The hormone 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) elicits the programmed pattern of differentiation in epidermal keratinocytes. Based on data indicating a potential role of phospholipase D (PLD) in mediating keratinocyte differentiation, we investigated the effect of 1,25(OH)₂D₃ on PLD expression. A 24-h exposure to 1,25(OH)₂D₃ stimulated PLD-1, but not PLD-2, mRNA expression. This 1,25(OH)₂D₃-enhanced expression was accompanied by increased total PLD and PLD-1 activity. Time course studies indicated that 1,25(OH)₂D₃ induced PLD-1 expression by 8 h, with a maximal increase at 20–24 h. Exposure to 1,25(OH)₂D₃ inhibited proliferation over the same time period with similar kinetics. Expression of the early (spinous) differentiation marker keratin 1 decreased in response to 1,25(OH)₂D₃ over 12–24 h. Treatment with 1,25(OH)₂D₃ enhanced the activity of transglutaminase, a late (granular) differentiation marker, by 12 h with a maximal increase after 24 h. In situ hybridization studies demonstrated that the highest levels of PLD-1 expression are in the more differentiated (spinous and granular) layers of the epidermis, with little expression in basal keratinocytes. Our results suggest a role for PLD expression/activity during keratinocyte differentiation.

The skin is a dynamic organ consisting of the dermis and epidermis, with the latter continuously undergoing regeneration to replace cells lost through normal exposure to the environment. The epidermis is composed of several cell layers. The deepest layer, located at the dermal-epidermal junction, is the basal layer, consisting of the undifferentiated basal keratinocytes, which continuously proliferate. As the cells migrate up through the epidermis, keratinocytes 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 cytokeratins 1 and 10 in the first differentiated layer of the epidermis, the spinous layer. Early differentiation in the spinous layer is followed by late differentiation in the granular layer accompanied by expression of proteins, including the enzyme transglutaminase, that are essential for the formation of the cornified envelope and corneocytes. The corneocytes are terminally differentiated and constitute the outer layer of the epidermis, the cornified layer, which gives skin its resistance to mechanical stresses (for reviews, see Refs. 1 and 2). Despite the importance of the keratinocyte differentiation program and intense investigation, both the extracellular and the intracellular signals controlling this process are largely unknown. Numerous studies have pointed to a role for 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in regulating keratinocyte differentiation (reviewed in Ref. 1). In fact, this compound and its structural analogs have been used successfully to treat psoriasis (reviewed in Ref. 3), a human disease characterized by hyperproliferation, abnormal differentiation, and inflammation of the skin. A physiologic role for 1,25(OH)₂D₃ in regulating keratinocyte differentiation in the epidermis in vivo is suggested by several lines of evidence: 1) keratinocytes express the 1α-hydroxylase, which converts the inactive 25-hydroxyvitamin D₃ to its active 1,25-dihydroxymetabolite (reviewed in Ref. 1; 2) receptors for 1,25(OH)₂D₃ are present in the skin and in epidermal keratinocytes (references cited in Ref. 4); and 3) 1,25(OH)₂D₃ triggers two major events in keratinocytes in vitro: inhibition of proliferation and induction of differentiation (reviewed in Ref. 1).

The mechanism by which 1,25(OH)₂D₃ inhibits proliferation and stimulates differentiation is still unclear. This hormone is thought to function through the vitamin D receptor, a transcription factor affecting expression of genes possessing vitamin D response elements. However, the keratin 1 gene is the only keratinocyte differentiation marker known to possess a 1,25(OH)₂D₃ response element (5, 6), and the expression of this marker is inhibited by 1,25(OH)₂D₃ (7). In addition, 1,25(OH)₂D₃ was recently shown to enhance the expression of several phosphoinositide-specific phospholipase C isoenzymes (8), the activity of which generates diacylglycerol (DAG). DAG, in turn, is known to regulate the activity of protein kinase C (PKC), and numerous data suggest the involvement of PKC in the regulation of keratinocyte growth and differentiation (reviewed in Refs. 1 and 9).

