Human involucrin (hINV) mRNA level and promoter activity increase when keratinocytes are treated with the differentiating agent, 12-O-tetradecanoylphorbol-13-acetate (TPA). This response is mediated via a p38 mitogen-activated protein kinase cascade-dependent pathway that targets activator protein 1 (AP1) that binds an AP1-binding site. This cascade includes protein kinase C (PKC); nPKC, novel PKCs; aPKCs, atypical PKCs; cPKC, conventional PKCs (cPKC); AP1, activator protein 1; PKC, protein kinase C. The role of PKC isoforms in the regulation of hINV gene expression is studied. Treatment of human keratinocytes with TPA increases expression of the endogenous hINV gene. hINV is a model for the study of gene expression in stratifying epithelia and involves a PKC-dependent response. In the present study, we demonstrate that novel PKC isoforms, which are not the conventional and atypical PKC isoforms, are involved in regulation of the hINV gene.
transfection efficiency as described (28). Pressing green fluorescent protein (CLONTECH) was used to monitor phosphorylation activity of PKC isozymes or empty expression vector (EV). After 24 h, the cultures were treated with (+) or without (−) 50 ng/ml TPA. At 48 h after transfection, the cells were harvested, and lysates were assayed for luciferase activity. C. keratinocytes were transfected with 4 µg of PKC8, δ, ε, or η. At 24 h post-transfection, the cells were harvested for preparation of total cell extracts. Equal quantities of extract were electrophoresed on an 8% denaturing/reducing polyacrylamide gel, transferred to nitrocellulose, and incubated with hINV-specific antibody (19).

Immunoblot Analysis—Cultured keratinocytes, grown in KSFM, were treated with or without 50 ng/ml TPA and/or indicated pharmacological agent (concentrations indicated in each experiment) for 24 h prior to preparation of total cell extracts. Equal quantities of protein were electrophoresed on denaturing polyacrylamide gels and transferred to nitrocellulose. The membranes were blocked and then incubated with the appropriate primary antibody followed by a goat anti-rabbit IgG secondary antibody. Secondary antibody binding was visualized using a chemiluminescent detection system (Amersham Pharmacia Biotech).

RESULTS

PKC Isoforms That Regulate hINV Promoter Activity—Fig. 1A shows that treatment of keratinocytes with phorbol ester increases human involucrin protein levels. This TPA-dependent increase in endogenous gene expression can be inhibited by BIS-IM, an agent that inhibits all PKC isoforms. To identify the specific PKC isoforms responsible for this regulation, we co-transfected keratinocytes with pINV-241 involucrin promoter-luciferase reporter construct (6, 7, 9) and expression plasmids encoding specific wild type PKC isoforms. The results, see Fig. 1B, indicate that novel PKC (nPKC) isoforms δ, ε, and η increase hINV promoter activity as efficiently as TPA treatment (>10-fold). In contrast, the conventional PKC (cPKC) isoforms α, β1, and γ, and the atypical isoform, PKCζ, produce minimal changes. As shown in Fig. 1C, the novel isoform-dependent increase is also observed for the endogenous gene, suggesting that the stimulation is physiologically relevant.

Failure of the cPKC isoforms to regulate hINV promoter activity in keratinocytes could result from a failure of the enzymes to be expressed or because they are expressed in an inactive form. To address these concerns, we transfected keratinocytes with PKCa, δ, ε, and η, and we monitored for expression of the corresponding protein by immunoblot. As shown in Fig. 2A, each PKC isoform is expressed at a comparable level. Although not evident from the exposure shown here, PKCa, δ, ε, and η are also expressed in non-transfected keratinocytes. To ensure that the transfected cPKC isoforms are active, we transfected the c-fos promoter (27), which responds to phorbol ester-sensitive PKC isoforms (29–32), with each cPKC isoform. Fig. 2B shows that PKCa, β1, and γ strongly increase c-fos promoter activity, confirming activity of these enzymes in keratinocytes. Thus, the lack of hINV promoter activation by the cPKC isoforms is not due to low expression or lack of activity of the expressed enzymes.

