

Phospholipase D Signaling and Extracellular Signal-Regulated Kinase-1 and -2 Phosphorylation (Activation) Are Required for Maximal Phorbol Ester-Induced Transglutaminase Activity, a Marker of Keratinocyte Differentiation

Wendy B. Bollag, Xiaofeng Zhong, M. Ernest Dodd, David M. Hardy, Xiangjian Zheng,¹ and W. Thomas Allred

Institute of Molecular Medicine and Genetics (W.B.B., X.Zho., M.E.D., D.M.H., X.Zhe., W.T.A.), Departments of Medicine (Dermatology) (W.B.B.) and Cellular Biology and Anatomy (W.B.B.), Medical College of Georgia, Augusta, Georgia

ABSTRACT

Protein kinase C (PKC)-activating 12-*O*-tetradecanoylphorbol 13-acetate (TPA) stimulates phospholipase D (PLD) activity in primary mouse epidermal keratinocytes. PLD catalyzes the hydrolysis of phosphatidylcholine to yield phosphatidic acid (PA), which can be dephosphorylated to produce PKC-activating diacylglycerol. In the presence of small amounts of a primary alcohol, PLD can instead produce novel phosphatidylalcohols at the expense of PA and diacylglycerol. Here, we have demonstrated that inhibiting PLD signal generation with 1-butanol reduced TPA-stimulated transglutaminase activity, a marker of keratinocyte differentiation. On the other hand, the structurally related tertiary alcohol *tert*-butanol, which cannot be used by PLD, had no effect on TPA-induced transglutaminase activity. Since TPA activates all conventional and novel PKC isoforms directly, yet cannot overcome 1-butanol-mediated inhibition, this result suggests that PLD mediates its effects on transglu-

taminase activity (and keratinocyte differentiation) through an effector enzyme system distinct from the conventional or novel PKC isoenzymes. Data in the literature suggest that PA can recruit Raf-1 to the membrane, where it can be activated and initiate the mitogen-activated protein kinase cascade that culminates in activation of extracellular signal-regulated kinase (ERK)-1 and -2. Indeed, we found that inhibition of ERK-1/2 phosphorylation (activation) inhibited TPA-induced transglutaminase activity. However, inhibition of PLD-mediated signal generation had only a small effect on TPA-elicited ERK-1/2 phosphorylation (activation), whereas inhibition of ERK-1/2 did not affect PLD activation, suggesting that these two pathways likely operate largely in parallel. Thus, our results suggest the independent involvement of the PLD and ERK-1/2 pathways in mediating transglutaminase activity and keratinocyte differentiation.

Phorbol esters, such as 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), are known to promote the formation of tumors in epidermis initiated with carcinogens (for review, see Rubin, 2002). The mechanism of this tumor promotion is unknown, but the identification of protein kinase C (PKC) as the predominant phorbol ester binding protein in cells (for review, see Nishizuka, 1995) suggests an involvement of this

enzyme family in the process. However, the acute effect of TPA both *in vitro* and *in vivo* is to induce keratinocyte differentiation (discussed in Bollag et al., 1993), a response seemingly inconsistent with its tumor-promoting action. The mechanism and downstream pathways activated in this biphasic TPA response are still at present unclear.

As mentioned above, the primary target of TPA in cells is thought to be the PKC family. PKC isoenzymes can be divided into the classical members PKC- α , - β I and - β II, and - γ , which are phospholipid-dependent, calcium-sensitive, and activated by phorbol ester, or the physiological activator diacylglycerol. The novel isoforms PKC- δ , - ϵ , - η , and - θ are also phospholipid-dependent and activated by phorbol ester or diacylglycerol but are not sensitive to calcium. The atypical PKCs PKC- ι / λ and - ζ are phospholipid-dependent but are neither phorbol ester/diacylglycerol- nor calcium-sensitive

This study was supported by the National Institutes of Arthritis, Musculoskeletal, and Skin Diseases Grant AR45212. This work was presented in part at the 64th Annual Meeting of the Society for Investigative Dermatology (Miami Beach, FL, May 2003) and the 37th Meeting of the Southeast Regional Lipid Conference (Cashiers, NC, November 2002).

¹ Current address: Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.104.075622.

ABBREVIATIONS: TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; SFKM, serum-free keratinocyte medium; DMSO, dimethyl sulfoxide; BisI, bisindolylmaleimide I.

(for review, see Nishizuka, 1995). Keratinocytes express many of these isoforms, including PKC- α , - β , - δ , - ϵ , - η , and - ζ (Dlugosz et al., 1992; Fischer et al., 1993). We have also recently proposed that TPA activates another phorbol ester/diacylglycerol-responsive protein kinase distinct from PKC with proliferative (or antidifferentiative) activity (for review, see Bollag et al., 2004). The enzyme suggested to occupy this role in keratinocytes is protein kinase D (also known as PKC- μ), based on recent pharmacological (Shapiro et al., 2002) and transient cotransfection data (M. E. Dodd, V. L. Ristich, S. Ray, R. M. Lober, and W. B. Bollag, manuscript submitted for publication) in keratinocytes, as well as information in the literature indicating a mitogenic role for this enzyme in fibroblasts and other cell types (for review, see Bollag et al., 2004). On the other hand, TPA may activate additional enzymes other than protein kinases, such as the Ras GTP-exchange protein RasGRP (for review, see Brose and Rosenmund, 2002), involved in keratinocyte growth and differentiation (Rambaratsingh et al., 2003).

One such possibility is phospholipase D (PLD), which is activated by TPA in a sustained manner in keratinocytes (Jung et al., 1999). PLD is an enzyme that hydrolyzes predominantly phosphatidylcholine to generate phosphatidic acid (PA) (for review, see Liscovitch et al., 2000). PA can, in turn, be dephosphorylated by lipid phosphate phosphatases to yield diacylglycerol. Like the diacylglycerol produced by phosphoinositide-specific phospholipase C, this PLD-generated diacylglycerol is thought to also activate PKC, and many biological actions of PLD are thought to be mediated by this ability to produce PKC-activating diacylglycerol. However, recent studies have begun to suggest additional actions of PLD, including those mediated by PA itself (for review, see Bollag and Zheng, 2005). Thus, both the Bell and Romero laboratories have shown that PA can recruit Raf-1 kinase to the plasma membrane where it can interact with and be activated by Ras (for review, see Bollag and Zheng, 2005). Raf-1 then goes on to phosphorylate and activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-1 (MEK-1), which phosphorylates and activates ERK-1/2. Indeed, Rizzo et al. (1999, 2000) have demonstrated that PLD activity is required for Raf-1 translocation to the membrane and activation of the MAPK pathway in insulin receptor-overexpressing 3T3 cells. In addition, McPhail et al. (1999) have identified a PA-activated protein kinase distinct from PKC in human neutrophils. Based on the importance of PLD activity to multiple cellular responses (for review, see Bollag and Zheng, 2005), we hypothesized that TPA-mediated PLD activation contributes to the keratinocyte differentiation induced by this agent.

