Calcium-dependent Involucrin Expression Is Inversely Regulated by Protein Kinase C (PKC)α and PKCδ*

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Calcium is an important physiologic regulator of keratinocyte function that may regulate keratinocyte differentiation via modulation of protein kinase C (PKC) activity. PKCα and PKCδ are two PKC isoforms that are expressed at high levels in keratinocytes. In the present study, we examine the effects of PKCα and PKCδ on calcium-dependent keratinocyte differentiation as measured by involucrin (hINV) gene expression. Our studies indicate that calcium increases hINV promoter activity and endogenous hINV gene expression. This response requires PKCδ, as evidenced by the observation that treatment with dominant-negative PKCδ inhibits calcium-dependent hINV promoter activity, whereas wild type PKCδ increases activity. PKCα, in contrast, inhibits calcium-dependent hINV promoter activation, a finding that is consistent with the ability of dominant-negative PKCα and the PKCδ inhibitor, Go6976, to increase hINV gene expression. The calcium-dependent regulatory response is mediated by an AP1 transcription factor-binding site located within the hINV promoter distal regulatory region that is also required for PKCδ-dependent regulation; moreover, both calcium and PKCδ produce similar, but not identical, changes in AP1 factor expression. A key question is whether calcium directly influences PKC isoform function. Our studies show that calcium does not regulate PKCα or δ levels or cause a marked redistribution to membranes. However, tyrosine phosphorylation of PKCδ is markedly increased following calcium treatment. These findings suggest that PKCα and PKCδ are required for, and modulate, calcium-dependent keratinocyte differentiation in opposing directions.

Calcium is an important regulator of keratinocyte differentiation. Incubation of cultured keratinocytes with calcium increases differentiation and expression of differentiation-associated genes (1–3). Moreover, the presence in vivo of an epidermal calcium gradient, with increasing calcium levels in the more differentiated layers, suggests a role for calcium in regulating epidermal differentiation (2, 4–6). However, the mechanism whereby the increase in extracellular free calcium triggers differentiation is not well understood. One possible mechanism involves the calcium-dependent activation of protein kinase C (PKC) isoforms (7–9). Keratinocytes express the PKCα, -δ, -ε, -η, and -ζ isoforms (10). These enzymes control a variety of signaling cascades and transcription factors and function as regulators of keratinocyte differentiation-dependent gene expression (11–15). In keratinocytes, PKCα and PKCδ are abundant PKC isoforms that have been implicated as regulators of differentiation (16–19). In the present study, we focus on the role of these isoforms and their effects on calcium-dependent regulation of differentiation.

Involucrin, a keratinocyte structural protein that functions as a precursor of the cornified envelope (20–22), is expressed in a tissue-specific and differentiation-appropriate manner in vivo (23). Moreover, agents that promote keratinocyte differentiation, including calcium, increase hINV levels and hINV promoter activity in cultured keratinocytes (24–27). A novel PKC, Ras, MEKK1, MEK3/MEK6, p38 pathway has been shown to mediate phorbol ester-dependent activation of hINV gene expression (28–30). This pathway targets AP1 transcription factors that, in turn, bind to sites within the hINV promoter to activate transcription (31–33). However, the events leading to calcium-dependent induction of hINV gene expression in normal keratinocytes are not well understood. The goal of the present study is to evaluate the role of PKC in mediating the calcium-dependent increase in hINV gene expression. Our findings suggest that PKCα inhibits and PKCδ enhances the calcium-dependent activation of hINV promoter activity and endogenous gene expression.

MATERIALS AND METHODS

Chemicals and Reagents—Keratinocyte serum-free medium (KSFM) was obtained from Invitrogen. Go6976, an inhibitor of classical PKC isoforms, was obtained from Calbiochem. The pGlu2-basic plasmid and the chemiluminescent luciferase assay system were purchased from Promega. Isoform-selective rabbit polyclonal antibodies for PKCα (sc-208) and PKCδ (sc-937) were obtained from Santa Cruz Biotechnology and used diluted 1:7500. Goat polyclonal Sp1-specific antibody (sc-59), obtained from Santa Cruz Biotechnology, was used for immunoblot at a dilution of 1:500. Rabbit anti-human involucrin polyclonal antibody, used for immunoblot at a dilution of 1:8000, has been described (34). The mouse monoclonal anti-phosphotyrosine (clone 4G10) was obtained from Upstate Biotechnology, Inc., and diluted 1:500 for immunoblot. Mouse monoclonal anti-human β-actin (Sigma, clone AC-15) was diluted 1:10,000 for immunoblot. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (NA934) was from Amersham Biosciences and used for immunoblot at a dilution of 1:7500.