Concentration-dependent Regulation of hINV Promoter Ac-
Figu
tivity by Individual PKC Isoforms—PKC-dependent responses can be concentration-dependent, and so we studied the effects of various concentrations of PKC expression plasmid on promoter activity. PKCα did not regulate promoter activity at any concentration examined (Fig. 3). Although not evident in this figure, because the responses are minimal, cPKC γ slightly stimulated promoter activity at high plasmid concentrations, and promoter activity was increased a modest 2-fold by 0.25 μg of PKCζ plasmid with no further increase at higher plasmid concentrations. In contrast to these minimal responses, the nPKC isoforms, -δ, -ε, and -η, produced similar dose-response curves and a 12–35-fold increase in promoter activity.

Rottlerin, but Not Go-6976, Suppresses hINV Promoter Activity—To examine this regulation further, we used isof orm-specific PKC inhibitors. Rottlerin specifically inactivates PKCδ (33), and Go-6976, a staurosporine-related compound, inactivates cPKC isoforms (34). pINV-241 reporter plasmid-transfected cells were treated with increasing concentrations of each inhibitor. Fig. 4A shows that rottlerin inhibits hINV promoter activity at concentrations that selectively inhibit PKCδ (33). In contrast, the cPKC inhibitor, Go-6976, which is normally active in the nanomolar range (34), did not inhibit promoter activity even at micromolar concentrations (Fig. 4B).

Dominant-negative PKC8 Suppresses PKCδ, -ε, and -η-dependent Promoter Activation—The previous experiment suggests that PKC8 may be the primary PKC controlling hINV gene expression. However, additional nPKC isoforms may also have a role. To confirm a role for PKC8 and to study the role of the other nPKC isoforms, we used a dominant-negative form of PKC8, dnδ(KR), in which the ATP-binding site is mutated (26). We show, in Fig. 5A, that dnδ(KR) completely inhibits TPA-dependent promoter activation. Thus, ligand-dependent activation of the promoter is inhibited by dnδ(KR). In Fig. 5B we show that dnδ(KR) completely inhibits PKCδ-dependent promoter activation. However, it is interesting that dnδ(KR) also inhibits wild type PKCη and PKCε-dependent promoter activation, although less efficiently compared with the dnδ(KR)-dependent inhibition of PKCδ-driven activity. This result suggests caution in assigning the sole regulatory role to PKCδ.

Activation by PKC Isoforms in the Presence of TPA—The results presented above indicate that nPKC isoforms activate basal hINV promoter activity. To examine the effects of TPA on PKC8 isoform-dependent activation, keratinocytes were co-transfected with pINV-241 reporter vector and PKC expression plasmid and treated with TPA. As shown in Fig. 6, none of the classical PKC isoforms (α, β1, and γ) or the atypical isoform (ζ) altered the TPA-dependent response. However, PKCε and PKCη produced a dramatic superinduction of hINV promoter activity in the presence of TPA (100-fold activation). Unexpectedly, and in contrast to the PKCδ-dependent activation observed in the absence of TPA (Fig. 3), PKC8 suppressed the TPA-dependent activation to TPA-nonstimulated levels.

To investigate further the nPKCδ effect, we transfected keratinocytes with a fixed amount of hINV reporter plasmid (2 μg) and increasing concentrations of nPKC δ expression plasmid (0.25–2 μg), and we treated with TPA (Fig. 7). PKCδ, at 0.25 μg expression plasmids per dish, produced a strong promoter activation. A comparable increase was observed at lower PKCδ concentrations (0.06 μg of PKCδ per dish, not shown).
Regulation of hINV Promoter Activity by nPKC Isoforms