In this report, we demonstrate that PKC inhibitors inhibit TPA-induced PLD activation with a profile that correlates with their ability to inhibit TPA-stimulated transglutaminase activity, a marker of late keratinocyte differentiation. Furthermore, we show that inhibition of PLD-mediated signal generation in response to TPA inhibits the transglutaminase activity induced by this agent. The inability of TPA, which activates all classical and novel PKC isoenzymes directly, to overcome the inhibition of PLD signaling suggests effects of PLD independent of these PKC isoforms. Additional experiments demonstrating TPA-induced MAPK phosphorylation (and presumed activation) and inhibition of TPA-triggered transglutaminase activity with the MAPK/ERK kinase

(MEK) inhibitor PD 98059 suggest a possible role for ERK-1/2 in keratinocyte differentiation. Furthermore, the additive effect of the inhibitor of PLD-mediated signal generation and PD 98059 suggests an additional, as yet undefined, target of PLD signaling.

Materials and Methods

Materials. The PKC inhibitors Ro 31-8220 {3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide], bisindolylmaleimide I (BisI) {2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide}, Gö 6976 {12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole}, and Gö 6983 {2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide}, as well as the mitogen-activated protein kinase kinase MEK-1 inhibitor PD 98059 (2'-amino-3-methoxyflavone) and the ERK-2 inhibitor 5-iodotubercidin {4-amino-5-iodo-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine} were obtained from Calbiochem (San Diego, CA). Calcium-free minimal essential medium was obtained from Biologos (Montgomery, IL). Trypsin, epidermal growth factor, bovine pituitary extract, and 0.05% bovine serum albumin were purchased from Invitrogen (Carlsbad, CA). ITS⁺ (6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 ng/ml selenous acid, 5.35 μ g/ml linoleic acid, and 0.125% bovine serum albumin) was obtained from Collaborative Research (Bedford, MA). [³H]Oleic acid was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), and [³H]putrescine was from Moravak Biochemicals (Brea, CA). Antibodies against total and phospho- (active) mitogen-activated protein kinase ERK-1/2 were obtained from Cell Signaling Technology Inc. (Beverly, MA), and horseradish peroxidase-coupled secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). IRDye 800- or AlexaFluor 680-coupled secondary antibodies were obtained from Rockland (Gilbertsville, PA) and Molecular Probes (Eugene, OR), respectively. 1-Butanol and *tert*-butanol were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation and Culture of Primary Mouse Epidermal Keratinocytes. Primary epidermal keratinocytes were prepared from newborn ICR mice after flotation overnight at 4°C on 0.25% trypsin and separation of the epidermis from the dermis. Keratinocytes were collected by centrifugation, plated in a 2% dialyzed fetal bovine serum-containing medium in six-well tissue culture plates (BD Biosciences Discovery Labware, Bedford, MA), and incubated at 37°C overnight in a humidified atmosphere of 95% air, 5% CO₂, as described in Griner et al. (1999). The medium was replaced with serum-free keratinocyte medium [SFKM: calcium-free minimal essential medium containing 25 μ M calcium, 5 ng/ml epidermal growth factor, 90 μ g/ml bovine pituitary extract, 2 mM glutamine, ITS⁺ (and antibiotic/antimycotic)], and the cells were grown an additional 3 to 5 days to confluence before use. Cells were refed every 1 to 3 days with fresh SFKM.

PLD Activity Assay. Keratinocytes prelabeled with 2.5 μ Ci/ml [³H]oleate in SFKM for 20 to 24 h were incubated with SFKM or SFKM containing TPA with and without inhibitors in the presence of 0.5% ethanol for 30 min. Inhibitor concentrations were selected based on previous data from our laboratory (Jung et al., 1999; Shapiro et al., 2002), and all samples contained the same concentration of vehicle (dimethyl sulfoxide; DMSO). Phospholipids were extracted with chloroform/methanol and separated by thin layer chromatography as described in Jung et al. (1999). Radiolabeled phosphatidylethanol, a unique marker of PLD activity (Thompson et al., 1991), was quantified by liquid scintillation spectrometry, also as described in Jung et al. (1999).

Transglutaminase Activity. Transglutaminase activity was monitored by the cross-linking of [³H]putrescine to casein as described previously (Griner et al., 1999). Briefly, keratinocytes were treated with SFKM or SFKM containing TPA in the presence and absence of various inhibitors for 6 h. Cells were scraped into homog-

enization buffer, collected by centrifugation, and subjected to one freeze-thaw cycle. Cells were then lysed by sonication, and the supernatants were incubated with casein and [^3H]putrescine at 37°C overnight (~18 h). Casein was precipitated by the addition of ice-cold trichloroacetic acid, and the precipitates were collected by filtration and quantified by liquid scintillation spectrometry. Counts were normalized to the protein content of each sample, using the Bio-Rad protein assay kit with bovine serum albumin as the standard.

Western Analysis of ERK-1/2 Activation. Keratinocytes treated as indicated were lysed in hot lysis buffer consisting of 1% SDS and 12.5% glycerol in 31.25 mM Tris, pH 6.8. Equal sample volumes were separated by standard SDS 5% β -mercaptoethanol polyacrylamide gel electrophoresis on an 8% gel and electrotransferred to Immobilon P polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). After blocking with 5% milk in phosphate-buffered saline lacking divalent cations and containing 1% Tween 20, membranes were incubated in the saline solution containing 2% milk and antibody to phospho-ERK-1/2. Membranes were then rinsed and incubated with a horseradish peroxidase-coupled secondary antibody and visualized with enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Parallel membranes were similarly probed for total ERK-1/2 to ensure equal loading. In an alternate protocol, membranes with transferred proteins were blocked with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) and probed simultaneously with mouse monoclonal anti-phospho-ERK-1/2 and a rabbit polyclonal anti-ERK-1/2 antibodies. Immunoreactive proteins were then visualized with IRDye800-coupled donkey anti-rabbit IgG or IR Alexa Fluor 680-coupled goat anti-mouse IgG on a Li-Cor Odyssey infrared imaging system.

Statistical Analysis. The significance of differences between mean values was determined using analysis of variance, with a Student-Newman-Keuls post hoc test, as performed by the program Instat (GraphPad Software Inc., San Diego, CA).

Results

PKC Inhibitors Inhibit TPA-Induced Transglutaminase Activity, a Marker of Keratinocyte Differentiation, in Concert with Their Ability to Inhibit PLD Activation. PLD is known to be activated by PKC, and numerous investigators have demonstrated inhibition of agonist-elicited PLD activation by various PKC inhibitors (for review, see Liscovitch et al., 2000). We examined multiple PKC inhibitors for their ability to inhibit both TPA-induced transglutaminase activity, a measure of keratinocyte differentiation, and PLD activation. A stimulation of PLD activity was measured using the unique property of PLD to catalyze a transphosphatidylation reaction using primary alcohols in place of water for phospholipid metabolism. Thus, activation of PLD in the presence of small quantities of primary alcohols, such as ethanol or 1-butanol, results in the production of the corresponding radiolabeled phosphatidylalcohol from prelabeled phospholipids. With this assay, we found that the bisindolylmaleimide PKC inhibitors Ro 31-8220 and BisI (also known as GF 109203X and Gö 6850), inhibited TPA-induced PLD activation to a comparable extent [by ~86% (Jung et al., 1999) and 69% (Fig. 1), respectively]. These agents also decreased TPA-stimulated transglutaminase activity by similar amounts [~66% (Fig. 2B) and 66% (Fig. 2A), respectively]. On the other hand, the indolocarbazole analog Gö 6976 was less efficacious than a similar compound, Gö 6983, in terms of its inhibition of TPA-elicited PLD activation, reducing PLD activity only ~36% versus the 80% inhibition observed with Gö 6983 (Fig. 3). In close correlation, Gö 6976 was also less efficient in inhibiting TPA-

