# Epithelial cell motility is triggered by activation of the EGF receptor through phosphatidic acid signaling

**Abigail R. Mazie, Julie K. Spix, Ethan R. Block, Hewa B. Achebe and Jes K. Klarlund***

Ophthalmology and Visual Sciences Research Center, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

*Author for correspondence (e-mail: klarlundjk@upmc.edu)

*Accepted 28 December 2005*
*Journal of Cell Science 119, 1645-1654 Published by The Company of Biologists 2006*
*doi:10.1242/jcs.02858*

## Summary

**Phospholipase D catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid, and there is currently much interest in elucidating messenger functions for this molecule. We report here that wounding sheets of corneal epithelial and Madin Darby canine kidney cells induces strong activation of phospholipase D, and we provide evidence that activation is amplified through a positive feed-back loop. Short-chain analogues of phosphatidic acid induce motility robustly in corneal and other epithelial cell types. The effects of these analogues were not the result of their conversion to the corresponding diacylglycerol or lysophosphatidic acid, implying that phosphatidic acid acts directly on one or more cellular targets. Strikingly, phosphatidic acid signaling was found to stimulate the epidermal growth factor receptor (EGFR) through a transactivation process. Healing of wounds in sheets of corneal epithelial cells is absolutely dependent on epidermal growth factor receptor signaling, and the present data suggest that its activation is a result of wound-induced phospholipase D activation.**

Key words: Phospholipase D, Wound healing, Cell migration, Epidermal growth factor, Transactivation, Positive feed-back

## Introduction

Epithelia serve as barriers that separate compartments of the body and provide protection from the external milieu. Powerful mechanisms have evolved to re-establish the integrity of epithelial sheets quickly after wounding. Cells at the edges of wounds acquire a motile phenotype which involves dramatic changes in their molecular organization, such as dissolution of cortical F-actin, formation of lamellipodia, and loosening or loss of intercellular adhesion structures (see Coulombe, 2003; Kuo, 2004; Lu et al., 2001; Martin, 1997; Suzuki et al., 2003). These changes are reminiscent of the epithelial-mesenchymal transitions that occur during development and malignant progression (Prindull and Zipori, 2004; Thiery, 2002; Thiery, 2003).

It is clearly important to identify signaling pathways that mediate the changes that occur in epithelia upon wounding. Many growth factors and hormones stimulate activation of phospholipase D (PLD), an enzyme that catalyzes conversion of phosphatidylcholine to phosphatidic acid (PA) (for reviews, see Exton, 2002a; Exton, 2002b; McDermott et al., 2004). Two mammalian isoforms of PLD are known, PLD1 and PLD2, which differ in cellular localization and regulation. PA is thought to be a secondary messenger and at present a number of targets have been identified, including protein kinases such as Raf-1 or mTOR (Andresen et al., 2002; Fang et al., 2001), the Src-homology-domain-containing protein tyrosine phosphatase-1 (SHP-1) (Frank et al., 1999), and enzymes involved in lipid metabolism such as sphingosine kinase and phosphatidylinositol 4-phosphate 5′-kinase (Delon et al., 2004; Jenkins et al., 1994). PA can be dephosphorylated to generate diacylglycerol (DAG) which is an activator of protein kinase C (PKC) (Parker and Murray-Rust, 2004; Spitaler and Cantrell, 2004), and it can be deacylated to generate lyso-PA which signals through G-protein-coupled cell surface receptors (Ishii et al., 2004; Moolenaar et al., 2004). As a consequence, it is necessary to clarify whether observed responses to increased cellular levels of PA, either produced by the endogenous reaction or added exogenously, are due to direct PA signaling or due to its conversion to other lipid species.

A number of reports have implicated PA signaling in regulation of the cytoskeleton. Addition of exogenous PA has been shown to enhance production of stress fibers in fibroblasts, and the use of dominant negative constructs of PLD has also supported a role for PLD in stress fiber formation (Cross et al., 1996; Ha and Exton, 1993; Kam and Exton, 2001). Wild-type PLD2 is constitutively active and induces altered cortical actin organization and numerous filopodia-like projections upon transfection into rat embryo fibroblasts (Colley et al., 1997). PLD2 is colocalized in ruffles and lamellipodia, together with probable activators of the enzyme (Honda et al., 1999; Powner et al., 2002). In a recent interesting study, overexpression of the ARF6 guanine nucleotide exchange factor ARNO was found to activate PLD and Rac1 and to induce migration of Madin Darby canine kidney (MDCK) cells (Santy and Casanova, 2001). A necessary role for PLD in cell migration was suggested because the selective PLD inhibitor 1-butanol blocked formation of lamellipodia.

Together, these results suggested that PA signaling might have important roles in healing of wounds in sheets of cells. Using a previously described model (Block et al., 2004) we found that PLD is indeed activated upon wounding, and that its activity is necessary for induction of motility. Furthermore,

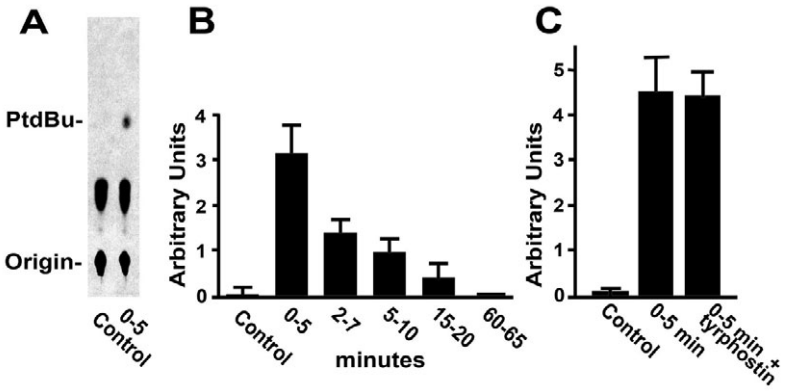


**Fig. 1.** Wounding induces activation of PLD. Corneal epithelial cells were seeded in the presence of agarose droplets (Block et al., 2004) and labeled with [$^3$H]myristic acid. The agarose was removed and 1-butanol added for 5-minute intervals, and the amounts of radioactivity in phosphatidylbutanol (PtdBu) quantified (for details, see Materials and Methods). (A) Autoradiogram of thin-layer plates. (B) Time course of activation. The values in this and the following figures are means ± s.d. of triplicate determinations, except where noted. All experiments were performed at least three times with consistent results. (C) Effect of tyrphostin AG 1478 on PLD activation. Corneal epithelial cells were wounded and activities measured at 0-5 minutes with 10 μM UO126 or 10 μM tyrphostin AG 1478, as indicated. Control blots showed that the phosphorylation of the EGFR was completely blocked (data not shown, but see Fig. 7C,D).

since activation of the epidermal growth factor receptor (EGFR) is absolutely required for healing of wounds in sheets of corneal epithelial cells, we examined the connection between PLD and epidermal growth factor (EGF) signaling.

## Results

### Activation of PLD upon initiation of migration in corneal epithelial cells

To examine whether wounding of epithelial cell layers induces PLD activation, we used a previously developed model (Block et al., 2004) and took advantage of the unique transphosphatidylation reaction of PLD, in which primary alcohols are used to generate phosphatidylalcohols. Cells were labeled with [$^3$H]myristic acid, wounding was induced, and at various times 1-butanol was added for 5-minute intervals. Labeled lipids were extracted, separated by thin-layer chromatography, and the radioactivity in phosphatidylbutanol quantified. As is seen in Fig. 1A, phosphatidylbutanol was readily detected and increased markedly within minutes after stimulation. Activation was transient and declined to background levels after 1-2 hours (Fig. 1B). Since induction of migration in corneal epithelial cells is strictly dependent on the activity of the EGFR (Block et al., 2004), we examined whether inhibition of the receptor with tyrphostin AG 1478 blocked activation of PLD. As is seen in Fig. 1C, the compound did not have any effect on PLD activation in response to wounding. Activation of PLD was also seen in MDCK cells after wounding (data not shown).

### Induction of a migratory phenotype by addition of exogenous PA

Stimulation of the EGFR activates PLD in corneal epithelial cells (Zhang and Akhtar, 1998) (data not shown), and addition of 100 ng/ml EGF was found to promote a migratory phenotype (Fig. 2A). To examine whether increasing cellular levels of PA also promotes the migratory phenotype, a water-soluble synthetic PA, 1,2-dioctanoyl-glycero-3-phosphate ($C_8$-PA), was added to the cultures. As is seen in Fig. 2A, addition of this compound caused greatly increased formation of lamellipodia and scattering of the cells, thus mimicking the effects of EGF.