Adenoviruses and Plasmids—The hINV promoter constructs used in this study have been described previously (31, 32). All nucleotide posi...
tions are defined relative to the hINV gene transcription start site (32). Expression vectors encoding wild type PKC isoforms, cloned into pcDNA3, were a generous gift of Dr. S. Ohno (35–37). Dominant-nega-
tive PKCa, cloned in pcDNA3 (K368R mutation in the ATP-binding site), was a gift from Dr. B. Weinstein (38). Adenoviruses encoding wild type PKCa or dominant-negative (dn) kinases were kindly provided by Dr. Dr. Kuroki (39). Wild type PKCδ and PKCa and dnPKCs, in which a Lys to Arg mutation was introduced in the ATP-binding site (39), are trans-
scribed, respectively, from the cytomegalovirus and chicken beta-actin promoter.

**Keratinocyte Transfection and Infection—**Normal human foreskin keratinocytes were cultured as described previously (35). For adenovirus infection, keratinocytes, in 9.5-cm² dishes, were transfected when ~25% confluent. FuGENE 6 transfection reagent was mixed with KSFM at a final concentration of 3% for 5 min at 25 °C. This mixture (100 μl) was then added to 1 μg of plasmid DNA, incubated for an additional 15 min, and then added dropwise to the cells in dishes containing 2 ml of KSFM. After 24 h, the medium was changed to KSFM containing 0.09 or 0.3 mM calcium chloride. After 48 h, the cells were harvested and assayed for luciferase activity. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Luciferase activity is normalized per μg of protein (28). As required, transfection efficiency was determined using a green fluorescent protein-expressing plasmid (29).

For adenovirus infection, keratinocyte cultures in 9.5-cm² dishes were transfected with 1 μg of pINV-2473 when 30% confluent and incubated for 24 h. The media were then removed, and the cells were incubated with the appropriate adenovirus for 24 h in 1 ml of KSFM containing 2.5 μM/ml Polybrene. The cells were then transferred to fresh medium containing 0.09 or 0.3 mM calcium chloride and incubated for 48 h prior to harvest and measurement of luciferase activity (30).

**PKCs Immunoprecipitation—**A confluent 50-cm² dish of keratinocytes was washed with phosphate-buffered saline, incubated for 15 min in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfon fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate), sonicated, and centrifuged at 12,000 × g at 4 °C for 5 min (40). The supernatant was preabsorbed with 100 μl of Pansorbin for 1 h at 4 °C. Rabbit polyclonal anti-PKCa or normal mouse IgG (1.5 μg of antibody with 400 μg of protein) was added, and the sample was incubated for 24 h at 4 °C with gentle agitation. The complex was precipitated by incubating with 40 μl of protein-A/G PLUS agarose (Santa Cruz Biotechnology) for 4 h at 4 °C. The mixture was then centrifuged, and the pellet was washed twice with 50 μl Triton-X100, 7.4, 500 mM NaCl, 1% Nonidet P-40, 0.05% sodium deoxycholate, 1 mM phenylmethylsulfon fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate, and twice with RIPA wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X, 0.1% SDS, 1% sodium deoxycholate, 1 mM phenylmethylsulfon fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate) (40). The pellet was resuspended in Laemmli buffer, boiled, electrophoresed on an 8% polyacrylamide gel, and transferred to nitrocellulose for immunoblot with anti-phospho-

**Cell Fractionation—**Cells were washed in cold phosphate-buffered saline and scraped into a minimal volume of extraction buffer (20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfon fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate) (41). The suspension was sonicated, and centrifuged at 100,000 × g for 1 h. The supernatant (cytosol) was removed, and the pellet was resuspended in extraction buffer containing 1% Triton X-100, sonicated, incubated on ice for 1 h, and centrifuged at 100,000 × g for 1 h to yield the Triton-soluble fraction. The high speed pellet was resuspended in sample buffer to yield the particulate fraction (41).

