Cell wounding activates phospholipase D in primary mouse keratinocytes

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Abstract  Plasma membrane disruptions occur in mechanically active tissues such as the epidermis and can lead to cell death if the damage remains unrepaired. Repair occurs through fusion of vesicle patches to the damaged membrane region. The enzyme phospholipase D (PLD) is involved in membrane trafficking; therefore, the role of PLD in membrane repair was investigated. Generation of membrane disruptions by lifting epidermal keratinocytes from the substratum induced PLD activation, whereas removal of cells from the substratum via trypsinization had no effect. Pretreatment with 1,25-dihydroxyvitamin D3, previously shown to increase PLD1 expression and activity, had no effect on, and a PLD2-selective (but not a PLD1-selective) inhibitor decreased, cell lifting-induced PLD activation, suggesting PLD2 as the isoform activated. PLD2 interacts functionally with the glycerol channel aquaporin-3 (AQP3) to produce phosphatidylglycerol (PG); however, wounding resulted in decreased PG production, suggesting a potential PG deficiency in wounded cells. Cell lifting-induced PLD activation was transient, consistent with a possible role in membrane repair, and PLD inhibitors inhibited membrane resealing upon laser injury. In an in vivo full-thickness mouse skin wound model, PG accelerated wound healing. These results suggest that PLD and the PLD2/AQP3 signaling module may be involved in membrane repair and wound healing.—Arun, S. N., D. Xie, A. C. Howard, Q. Zhong, X. Zhong, P. L. McNeil, and W. B. Bollag. Cell wounding activates phospholipase D in primary mouse keratinocytes. J. Lipid Res. 2013. 54: 581–591.

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Keratinocytes form the epithelium of the skin, the epidermis, and comprise several cell layers. As keratinocytes migrate up from the stratum basale, they undergo a distinct pattern of differentiation that is essential for the function of the skin as a protective barrier. This pattern is characterized by growth arrest and expression of the mature keratins 1 and 10 in the first differentiated layer of the epidermis, the spinous layer. Early differentiation in the spinous layer is followed by further differentiation in the granular layer, which is accompanied by expression of proteins that are essential for the formation of the cornified envelope and corneocytes. The corneocytes constitute the outer layer of the epidermis, the stratum corneum, and give skin its resilience to mechanical stress (as reviewed in Ref. 1). Deficiencies in the mechanical barrier function of the epidermis result in skin diseases. For example, epidermolysis bullosa simplex and epidermolytic hyperkeratosis arise through mutations in keratins comprising the intermediate filaments and are characterized by extensive blistering and epidermal sloughing as a result of the mechanical stresses encountered by routine interactions with the environment (as reviewed in Ref. 2).

Many tissues of the body in addition to the skin are exposed to mechanical stresses that result in tearing, or disrupting, the plasma membrane of the constituent cells. These disruptions will result in cell death if left unrepaired. However, cells possess an active plasma membrane repair process that can restore plasma membrane integrity if the disruption is not too extensive (as reviewed in Ref. 3). For example, intestinal cells in the gastrointestinal tract are subjected to mechanical perturbations during the transit of a food bolus; these plasma membrane disruptions can be repaired to allow cell survival (4–6). Similarly, eccentric contraction of skeletal muscle as a result of downhill treadmill running induces plasma membrane disruptions that are largely repaired (7). Routine ambulation also appears to lead to plasma membrane disruptions in the epidermis of the digits (8). Therefore, it is critical that cells in these mechanically active tissues be able to repair membrane

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tears in order to prevent extensive tissue damage, or disease can result. As an example, some forms of muscular dystrophy may be caused in part by a reduced ability of skeletal muscle cells to repair membrane disruptions. Indeed, muscle fibers from the dysferlin knockout mouse, a mouse model of limb-girdle muscular dystrophy and Miyoshi myopathy, fail to properly repair cell wounds (9). In addition, patients with a form of Miyoshi myopathy in which mutations in dysferlin are not observed nevertheless show a skeletal muscle cell membrane repair defect, which is correlated with the severity of their disease (10), indicating the importance of this process to tissue health.