When corneal epithelium is wounded in vivo, a significant redirection of cellular protein synthesis occurs, and a marked increase in cellular content of the focal adhesion-associated protein vinculin is observed (Zieske et al., 1989). Cell extracts

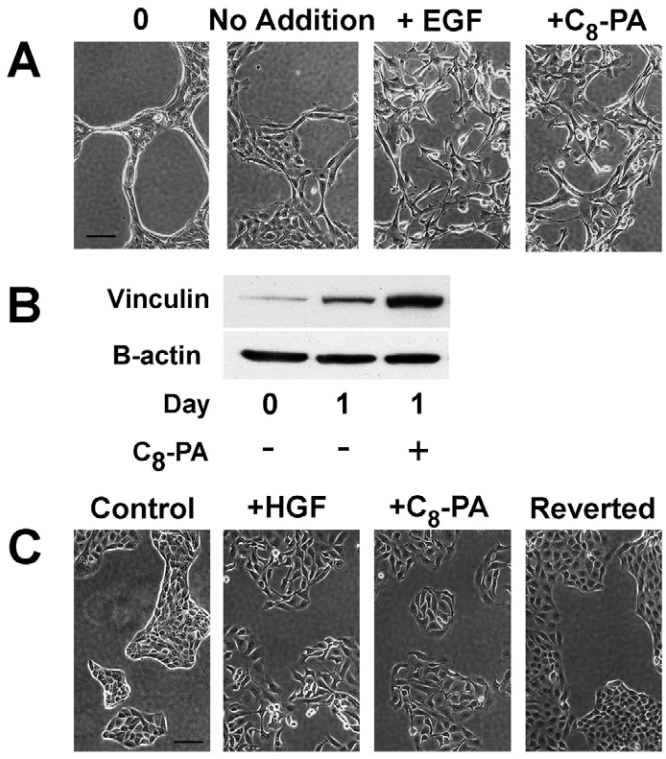


**Fig. 2.** $C_8$-PA promotes a motile phenotype. (A) Corneal epithelial cells were seeded in the presence of agarose droplets and photographed using phase contrast optics. '0' is before removal of agarose droplets. 'Untreated', '+EGF' and '+$C_8$-PA' are 1 day after removal of the agarose droplets with no treatment, or in the presence of 100 ng/ml EGF or 250 μM $C_8$-PA, as indicated. Bars, 50 μm. (B) Induction of vinculin by wounding. Corneal epithelial cells were seeded as in A. The agarose droplets were removed, and the cells were harvested at the indicated times. Extracts were then subjected to SDS-PAGE and blotted with an anti-vinculin antibody, and the same samples blotted with an anti-β-actin antibody as a loading control. (C) MDCK cells were treated with 10 ng/ml HGF or 250 μM $C_8$-PA overnight, as indicated. 'Reverted' refers to cells that had been treated with $C_8$-PA overnight and transferred to fresh medium without $C_8$-PA for 8 hours.

were made at different times after primary corneal epithelial cells were stimulated to become motile and immunoblotted with an anti-vinculin antibody. Vinculin was upregulated 5- to 20-fold after 2 days as determined by densitometry of the autoradiograms. Importantly, a further enhancement was seen upon treatment with $C_8$-PA (Fig. 2B), demonstrating that PA signaling also enhances the motile phenotype according to this criterion.

To determine whether phosphatidic acid-induced migration is a general phenomenon, other epithelial cell types were also studied. In contrast to corneal epithelial cells, most epithelial cells grow as coherent colonies when seeded in tissue culture. MDCK cells are the prototype for studying agonist-induced epithelial cell migration. They form tight colonies and upon stimulation with hepatocyte growth factor (HGF) cells at the edges migrate and the colonies consequently scatter. As expected, HGF was found to activate PLD in MDCK cells (Adachi et al., 1996) (data not shown). Addition of $C_8$-PA resulted in formation of lamellipodia at the edges of colonies after 10-15 minutes and caused scattering of colonies that was similar to that seen upon addition of HGF (Fig. 2C). The effects of $C_8$-PA were clearly visible at concentrations down to 50 μM. Other dioctanoyl-glycero-phosphate derivatives were found to be either insoluble or toxic to the cells. Addition of di-hexanoyl PA ($C_6$-PA) also induced scattering, whereas di-myristoyl PA was ineffective, possibly because of its lower solubility. $C_8$-PA also caused scattering in human skin keratinocytes and in Vero cells. When MDCK cells were transferred to medium without $C_8$-PA, the cells reverted to a normal phenotype after approximately 8 hours (Fig. 2C), indicating that PA signaling is continuously required for maintenance of the motile state.

To enhance cellular levels of PA by independent means, we overexpressed wild-type PLD2, which is constitutively active, and as shown in Fig. 3A, verified that it caused a marked elevation of PLD activity in MDCK cells (Colley et al., 1997; Kodaki and Yamashita, 1997; Lopez et al., 1998). Increasing cellular levels of PA by this procedure also promoted formation of lamellipodia at the edges of the colonies, although the scattering of the cells was somewhat less pronounced than after addition of $C_8$-PA (Fig. 3A). Expression of a catalytically inactive mutant PLD2(K758R) did not elevate cellular PLD activity and did not induce scattering. To further verify the motile phenotype, we examined additional markers. In stationary epithelia, E-cadherin exists in intracellular vesicles and at cell-cell contacts, and this distribution was evident in uninfected MDCK cells (not shown) and in cells infected with PLD2(K758R) (Fig. 3B). However, in cells treated with $C_8$-PA or in cells infected with PLD2(wt) E-cadherin was totally internalized as cells scattered, as is typically seen in motile epithelial cells. As previously observed in uninfected cells

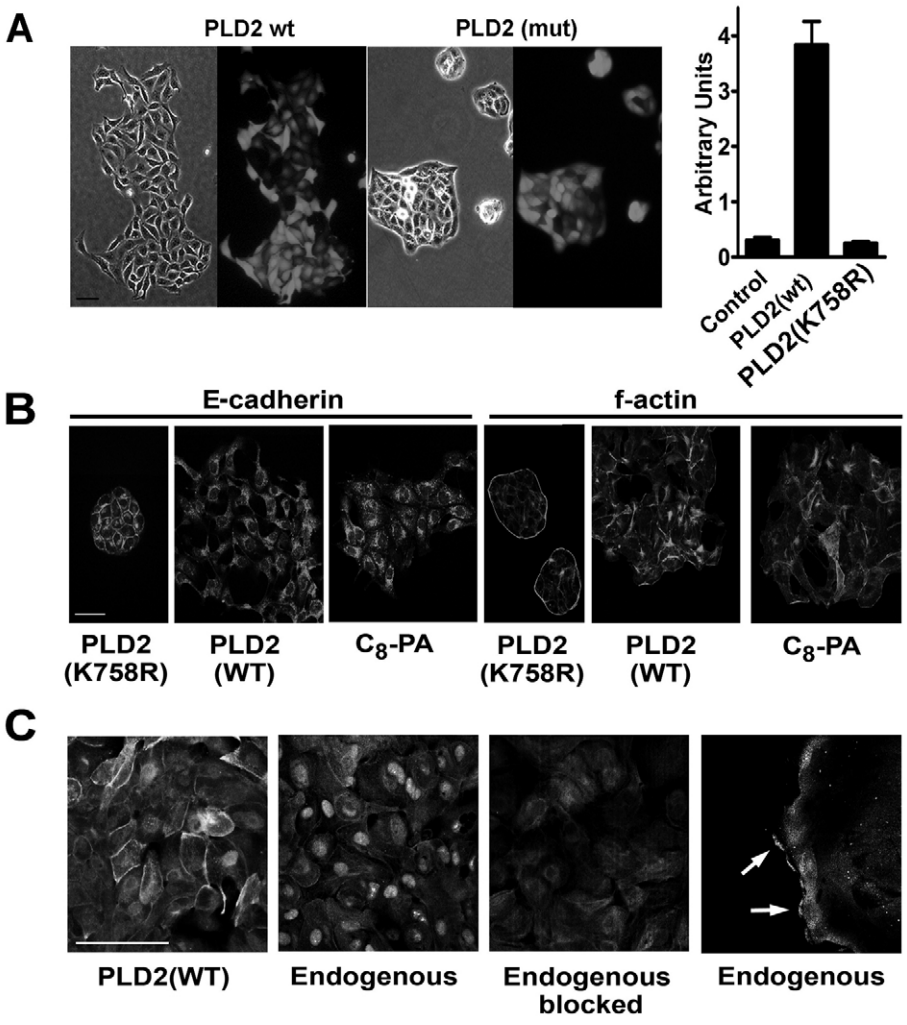


**Fig. 3.** Analysis of phenotype induced by overexpression of PLD2, and localization of endogenous PLD2. (A) MDCK cells were infected with the adenovirus coding for wild-type or an enzymatically inactive mutant of PLD2 and photographed using phase contrast optics. The vector also codes for the green fluorescent protein, and the infected cells are shown. The graph shows PLD activities in infected cultures. Bars, 20 μm. (B) Cells were infected overnight with adenovirus coding for the indicated construct, or treated with $C_8$-PA for 5 hours and stained with Alexa Fluor® 546 phalloidin or immunostained for E-cadherin. As in A, virtually all cells in the fields were infected.
(C) Localization of PLD2. Corneal epithelial cells infected with adenovirus coding for PLD2(wt) or uninfected cells were stained with an anti-PLD2 antibody. Unspecific staining of uninfected cells was determined by pre-incubating the antiserum with the immunizing peptide ('blocked'). Uninfected cells 8 hours after induction of wounding were also stained (right panel). Arrows indicate staining of lamellipodia. The lamellipodial staining was blocked by preincubation of the antibody with the peptide (data not shown). A confocal section close to the tissue culture plastic is depicted, so the nuclei are not visible.