**Immunofluorescence Microscopy—**Keratinocytes were plated on glass coverslips and grown in KSFM containing 0.09 mM calcium. Cells were then incubated for various times in KSFM containing 0.3 mM calcium or 500 mM 12-O-tetradecanoylphorbol-13-acetate (TPA). The cells were then fixed at 4 °C for 12 h in 2% paraformaldehyde, permeabilized with 100% methanol for 30 min, blocked with 10% goat serum, and then incubated for 30 min in primary PKC antibody at 4 °C. An equal dilution in the presence or absence of isosform-specific blocking peptide (PKCa peptide, Santa Cruz Biotechnology, sc-957P). The sections were then incubated for 30 min with Oregon Green 514-linked goat anti-rabbit IgG (Molecular Probes) at a dilution of 1:500. The coverslips were mounted using Gel Mount Media (Biomedia), and fluorescent images were obtained at 100× using a digital Nikon Optiphot microscope.

**Nuclear Extract Preparation and Detection of AP1 and Sp1—**Kera-
tinocytes were plated in 100-mm dishes at 30% confluence. After attach-
ment the cells were treated with 0.09 or 0.3 mM calcium for 48 h. In a parallel experiment, cells were treated with 8 μM of either empty adenovirus or PKCa-encoding adenovirus for 48 h. After treatment, the cells were harvested for preparation of nuclear extracts. Briefly, keratinocytes (one 56-cm² dish) were scraped into 400 μl of cold buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenethylsulfon fluoride), and the cells were allowed to swell on ice for 15 min. Twenty five microliters of 10% Nonident P-40 was added, and the sample was vortexed for 10 s prior to centrifugation. The resulting pellet was suspended in 50 μl of buffer C (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenethylsulfon fluoride) for 15 min at 4 °C and centrifuged for 5 min at 15,000 × g. The resulting supernatant was collected as the nuclear fraction (42). Samples of the nuclear fraction (15 μg) were electrophoresed on a 10% polyacrylamide gel, transferred to Immobilon-P, and incubated with anti-c-Fos (Santa Cruz Biotechnology, sc-52x) at 1:500, anti-Fra 1 (San-
ta Cruz Biotechnology, sc-605x) at 1:2500, anti-Fra 2 (Santa Cruz Biotechnology, sc-17lx) at 1:500, anti-c-Jun (Santa Cruz Biotechnology, sc-45x) at 1:5000, anti-Jun B (Santa Cruz Biotechnology, sc-46x) at 1:500, anti-Jun D (Santa Cruz Biotechnology, sc-74x) at 1:2500, anti-Sp1 (Santa Cruz Biotechnology, sc-59) at 1:500, or anti-β-actin (Sigma A5441) at 1:10,000. To visualize primary antibody binding, the appro-
priate species-specific horseradish peroxidase-linked secondary antibody (Amersham Biosciences) was added, followed by ECL (Amersham Biosciences).

**RESULTS**

**Calcium Regulates Keratinocyte Differentiation—**Involucrin is a well characterized marker of keratinocyte differentiation (27, 43) that has been extensively used as a model to identify mechanisms that regulate differentiation (23, 28, 29, 31, 44). We began our studies by confirming that the hINV gene ex-
pression is regulated by calcium in our culture system. Keratinocytes were cultured in medium containing 0.09 or 0.3 mM calcium for 48 h, and hINV levels were then monitored by immunoblot. The inset in Fig. 1A shows that calcium treatment causes a 5-fold increase in endogenous hINV expression. We confirmed this response by examining the effects of calcium on hINV promoter activity. Keratinocytes were transfected with the hINV promoter reporter plasmids, pINV-41 or pINV-2473 (32), and then grown for 48 h in 0.09 or 0.3 mM calcium-containing medium. The activity of the full-length hINV pro-
moter construct, pINV-2473, is increased 5-fold by calcium treatment. In contrast, activity of the minimal promoter con-
struct, pINV-41, which encodes only the hINV gene TATA box (32), is not regulated. We also confirmed that the appropriate PKC isoforms are expressed in the cultured human keratinocytes. Cell extracts were prepared from keratinocytes growing in medium containing 0.09 mM calcium, and samples were electrophoresed for immunodetection using PKC-specific anti-
bodies. Fig. 1B confirms that PKCa, -δ, -ε, -η, and -ζ are ex-
pressed in our model system, as has been reported elsewhere (10, 14, 29, 45–48). Moreover, although the results cannot be regarded as quantitative, the film exposure times and protein loading densities required for visualization suggest that PKCa and -δ are the most abundant isoforms.