Although PKC-activating DAG can be generated directly by phosphoinositide turnover via phospholipase C, such DAG can also be generated indirectly by an additional pathway. Diacylglycerol is generated by the combination of phospholipase D (PLD), which hydrolyzes phospholipids to generate phosphatic acid (PA), and PA phosphohydrolase, which dephosphorylates PA to yield DAG (reviewed in Ref. 10). Indeed, in several cell systems, PLD activity has been shown to underlie at least

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† To whom correspondence should be addressed: Dept. of Medicine and Cellular Biology and Anatomy, Inst. of Molecular Medicine and Genetics, Medical College of Georgia, 1120 15th St., Augusta, GA 30912.

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1 The abbreviations used are: 1,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; DAG, sn-1,2-diacylglycerol; PA, phosphatic acid; PKC, protein kinase C; PLD, phospholipase D; ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-3-O-(thio)triphosphate; K1, cytokeratin K1; GQ₃(1→8), II₃(NeuAc)₂, IV₃(NeuAc)₂-GpOseCer.
a portion of agonist-induced sustained DAG production (reviewed in Ref. 10), and it has been proposed that chronic elevations in DAG content are the product of the combined activities of PLD and PA phosphohydrolase (11).

Several lines of evidence support a role for PLD in differentiation of keratinocytes. For example, the tetrasiloxiganglioside GQ1b induces PLD activation, a sustained elevation in DAG content, and induction of keratinocyte differentiation (12, 13). Studies in our laboratory also provide evidence for an involvement of PLD in keratinocyte growth inhibition and differentiation. Specifically, incubation of epidermal keratinocytes with bacterial PLD results in inhibition of proliferation and an increase in the activity of a marker of differentiation, transglutaminase (14). In addition, staurosporine and 12-O-tetradecanoylphorbol-13-acetate elicit sustained PLD activation and also induce transglutaminase activity in primary mouse keratinocytes.2 Finally, previous findings with two microbial toxins, also induce transglutaminase activity in primary mouse keratinocytes.2

Because 1,25(OH)2D3 increases the expression of phosphoinositide-specific phospholipase, which generates PKC-activating DAG, and because PLD not only generates DAG but also may be an important signaling enzyme during keratinocyte differentiation, we hypothesized that 1,25(OH)2D3 may also exert its effects on keratinocyte differentiation by modulating PLD expression and/or activity. We report that 1,25(OH)2D3 induces PLD-1, but not PLD-2, expression. The current work establishes the time course of the induction of PLD-1 expression in primary mouse epidermal keratinocytes in response to 1,25(OH)2D3 and demonstrates a concomitant enhancement of PLD activity. Furthermore, the data correlate the activation of this signaling pathway with the induction and progression of keratinocyte differentiation.

EXPERIMENTAL PROCEDURES

Materials—Calcium-free minimal essential medium and antibiotics were obtained from Biologos, Inc. (Maperville, IL). Bovine pituitary extract, epidermal growth factor, and Trizol reagent were purchased from Life Technologies, Inc. ITS + (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 8.25 ng/ml selenic acid, 5.35 μg/ml linoleic acid, and 0.125% bovine serum albumin) was supplied by Collaborative Biomedical Prod-

FIG. 1. Effect of 1,25(OH)2D3 on the expression of PLD-1 and -2 in primary cultures of mouse keratinocytes. Near confluent cultures of primary mouse keratinocytes were treated with 250 nM 1,25(OH)2D3 (D3) or vehicle control (C) for 24 h, and Northern blot analysis was performed. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands are shown to indicate approximately equal loading of 15 μg of total RNA. Also shown are molecular size markers to indicate the approximate size of each transcript.