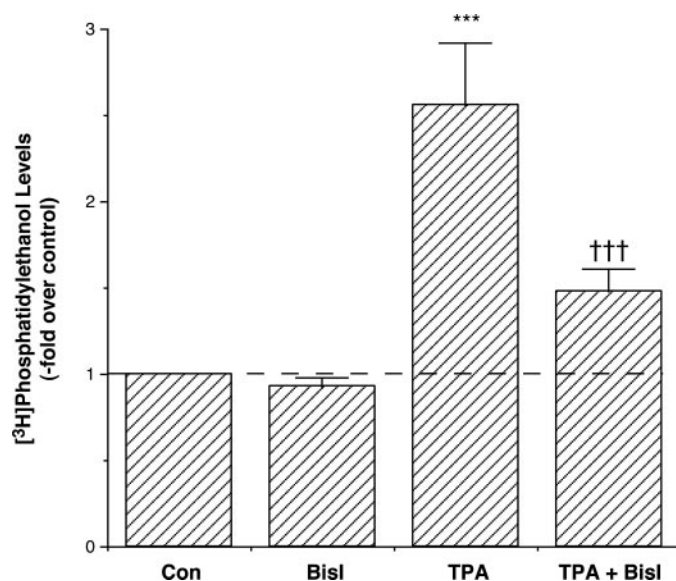


Fig. 1. BisI inhibits TPA-stimulated PLD activity. Near-confluent keratinocytes were prelabeled for 20 to 24 h with 2.5 $\mu\text{Ci/ml}$ [^3H]oleate before measurement of PLD activity with or without 100 nM TPA in the presence or absence of 1 μM BisI in the presence of 0.5% ethanol. All samples also contained 0.1% DMSO. Radiolabeled phospholipids, including PA and phosphatidylethanol, were extracted into chloroform, separated by thin layer chromatography, and quantified as described under *Materials and Methods*. Values represent the means \pm S.E.M. of six experiments performed in duplicate; ***, $p < 0.001$ versus the control value; †††, $p < 0.001$ versus TPA alone.

stimulated transglutaminase activity, decreasing values by ~35% in comparison with the 96% Gö 6983-induced inhibition (Fig. 4). Thus, the ability of these compounds to inhibit TPA-elicited PLD activation corresponds with their ability to inhibit transglutaminase activity, a marker of keratinocyte differentiation, in response to this agent.

Inhibition of PLD Signaling Inhibits TPA-Induced Transglutaminase Activity, a Marker of Keratinocyte Differentiation. The ability of PLD to effect the transphosphatidylation reaction discussed above can also be used to inhibit PLD-mediated lipid signal generation (for review, see Liscovitch et al., 2000). Thus, primary alcohols can be used to divert phospholipid metabolism away from the production of PA and toward the phosphatidylalcohol. Since the phosphatidylalcohols are also not readily metabolized (for review, see Liscovitch et al., 2000), the result of treatment in the presence of a low concentration of a primary alcohol is a reduction in both potential lipid messengers derived from PLD activity, PA and diacylglycerol, as has been demonstrated by numerous investigators (for review, see Liscovitch et al., 2000). To determine the potential involvement of PLD signaling induced by TPA in phorbol ester-induced keratinocyte differentiation, we used the primary alcohol 1-butanol to determine its effect on transglutaminase activity. This primary alcohol has the advantage of having a closely related analog, *tert*-butanol, which, as a tertiary alcohol, cannot be used by PLD in the transphosphatidylation reaction. Thus, *tert*-butanol can be used to control for potential nonspecific effects of the organic alcohol.

As shown in Fig. 5, neither 1-butanol nor *tert*-butanol affected basal transglutaminase activity, arguing against cytotoxicity at this concentration [0.3% (v/v)]. As observed previously, TPA stimulated transglutaminase activity, and this

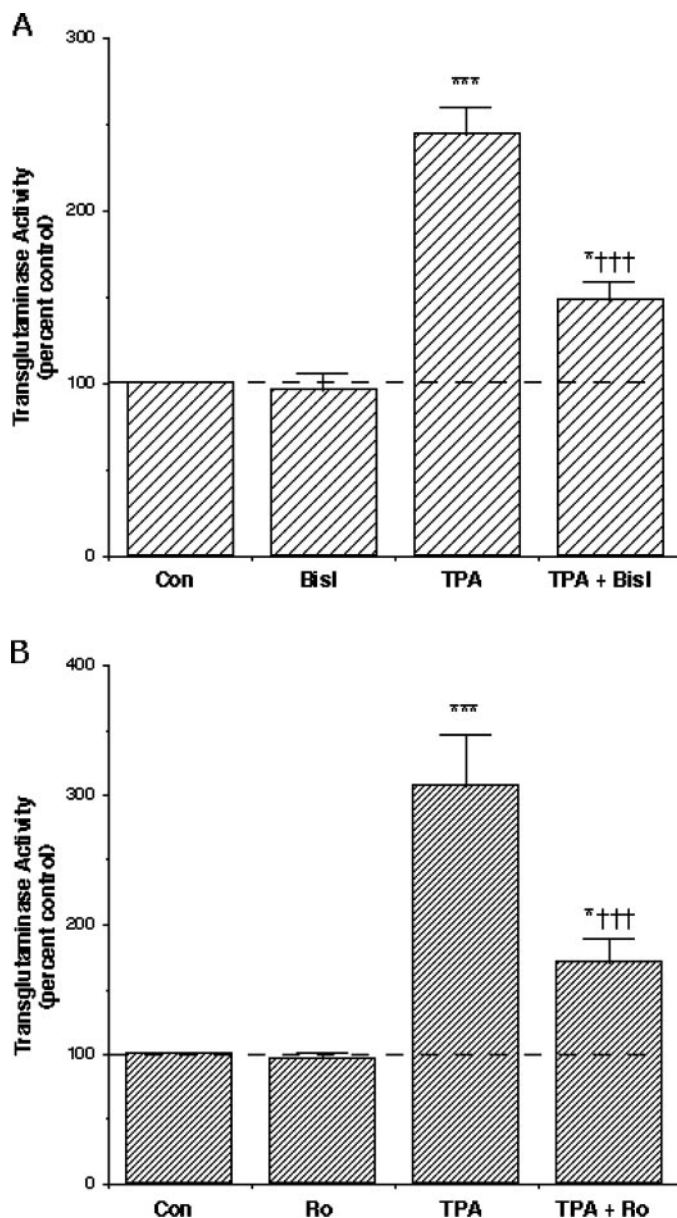


Fig. 2. BisI and Ro 31-8220 inhibit TPA-stimulated transglutaminase activity with similar efficacy. Near-confluent keratinocytes were incubated with 0.1% DMSO (Con) or 100 nM TPA in the presence or absence of 1 μ M BisI (A) or Ro 31-8220 (B) for 6 h. The cells were then scraped and transglutaminase activity measured as described under *Materials and Methods*. Values represent the means \pm S.E.M. of six experiments performed in duplicate or triplicate; *, $p < 0.05$; ***, $p < 0.001$ versus the control value; *+, $p < 0.001$ versus TPA alone.

increase was not significantly altered by *tert*-butanol, again suggesting a lack of toxicity. However, 1-butanol decreased TPA-stimulated transglutaminase activity by \sim 50%, returning the activity to a level not significantly different from the control value. Thus, diversion of PLD-mediated PA production to phosphatidylbutanol inhibited the ability of TPA to induce transglutaminase activity, a marker of keratinocyte differentiation. Since TPA can itself directly stimulate classical and novel PKC isoenzymes, the inability of TPA to overcome the inhibition of PLD-mediated lipid signal generation indicates at least a partial PKC-independent mode of action for this signaling system.