Figure 5. Dominant-negative PKCδ inhibits novel PKC-dependent promoter activation. A, keratinocytes were transfected with 2 μg of pINV-241 in the presence (+) or absence of (− dnδKR) and treated with 50 ng/ml TPA. After 24 h, extracts were assayed for promoter activity. B, keratinocytes were transfected with 2 μg of pINV-241 and 1 μg of PKCδ, −η, or −ε in the presence (+) or absence (−) of 1 μg of dominant-negative PKCδ (dnδKR). At 24 h after transfection extracts were prepared and assayed for luciferase activity.

indicating that very small concentrations of this isoform can activate transcription. Interestingly, a smaller increase is observed at intermediate plasmid concentrations, and inhibition is observed at high (2 μg) plasmid levels. In contrast, nPKCε and nPKCη (Fig. 7) markedly enhance the TPA-induced hINV promoter activity at all concentrations tested.

One mechanism whereby PKC is inactivated is via degradation (10, 35). We therefore determined whether PKCδ, ε and η levels change in response to TPA treatment. The results, shown in Fig. 8, indicate that PKCε is markedly decreased by TPA treatment; PKCδ is decreased by 50%; and PKCη is slightly increased. These results suggest that PKC level is not correlated with ability to drive TPA/PKC-dependent hINV promoter activation.

DISCUSSION

Involucrin Promoter Activity and Endogenous hINV Gene Expression Are Regulated by Novel PKC Isoforms—We have previously presented evidence indirectly implicating PKC in the signal transduction pathway leading to hINV promoter activation (7, 9). Evidence includes the finding that TPA increases hINV mRNA levels and promoter activity (7), and BIS-IM, a general PKC inhibitor, blocks these responses (9). The present studies were designed, in part, to identify which PKC isoform(s) are involved in this regulation. Keratinocytes express classical, novel, and atypical PKC isoforms, including cPKCα, nPKCδ, nPKCε, nPKCη, and αPKCγ (14–18, 36). All of these forms, with the exception of αPKCγ, can be activated by TPA (25). Because of their distinct pattern of expression, primary sequence, differing response to stimuli, and differing cofactor dependence, each PKC is expected to have a different function (10, 13). Therefore, it is important to determine which isoforms regulate keratinocyte target gene expression and which pathways convey the regulatory signal. To address these issues, we expressed wild type PKC isoforms in normal human keratinocytes and monitored the effects on basal hINV promoter activity. These experiments identify the novel PKC isoforms δ, ε, and η as potent inducers of promoter activity. In contrast, atypical PKCζ, the conventional PKC isoforms α, β1, and γ, failed to regulate activity. The lack of activation by PKCα, β1, and γ was due to inadequate expression of these kinases, as each was detected at high level by immunoblot. Moreover, activity of these kinases was confirmed by demonstrating PKCα, β1, and γ-dependent regulation of the c-fos promoter. c-fos is known to be regulated by TPA-dependent PKC isoforms (29–32). A regulatory role for the novel PKC isoforms is further supported by the finding that Go-6976, an inhibitor of conventional but not novel PKC isoforms (34), does not inhibit promoter activity.

The Role of PKCδ—An important role for PKCδ is suggested by the finding that promoter activity is inhibited by concentrations of rottlerin (33) that selectively inhibit PKCδ. However, our results also support a role for PKCδ and PKCγ in two ways. First, transfection of keratinocytes with PKCδ or −γ activates hINV promoter activity and expression of the endogenous hINV gene. Second, dominant-negative PKCδ inhibits PKCε- and PKCγ-dependent activity. Dominant-negative mutants have been extensively utilized to map signal transduction cascades. These proteins function to inhibit the activity of the endogenous wild type enzymes by a variety of mechanisms. For PKCs, dominant-negative mutants have been constructed by mutating threonine phosphorylation sites in the activation loop of the kinase domain (37) or by mutating the site that binds ATP, a necessary cofactor for enzyme activity (26, 29, 38–41).
There are several possible mechanisms whereby dnPKC<sub>d</sub> and increasing concentrations of PKC<sub>δ</sub> inhibit PKC<sub>δ</sub>- and PKC<sub>ε</sub>-dependent promoter activation, a result that is consistent with a role for PKC<sub>δ</sub> in regulating hINV gene expression. Our experiments show that dominant-negative PKC<sub>δ</sub> inhibits PKC<sub>δ</sub>-protein concentration, were electrophoresed on an 8% acrylamide gel and transferred to nitrocellulose for detection using PKC<sub>δ</sub>-specific antibodies. Binding of the primary antibody was detected by incubation with an appropriate secondary antibody, and binding was visualized using ECL technology.