1,25(OH)2D3 Increases PLD Expression and Activity

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2 E. M. Jung, S. Betancourt-Calle, R. D. Griner, R. Mann-Blakeney, and W. B. Bollug, Carcinogenesis, in press.
FIG. 2. The time-dependent effect of 1,25(OH)_{2}D_{3} on the expression of PLD-1 in primary cultures of mouse keratinocytes. Near confluent cultures of primary mouse keratinocytes were treated with 250 nm 1,25(OH)_{2}D_{3} (D_{3}) or vehicle control (C) for the indicated time periods, and Northern blot analysis was performed. Two bands are typically recognized by the PLD-1 probe; the size differential of these two bands (the upper band is 7.5 kilobase pairs, and the lower band is 5 kilobase pairs) suggests that they cannot represent the two splice variants of PLD-1 described previously, PLD1a and PLD1b, which differ by only 114 base pairs (20). Although the precise identities of the two bands are unclear, the lower band was used for quantitation, since the 5-kilobase pair size matches that defined as the cDNA coding region (20). The density of the PLD-1 band was normalized to the density of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band within each lane, and the level of PLD-1 expression by treated keratinocytes is expressed as a percentage of the control PLD-1 expression for each time point. The compiled results of multiple experiments are expressed graphically, and one representative blot containing a range of time points is shown. Asterisks indicate that PLD-1 expression by treated keratinocytes is significantly greater than the control PLD-1 expression at the given time point (means ± S.E., n = 3–5, p < 0.05).

Measurement of Phospholipase D-1 Activity in Vitro—Rho- and ADP-ribosylation factor (ARF)-stimulable PLD-1 activity was assayed in vitro essentially as in Ref. 27. Near confluent epidermal keratinocytes were treated for 24 h with control vehicle (0.05% ethanol) or 250 nm 1,25(OH)_{2}D_{3}. Cells were rinsed once with phosphate-buffered saline and scraped from the plate in 300 μl of buffer A (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl_{2}, 1 mM EGTA, 5 mM dithiothreitol, and protease inhibitors (0.5 mM 4-(2-aminooethyl)-benzenesulfonyl fluoride, 2.1 μM leupeptin, 1.8 μM/ml aprotinin, 1 μM/ml peptatin A with or without 2.5 μg/ml trypsin inhibitor and 1 μM/ml chymostatin)). Keratinocytes were then homogenized using a pellet pestle (Kontes, Vineland, NJ) and centrifuged to remove unlysed cells. The resulting supernatant (40 μl) was assayed in vitro for PLD activity in the presence and absence of 1 μM each of GTPγS-loaded Rho and ARF in a final volume of 150 μl of 50 mM HEPES, pH 7.4, 3 mM EGTA, 80 mM KCl, 1 mM dithiothreitol, 3 mM MgCl_{2}, and 2 mM CaCl_{2}. The substrate consisted of phospholipid vesicles composed of 320 μg of phosphatidylethanolamine, 20 μg of phosphatidylcholine, 14 μg of phosphatidylinositol 4,5-bisphosphate, and 50 μCi of [chlone-methyl-3H]dipalmitoylphosphatidylcholine. The vesicle mixture (1.3 μl/sample) and proceeded for 30 min at 37°C with shaking. Reactions were terminated, and unreacted [3H]phosphatidylcholine was precipitated by the addition of ice-cold 20% trichloroacetic acid and bovine serum albumin. Samples were centrifuged at 4°C, and [3H]choline released into the supernatant was quantified by liquid scintillation spectrometry. Blanks to which no cell lysate was added were performed, and the determined radioactivity was subtracted from all samples. Values were then expressed as the difference between PLD activity assayed in the presence of Rho and ARF versus in the absence of these two small GTP-binding proteins. Controls in which human PLD-1- or mouse PLD-2-overexpressing (baculovirus-infected) Sf9 membranes were assayed as above showed that PLD-1 activity was stimulation of the presence of Rho and ARF, whereas PLD-2 activity was unaffected (and/or slightly inhibited), as previously reported (22). Purified recombinant, baculovirus-expressed human Rho and ARF and PLD-overexpressing insect cell membranes were generous gifts of Dr. Nancy Pryer (Onyx Pharmaceuticals, Richmond, CA).