**PKC Activity Is Required for Calcium-dependent Regulation of hINV Gene Expression—**Because of their relative abundance, and the fact that they have been implicated as mediating differentiation-dependent regulation in keratinocytes (14, 18, 28, 29, 39, 40, 49), we focused on the PKCδ and PKCa isoforms. We began by studying the role of PKCa. Keratinocytes were co-transfected with pINV-2473 and PKCa-encoding vector and then treated with 0.09 or 0.3 mM calcium for 48 h. Cell extracts were then prepared and assayed for hINV promoter activity. As shown in Fig. 2A, both basal and calcium-stimulated hINV promoter activity is increased by PKCa. This suggests that cotreatment with calcium and PKCa can enhance promoter
activity but does not indicate whether PKCε activity is required for the calcium response. To determine whether PKCε activity is required for calcium regulation, we used a dominant-negative form of PKCε. In this experiment cells were transfected with pINV-2473 and 24 h later with dnPKCε-encoding virus and then incubated with 0.09 or 0.3 mM calcium for 48 h. Fig. 2B shows that dnPKCε nearly completely inhibits the calcium-dependent increase in hINV promoter activity. In contrast, dnPKCδ expression does not alter base-line promoter activity. To confirm that PKCδ and dnPKCδ isoforms are expressed, we treated cells with empty vector (EV) or expression vectors encoding PKCδ or dnPKCδ. Extracts were then prepared for immunoblot. As shown in Fig. 2C, this analysis confirms that the PKCδ and dnPKCδ expression vectors produce each respective protein in keratinocytes and that these products co-migrate with the endogenous PKCδ. These results suggest that calcium-associated regulation of hINV promoter activity requires PKCδ activity. We next determined whether the endogenous gene displays a similar sensitivity. In Fig. 2D, cells were incubated with 0.09 (−) or 0.3 mM (+) calcium in the presence of empty vector (EV) or PKCδ-encoding adenovirus. After 48 h, hINV protein levels were measured by immunoblot. Treatment with 0.3 mM calcium or PKCδ causes a 2.5-fold increase in hINV protein level. Stimulation with both 0.3 mM calcium and PKCδ results in a 5.5-fold increase. To determine whether PKCδ activity is required for calcium regulation of endogenous hINV level, we infected keratinocytes with empty vector (EV) or vector encoding dominant-negative PKCδ (dnPKCδ), and then treated with 0.3 mM calcium for 48 h. Fig. 2E shows that dnPKCδ efficiently inhibited the calcium-dependent increase in endogenous hINV levels.

PKCa Suppresses Basal and Calcium-dependent Promoter Activity—PKCa is a classical PKC isoform that has an important regulatory role in mouse keratinocytes (18, 19). To determine whether PKCα influences calcium-dependent regulation of differentiation, we transfected normal keratinocytes with pINV-2473 and increasing concentrations of PKCa expression plasmid and incubated for 48 h in the presence of 0.09 or 0.3 mM calcium. As shown in Fig. 3A, PKCa causes a concentration-dependent reduction in calcium-dependent promoter activity.
PKC isoform. Particulate fraction β-actin levels were monitored as a control. We next performed a calcium concentration-response curve to determine whether higher levels of calcium may cause PKC transloca- tion to membranes. As a positive control for PKC mobilization, we treated keratinocytes for 30 min with 500 nM TPA. Our results confirm, as reported previously (41), that TPA mobilizes PKCδ and PKCα from the cytosol to the Triton-soluble fraction (Fig. 4D). To confirm the above results visually, we treated keratinocytes for various times with 0.3 mM calcium, and we monitored PKCα and PKCδ subcellular localization by fluorescence microscopy. As shown in Fig. 4E, elevated calcium did not promote detectable translocation of PKCδ. However, a 30-min treatment with 500 nM TPA caused mobilization of PKCδ. We could not monitor PKCα movement by immunohistochemistry due to technical difficulties with the antibody; however, the biochemical analysis clearly showed redistribution from cytosol to membrane (see Fig. 4D).

