A Potential Role for the Phospholipase D2-Aquaporin-3 Signaling Module in Early Keratinocyte Differentiation: Production of a Phosphatidylglycerol Signaling Lipid

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In keratinocytes aquaporin-3 (AQP3), an efficient glycerol transporter, is associated with phospholipase D2 (PLD2) in caveolin-rich membrane microdomains. PLD catalyzes both phospholipid hydrolysis to produce phosphatidate and a transphosphatidylation reaction using primary alcohols to generate phosphatidylalcohols. As PLD2 can utilize the physiological alcohol glycerol to form phosphatidylglycerol (PG), we hypothesized that AQP3 provides glycerol to PLD2 for PG synthesis, which then modulates keratinocyte function. Acidic medium inhibits AQP3 transport activity; both glycerol uptake and PG synthesis were inhibited by low *versus* physiological pH. Co-transfection experiments were performed in which AQP3 or empty vector was introduced into keratinocytes simultaneously with reporter constructs in which differentiation or proliferation promoters directed expression of a luciferase reporter gene. AQP3 coexpression decreased the promoter activity of keratin 5, increased that of keratin 10 and enhanced the effect of a differentiating agent on the promoter activity of involucrin, consistent with promotion of early differentiation. Glycerol inhibited DNA synthesis, whereas equivalent concentrations of xylitol or sorbitol, as osmotic controls, had no effect. Direct provision of PG, but not phosphatidylpropanol, inhibited DNA synthesis in proliferative cells. Thus, our results support the idea that AQP3 supplies PLD2 with glycerol for synthesizing PG, a lipid signal that promotes early keratinocyte differentiation.

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INTRODUCTION

Phospholipase D (PLD) is a lipolytic enzyme that has been implicated in multiple cellular processes including growth, differentiation, vesicle trafficking and cytoskeletal rearrangement (reviewed by Bollag and Zheng, 2005). In the presence of water, PLD hydrolyzes phosphatidylcholine to produce phosphatidic acid, which serves as a second messenger itself or can, in turn, be converted to the lipid messengers, diacylglycerol or lysophosphatidic acid. However, in the presence of small amounts of a primary alcohol, PLD can also catalyze a transphosphatidylation reaction to produce phosphatidylalcohols (reviewed by Morris *et al.*, 1997). This characteristic has been utilized to specifically track the activity of PLD, but the question arises: is there a physiological primary alcohol substrate for which PLD has retained this transphosphatidylation capacity throughout the evolutionary process?

In keratinocytes, PLD activation in response to several agents has been associated with differentiation. Indeed, the keratinocyte-differentiating agent 1,25-dihydroxyvitamin D_3, increases the expression and activity of one isoform of PLD, PLD-1, without affecting the other, PLD-2 (Griner *et al.*, 1999). Phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) also activate PLD (e.g., Jung *et al.*, 1999), and we have shown that 1-butanol, which is used by PLD in the transphosphatidylation reaction to divert production away from phosphatidic acid, inhibits phorbol ester-induced transglutaminase activity, a marker of late keratinocyte differentiation (Bollag *et al.*, 2005). On the other hand, tert-butanol, an organic alcohol and 1-butanol analog that is not readily utilized by PLD in the transphosphatidylation reaction, does not inhibit transglutaminase activity, suggesting that the inhibitory effect of 1-butanol is related to its ability to inhibit PLD-mediated lipid signal generation (Bollag *et al.*, 2005).

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Abbreviations: AQP3, aquaporin-3; PG, phosphatidylglycerol PLD, phospholipase D; PP, phosphatidylpropanol; SFKM, serum-free keratinocyte medium

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In other experiments, we have shown that PLD2 can utilize glycerol *in vitro* in the transphosphatidylation reaction to generate phosphatidylglycerol (PG), and this production can occur at the expense of both phosphatidic acid and the phosphatidylalcohol, phosphatidylethanol (Zheng *et al.*, 2003). Furthermore, in intact cells, elevated extracellular calcium levels, a treatment that triggers differentiation of keratinocytes, could both activate PLD and increase PG synthesis. This enhanced PG synthesis could be inhibited by ethanol, indicating that it was mediated by PLD (Zheng *et al.*, 2003). In addition, PG production exhibited a dose dependence, with maximal synthesis observed at a concentration of extracellular calcium reported to be essentially maximal for expression of keratins 1 and 10, markers of early keratinocyte differentiation (Zheng *et al.*, 2003).

How might the glycerol have access to the intracellular PLD enzyme? The aquaglyceroporin, aquaporin-3 (AQP3) is expressed in the epidermis (Matsuzaki *et al.*, 1999; Sugiyama *et al.*, 2001; Sougrat *et al.*, 2002) and is known to be an efficient transporter of glycerol, but relatively inefficient in its transport of water (Yang and Verkman, 1997). We have previously shown that, in keratinocytes, AQP3 and PLD2 associate in caveolin-rich membrane microdomains (Zheng and Bollag, 2003). Furthermore, the presence of the AQP3 glycerol channel is required for normal epidermal function. Thus, AQP3 null mutant mice have an epidermal phenotype characterized by decreased epidermal glycerol content, dry skin, delayed wound healing and barrier recovery and decreased elasticity and water-holding capacity (Hara *et al.*, 2002; Ma *et al.*, 2002; Hara and Verkman, 2003). This phenotype can be corrected by topical or oral application of glycerol but not other osmotically active molecules (Hara and Verkman, 2003), suggesting that the effect is not simply a function of glycerol's hydrophilic properties. Asebia mice, mice possessing a stearoyl-CoA desaturase-1 mutation that results in a lack of sebaceous glands, also show a reduced epidermal glycerol content and a phenotype of dry skin and hyperproliferation (Fluhr *et al.*, 2003). In these mice also, the phenotype can be corrected by topical application of glycerol but not other humectants (Fluhr *et al.*, 2003). Thus, we have hypothesized that AQP3 transports glycerol to PLD2 for use in the transphosphatidylation reaction to produce PG and that PG, in turn, acts as a lipid second messenger to modulate keratinocyte function (reviewed by Bollag and Zheng, 2005)]. Indeed, we have proposed the existence of a novel signaling module comprised of AQP3, PLD2, glycerol and PG.