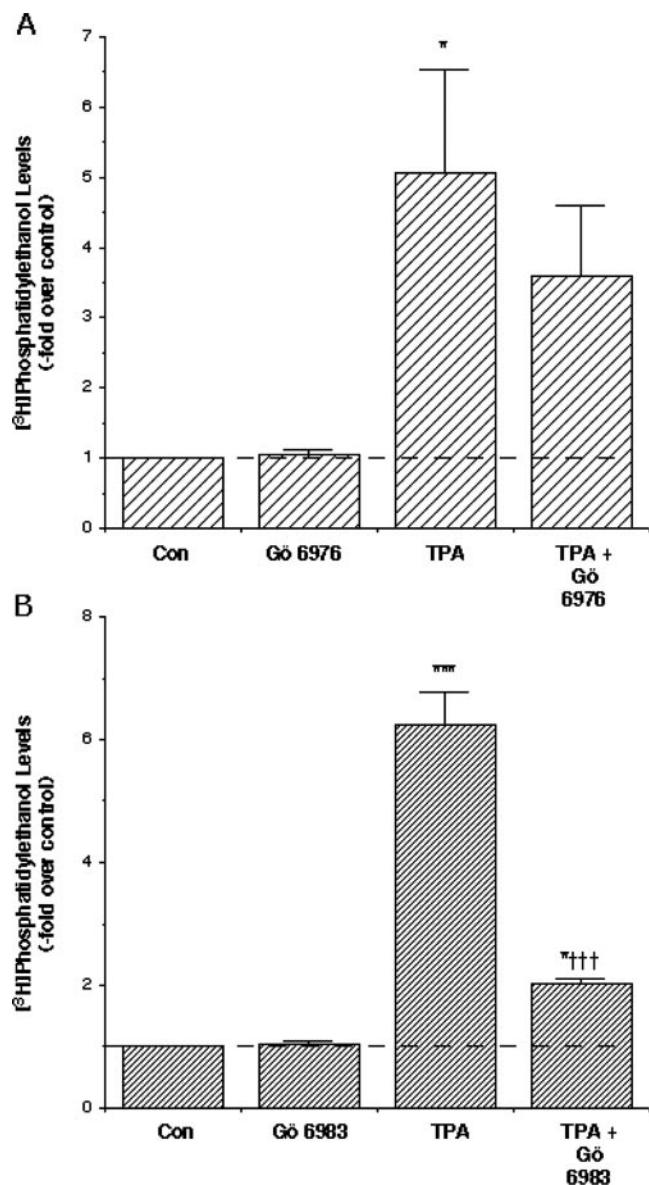


Fig. 3. G6 6983 Inhibits TPA-stimulated PLD activity more efficiently than G6 6976. Near-confluent keratinocytes were prelabeled for 20 to 24 h with 2.5 μ Ci/ml [³H]oleate before measurement of PLD activity with or without 100 nM TPA in the presence or absence of 1 μ M G6 6976 (A) or 6983 (B) in the presence of 0.5% ethanol. All samples also contained 0.1% DMSO. Radiolabeled phospholipids, including PA and phosphatidylethanol, were extracted into chloroform, separated by thin layer chromatography, and quantified as described under *Materials and Methods*. A, values represent the means \pm S.E.M. of three experiments performed in triplicate; *, $p < 0.05$ versus the control value. B, values represent the means \pm S.E.M. of three experiments performed in duplicate; *, $p < 0.05$; ***, $p < 0.001$ versus the control value; *+, $p < 0.001$ versus TPA alone.

TPA-Stimulated ERK-1/2 Activation Mediates in Part Phorbol Ester-Induced Transglutaminase Activity, a Marker of Keratinocyte Differentiation. Reports in the literature (Rizzo et al., 1999, 2000) suggest that PLD-generated PA may play a role in activating the MAPK pathway by recruiting Raf-1 kinase to the membrane where it can interact with and be activated by Ras. Raf-1 then phosphorylates and activates MEK-1, which phosphorylates and activates the MAPKs ERK-1/2 (for review, see Hagemann and Blank, 2001). On the other hand, investigators have also demonstrated that PLD activation is mediated by ERK-1/2

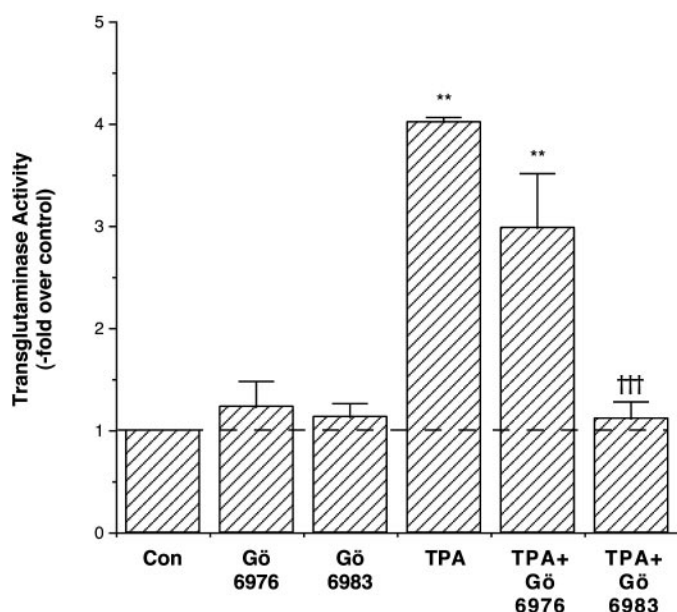


Fig. 4. Gö 6983 also inhibits TPA-stimulated transglutaminase activity more efficiently than Gö 6976. Near-confluent keratinocytes were incubated with 0.1% DMSO (Con) or 100 nM TPA in the presence or absence of 1 μ M Gö 6983 or 6976 for 6 h. The cells were then scraped, and transglutaminase activity was measured as described under *Materials and Methods*. Values represent the means \pm S.E.M. of five experiments performed in duplicate or triplicate; **, $p < 0.01$ versus the control value; †††, $p < 0.001$ versus TPA alone.