Measurement of DNA Synthesis—Near confluent cultures of primary keratinocytes were refed with keratinocyte growth medium supplemented with the indicated concentrations of 1,25(OH)_{2}D_{3} or vehicle control. After the indicated time periods, the cells were labeled with 1 μCi/ml [3H]thymidine for approximately 1 h. Cells were washed twice with phosphate-buffered saline, and reactions were terminated using ice-cold 5% trichloroacetic acid. Cells were washed sequentially with 5% trichloroacetic acid followed by deionized water and were solubilized in 0.3 M NaOH. An aliquot of this NaOH extract was counted in a liquid scintillation spectrometer.

Measurement of Cellular Transglutaminase Activity—Near confluent cultures of primary keratinocytes were refed with keratinocyte growth medium supplemented with 250 nm 1,25(OH)_{2}D_{3} or vehicle control. Cells were harvested, and transglutaminase activity was measured according to the method of Folk and Chung (28) with minor modifications. Specifically, dimethylcasein was substituted for α-casein, and [3H]putrescine dimethylcasein product on filters with ice-cold trichloroacetic acid. The radiolabeled product was then counted in a liquid scintillation spectrometer and normalized to protein content of the samples as determined using the micro-BCA protein assay with bovine serum albumin as the standard.
1,25(OH)_2D_3 Increases PLD Expression and Activity

**RESULTS**

**Induction of Phospholipase D-1 Expression and Activity by 1,25(OH)_2D_3—** To determine the ability of 1,25(OH)_2D_3 to modulate the expression of PLD, Northern blot analysis was performed. Near confluent cultures of primary mouse keratinocytes were treated with 250 nM 1,25(OH)_2D_3 (a maximal growth-inhibitory concentration, as shown in Fig. 4A) or vehicle for 24 h, and total RNA was isolated. RNA was analyzed by Northern hybridization with probes for PLD-1 and PLD-2 as described under “Experimental Procedures.” PLD-1 mRNA levels were specifically increased by 250 nM 1,25(OH)_2D_3, while PLD-2 levels were unchanged (Fig. 1). Subsequently, treatment of near confluent cultures of primary mouse keratinocytes with 250 nM 1,25(OH)_2D_3 for various times revealed that PLD-1 expression increased to 167% of control within 8 h and reached a peak increase of 289% of control at 24 h (Fig. 2).

To establish that the increase in PLD-1 mRNA levels resulted in increased enzyme activity, total PLD activity was measured in near confluent cultures of primary mouse keratinocytes following both acute (30-min) and chronic (24-h) exposures to 250 nM 1,25(OH)_2D_3. After 30 min, there was no significant effect of 1,25(OH)_2D_3 on PLD activity (Fig. 3A). However, after 24 h the PLD activity was increased to 140% of control (Fig. 3A). Thus, 1,25(OH)_2D_3 increased both PLD-1 expression and PLD activity within 24 h in primary cultures of mouse keratinocytes.

Previous reports indicate that PLD-1 activity is enhanced by the small GTP-binding proteins, Rho and ARF (reviewed in Ref. 16). To verify that the observed increase in PLD activity represented PLD-1, we measured Rho- and ARF-stimulatable PLD activity in vitro in homogenates of cells preexposed for 24 h to control vehicle or 250 nM 1,25(OH)_2D_3. We observed an approximate 3-fold increase in Rho- and ARF-stimulated activity in 1,25(OH)_2D_3-pretreated cell homogenates (Fig. 3B), consistent with our observations of an approximate 3-fold increase in PLD-1 expression, as well as enhanced total PLD activity, in response to 1,25(OH)_2D_3.

