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Regulation of Protein Kinase D During Differentiation and Proliferation of Primary Mouse Keratinocytes

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Diseased skin often exhibits a deregulated program of the keratinocyte maturation necessary for epidermal stratification and function. Protein kinase D (PKD), a serine/threonine kinase, is expressed in proliferating keratinocytes, and PKD activation occurs in response to mitogen stimulation in other cell types. We have proposed that PKD functions as a pro-proliferative and/or anti-differentiative signal in keratinocytes and hypothesized that differentiation inducers will downmodulate PKD to allow differentiation to proceed. Thus, changes in PKD levels, autophosphorylation, and activity were analyzed upon stimulation of differentiation and proliferation in primary mouse keratinocytes. Elevated extracellular calcium and acute 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatments induced differentiation and triggered a downmodulation of PKD levels, autophosphorylation at serine 916, and activity. Chronic TPA treatment stimulated proliferation and resulted in a recovery of PKD levels, autophosphorylation, and activity. Immunohistochemical analysis demonstrated PKD localization predominantly in the proliferative basal layer of mouse epidermis. Co-expression studies revealed a pro-proliferative, anti-differentiative effect of PKD on keratinocyte maturation as monitored by increased and decreased promoter activities of keratin 5, a proliferative marker, and involucrin, a differentiative marker, respectively. This work describes the inverse regulation of PKD during keratinocyte differentiation and proliferation and the pro-proliferative/anti-differentiative effects of PKD co-expression on keratinocyte maturation.

Key words: calcium/12-*O*-tetradecanoylphorbol-13-acetate/protein kinase C/skin/tumorigenesis
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The skin is the largest organ of the body and acts as a protective barrier against environmental insults, such as ultraviolet radiation, mechanical stress, and water permeation. The skin is also an integral component of the immune, nervous, and endocrine systems. Keratinocytes, the major cell type of the epidermis, produce the proteins and lipids necessary for the barrier function of the skin. Keratinocyte maturation encompasses a tightly regulated program of proliferation and terminal differentiation that facilitates epithelial stratification. As keratinocytes differentiate, they migrate upward through the four layers of the epidermis, the strata basalis, spinosum, granulosum, and corneum, and express proteins indicative of their differentiative status. For example, within the stratum basalis, keratinocytes maintain a proliferative status and express keratin 5 (Fuchs, 1995). Within the stratum spinosum, keratinocytes are committed to terminal differentiation and begin to express cornified envelope components, such as involucrin (Warhol *et al*, 1985). Thus, keratin 5 expression is a marker of proliferation, whereas involucrin expression is a marker of differentiation. Another hallmark of differentiation is transglutaminase activity, which is maximal in the stratum granulosum and catalyzes the formation of γ-glutamyl-ε-lysine bonds that cross-link the cornified envelope proteins (Thacher and Rice, 1985).

One regulator of the differentiation program of keratinocytes is thought to be elevated extracellular calcium concentrations ($[Ca^{2+}]_e$). Indeed, keratinocytes are known to possess a calcium-sensing receptor (CaR), a G protein-coupled receptor identical to the parathyroid CaR (Hebert and Brown, 1995; Chattopadhyay *et al*, 1996). Not only does the CaR mediate elevated $[Ca^{2+}]_e$-induced differentiation *in situ* (Tu *et al*, 1999), but the full-length version of the CaR is necessary for normal epidermal differentiation (Komuves *et al*, 2002). Keratinocytes downregulate the CaR by alternative splicing, which yields an inactive form (Oda *et al*, 1998, 2000) as a result of differential glycosylation that prevents transport to the plasma membrane (Tu *et al*, 2004). In normal epidermis, a calcium gradient is maintained such that the lowest levels exist in the basal layer and the highest levels are present in the granular layer (Tu *et al*, 2004). *In situ* data correlate keratinocyte growth capacity with this *in vivo* gradient. Specifically, keratinocyte cultures maintain a proliferative status when cultured in low calcium concentrations (0.05–0.09 mM), but intermediate calcium concentrations

Abbreviations: $[Ca^{2+}]_e$, extracellular calcium concentration; $125(OH)^2D_3$, 1,25-dihydroxyvitamin D_3; C, carboxy; CaR, calcium-sensing receptor; DMSO, dimethyl sulfoxide; Gö, Gödecke; ITS+, insulin, transferrin, selenous acid, linoleic acid; N, amino; PBS-, phosphate-buffered saline lacking divalent cations; PKC, protein kinase C; PKD, protein kinase D; SEM, standard error of the mean; SFKM, serum-free keratinocyte medium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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(0.14–0.29 mM) stimulate focal areas of stratification, whereas higher calcium concentrations (above 1 mM) induce terminal differentiation (Hennings *et al*, 1980). Although only qualitative data exist describing the calcium concentrations in the epidermal layers and the exact mechanisms of action of this ion have not been elucidated, the presence of an extracellular CaR, an *in vivo* calcium gradient, and *in situ* effects of elevated calcium concentrations strongly support the idea that calcium acts as a key regulator of keratinocyte maturation *in vivo*.

12-*O*-tetradecanoylphorbol-13-acetate (TPA), a complete tumor promoter and phorbol ester, causes biphasic responses in keratinocytes, stimulating differentiation acutely and proliferation chronically, *in situ* (Yuspa *et al*, 1982a) and *in vivo* (Raick *et al*, 1972; Argyris, 1980; Toftgard *et al*, 1985). Data supporting the ability of TPA to stimulate terminal differentiation include demonstrations of enhancement of transglutaminase activity (Yuspa *et al*, 1982a), induction of cornified envelope formation (Parkinson *et al*, 1984), augmentation of expression of the late differentiative markers, involucrin (Poumay *et al*, 1999), loricrin and filaggrin (Dlugosz and Yuspa, 1993), repression of early differentiation markers, such as Keratins 1 and 10 (Dlugosz and Yuspa, 1993), and activation of several protein kinase C (PKC) isoforms (PKC-α, -δ, -ε, or -η) implicated in differentiation (Bollag and Bollag, 2001). Conversely, chronic treatment of keratinocytes with TPA stimulates proliferation *in situ* (Yuspa *et al*, 1976, 1982a), and in conjunction with a DNA-damaging agent, leads to tumor formation *in vivo* (Goerttler *et al*, 1979). This chronic proliferative response has allowed the use of keratinocyte cultures treated with TPA as *in situ* models for mouse tumor formation (Yuspa *et al*, 1982b). Yuspa *et al* (1982a) discovered that approximately half of a population of cultured mouse keratinocytes are resistant to the differentiative effects of TPA, such that differentiating cells are sloughed to the environment whereas proliferating cells are retained. This suggests that the response of keratinocytes to TPA treatment varies because of differences in cell cycle or commitment to differentiation. Also, chronic TPA stimulation causes phorbol ester-responsive PKC to undergo proteolytic degradation, a characteristic not shared by the distantly related enzyme, protein kinase D (PKD) (Rennecke *et al*, 1996). Whether chronic TPA promotes proliferation or tumor formation through the proteolysis of differentiative PKC, through the activation of other phorbol ester-responsive enzymes, such as PKD, or through a combination of these actions, is not known.

PKD, also known as PKCμ, was cloned and identified by two groups using PCR-based strategies to identify novel protein kinases (Johannes *et al*, 1994; Valverde *et al*, 1994). PKD binds diacylglycerol and phorbol esters, leading to activation, *in vitro* and *in situ* (Valverde *et al*, 1994; Van Lint *et al*, 1995; Dieterich *et al*, 1996). Sequence analysis identified regions of similarity to PKC (Johannes *et al*, 1994), but the unique characteristics of PKD have led to its distinction as the first member (i.e., PKD1) of a new family of enzymes, the PKD family (Valverde *et al*, 1994; Van Lint *et al*, 1995), which now consists of PKCμ/PKD1, PKD2, and PKCν/PKD3. Some of the characteristics that distinguish PKD from PKC include: the presence of an amino (N) terminal hydrophobic region, the lack of an autoinhibitory pseudosubstrate domain, and the increased length of the intervening sequence separating the two C1 domains that also lack some of the conserved residues found in PKC (Bollag *et al*, 2004). Additionally, PKD does not bind or become activated by calcium, as do classical PKCs, and it contains a pleckstrin homology domain, which is involved in regulating enzymatic activity, binding of effector proteins, and maintaining subcellular localization (Bollag *et al*, 2004). The catalytic domain of PKD is more homologous to that of the calcium/calmodulin-dependent protein kinases; therefore, PKD is able to efficiently phosphorylate the calcium/calmodulin-dependent protein kinase synthetic substrate, syntide-2, *in vitro* (Van Lint *et al*, 1995; Dieterich *et al*, 1996). At the carboxy (C) terminus within the catalytic domain, PKD is autophosphorylated on the serine residue at position 916, which correlates with PKD activation (Matthews *et al*, 1999), and once activated *in situ*, PKD retains enzymatic activity following immunoprecipitation (Rozengurt *et al*, 1997). The function of PKD is the subject of active research, but few bona fide *in vivo* PKD substrates or downstream effector proteins have been convincingly demonstrated (Rykx *et al*, 2003). Even less is known about the function of the PKD family members, PKD2 and PKD3.

PKD (i.e., PKD1) has been implicated in mitogenesis in multiple cell types. PKD is activated following exposure of cells to a variety of mitogens, such as platelet-derived growth factor, bombesin, vasopressin, endothelin, bradykinin, lysophosphatidic acid, thrombin, angiotensin II, and neurotensin (Bollag *et al*, 2004). When overexpressed in Swiss 3T3 cells, PKD potentiates DNA synthesis and cell proliferation in response to treatment with phorbol esters and the G_q-protein-coupled receptor agonists, bombesin and vasopressin (Zhukova *et al*, 2001). This potentiation of DNA synthesis in response to G protein-coupled receptor agonists is thought to be facilitated by the ability of active PKD to increase the duration of extracellular-regulated protein kinase-1 and -2 activation (Sinnett-Smith *et al*, 2004). In mouse keratinocytes, PKD has been discovered at higher concentrations in proliferative cell fractions, and PKD levels are increased in carcinomas isolated from mouse epidermis (Rennecke *et al*, 1999). Studies using the classical PKC/PKD inhibitor Gödecke (Gö) 6976 have demonstrated that the inhibition of both classical PKC and PKD stimulates keratinocyte differentiation. In contrast, Gö 6983, which inhibits classical PKC but not PKD at the concentrations used, does not stimulate differentiation (Shapiro *et al*, 2002). These results support the idea that Gö 6976, but not Gö 6983, induces differentiation because of its inhibition of a pro-proliferative and/or anti-differentiative signal. The use of a panel of inhibitors suggests that the pro-proliferative/anti-differentiative signal is PKD (Shapiro *et al*, 2002), as we have recently proposed (Bollag *et al*, 2004). We also predict that agents that stimulate keratinocyte differentiation, such as elevated $[Ca^{2+}]_e$ or acute TPA treatment, will downregulate PKD expression and/or activity to allow differentiation to proceed.