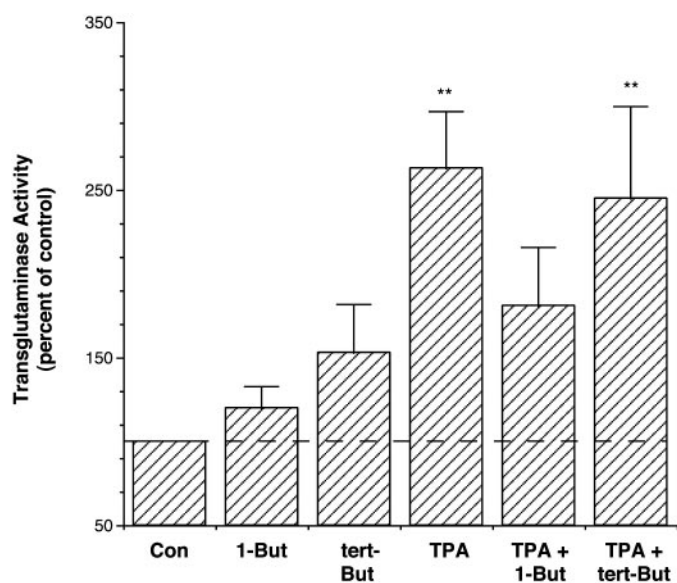


Fig. 5. 1-Butanol, but not *tert*-butanol, inhibits TPA-induced transglutaminase activity. Keratinocytes were incubated with 0.1% DMSO (Con) or 100 nM TPA in the presence or absence of 0.3% 1-butanol or *tert*-butanol for 6 h. The cells were then scraped and transglutaminase activity measured as described under *Materials and Methods*. Values represent the means \pm S.E.M. of five experiments performed in duplicate or triplicate; **, $p < 0.01$ versus the control value.

activation in several cell types (for review, see Banno, 2002), suggesting that ERK-1/2 can lie both upstream and downstream of PLD activation. Therefore, we first sought to determine whether TPA activates ERK-1/2 in keratinocytes, as has been observed in many cell systems (Koike et al., 2003). Indeed, incubation of primary epidermal keratinocytes with TPA increased the amount of active phospho-ERK-1/2 de-

tected by Western blot analysis, without affecting total ERK levels (Fig. 6). The question arose, Does this TPA-induced ERK-1/2 phosphorylation (activation) contribute to phorbol ester-stimulated transglutaminase activity and keratinocyte differentiation?

To address this question, we used an inhibitor, PD 98059, of the ERK-1/2 kinase, MEK-1, to block ERK-1/2 phosphorylation and activation. Figure 6 demonstrates that PD 98059 was able to inhibit TPA-induced ERK-1/2 activation, in that PD 98059 prevented the phorbol ester-elicited increase in phospho- (active) ERK-1/2 after a 5-min exposure to the phorbol ester, without affecting basal ERK-1/2 phosphorylation. PD 98059 also inhibited ERK-1/2 phosphorylation (activation) after a 30-min exposure (data not shown). Both high (50 μ M) and low (1–25 μ M) concentrations of PD 98059 also dose dependently inhibited TPA-induced transglutaminase activity after a 6-h incubation, without affecting basal transglutaminase activity (Figs. 7 and 8A). In addition, 5-iodotubercidin, a compound reported to inhibit ERK-2 (Gambelli et al., 2004), also inhibited TPA-induced transglutaminase activity by approximately 66% without affecting basal activity (Fig. 8B). Together, these results suggest that the phorbol ester-mediated increase in ERK-1/2 activity is required for maximal stimulation of transglutaminase activity.

ERK-1/2 Phosphorylation (Activation) Is Not Required for TPA-Induced PLD Activation. We next addressed the question of whether PLD activation contributed to ERK-1/2 activation or vice versa, that is, whether ERK-1/2 might act either upstream or downstream of PLD. To inhibit PLD-mediated signal generation we again used 1-butanol and found that neither 1-butanol nor *tert*-butanol significantly inhibited basal or TPA-induced ERK-1/2 phosphorylation (activation) after a 5-min stimulation (Fig. 9A). On the other hand, a 30-min exposure to TPA in the presence of 1-butanol resulted in a small (24%) but statistically significant decrease in TPA-induced ERK-1/2 phosphorylation (Fig. 9B). This result suggests that PLD-mediated signaling may be involved to a small extent in ERK-1/2 phosphorylation (activation) in response to TPA. Also, inhibition of ERK-1/2 activation with PD 98059 had no effect on TPA-induced PLD activation, as shown by the lack of effect of PD 98059 on radiolabeled phosphatidylethanol levels in the presence or

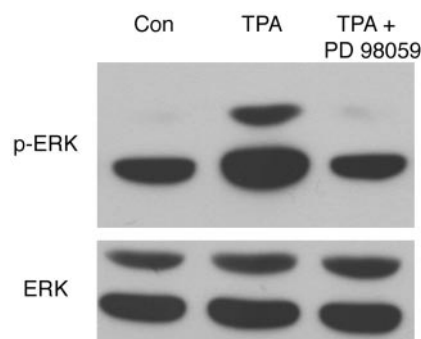


Fig. 6. TPA induces ERK phosphorylation (activation) and this phosphorylation (activation) is inhibited by the MEK inhibitor PD 98059. Keratinocytes were incubated with or without 100 nM TPA in the presence and absence of 25 μ M PD 98059 for 5 min. Cells were solubilized in sample buffer, and equal volumes were analyzed by Western blotting using enhanced chemiluminescence as described under *Materials and Methods*. Shown is a representative blot, with similar results obtained from at least three separate experiments.