In this report we provide additional evidence for this module in keratinocytes, demonstrating that glycerol entering through an acid-sensitive aquaglyceroporin is utilized by PLD to form PG. In transient co-transfection studies AQP3 was coexpressed with reporter constructs in which promoters for markers of keratinocyte proliferative or differentiative status drive luciferase expression. These studies indicated that AQP3 coexpression inhibited the promoter activity of keratin 5, a marker of basal, proliferative keratinocytes, increased the promoter activity of keratin 10, a marker of early keratinocyte differentiation, and enhanced the effect of an elevated extracellular calcium level on the promoter activity of involucrin, a marker of intermediate differentiation. Glycerol and 1,2-propylene glycol (glycerol missing one hydroxyl group on the number 3 terminal carbon) inhibited DNA synthesis in a dose-dependent manner both in a low (25 μM) and an intermediate (125 μM) calcium concentration, whereas equivalent concentrations of the osmotically active agents, xylitol and sorbitol, had little or no effect. Direct provision of PG liposomes also inhibited DNA synthesis in a dose-dependent fashion in rapidly dividing keratinocytes, although in growth-inhibited cells PG liposomes dose dependently enhanced [^{3}H]-thymidine incorporation into DNA. These data support the idea of a signaling module consisting of AQP3, PLD2, glycerol and PG and involved in promoting growth inhibition and/or early differentiation of proliferating keratinocytes.

RESULTS

Inhibition of glycerol uptake with acidic medium inhibits PG synthesis

We have previously shown that PLD2 and AQP3 colocalize in caveolin-rich membrane microdomains in keratinocytes (Zheng and Bollag, 2003). In addition, PLD-mediated PG synthesis is stimulated by elevated extracellular calcium levels in keratinocytes (Zheng *et al.*, 2003), and we hypothesized that AQP3 provides glycerol to PLD2 for the transphosphatidylation reaction to produce PG (reviewed by Bollag and Zheng, 2005). Because in lung cells AQP3 is inhibited by acidic medium (Zelenina *et al.*, 2003), we investigated whether a medium of low pH would inhibit glycerol uptake and PG synthesis. Keratinocytes were incubated for 24 hours with control serum-free keratinocyte medium (SFKM) (25 μM Ca^{2+}) or SFKM containing 125 μM Ca^{2+} before measurement of [^{3}H]-glycerol uptake and [^{14}C]PG production in SFKM of pH 4 or 7.4. As shown in Figure 1a, 125 μM Ca^{2+} significantly stimulated glycerol uptake in control medium. Low-pH medium significantly inhibited glycerol uptake both under basal conditions and upon stimulation with the intermediate calcium concentration (Figure 1a). Similarly, pH 4 medium significantly inhibited radiolabeled PG synthesis after a 10-minute incubation with [^{14}C]glycerol, both in cells incubated with control medium and 125 μM Ca^{2+} medium (Figure 1b). To ensure that the inhibition of glycerol uptake and/or PG production by pH 4 medium was not related to toxicity, some cells were also preincubated for 5 minutes with pH 4 medium before measurement of glycerol uptake or PG synthesis in control pH 7.4 medium (pH 4/7). Note that preincubation with pH 4 medium had essentially no effect on glycerol uptake or PG production (Figure 1).

Coexpression of AQP3 inhibits keratin 5 promoter activity, stimulates keratin 10 promoter activity and enhances the effect of an elevated extracellular calcium level on involucrin promoter activity

Primary mouse epidermal keratinocytes are difficult to transfect with high efficiency. To overcome this limitation, we used the strategy of co-transfecting cells with AQP3 or the empty vector and reporter constructs in which promoters for markers of keratinocyte proliferation or differentiation control luciferase expression as described previously (Dodd *et al.*,

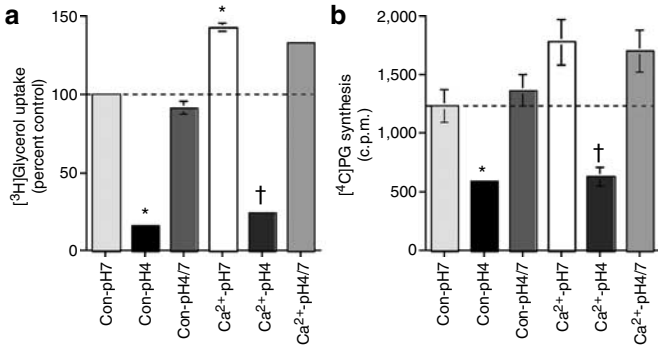

Figure 1. An extracellular medium of pH 4 inhibits radiolabeled glycerol uptake and PG synthesis. Keratinocytes were pretreated for 24 hours with control medium [25 μM Ca^{2+} (Con)] or 125 μM Ca^{2+} (Ca^{2+})-containing medium. Some cells were then incubated for 5 minutes (panel A) with medium of pH 4 before (**a**) measurement of [^{3}H]-glycerol uptake for 5 minutes, as described by Zheng *et al.* (2003), or (**b**) [^{14}C]PG synthesis for 10 minutes, also as described by Zheng *et al.* (2003), at pH 4 or 7 (7.4) as indicated. Results represent the means ± SEM of 4 experiments performed in duplicate; *$P<0.05$ *versus* the control value (glycerol uptake or PG synthesis in control cells measured at pH 7); $^{\dagger}P<0.01$ *versus* the Ca^{2+} value measured at pH 7 (7.4). Note that the effects of low pH on [^{3}H]-glycerol uptake (**a**) and [^{14}C]PG synthesis (**b**) were essentially reversible (compare pH 7 to 4/7).