We have used a form of PKC<sub>δ</sub> in which a conserved lysine at the ATP-binding site is converted to arginine to inactivate the enzyme (26). Our experiments show that dominant-negative PKC<sub>δ</sub> may interfere with chaperone "docking" proteins that may regulate the function of multiple PKC isoforms. Experiments that suggest these possibilities have been noted using activation-loop mutants of PKC (10, 37). The dnPKC<sub>δ</sub> used in the present studies is an ATP-binding site mutant (26). ATP-binding site mutants may be more specific inhibitors of the corresponding wild type PKC isoform than activation-loop mutants; however, this has not been rigorously tested. Third, PKC<sub>δ</sub>, -ε, and -η may indirectly regulate the level/activity of each other by regulating expression of the corresponding genes. This interesting possibility is not unprecedented, as a recent study in mouse lymphoma cells shows that PKC<sub>α</sub> increases PKC<sub>δ</sub>-protein level by regulating PKC<sub>δ</sub>-mRNA level (42). Fourth, PKC<sub>δ</sub>, -ε, and -η may share a common substrate(s). Fifth, overexpression of individual PKC isoforms could lead to non-selective activation of downstream targets of other PKC isoforms. Thus, although our present studies strongly point to a role for PKC<sub>δ</sub>, it is likely that PKC<sub>ε</sub> and -η also play an important role.

Function of PKCs in the Presence of the PKC Activator, Phorbol Ester—Diaoyglycerol is a ligand that directly activates PKC isoforms (11). TPA is a stable diacylglycerol analog that mimics the effects of diacylglycerol and strongly activates PKC (43–45) and is a potent inducer of keratinocyte differentiation (46). Treating cultured keratinocytes with TPA increases cell differentiation, and this change is correlated with an increase in hINV mRNA and protein (7, 9, 47, 48). To study the effects of TPA-dependent activation of individual PKC isoforms on hINV promoter activity, we transfected cells with PKC expression constructs and then treated with TPA. The results indicate a synergistic activation (>100-fold, Fig. 6) of promoter activity when PKC<sub>ε</sub>- or -η-treated cells are incubated with TPA. This increase depends directly on the concentration of PKC<sub>ε</sub> or -η expression plasmid transfected. In contrast, no potentiation was observed for PKC<sub>α</sub>, -β1, -γ, or -ζ. PKC<sub>δ</sub>, however, caused synergistic promoter activation at moderate plasmid concentrations and inhibition at higher plasmid concentrations. It is not clear why the response to δ is bhipasic; however, this result suggests that the PKC-dependent regulation is complex. One possible explanation is that PKC<sub>δ</sub> levels are reduced by TPA treatment. However, immunoblot results suggest that PKC<sub>δ</sub> levels are decreased by only 30–50% in response to TPA treatment. In contrast, PKC<sub>ε</sub> levels are reduced substantially, and PKC<sub>η</sub> levels are relatively unchanged. These results suggest that TPA-dependent regulation of PKC level does not explain the difference in activity. There are other possible explanations. For example, PKC<sub>δ</sub> undergoes tyrosine phosphorylation in response to various stimuli, including epidermal growth factor, platelet-derived growth factor, transforming growth fac-

![Fig. 7. Concentration dependence of PKC-dependent activation in the presence of TPA.](image)