Several agents are known to stimulate phosphorylation of PKCδ on tyrosine (52, 53), and tyrosine phosphorylation can regulate PKCδ activity and substrate specificity (54, 55). We were interested to determine whether calcium treatment produces a covalent modification of PKCδ. To detect tyrosine phosphorylation, endogenous PKCδ was immunoprecipitated with anti-PKCδ, and phosphotyrosine was assayed by immunoblot. As shown in Fig. 5, anti-PKCδ precipitates endogenous PKCδ from cells treated with low or high calcium (PKCδ blot). Non-specific anti-IgG, in contrast, does not precipitate PKCδ. The phosphotyrosine blot of the precipitated material demonstrates that calcium treatment increases PKCδ tyrosine phosphorylation. We confirmed this finding using extracts prepared from keratinocytes transfected with PKCδ-encoding plasmid (expressed PKCδ). Keratinocytes were transfected with empty plasmid or PKCδ-encoding plasmid, and extracts were prepared at 72 h post-transfection. Fig. 5 shows that expressed PKCδ can be precipitated and, as with the endogenous enzyme, is tyrosine-phosphorylated following calcium treatment.

**Location of hINV Promoter Calcium- and PKC-response Elements**—The above results indicate that PKCδ and α influence calcium-dependent regulation of hINV gene expression. To identify the region of the hINV promoter responsible for this regulation, keratinocytes were transfected with the constructs shown in Fig. 6A and then treated with 0.09 or 0.3 mM calcium for 48 h. As shown in Fig. 6B, calcium increased pINV-2473 and pINV-2216 promoter activity by 4.3- and 3-fold, respectively. In contrast, shorter constructs displayed a reduced calcium-dependent response. This suggests that the −2473/−2100 seg-
**Calcium Regulation of Involucrin Expression**

**Calcium and PKC Regulation of AP1 Factor Expression**—The common requirement for an intact AP1–5 site for both PKCδ and calcium-dependent regulation of hINV gene expression suggests that each stimulus may regulate AP1 factor expression. To evaluate this possibility, we infected keratinocytes with empty vector or PKCδ-encoding adenovirus, and after 48 h we prepared nuclear extracts to assay for AP1 factor levels by immunoblot. Fig. 8A shows that PKCδ expression increases JunB, c-Fos, and Fra-2 expression and decreases Fra-1 and c-Jun expression. In contrast, JunD levels are not altered. In parallel experiments, we treated keratinocytes for 48 h in medium containing 0.09 or 0.3 mM calcium. As shown in Fig. 8B, although calcium produces similar changes as compared with those observed with PKCδ, Fra-2 levels are increased by PKCδ but not by calcium. The distal regulatory region of the hINV promoter also includes a functionally important Sp1-binding site (23). Sp1 binds at this site and cooperates with AP1 factors to regulate gene expression (31). We therefore evaluated whether calcium alters Sp1 expression. Fig. 8C shows that nuclear Sp1 levels are substantially elevated in response to a 48-h treatment with 0.3 mM calcium. To further confirm a role for PKCδ in the regulation of transcription factor levels, we treated cells with dnPKCδ-encoding virus and then treated for 48 h with 0.09 or 0.3 mM calcium prior to preparation of nuclear extracts. As shown in Fig. 8D, the calcium-dependent changes in AP1 factor and Sp1 factor levels are completely inhibited in the presence of dnPKCδ.

**DISCUSSION**

Calcium is an important regulator of human and mouse keratinocyte differentiation (15, 56). Calcium regulation is manifest in vivo by the presence of an epidermal calcium gradient in which free calcium levels increase in the superficial epidermal layers (6, 57). In cultured keratinocytes, intracellular diacylglycerol and intracellular free calcium levels increase with keratinocyte differentiation (58, 59), suggesting that these agents may drive differentiation via activation of downstream signaling (28). Because these agents are known activators of PKC, it is likely that some of the calcium-dependent regulation is transmitted via a protein kinase C-dependent mechanism (17). However, detailed information regarding the signal transduction mechanisms mediating this response is limited. A major goal of the present study is to assess the role of PKCα and -β as mediators of calcium-dependent regulation.

PKC and Calcium Regulate hINV Gene Expression—Previous studies (29, 60, 61) suggest that calcium regulates hINV gene expression at the mRNA and protein level and suggest that novel PKC isoforms mediate the phorbol ester-dependent increase in hINV gene expression. The PKC regulation is transmitted via a pathway that includes novel PKC, Ras, MEKK1, MEKK3, and p38 MAPK (28, 29). Because addition of exogenous calcium results in an increase in intracellular keratinocyte diacylglycerol levels (62), it is possible that calcium activates the novel PKC isoforms via a diacylglycerol-dependent mechanism that targets this pathway. Thus, we have investigated whether PKCδ activity is required for calcium-dependent regulation of hINV gene expression. Our studies, using a dominant-negative mutant of PKCδ, show that inactivation of PKCδ results in a loss of calcium-dependent hINV promoter activity. In contrast to the PKCδ-associated regulation, PKCs suppresses the calcium-associated increase in hINV promoter activity. Consistent with this, an inhibitor of classical PKC isoform function, Go6976, promotes an increase in endogenous hINV gene expression, and inhibition of PKCδ by dominant-negative PKCδ inhibits this increase.

