) Densitometry of Western blots shown in D. Analysis of variance followed by Bonferroni's multiple comparison test showed that either Src inhibitor reduced EGFR activation and AR release significantly after wounding ($p < 0.001$). Data points in this figure are the means of at least four determinations; error bars, SDs.

pathway, we noted that elimination of extracellular ATP with apyrase did not abolish activation of SFKs as monitored by immunoblotting with an antibody that recognizes the SFKs phosphorylated on tyr-416 (Roskoski, 2005; Figure 8, A and B). Blocking the EGFR with tyrphostin AG 1478 did not block SFK activation in HCLE cells, in agreement with the notion that SFK is upstream of EGFR activation (Xu *et al.*, 2006 and data not shown). Importantly, incubation with either inhibitor blocked release of AR and activation of the EGFR in our standard wounding model in the presence of apyrase (Figure 8, C–E), although they did not inhibit EGFR activation by exogenous ligand (Supplemental Figure S7). Together these data indicate that one or more SFKs are required for EGFR transactivation in both the ATP/PLD2-dependent and -independent pathways.

Healing of Wounds in the Absence of Signaling by Extracellular ATP

Cells within monolayers typically move spontaneously, and according to some models, cells do not move into wounds as a result of increases in speeds of migration, but rather as a result of the lack of mechanical constraints in the denuded area (Sherratt and Dallon, 2002; Bindschadler and McGrath, 2007). According to such models, it is not necessary to postulate the existence of any biochemical signals that affect cell behavior. To determine whether wounding induces increased velocities, positions of cells were recorded at various times by time-lapse microscopy. As is seen in Figure 9A, cells within 50 μ m from the agarose barrier exhibited basal motility irrespective of induction of wounds. However, wounding induced significant increases of the velocities of the cells at the edge of wounds, and this was not affected by

the presence of apyrase. We conclude that wounding does indeed elicit a biological response in the HCLE cells in that they move faster.

Figure 9. Epithelial wound healing proceeds independently of ATP signaling. (A) HCLE cells were cultured to confluence around agarose strips. The strips were left in place (NT) or removed (Wound) in the presence of 30 U/ml apyrase, as indicated. The velocities of individual cells within 50 μ m of the wound edge (Edge cells) or >2 mm from the wound edge (Distal cells) were calculated as described in *Materials and Methods*. Data points are means of 12 cells; error bars, SDs. Analysis of variance followed by Bonferroni's multiple comparison test showed that velocity was significantly increased by wounding ($p < 0.001$), and this was not significantly changed by apyrase. (B) HCLE cells were subjected to a wound healing assay as described in *Materials and Methods* with no treatment (NT) or 30 U/ml apyrase (APY). Data points are the means of 12 determinations; error bars, SDs. Student's *t*-test indicated that the healing rates were not significantly different.

We next tested whether signaling by ATP is necessary for healing wounds in sheets of HCLE cells by examining whether healing is influenced by the presence of 30 U/ml apyrase, which is a sixfold excess of that found to reduce extracellular ATP to undetectable levels (Figure 1A). As shown in Figure 9B, no significant difference in wound healing was observed between the untreated control and enzyme-treated groups. Similar results were obtained with secondary rabbit corneal epithelial cells (not shown). To verify that the apyrase was functional, we tested its ability to degrade [α - 32 P]ATP and [α - 32 P]ADP. As little as 0.3 U/ml apyrase was sufficient to degrade ATP and ADP completely, and 30 U/ml was fully active even after 14 h in cell culture (Supplemental Figure S8A). From these data, we conclude that extracellular ATP signaling is not necessary for wound healing in sheets of HCLE cells. Other reports have suggested that ATP signaling is critical for wound healing because healing and wound-induced EGFR activation are inhibited by the general P2 receptor antagonist reactive blue 2 (RB2; Klepeis *et al.*, 2004; Boucher *et al.*, 2007; Yin *et al.*, 2007). We have confirmed these findings, but we have also observed that the same concentration of RB2 inhibited activation of the EGFR by AR, which makes RB2 an unsuitable reagent for this type of study (Supplemental Figure S8, B and C).

DISCUSSION

Previous reports have shown that wounding sheets of corneal epithelial cells results in release of ATP, and that extracellular ATP can activate the EGFR (Boucher *et al.*, 2007; Yin *et al.*, 2007). We report here the existence of a distinct mode of activation of the EGFR after wounding, which is not dependent on ATP signaling, based on the following observations: 1) Activation of the EGFR is unaffected by removal of extracellular ATP with apyrase in our standard wounding assay. 2) Extracellular ATP activates the EGFR through PLD2 signaling; however, knock-down of PLD2 does not inhibit EGFR activation in our standard wounding assay. 3) Visualization by immunofluorescence shows that the EGFR/ERK1/2 pathway is stimulated in cells near wounds in the presence of apyrase. 4) The enhanced velocity of cells near wounds is not affected by the presence of apyrase.