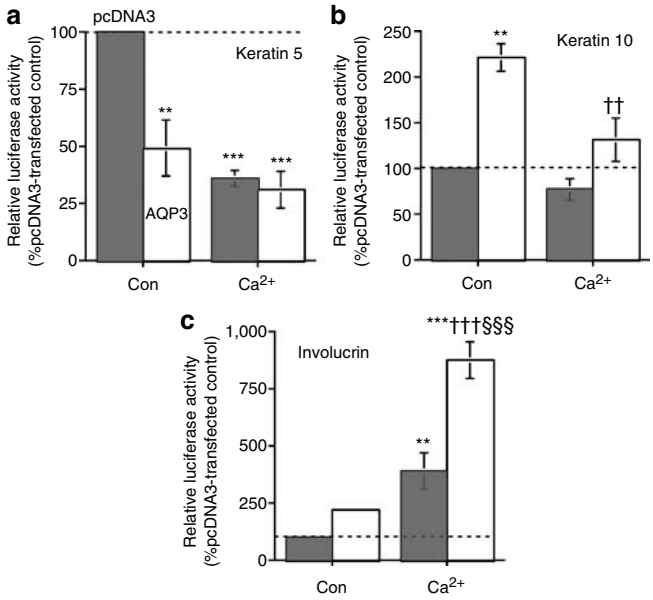

Figure 2. Effects of AQP3 overexpression. AQP3 coexpression decreases keratin 5 promoter activity, increases keratin 10 promoter activity and enhances the effect of elevated extracellular calcium concentration on involucrin promoter activity. Primary keratinocytes were co-transfected with pcDNA3 vector alone (control) or the vector possessing AQP3 and (**a**) the keratin 5 promoter/reporter gene construct, (**b**) the keratin 10 promoter/reporter gene construct, or (**c**) the involucrin promoter/reporter gene constructs (and pRL-SV40 for normalization purposes) using TransIT keratinocyte as described by the manufacturer. After 24 hours cells were refed with medium containing 25 μM (control) or 1 mM-Ca^{2+} for an additional 24 hours. Luciferase activity was then measured using a Dual Luciferase kit as directed by the manufacturer. Activity is expressed relative to the pcDNA3-transfected control cells and represents the mean ± SEM of three experiments performed in triplicate; **$P<0.01$, ***$P<0.001$ *versus* the control (untreated pcDNA3 vector) value, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ *versus* the AQP3-transfected value under control conditions and $^{\S\S\S}P<0.001$ *versus* the Ca^{2+}-treated pcDNA3 vector control value.

2005). As vectors are mixed thoroughly before transfection, cells that take up one vector should incorporate the other, allowing measurement of reporter luciferase activity only in cells that also possess AQP3 or the empty vector. Whereas keratin 5 expression characterizes basal proliferating keratinocytes, keratin 10 and involucrin mark the differentiating spinous cells, with keratin 10 serving as a marker for early differentiation and involucrin as a marker for intermediate differentiation (reviewed by Eckert *et al.*, 2005). Figure 2a illustrates the effect of AQP3 coexpression on keratin 5 promoter activity under basal conditions and after a 24-hour incubation with the differentiating agent, 1 mM calcium. AQP3 coexpression induced a significant decrease (to 49 ± 12% of the empty vector-transfected control) in keratin 5 promoter activity. Calcium (1 mM) also inhibited keratin 5 promoter activity (by 64%) and there was no significant additional effect of AQP3 coexpression. On the other hand, AQP3 coexpression stimulated keratin 10 promoter activity (Figure 2b). Treatment with 1 mM calcium slightly inhibited keratin 10 expression (by 22%), and this effect was partially reversed by AQP3 coexpression. As a differentiating agent, 1 mM calcium might be expected to increase keratin 10 promoter activity; however, such high calcium concentrations drive keratinocytes towards later differentiation and actually reduce the expression of early differentiation markers (Dlugosz and Yuspa, 1993). Finally, AQP3 coexpression had no significant effect on involucrin promoter activity alone, but enhanced the stimulation induced by 1 mM calcium (Figure 2c). These results are consistent with AQP3 coexpression promoting early keratinocyte differentiation.

Glycerol, but not xylitol or sorbitol, inhibits DNA synthesis

We hypothesize that the AQP3 and PLD2 colocalize to provide glycerol for use by PLD2 in the transphosphatidylation reaction to generate PG (reviewed by Bollag and Zheng, 2005), which then acts to promote early keratinocyte differentiation. This idea would predict that increasing the delivery of glycerol through the AQP3 channel should also trigger early differentiation. As one of the first hallmarks of early differentiation is exit from the cell cycle and a reduction in DNA synthesis (Denning, 2004), we investigated the effect of exogenous glycerol (to enhance flux through the channel) on [^{3}H]-thymidine incorporation into DNA, a measure of DNA synthesis. As shown in Figure 3a, concentrations of glycerol as low as 0.02% (2.73 mM) significantly inhibited keratinocyte DNA synthesis. We also investigated the effects of higher concentrations of glycerol. However, because osmotic stress is known to regulate keratinocyte function (Dascalu *et al.*, 2000), to control for any osmotic effects of glycerol, equivalent concentrations of two other osmolytes, xylitol and sorbitol, were also used as controls. As shown in Figure 3b, glycerol at concentrations from 0.1–1% inhibited DNA synthesis and enhanced the inhibitory effect of 125 μM Ca^{2+}. On the other hand, xylitol had no significant effect on basal or 125 μM Ca^{2+}-inhibited DNA synthesis. Similarly, we