**Fig. 3.** PKCα inhibits calcium-dependent hINV gene expression. A, keratinocytes, growing in 0.09 mM calcium medium, were transfected with 1 μg of pINV-2473 and 0–2.5 μg of PKCα expression vector at a total plasmid concentration of 3.5 μg (maintained by addition of empty expression vector). After 24 h, the cells were shifted to medium containing 0.09 or 0.3 mM calcium. At 48 h after calcium addition, cell extracts were prepared for assay of luciferase activity. The values present the mean ± S.D. Similar results were observed in three separate experiments. B, keratinocytes were grown and treated exactly as in A, except that they were transfected with 0–2 μg of dnPKCα. The inset shows an immunoblot, using anti-PKCα, demonstrating that the PKCα and dnPKCα expression vectors produce the corresponding proteins. Endogenous PKCα is detected in cells transfected with empty vector (EV). Extracts were isolated 48 h after transfection with 2 μg of empty plasmid or plasmid encoding PKCα or dnPKCα. Whole cell lysates were prepared, and equivalent amounts of protein were electrophoresed on an 8% gel, transferred to membrane, and blotted with anti-PKCα. C, keratinocytes were grown for 48 h in medium containing 0.09 or 0.3 mM calcium in the presence or absence of 1 μM Go6976. Go6976 treatment was initiated 45 min prior to calcium treatment. At 48 h, the cells were harvested in sample buffer, and 20 μg of whole cell lysate was electrophoresed on an 8% polyacrylamide gel. hINV protein level was assessed by immunoblot, and β-actin was used as a loading control. D, keratinocytes were infected with 8 m.o.i. of EV or PKCα-encoding adenovirus and then treated in the presence of 0.09 or 0.3 mM calcium for 48 h. The cells were then harvested in sample buffer, and 20 μg of whole cell lysate was electrophoresed on an 8% polyacrylamide gel. hINV and β-actin protein levels were assessed by immunoblot.

ment contains the calcium-responsive element(s). We next determined whether the AP1 and Sp1 sites, previously shown to be present in this region (Fig. 6A) (31), are required for regulation. As shown in Fig. 6C, mutation of either the AP1 or Sp1, or both sites, reduces basal transcription and eliminates or reduces the calcium-dependent increase.

An important issue is whether the PKCδ-associated hINV promoter activation is mediated via these same elements. To evaluate this, keratinocytes were transfected with each reporter construct in the presence of empty expression vector (−PKCδ) or PKCδ-encoding expression vector (+PKCδ). As shown in Fig. 7A, PKCδ markedly increases the activity of constructs pINV-2473 and pINV-2216, suggesting that this region contains a response element. To determine whether the AP1 site is required for activity, we tested a construct in which this site is mutated, pINV-2473(AP1–5m) (31). The results presented in Fig. 7B indicate that the AP1–5 site is required for PKCδ-dependent regulation. A parallel experiment using pINV-2473(Sp1m) shows that mutation of the hINV promoter Sp1 site results in a smaller reduction in calcium-dependent activation (Fig. 7B).
PKCδ and -α on hINV promoter activity and endogenous gene expression are an interesting finding, as PKCδ and -α have been shown to oppose each other in other contexts. For example, PKCδ is shown to be an activator of apoptosis in keratinocytes and other cell types (63, 64), whereas PKCα produces anti-apoptotic responses in several cell types (65–68). Because calcium addition induces both hINV expression and other changes in keratinocytes leading to differentiation-related cell death, our results are consistent with the idea that PKCδ is a downstream mediator of these effects. This also supports the general hypothesis that PKCδ and PKCα play opposing regulatory roles, i.e. PKCδ is a pro-apoptosis, pro-differentiation mediator, whereas PKCα is a pro-proliferation regulator. This concept is supported by several additional studies (39, 41, 63, 68–71) but is not supported by others (12, 17), pointing to the complexity of the regulation.