Herein, we describe the regulation of PKD protein levels, autophosphorylation, and activity following treatment of primary mouse keratinocyte cultures with the differentiation-promoting agents, elevated $[Ca^{2+}]_e$ and acute TPA treatment ($\leqslant$8 h), and the proliferation-promoting agent, chronic

TPA treatment (>24 h). PKD protein levels, autophosphorylation, and activity were downmodulated upon commitment to the differentiation program. Conversely, a recovery of PKD occurred in conjunction with proliferation. By immunohistochemical analysis, the distribution of PKD protein expression in intact neonatal mouse epidermis was found to be highest in the basal layer, the proliferative layer. Finally, PKD co-expression affected keratinocyte maturation status in a pro-proliferative/anti-differentiative manner.

Results

Treatment of keratinocytes with elevated extracellular calcium and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) causes apparent decreases in PKD protein levels In order to evaluate the regulation of PKD in mouse keratinocytes, PKD protein levels were analyzed following treatment of cultures with the differentiation-promoting agents, elevated $[Ca^{2+}]_e$ and $1,25(OH)_2D_3$. We have proposed that PKD is a pro-proliferative and/or anti-differentiative signal in keratinocytes (Bollag *et al*, 2004) and have hypothesized that agents that stimulate keratinocytes to differentiate might also initiate a reduction in PKD expression and/or activation to allow differentiation to proceed. Therefore, near-confluent cultures of keratinocytes were incubated for 24 h in serum-free keratinocyte medium (SFKM) (25 μM $CaCl_2$) containing vehicle (0.05% ethanol), 1 mM $CaCl_2$ (+0.05% ethanol), or 250 nM $1,25(OH)_2D_3$. After treatment, samples were collected for western blot analysis, and the immunoreactive signals obtained using a C-terminus-specific PKD antibody, SC-935, and an ^{125}I-labeled secondary antibody were quantified. Both calcium and $1,25(OH)_2D_3$ treatment caused significant decreases in the PKD signal, 27% ± 2.32% and 25% ± 8.76%, respectively (Fig 1*A,B*). These results could be interpreted as a reduction of PKD in conjunction with differentiation, or alternatively, as reduced antigen recognition by the SC-935 antibody for PKD because of a change in the conformation or phosphorylation status of PKD following treatment. Another C-terminus-specific PKD antibody, SC-639, is known to exhibit reduced recognition of PKD once PKD becomes phosphorylated/activated (Rennecke *et al*, 1996).

Acute treatment of keratinocytes with TPA causes apparent decreases in PKD protein levels but increases PKD activity To investigate the possibility that the recognition of PKD by the SC-935 antibody is affected by the phosphorylation/activation status of PKD, PKD protein levels were analyzed following short-term treatment of cultures with TPA. TPA is a phorbol ester known to activate and increase the autophosphorylation status of PKD (Rennecke *et al*, 1996; Zugaza *et al*, 1996). Near-confluent cultures of keratinocytes were incubated for 10 min in SFKM (25 μM $CaCl_2$) containing vehicle (0.1% dimethyl sulfoxide (DMSO)) or 100 nM TPA, and samples were collected for western blot analysis and signal quantification. Surprisingly, the TPA treatment caused a 53% ± 7.8% decrease in the PKD signal, suggesting an apparent reduction in total PKD levels at this early time point (Fig 2*A,B*). The data were interpreted to mean that SC-935 exhibits reduced recognition of phosphorylated/activated PKD for the following reasons: (1) the short treatment time suggests that downregulation is unlikely; (2) reports describe the lack of downregulation of PKD following TPA treatment (Rennecke *et al*, 1996); and (3) TPA is known to activate and induce the autophosphorylation of PKD in keratinocytes (Rennecke *et al*, 1996) and in other cell types (Zugaza *et al*, 1996).

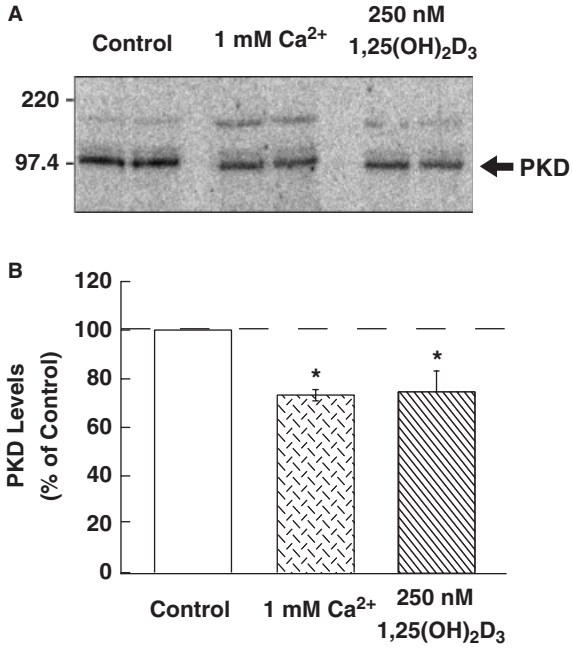

Figure 1
Elevated extracellular calcium and 1,25-dihydroxyvitamin D_3 induce apparent decreases in protein kinase D (PKD) protein levels. Primary keratinocytes were incubated for 24 h in serum-free keratinocyte medium containing vehicle (0.05% ethanol) (Control, *open bar*), 1 mM $CaCl_2$ (+0.05% ethanol) (1 mM Ca^{2+}, *hatched bar*) or 250 nM (250 nM D_3, *striped bar*). After lysis and collection, samples were loaded according to protein concentration and subjected to western blot analysis. (*A*) The immunoreactive signals obtained using SC-935 and an ^{125}I-labeled secondary antibody were visualized using a Phosphorimager SI and (*B*) were quantified by ImageQuant software and analyzed using Instat 2.0. Values represent the means ± standard error of the mean from four separate experiments *versus* the control and were analyzed by ANOVA with significance at $p<0.05$ (*).

To verify that TPA activates PKD in our system, keratinocyte cultures were incubated in SFKM (25 μM $CaCl_2$) containing vehicle (0.1% DMSO) or 100 nM TPA for 10 min. PKD was immunoprecipitated using two primary antibodies simultaneously: one targeting the N-terminus, SC-638, and one targeting the C-terminus, SC-935. TPA stimulation followed by incubation of immunoprecipitated PKD with syntide-2 in the presence of [γ-^{32}P]adenosine triphosphate ([γ-^{32}P]ATP) produced a significant 3.3 ± 0.41-fold increase in phosphorylation of syntide-2 (Fig 2*C*), as well as an increase in PKD autophosphorylation (data not shown). The data verified that PKD is indeed activated by TPA in keratinocytes and support the interpretation that SC-935 exhibits reduced recognition of phosphorylated/activated PKD.

Commercially available PKD-specific antibodies vary in their recognition of PKD Because the SC-935 antibody seemed inappropriate for our studies, we investigated the antigen recognition of several alternative commercially

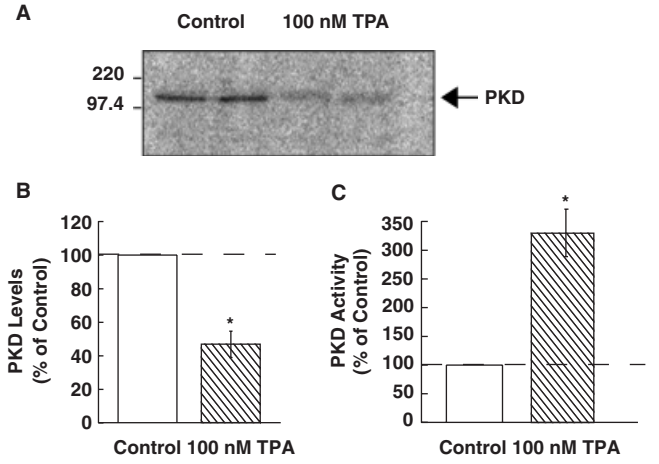

Figure 2
12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces apparent decreases in protein kinase D (PKD) protein levels but stimulates PKD activity. Primary keratinocytes were incubated for 10 min in serum-free keratinocyte medium containing vehicle (0.1% dimethyl sulfoxide) (Control, *open bar*) or 100 nM TPA (100 nM TPA, *striped bar*). (*A,B*) After lysis and collection, samples were loaded according to protein concentration and subjected to western blot analysis. (*A*) The immunoreactive signals obtained using SC-935 and an ^{125}I-labeled secondary antibody were visualized using a Phosphorimager SI and (*B*) were quantified by ImageQuant software and analyzed using In Stat 2.0. Values represent the means ± standard error of the mean (SEM) from four experiments *versus* the corresponding control and were analyzed by a Student's *t* test with significance at $p < 0.05$ (*). (*C*) PKD was immunoprecipitated using both SC-638 and SC-935, simultaneously, and protein-A agarose. The immunoprecipitate was incubated with syntide-2 in the presence of [γ-^{32}P]adenosine triphosphate, and the resulting phosphorylation was measured in a scintillation counter. Values represent the means ± SEM from three separate experiments *versus* the respective corresponding control and were analyzed by a Student's *t* test with significance at $p < 0.05$ (*).

available PKD-specific antibodies. COS-7 cells were transfected with either the empty vector control, pcDNA3, or the PKD expression vector, pcDNA3–PKD. After lysis and collection, 10 μg of protein were loaded in quadruplicate and was subjected to western blot analysis using the PKD-specific antibodies: SC-935, SC-638, CS-2052, and CS-2051. (The SC-639 antibody was not tested because of previous reports of this antibody exhibiting reduced recognition of activated/phosphorylated PKD (Rennecke *et al*, 1996)). Each antibody tested (SC-935, SC-638, CS-2052, and CS-2051) produced a strong immunoreactive signal when PKD was transiently overexpressed compared with empty vector control (pcDNA3) (Fig 3). The SC-638 antibody produced the weakest immunoreactive signal, which suggested a less efficient recognition of PKD, and previous results (Fig 2) rendered the SC-935 antibody suspect for western analyses. Based on these results, the CS-2052 and CS-2051 antibodies were used for our subsequent western analyses.