Elimination of extracellular ATP with apyrase did not affect the rate of wound healing, suggesting that the ATP/PLD2-dependent pathway is not required for healing of wounds. This is in agreement with one previous report describing similar results with Madin-Darby canine kidney epithelial cells (Farooqui and Fenteany, 2005), but differs from the conclusions reached by the use of the purinergic receptor antagonist RB2 (Klepeis *et al.*, 2004; Boucher *et al.*, 2007; Yin *et al.*, 2007). We have found that RB2 also blocks EGFR activation by exogenous ligands. RB2 is therefore not a suitable reagent for determining the role of purinergic signaling in healing of wounds in corneal epithelial cells because EGFR activation is an absolute prerequisite for induction of motility.

A notable difference in the two pathways is their range of action. ATP is diffusible, and we found both by direct immunoblotting of cells at a distance from wounds and by immunofluorescence studies that ATP signaling activates the EGFR at least 0.5 cm from the wound edge. In contrast, the ATP/PLD2-independent signaling pathway acts only in cells near wounds. ATP is found in conditioned media after wounding at a concentration of 1–2 μ M, which is sufficient to activate the EGFR (Yin *et al.*, 2007) and PLD (Block and Klarlund, unpublished observations). ATP is now recog-

Figure 10. A hypothetical model for mechanisms of EGFR activation in cells near a wound. See text for details and discussion. P2Y: P2Y-type purinergic receptor.

nized as an extracellular messenger with numerous functions and has the potential of influencing other processes related to wound healing such as induction of inflammation, regeneration of nerves, and perhaps communication with underlying stroma (Bours *et al.*, 2006; Burnstock, 2006, 2007a,b).

PLD activation and release of extracellular ATP have both been reported to be upstream events that lead to EGFR transactivation after wounding (Klepeis *et al.*, 2001, 2004; Mazie *et al.*, 2006; Boucher *et al.*, 2007; Yin *et al.*, 2007). In this communication we report that these two signaling events are related by showing that ATP signals through PLD2 to activate the EGFR. The results reported here are to our knowledge the first descriptions of a role for PLD in ATP-stimulated EGFR transactivation. There is a growing list of cytokines and growth factors that stimulate EGFR transactivation (Higashiyama and Nanba, 2005; Ohtsu *et al.*, 2006; Sanderson *et al.*, 2006), and many of these, such as angiotensin II, bradykinin, endothelin-I, and lysophosphatidic acid also stimulate PLD activity (Martin *et al.*, 1989; Bollag *et al.*, 1990; Liu *et al.*, 1992; van der Bend *et al.*, 1992). A role for PLD2 has previously been reported for angiotensin II-mediated transactivation of the EGFR (Li and Malik, 2005), and it therefore seems reasonable to hypothesize that PLD2 mediates EGFR transactivation by stimuli other than ATP.

Our experiments suggest that one or more members of the SFKs are part of the signaling that leads to EGFR activation. SFK activity is necessary for EGFR transactivation by various stimuli, possibly by being closely coupled to sheddases of the ADAM family (Zhang *et al.*, 2004, 2006). We have found that addition of PA to cells causes activation of SFKs (Block and Klarlund, unpublished observations), and overexpression of PLD2 has been shown to increase SFK activity (Ahn *et al.*, 2003). This favors a model in which one or more of the SFKs are downstream of PLD2 activation after wounding.

In summary, our data indicate that two distinct mechanisms exist for EGFR activation. One depends on release of ATP, which can elicit EGFR activation at least 0.5 cm from wounds and which uses PLD2 as a signaling intermediary. This pathway undoubtedly contributes to induction of motility since addition of extracellular ATP or PA has been shown to enhance wound healing in many different epithelial cell lines (Sponsel *et al.*, 1995; Dignass *et al.*, 1998; Klepeis *et al.*, 2004; Allen-Gipson *et al.*, 2006; Mazie *et al.*, 2006; Wesley *et al.*, 2007; Yin *et al.*, 2007), but it is not required for healing of wounds. The other pathway, which is not dependent on ATP/PLD2 signaling, acts locally at edges of wounds. The results of these and prior (Block *et al.*, 2004; Mazie *et al.*, 2006; Xu *et al.*, 2006; Boucher *et al.*, 2007; Yin *et al.*, 2007) studies can be summarized by the model depicted in Figure 10. In this model, an unknown wound sensor (or sensors) causes activation of one or more SFKs and subsequent transactivation of the EGFR, either through ATP/PLD2 signaling or by a distinct intracellular mechanism.

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