(Imamura et al., 1998; Ridley et al., 1995) and in PLD2(K758R) infected cells, F-actin was most notable as heavy bands at the periphery of the colonies, and was also apparent in cortical positions in the cells and as fine stress fibers. Upon stimulation with $C_8$-PA, major changes in the distribution of F-actin occurred: the peripheral bundles disappeared, cortical actin dissolved, and stress fibers tended to appear with a stellate organization (Fig. 3B). Very similar changes have previously been observed after stimulation with phorbol ester or HGF (Imamura et al., 1998; Ridley et al., 1995).

In order to obtain quantitative measurements, motility was measured by a wound-healing assay. Healing is very rapid in corneal epithelial cells (Block et al., 2004), and it was difficult to observe any effect of added $C_8$-PA. However, healing in monolayers of MDCK cells is slow, and a clear dose-dependent enhancement of healing of wounds was observed (Fig. 4A). Control experiments with concentrations of mitomycin C that blocked cell division demonstrated that enhancement of healing by $C_8$-PA was not due to enhanced cell proliferation in the presence of $C_8$-PA (data not shown).

In view of the fact that the PLD2 isoform induced cell scattering and that this isoform has been extensively associated with alterations in the actin cytoskeleton (for reviews, see Exton, 2002a; Exton, 2002b; McDermott et al., 2004), we also examined the subcellular distribution of endogenous PLD2. Overexpressed PLD2 was clearly seen in cell-cell junctions in stationary epithelial cells (Fig. 3C). We also examined endogenous PLD2 in stationary cells and found it to be present at a similar location, although the signals were, as expected, weaker. Also, PLD2 was detected in nuclei, as has been reported previously in macrophages with this antibody (Iyer et al., 2004). When cells were stimulated to move, there were some increases, in expression of PLD2 at the wound edges, and it was clearly detected at the leading edges, of lamellipodia. There seemed to be some PLD2 present in the cytoplasm, but since there was some nonspecific binding of the antibody it is difficult to locate PLD2 more precisely.

## The effects of $C_8$-PA are not due to activation of PKC

Experiments that rely on addition of PA are complicated by the fact that it may be further metabolized to diacylglycerol (DAG) or lyso-PA, both of which are bioactive compounds. We analyzed the effects of the dephosphorylation product of $C_8$-PA, dioctanoyl glycerol ($C_8$-DAG), which is an activator of PKC. Stimulation of PKC by the potent activator phorbol 12-myristate 13-acetate (PMA) induced scattering of MDCK cells, as expected (Rosen et al., 1990) (data not shown). In accord with a previous report that $C_8$-DAG is a weak scattering agent for MDCK cells (Rosen et al., 1990), we found that addition of $C_8$-DAG induced similar, but less prominent effects than $C_8$-PA or PMA, and the effects were only seen at concentrations of 250 μM or more, which is five times higher than required for $C_8$-PA. Furthermore, whereas $C_8$-PA treated cells exhibited the migratory phenotype for days, the effects of $C_8$-DAG were transient, and after 5-8 hours the cells were indistinguishable from untreated cells.

To determine whether $C_8$-PA activates PKC the phosphorylation state of the myristoylated alanine-rich C kinase substrate (MARCKS), which is a recognized read-out for PKC activation in intact cells, was also examined (Arbuzova et al., 2002; Sundaram et al., 2004). Stimulation with PMA or $C_8$-DAG clearly induced phosphorylation of MARCKS, whereas no stimulation was seen in MDCK or corneal epithelial cells with $C_8$-PA (Fig. 5, and data not shown). This, together with data reported below, strongly suggests that $C_8$-PA does not act by conversion to $C_8$-DAG.

It seems unlikely that the effects of $C_8$-PA are mediated through lyso-PA signaling because most of the experiments were performed in the presence of 10% fetal calf serum which contains biologically saturating amounts of lyso-PA (Eichholtz et al., 1993). The lyso derivative of $C_8$-PA is not commercially available; however, addition of lyso$C_6$-PA did not cause scattering of MDCK cells, whereas addition of di-hexanoyl PA ($C_6$-PA) was almost as active as $C_8$-PA (data not shown). Also, the lyso-lipid, 1-oleoyl-glycero-3-phosphate, did not induce scattering of the cells.

## A role for PLD in EGF-induced cell motility

Wounds in sheets of corneal epithelial cells heal rapidly, and we examined whether inhibition of PLD blocks healing. PLD utilizes primary alcohols more efficiently than water, and the resulting phosphatidylalcohols are considered to have no signaling capability (Exton, 2002a). When 1-butanol was included in the wounding assays, significant inhibition of healing was observed (Fig. 6A). By contrast, little inhibition

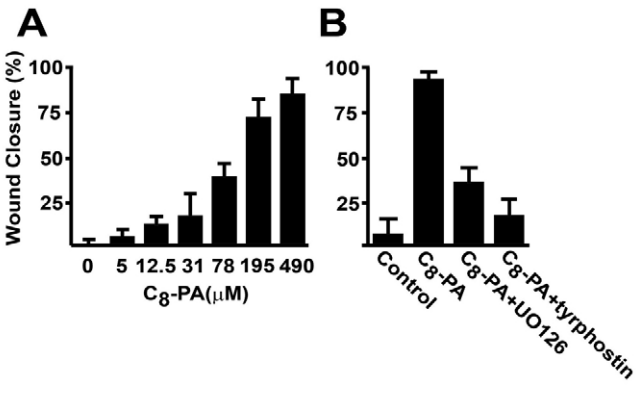


**Fig. 4.** $C_8$-PA enhances wound healing in MDCK cells. (A) Dose response. Wounds were induced as described previously (Block et al., 2004), and healing allowed to proceed for 14 hours in the presence of the indicated concentrations of $C_8$-PA. (B) Healing in the presence of 375 μM $C_8$-PA, 10 μM UO126 or 10 μM tyrphostin AG 1478, as indicated.

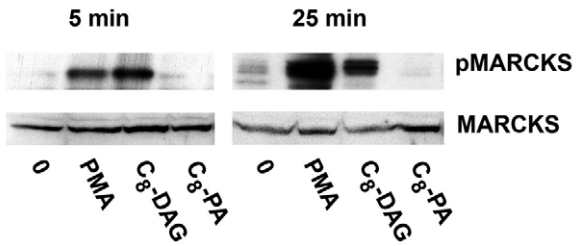


**Fig. 5.** $C_8$-PA does not induce phosphorylation of MARCKS. MDCK cells were stimulated with 100 nM PMA, 500 μM $C_8$-DAG, or 500 μM $C_8$-PA for 5 or 25 minutes, and extracts were immunoblotted with an anti-phospho-MARCKS antibody. Lower panel is a parallel blot probed with an anti-MARCKS antibody.