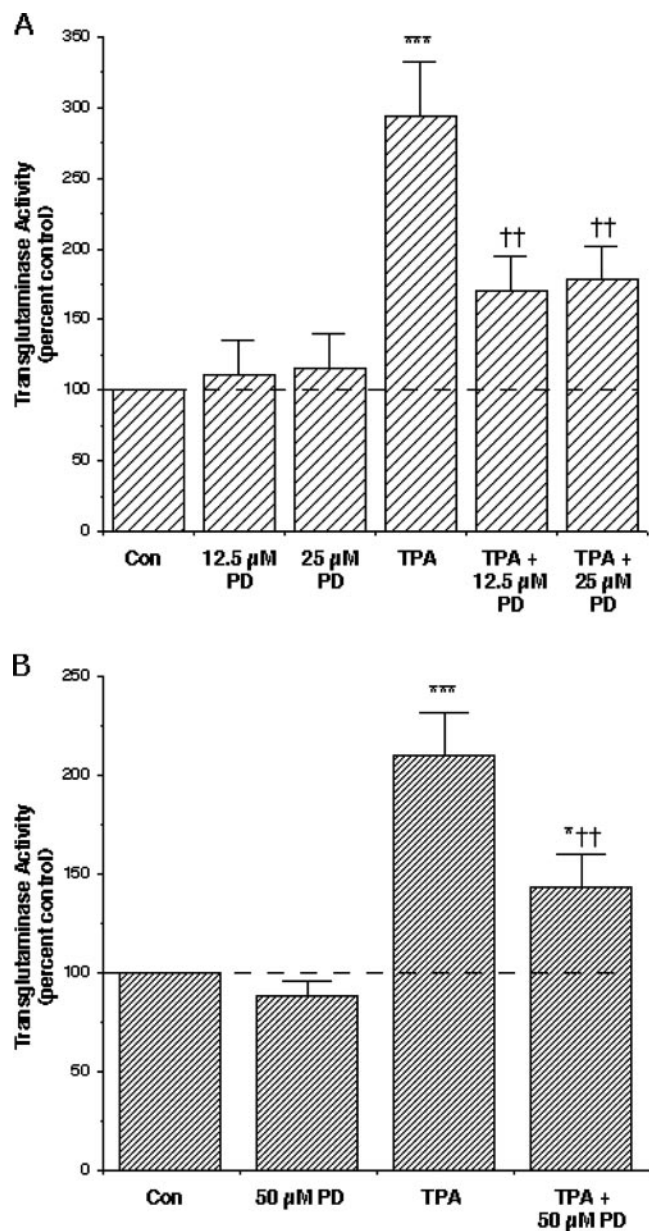


Fig. 7. ERK-1/2 pathway inhibitor PD 98059 inhibits TPA-induced transglutaminase activity. Keratinocytes were incubated with 0.2% (A) or 0.4% (B) DMSO (Con) or 100 nM TPA in the presence or absence of 12.5 or 25 μM PD 98059 (A) or 50 μM PD 98059 (B) for 6 h. The cells were then scraped and transglutaminase activity measured as described under *Materials and Methods*. A, values represent the means ± S.E.M. of four experiments performed in duplicate or triplicate; ***, $p < 0.001$ versus the control value; ††, $p < 0.01$ versus TPA alone. B, values represent the means ± S.E.M. of six experiments performed in duplicate or triplicate; *, $p < 0.05$; ***, $p < 0.001$ versus the control value; ††, $p < 0.01$ versus TPA alone.

absence of TPA (Fig. 10). Thus, these two signaling systems (ERK-1/2 and PLD) likely have distinct roles in mediating long-term keratinocyte differentiation. This interpretation is supported by the finding that 1-butanol and PD 98059 additively inhibited TPA-induced transglutaminase activity after 6 h (Fig. 11). Thus, PD 98059 inhibited TPA-stimulated transglutaminase activity by approximately 35%, 1-butanol reduced the response by about 63%, and the combined treatment resulted in a complete blockade of the induction of transglutaminase activity by TPA.

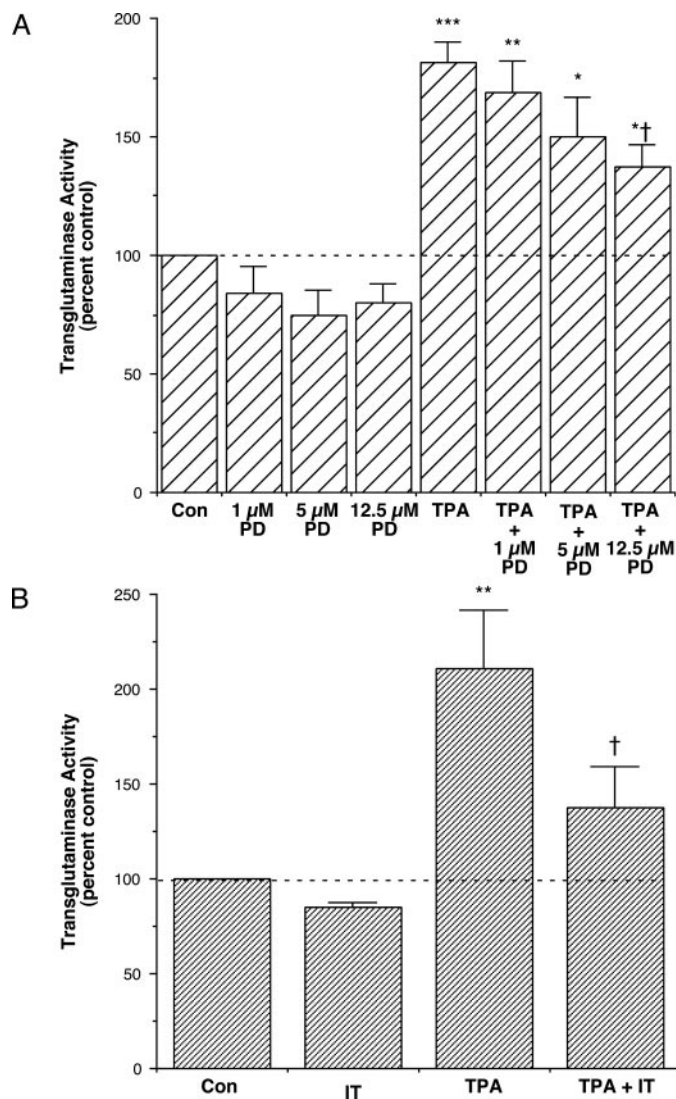


Fig. 8. Effect of PD 98059 is dose-dependent and is reproduced by the ERK-2 inhibitor 5-iodotubercidin. Keratinocytes were incubated with 0.1% DMSO (Con) or 100 nM TPA in the presence or absence of 1 to 12.5 μM PD 98059 (A) or 2 μM 5-iodotubercidin (IT) (B) for 6 h. The cells were then scraped and transglutaminase activity measured as described under *Materials and Methods*. A, values represent the means ± S.E.M. of three experiments performed in duplicate; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus the control value; †, $p < 0.05$ versus TPA alone. B, values represent the means ± S.E.M. of five experiments performed in triplicate; **, $p < 0.01$ versus the control value; †, $p < 0.05$ versus TPA alone.

Discussion

The ability of PKC inhibitors to inhibit PLD activation is well established (for reviews, see Liscovitch et al., 2000; Bollag and Zheng, 2005), although the mechanism by which they do so is unclear. PKC is known to bind to and activate PLD *in vitro* (for reviews, see Liscovitch et al., 2000; Bollag and Zheng, 2005); however, the question remains as to whether this activation requires PKC activity or simply association of the two enzymes. It is likely that binding of the inhibitors to the ATP binding domain of PKC induces a conformational change in PKC that alters its association with, and therefore its ability to activate, PLD and/or that the inhibitors prevent phosphorylation and activation of PLD or a PLD activity modulator (or a combination of these actions). In this study, we also demonstrate a correlation be-

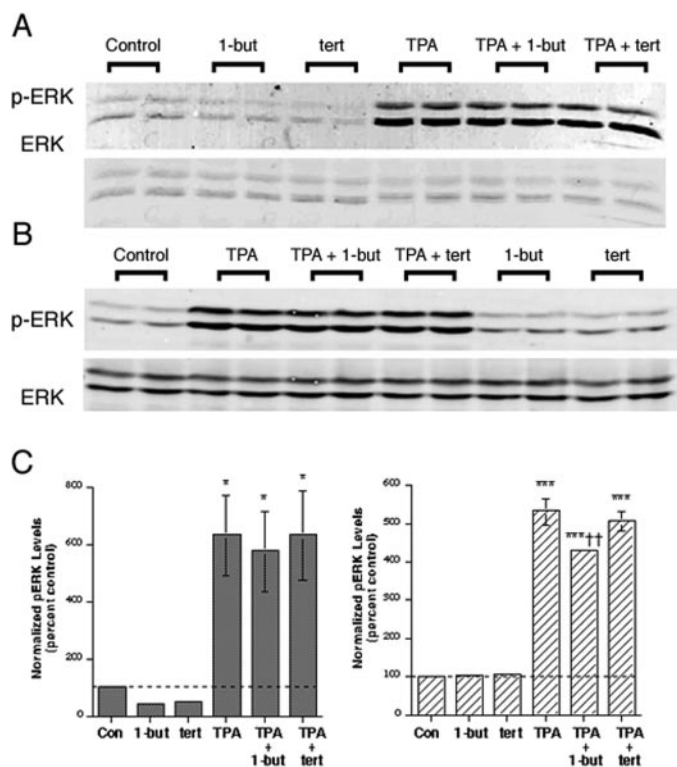


Fig. 9. 1-Butanol has a minimal effect on TPA-induced ERK phosphorylation (activation). Keratinocytes were incubated with or without 100 nM TPA in the presence and absence of 0.3% 1-butanol or *tert*-butanol for 5 (A) or 30 (B) min. Cells were solubilized in sample buffer, and equal volumes were analyzed by Western blotting for total and phospho-ERK simultaneously using the Licor infrared imaging system as described under *Materials and Methods*. Phospho-ERK levels in multiple experiments were normalized using the measured total ERK levels and expressed as the percentage of the control value. Shown to the left is a representative blot and to the right cumulative results representing the means \pm S.E.M. of three (A) or four (B) separate experiments performed in duplicate; *, $p < 0.05$; ***, $p < 0.001$ versus the control value; **, $p < 0.05$ versus TPA alone.