The enzyme phospholipase D (PLD) hydrolyzes phospholipids, primarily phosphatidylcholine, to generate phosphorylcholine, which can be dephosphorylated by lipid phosphate phosphatases to yield diacylglycerol. Two isoforms of PLD, PLD1 and PLD2, have been well-characterized (as reviewed in Ref. 11). Interestingly, both PLD isoforms can also, in the presence of primary alcohols, catalyze a transphosphatidylation reaction to generate a phosphatidylalcohol. In fact, PLD utilizes alcohols such as ethanol and butanol to yield phosphatidylethanol (PEt) or phosphatidylbutanol (12), even at a low alcohol concentration. PLD’s preferential use of alcohols has been puzzling: in the absence of evolutionary pressure to utilize these alcohols, why should PLD retain the ability to catalyze this transphosphatidylation reaction? We have proposed that PLD has retained this function in order to use the physiological alcohol glycerol to synthesize phosphatidyglycerol (PG) (as reviewed in Refs. 13, 14). Indeed, our data indicate that in keratinocytes, PLD2 colocalizes and coimmunoprecipitates with the glycerol channel aquaporin-3 (AQP3) (15), and glycerol can be utilized by PLD to generate PG in intact keratinocytes [(16) and as reviewed in Refs. 13, 14]. Thus, we have proposed that AQP3 provides glycerol to PLD2 for the production of PG via the transphosphatidylation reaction, and that this PG acts as a novel lipid signaling molecule to regulate early keratinocyte differentiation (as reviewed in Refs. 13, 14). In a recent study, we have shown that manipulations that alter the function of this PLD2/AQP3/PG signaling module can inhibit epidermal keratinocyte proliferation and promote differentiation (17).

PLD also regulates vesicle trafficking and membrane fusion, as well as actin cytoskeleton rearrangements (as reviewed in Refs. 11, 18). Because both vesicle fusion and disintegration of cortical actin are required for membrane resealing of plasma membrane disruptions (3), we hypothesized that PLD would be activated by cell wounding and mediate, at least in part, membrane repair. In this report, we demonstrate that, in fact, cell wounding, induced by lifting of the cells from the extracellular matrix, activated PLD. Treatment with 1,25-dihydroxyvitamin D$_3$[1,25(OH)$_2$D$_3$], which we have previously shown to increase PLD1 expression and activity (19), did not affect PLD activity elicited by wounding, suggesting that PLD2 was the PLD isoform activated by plasma membrane disruption. This result was also consistent with the observed ability of a PLD2-selective, but not a PLD1-selective, inhibitor to decrease cell lifting-induced PLD activity. Interestingly, however, despite our previous finding that PLD2 activity appears to mediate PG production in response to elevated extracellular calcium levels (16), there was no increase in the production of PG upon wounding. This result suggests the possibility that the functional interaction between PLD2 and AQP3 was disturbed and therefore suggests that a potential deficiency in PG levels might accompany wounding. In addition, we showed that PLD1 and PLD2 inhibitors tended to inhibit the repair of laser-induced plasma membrane wounds, whereas the PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) significantly inhibited this process. This result indicates the importance of PLD’s function in mediating the repair of plasma membrane disruptions caused by mechanical stresses. Because plasma membrane wound repair is one aspect of macroscopic wound healing, we also examined the role of PG in the healing of a full-thickness skin wound and found that PG promoted wound healing.

MATERIALS AND METHODS

Materials

Calcium-free minimum essential medium (MEM)-a was from Biologos, Inc. (Montgomery, IL), ITS+ (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 µg/ml BSA, and 5.35 µg/ml linoleic acid) from Collaborative Biomedical Products (Bedford, MA), and bovine pituitary extract and epidermal growth factor from Gibco Invitrogen (Grand Island, NY). DC protein assay reagents were from Bio-Rad (Hercules, CA). BSA and FIPI were from Sigma (St. Louis, MO), and [3H]oleic acid, [14C]glycerol, [3H]leucine, and [3H]thymidine from Perkin Elmer NEN (Waltham, MA). Hank’s buffered salt solution (HBSS) was obtained from Mediatech (Manassas, VA). Phosphatidic acid, PEt, and PG derived from egg (egg PG) were obtained from Avanti Polar Lipids (Alabaster, AL), FM1-43 from Invitrogen, and the PLD1- (CAY10593) and PLD2-selective (CAY10594) inhibitors from Cayman Chemical (Ann Arbor, MI). Commercial keratinocyte serum-free medium (K-SFM) and the appropriate supplements were obtained from Gibco Invitrogen. PBS contained 137.9 mM NaCl, 2.7 mM KCl, 15.2 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, and 1 mM MgCl$_2$ (with or without 1.2 mM CaCl$_2$), all from Sigma.

Keratinocyte culture

Mouse epidermal keratinocytes were isolated from newborn ICR CD1 outbred mice and cultured as described previously (19). Briefly, skins were harvested and floated overnight on 2.5% trypsin at 4°C prior to a brief incubation at 37°C. The dermis and epidermis were then mechanically separated, and the keratinocytes gently scraped from the underside of the epidermis. Cells were collected by centrifugation and seeded overnight in a dialyzed serum-containing plating medium, followed by refedding with serum-free keratinocyte medium (K-SFM) as in (19). In some experiments, after incubation overnight in plating medium, cells were refed with K-SFM (containing 50 µM calcium chloride) and cultured until confluence.