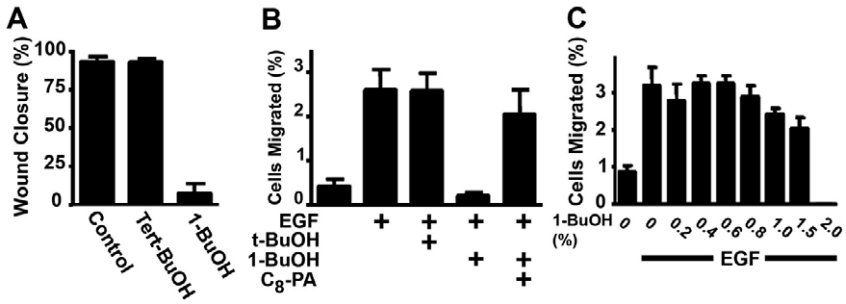


**Fig. 6.** Inhibition of PLD blocks cell motility in corneal epithelial cells. (A) Inhibition of healing in wounds of confluent layers of cells. Healing was allowed to proceed in the presence of EGF (200 ng/ml), and alcohols [1-butanol (1-BuOH) and tert-BuOH] (0.45%). (B) Inhibition of EGF-induced chemokinesis. Cells were seeded in Transwell® migration chambers in the presence of the indicated compounds in both the top and bottom chambers. Alcohols were used at 0.40%, and $C_8$-PA at 125 μM. (C) Cells were pre-treated with the indicated concentrations of 1-butanol for 8 hours. The 1-butanol was washed out, and the cells were allowed to migrate for another 8 hours in the presence of EGF.

was seen with the structurally similar tert-butanol, which is not a substrate for PLD.

To see whether EGF-stimulated migration was dependent on PLD, we analyzed chemokinesis of corneal epithelial cells in modified Boyden chambers and found that 1-butanol inhibited EGF-induced cell movement, whereas tert-butanol was ineffective (Fig. 6B). Importantly, when $C_8$-PA was included in the assays, motility was largely restored, strongly suggesting that the effects of 1-butanol were not due to nonspecific toxicity. The concentrations of 1-butanol that had to be used to achieve complete inhibition of movement are in the high range of what is customarily employed, and therefore we further verified that no toxic effects complicated the assay. We examined whether pre-treatment with 1-butanol inhibited the responsiveness to EGF, and as is shown in Fig. 6C, the corneal epithelial cells tolerated pre-treatment with up to 0.8% 1-butanol without any significant decline in response. Also we note that the presence of 1-butanol at up to 1.5% did not abrogate the ability of added EGF to activate the receptor and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (data not shown).

## $C_8$-PA induces motility through EGFR signaling

Since scattering of MDCK cells by HGF requires signaling through both PI 3-kinase and ERK (Potempa and Ridley, 1998), we examined the possible roles of these pathways on scattering induced by $C_8$-PA. We confirmed that addition of the cell permeable inhibitors of PI 3-kinase, wortmannin and LY294002, blocks scattering induced by HGF. These compounds did not inhibit scattering by $C_8$-PA, suggesting that PI 3-kinase is not required for scattering induced by $C_8$-PA (Fig. 7A, and data not shown). Addition of $C_8$-PA enhanced activation of ERK1/2 as demonstrated by blotting with an antibody against the activated

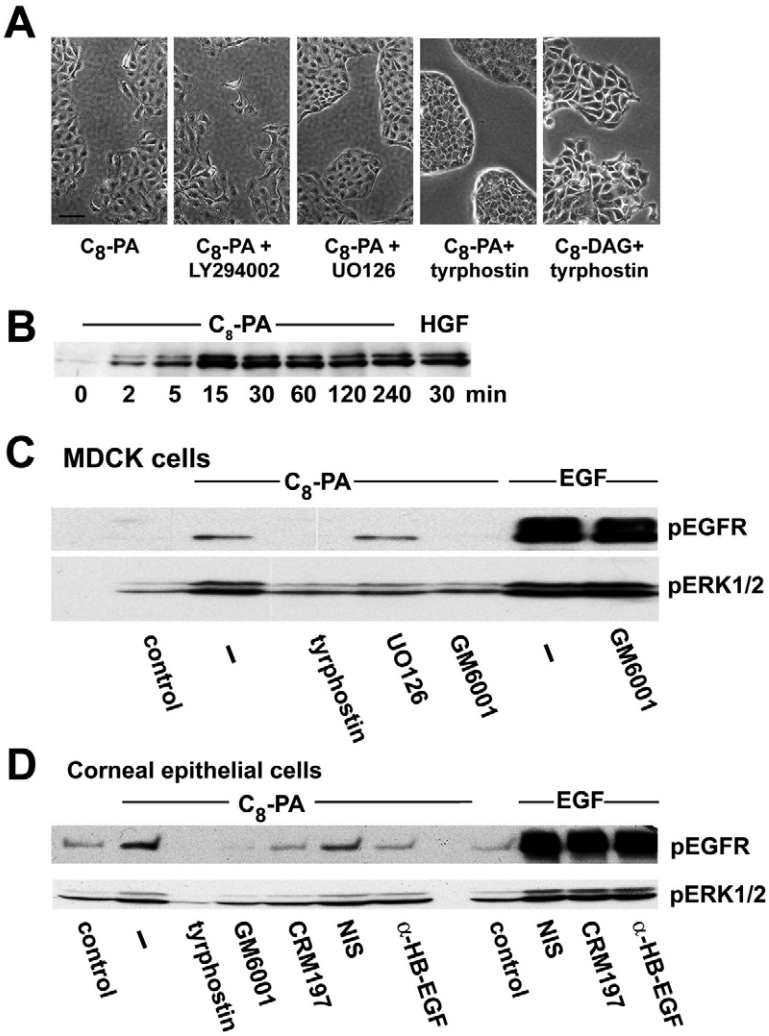


**Fig. 7.** $C_8$-PA-induced scattering requires ERK1/2 and EGFR activities. (A) MDCK cells were incubated overnight with 250 μM $C_8$-PA and 50 μM LY294002, 10 μM UO126, or 10 μM tyrphostin AG 1478 as indicated, and photographed using phase contrast optics. Bar, 50 μm. (B) Time-course for ERK1/2 activation. MDCK cells were treated with 375 μM $C_8$-PA or 10 ng/ml HGF for the indicated periods of time and extracts were prepared and immunoblotted with an antibody against the activated forms of ERK1/2. (C) Activation of the EGFR by $C_8$-PA in MDCK cells. Cells were pre-incubated with 10 μM UO126, 10 μM tyrphostin AG 1478, 50 μM GM6001, and then treated with $C_8$-PA for 15 minutes or 10 ng/ml EGF for 10 minutes. Equal loads in the lanes were verified by staining the blots with Ponceau S (see Materials and Methods). (D) Activation of the EGFR by $C_8$-PA in human corneal epithelial cells. Conditions were as in C. Preincubation with 50 μg/ml CRM 197 or 20 μg/ml antibody was for 6 hours.

forms of the kinases (Fig. 7B). Activation was evident down to 15 μM of added lipid. Incubation overnight with 0.2 μg/ml pertussis toxin did not block activation of ERK by $C_8$-PA (data not shown). Two inhibitors of the activating kinase for ERK1/2, PD98059 and U0126, inhibited scattering induced by $C_8$-PA, indicating a role for ERK1/2 in the response to $C_8$-PA (Fig. 7A, and data not shown).

Wounding of corneal epithelial cells activates the EGFR (Block et al., 2004; Xu et al., 2004). Importantly, the EGFR and its down-stream targets, ERK1/2, were found to be activated by $C_8$-PA in both MDCK and corneal epithelial cells, as is illustrated in Fig. 7C. This was surprising since PLD activation is usually considered to be down-stream of EGFR activation (for reviews, see Exton, 2002a; Exton, 2002b; McDermott et al., 2004). We have previously shown that blocking the EGFR signaling pathway in corneal epithelial cells completely blocks induction of migration (Block et al., 2004). Remarkably, addition of tyrphostin AG 1478 to MDCK cells similarly blocked scattering in response to $C_8$-PA (Fig. 7A), whereas scattering induced by $C_8$-DAG was not blocked by the inhibitor. Tyrphostin AG 1478 also blocked scattering induced by overexpression of PLD2(wt) (data not shown) and healing induced by $C_8$-PA in monolayers of MDCK cells (Fig. 4B). Wounding activates the EGFR through a transactivation process, which depends on cleavage of a precursor of heparin binding epidermal growth factor-like growth factor (HB-EGF) (Block et al., 2004; Xu et al., 2004). As is seen in Fig. 7C, incubation with the metalloproteinase protease inhibitor GM6001 inhibited activation of the EGFR by $C_8$-PA. That this was not a nonspecific effect was demonstrated by the fact that the inhibitor did not affect EGF-induced activation of the EGFR or ERK1/2.

Furthermore, we examined whether HB-EGF was involved. Since the relevant reagents are human specific, we used an immortalized human corneal epithelial cell line for these studies (Gipson et al., 2003). In this cell line tyrphostin AG 1478 and GM6001 also blocked activation of the EGFR (Fig. 7D). We used a mutated form of diphtheria toxin, CRM 197, which binds to proHB-EGF and neutralizes its actions (Mitamura et al., 1995). As is seen in Fig. 7D this effectively blocked activation of the EGFR by $C_8$-PA. In addition, an antibody against human HB-EGF also blocked activation of the EGFR by $C_8$-PA. These reagents did not interfere with the ability of EGFR to be activated by exogenously added EGF. We conclude that $C_8$-PA causes trans-activation of the EGFR through proteolytic cleavage of the HB-EGF precursor.