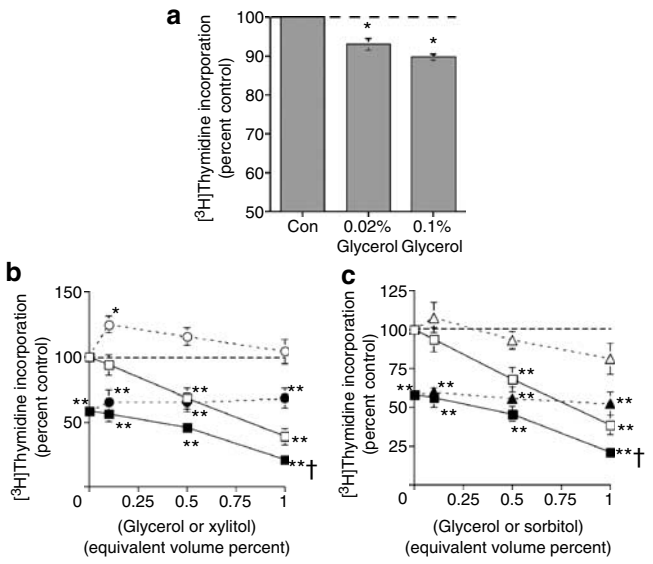

Figure 3. Glycerol, but not xylitol or sorbitol, inhibits DNA synthesis and enhances the inhibitory effect of an elevated extracellular Ca^{2+} concentration. (**a**) Near-confluent keratinocytes were incubated for 24 hours with 0.02 or 0.1% glycerol and DNA synthesis measured as the incorporation of [^{3}H]-thymidine incorporation into DNA. (**b**) Near-confluent keratinocytes were incubated for 24 hours with the indicated concentrations of glycerol (G) or equivalent concentrations of xylitol (X) in SFKM containing 25 μM (control; open symbols) or 125 μM Ca^{2+} (Ca^{2+}; closed symbols). (**c**) Near-confluent keratinocytes were incubated for 24 hours with the indicated concentrations of glycerol (G) or equivalent concentrations of sorbitol (S) in SFKM containing 25 μM (control; open symbols) or 125 μM Ca^{2+} (Ca^{2+}; closed symbols) for 24 hours. [^{3}H]-Thymidine incorporation into DNA was then determined as described by Griner *et al.* (1999). Values represent the means ± SEM of 4 to 5 separate experiments performed in duplicate; $^*P<0.05$, $^{**}P<0.01$ *versus* the control value, $^{\dagger}P<0.05$ *versus* the value in the presence of Ca^{2+} alone.

observed no significant effect of sorbitol on either control or 125 μM Ca^{2+}-reduced [^{3}H]-thymidine incorporation into DNA (Figure 3c).

AQP3 transfection sensitized keratinocytes to the inhibitory effect of glycerol on keratin 5 promoter activity

To determine whether glycerol's inhibition of proliferative status was related to AQP3, we examined the effect of transfection with AQP3, with and without glycerol treatment, on keratin 5 promoter activity. As shown in Figure 4, transfection of keratinocytes with AQP3 again (as in Figure 3a) inhibited (by approximately 32%) keratin 5 promoter activity relative to the empty vector. Glycerol also induced a small but significant (about 9%) reduction in keratin 5 promoter activity. However, keratinocytes treated with the combination of AQP3 transfection and glycerol treatment exhibited an enhanced response to glycerol, with a 47% decrease in keratin 5 promoter activity observed. This result suggests that AQP3 expression enhanced the inhibitory effect of glycerol on the basal-like phenotype of keratinocytes.

1,2-Propylene glycol inhibits DNA synthesis

In studies of the AQP3 null mutant mouse, Verkman's group demonstrated that glycerol, but not xylitol or 1,2-propylene

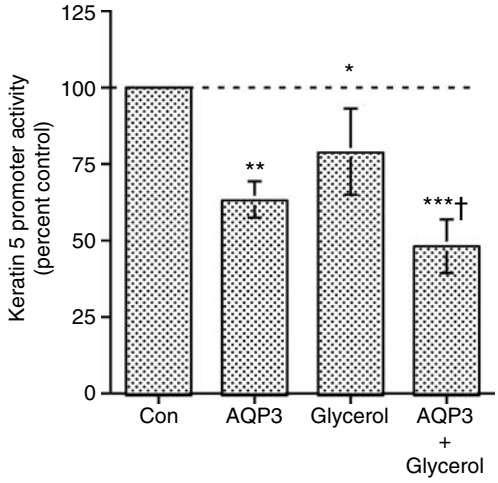

Figure 4. AQP3 expression enhances the inhibitory effect of glycerol on keratin 5 promoter activity. Primary keratinocytes were co-transfected with pcDNA3 vector alone (control) or the vector possessing AQP3 and the keratin 5 promoter/reporter gene construct (and pRL-SV40 for normalization purposes) using TransIT Keratinocyte as described by the manufacturer. After 24 hours cells were refed with medium containing no addition (control) or 0.2% glycerol for an additional 24 hours. Luciferase activity was then measured using a Dual Luciferase kit as directed by the manufacturer. Activity is expressed relative to the pcDNA3-transfected control cells and represents the mean ± SEM of five experiments performed in triplicate; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* the control (untreated pcDNA3 vector) value, $^{\dagger}P<0.05$ *versus* the AQP3-transfected value under control conditions.

glycol (or 1,3-propylene glycol), could correct the epidermal phenotype of this knockout model (Hara and Verkman, 2003). Therefore, 1,2-propylene glycol was also tested for its ability to inhibit DNA synthesis basally and upon differentiation with 125 μM Ca^{2+}. The effect of 1,2-propylene glycol was analogous to that of glycerol, exhibiting dose-dependent inhibition of [^{3}H]-thymidine incorporation under control (25 μM Ca^{2+}) conditions and upon differentiation with 125 μM Ca^{2+} (Figure 5). Also shown in Figure 5 (inset) are the structures of glycerol and 1,2-propylene glycol to demonstrate their similarity.

PG liposomes, but not phosphatidylpropanol liposomes, inhibit DNA synthesis in rapidly dividing keratinocytes and increase involucrin levels

Our hypothesis would also predict that direct provision of PG itself should also trigger early differentiation. Therefore, we determined the effect of providing PG in the form of liposomes directly to keratinocytes and found that in highly proliferative cells, PG inhibited DNA synthesis (Figure 6a). Maximal inhibition was observed at 25 μg/ml with a plateau from 50 to 100 μg/ml. This effect is not likely to represent toxicity as morphological changes characteristic of cell death were not observed (data not shown). There existed the possibility that PG liposomes inhibited DNA synthesis nonspecifically. To test this idea, keratinocytes were treated with liposomes formed from dioleoyl-phosphatidylpropanol (PP) and [^{3}H]-thymidine incorporation into DNA measured. Whereas PG liposomes inhibited DNA synthesis, PP