PLD activity assay

Cells were prelabeled with 5 µCi/ml [3H]oleic acid in SFKM for 20–24 h prior to refedding with SFKM or HBSS. Ethanol
In vivo wound healing

Male and female ICR CD1 mice were anesthetized with isoflurane and their flanks shaved. Two approximately 4 mm diameter full-thickness wounds were made using a punch biopsy. The wounds were untreated or treated daily with 2 M glycerol in water, PBS lacking divalent cations (PBS−), or 100 µg/ml PG, prepared as liposomes by sonication in PBS−. The wounds were digitally photographed and analyzed using ImageJ imaging software in comparison with a simultaneously photographed circle of known size. The experiment was repeated on a second group of mice, with the opposite side exposed to the treatment of interest (glycerol or PG liposomes). No difference was observed between male and female mice, so the results were pooled.

Statistical analysis

All experiments were performed a minimum of three times. Statistical analysis was performed as indicated using Graphpad Prism or Instat (La Jolla, CA).

RESULTS

Activation of PLD by cell wounding induced upon cell lifting

Scraping or lifting cells from tissue culture dishes with a rubber policeman induces plasma membrane disruptions and cell wounding (22). We tested PLD activity after plasma membrane disruptions in epidermal keratinocytes by monitoring changes in radiolabeled PEt levels in [3H]oleate-prelabeled cells scraped or lifted from the culture dish in the presence of 1% ethanol. Controls included cells exposed to ethanol without scraping or lifting as well as cells released from the dish by trypsinization. As shown in Fig. 1A, we found that in SFKM cell wounding by gently lifting the cells from the culture dish with a rubber policeman, but not trypsinization, activated PLD, resulting in increased [3H]PEt levels. This PLD activation was not the result of growth factors present in the medium, because similar results were obtained when the wounding was performed in HBSS (Fig. 1B). Similar results were also obtained when cells were more vigorously scraped from the culture substratum with a plastic cell lifter (data not shown).

We then determined the time course of the PLD activation upon cell lifting. To do so, 1% ethanol was added to the cultures at various times after the lifting (immediately before and 15 min after), and PLD activity was monitored by radiolabeled PEt levels. As previously, cell lifting in the presence of 1% ethanol activated PLD. However, when ethanol was added 15 min after lifting of the cells with a rubber policeman (for 15 minutes), PLD activity had returned to a basal, nonlifted level (Fig. 2). Because membrane repair occurs rapidly in the presence of calcium [e.g., (20, 21) and see below], this result indicates that upon membrane repair, PLD activity returned to basal levels, suggesting a possible role for this enzyme in the repair process.

Effect of 1,25-dihydroxyvitamin D3, an inducer of PLD-1 expression and activity, on wounding-induced PLD activation

In previous experiments, we have demonstrated that a 24 h pretreatment with 250 nM 1,25(OH)2D3 increases PLD1 expression and activity (19) and can enhance PLD activation measured in response to some agonists (23). To determine whether the PLD isoform activated in response to cell wounding was PLD1, we pretreated keratinocytes with a rubber policeman or exposed to 0.5% trypsin for 15 min. Reactions were terminated with 0.2% SDS containing 5 mM EDTA, and the lipids were extracted and separated as above.

PG production

Radiolabeled PG production was measured as in (16). Briefly, cells were lifted gently from the culture dish with a rubber policeman in the presence of [14C]glycerol for 15 min. Reactions were terminated with 0.2% SDS containing 5 mM EDTA, and the lipids extracted and separated as above.

Membrane repair assay

Plasma membrane repair following cell wounding was measured as in (20, 21). Keratinocytes were incubated with PLD inhibitors or DMSO (vehicle control) in SFKM or KSFM prior to washing and addition of PBS with or without calcium. FM 1-43 dye (2.5 µM) was added to the cells immediately prior to wounding by a Zeiss LSM 510 confocal laser scanning microscope containing a Meta System equipped with a Coherent Mira 900 tunable Ti:Sapphire laser for multi-photon excitation, with the laser used at full power at a wavelength of 895 nm. Intracellular uptake of FM 1-43 after two-photon injury was measured by analyzing fluorescence intensity (FI) of each cell using Zeiss LSM 510 imaging software. Percent recovery was calculated using the following equation:

\[
\text{% recovery} = \frac{\text{FI in the absence of calcium} - \text{FI of treated cells}}{\text{FI in the absence of calcium} - \text{FI in the presence of calcium}} \times 100\%
\]

This equation sets the percent recovery in the absence of calcium at 0% and in presence of calcium at 100%.

Cytotoxicity assay

The cytotoxicity of PLD inhibitors was monitored as effects on protein synthesis and DNA synthesis by measuring the incorporation of radiolabeled leucine or thymidine, respectively, into TCA-precipitable macromolecules. Cell exposure to PLD inhibitors was set to 3 h, coinciding with the maximal length of time that keratinocytes were incubated with PLD inhibitors during the cell wounding assays. In these experiments, near-confluent to confluent keratinocytes were incubated for 2 h with the PLD inhibitors, followed by addition of 1 µCi/ml [3H]leucine (or [3H]thymidine) and incubation for an additional 60 min. Reactions were terminated and macromolecules precipitated by the addition of cold 5% TCA. After washing, cells were solubilized in NaOH, and an aliquot was counted by liquid scintillation spectrometry.