### Stimulation of endogenous PLD activity by addition of exogenous PA

The experimental system made it possible to test whether PA induces its own synthesis. $C_8$-PA was added to corneal epithelial or MDCK cells and endogenous PLD activity assayed by metabolic labeling as above. In both systems, this resulted in rapid activation of endogenous PLD, indicating the presence of a potent positive feed-back mechanism (Fig. 8A). The response was of a magnitude similar to that induced by PMA, and the effects of PMA and $C_8$-PA were not additive (Fig. 8B). Pre-treatment with brefeldin A did not abrogate the response, although the treatment clearly disrupted Golgi structure (data not shown). Interestingly, tyrphostin AG 1478, which inhibits activation of ERK1/2 in response to $C_8$-PA (Fig. 7C), does not inhibit activation of the endogenous PLD reaction by $C_8$-PA. Addition of lyso-$C_6$-PA or 1-oleoyl-glycero-3-phosphate did not induce activation of endogenous PLD in MDCK cells. The biological activity of our preparations of 1-oleoyl-glycero-3-phosphate was verified by its ability to induce ERK1/2 activation in corneal epithelial cells (data not shown).

It has been suggested that a positive feed-back mechanism might result from the following: PA is a powerful stimulator of

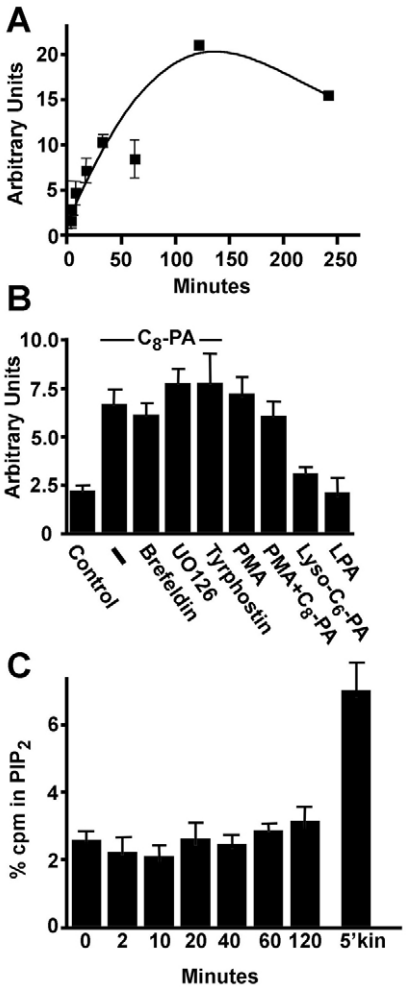


**Fig. 8.** Addition of exogenous PA activates cellular PLD. (A) Time course. 375 μM $C_8$-PA was added to MDCK cells and the cellular PLD activities assayed as described in Materials and Methods at the indicated times after addition. The values are means of duplicate determinations and the error bars are the ranges of the values. (B) Effects of various agents on PLD activation by $C_8$-PA. Cells were stimulated with 375 μM $C_8$-PA after 4 hours pre-incubation with 50 μM brefeldin A, or 15 minutes preincubation with 10 μM UO126 or 10 μM tyrphostin AG 1478. PMA was used at 0.1 μM in the presence or absence of 10 μM $C_8$-PA. Lyso$C_6$-PA was used at 10 μM and 1-oleoyl-glycero-3-phosphate (lyso phosphatidic acid; LPA) complexed with lipid-free bovine serum albumin was used at 2 μM. This experiment was performed in the absence of serum. (C) Lack of increase of PtdIns(4,5)$P_2$ levels by $C_8$-PA was assayed at the indicated times after addition of 500 μM $C_8$-PA to MDCK cells. '5′kin' cells were infected with adenovirus coding for phosphatidylinositol 4-phosphate 5′-kinase as a positive control.

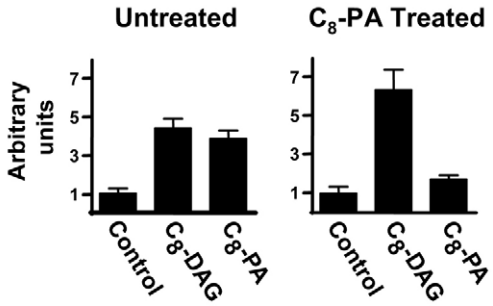


**Fig. 9.** Down-regulation of response to $C_8$-PA does not down-regulate response to $C_8$-DAG. MDCK cells were untreated or pre-treated with 500 μM $C_8$-PA overnight. The cells were then untreated or stimulated with 375 μM $C_8$-PA or $C_8$-DAG, as indicated, for 15 minutes and PLD activities were measured.

phosphatidylinositol 4-phosphate 5′-kinase, and its reaction product, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)$P_2$), is a stimulator of PLD (Czech, 2000; Liscovitch and Cantley, 1995; Martin, 1998). However, little increase was seen in cellular levels of PtdIns(4,5)$P_2$ in response to treatment of corneal epithelial or MDCK cells with $C_8$-PA, making this scenario unlikely (Fig. 8C).

The activation of endogenous PLD by added PA provided a further test of whether $C_8$-PA signals through its dephosphorylation to $C_8$-DAG, using down-regulation experiments. The endogenous PLD activity reverted to basal levels in MDCK cells after treatment with $C_8$-PA for 8-10 hours. As expected, little stimulation of endogenous PLD activity was seen after an additional treatment with $C_8$-PA. By contrast, both PMA and $C_8$-DAG activated endogenous PLD activities to extents similar to those seen in untreated cells (Fig. 9, and data not shown). The fact that desensitization to $C_8$-PA fails to desensitize to $C_8$-DAG strongly supports the notion that they are on separate signaling pathways.

## Discussion

We have previously reported that, wounding sheets of corneal epithelial cells trans-activates the EGFR through proteolytic cleavage of proHB-EGF and, that, activation through this mechanism is absolutely required for induction of motility. We report in this paper the novel observation that wounding epithelial cell layers activates PLD, and we provide evidence that activation is enhanced through a positive feed-back loop. Increasing cellular levels of PA by addition of exogenous $C_8$-PA or overexpression of PLD2 promotes a motile phenotype and we find that PA signaling is critical for motility. Finally we have made the unexpected observation that PA signaling trans-activates the EGFR, placing PLD up-stream of EGFR activation.

One complication of studying the functions of PA, DAG and lyso-PA is that they are interconvertible. Our studies relied to a significant extent on adding the short chain $C_8$-PA to cells. It is unlikely that it acts by being deacylated to lyso$C_8$-PA, because most assays were performed in 10% serum which contains biologically saturating amounts of lyso-PA, and because both lyso$C_6$-PA and 1-oleoyl-glycerophosphate were inactive in inducing scattering in MDCK cells and in activating endogenous PLD activities. We also consider it unlikely that $C_8$-PA acts after hydrolysis to $C_8$-DAG for the following reasons: (1) $C_8$-DAG was less active than $C_8$-PA or inactive in many of our assays; (2) the scattering effect of $C_8$-DAG was transient whereas that of $C_8$-PA was not; (3) $C_8$-PA did not induce phosphorylation of the PKC substrate MARCKS, whereas this was clearly seen upon addition of $C_8$-DAG; (4) down-regulation of activation of endogenous PLD by $C_8$-PA did not abrogate the response to $C_8$-DAG; (5) $C_8$-DAG-induced scattering was not dependent on EGFR signaling. It is important to distinguish the actions of $C_8$-PA from those of its dephosphorylation product, $C_8$-DAG, in our systems because activation of PKC induces cell motility (Rosen et al., 1990) and $C_8$-PA has been observed to be dephosphorylated when added to neutrophils, especially in the absence of calcium in the culture medium (Perry et al., 1993). Our data imply that $C_8$-PA acts directly on cellular targets, independently of PKC in MDCK and corneal epithelial cells.

Addition of exogenous PA stimulated synthesis of endogenous PA, indicating that production of PA is regulated through a positive feed-back loop. This is reminiscent of the better characterized lipid messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(4,5)$P_3$], which has also been shown to stimulate its own synthesis (Weiner et al., 2002). A positive feed-back loop is thought to be instrumental in producing large quantities of phosphatidylinositol 3,4,5-trisphosphate locally, which generates polarity in cells. This may also be relevant in PA signaling since we have observed that exogenous PA serves as a chemoattractant (J.K.K., unpublished observations). Activation of endogenous PLD activity by $C_8$-PA was not inhibited by tyrphostin AG 1478, and it is therefore improbable that the positive feedback loop depends on activation of the EGFR.