PKD protein levels are not decreased following acute treatment of keratinocytes with TPA Previous reports have demonstrated that serine 916 of PKD becomes phosphorylated in response to phorbol esters, and this phosphorylation status correlates with enzymatic activity (Matthews *et al*, 1999). This was corroborated in our system by syntide-2 phosphorylation assays; therefore, the

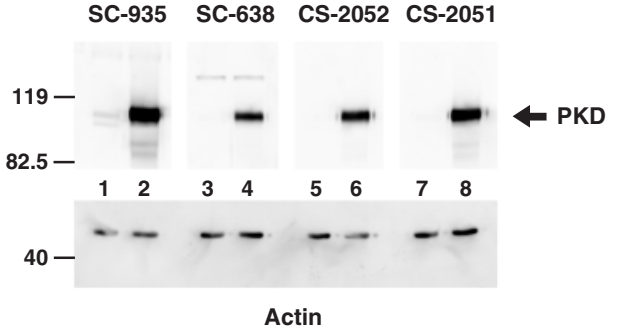

Figure 3
Commercial protein kinase D (PKD)-specific antibodies recognize PKD overexpressed in COS-7 cells. COS-7 cells were transfected with pcDNA3 (*lanes 1, 3, 5*, and *7*) or pcDNA3-PKD (*lanes 2, 4, 6,* and *8*). After lysis and collection, equal protein amounts (10 μg) from each sample were loaded in quadruplicate and subjected to western blot analysis. The immunoreactive signals were obtained using PKD-specific antibodies (SC-935, SC-638, CS-2052, or CS-2051) and a secondary antibody conjugated with alkaline phosphatase. The signals were visualized using a Storm Phosphorimager. Actin was used as a control to verify equal loading.

decreased signals corresponding to PKD protein levels were interpreted as decreases in the recognition of the SC-935 antibody for phosphorylated activated PKD. Therefore, we re-examined the effects of acute 100 nM TPA treatments on PKD protein levels using two alternate primary antibodies obtained from Cell Signaling Technology: one targeting the C-terminus of PKD, CS-2052, and one targeting the autophosphorylated serine 916 residue, CS-2051. Near-confluent primary keratinocyte cultures were treated with 100 nM TPA for 10 min, and samples were collected for western blot analysis. Interestingly, no apparent decrease in PKD protein levels was observed when using CS-2052; yet, a large increase in signal occurred when using CS-2051, suggesting an increase in phosphorylation at serine 916 (Fig 4). These data suggest that the SC-935 antibody preferentially recognizes the unphosphorylated form of PKD, as previously reported of the SC-639 antibody (Rennecke *et al*, 1996). Thus, the previous conclusion that PKD protein levels were downmodulated following elevated $[Ca^{2+}]_e$- and $1,25(OH)_2D_3$-induced differentiation of primary keratin-

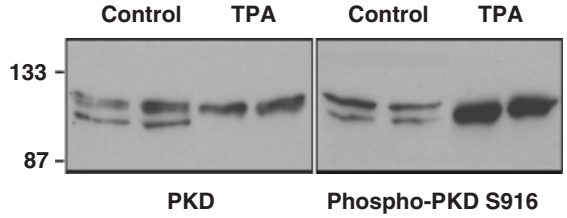

Figure 4
Acute 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment does not induce decreases in protein kinase D (PKD) protein levels. Primary keratinocytes were incubated for 10 min in serum-free keratinocyte medium containing vehicle (0.005% dimethyl sulfoxide) (Control) or 100 nM TPA (TPA). After lysis and collection, samples were loaded according to protein concentration and subjected to western blot analysis. The immunoreactive signals were obtained using either CS-2052 (PKD) or CS-2051 (Phospho-PKD S916) and a secondary antibody conjugated with horseradish peroxidase. The signals were visualized after exposure to film using enhanced chemiluminescence, and the figure is a representative blot of four experiments.

ocytes was rendered suspect. Additionally, the results suggested that the recognition of PKD by the CS-2052 antibody is not affected by PKD phosphorylation status (Fig 4).

Treatment of keratinocytes with elevated extracellular calcium causes a reduction in PKD protein levels, autophosphorylation, and activity Because the SC-935 antibody exhibited reduced recognition of activated PKD, the apparent decrease in PKD protein levels observed in response to both elevated $[Ca^{2+}]_e$ and $1,25(OH)_2D_3$ could be interpreted as an actual decrease in PKD protein levels or an increase in PKD activation. In order to determine which of these alternate events occurred, the effects of elevated $[Ca^{2+}]_e$-induced keratinocyte differentiation on PKD protein levels were re-evaluated using the CS-2052 antibody. The subsequent experiments focused on the effects of elevated $[Ca^{2+}]_e$ rather than $1,25(OH)_2D_3$ because of greater and more consistent effects of this differentiating agent. Western blot analyses were performed on samples obtained from primary keratinocytes incubated in SFKM (25 μM $CaCl_2$) or SFKM containing 1 mM $CaCl_2$ using CS-2052 and CS-2051 to analyze changes in PKD protein levels and autophosphorylation/activation status, respectively (Fig 5*B*). The technique of enhanced chemifluorescence (ECF) was used to quantitatively measure the changes observed. To investigate the effects of calcium-induced differentiation on PKD activity, PKD was immunoprecipitated from keratinocyte cultures incubated for 10 min or 16 h in SFKM (25 μM $CaCl_2$) or SFKM containing 1 mM $CaCl_2$ using CS-2052, and *in vitro* kinase assays were performed. The changes in PKD protein levels and serine 916 phosphorylation were measured at the time points of 8, 16, and 24 h because these time points encompass the rising phase (8 h), the maximal observed transglutaminase activity (16 h), and the plateau phase (24 h) after stimulation with 1 mM $CaCl_2$ (Fig 5*A*). These time points also correspond to the previous report that elevated $[Ca^{2+}]_e$ (1 mM) treatment inhibits the proliferative response of keratinocytes as shown by the significant and maximal inhibition of DNA sythesis after 8 and 24-h calcium treatments, respectively (Bollag *et al*, 1994). The data indicate that PKD protein levels and serine 916 phosphorylation were significantly downmodulated after 8, 16, and 24 h of elevated $[Ca^{2+}]_e$ treatment when compared with control (Fig 5*B,C*). Also, significant decreases in PKD activity were discovered in cultures treated with 1 mM $CaCl_2$ for 16 h (Fig 5*D*). These results are consistent with our hypothesis that PKD is pro-proliferative and/or anti-differentiative; therefore, agents, like elevated $[Ca^{2+}]_e$, that trigger keratinocytes to differentiate also decrease PKD protein expression and/or activation.

TPA treatment of keratinocytes induces a biphasic response in terms of DNA-specific activity and transglutaminase activity consistent with an initiation of differentiation followed by proliferation TPA is known

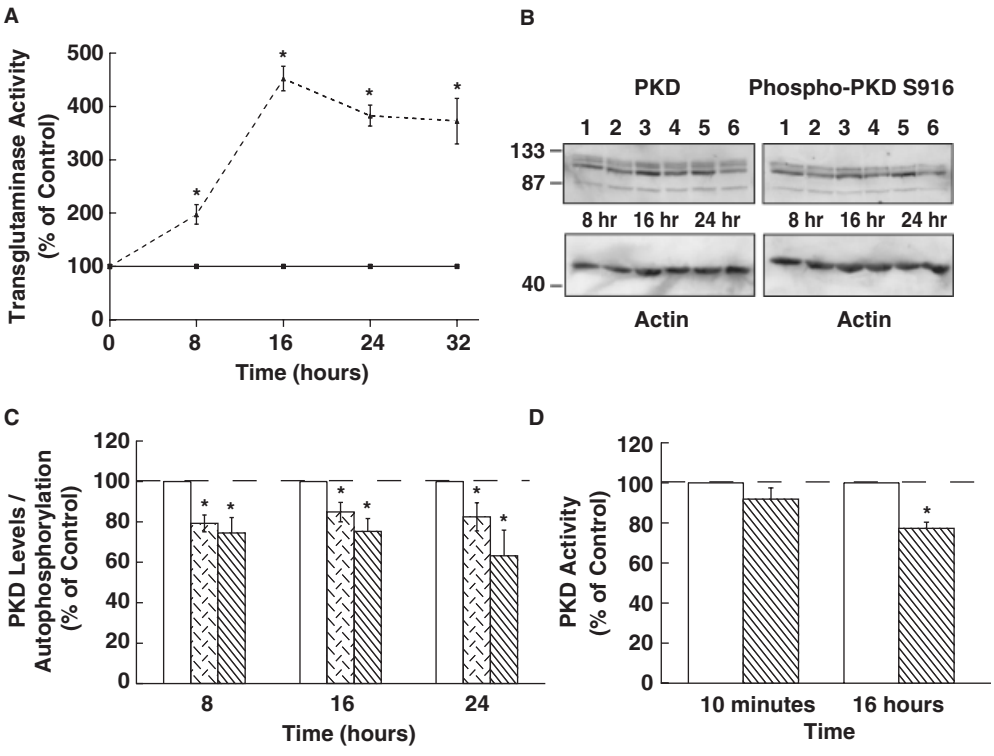

Figure 5
Elevated extracellular calcium stimulates transglutaminase activity and induces a downmodulation of protein kinase D (PKD) protein levels, serine 916 autophosphorylation, and activity. (*A*) Primary keratinocytes were incubated for 8, 16, 24, or 32 h in serum-free keratinocyte medium (SFKM) (25 μM $CaCl_2$) (*solid line*) or SFKM containing 1 mM $CaCl_2$ (*dashed line*). The samples were collected and transglutaminase activity assays were performed by incubating cell sonicates overnight in a reaction mixture containing [^{3}H]-putrescine and dimethylcasein, followed by precipitating the cross-linked product and collecting by filtration. The cpm of the radiolabeled product were determined using liquid scintillation spectrometry, and data were normalized to protein content and were analyzed using Instat 2.0. Values represent the means ± standard error of the mean (SEM) from three experiments and were analyzed by ANOVA with significance at $p<0.05$ (*). (*B,C*) Primary keratinocytes were incubated for 8, 16, or 24 h in SFKM (25 μM $CaCl_2$) (*lanes 1, 3,* and *5*, *open bars*) or SFKM containing 1 mM $CaCl_2$ (*lanes 2, 4,* and *6*, *hatched bars*, *striped bars*). After lysis and collection, samples were loaded according to protein concentration and subjected to western blot analysis. The immunoreactive signals were obtained using either CS-2052 (PKD, *hatched bars*) or CS-2051 (Phospho-PKD S916, *striped bars*) and a secondary antibody conjugated with alkaline phosphatase. (*B*) The signals were visualized using a Storm Phosphorimager and (*C*) were quantified by ImageQuant software and expressed relative to actin levels. Values represent the means ± SEM from five to six separate experiments *versus* the respective matched control and were analyzed by a Student's *t* test with significance at $p<0.05$ (*). (*D*) Primary keratinocytes were incubated for 10 min or 16 h in SFKM (25 μM $CaCl_2$) (*open bars*) or SFKM containing 1 mM $CaCl_2$ (*striped bars*), and PKD was immunoprecipitated using CS-2052 and protein-A agarose. The immunoprecipitate was incubated with syntide-2 in the presence of [γ-^{32}P]adenosine triphosphate, and the resulting phosphorylation was measured in a scintillation counter. Values represent the means ± SEM from three separate experiments *versus* the respective matched control and were analyzed by a Student's *t* test with significance at $p<0.05$ (*).

to stimulate a biphasic response in keratinocytes, with acute treatment inducing differentiation and chronic treatment stimulating proliferation *in vitro* (Yuspa *et al*, 1982a) or tumor promotion *in vivo* in conjunction with a carcinogen (Goerttler *et al*, 1979). Some of these previous studies were performed under slightly different culture conditions (i.e., containing serum) (Yuspa *et al*, 1982a). To confirm the biphasic nature of the TPA response in our system, near-confluent keratinocytes were incubated in SFKM (25 μM $CaCl_2$) containing vehicle control (0.005% DMSO) or 100 nM TPA for 8, 24, 48, and 72 h and assayed for both DNA-specific activity, as a measure of proliferation, and transglutaminase activity, as a measure of differentiation. As expected, significant increases in transglutaminase activity were observed after acute TPA treatment (8 h) while significant reductions were observed after chronic TPA treatments (48 and 72 h) (Fig 6). Conversely, a reduction in DNA synthesis below that of the vehicle control was observed after acute TPA treatment (8 h), whereas significant increases occurred after chronic TPA treatment (Fig 6). These data demonstrate the ability of acute TPA treatment to induce differentiation and of chronic TPA treatment to stimulate proliferation in our system.