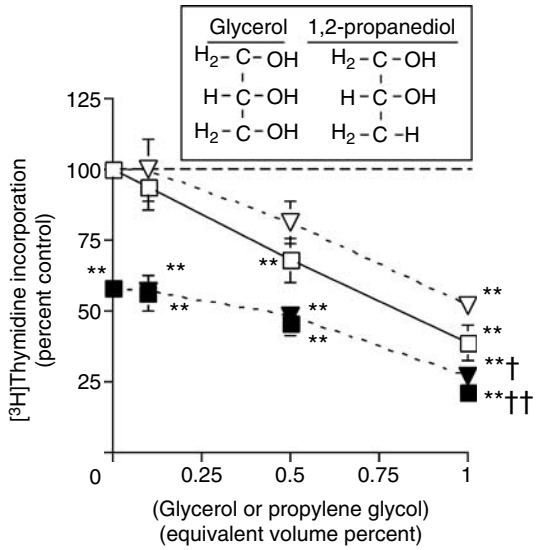

Figure 5. 1,2-Propylene glycol (1,2-propanediol) inhibits DNA synthesis and enhances the inhibitory effect of an elevated extracellular Ca^{2+} concentration. Near-confluent keratinocytes were incubated for 24 hours with the indicated concentrations of glycerol (G; squares) or equivalent concentrations of 1,2-propylene glycol (1,2-propanediol; triangles) in SFKM containing 25 μM (control; open symbols) or 125 μM Ca^{2+} (Ca^{2+}; closed symbols). [^{3}H]-Thymidine incorporation into DNA was then determined as described by Griner *et al.* (1999). Values represent the means ± SEM of 3 to 5 separate experiments performed in duplicate; $^{**}P<0.01$ *versus* the control value, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *versus* the value in the presence of Ca^{2+} alone. Shown in the inset are the structures of glycerol and 1,2-propylene glycol to demonstrate the similarity of their configuration.

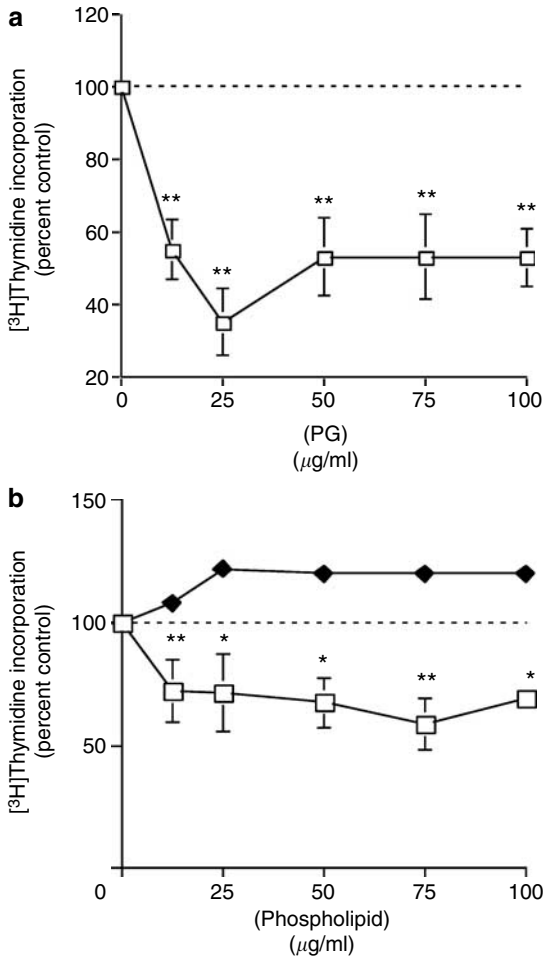

Figure 6. PG liposomes, but not phosphatidylpropanol liposomes inhibit DNA synthesis in proliferating keratinocytes. (**a**) Near-confluent keratinocytes were treated for 24 hours with the indicated concentrations of phosphatidylglycerol (PG), prepared via bath sonication of PG in SFKM. [^{3}H]-Thymidine incorporation into DNA was then determined as above. [^{3}H]-Thymidine incorporation into DNA in the control was 85,550 ± 5,730 cpm/well. Values represent the means ± SEM of 7–9 separate experiments performed in duplicate; $^{*}P<0.01$ *versus* the control value. (**b**) Near-confluent keratinocytes were treated for 24 hours with the indicated concentrations of phosphatidylglycerol (PG) or dioleoyl-phosphatidylpropanol (PP), prepared via bath sonication of PG or PP in SFKM. [^{3}H]-Thymidine incorporation into DNA was then determined as in (Griner *et al.*, 1999). Values represent the means ± SEM of 5–6 separate experiments performed in duplicate; $^{*}P<0.05$, $^{**}P<0.01$ *versus* the control value.

liposomes had no effect on this parameter (Figure 6b). The egg PG used in these experiments is composed primarily of the dioleoyl and dipalmitoyl species; therefore, we also determined that there was also no effect of dipalmitoyl-PP on DNA synthesis (data not shown).

PG liposomes enhance the effect of an elevated extracellular calcium concentration on involucrin levels

To determine if PG could stimulate a marker of differentiation, as well as inhibit proliferation, we treated keratinocytes with or without PG liposomes in 25 μM calcium- (control) or 125 μM calcium-containing medium for 24 hours and analyzed cell lysates for involucrin protein levels. Both PG itself and 125 μM calcium alone (at this concentration and time point) had no significant effect on involucrin levels normalized with actin as a loading control (Figure 7). However, the combination of PG liposomes and a moderately elevated calcium concentration produced a significant increase in involucrin levels. This result suggests that PG and the PLD2/AQP3 signaling module participate in early keratinocyte differentiation, but that later differentiation requires the provision of additional signals.

PG liposomes stimulate DNA synthesis in slowly proliferating cells

Additional evidence for a lack of toxicity was provided by the observed effects of the PG liposomes on keratinocytes exhibiting reduced proliferation, presumably as the result of contact inhibition. Thus, if PG liposomes were applied to keratinocytes with decreased proliferative capacity (as indicated by reduced [^{3}H]-thymidine incorporation into DNA under control conditions; Figure 8), DNA synthesis was stimulated in a dose-dependent manner, with a half-maximal effect at a concentration of approximately 35 μg/ml and a maximal stimulation at 100 μg/ml (Figure 8). This result suggests that PG has the capacity to normalize keratinocyte proliferation, inhibiting the proliferation of rapidly dividing cells and increasing proliferation in a setting of reduced growth.