There has been some debate about the subcellular localization of PLD2 (for discussion, see Du et al., 2004). Many studies have indicated predominant plasma membrane localization, but its localization has not been extensively described in epithelial cells. We found it at cell-cell contacts in corneal epithelial cells, which is interesting because disruption of adherens junctions at this localization through a variety of mechanisms is considered an important trigger for induction of motility (D'Souza-Schorey, 2005). Once the cells were induced to move, concentrations of PLD2 were upregulated at wound margins and were concentrated at the leading edges of lamellipodia, which is reminiscent of its localization in membrane ruffles (Honda et al., 1999). These observations further suggest a role of PLD2 in motility.

A major finding in the present study is that $C_8$-PA activates the EGF receptor. This was unexpected because previous studies have focused on the converse phenomenon, EGF activating PLD (Exton, 2002a; Exton, 2002b; McDermott et al., 2004). Our studies, using the protease inhibitor GM6001 and the HB-EGF neutralizing agents, strongly suggest that the EGFR is activated by $C_8$-PA through a triple membrane-passing transactivation pathway. The basis of these pathways is activation of a cellular protease of the disintegrin and metalloprotease (ADAM) or metalloprotease families, which results in cleavage of precursor forms of EGF ligands and subsequent activation of the EGFR (Fischer et al., 2003). The activation of the EGFR was not as great as seen with high doses of EGF (Fig. 7C), however activation of ERK1/2 in response

to $C_8$-PA was almost as strong. The observation that a modest level of EGFR activation leads to strong activation of ERK1/2 and subsequent robust cellular responses is unsurprising (see Swindle et al., 2001 and references therein), and we have observed almost full activation of ERK1/2 at low levels of EGF that induced no detectable activation of the EGFR.

Some agents such as DMSO, unsaturated lipids, sphingosine and cholesterol depletion may activate the EGFR in a ligand-independent fashion, either by direct binding to the receptor or by increasing membrane fluidity (Chen and Resh, 2002; Davis et al., 1988; Rubin and Earp, 1983; Vacaresse et al., 1999). However, the observations that the protease inhibitor GM6001 and inhibitors of HB-EGF signaling block activation of the EGFR by $C_8$-PA are not easily reconcilable with such a mechanism.

Activation of ERK1/2 in response to $C_8$-PA was blocked by tyrphostin AG 1478 and GM6001, which is consistent with the notion that ERK1/2 is down-stream of the transactivated EGFR. We suggest that the failure of the HB-EGF neutralizing agents to block ERK1/2 activation may be due to incomplete blockage of EGFR activation. At least seven ligands of the EGFR are synthesized as precursor forms that can be cleaved by proteases, and although HB-EGF appears to be a major contributor to EGFR activation, others may also play a role. Owing to the nonlinear relationship of EGFR and ERK1/2 activation, a residual EGFR activation, though difficult to detect by western-blotting, may be sufficient to activate ERK1/2.

We and others have previously found that EGFR signaling is absolutely required for healing of wounds in sheets of corneal epithelial cells, and that activation of the EGF receptor is predominantly induced by transactivation via cleavage of the precursor for HB-EGF (Block et al., 2004; Xu et al., 2004). The data reported here suggest that wound-induced activation of the EGFR is mediated through PA signaling. It would be interesting to see whether transactivation of the EGFR induced by other signals similarly acts through PLD activation.

We found that inhibition of the EGFR/ERK1/2 pathway blocked motility in both MDCK and corneal epithelial cells according to the assays described in this paper. One important down-stream target for PA signaling therefore appears to be the EGFR. However, our data show that PA must have other signaling functions as well. Activation of endogenous PLD was not inhibited by the EGFR and MEK inhibitors tyrphostin AG 1478 and UO126. Also, inhibition of PLD signaling by 1-butanol abrogated EGF-stimulated chemokinesis but not EGFR activation, and addition of $C_8$-PA restored motility of the cells (Fig. 6B). This strongly suggests that PA has signaling functions in addition to activating the EGFR/ERK1/2 pathway.

A limited number of targets have been described for PA. PtdIns(4,5)$P_2$ binds to many proteins that interact with the actin cytoskeleton, and high concentrations of PtdIns(4,5)$P_2$ are generally thought to promote actin polymerization (Doughman et al., 2003; Martin, 1998; Yin and Janmey, 2003). PtdIns(4,5)$P_2$ is primarily synthesized by phosphatidylinositol 4-phosphate 5′-kinase, which is activated by PA (Jenkins et al., 1994). We did not find increased overall levels of PtdIns(4,5)$P_2$ in cells treated with $C_8$-PA, making this mechanism unlikely, but we cannot exclude the possibility that localized pools of PtdIns(4,5)$P_2$ may be increased. Sphingosine kinase has recently been found to be an effector of PA (Delon et al., 2004).

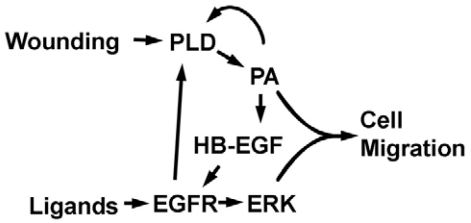


**Fig. 10.** Model of PLD signaling in cell motility in corneal epithelial cells. Wounding stimulates PLD and/or EGFR through unknown mechanisms. PA synthesis is amplified through a positive feed-back loop. Increased PA leads to activation of the EGFR, and PLD can conversely be activated by EGFR signaling. Both EGFR and PA signaling are necessary for epithelial cell motility.

Movement of corneal epithelial cells is dependent on transactivation of the EGF receptor (Block et al., 2004; Xu et al., 2004), and recently a role for sphingosine 1-phosphate was established in EGF-induced cell migration (Le Stunff et al., 2004). Finally, PA is reported to facilitate recruitment of Raf-1 to the plasma membrane, which may accentuate the activation of the EGFR/ERK1/2 signaling pathway in response to PA (Andresen et al., 2002; Rizzo et al., 2000). How any of these or other targets might result in activation of a member of the ADAM family of proteases to transactivate the EGFR remains unknown.

In conclusion, we present a hypothetical outline for the role of PA signaling in epithelial cell migration (Fig. 10). The initial stimulatory signal is still unknown but may relate to interactions with the extracellular matrix and/or to mechanical processes in cells, induced by wounding. Wounding induces activation of PLD through a process that does not depend on EGFR activation and synthesis of PA is enhanced through a positive feed-back loop. Production of PA triggers activation of the EGFR through the classical triple-membrane pass transactivation process (Fig. 7C). In addition, the EGFR can be activated by soluble ligands present in the wound milieu or by stimulation by the EGF-like repeats that are present in certain extracellular matrix proteins (Tran et al., 2004), which provides a second possible signaling input. Activation of the EGFR/ERK1/2 pathway is absolutely required for induction of motility, but additional PA signaling is required as well.

## Materials and Methods

### Materials

Antibodies against phospho-ERK1/2 were from Santa Cruz Biotechnology, antibodies against vinculin were from Sigma, Alexa Fluor® 546 phalloidin was from Invitrogen, antibodies against E-cadherin were from BD Biosciences and antibodies against β-actin were from Sigma. Antibodies against phospho-MARCKS (Ser152/156), inhibitors of ERK kinase and PI 3-kinase, Pertussis toxin, and PMA were from EMD Biosciences. Recombinant human EGF and HGF were from Cell Sciences. [$^3$H]Myristic acid was from Perkin Elmer Life Sciences. Tissue culture reagents were from Mediatech (Herndon, VT). Lipid-free bovine serum albumin was from Roche Diagnostics. Synthetic lipids were from Avanti Polar Lipids (Alabaster, AL). Other reagents were from Sigma or Fisher Scientific, except where indicated.