Treatment of keratinocytes with TPA induces biphasic changes in PKD protein levels, serine 916 autophosphorylation and activity PKD protein levels were downmodulated upon keratinocyte differentiation induced by elevated $[Ca^{2+}]_e$ treatment. These results support our hypothesis that in keratinocytes, PKD is a pro-proliferative and/or anti-differentiative enzyme that may require downmodulation to allow differentiation to proceed. To determine whether the results are specific to elevated $[Ca^{2+}]_e$-induced differentiation or also characteristic of TPA-induced differentiation, the effects of acute and chronic TPA treatments on PKD protein expression and activation were examined. Specifically, near-confluent primary mouse keratinocytes were incubated in SFKM (25 μM $CaCl_2$) containing vehicle (0.005% DMSO) or 100 nM TPA for 1, 8, 16, 24, and 48 h, and western analyses were performed using CS-2052 to detect PKD protein levels and CS-2051 to detect autophosphorylation. To investigate the effects of acute and chronic TPA treatments on PKD activity, PKD was immunoprecipitated from keratinocyte cultures incubated for 1, 16, and 48 h in SFKM (25 μM $CaCl_2$) containing vehicle (0.005% DMSO) or 100 nM TPA using CS-2052, and *in vitro* kinase assays were performed. There were significant increases in serine 916 autophosphorylation after 1 h of TPA treatment and significant reductions in autophosphorylation at 8, 16, and 24 h post-treatment (Fig 7). Interestingly, the PKD protein levels were significantly reduced to 69% ± 7.8% after 8 h of acute TPA treatment (Fig 7*A,B*), and this time point correlates with maximal transglutaminase activity and minimal DNA-specific activity observed following acute TPA-stimulated differentiation (Fig 6). After 48 h of TPA treatment, PKD protein levels and serine 916 autophosphorylation recovered to values similar to those of control (Fig 7*A,B*). Furthermore, PKD activity was significantly increased after 1 h of TPA treatment, significantly decreased after 16 h of TPA treatment, and recovered to that of control after 48 h of TPA treatment (Fig 7*C*). These results suggest that differentiation in response to acute TPA also decreases PKD protein levels, autophosphorylation status and activity, consistent with our idea that the pro-proliferative and/or anti-differentiative signal of PKD may need to be downmodulated during differentiation in keratinocytes.

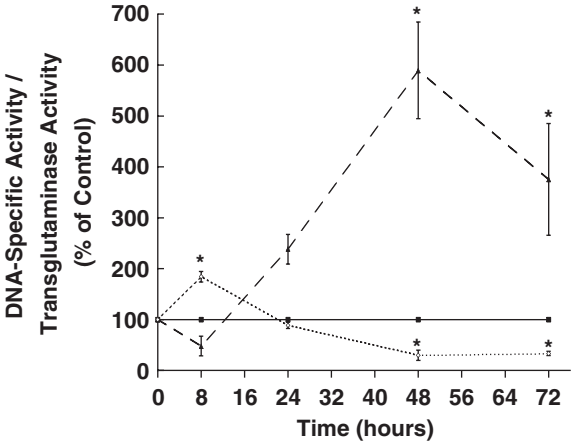

Figure 6
12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulates a biphasic response of differentiation followed by proliferation as monitored using DNA-specific activity and transglutaminase activity. Primary keratinocytes were incubated for 8, 24, 48, or 72 h in serum-free keratinocyte medium containing vehicle (0.005% dimethyl sulfoxide) (*solid line*) or 100 nM TPA (*large dashed line, small dashed line*). The DNA-specific activity assay (*large dashed line*) was performed by incubating cultures with 1 μCi per mL [^{3}H]-thymidine for 1 h after treatment and solubilizing the DNA. [^{3}H]-Thymidine incorporation was measured in a scintillation counter whereas DNA concentrations were determined using Hoescht dye, and values were calculated as described in Experimental Procedures. The transglutaminase activity assay (*small dashed line*) was performed by incubating cell sonicates overnight in a reaction mixture containing [^{3}H]-putrescine and dimethylcasein, followed by precipitating the cross-linked product and collecting by filtration. The cpm of the radiolabeled product were determined using liquid scintillation spectrometry, and data were normalized to protein content. Values represent the means ± standard error of the mean from at least three experiments of either assay and were analyzed by ANOVA with significance at $p < 0.05$ (*).

PKD protein distribution in neonatal mouse epidermis is highest in the stratum basalis In order to investigate whether PKD is present in the proliferative layers of the epidermis, immunohistochemical analyses of PKD protein expression were performed on neonatal mouse epidermis using the PKD-specific primary antibodies, SC-935, which targets the C-terminus, and SC-638, which targets the N-terminus. Unfortunately, CS-2052 is not suitable for immunohistochemical analysis. Both SC-935 and SC-638 antibodies showed similar results: although PKD was detected in suprabasal layers, PKD protein levels were greatest in the basal layer of the epidermis, the proliferative layer (Fig 8). These results correlate with the previous findings of Rennecke *et al* (1999) that PKD expression is highest in the proliferating cell nuclear antigen-expressing fraction of cells isolated from mouse epidermis and are consistent with the hypothesis that PKD is a pro-proliferative and/or anti-differentiative signal in keratinocytes.

Co-expression of PKD has a pro-proliferative and anti-differentiative effect on the maturation status of keratinocytes as evidenced by an increase in keratin 5 promoter activity and decrease in involucrin promoter

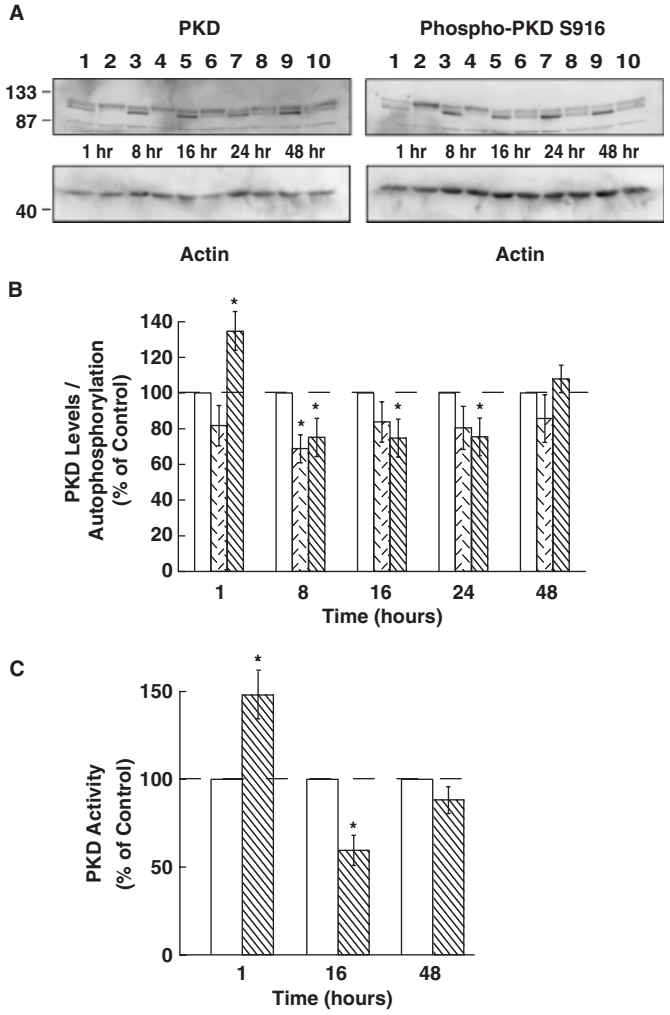

Figure 7
12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces biphasic changes in protein kinase D (PKD) protein levels, serine 916 autophosphorylation, and activity. Primary keratinocytes were incubated for 1, 8, 16, 24, or 48 h in serum-free keratinocyte medium (SFKM) containing vehicle (0.005% dimethyl sulfoxide (DMSO)) (*lanes 1, 3, 5, 7*, and *9, open bars*) or 100 nM TPA (*lanes 2, 4, 6, 8*, and *10*, *hatched bars, striped bars*). After lysis and collection, samples were loaded according to protein concentration and subjected to western blot analysis. (*A,B*) The immunoreactive signals obtained using either CS-2052 (PKD, *hatched bars*) or CS-2051 (Phospho-PKD S916, *striped bars*) and a secondary antibody conjugated with alkaline phosphatase (*A*) were visualized using a Storm Phosphorimager and (*B*) were quantified by ImageQuant software and expressed in *Panel B* relative to actin levels. Values represent the means ± standard error of the mean (SEM) from four to six experiments *versus* the respective matched control and were analyzed by a Student's *t* test with significance at $p < 0.05$ (*). (*C*) PKD was immunoprecipitated using CS-2052 and protein-A agarose. The immunoprecipitate was incubated with syntide-2 in the presence of [γ-^{32}P]adenosine triphosphate, and the resulting phosphorylation was measured in a scintillation counter. Values represent the means ± SEM from three experiments *versus* the respective matched control and were analyzed by a Student's *t* test with significance at $p < 0.05$ (*).