In vivo wound healing

Male and female ICR CD1 mice were anesthetized with isoflurane and their flanks shaved. Two approximately 4 mm diameter full-thickness wounds were made using a punch biopsy. The wounds were untreated or treated daily with 2 M glycerol in water, PBS lacking divalent cations (PBS−), or 100 µg/ml PG, prepared as liposomes by sonication in PBS−. The wounds were digitally photographed and analyzed using ImageJ imaging software in comparison with a simultaneously photographed circle of known size. The experiment was repeated on a second group of mice, with the opposite side exposed to the treatment of interest (glycerol or PG liposomes). No difference was observed between male and female mice, so the results were pooled.
with $1,25(OH)_2D_3$ for 24 h before monitoring PLD activation in lifted cells as in Fig. 1. Although PLD was still activated by cell lifting in the $1,25(OH)_2D_3$-pretreated keratinocytes, radiolabeled PEt levels were not enhanced in these cells; in fact, PEt levels were not even increased to as great an extent with $1,25(OH)_2D_3$ pretreatment as without (Fig. 3). This result suggests that PLD2, rather than PLD1, is the isoform activated upon cell wounding induced by lifting of keratinocytes from the culture dish.

**Effect of PLD1- and PLD2-selective inhibitors on wounding-induced PLD activation**

The results shown in Fig. 3 suggest that PLD2 is the PLD isoform activated by cell wounding. We, therefore, determined the effect of PLD-selective inhibitors on cell lifting-elicted PLD activation. Radiolabeled cells were pretreated with the indicated concentrations of the PLD1-selective inhibitor CAY10593 and the PLD2-selective inhibitor CAY10594 (24) prior to lifting and monitoring of PLD activity. The concentrations of the PLD inhibitors were selected based on the data shown in (24), as determined in intact cells (i.e., Fig. 6 of the cited reference, with CAY10593 corresponding to compound #69 and CAY10594 to compound #72 in this article). Our results demonstrate that the PLD2-selective, but not the PLD1-selective, inhibitor decreased radiolabeled PEt production in cells lifted from the substratum in the presence of ethanol (Fig. 4). These data strongly suggest that PLD2 is the PLD isoform activated by cell wounding.

**Effect of wounding-induced PLD activation on PG levels**

We have previously shown that the PLD2 isoform colocalizes and coinmunoprecipitates with the glycerol channel AQP3 (25). Furthermore, radiolabeled PG levels are increased upon the addition of radiolabeled glycerol in a PLD-mediated manner in keratinocytes stimulated to differentiate with elevated extracellular calcium levels (16), and our data suggest that this PG increase is mediated by PLD2 (16). Because the current study suggested that PLD2 was the isoform activated by cell wounding, we examined the effect of cell lifting on PG levels. Despite the increase in PLD activity, cell wounding decreased the levels of radiolabeled PG (Fig. 5). On the other hand, lifting the cells from the substratum by trypsinization had no effect on $[^{14}C]$PG levels. Together, these results suggest that 1) the functional interaction between PLD2 and AQP3 is disrupted by cell wounding, and 2) PG levels may be deficient in wounded cells.

**Effect of PLD inhibition on wound repair**

Our results indicate that PLD, in particular PLD2, is activated upon plasma membrane disruptions induced by cell lifting from the substratum. To determine the role of this PLD activation, we first determined the effect of inhibiting PLD1 or PLD2 activity on the ability of keratinocytes to repair plasma membrane disruptions. A laser was used to disrupt cell membranes in the presence of a lipid-soluble fluorescent dye. This dye enters the cell and binds to membrane-delimited cell organelles such that the fluorescence intensity of the cell continues to increase until the membrane disruption is repaired, thereby halting dye entry (20, 21). Because membrane repair requires calcium...
Q1 Cell wounding activates phospholipase D in keratinocytes caused a dose-dependent inhibition of plasma membrane resealing upon laser-induced cell wounding. Cumulative data from three experiments showed a trend toward inhibition with the 0.1 µM dose of the PLD1 inhibitor and the 1 µM concentration of the PLD2 inhibitor; thus, the fluorescence intensity under these two conditions was not significantly different from that measured in the absence of calcium (Fig. 6B).