### Cell culture

Rabbit eyes were purchased from Pel-Freez (Rogers, AZ). Corneas with adjacent limbus were excised and grown as explants according to an established protocol (Ebato et al., 1988) in supplemented hormonal epithelial medium consisting of base medium [Ham's F12 and Dulbecco's modified Eagle's medium (DMEM; 1:1)], 0.5% DMSO, 50 IU/ml penicillin, and 50 μg/ml streptomycin) supplemented with 15% fetal bovine serum (FBS), 0.1 μg/ml cholera toxin, 10 ng/ml human

recombinant EGF, 5 μg/ml insulin, and 40 μg/ml gentamycin, and were used at passage one. MDCK cells were kindly provided by Ora A. Weisz (University of Pittsburgh, PA) and grown in DMEM supplemented with 10% FBS. Human neonatal keratinocytes were from Cascade Biologicals and were cultured in EpiLife® medium with human keratinocyte growth supplement. Immortalized human corneal epithelial cells were provided by Irene Gipson (Harvard Medical School, Boston, MA) and grown as described previously (Gipson et al., 2003). Wild-type PLD2 and a catalytically inactive PLD2(K758R) mutant (Sung et al., 1997) cloned into adenovirus vectors (He et al., 1998) were a generous gift from Michael P. Czech (University of Massachusetts, Worcester, MA). These vectors also code for the green fluorescent protein. For infection, MDCK cells were seeded at 15,000 per 3.5-cm tissue culture plate. Two days later, they were infected at a multiplicity of 25 for 4 hours, and photographs were taken after 14-16 hours. Vero cells were a kind gift from Robert L. Hendricks (University of Pittsburgh, PA).

### Immunofluorescence microscopy and western blotting

Immunofluorescence labeling was performed as described using formaldehyde fixed cells, and microscopy was performed as described previously (Block et al., 2004). Western blotting was performed according to standard procedures, and blots were developed using the SuperSignal® Dura detection kit (Pierce). Equal loading of the lanes in gels was routinely monitored by protein staining. Briefly: after autoradiography, the blots were immersed in 0.1% Ponceau S in 5% acetic acid for 5 minutes. The blots were then destained in 40% ethanol, 10% acetic acid.

### Wounding model and assay for PLD activity

Cells were seeded on plates containing agarose droplets, and cell migration was initiated by removal of droplets (Block et al., 2004). To determine PLD activities, cells were labeled with 15 μCi [$^3$H]myristic acid for 6 hours, stimulated as indicated and 1-butanol was added to a final concentration of 0.5% for 5 minutes. The dishes were placed on ice and rinsed with cold phosphate-buffered saline (PBS: 171 mM NaCl, 10.1 mM $Na_2HPO_4$, 3.35 mM KCl, 1.84 mM $KH_2PO_4$, pH 7.2). The lipids were then extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). The dried samples were resuspended in chloroform:methanol (2:1), and the phospholipids were resolved on oxalate-treated silica gel G/UV TLC plates (Whatman) by developing with $CHCl_3$:methanol:glacial acetic acid (50:15:2). The plates were air-dried, treated with EN$^3$HANCE® (PerkinElmer Life and Analytical Sciences, Boston, MA), and exposed to a Kodak Biomax XAR film at –80°C. Following autoradiography, phosphatidylbutanol and phosphatidylcholine spots were cut from the chromatography plates, and the radioactivity was measured in a Beckman LS5000 β counter. Labeled phosphatidylbutanol was identified by co-migration with authentic phosphatidylbutanol, by its absence without addition of 1-butanol, and by its increase upon stimulation of cells with PMA. Labeled phosphatidylcholine was identified by co-migration with authentic phosphatidylcholine. The amount of radioactivity in phosphatidylbutanol was normalized to the amount in phosphatidylcholine for each sample. To measure the rates of wound closure, cells were seeded in the presence of agarose strips (Block et al., 2004), and healing was allowed to proceed in DMEM with 10% FBS (MDCK cells) or base medium with 10% FBS (corneal epithelial cells). Wound healing was monitored by measuring the widths of wounds as described previously (Block et al., 2004).

### Assay for PtdIns(4,5)$P_2$

Cells were labeled for 24 hours with 5 μCi $^{32}P_i$/ml in phosphate-free DMEM and 10% FBS. The lipids were extracted (Bligh and Dyer, 1959) and resolved on oxalate-treated silica gel plates using $CHCl_3$:methanol:4 M $NH_4OH$ (9:7:2) as the solvent. The radioactive PtdIns(4,5)$P_2$ spot was identified by co-migration with authentic PtdIns(4,5)$P_2$ standard. The amount of radioactivity in PtdIns(4,5)$P_2$ was normalized to the amount in phosphatidylcholine for each sample. As a positive control, cells were infected with adenovirus coding for the murine α-type I phosphatidylinositol 4-phosphate 5′-kinase (kindly provided by Ora A. Weisz) together with tet-off™ adenovirus (BD Biosciences), both at a multiplicity 25.

### Migration through Transwell® membranes

Corneal epithelial cells were seeded at 90,000 per Transwell® (Corning) insert (8 μm pore size) in base medium with 10% FBS and used after 2 days. Migration was allowed to proceed for 8 hours in base medium with 2% FBS in the presence of the indicated compounds. The cells that had not migrated through the filters were removed with a fine brush and the inserts were fixed in 3.7% formaldehyde and 0.5% Triton X-100. Nuclei were subsequently stained with ethidium bromide, 2 μg/ml in PBS. Ten or twenty randomly chosen fields of each insert were counted using a fluorescent microscope.

We thank Irene Gipson for providing the immortalized human corneal epithelial cell line, and Sylvain G. Bourgoin for the anti-PLD2 antibody and immunizing peptide. We thank Michelle Rigatti for assistance in preparing the epithelial cell explants and Kira Lathrop for help in preparing the figures. This work was supported by the National Institutes of Health Grants EY013463 and EY08098, and grants from Research to Prevent Blindness and The Eye and Ear Foundation (Pittsburgh, PA, USA).

## References

**Adachi, T., Nakashima, S., Saji, S., Nakamura, T. and Nozawa, Y.** (1996). Phospholipase D activation in hepatocyte growth factor-stimulated rat hepatocytes mediates the expressions of c-jun and c-fos: involvement of protein tyrosine kinase, protein kinase C, and Ca2+. *Hepatology* **24**, 1274-1281.

**Andresen, B. T., Rizzo, M. A., Shome, K. and Romero, G.** (2002). The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade. *FEBS Lett.* **531**, 65-68.

**Arbuzova, A., Schmitz, A. A. and Vergeres, G.** (2002). Cross-talk unfolded: MARCKS proteins. *Biochem. J.* **362**, 1-12.

**Bligh, E. G. and Dyer, W. J.** (1959). A rapid method of total lipid extraction and purification. *Can. J. Med. Sci.* **37**, 911-917.

**Block, E. R., Matela, A. R., SundarRaj, N., Iszkula, E. R. and Klarlund, J. K.** (2004). Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints: role of heparin-binding epidermal growth factor-like growth factor signaling. *J. Biol. Chem.* **279**, 24307-24312.

**Chen, X. and Resh, M. D.** (2002). Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor. *J. Biol. Chem.* **277**, 49631-49637.

**Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altshuller, Y., Bar-Sagi, D., Morris, A. J. and Frohman, M. A.** (1997). Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7**, 191-201.

**Coulombe, P. A.** (2003). Wound epithelialization: accelerating the pace of discovery. *J. Invest. Dermatol.* **121**, 219-230.

**Cross, M. J., Roberts, S., Ridley, A. J., Hodgkin, M. N., Stewart, A., Claesson-Welsh, L. and Wakelam, M. J.** (1996). Stimulation of actin stress fibre formation mediated by activation of phospholipase D. *Curr. Biol.* **6**, 588-597.

**Czech, M. P.** (2000). PIP2 and PIP3: complex roles at the cell surface. *Cell* **100**, 603-606.

**Davis, R. J., Girones, N. and Faucher, M.** (1988). Two alternative mechanisms control the interconversion of functional states of the epidermal growth factor receptor. *J. Biol. Chem.* **263**, 5373-5379.

**Delon, C., Manifava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S. and Ktistakis, N. T.** (2004). Sphingosine kinase 1 is an intracellular effector of phosphatidic Acid. *J. Biol. Chem.* **279**, 44763-44774.

**Doughman, R. L., Firestone, A. J. and Anderson, R. A.** (2003). Phosphatidylinositol phosphate kinases put PI4,5P(2) in its place. *J. Membr. Biol.* **194**, 77-89.

**D'Souza-Schorey, C.** (2005). Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* **15**, 19-26.

**Du, G., Huang, P., Liang, B. T. and Frohman, M. A.** (2004). Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. *Mol. Biol. Cell* **15**, 1024-1030.

**Ebato, B., Friend, J. and Thoft, R. A.** (1988). Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Invest. Ophthalmol. Vis. Sci.* **29**, 1533-1537.

**Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W. H.** (1993). The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* **291**, 677-680.

**Exton, J. H.** (2002a). Phospholipase D-structure, regulation and function. *Rev. Physiol. Biochem. Pharmacol.* **144**, 1-94.