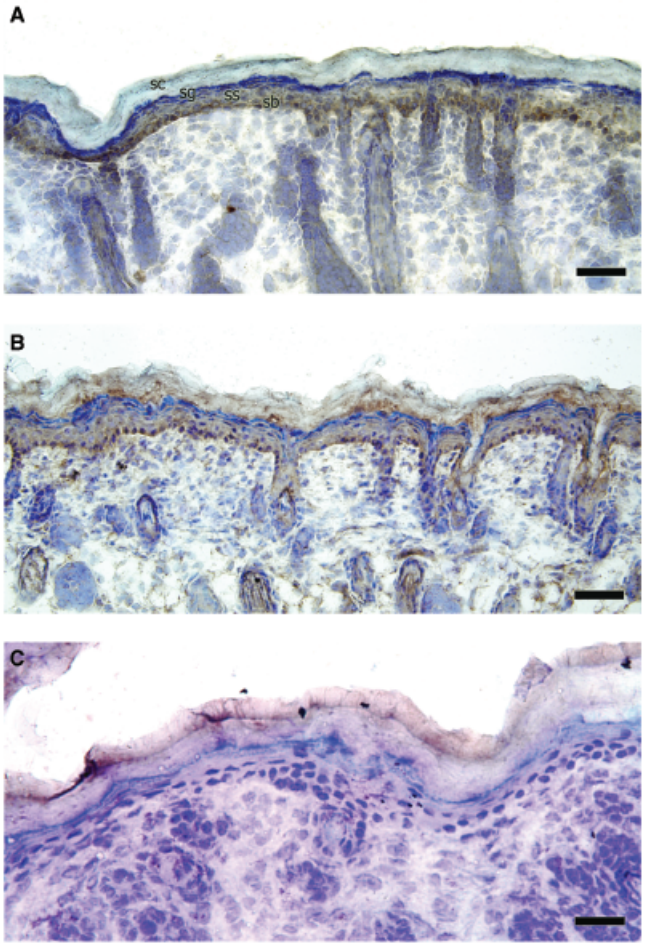

Figure 8
Protein kinase D (PKD) predominates in the stratum basalis of neonatal mouse epidermis. Shown here are representative tissue sections, prepared as described in the Methods section, that demonstrate the immunohistochemical distribution of PKD protein expression in newborn murine epidermis. (*A*) The epidermis was incubated and stained using SC-638, which targets the N-terminus of PKD. (*B*) The epidermis was incubated and stained using SC-935, which recognizes the C-terminus of PKD. (*C*) The negative control was obtained by incubating sections with the secondary biotinylated antibody only. Abbreviations used are as follows: sc, stratum corneum; sg, stratum granulosum; ss, stratum spinosum; sb, stratum basalis. *Scale bar* = 50 μm.

activity To test the effects of PKD on the maturation status of keratinocytes, the promoter activities of well-established markers of proliferation and differentiation were monitored following PKD co-expression using the dual-luciferase assay. In various transfection experiments using several different protocols and reagents (including TransIT Keratinocyte), the maximal transfection efficiency observed for primary mouse keratinocytes was about 10%–20%. Therefore, to circumvent the low transfection efficiency, the dual-luciferase assay was used to obtain data from the small population of transfected cells. The empty vector or PKD expression vector is co-transfected with constructs in which the keratin 5 or involucrin promoter drives the expression of firefly luciferase, which is used as a reporter to quantify the promoter activities. Keratin 5 is expressed in the basal layer of the epidermis and is a marker of the proliferative stage of the keratinocyte life cycle (Byrne *et al*, 1994; Fuchs, 1995). Conversely, involucrin, a marker for intermediate-to-late differentiation in keratinocytes, begins to be expressed in the upper spinous layer and increases in expression as cells migrate upward toward the stratum corneum (Banks-Schlegel and Green, 1981; Warhol *et al*, 1985). A transfection control is provided by co-transfecting an expression construct in which the Renilla luciferase gene is under the control of the SV40 promoter, which does not

respond to calcium (Xie and Bikle, 1997). Prior to transfection, the three constructs: (1) 1 μg of the pcDNA3 or PKD expression construct; (2) 1 μg of the keratin 5 or involucrin–firefly luciferase construct; and (3) 0.25 μg of the control SV40–Renilla luciferase construct (pRL–SV40), are thoroughly mixed. Thorough mixing increases the likelihood that cells that are transfected with the reporter construct are also transfected with the pcDNA3 or PKD expression construct and the pRL–SV40 control construct. In addition, the control Renilla construct is present at only one-quarter of the amount of the other two vectors, which renders the possibility unlikely that a cell could be transfected with only the control construct and not at least one of the other two constructs. Finally, the data are expressed as a ratio of firefly luciferase activity to Renilla luciferase activity. In the unlikely event that the cells become transfected with the reporter vector but not the PKD expression vector or vice versa, such scenarios would lead to an underestimation of the maturation state changes resulting from the expression of exogenous PKD. Co-expression of PKD significantly increased keratin 5 promoter activity by 90% ± 10.4% and significantly reduced involucrin promoter activity by 43% ± 2.3% (Fig 9*A*) when compared with co-expression of pcDNA3 (control). These data suggest that PKD is a pro-proliferative and anti-differentiative signal in keratinocytes.

In addition to testing the effects of PKD co-expression under basal conditions, the effects of PKD co-expression on keratinocyte maturation status were also examined under differentiating conditions (i.e., elevated $[Ca^{2+}]_e$). Thus, keratinocytes were transfected with each of the three constructs and were incubated in SFKM containing 1 mM $CaCl_2$ for 24-h post-transfection. As expected, elevated $[Ca^{2+}]_e$ stimulated differentiation as shown by the significant reduction of keratin 5 promoter activity by 24% ± 4.1% and significant increase of involucrin promoter activity by 500% ± 61.5% in cultures co-expressing pcDNA3 (data not shown). Interestingly, when PKD was co-expressed in cultures treated with elevated $[Ca^{2+}]_e$, keratin 5 promoter activity was increased by 32% ± 11.7% above that found in elevated $[Ca^{2+}]_e$-treated cultures co-expressing pcDNA3 (Fig 9*B*). Conversely, when PKD was co-expressed in cultures treated with elevated $[Ca^{2+}]_e$, involucrin promoter activity was significantly decreased by 32% ± 4.7% when compared with elevated $[Ca^{2+}]_e$-treated vector-transformed cells (Fig 9*B*). Thus, PKD co-expression opposed the elevated $[Ca^{2+}]_e$-induced differentiation effects on both keratin 5 and involucrin promoter activities. These data suggest that PKD is a pro-proliferative and anti-differentiative signal in keratinocytes even in the presence of the differentiating signal of elevated $[Ca^{2+}]_e$.

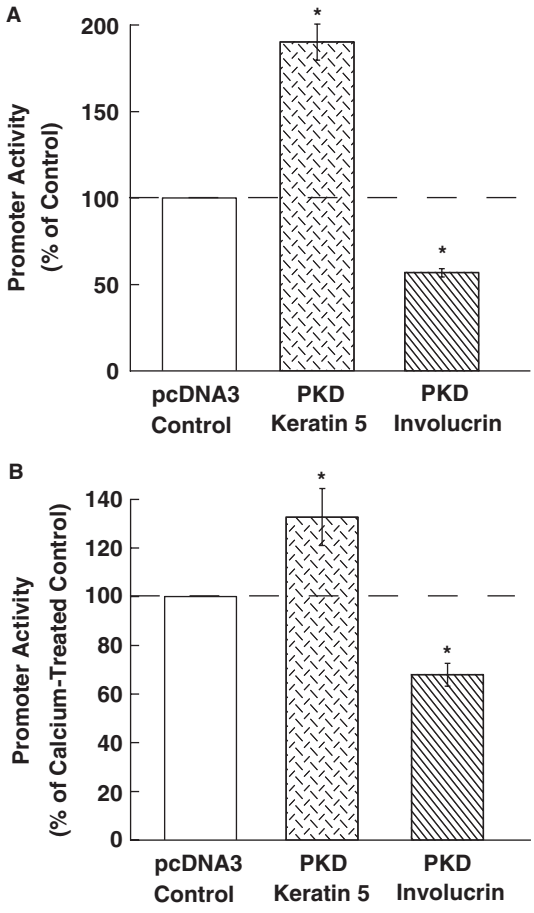

Figure 9
Co-expression of protein kinase D (PKD) stimulates keratin 5 promoter activity but inhibits involucrin promoter activity. Primary keratinocytes were co-transfected with three constructs: (1) pcDNA3 control vector (*open bar*) or pcDNA3–PKD expression construct (*hatched bar, striped bar*); (2) firefly–luciferase reporter construct under control of the minimal promoter of keratin 5 (*hatched bar*) or involucrin (*striped bar*); and (3) pRL–SV40 *Renilla* luciferase construct under control of the SV40 promoter, which serves as an internal transfection control. Cells were harvested after 48 h following the transfections and 24 h following exposure to (*A*) basal conditions or (*B*) differentiating conditions (i.e., 1 mM $CaCl_2$). After lysis, the resulting luciferase activity was measured in a luminometer. Values represent the means ± standard error of the mean from four keratin 5 and three involucrin experiments and were analyzed by ANOVA with significance at $p < 0.05$ (*).

Discussion

Data in the literature suggest that PKD is involved in mitogenesis in multiple systems, including keratinocytes (Abedi *et al*, 1998; Chiu and Rozengurt, 2001; Guha *et al*, 2002; Tan *et al*, 2003; Yuan *et al*, 2003; Bollag *et al*, 2004; Kam and Exton, 2004; Sinnett-Smith *et al*, 2004). Therefore, our laboratory investigated the role that PKD plays in keratinocyte maturation by analyzing the changes in PKD protein levels, autophosphorylation, and activity in response to differentiating and proliferating agents. We hypothesized that PKD is a pro-proliferative/anti-differentiative signal in keratinocytes, which may require downmodulation to allow differentiation to proceed.

Initial observations using the SC-935 antibody suggested that PKD levels were downmodulated following induction of keratinocyte differentiation in response to 24-h elevated $[Ca^{2+}]_e$ and $1,25(OH)_2D_3$ treatments. Western analyses with SC-935 also demonstrated reduced PKD protein levels following 10-min TPA treatments. But western analyses using the CS-2052 antibody, unlike analyses with the SC-935 antibody, showed no reduction in PKD protein levels after 10-min TPA-treatment, suggesting that the recognition of PKD by the SC-935 antibody is reduced once PKD is activated/autophosphorylated. Specifically, the autophosphorylation

status and activity of PKD were increased following 10-min acute TPA-treatment as verified by western analyses with the CS-2051 antibody and *in vitro* kinase assays, respectively.