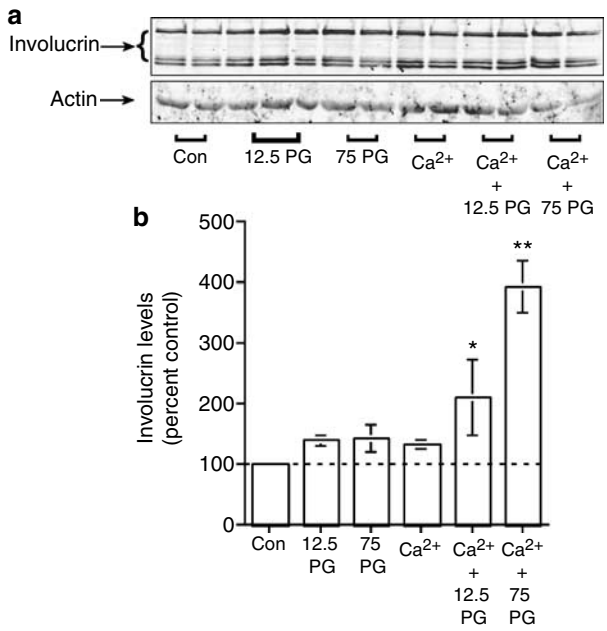

Figure 7. PG liposomes enhance elevated extracellular calcium concentration-induced involucrin protein expression. Near-confluent keratinocytes were treated for 24 hours with the indicated concentrations of phosphatidylglycerol (PG), prepared via bath sonication of PG in SFKM, in 25 μM Ca^{2+}-containing (control) medium or medium containing 125 μM Ca^{2+}. Cells were harvested and subjected to western analysis using an anti-involucrin antibody (Covance) and the LiCor Odyssey system. (**a**) Shown is a representative immunoblot. Multiple immunoblots were quantified using the KODAK molecular imaging software, with values shown in (**b**) representing the means ± SEM of 3–4 separate experiments performed in duplicate; $*P<0.05$, $**P<0.01$ *versus* the control value.

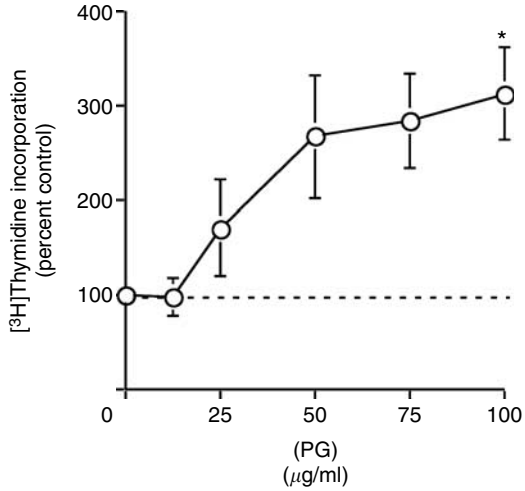

Figure 8. PG liposomes increase DNA synthesis in growth-inhibited keratinocytes. Confluent keratinocytes were treated for 24 hours with the indicated concentrations of phosphatidylglycerol (PG), prepared via bath sonication of PG in SFKM. [^{3}H]-Thymidine incorporation into DNA was then determined as above. [^{3}H]-Thymidine incorporation into DNA under control conditions was 12,880 ± 1040 cpm/well. Values represent the means ± SEM of 3 separate experiments performed in duplicate; $*P<0.05$ versus the control value.

DISCUSSION

Our previous studies indicated that PLD can utilize glycerol in a transphosphatidylation reaction to synthesize PG (Zheng *et al.*, 2003); furthermore, an interaction between PLD2 and an aquaglyceroporin, AQP3 (Zheng and Bollag, 2003), suggested a mechanism by which glycerol could reach this isoenzyme for transphosphatidylation. If our idea is correct, inhibition of the glycerol uptake function of AQP3 should reduce PG synthesis as well. Figure 1 shows that acidic medium induces a concomitant decrease in 125 μM Ca^{2+}-elicited glycerol uptake and PG synthesis. However, it is known that other aquaporins capable of transporting glycerol, such as AQP9, are expressed by keratinocytes (Sugiyama *et al.*, 2001; Pisarchik *et al.*, 2004). It is not known whether the transport function of these aquaporins is also affected by acidic medium and also whether they might associate with PLD. Thus, our results do not eliminate the possibility that these other aquaglyceroporins may also contribute to glycerol uptake and PG synthesis in keratinocytes.

We have further hypothesized that the PG synthesized by the PLD2/AQP3 signaling module serves as a lipid messenger to regulate keratinocyte and epidermal function. This idea was based on the literature indicating that AQP3 null mutant mice exhibit an epidermal phenotype that can be corrected by glycerol, but not other osmotically active agents (Hara *et al.*, 2002; Ma *et al.*, 2002; Hara and Verkman, 2003). Indeed, our coexpression studies suggested that AQP3 promotes early keratinocyte differentiation: AQP3 decreased the promoter activity of keratin 5 (Figure 2a), a marker of the basal proliferative layer. Downregulation of keratin 5 expression characterizes the transition of basal keratinocytes into the first suprabasal cells in the spinous layer (Dlugosz and Yuspa, 1993). Also characteristic of spinous keratinocytes is an increase in the expression of keratin 10; coexpression of AQP3 increased keratin 10 promoter activity (Figure 2b). It was perhaps somewhat unexpected that the elevated extracellular calcium level did not increase keratin 10 promoter activity; however, it is known that this high calcium concentration (1 mM) is less effective than intermediate calcium concentrations at increasing early keratinocyte differentiation marker expression (Yuspa *et al.*, 1989). Thus, this high calcium level may propel keratinocytes past early differentiation steps to a later differentiation stage, resulting in a slight reduction in keratin 10 promoter activity (Figure 2b). As keratinocytes proceed to migrate up through the multiple spinous layers, they begin to express involucrin (reviewed by Eckert *et al.*, 2005). Although AQP3 coexpression alone did not significantly increase involucrin promoter activity, AQP3 did enhance the effect of an elevated extracellular calcium concentration on the promoter activity of this intermediate differentiation marker (Figure 2c). It should be noted that it seems unlikely that AQP3 is directly affecting the promoter activities of these various markers, that is, via interactions with other transcription factors and/or the promoters themselves. Rather, we suggest that the results are consistent with AQP3 expression inducing an early differentiation phenotype, and that the differentiation status of the cells then controls the activities of these promoters.