The PLD inhibitor FPI decreased PLD activity in keratinocytes stimulated with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate

Our results with the PLD1- and PLD2-selective inhibitors suggested that both isoforms could play a role in membrane repair, although our earlier results (Figs. 3, 4) suggested that PLD2 is the isoform activated by cell lifting. We therefore wished to use an inhibitor of PLD that targets both isoforms. FPI has previously been shown to completely inhibit PLD activity in intact Chinese hamster ovary cells overexpressing PLD isoforms in the 750 nM to 7.5 µM concentration range, as well as PLD-mediated cellular responses in human embryonic kidney, COS-7, FMLP-treated HL-60 (neutrophil), and Min-6 (pancreatic β) cells (26). We determined the ability of FPI, an agent that inhibits both PLD1 and PLD2 (26), to inhibit PLD activity in intact keratinocytes by measuring the effect of FPI pretreatment on PLD activation in response to the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA). As shown previously (27), TPA stimulated PLD activity in keratinocytes, as measured by an approximate 4.5-fold increase in radiolabeled PEt levels (Fig. 7). Whereas FPI alone had no significant effect on PEt levels, pretreatment with this compound inhibited the TPA-induced PLD activation, indicating the inhibitory efficacy of FPI in intact keratinocytes.

We then performed the laser-wounding membrane repair assay in keratinocytes pretreated with 750 nM or 7.5 µM FPI. As shown in Fig. 8A, B, FPI inhibited membrane repair, such that the rate of increase in fluorescence intensity in the cells incubated with extracellular calcium and FPI was intermediate between cells incubated in the absence of calcium and cells incubated in the presence of calcium without FPI. Data analysis of multiple separate experiments to determine the degree of membrane resealing indicates that FPI inhibited membrane repair in a dose-dependent manner (Fig. 8C), indicating the importance of PLD in this process.

PLD inhibitors exerted little or no cytotoxic effect

Despite the prior report of the lack of toxicity of FPI (26), it was important to exclude the possibility that the inhibitory effects of the compound on membrane resealing were the result of a generalized cytotoxicity. Because of similar concerns about potential toxicity of the other PLD inhibitors as well, protein and DNA synthesis after exposure to the PLD inhibitors were monitored as measures of cell health. A total incubation of 3 h with the PLD inhibitors was used, because at no time were cells exposed to inhibitors for longer than this time period prior to monitoring of

Fig. 3. Pretreatment with 1,25(OH)2D3 had no enhancing effect on PLD activation induced by cell wounding. Cells were pretreated with or without 250 nM 1,25(OH)2D3 and prelabeled with 1H]oleate for 24 h in SFKM prior to assay of PLD activity upon cell lifting in Fig. 1. Values are expressed as fold over the control (with or without 1,25(OH)2D3 pretreatment) and represent the means ± SEM from four separate experiments performed in duplicate; *P < 0.01 versus the control value.

Fig. 4. A PLD2-selective but not a PLD1-selective inhibitor inhibited PLD activation induced by cell wounding. [1H]oleate-prelabeled cells were pretreated with or without the indicated concentrations of each inhibitor for 30 min in K-SFM prior to assay of PLD activity upon cell lifting as in Fig. 1. Values are expressed as fold over the lifted control and represent the means ± SEM of three separate experiments performed in duplicate; *P < 0.05, **P < 0.01 versus the lifted control value.
PG was extracted, separated by TLC, and quantified. Values are after cell lifting and incubation for 15 min. Reactions were terminated by the addition of 0.2% SDS containing 5 mM EDTA, and \(^{14}C\)PG was extracted, separated by TLC, and quantified. Values are expressed as fold over the control and represent the means ± SEM of three separate experiments performed in duplicate; \(P < 0.05\), versus the control value. Similar results were obtained when cells were more forcefully lifted from the dish using a plastic cell lifter.

membrane resealing. As shown in Fig. 9, no significant effect was found for any of the PLD inhibitors, such that protein and DNA synthesis remained at control levels after a 3 h exposure to the compounds.

**Effect of provision of PG on wound healing in vivo**

The involvement of PLD activity in membrane repair suggests that a lipid signal generated by the enzyme could mediate the process. The data shown in Fig. 5 suggest that PG levels will be reduced upon keratinocyte wounding, suggesting the possibility that if PG is important in cell membrane repair, increasing the levels of PG might accelerate the process. However, the hydrophobicity of the FM 1-43 dye used for assessment of plasma membrane repair in the laser wounding assay prevented an investigation of the effect of PG liposomes on plasma membrane repair in vitro using this technique. Nevertheless, repair of cell wounding is one aspect of macroscopic wound repair; therefore, we sought to determine the importance of PG in repair by examining the effect of PG on skin wound healing, with the expectation that increasing the levels of PG might accelerate the process. To test this idea, we made two full-thickness skin punch biopsies on either flank of two groups of mice. For one group, the wounds were treated with either PBS lacking divalent cations (PBS−) or 100 µg/ml PG liposomes in PBS−. Wound healing was followed over 4 days using digital photography and computer image analysis. Figure 10A, B show a representative mouse from each group, and Fig. 10C represents the cumulative results from eight mice per group, expressed as the percent of wound healing at day 4 relative to day 1 (to control for possible slight differences in the size of the initial wounds and wound contraction). Glycerol is known to improve skin function, and, as anticipated based on the literature (as reviewed in Ref. 28), glycerol treatment accelerated wound healing. More importantly, PG liposomes also significantly increased the rate of wound healing to a comparable degree.