After validating the experimental design, the changes in PKD were monitored upon induction of keratinocyte differentiation. Because the effects of $1,25(OH)_2D_3$ on keratinocyte maturation were more variable, potentially because of both genomic and non-genomic effects, we focused on the effects of elevated $[Ca^{2+}]_e$. Elevated $[Ca^{2+}]_e$ is known to induce keratinocyte differentiation *in situ* (Hennings *et al*, 1980), and this was verified in our system by a time course analysis of transglutaminase activity after 8, 16, and 24 h of 1 mM $CaCl_2$ treatments. It should be noted that transglutaminase is a marker of late differentiation because although it is expressed first in the spinous layer, its activity is regulated at the levels of transcription, post-transcription, and post-translation (Gibson *et al*, 1996) and is maximal in the granular layer (Buxman and Wuepper, 1975). Western analyses showed that PKD protein levels and serine 916 autophosphorylation were downmodulated after 8-, 16-, and 24-h treatments with elevated $[Ca^{2+}]_e$. Immunoprecipitation followed by *in vitro* kinase assays showed that PKD activity was downmodulated after 16 h but not after 10 min of elevated $[Ca^{2+}]_e$ treatment, and this 16-h time point coincided with the time of maximal observed transglutaminase activity in response to elevated $[Ca^{2+}]_e$ (Fig 5). Because PKD lacks the C2 domains that are necessary for calcium binding and are found in classical PKC, we believe that the downmodulation of PKD is because of the calcium-induced differentiation and not because of direct binding of calcium. Although the reductions of PKD protein levels, autophosphorylation, and activity were statistically significant following elevated $[Ca^{2+}]_e$ treatment, the changes were small, within the range of 20%–30%. PKD, however, is known to associate with specific cellular compartments; thus, global changes in protein levels, phosphorylation and activity may not be reflective of the events occurring in a critical site within the cell. Future analyses of changes within particular compartments (e.g., the nucleus or the Golgi apparatus) might yield more information as to the specific regulation of PKD in response to keratinocyte maturation.

Next, the effects of TPA-induced differentiation and proliferation on PKD were monitored in order to determine whether the downmodulation of PKD is specific to calcium-induced differentiation or whether it occurs during keratinocyte differentiation in general. Previous reports by Yuspa *et al* (1982a) describe the biphasic effects of TPA on keratinocyte differentiation and proliferation *in situ*, and this shift from differentiation to proliferation occurred well after 24 h.This early work, however, was performed in the presence of serum. In our system, keratinocytes were incubated with TPA in low calcium concentrations (25 μM $CaCl_2$) in the absence of serum, and our results identify the shift from differentiation to proliferation as occurring between 16 and 24 h. Specifically, an acute 8-h TPA treatment stimulated transglutaminase activity to 184% $\pm$ 10.3% of control while causing a reduction of DNA-specific activity below that of control. Conversely, by 24-h, DNA-specific activity was increased to 238% $\pm$ 29.1% of control whereas transglutaminase activity was reduced below that of control between 24 and 48 h. These data are consistent with the model that acute TPA treatment promotes differentiation but that chronic TPA treatment enhances proliferation.

Once the experimental system was validated in terms of TPA inducing a biphasic response of differentiation followed by proliferation in keratinocytes, we focused on the effects of differentiation and proliferation on PKD protein levels, autophosphorylation, and activity. Because of direct binding of TPA to the C1 domain, PKD autophosphorylation of serine 916 and enzymatic activity were stimulated following TPA treatments of 10 min and 1 h. Corresponding with TPA-induced differentiation, PKD levels were downmodulated after an 8-h treatment but not after exposure times of 10 min to 1 h, consistent with the findings of Rennecke *et al* (1996). Furthermore, PKD autophosphorylation was significantly reduced below that of control after 8, 16, and 24 h of TPA treatment, with a corresponding decrease in PKD activity after 16 h of TPA treatment. We believe that PKD downmodulation occurs as a result of the differentiative effects of acute TPA treatment and not as a result of a direct-binding effect or proteolytic degradation by the tumor promoter. Chronic TPA treatment, which results in keratinocyte proliferation, resulted in recovery of PKD protein levels, autophosphorylation status and activity to those of control. Why these values did not increase significantly above control is somewhat puzzling given the proliferative response at these times (Fig 7); however, it may be related to timing and/or experimental limitations in the sensitivity of the western blot analyses and *in vitro* kinase assays.

PKD1 is the first member of a family of protein kinases that now also includes PKD2 and PKD3/PKCν. Comparative western analyses using the four commercially available PKD-specific antibodies described in this work definitively showed that each antibody produces a strong immunoreactive signal corresponding to PKD1 overexpression (Fig 3). All three family members, however, share a similar domain structure with some sequence homology (Rykx *et al*, 2003), and the SC-935 antibody, a C-terminal-specific PKD antibody, has been reported to cross-react with PKD2 in western analyses (Sturany *et al*, 2001). In addition, the phospho-PKD/PKCμ Ser744-Ser748 antibody, which is directed against the serines located in the activation loop, cross-reacts with all three members (Rey *et al*, 2003). At this point, we are unsure whether the CS-2052 or CS-2051 antibodies cross-react with other PKD family members and are actively investigating the role of PKD2 in keratinocyte maturation. Although neither PKD2 nor PKD3 have been reported in keratinocytes, we have detected PKD2 expression by RT-PCR analysis in RNA isolated from our primary keratinocytes (unpublished results). Our results concerning PKD protein levels and autophosphorylation are thought to reflect the changes in PKD1, but because of the limitations of antibody specificity, changes in PKD2 and/or PKD3 may also contribute to the observed effects.

The epidermis is a stratified epithelium, and cellular localization within the epidermal tissue is indicative of maturation status. Therefore, immunohistochemical analysis was utilized to discern whether the expression pattern of PKD corresponded with the proliferative keratinocytes. Two separate antibodies, SC-638 and SC-935, which target the N- and C-terminus, respectively, produced staining patterns

indicating that PKD protein expression was highest in the basal layer, the layer consisting of proliferating progenitor cells. Because similar staining was observed with two different PKD-specific antibodies, we believe that the results accurately depict the expression pattern of PKD. This staining pattern is consistent with our hypothesis that PKD has a pro-proliferative and/or anti-differentiative role in keratinocytes. Interestingly, both antibodies also labeled the nuclear compartment, which is consistent with reports that PKD, as well PKD3, can translocate to the nucleus (Rey *et al*, 2001, 2003).

Finally, the effect of PKD on keratinocyte maturation was tested by co-expression studies under basal (25 μM calcium) and differentiating conditions (1 mM calcium) using the keratin 5 and involucrin promoters as markers of proliferation and differentiation, respectively. Under basal conditions, PKD co-expression enhanced the promoter activity of keratin 5 and reduced the promoter activity of involucrin. Under differentiating conditions, PKD co-expression opposed the effects of elevated $[Ca^{2+}]_e$, also increasing the promoter activity of keratin 5 and reducing the promoter activity of involucrin when compared with calcium-treated cultures co-transfected with pcDNA3. In this experiment, we have used the dual-luciferase assay in a novel way to monitor changes in keratinocyte maturation in response to PKD co-expression. Although there are reports that PKD can directly phosphorylate c-Jun (Hurd *et al*, 2002) and histone deacetylase (Vega *et al*, 2004), which could affect transcription, we believe that PKD is affecting the maturation status of keratinocytes, thereby modulating the promoter activities of keratin 5 and involucrin and not by directly influencing the promoter activities of keratin 5 or involucrin. These data suggest that PKD acts as a pro-proliferative and anti-differentiative signal in keratinocytes and are consistent with our proposed hypothesis. These data also support the need for further research into the potential role of PKD in the tumor-promoting effects of phorbol esters on keratinocytes.

Materials and Methods

Materials Calcium-free minimum essential medium (MEMα) was from Biologos (Montgomery, Illinois), and ITS+ (insulin, transferrin, selenous acid, linoleic acid) was from Collaborative Biomedical Products (Bedford, Massachusetts). $1,25(OH)_2D_3$ was a generous gift from Dr Maurice Pechet (Harvard, Cambridge, Massachusetts). TPA, Hoechst 33258 dye, calf-thymus DNA and the secondary antibodies, goat anti-rabbit conjugated with alkaline phosphatase and rabbit anti-goat conjugated with alkaline phosphatase, were from Sigma (St Louis, Missouri). DC protein assay reagents were from Bio-Rad (Hercules, California). Bovine serum albumin (BSA) was from Pierce (Rockford, Illinois), and Immobilon-P polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, Massachusetts). Opti-MEM and Lipofectin were from Invitrogen (Carlsbad, California). Dulbecco's modification of Eagle's medium (DMEM + 4.5 g per liter of glucose) was from Fisher Scientific (Hampton, New Hampshire). In addition to the ABC Immunohistochemistry detection system, the following antibodies were from Santa Cruz Biotech (Santa Cruz, California): the anti-PKD antibodies, SC-638 and SC-935; the anti-actin antibody, SC-1616; the protein-A agarose-conjugated antibody, SC-2001; and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody, SC-2004. The anti-phospho-PKD S916 antibody, CS-2051, and the anti-PKD antibody, CS-2052, were from Cell Signaling Technology (Beverly, Massachusetts). O.C.T.4583 compound was from Sakura Finetek USA (Torrance, California), and ^{125}I-labeled goat anti-rabbit secondary antibody was from Amersham Biosciences (Piscataway, New Jersey). Hematoxylin solution was from Richard-Allan Scientific (Kalamazoo, Michigan), and Permount was from Biomedia (Foster City, California). TranslT-Keratinocyte transfection reagent was from Mirus (Madison, Wisconsin). P-81 paper and GF/A glass microfiber filter circles were from Whatman (Clifton, New Jersey). PKD was cloned from CBA mouse brain cDNA using PCR and was subcloned into the pcDNA3 expression vector. The pRL–SV40 construct was included in the Dual-Luciferase Reporter assay kit from Promega (Madison, Wisconsin). Dr Bogi Andersen (University of California, San Diego, California) kindly provided the keratin 5–luciferase construct (Sugihara *et al*, 2001) while Dr Daniel Bikle (University of California, San Francisco, California) kindly provided the involucrin–luciferase construct (Ng *et al*, 1996).

Keratinocyte culture Primary epidermal keratinocytes were prepared from 1- to 3-d-old neonatal ICR mice by first removing the skins as single sheets. After incubating the mouse skins in 0.25% trypsin overnight at 4°C, the epidermis and dermis were mechanically separated using forceps, and cells were released by gentle scraping. The cells were then collected by centrifugation and plated at a density of 25,000 cells per cm^2 in six-well tissue-culture plates in plating medium consisting of calcium-free MEM α supplemented with 2% dialyzed fetal bovine serum (FBS), 25 μM $CaCl_2$, 5 ng per mL epidermal growth factor, 2 mM glutamine, ITS+, 100 U per mL of penicillin, 100 μg per mL streptomycin, and 0.25 μg per mL fungizone. After approximately 24 h, the plating media was replaced with SFKM containing 25 μM $CaCl_2$, 90 μg per mL bovine pituitary extract, ITS+, 5 ng per mL epidermal growth factor, 2 mM glutamine, 0.05% BSA, 100 U per mL penicillin, 100 μg per mL streptomycin, and 0.25 μg per mL fungizone. The media was replaced with fresh SFKM every 1–2 d. All animal studies were performed using protocols approved by the institutional animal care and use committee.