Our hypothesis predicts that increasing glycerol influx should increase PG synthesis and promote this early differentiation phenotype, a primary event of which is growth arrest. Indeed, glycerol inhibited DNA synthesis and this inhibition was not reproduced by equivalent concentrations of two other osmotically active compounds, xylitol and sorbitol (Figure 3), suggesting that the inhibition was not the result of increased osmolarity. Furthermore, this effect seemed to be related to AQP3, as AQP3 expression sensitized keratinocytes to the inhibitory effect of a low concentration of glycerol on keratin 5 promoter activity (Figure 4). Interestingly, 1,2-propylene glycol (1,2-propanediol) produced an essentially identical effect as glycerol on DNA synthesis (Figure 5). This result contrasts with data from Hara *et al.* (Hara and Verkman, 2003), who reported that 1,2-propylene glycol did not correct the epidermal phenotype in AQP3 null mutant mice. It is unclear why 1,2-propylene glycol should be able to mimic glycerol's effect in intact cells *in vitro* but not *in vivo*. It is possible that the phospholipid formed by transphosphatidylation with 1,2-propylene glycol (PG missing the hydroxyl group on the terminal carbon) is similar enough to PG to activate PG effector enzymes; however, perhaps 1,2-propylene glycol does not readily permeate the epidermis *in vivo* and/or is metabolized before entry into the appropriate (basal) keratinocytes. Clearly, further studies are necessary to address this issue.

If glycerol functions to alter keratinocyte proliferation by serving as a substrate for PG formation, then direct provision of PG should also inhibit DNA synthesis. Indeed, in rapidly growing cells (as determined by high [^{3}H]-thymidine incorporation into DNA under basal conditions), PG dose dependently decreased DNA synthesis (Figure 6). This effect did not seem to be the result of nonspecific toxicity as no morphological correlates of toxicity were observed (data not shown). In addition, a related phospholipid, phosphatidylpropanol, had no effect on DNA synthesis. PG liposomes alone exhibited no or a minimal effect on involucrin protein expression; however, PG increased the effect of an elevated extracellular calcium concentration on involucrin levels (Figure 7). This result is similar to our finding that AQP3 coexpression had little effect itself on involucrin promoter activity, but enhanced the effect of an elevated extracellular calcium concentration (Figure 2C). However, unexpectedly, in keratinocytes that exhibited reduced DNA synthesis, likely as the result of contact inhibition, PG dose dependently stimulated DNA synthesis (Figure 8). The mechanism of this biphasic response is unknown (although possibilities are discussed below), but in cases where the epidermis is hyperproliferative, PG liposomes would be expected to inhibit keratinocyte growth, whereas under conditions of too little proliferation (e.g., with age; Gilhar *et al.*, 2004) the liposomes should increase growth. Thus, the results suggest that PG liposomes might be an ideal treatment to normalize skin function under both pathological and physiological conditions.

A major question remains concerning this signaling module: namely, the effector enzyme for the PG signal is unknown, although there are several possibilities, including protein kinase C-βII, protein kinase C-θ and PK-P, all known PG-sensitive protein kinases (Murray and Fields, 1998; Klemm *et al.*, 1988; Pietromonaco *et al.*, 1998; Klemm and Elias, 1988a, b). Alternatively, PG may be incorporated into the plasma membrane and/or specific microdomains and influence membrane protein assembly and/or microdomain function, as in thylakoid membranes of cyanobacteria and spinach (Kruse *et al.*, 2000; Sato *et al.*, 2000). PG is also a precursor of cardiolipin (diphosphatidylglycerol), and both PG and cardiolipin are important in mitochondrial function (Piccotti *et al.*, 2002). Thus, we speculate that PG may induce growth inhibition of rapidly proliferating keratinocytes (as in Figure 6) through activation of a protein kinase pathway, whereas this phospholipid may promote proliferation in inhibited cells (as in Figure 8) by improving mitochondrial function and energy production, as illustrated in Figure 9. Interestingly, in keratinocytes exposure to ultraviolet light results in an upregulation of AQP3 expression (Pisarchik *et al.*, 2004), and we propose that this increase represents a cellular response to promote PG production, mitochondrial health and recovery from the stress of the irradiation. Obviously, additional experiments are necessary to investigate these possibilities.

Finally, another major question to be addressed is the source of the glycerol transported by AQP3. Based on data in the literature, there appear to be both endogenous and exogenous sources that can supply glycerol to keratinocytes

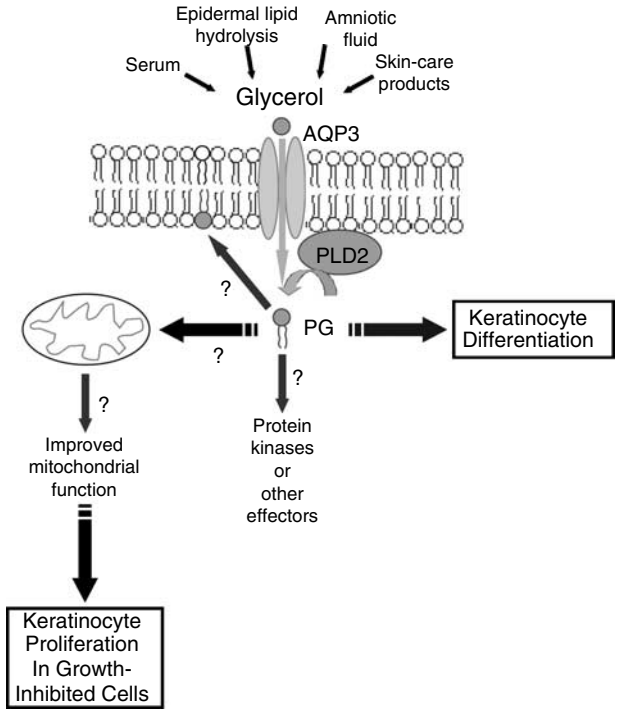

Figure 9. A proposed model for the PLD2/AQP3 signaling module's effects on keratinocyte function. Illustrated is our proposal for the mechanism by which the PLD2/AQP3 signaling module produces PG from glycerol and induces keratinocyte differentiation or proliferation. As indicated, glycerol originates from serum, hydrolysis of epidermal lipids, amniotic fluid and skin-care products.

in vivo, as reviewed by Bollag and Zheng (2005). These include serum (Graham *et al.*, 2000) and lipase-mediated release from epidermal lipids (reviewed by Madison, 2003), as well as amniotic fluid (Herold and Reed, 1988) and skin care products (Goldsmith, 2003). Thus, our results provide a mechanism through which glycerol can improve skin function (Fluhr *et al.*, 1999) and support the idea that the PLD2/AQP3 signaling module is an important regulator of early keratinocyte differentiation.