**Exton, J. H.** (2002b). Regulation of phospholipase D. *FEBS Lett.* **531**, 58-61.

**Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A. and Chen, J.** (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **294**, 1942-1945.

**Fischer, O. M., Hart, S., Gschwind, A. and Ullrich, A.** (2003). EGFR signal transactivation in cancer cells. *Biochem. Soc. Trans.* **31**, 1203-1208.

**Frank, C., Keilhack, H., Opitz, F., Zschornig, O. and Bohmer, F. D.** (1999). Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry Mosc.* **38**, 11993-12002.

**Gipson, I. K., Spurr-Michaud, S., Argueso, P., Tisdale, A., Ng, T. F. and Russo, C. L.** (2003). Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest. Ophthalmol. Vis. Sci.* **44**, 2496-2506.

**Ha, K. S. and Exton, J. H.** (1993). Activation of actin polymerization by phosphatidic acid derived from phosphatidylcholine in IIC9 fibroblasts. *J. Cell Biol.* **123**, 1789-1796.

**He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. and Vogelstein, B.** (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95**, 2509-2514.

**Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A. et al.** (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521-532.

**Imamura, H., Takaishi, K., Nakano, K., Kodama, A., Oishi, H., Shiozaki, H., Monden, M., Sasaki, T. and Takai, Y.** (1998). Rho and Rab small G proteins coordinately reorganize stress fibers and focal adhesions in MDCK cells. *Mol. Biol. Cell* **9**, 2561-2575.

**Ishii, I., Fukushima, N., Ye, X. and Chun, J.** (2004). Lysophospholipid receptors: signaling and biology. *Annu. Rev. Biochem.* **73**, 321-354.

**Iyer, S. S., Barton, J. A., Bourgoin, S. and Kusner, D. J.** (2004). Phospholipases D1 and D2 coordinately regulate macrophage phagocytosis. *J. Immunol.* **173**, 2615-2623.
**Jenkins, G. H., Fisette, P. L. and Anderson, R. A.** (1994). Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.* **269**, 11547-11554.
**Kam, Y. and Exton, J. H.** (2001). Phospholipase D activity is required for actin stress fiber formation in fibroblasts. *Mol. Cell. Biol.* **21**, 4055-4066.
**Kodaki, T. and Yamashita, S.** (1997). Cloning, expression, and characterization of a novel phospholipase D complementary DNA from rat brain. *J. Biol. Chem.* **272**, 11408-11413.
**Kuo, I. C.** (2004). Corneal wound healing. *Curr. Opin. Ophthalmol.* **15**, 311-315.
**Le Stunff, H., Mikami, A., Giussani, P., Hobson, J. P., Jolly, P. S., Milstien, S. and Spiegel, S.** (2004). Role of sphingosine-1-phosphate phosphatase 1 in epidermal growth factor-induced chemotaxis. *J. Biol. Chem.* **279**, 34290-34297.
**Liscovitch, M. and Cantley, L. C.** (1995). Signal transduction and membrane traffic: the PITP/phosphoinositide connection. *Cell* **81**, 659-662.
**Lopez, I., Arnold, R. S. and Lambeth, J. D.** (1998). Cloning and initial characterization of a human phospholipase D2 (hPLD2). ADP-ribosylation factor regulates hPLD2. *J. Biol. Chem.* **273**, 12846-12852.
**Lu, L., Reinach, P. S. and Kao, W. W.** (2001). Corneal epithelial wound healing. *Exp. Biol. Med. (Maywood)* **226**, 653-664.
**Martin, P.** (1997). Wound healing–aiming for perfect skin regeneration. *Science* **276**, 75-81.
**Martin, T. F.** (1998). Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. *Annu. Rev. Cell Dev. Biol.* **14**, 231-264.
**McDermott, M., Wakelam, M. J. and Morris, A. J.** (2004). Phospholipase D. *Biochem. Cell Biol.* **82**, 225-253.
**Mitamura, T., Higashiyama, S., Taniguchi, N., Klagsbrun, M. and Mekada, E.** (1995). Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem.* **270**, 1015-1019.
**Moolenaar, W. H., van Meeteren, L. A. and Giepmans, B. N.** (2004). The ins and outs of lysophosphatidic acid signaling. *BioEssays* **26**, 870-881.
**Parker, P. J. and Murray-Rust, J.** (2004). PKC at a glance. *J. Cell Sci.* **117**, 131-132.
**Perry, D. K., Stevens, V. L., Widlanski, T. S. and Lambeth, J. D.** (1993). A novel ecto-phosphatidic acid phosphohydrolase activity mediates activation of neutrophil superoxide generation by exogenous phosphatidic acid. *J. Biol. Chem.* **268**, 25302-25310.
**Potempa, S. and Ridley, A. J.** (1998). Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. *Mol. Biol. Cell* **9**, 2185-2200.
**Powner, D. J., Hodgkin, M. N. and Wakelam, M. J.** (2002). Antigen-stimulated activation of phospholipase D1b by Rac1, ARF6, and PKCalpha in RBL-2H3 cells. *Mol. Biol. Cell* **13**, 1252-1262.
**Prindull, G. and Zipori, D.** (2004). Environmental guidance of normal and tumor cell plasticity: epithelial mesenchymal transitions as a paradigm. *Blood* **103**, 2892-2899.
**Ridley, A. J., Comoglio, P. M. and Hall, A.** (1995). Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. *Mol. Cell. Biol.* **15**, 1110-1122.
**Rizzo, M. A., Shome, K., Watkins, S. C. and Romero, G.** (2000). The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J. Biol. Chem.* **275**, 23911-23918.
**Rosen, E. M., Meromsky, L., Goldberg, I., Bhargava, M. and Setter, E.** (1990). Studies on the mechanism of scatter factor. Effects of agents that modulate intracellular signal transduction, macromolecule synthesis and cytoskeleton assembly. *J. Cell Sci.* **96**, 639-649.
**Rubin, R. A. and Earp, H. S.** (1983). Dimethyl sulfoxide stimulates tyrosine residue phosphorylation of rat liver epidermal growth factor receptor. *Science* **219**, 60-63.
**Santy, L. C. and Casanova, J. E.** (2001). Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J. Cell Biol.* **154**, 599-610.
**Spitaler, M. and Cantrell, D. A.** (2004). Protein kinase C and beyond. *Nat. Immunol.* **5**, 785-790.
**Sundaram, M., Cook, H. W. and Byers, D. M.** (2004). The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. *Biochem. Cell Biol.* **82**, 191-200.
**Sung, T. C., Roper, R. L., Zhang, Y., Rudge, S. A., Temel, R., Hammond, S. M., Morris, A. J., Moss, B., Engebrecht, J. and Frohman, M. A.** (1997). Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. *EMBO J.* **16**, 4519-4530.
**Suzuki, K., Saito, J., Yanai, R., Yamada, N., Chikama, T., Seki, K. and Nishida, T.** (2003). Cell-matrix and cell-cell interactions during corneal epithelial wound healing. *Prog. Retin. Eye Res.* **22**, 113-133.
**Swindle, C. S., Tran, K. T., Johnson, T. D., Banerjee, P., Mayes, A. M., Griffith, L. and Wells, A.** (2001). Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. *J. Cell Biol.* **154**, 459-468.
**Thiery, J. P.** (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2**, 442-454.
**Thiery, J. P.** (2003). Epithelial-mesenchymal transitions in development and pathologies. *Curr. Opin. Cell Biol.* **15**, 740-746.
**Tran, K. T., Griffith, L. and Wells, A.** (2004). Extracellular matrix signaling through growth factor receptors during wound healing. *Wound Repair Regen.* **12**, 262-268.
**Vacaresse, N., Lajoie-Mazenc, I., Auge, N., Suc, I., Frisach, M. F., Salvayre, R. and Negre-Salvayre, A.** (1999). Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. *Circ. Res.* **85**, 892-899.
**Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C. and Bourne, H. R.** (2002). A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat. Cell. Biol.* **4**, 509-513.
**Xu, K. P., Ding, Y., Ling, J., Dong, Z. and Yu, F. S.** (2004). Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **45**, 813-820.
**Yin, H. L. and Janmey, P. A.** (2003). Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* **65**, 761-789.
**Zhang, Y. and Akhtar, R. A.** (1998). Epidermal growth factor stimulates phospholipase D independent of phospholipase C, protein kinase C or phosphatidylinositol-3 kinase activation in immortalized rabbit corneal epithelial cells. *Curr. Eye Res.* **17**, 294-300.
**Zieske, J. D., Bukusoglu, G. and Gipson, I. K.** (1989). Enhancement of vinculin synthesis by migrating stratified squamous epithelium. *J. Cell Biol.* **109**, 571-576.