**Induction of Keratinocyte Growth Arrest by 1,25(OH)_2D_3—** To confirm that 1,25(OH)_2D_3 induces growth arrest of primary cultures of mouse keratinocytes, we examined the ability of 1,25(OH)_2D_3 to inhibit proliferation by examining the incorporation of [³H]thymidine into DNA. 1,25(OH)_2D_3 inhibited proliferation of primary mouse keratinocytes in both a concentration- and time-dependent manner (Fig. 4, A and B). Following a 24-h exposure, only 0.1 nM 1,25(OH)_2D_3 was required to significantly inhibit [³H]thymidine incorporation, and the inhibition was maximal between 100 and 250 nM (Fig. 4A). Thus, 250 nM 1,25(OH)_2D_3 was established as the concentration to be used in all subsequent experiments. At this concentration, [³H]thymidine incorporation was significantly decreased after only 8 h (75% of control), and maximal inhibition of [³H]thymidine incorporation was achieved by 24 h (18% of control) (Fig. 4B).

**Induction of Keratinocyte Differentiation by 1,25(OH)_2D_3—** Expression of cytokeratin K1, a marker of the early (spinous) stage of keratinocyte differentiation, was measured by Northern blot analysis in order to determine the ability of 1,25(OH)_2D_3 to induce cultured basal keratinocytes to differentiate into spinous keratinocytes. Near confluent cultures of primary mouse keratinocytes were treated with 250 nM 1,25(OH)_2D_3 or vehicle for the indicated time periods, and total RNA was isolated. Cytokeratin K1 mRNA levels began to decrease after 8 h, and K1 mRNA levels were significantly de-
creased by 12 h to 41% of control (Fig. 5). Thus, 250 nM 1,25(OH)2D3 decreased the expression of a spinous stage marker in primary mouse keratinocytes.

Activity of transglutaminase, a late (granular) stage marker of keratinocyte differentiation, was measured using a radiolabeled substrate in order to determine the ability of 1,25(OH)2D3 to induce cultured keratinocytes to differentiate to the granular stage. Near confluent cultures of primary mouse keratinocytes were treated with 250 nM 1,25(OH)2D3 or vehicle for the indicated time periods, and transglutaminase activity was measured as described under “Experimental Procedures.” Transglutaminase activity was increased to 160% of control by 1,25(OH)2D3 after 12 h, and after 24 h transglutaminase activity was maximally increased to 214% of control (Fig. 6). Thus, 1,25(OH)2D3 increased the activity of a late stage marker of differentiation in primary cultures of mouse keratinocytes. Furthermore, the increase in transglutaminase activity closely followed the increase in expression of PLD-1.

Expression of PLD-1 by Neonatal Mouse Epidermis—To establish that PLD-1 expression is important for the intermediate stages of mouse keratinocyte differentiation in vivo, in situ hybridization using sense and antisense probes for PLD-1 was performed on neonatal mouse skins as described under “Experimental Procedures.” The cells between the basal and cornified layers are in the spinous and granular stages of differentiation, and this region showed strong staining for PLD-1 (Fig. 7, A and C). The undifferentiated basal cells and the terminally differentiated and metabolically inactive cornified cells exhibited relatively light staining. Hybridization with the control probe showed light and diffuse staining throughout the layers (Fig. 7, B and D). Thus, the importance of PLD-1 expression during the intermediate stages of differentiation of cultured keratinocytes is supported by the expression of PLD-1 in the actively differentiating cells of neonatal mouse epidermis.

**DISCUSSION**

The ability of 1,25(OH)2D3 to increase the expression of phosphoinositide-specific phospholipase C isoforms (8) led us to examine the effect of this sterol hormone on another DAG-generating signaling enzyme, PLD. We found that 1,25(OH)2D3 specifically increased the expression of the PLD-1 isoform of PLD without affecting PLD-2 expression (Fig. 1). The specificity of this stimulation suggests that the observed increase in expression is not due to a global enhancement of transcription and/or message stability.