tween the inhibition of PLD activation and of increased transglutaminase activity in response to TPA, suggesting a role for PLD in this process. It should be noted that transglutaminase activity is considered a marker of late keratinocyte differentiation, with enzymatic activity regulated at multiple levels, including transcriptionally, translationally, and post-translationally (Gibson et al., 1996). Therefore, an ability of the agents used in this study to inhibit TPA-induced transglutaminase activity suggests a more general effect on keratinocyte differentiation.

Nevertheless, the lack of specificity of these inhibitors complicates the interpretation of our results. Thus, for example, Gö 6976, but not Gö 6983, BisI, or Ro 31-8220, is known to inhibit the phorbol ester-responsive serine/threonine protein kinase protein kinase D (discussed in Shapiro et al., 2002). Indeed, we have previously shown that Gö 6976, but not the other PKC inhibitors mentioned above, stimulates transglutaminase activity and enhances the effect of 1,25-dihydroxyvitamin D₃ in keratinocytes (Shapiro et al., 2002). Similarly, we (Shapiro et al., 2002) and others (Rennecke et al., 1999) have shown that Gö 6976, but not Gö 6983, inhibits DNA synthesis in keratinocytes as well. Furthermore, we have proposed that PKD serves as a proproliferative and/or antidifferentiative signaling enzyme in these cells (Shapiro

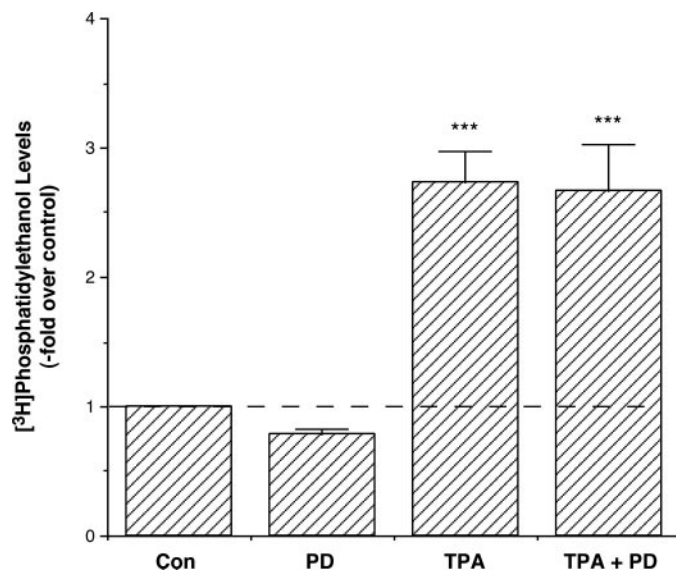


Fig. 10. ERK-1/2 pathway inhibitor PD 98059 does not inhibit TPA-induced PLD activation. Near-confluent keratinocytes were prelabeled for 20 to 24 h with 2.5 μ Ci/ml [³H]oleate before measurement of PLD activity with or without 100 nM TPA in the presence or absence of 50 μ M PD 98059 in the presence of 0.5% ethanol. All samples also contained 0.4% DMSO. Radiolabeled phospholipids, including PA and phosphatidylethanol, were extracted into chloroform, separated by thin layer chromatography, and quantified as described under *Materials and Methods*. Values represent the means \pm S.E.M. of five experiments performed in duplicate; ***, $p < 0.001$ versus the control value.

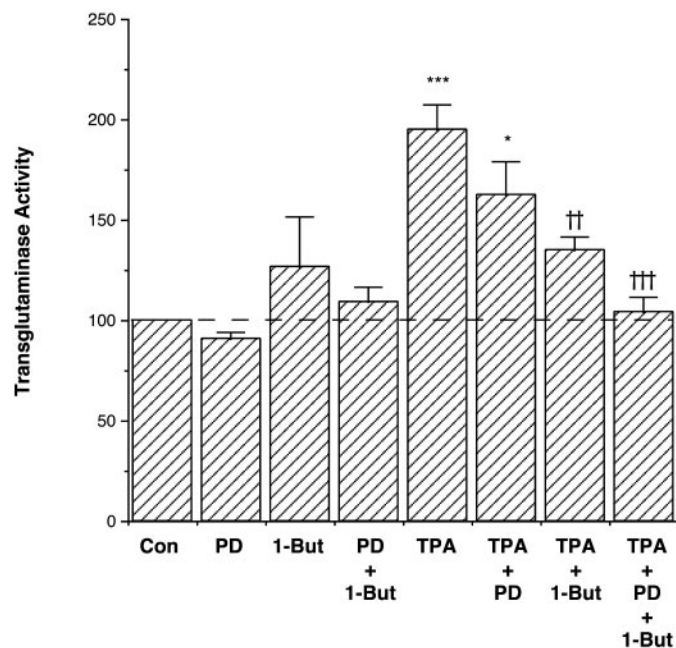


Fig. 11. 1-Butanol and the ERK-1/2 pathway inhibitor PD 98059 additively inhibit TPA-induced transglutaminase activity. Keratinocytes were incubated with 0.4% DMSO (Con) or 100 nM TPA in the presence or absence of 50 μ M PD 98059 with or without 0.3% 1-butanol for 6 h. The cells were then scraped, and transglutaminase activity was measured as described under *Materials and Methods*. Values represent the means \pm S.E.M. of four experiments performed in duplicate or triplicate; *, $p < 0.05$; ***, $p < 0.001$ versus the control value; †, $p < 0.01$; ††, $p < 0.001$ versus TPA alone.

et al., 2002; M. E. Dodd, V. L. Ristich, S. Ray, R. M. Lober, and W. B. Bollag, manuscript submitted for publication; for review, see Bollag et al., 2004). Therefore, it is possible that

the reduced efficacy of Gö 6976 stems from its ability to inhibit PKD and is unrelated to its effects on conventional PKC isoforms and/or PLD. On the other hand, PD 98059 has been shown to inhibit MEK activation of ERK selectively, although both the MEK-1/ERK-1/2 and MEK-5/ERK-5 pathways are reportedly affected (Davies et al., 2000). However, a recent study suggests that in keratinocytes PD 98059 also inhibits 1,25-dihydroxyvitamin D₃-induced MEK activation of c-Jun NH₂-terminal kinase (Johansen et al., 2003), and it is possible that c-Jun NH₂-terminal kinase may play a role in the stimulation of transglutaminase activity in response to TPA. On the other hand, the ability of the ERK-2 inhibitor 5-iodotubercidin also to inhibit TPA-elicited transglutaminase activity (Fig. 8B) provides further support for a role of ERK itself in mediating maximal phorbol ester-stimulated transglutaminase activity.