Studies in cultured mouse keratinocytes suggest that PKC inhibits calcium-dependent activation of genes that are normally expressed early (K1, K10) in differentiation (49). In contrast, PKC activation appears to increase expression of the late markers, loricrin and filaggrin (49). In addition, PKCα positively regulates calcium-dependent induction of loricrin and filaggrin gene expression in mouse cells but does not influence calcium-dependent K1 expression (19). This suggests that PKC activation produces differential effects on different classes of genes during differentiation. Our present study suggests that PKCα inhibits expression of involucrin in human keratinocytes, suggesting a role of PKCα in inhibiting spinous layer markers. Because, hINV is first expressed in the late spinous layer, it is possible that PKCα functions to keep hINV gene expression off during early spinous differentiation. PKCδ, in contrast, may activate hINV gene expression in the late spi-
ous and granular layers. One previous study (72) examined the role of PKC as a regulator of hINV gene expression. In contrast to our findings, these investigators showed that TPA-dependent hINV promoter activity is increased by PKCα and is not influenced by PKCδ. However, this study differs from the present study in several important respects. First, the cells used were SV40 large T antigen-immortalized keratinocytes. Second, the hINV promoter construct used in this study did not contain the sequences identified in the present report. In addition, studies in our laboratory, using an extensive set of immortalized keratinocyte cell lines, suggest that regulation of hINV gene expression is markedly attenuated and abnormal in most transformed cell lines.

Our studies also indicate that calcium treatment is associated with enhanced phosphorylation of PKCδ. This result is in agreement with a recent report (40) in mouse keratinocytes showing a calcium-dependent increase in phosphorylated PKCδ in cultured keratinocytes. Phosphorylated PKCδ was also detected in vivo in the mouse epidermis (40). PKCδ phosphorylation can activate or inhibit the enzyme, depending upon the stimulus (52, 53, 73, 74). Moreover, the direction of change in catalytic activity may be substrate-dependent (54). Thus, although our studies clearly show that calcium treatment produces covalent changes in PKCδ, further studies will be necessary to determine whether the tyrosine phosphorylation of PKCδ in our system activates or inhibits the enzyme.

In addition, a surprising finding from our study is that calcium addition did not induce significant mobilization of PKCα or PKCδ to membrane fractions. Membrane mobilization is usually thought to be necessary for PKC activity but may not be absolutely required. The apparent lack of calcium-dependent PKC mobilization in our study is not an artifact, because TPA treatment did, as reported previously (41), mobilize PKCδ and PKCα. It is possible that the PKC isoforms are cycling to and from the membrane at a steady rate that is not detected in our assays and that active, membrane-associated, forms are thus generated continually. Such cycling has been reported for PKCδ in ceramide-treated cells (75). Increased membrane-associated PKC activity has also been reported in calcium-treated mouse keratinocytes (76), and this is associated with PKCα and -δ movement to membranes (77). It is also possible that PKC, resident at the membrane before calcium treatment, simply becomes active in the presence of calcium. For example, PKCδ is activated by H2O2 in Chinese hamster ovary cells in the absence of membrane translocation (78). Moreover, this translocation-independent activation is associated with tyrosine phosphorylation of PKCδ (78). Thus, the phosphorylation of PKCδ described in the present study may be important in this context. Although tyrosine phosphorylation has been reported to reduce PKCδ activity in mouse keratinocytes (79), the effect of this modification is likely to be context-dependent. Additional studies will be required to understand the effect of this modification in our system.
tinocytes were infected with 8 m.o.i. of dnPKC
immunoblot using an Sp1-specific antibody (dilution
above. Quantities of protein were electrophoresed on an 8% gel and transferred
calcium treatment as described in Fig. 7
virus (B
Similar results were observed in each of five separate experiments.
and empty vector produced the same regulatory responses shown in
B
PKC Regulation Targets the hINV Promoter Distal Regulatory Region—In some systems, AP1 transcription factors are
required for tissue-specific (epidermis) and differentiation-ap-
appropriate (suprabasal layers) expression of hINV in transgenic mice (23, 32, 81).