MATERIALS AND METHODS

Keratinocyte preparation and cell culture

Keratinocytes were prepared from ICR CD-1 outbred mice in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Briefly, the skins were harvested and incubated overnight in 0.25% trypsin at 4°C. The epidermis and dermis were separated and basal keratinocytes scraped from the underside of the epidermis. The cells were collected by centrifugation and incubated overnight in an atmosphere of 95% air/5% carbon dioxide at 37°C in plating medium as described previously (Dodd *et al.*, 2005). The plating medium was replaced with SFKM, and the cells were refed every 1–2 days with fresh medium until use.

[^{3}H]-Glycerol uptake assay

Near-confluent keratinocyte cultures were incubated for 24 hours in SFKM (25 μM calcium) or SFKM containing 125 μM calcium (125 μM Ca^{2+}-SFKM) and the glycerol uptake assay performed as described previously (Zheng and Bollag, 2003; Zheng *et al.*, 2003). Briefly, cells were incubated with SFKM containing 20 mM HEPES (for additional pH buffering) and 1 μCi/ml [^{3}H]-glycerol for 5 minutes, as we have previously shown that this time point is in the linear range of [^{3}H]-glycerol uptake (Zheng and Bollag, 2003; Zheng *et al.*, 2003). Reactions were terminated by rapidly washing three times with ice-cold phosphate-buffered saline lacking divalent cations (PBS-). Cells were then solubilized in 0.3 M NaOH and [^{3}H]-glycerol uptake quantified by liquid scintillation spectroscopy.

PG synthesis

After incubation of near-confluent keratinocytes for 24 hours in SFKM (25 μM calcium) or SFKM containing 125 μM calcium (125 μM Ca^{2+}-SFKM), 0.5–1 μCi/ml [^{14}C]glycerol was added for 10 minutes and PG synthesis determined as described by Zheng *et al.* (2003). Briefly, radiolabeled PG was extracted into chloroform/methanol. Phospholipids were then separated by thin-layer chromatography on silica gel 60 plates using the upper phase of a mobile solvent consisting of ethyl acetate:iso-octane:acetic acid:water (13:2:3:10 vol:vol:vol:vol), as described by Zheng *et al.* (2003).

Co-transfection analysis

Co-transfection experiments were performed as described previously (Dodd *et al.*, 2005) using 1 μg of the pcDNA3 empty vector or a construct possessing AQP3, 0.5 μg of one of the reporter constructs in which the promoters for keratin 5, keratin 10, or involucrin drive expression of luciferase and 25 ng of the pRL-SV40 control vector (included in the Promega Dual Luciferase Reporter Assay kit) to normalize for transfection efficiency. The keratin 5- and keratin 10 promoter-luciferase constructs (Sugihara *et al.*, 2001) were a kind gift of Dr Bogi Andersen (University of California, Irvine, CA); the involucrin promoter-luciferase construct (Ng *et al.*, 1996) was generously provided by Dr Daniel Bikle (University of California, San Francisco, CA). Subconfluent (approximately 30%) keratinocytes were transfected using TransItKeratinocyte according to the manufacturer's instructions. After 24 hours cells were refed with medium containing 25 μM (control) or 1 mM-Ca^{2+} for an additional 24 hours. In additional experiments, after co-transfection and a 24-hour incubation, the cells were treated for an additional 24 hours with or without 0.2% glycerol before collection of cell lysates. Luciferase activity was then measured using the Dual Luciferase Reporter Assay kit (Promega, Madison, WI) as directed by the manufacturer.

Assay of DNA synthesis

[^{3}H]-Thymidine incorporation into DNA was determined as a measure of DNA synthesis as described previously (Griner *et al.*, 1999). Near-confluent keratinocyte cultures were incubated for 24 hours in SFKM containing the indicated additions. PG or phosphatidylpropanol (dioleoyl- or dipalmitoyl-) was added in the form of liposomes prepared by bath sonication of dried lipid in SFKM to make a stock solution of 2 mg/ml. [^{3}H]-Thymidine at a final concentration of 1 μCi/ml was then added to the cells for an additional 1-hour incubation. Reactions were terminated by washing with PBS- and macromolecules precipitated with ice-cold 5% trichloroacetic acid. Cells were solubilized in 0.3 M NaOH and the radioactivity incorporated into DNA quantified by liquid scintillation spectroscopy.

Western blot analysis of involucrin levels

Keratinocytes were treated with or without PG liposomes in 25 μM calcium- (control) or 125 μM calcium-containing medium for 24 hours. Cell lysates were collected and analyzed by western blotting as described previously (Bollag *et al.*, 2005), using infrared dye-coupled secondary antibodies and a LiCor Odyssey Infrared Imaging System for visualization. Three major bands were detected using two different anti-involucrin antibodies: one was a generous gift from Dr Richard Eckert (University of Maryland Medical School, Baltimore, MD) and the other is commercially available (Covance Research Products, Berkeley, CA). Blots were converted to black-and-white images and quantified using KODAK Molecular Imaging software. Values for involucrin protein levels were normalized using actin as a loading control.

Statistics

Experiments were performed a minimum of three times as indicated. Values were analyzed for statistical significance by analysis of variance or repeated measures analysis of variance, with a Student-Newmann-Keuls or Dunn's *post-hoc* test using Instat (GraphPad Software, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflicts of interest.

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