Our data suggest that PLD participates in regulating TPA-induced keratinocyte differentiation. Thus, the PLD signaling inhibitor 1-butanol is able to reduce TPA-stimulated transglutaminase activity to a value not significantly different from the control level. This primary alcohol diverts lipid production away from PA (and diacylglycerol) to phosphatidylbutanol (for review, see Liscovitch et al., 2000), and inhibition of cellular responses by 1-butanol but not the negative control *tert*-butanol is taken as strong evidence of an involvement of PLD signaling (for review, see Liscovitch et al., 2000). Thus, the inability of *tert*-butanol to inhibit TPA-stimulated transglutaminase activity, as well as the lack of effect of 1-butanol on basal activity, argues against a simple toxic effect of 1-butanol. However, it should be noted that 1-butanol did not completely inhibit the cellular response, nor would it be expected to, since 1-butanol functions to divert some, but not all, PLD-mediated production of PA to phosphatidylbutanol. The volatility of 1-butanol would also hinder a complete inhibition of the cellular response, since this organic alcohol will not likely be present throughout the entire TPA stimulation period of 6 h. Thus, our data indicating that 1-butanol, but not *tert*-butanol, inhibits TPA-induced transglutaminase activity, supports a role for PLD in keratinocyte differentiation.

Since TPA directly activates classical and novel PKC isoforms, the ability of 1-butanol to inhibit transglutaminase activity in the presence of the TPA indicates a diacylglycerol/phorbol ester-independent target for the PLD-generated lipid signals. Since TPA itself can activate classical and novel PKC isoforms, as well as the nucleotide exchange factor for Ras, RasGRP (for review, see Brose and Rosenmund, 2002), these enzymes are unlikely to be the effector enzymes for the PLD-produced lipid signal. Diacylglycerol may have actions unrelated to PKC activation; for instance, as a cosubstrate in ceramide metabolism by sphingomyelin synthase (Luberto and Hannun, 1998) and/or in vesicle trafficking (Kearns et al., 1997). PLD has also been reported to be involved in vesicle trafficking (for review, see Jones et al., 1999), as well as to alter cytoskeleton dynamics (for review, see Bollag and Zheng, 2005) and activate an as yet unknown PA-dependent protein kinase (McPhail et al., 1999). In addition, PA has been demonstrated to recruit Raf-1 to the plasma membrane (Rizzo et al., 1999, 2000) where it can presumably be activated by Ras and initiate the activation of the ERK-1/2 MAPK pathway. However, our results indicating only a minimal effect of 1-butanol on TPA-induced sustained ERK-1/2

phosphorylation (activation) suggest that PLD-generated signals are relatively unimportant in this response to TPA. Thus, the MAPK pathway may be directly activated by TPA-stimulated PKC isoforms (Hamilton et al., 2001) and/or by TPA-mediated activation of RasGRP (Rambaratsingh et al., 2003).

Finally, our results support not only a possible role for PLD but also for ERK-1/2 in TPA-induced keratinocyte differentiation. Nevertheless, data in the literature suggest a role for this MAPK pathway in *proliferation* in response to mitogens, phorbol esters, and cell adhesion, both in other cell systems (for review, see Howe et al., 2002) and in keratinocytes (Zhu et al., 1999; Haase et al., 2001). For instance, Gniadecki (1996) demonstrated a 1,25-dihydroxyvitamin D₃-induced activation of ERK-1/2 associated with a hormone-elicited increase in cell proliferation. Furthermore, Watt and colleagues have shown that targeted overexpression of β 1-integrin to suprabasal keratinocytes in transgenic mice results in activation of ERK-1/2 in the epidermis and the development of a sporadic psoriatic phenotype (Haase et al., 2001). Moreover, in human keratinocytes transient overexpression of constitutively active MEK-1 (the ERK-1/2 kinase) increases and a dominant-negative MEK-1 decreases the growth rate (Zhu et al., 1999; Haase et al., 2001). On the other hand, epidermis reconstituted with keratinocytes expressing the dominant negative MEK-1 demonstrates not only a hypocellularity but also an incomplete terminal differentiation (Haase et al., 2001), suggesting a possible role for this pathway in keratinocyte differentiation as well. The finding that elevated extracellular calcium concentrations induce a transient activation of this enzyme (Schmidt et al., 2000), as well as the data shown in Figs. 7 and 8 of this report, provides additional support for a possible involvement of ERK-1/2 in keratinocyte differentiation. Nevertheless, it is unclear whether the requirement for ERK-1/2 activity for maximal transglutaminase activity and keratinocyte differentiation results from a direct role of this enzyme in mediating differentiation. Indeed, Lane and colleagues have reported that mitotic clonal expansion is necessary for complete differentiation of preadipocytes to adipocytes (Tang et al., 2003). Thus, it is possible that the requirement for ERK-1/2 activity may instead arise from a cell cycle- and/or proliferation dependence of the differentiation process, although a recent report suggests that a G₀ or G₁ arrest is not required for triggering terminal differentiation of keratinocytes (Gandarillas et al., 2000). On the other hand, these authors also suggested that transit through the cell cycle may be necessary for keratinocyte differentiation to proceed (Gandarillas et al., 2000).

In summary, we provide the first direct evidence for a potential role of PLD, as well as the ERK-1/2 MAPK pathway, in mediating TPA-elicited transglutaminase activity and keratinocyte differentiation. In addition, our results point to an involvement of (classical and novel) PKC-independent targets of PLD-mediated signal generation in this process. Our data also suggest that PLD and ERK-1/2 function through parallel pathways of signal transduction. The targets of these two pathways are unknown, although data in the literature point to the ability of both the MAPK and the PKC pathways to influence the activity of the activator protein-1 transcription factor, a known modulator of the expression of numerous keratinocyte differentiation markers (for

review, see Angel et al., 2001), via changes in expression and/or phosphorylation of activator protein-1 family members. Research is in progress to determine the signal transduction system that is initiated with the TPA-mediated activation of PKC and PLD and culminates in the expression/activity of multiple late keratinocyte differentiation markers, as well as the role of these pathways in tumorigenesis. The results of this research should provide a better understanding of the pathways regulating keratinocyte differentiation and allow the identification of potential targets for therapeutic intervention in hyperproliferative skin disorders.

Note Added in Proof

In a recent article [Seo HR, Kwan Y-W, Cho C-K, Bae S, Lee S-J, Soh J-W, Chung H-Y, Lee Y-S (2004) PKC α induces differentiation through ERK1/2 phosphorylation in mouse keratinocytes. *Exp Mol Med* 36:292–299], inhibition of ERK-1/2 activation using PD 98059 or a dominant-negative ERK-1 was reported to inhibit the protein expression of the differentiation markers keratins 1 and 10, loricrin, and filaggrin induced in response to high extracellular calcium levels.

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Address correspondence to: Dr. Wendy B. Bollag, Institute of Molecular Medicine and Genetics/CB-2803, Medical College of Georgia, 1120 15th St., Augusta, GA 30912-2630. E-mail: wbollag@mail.mcg.edu