Western blot analysis Near-confluent (60%–80%) cultures of keratinocytes were incubated in SFKM (25 μM $CaCl_2$) containing vehicle (0.05% ethanol, 0.1% DMSO, or 0.005% DMSO) or the indicated treatment: 1 mM $CaCl_2$, 250 nM $1,25(OH)_2D_3$, or 100 nM TPA. The cells were then harvested at the desired time points by washing once with phosphate-buffered saline lacking divalent cations (PBS−), followed by the addition of 300 μL of heated lysis buffer (0.1875 M Tris-HCl (pH 8.5), 3% sodium dodecyl sulfide (SDS), 1.5 mM ethylenediaminetetraacetic acid (EDTA)). Thirty microliters of lysate was used to determine protein concentration using the DC protein assay with BSA as the standard, and 270 μL of the lysate was added to 135 μL of 3 × sample buffer (30% glycerol, 15% β-mercaptoethanol, 1% bromophenol blue) to constitute Laemmli buffer (Laemmli, 1970). Samples were stored at −70°C until western analysis was performed. After boiling, equal protein amounts from each sample were loaded, separated by electrophoresis through 8% SDS polyacrylamide gels, and transferred to PVDF membrane. Membranes were incubated in blocking buffer (0.1% Tween-20, 4% milk in PBS− or Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 8)) prior to incubation at 4°C in blocking buffer containing one of the following primary antibodies: rabbit anti-human PKD, SC-935 (1:500); and rabbit anti-mouse PKD, CS-2052 (1:1000); rabbit anti-mouse phospho-PKD S916, CS-2051 (1:1000), or goat anti-human actin (I-19), SC-1616 (1:1000). After washing in PBS−/0.1% Tween-20 solution or TBS/0.1% Tween-20 solution, the membranes were incubated for 1–2 h in blocking buffer containing one of the following secondary antibodies: ^{125}I-labeled goat anti-rabbit (0.1 μCi per mL or 1:500); goat anti-rabbit conjugated with HRP, SC-2004 (1:1000); goat anti-rabbit conjugated with alkaline phosphatase (1:7500) or rabbit anti-goat conjugated with alkaline phosphatase (1:30,000). Immunoreactive bands corresponding to the proteins of interest were

visualized by enhanced chemiluminescence using film or by autoradiography (^{125}I) or ECF using a Phosphorimager SI (Molecular Dynamics, Sunnyvale, California) or a Storm Phosphorimager (Molecular Dynamics), respectively. Immunoreactive signals were quantified using ImageQuant software (Molecular Dynamics), and the data reported were normalized according to actin levels.

COS-7 transfection COS-7 cells were grown to 40%–60% confluence in 60-mm dishes in 10% FBS/DMEM and were transfected with 6 μg per dish of pcDNA3 or pcDNA3–PKD. Transfections were performed using 2 mL per dish of Opti-MEM and 10 μL per dish of Lipofectin as described in the manufacturer's protocol. After 5 h of incubation in transfection mix, 2 mL of 10% FBS/Opti-MEM was added to each dish. After 72 h of incubation, cells were scraped in heated lysis buffer and were subjected to western analysis as described above using the PKD-specific antibodies (SC-935, SC-638, CS-2052, and CS-2051) and the actin-specific antibody (I-19). Equal amounts of protein (10 μg) from each sample were loaded in quadruplicate, and actin was used to verify equal loading.

***In vitro* kinase assay** Near-confluent cultures of primary keratinocytes were incubated in SFKM (25 μM $CaCl_2$) or SFKM containing 1 mM $CaCl_2$, vehicle (0.1% DMSO or 0.005% DMSO), or 100 nM TPA for the desired time periods at 37°C. Cells were scraped in immunoprecipitation buffer [50 mM Tris-HCl (pH 7.4), 2 mM ethyleneglycoltetraacetic acid, 2 mM EDTA, 1 mM dithiothreitol, 10 μg per mL aprotinin, 10 μg per mL leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 1% Triton-X 100] and disrupted by sonication. Thirty microliters of lysates were used to determine protein concentration prior to immunoprecipitation. Equal protein amounts were precleared with protein-A agarose (SC-2001; 1:250) to remove any proteins that may immunoprecipitate because of non-specific binding. PKD was then immunoprecipitated using both SC-638 (1:200) and SC-935 (1:200) or CS-2052 alone followed by incubation with protein-A agarose (1:250) at 4°C. The immunoprecipitates were washed twice, resuspended in 30 μL of kinase buffer (30 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM dithiothreitol) and incubated with 2.5 mg per mL (1.66 mM) syntide-2, 100 μM ATP, and 10 μCi [γ-^{32}P]ATP at 30°C for 10 min. The reactions were terminated by the addition of 350 mM H_3PO_4 (final concentration) and were spotted onto P-81 paper. Finally, the P-81 papers were washed in 75 mM H_3PO_4 and dried overnight. The radioactivity was quantified using an LS 6500 scintillation counter (Beckman Coultier, Fullerton, California).

Transglutaminase assay Transglutaminase activity assays were performed as described (Griner *et al*, 1999). Briefly, cultures of primary keratinocytes were incubated in SFKM (25 μM $CaCl_2$) or SFKM containing 1 mM $CaCl_2$, vehicle (0.005% DMSO), or 100 nM TPA for the desired time periods. Subsequently, intact cells were harvested, and transglutaminase activity was measured according to the method of Folk and Chung (1985) with minor modifications. Specifically, dimethylcasein was substituted for α-casein, and reactions were permitted to continue overnight before ice-cold trichloroacetic acid was used to precipitate the [^{3}H]-putrescine/dimethylcasein cross-linked product onto GF/A glass microfiber filter circles. The cpm of the radiolabeled products were determined using liquid scintillation spectrometry, and data were normalized to protein content.

DNA-specific assay The DNA-specific assay was performed as described (Bollag *et al*, 1993). Briefly, cultures of primary keratinocytes were incubated in SFKM (25 μM $CaCl_2$) containing vehicle (0.005% DMSO) or 100 nM TPA for the indicated duration followed by incubation with 1 μCi per mL [^{3}H]-thymidine for 1 h at 37°C. Cells were harvested by scraping, washed, and resuspended in 500 μL of PEN buffer (0.05 M NaH_2PO_4/Na_2HPO_4 (pH 7.4), 2 mM EDTA, and 2 M NaCl). Samples were stored at −20°C until all time points were collected. Samples were thawed and sonicated on ice, and unbroken cells were pelleted by centrifugation. The supernatant containing the solubilized DNA was recovered, and a 200 μL aliquot was subjected to liquid scintillation spectrometry. A second aliquot was used to quantify the DNA concentration of the samples using 0.1 μg per mL Hoechst 33258 dye and calf-thymus DNA as the standard. Finally, the DNA-specific activity of each sample was expressed as average dpm multiplied by 2.5 and divided by the DNA concentration ((average dpm × 5/2)/DNA concentration) to represent the total DNA-specific activity of each well.

Immunohistochemistry Murine epidermal samples were harvested following sacrifice, in compliance with animal care and use committee guidelines. The tissue specimens were quick-frozen in O.C.T.4583 compound in a dry ice/isopentane bath, cryosectioned onto glass microscope slides, and stored at −70°C until analysis. The ABC immunohistochemistry detection system was used for the analysis of 7 μm-thick transverse cryostat sections. Cryostat sections were fixed in cold acetone, followed by the quenching of endogenous peroxidases by incubation in 3% H_2O_2/PBS− and the blocking of non-specific binding sites by incubation in 5% goat serum/PBS−. The sections were incubated with primary antibody, SC-935 (1:200) or SC-638 (1:200), followed by incubation with a biotinylated secondary antibody, SC-2018 (1:200). Samples were immersed in the ABC reagent to allow binding of avidin to the biotinylated secondary antibody and binding of biotinylated HRP to the avidin. The target protein was visualized by incubation of samples in the diaminobutane-chromogen, which produces a distinctive brown stain. Nuclei were counterstained with a hematoxylin solution, and negative controls were prepared similarly, except that the primary antibody was omitted. Finally, sections were dehydrated by immersion in solutions with increasing concentrations of ethanol and cover slip-mounted with Permount. Observation and imaging of stained samples was performed using bright field analysis on a Zeiss (Carl Zeiss International, Thornwood, New York) Axiophot microscope (magnifications of × 10 and × 20) coupled to an imaging station.

Luciferase assay PKD was cloned from CBA mouse brain cDNA using PCR and was subcloned into the pcDNA3 expression vector. Sub-confluent (∼ 30%) cultures of primary keratinocytes in SFKM were co-transfected with three constructs: (1) 1 μg per well of pcDNA3 control vector or pcDNA3–PKD expression construct; (2) 1 μg per well of firefly–luciferase reporter construct under control of the minimal promoter of keratin 5, kindly provided by Dr Bogi Andersen (University of California, San Diego, California) (Sugihara *et al*, 2001) or involucrin, kindly provided by Dr Daniel Bikle (University of California, San Francisco, California) (Ng *et al*, 1996); and (3) 25 ng per well of pRL–SV40 *Renilla* luciferase construct under control of the SV40 promoter, which serves as an internal transfection control. The constructs were mixed thoroughly prior to addition of the transfection reagents. Transfections were performed using the TransIT–Keratinocyte transfection reagent according to the manufacturer's protocol. After 24 h, the transfection media were replaced with SFKM (25 μM $CaCl_2$) or SFKM containing 1 mM $CaCl_2$, and cultures were incubated for an additional 24 h. The luciferase assays were performed as described in the Dual-Luciferase Reporter Assay protocol, and measurements were obtained using a luminometer (Turner Designs, Sunnyvale, California). Data are expressed as the ratio of promoter-driven firefly luciferase expression to control SV40-driven *Renilla* luciferase expression.

Statistical analysis The significance of differences between mean values was determined using ANOVA with a Student–Neumann–Keuls *post hoc* test or a Student's *t* test with a two-tailed p value as indicated and as performed by Instat 2.0 (GraphPad Software, San Diego, California).