**DISCUSSION**

In this study, we demonstrate that plasma membrane disruptions, as induced by cell lifting from the substratum, resulted in the activation of PLD in a time-dependent manner (Figs. 1, 2). This activation did not appear to be the result of specific factors in the medium, inasmuch as similar activation was observed in HBSS (Fig. 1B) or SFKM (Fig. 1A). In the presence of calcium, plasma membrane disruptions are known to repair within minutes [e.g., (20, 21)], if the wound is not too extensive. The time course data in Fig. 2, therefore, are consistent with a role for this PLD activation in membrane repair, inasmuch as PLD activity returned to a basal value within 15 min of the wounding by cell lifting.

The generation of radiolabeled PEt in the presence of small amounts of ethanol is a measure of PLD activation; however, this assay does not distinguish between the PLD1 and PLD2 isoforms. Our previous results indicated that a 24 h treatment with 250 nM 1,25-(OH)\(_2\)D\(_3\) induced an increase in PLD1 expression and activity (19). In the current study, 1,25(OH)\(_2\)D\(_3\) pretreatment actually slightly inhibited the PLD activity induced upon cell wounding (Fig. 3), suggesting that PLD1 was not the isoform activated. Although we have previously shown that 1,25(OH)\(_2\)D\(_3\) increases PLD1 expression and activity (19), both PLD1 and PLD2 require phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) such that decreasing PIP\(_2\) levels or availability can inhibit PLD activity (29) and as reviewed in Ref. 30. 1,25-(OH)\(_2\)D\(_3\) is known to increase PIP\(_2\) hydrolysis in keratinocytes (31, 32); therefore, the reduced cell lifting-induced PLD activation may be the result of decreased PIP\(_2\) levels inhibiting PLD2 activity. The idea that PLD2 is the isoform activated by cell wounding is consistent with the intracellular distribution and function of PLD2 versus PLD1, in that PLD2 is reported to localize predominantly to the plasma membrane in many cell types (33), whereas PLD1 is typically found on intracellular membrane compartments (34). The involvement of PLD2 was further indicated by the data showing that a PLD2-selective but not a PLD1-selective inhibitor inhibited cell wounding-induced PLD activation (Fig. 4).

We have also previously proposed that the PLD-mediated increase in radiolabeled PG levels in response to elevated calcium concentrations is related to PLD2 activity (16). This view was based in part on the inability of 1,25-(OH)\(_2\)D\(_3\) pretreatment to influence calcium-induced changes in PG levels and in part on the inhibitory (rather than stimulatory) effect on PG levels of the phospholipase TPA, which is purportedly a better activator of PLD1 than PLD2 (35). In the current study, however, PLD activation of cell wounding was not accompanied by increased PG levels (Fig. 5) despite evidence from the 1,25-(OH)\(_2\)D\(_3\) pretreatment and PLD isoform-selective inhibitor experiments (Figs. 3, 4) suggesting that PLD2 rather than PLD1 was the isoform activated. In fact, cell wounding induced a decrease in PG levels (Fig. 5). The inhibitory effect of wounding on PG levels suggests two possibilities: the first of these is that the functional association between PLD2 and AQP3 may
be disrupted upon cell wounding. The mechanism by which the functional interaction between PLD2 and AQP3 is disrupted by cell wounding is unclear, but we have demonstrated an apparent protein kinase C-mediated decrease in AQP3 activity/glycerol transport (16). Thus, protein kinase C activation in response to the increase in cytosolic calcium levels induced by cell wounding could potentially trigger a reduction in AQP3-mediated glycerol transport and thus PG production. Second, the decreased radiolabeled PG levels with cell lifting suggested that PG levels in wounded cells might also be deficient.

This latter possibility prompted us to examine the effect of PG on skin wound healing in vivo. Our results showed that PG, at a fairly low dose (100 µg/ml), promoted healing of a full-thickness skin wound (Fig. 10). Hara, Ma, and Verkman (36) have observed that AQP3 knockout mice exhibit delayed wound healing as well as other epidermal abnormalities, and we have hypothesized that one mechanism underlying the abnormalities in this mouse model might be the lack of generation of the PG lipid signal. Hara and Verkman (37) also reported that they could correct the phenotype of the AQP3 knockout by topical application of glycerol. Although aquaglyceroporins such as AQP3 facilitate the entry of glycerol into the cell, they are not strictly required, and even in the absence of AQP3, glycerol can gain entrance and presumably serve as a precursor for the generation of PG. However, if the functional coupling between PLD2 and AQP3 is lacking, pharmacological doses of glycerol would probably be necessary. In this regard, the concentration of 100 µg/ml PG used in the wound-healing experiments shown in Fig. 10 is roughly equivalent to a concentration of 100 µM, which is approximately 10,000-fold less than the 2 M glycerol concentration that yielded an essentially equal acceleration of wound healing. This result implies that direct provision of PG might be more effective and/or potent in terms of stimulating...
Therefore, dermal fibroblasts and/or immune cells may also produce PG through the PLD2/AQP3 signaling module, suggesting that PG could potentially contribute to full-thickness skin wound healing by affecting the function of multiple cell types in the skin.