The time course of PLD-1 expression (Fig. 2) is consistent with several previous studies that have documented the effect of 1,25(OH)2D3 on the expression of inducible genes. While the mRNA levels of some 1,25(OH)2D3-responsive genes increase within 1–2 h (e.g. tumor necrosis factor-α (30)), other genes require longer time periods for their induction. For example, the expression of transforming growth factor-β is increased after a 6-h exposure of human keratinocytes to 1,25(OH)2D3 (31). Similarly, two genes that respond to 1,25(OH)2D3 at later time points are known to contain vitamin D response elements in their respective promoters (32). Specifically, in mouse epidermal cells osteopontin expression is increased within 1–2 h (e.g. tumor necrosis factor-α (30)), other genes require longer time periods for their induction. For example, the expression of transforming growth factor-β is increased after a 6-h exposure of human keratinocytes to 1,25(OH)2D3 (31).

The 1,25(OH)2D3-stimulated PLD-1 expression translates into an increase in PLD activity as well. The hormone has no acute effect on PLD activity (unlike hormones that stimulate cells through tyrosine kinase or G-protein-coupled receptors) but does activate PLD over 24 h (Fig. 3 A), consistent with the time course of enhanced PLD-1 expression. On the other hand, the pronounced change in PLD-1 expression results in only a relatively small increase in 1,25(OH)2D3-stimulated total PLD activity (approximately 1.4-fold over control) at 24 h. However, significant levels of PLD-2 mRNA are detected in the keratins.
Although PLD-2 expression is unaltered by 1,25(OH)₂D₃, PLD-2 is likely to contribute to the basal PLD activity, thus masking the extent of the 1,25(OH)₂D₃-induced change in PLD activity. In fact, when we measured Rho- and ARF-stimulatable PLD-1 activity in 1,25(OH)₂D₃-pretreated keratinocyte homogenates, we found an approximate 3-fold increase in PLD-1 activity (Fig. 3B), consistent with our observed ~3-fold increase in PLD-1 message. Thus, the observed
increase in PLD-1 mRNA is translated into elevated PLD-1 activity.

To establish a potential participation of 1,25(OH)2D3-induced PLD-1 expression/activation in the various stages of keratinocyte differentiation, we examined the time course of changes in markers of each of three stages of keratinocyte differentiation. If PLD-1 activity is to be involved in a particular stage of keratinocyte differentiation, PLD-1 expression/activation should precede changes in the appropriate marker. DNA synthesis is arrested in cells undergoing the basal to spinous transition. A significant growth arrest of proliferating keratinocytes is first induced by 1,25(OH)2D3 within approximately 8 h of exposure (Fig. 5). The expression of PLD-1 is first elevated at 8 h also. Since alterations in 1,25(OH)2D3-induced PLD-1 expression are coincident with growth arrest rather than preceding it, our result argues against a role for PLD-1 in the basal to spinous transition. We should note that our observation of a requirement for an 8-h exposure to 1,25(OH)2D3 conflicts with a previous report in mouse keratinocytes in which 1,25(OH)2D3 inhibited [3H]thymidine incorporation into DNA within 3 h (34). These investigators used as their control DNA synthesis at time 0. However, we have found changes in [3H]thymidine incorporation in vehicle-exposed keratinocytes over time and therefore have used [3H]thymidine incorporation in cells incubated with vehicle for the same period of time as the 1,25(OH)2D3-treated cells as the corresponding control value.