PKC6 and Calcium Regulate AP1 and Sp1 Factor Expression—One common mechanism whereby calcium and PKC6 may regulate hINV gene expression is through alteration of transcription factor levels. Our results show that treatment with either calcium or PKC6 increases c-Fos and JunB and decreases c-Jun and Fra-1 levels. Fra-2 levels, in contrast, are increased by PKC6 but not by calcium. In addition, the calcium-associated change in AP1 factor level requires PKC6 activity. These results suggest that regulation via both upstream modulators converges on AP1 factors. Because the extent of transcriptional activation or repression is a function of the partic-
ular AP1 heterodimers that are formed, any relative change in AP1 factor level may alter gene expression (82, 83). Interestingly, the PKC6 and calcium treatment produce similar changes in AP1 factor expression. In mouse keratinocytes, increased AP1 factor expression is also associated with cell con-
fluence and enhanced differentiation (17). In addition to the increase in AP1 levels, Sp1 levels also increase in the presence of calcium. Moreover, as measured using dominant-negative PKC6, the increase in Sp1 requires PKC6 activity. This sug-
st that Sp1 transcription factors may help mediate calcium-
dependent gene expression via a PKC6-dependent mechanism. Sp1 has been reported to be a key participant in the phorbol ester-dependent induction of gene expression (80, 84). Additional studies will be required to determine the mechanism

PKC6 and Calcium Regulate AP1 and Sp1 Factor Expression—One common mechanism whereby calcium and PKC6 may regulate hINV gene expression is through alteration of transcription factor levels. Our results show that treatment with either calcium or PKC6 increases c-Fos and JunB and decreases c-Jun and Fra-1 levels. Fra-2 levels, in contrast, are increased by PKC6 but not by calcium. In addition, the calcium-associated change in AP1 factor level requires PKC6 activity. These results suggest that regulation via both upstream modulators converges on AP1 factors. Because the extent of transcriptional activation or repression is a function of the particular AP1 heterodimers that are formed, any relative change in AP1 factor level may alter gene expression (82, 83). Interestingly, the PKC6 and calcium treatment produce similar changes in AP1 factor expression. In mouse keratinocytes, increased AP1 factor expression is also associated with cell confluence and enhanced differentiation (17). In addition to the increase in AP1 levels, Sp1 levels also increase in the presence of calcium. Moreover, as measured using dominant-negative PKC6, the increase in Sp1 requires PKC6 activity. This suggests that Sp1 transcription factors may help mediate calcium-dependent gene expression via a PKC6-dependent mechanism. Sp1 has been reported to be a key participant in the phorbol ester-dependent induction of gene expression (80, 84). Additional studies will be required to determine the mechanism

PKC Regulation Targets the hINV Promoter Distal Regulatory Region—In some systems, AP1 transcription factors are the downstream targets of PKC-dependent regulation (16, 17). For example, recent studies (28–30, 80) show that a Ras, MEKK1, MEK3, p38 MAPK cascade mediates the phorbol ester-dependent increase in hINV gene expression and that this
cascade targets AP1, Sp1, and C/EBP transcription factors. These factors, in turn, interact with selected binding motifs within the hINV promoter to regulate expression (31, 32). These motifs are localized in two major regions, the proximal regulatory region and the distal regulatory region (23, 32). Our present promoter truncation studies identify the distal regulatory region as containing the calcium-response elements. Targeted mutation of the Sp1 and AP1–5 sites reveals that both sites are required for the calcium-dependent response. Mutation of the AP1–5 site results in the complete elimination of calcium-dependent regulation. Mutation of the Sp1 site results in partial loss of the calcium-dependent response. These find-
ings are particularly interesting, as they suggest that calcium-
dependent regulation of hINV gene expression shares common features with phorbol ester-dependent regulation. Moreover, this segment encompasses a DNA regulatory region that is required for tissue-specific (epidermis) and differentiation-appropriate (suprabasal layers) expression of hINV in transgenic mice (23, 32, 81).
whereby AP1 and Sp1 factors regulate calcium-dependent hINV gene expression; however, it is possible that Sp1 may facilitate the response by assisting AP1 factor binding to DNA (31).

In summary, our results are consistent with the hypothesis that PKCδ and calcium activate keratinocyte differentiation via a mechanism that results in increased expression of AP1 and Sp1 transcription factors. Moreover, PKCδ and PKCε appear to produce opposing effects on calcium-dependent keratinocyte differentiation, PKCδ being an activator and PKCε functioning as an inhibitor of involucrin gene activation.

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6. In summary, our results are consistent with the hypothesis that PKCδ and calcium activate keratinocyte differentiation via a mechanism that results in increased expression of AP1 and Sp1 transcription factors. Moreover, PKCδ and PKCε appear to produce opposing effects on calcium-dependent keratinocyte differentiation, PKCδ being an activator and PKCε functioning as an inhibitor of involucrin gene activation.

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