Conversely, PLD activity results in second messengers in addition to PG, including phosphatidic acid and, indirectly, diacylglycerol and lysophosphatidic acid (and FFAs). Unfortunately, FM 1-43 interacts with lipids such as PG, and we were not able to examine the effect of PG liposomes on plasma membrane wound repair using the laser wounding assay. Thus, the exogenous, extracellular PG could not be removed from the cells once added, even upon extensive washing, leading to a high background fluorescence that made assay of membrane repair impossible (data not shown). Nevertheless, experiments using the PLD isoform-selective inhibitors as well as the nonisoform-selective PLD inhibitor FPI indicated that PLD activity was important in membrane repair. Both the PLD1- and PLD2-selective inhibitors reduced plasma membrane resealing in a representative experiment (Fig. 6A) and

wound healing than providing glycerol, inasmuch as the ability of AQP3 to “feed” the glycerol to PLD2 for PG production would seem to be impaired. Dermal fibroblasts also appear to express AQP3, the expression of which is induced by epidermal growth factor and modulates fibroblast migration (38), as do immune cells (39, 40). Therefore, dermal fibroblasts and/or immune cells may also produce PG through the PLD2/AQP3 signaling module, suggesting that PG could potentially contribute to full-thickness skin wound healing by affecting the function of multiple cell types in the skin.

Conversely, PLD activity results in second messengers in addition to PG, including phosphatidic acid and, indirectly, diacylglycerol and lysophosphatidic acid (and FFAs). Unfortunately, FM 1-43 interacts with lipids such as PG, and we were not able to examine the effect of PG liposomes on plasma membrane wound repair using the laser wounding assay. Thus, the exogenous, extracellular PG could not be removed from the cells once added, even upon extensive washing, leading to a high background fluorescence that made assay of membrane repair impossible (data not shown). Nevertheless, experiments using the PLD isoform-selective inhibitors as well as the nonisoform-selective PLD inhibitor FPI indicated that PLD activity was important in membrane repair. Both the PLD1- and PLD2-selective inhibitors reduced plasma membrane resealing in a representative experiment (Fig. 6A) and

Fig. 7. The PLD inhibitor FPI decreased PLD activity stimulated by TPA. [3H]oleate-prelabeled keratinocytes in SFKM were pre-treated for 20 min with 750 nM FPI prior to stimulation with or without 100 nM TPA in the presence of 0.5% ethanol for 30 min. Reactions were terminated by the addition of 0.2% SDS containing 5 mM EDTA, and [3H]PEt was extracted, separated by TLC, and quantitated. Values represent the means ± SEM of three separate experiments performed in triplicate; ***P < 0.001 versus the control; †††P < 0.01 versus TPA alone.

Fig. 8. The PLD inhibitor FPI delayed membrane repair following laser-induced membrane disruption. Keratinocytes were treated with FPI (750 nM or 7.5 µM), or 0.1% DMSO (vehicle control) for 30 min and then wounded using a sapphire laser at a wavelength of 835 nm. The control cells were wounded in PBS in the presence (Ca2+) or absence (no Ca2+) of calcium as positive and negative controls, respectively. The FPI-treated cells were wounded in PBS containing calcium. A: Shown are the means ± SEM of the fluorescence intensity of a minimum of six cells from a representative experiment; *P < 0.05 versus the values in PBS in the absence of calcium; †P < 0.05 versus the values in PBS in the presence of calcium determined using a Tukey’s multiple comparison test. B: Shown are the cumulative means ± SEM from six experiments in which the fluorescence intensity at 164 s after wounding was monitored under each condition in a minimum of six cells; *P < 0.05, **P < 0.01, ***P < 0.001 versus the values obtained in PBS lacking calcium; †P < 0.05, ††P < 0.01 versus in PBS without calcium. In panel C the data are replotted as the percentage resealing, with the recovery in the presence of calcium set to 100% and that in the absence of calcium to 0%; *P < 0.05, **P < 0.01, ***P < 0.001 versus the values obtained in PBS lacking calcium; †P < 0.05, ††P < 0.01, †††P < 0.001 versus in PBS without calcium.
Q1 Cell wounding activates phospholipase D in keratinocytes. From studies in skeletal muscle, it is known that the ability to repair plasma membrane wounding is important for the health of these cells (9, 10). Thus, a defect in plasma membrane wound healing in skeletal muscle cells underlies some forms of muscular dystrophy, in which the cells die, leading to progressive muscle weakness (as reviewed in Ref. 44). Similarly, some skin disorders, such as epidermolysis bullosa simplex and epidermolytic hyperkeratosis, are caused by mutations in the keratins comprising intermediate filaments (as reviewed in Ref. 2). These mutations decrease the resistance of the cells to mechanical perturbations such that mechanical stress triggers cell rupture and blistering of the skin (2). The common characteristic of these diseases is that the death of the mechanically wounded cells results in the appearance of macroscopic wounds. Thus, these defective wound healing experiments of nature suggest a connection between plasma membrane wound healing and tissue wounding.