The expression of the cytokeratin K1 is known to mark early (spinous) keratinocyte differentiation. However, in our studies 1,25(OH)2D3 decreased K1 expression (Fig. 5); thus, our results argue against a role for PLD-1 in mediating early (spinous) differentiation, because K1 expression was not increased. However, these findings may not accurately reflect the true involvement of PLD-1 in mediating the events of early differentiation, because the results of others indicate that cytokeratin K1 expression may be decreased directly by 1,25(OH)2D3, and therefore, any effect of PLD-1 on K1 expression may be masked. Specifically, Su et al. (35) demonstrated K1 message destabilization by 1,25(OH)2D3. In addition, there exists a 1,25(OH)2D3 inhibitory element in the 3′-untranslated region of the K1 gene (7). Further experiments using lower concentrations of 1,25(OH)2D3 and longer exposures may reveal a correlation of PLD-1 expression with early keratinocyte differentiation.

Exposure to 1,25(OH)2D3 also increased transglutaminase activity, a marker of late (granular) differentiation, with maximal stimulation observed at 24 h (Fig. 6). Our result is consistent with a previous report (35), in which the authors described a peak increase of approximately 2-fold after a 24-h 1,25(OH)2D3 treatment and a slight decline by 48 h (35). These data suggest that the mouse keratinocyte behaves similarly to the human and that transglutaminase activity can serve as a marker of differentiation in our primary mouse keratinocyte system. Our time course is also consistent with a potential role for PLD activity in regulating differentiation in that PLD-1 is expressed in response to 1,25(OH)2D3 prior to the measured increase in transglutaminase activity. Additional evidence for a role for PLD-1 in differentiation is provided by our in situ hybridization study (Fig. 7). The finding that little or no expression of PLD-1 is detected in basal keratinocytes argues against its involvement in growth arrest. Instead, expression is detected strongly in the upper layers (the spinous and granular layers), consistent with PLD-1 mediating keratinocyte differentiation.

There are numerous published data supporting a role for PKC in regulating keratinocyte growth and differentiation (reviewed in Refs. 1 and 9). However, the exact role of PKC is controversial, since agents that stimulate proliferation and those that induce differentiation have each been reported to increase phosphoinositide turnover and DAG levels (36–38) and/or activate PKC (13, 39–42). The explanation for these conflicting results may reside in the fact that PKC is a family of isoenzymes (reviewed in Ref. 11), with different PKC isoenzymes involved in proliferative versus differentiative events. Thus, two agents that both stimulate PKC yet produce different results may be activating two different PKC isoenzymes. In additional studies, treatment of keratinocytes with phorbol esters yielded paradoxical results of initial differentiation induction and subsequent growth promotion (43). These results are probably due to the ability of phorbol esters to activate multiple isoforms of PKC.

Because it is derived from phosphatidylcholine rather than from phosphoinositides, the DAG species generated by PLD (and PA phosphohydrolase) is composed of different acyl groups than DAG generated by other phospholipid-hydrolyzing enzymes. Thus, DAG generated by PLD may activate distinct PKC isoforms (reviewed in Ref. 10), leading to the expression of specific markers of keratinocyte differentiation. Numerous studies in other systems have suggested that individual PKC isoenzymes regulate specific cellular processes (reviewed in Ref. 11). For example, in a transfected rat keratinocyte cell line, PKC-γ has been shown to increase the transcription of transglutaminase, a marker of late keratinocyte differentiation, more effectively than other PKC isoforms (44). Thus, individual PKC isoenzymes, activated by distinct DAG species, may be responsible for determining the patterned expression of genes during keratinocyte differentiation. Alternatively, DAG derived from PLD activity may be differentially metabolized (45, 46), and/or the PA produced directly by PLD activity may itself serve a second messenger function (reviewed in Ref. 10).

Our present results are indicative of a role for 1,25(OH)2D3-induced PLD-1 expression/activity in mediating keratinocyte differentiation. Specifically, our time course studies, as well as our in situ hybridization data, support an involvement of PLD-1 in the spinous to granular transition in keratinocytes. Current experiments in our laboratory are directed toward attempting to prove this hypothesis through other methods of regulating PLD-1 expression and/or activity. Finally, our report is the first to document a 1,25(OH)2D3-elicted increase in the expression of the important phospholipid-metabolizing enzyme PLD.

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