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References

Abedi H, Rozengurt E, Zachary I: Rapid activation of the novel serine/threonine protein kinase, protein kinase D by phorbol esters, angiotensin II and PDGF-BB in vascular smooth muscle cells. FEBS Lett 427:209–212, 1998

Argyris TS: Epidermal growth following a single application of 12-*O*-tetradecanoyl-phorbol-13-acetate in mice. Am J Pathol 98:639–648, 1980

Banks-Schlegel S, Green H: Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J Cell Biol 90:732–737, 1981

Bollag WB, Bollag RJ: 1,25-Dihydroxyvitamin $D^{(3)}$, phospholipase D and protein kinase C in keratinocyte differentiation. Mol Cell Endocrinol 177:173–182, 2001

Bollag WB, Dodd ME, Shapiro BA: Protein kinase D and keratinocyte proliferation. Drug News Perspect 17:117–126, 2004

Bollag WB, Ducote J, Harmon CS: Effects of the selective protein kinase C inhibitor, Ro 31–7549, on the proliferation of cultured mouse epidermal keratinocytes. J Invest Dermatol 100:240–246, 1993

Bollag WB, Xiong Y, Ducote J, Harmon CS: Regulation of fos–lacZ fusion gene expression in primary mouse epidermal keratinocytes isolated from transgenic mice. Biochem J 300 (Part 1):263–270, 1994

Buxman MM, Wuepper KD: Keratin cross-linking and epidermal transglutaminase. A review with observations on the histochemical and immunochemical localization of the enzyme. J Invest Dermatol 65:107–112, 1975

Byrne C, Tainsky M, Fuchs E: Programming gene expression in developing epidermis. Development 120:2369–2383, 1994

Chattopadhyay N, Mithal A, Brown EM: The calcium-sensing receptor: A window into the physiology and pathophysiology of mineral ion metabolism. Endocr Rev 17:289–307, 1996

Chiu T, Rozengurt E: PKD in intestinal epithelial cells: Rapid activation by phorbol esters, LPA, and angiotensin through PKC. Am J Physiol Cell Physiol 280:C929–C942, 2001

Dieterich S, Herget T, Link G, Bottinger H, Pfizenmaier K, Johannes FJ: *In vitro* activation and substrates of recombinant, baculovirus expressed human protein kinase C mu. FEBS Lett 381:183–187, 1996

Dlugosz AA, Yuspa SH: Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. J Cell Biol 120:217–225, 1993

Folk JE, Chung SI: Transglutaminases. Methods Enzymol 113:358–375, 1985

Fuchs E: Keratins and the skin. Annu Rev Cell Dev Biol 11:123–153, 1995

Gibson DF, Ratnam AV, Bikle DD: Evidence for separate control mechanisms at the message, protein, and enzyme activation levels for transglutaminase during calcium-induced differentiation of normal and transformed human keratinocytes. J Invest Dermatol 106:154–161, 1996

Goerttler K, Loehrke H, Schweizer J, Hesse B: Systemic two-stage carcinogenesis in the epithelium of the forestomach of mice using 7,12-dimethylbenz(a)anthracene as initiator and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate as promoter. Cancer Res 39:1293–1297, 1979

Griner RD, Qin F, Jung E, *et al*: 1,25-dihydroxyvitamin D^3 induces phospholipase D-1 expression in primary mouse epidermal keratinocytes. J Biol Chem 274:4663–4670, 1999

Guha S, Rey O, Rozengurt E: Neurotensin induces protein kinase C-dependent protein kinase D activation and DNA synthesis in human pancreatic carcinoma cell line PANC-1. Cancer Res 62:1632–1640, 2002

Hebert SC, Brown EM: The extracellular calcium receptor. Curr Opin Cell Biol 7:484–492, 1995

Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH: Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19:245–254, 1980

Hurd C, Waldron RT, Rozengurt E: Protein kinase D complexes with C-Jun N-terminal kinase via activation loop phosphorylation and phosphorylates the C-Jun N-terminus. Oncogene 21:2154–2160, 2002

Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K: PKCu is a novel, atypical member of the protein kinase C family. J Biol Chem 269: 6140–6148, 1994

Kam Y, Exton JH: Role of phospholipase D in the activation of protein kinase D by lysophosphatidic acid. Biochem Biophys Res Commun 315:139–143, 2004

Komuves L, Oda Y, Tu CL, Chang WH, Ho-Pao CL, Mauro T, Bikle DD: Epidermal expression of the full-length extracellular calcium-sensing receptor is required for normal keratinocyte differentiation. J Cell Physiol 192:45–54, 2002

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970

Matthews SA, Rozengurt E, Cantrell D: Characterization of serine 916 as an *in vivo* autophosphorylation site for protein kinase D/protein kinase Cmu. J Biol Chem 274:26543–26549, 1999

Ng DC, Su MJ, Kim R, Bikle DD: Regulation of involucrin gene expression by calcium in normal human keratinocytes. Front Biosci 1:a16–a24, 1996

Oda Y, Tu CL, Chang W, *et al*: The calcium sensing receptor and its alternatively spliced form in murine epidermal differentiation. J Biol Chem 275: 1183–1190, 2000

Oda Y, Tu CL, Pillai S, Bikle DD: The calcium sensing receptor and its alternatively spliced form in keratinocyte differentiation. J Biol Chem 273:23344–23352, 1998

Parkinson EK, Pera MF, Emmerson A, Gorman PA: Differential effects of complete and second-stage tumour promoters in normal but not transformed human and mouse keratinocytes. Carcinogenesis 5:1071–1077, 1984

Poumay Y, Herphelin F, Smits P, De Potter IY, Pittelkow MR: High-cell-density phorbol ester and retinoic acid upregulate involucrin and downregulate suprabasal keratin 10 in autocrine cultures of human epidermal keratinocytes. Mol Cell Biol Res Commun 2:138–144, 1999

Raick AN, Thumm K, Chivers BR: Early effects of 12-*O*-tetradecanoyl-phorbol-13-acetate on the incorporation of tritiated precursor into DNA and the thickness of the interfollicular epidermis, and their relation to tumor promotion in mouse skin. Cancer Res 32:1562–1568, 1972

Rennecke J, Johannes FJ, Richter KH, Kittstein W, Marks F, Gschwendt M: Immunological demonstration of protein kinase C mu in murine tissues and various cell lines. Differential recognition of phosphorylated forms and lack of down-regulation upon 12-*O*-tetradecanoylphorphol-13-acetate treatment of cells. Eur J Biochem 242:428–432, 1996

Rennecke J, Rehberger PA, Furstenberger G, Johannes FJ, Stohr M, Marks F, Richter KH: Protein-kinase-Cmu expression correlates with enhanced keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture. Int J Cancer 80:98–103, 1999

Rey O, Sinnett-Smith J, Zhukova E, Rozengurt E: Regulated nucleocytoplasmic transport of protein kinase D in response to G protein-coupled receptor activation. J Biol Chem 276:49228–49235, 2001

Rey O, Yuan J, Young SH, Rozengurt E: Protein kinase C nu/protein kinase D3 nuclear localization, catalytic activation, and intracellular redistribution in response to G protein-coupled receptor agonists. J Biol Chem 278:23773–23785, 2003

Rozengurt E, Sinnett-Smith J, Zugaza JL: Protein kinase D: A novel target for diacylglycerol and phorbol esters. Biochem Soc Trans 25:565–571, 1997

Rykx A, De Kimpe L, Mikhalap S, Vantus T, Seufferlein T, Vandenheede JR, Van Lint J: Protein kinase D: A family affair. FEBS Lett 546:81–86, 2003

Shapiro BA, Ray S, Jung E, Allred WT, Bollag WB: Putative conventional protein kinase C inhibitor Gödecke 6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)py rrolo(3,4-c)-carbazole] stimulates transglutaminase activity in primary mouse epidermal keratinocytes. J Pharmacol Exp Ther 302:352–358, 2002

Sinnett-Smith J, Zhukova E, Hsieh N, Jiang X, Rozengurt E: Protein kinase D potentiates DNA synthesis induced by G_q-coupled receptors by increasing the duration of ERK signaling in Swiss 3T3 cells. J Biol Chem 279:16883–16893, 2004

Sturany S, Van Lint J, Muller F, *et al*: Molecular cloning and characterization of the human protein kinase D2. A novel member of the protein kinase D family of serine threonine kinases. J Biol Chem 276:3310–3318, 2001

Sugihara TM, Kudryavtseva EI, Kumar V, Horridge JJ, Andersen B: The POU domain factor skin-1a represses the keratin 14 promoter independent of DNA binding. A possible role for interactions between Skn-1a and CREB-binding protein/p300. J Biol Chem 276:33036–33044, 2001

Tan M, Xu X, Ohba M, Ogawa W, Cui MZ: Thrombin rapidly induces protein kinase D phosphorylation, and protein kinase C delta mediates the activation. J Biol Chem 278:2824–2828, 2003

Thacher SM, Rice RH: Keratinocyte-specific transglutaminase of cultured human epidermal cells: Relation to cross-linked envelope formation and terminal differentiation. Cell 40:685–695, 1985

Toftgard R, Yuspa SH, Roop DR: Keratin gene expression in mouse skin tumors and in mouse skin treated with 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res 45:5845–5850, 1985

Tu CL, Oda Y, Bikle DD: Effects of a calcium receptor activator on the cellular response to calcium in human keratinocytes. J Invest Dermatol 113: 340–345, 1999

Tu CL, Oda Y, Komuves L, Bikle DD: The role of the calcium-sensing receptor in epidermal differentiation. Cell Calcium 35:265–273, 2004

Valverde AM, Sinnett-Smith J, Van Lint J, Rozengurt E: Molecular cloning and characterization of protein kinase D: A target for diacylglycerol and phorbol esters with a distinctive catalytic domain. Proc Natl Acad Sci USA 91:8572–8576, 1994

Van Lint JV, Sinnett-Smith J, Rozengurt E: Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. J Biol Chem 270:1455–1461, 1995

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, McKinsey TA: Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol 24:8374–8385, 2004

Warhol MJ, Roth J, Lucocq JM, Pinkus GS, Rice RH: Immuno-ultrastructural localization of involucrin in squamous epithelium and cultured keratinocytes. J Histochem Cytochem 33:141–149, 1985

Xie Z, Bikle DD: Cloning of the human phospholipase C-gamma1 promoter and identification of a DR6-type vitamin D-responsive element. J Biol Chem 272:6573–6577, 1997

Yuan J, Slice LW, Gu J, Rozengurt E: Cooperation of G_q, G_i, and $G_{12/13}$ in protein kinase D activation and phosphorylation induced by lysophosphatidic acid. J Biol Chem 278:4882–4891, 2003

Yuspa SH, Ben T, Hennings H, Lichti U: Divergent responses in epidermal basal cells exposed to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res 42:2344–2349, 1982a

Yuspa SH, Ben T, Patterson E, Michael D, Elgjo K, Hennings H: Stimulated DNA synthesis in mouse epidermal cell cultures treated with 12-*O*-tetradecanoyl-phorbol-13-acetate. Cancer Res 36:4062–4068, 1976

Yuspa SH, Hennings H, Kulesz-Martin M, Lichti U: The study of tumor promotion in a cell culture model for mouse skin—a tissue that exhibits multistage carcinogenesis *in vivo*. Carcinog Compr Surv 7:217–230, 1982b

Zhukova E, Sinnett-Smith J, Rozengurt E: Protein kinase D potentiates DNA synthesis and cell proliferation induced by bombesin, vasopressin, or phorbol esters in Swiss 3T3 cells. J Biol Chem 276:40298–40305, 2001

Zugaza JL, Sinnett-Smith J, Van Lint J, Rozengurt E: Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. EMBO J 15:6220–6230, 1996