In summary, our results demonstrate that plasma membrane disruptions triggered by lifting keratinocytes from the substratum induce transient PLD activation, with a time course suggestive of a potential role for this activity in membrane repair. It is not clear whether this result suggests a slightly different mechanism by which membrane tears are repaired versus membrane holes induced by a laser, or if it represents an off-target effect of the PLD1 inhibitor other than cytotoxicity, inasmuch as no toxicity was observed (Fig. 9).

The nonisoform-selective PLD inhibitor FIPI also inhibited the process of membrane repair in a dose-dependent fashion (Fig. 8). It should be noted that FIPI has previously been shown to be effective in the 750 nM- to 7.5 µM-dose range, but is irreversible (26); and indeed, we demonstrated the ability of 750 nM FIPI to inhibit TPA-induced PLD activation (Fig. 7). This inhibition of membrane resealing by FIPI also did not appear to be the result of nonspecific cytotoxicity, because neither DNA nor protein synthesis was affected by the compound (Fig. 9), consistent with previous reports of its lack of toxicity (26). Inhibition of protein synthesis has previously been reported to be a measure of the cytotoxic effects of chemical agents [e.g., (41, 42)] and, in some cases, has been found to be at least as sensitive as or more sensitive than the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (43). Thus, our data indicate that the activation of PLD in response to cell wounding probably plays a key role in allowing the cell to repair plasma membrane disruptions.

On the other hand, the relationship between plasma membrane wound healing and skin wound healing is not obvious. From studies in skeletal muscle, it is known that the ability to repair plasma membrane wounding is important for the health of these cells (9, 10). Thus, a defect in plasma membrane wound healing in skeletal muscle cells underlies some forms of muscular dystrophy, in which the cells die, leading to progressive muscle weakness (as reviewed in Ref. 44). Similarly, some skin disorders, such as epidermolysis bullosa simplex and epidermolytic hyperkeratosis, are caused by mutations in the keratins comprising intermediate filaments (as reviewed in Ref. 2). These mutations decrease the resistance of the cells to mechanical perturbations such that mechanical stress triggers cell rupture and blistering of the skin (2). The common characteristic of these diseases is that the death of the mechanically wounded cells results in the appearance of macroscopic wounds. Thus, these defective wound healing experiments of nature suggest a connection between plasma membrane wound healing and tissue wounding.

In summary, our results demonstrate that plasma membrane disruptions triggered by lifting keratinocytes from the substratum induce transient PLD activation, with a time course suggestive of a potential role for this activity in membrane repair. Further, the data suggest that PLD2 rather than PLD1 is the isoform activated. However, in contrast to the PLD2 activity elicited in keratinocytes by elevated extracellular calcium concentrations (16), this PLD2 activation does not result in enhanced PG production, suggesting a possible interruption of the AQP3 supply of glycerol to PLD2 and thus a potential disruption of the functional association between PLD2 and AQP3. This disturbance would predict PG deficiency in wounded cells, and our studies in vivo supported the ability of PG...
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liposomes, at a relatively low dose, to promote epidermal wound healing. Our results thus suggest that further studies are warranted to explore the role of PLD, the PLD2-AQP3 signaling module, and PG in wound healing.

Fig. 10. PG liposomes accelerated wound healing of full-thickness punch biopsies of mouse skin. Two full-thickness skin punch biopsies of ~4 mm were made on the backs of ICR CD-1 mice. For each mouse, one wound was either (A) untreated (left) or treated with 2 M glycerol (as a positive control) in water (right) or (B) treated with PBS lacking divalent cations (PBS, left) or PBS containing 100 µg/ml PG, prepared as liposomes via bath sonication (right). The rate of wound healing was then monitored. Shown is the extent of wound healing on day 4 for two representative mice. Both male and female mice were used and the results pooled. C: Two full-thickness skin punch biopsies were made as described. The rate of wound healing was then followed over 4 days by digital photography and computer image analysis, and percentage of wound healing on day 4 relative to day 1 for each of the four groups is shown. The experiment was repeated on a second group of mice, with the opposite side exposed to glycerol or PG liposomes. No difference was observed between male and female mice, so the results were pooled. Results represent the means ± SEM of eight mice for each condition; *P < 0.02 versus treatment with PBS; **P < 0.001 versus no treatment.
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