![Fig. 8. Regulation of PKC isoform level by TPA.](image)
tor-α, carbachol, extracellular ATP or UTP, and hydrogen peroxide (35). Moreover, tyrosine kinases of the Src family phospholipid PKCs in vitro, although the functional significance of this phosphorylation has not been clearly established (45). Several reports in keratinocytes suggest that phosphorylation of PKCδ on tyrosine residues in the regulatory domain diminishes activity (49–51), although studies in other systems report increased PKC activity following tyrosine phosphorylation (52–54). Thus, high level overexpression of PKCδ may result in tyrosine phosphorylation-dependent inactivation of PKCδ. However, although PKCδ could be inactivated by phosphorylation, it is difficult to understand the unique plasmid concentration dependence of the inhibition. As noted above, PKCδ is expressed at high levels in keratinocytes. It is possible that very high plasmid concentrations inhibit promoter activity by saturating the system with PKCδ which “squelches” the response.

**PKC Isoforms and Keratinocyte Function**—Our results suggest that PKCδ, ε, and η regulate hINV gene expression. Takahashi et al. (55) showed that PKCγ increases basal hINV promoter activity by 2–3-fold in the absence of TPA, whereas PKCa and η increase activity by 2-fold in TPA-treated cells. These studies differ from ours in that we do not observe PKCa-dependent regulation. Moreover, the magnitude of our responses is much larger. We attribute the different findings to the fact that we use normal human keratinocytes, whereas Takahashi et al. (55) used SV40-immortalized keratinocytes. Signal transduction is known to be altered in immortalized cell lines, and a significant amount of circumstantial evidence indicates that the involucrin gene is not always appropriately regulated in immortalized keratinocyte cell lines. For example, in cell lines, the level of hINV gene expression and the response to stimuli is significantly reduced compared with normal cells.2 However, the studies in both cell types support a role for PKCη as a regulator of hINV gene expression.

In epidermis, involucrin is expressed in the late spinous and granular layers but not in the basal layer (1, 3, 5). Type I transglutaminase is another marker of keratinocyte differentiation that displays a similar spatial and temporal pattern of expression (56, 57). Thus, it is useful to compare mechanisms that regulate expression of the involucrin and transglutaminase type 1 (TG1) genes. Recent studies indicate that overexpression of the η and δ PKC isoforms in human keratinocytes causes an increase in TG1-encoding mRNA. This is correlated with growth inhibition and morphological changes (58). In contrast, the α and ζ PKC isoforms do not regulate TG1 expression. Regulation in response to PKCε was not studied. In addition, it has been reported that expression of exogenous PKCζ in a rat keratinocyte cell line efficiently induces TG1 transcription, but PKCa, PKCζII, PKCγ, and PKCδ did not regulate activity (59). Yuspa and co-workers (60) have shown that TPA blocks the calcium-dependent increase in K1 and K10 (spinous layer markers) and simultaneously increases filaggrin and loricrin expression (granular layer markers). This TPA-dependent response is blocked by bryostatin, a PKC inactivating agent or cycloheximide, a protein synthesis inhibitor agent. This suggests that PKC regulates genes in the transition from spinous to granular layers (60). These results are consistent with ours, as involucrin is predominantly a granular cell marker (2). Moreover, PKCη is known to be localized in the epidermal granular layer (61). Thus, our studies suggest a role for novel PKCs as regulators of hINV gene expression.

**Acknowledgment**—The Skin Diseases Research Center of Northeast Ohio was supported by National Institutes of Health Grant AR39750.

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2 R. L. Eckert, unpublished observations.
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53. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11233–11237
54. Kadotani, M., Nishi, T., Nanahoshi, M., Tsujishita, Y., Ogita, K., Nakamura, S., Kikkawa, U., and Asaoka, Y. (1997) J. Biochem. (Tokyo) 121, 1047–1053
55. Takahashi, H., Asano, K., Manabe, A., Kinouchi, M., Ishida-Yamamoto, A., and Iizuka, H. (1998) J. Invest. Dermatol. 110, 218–223
56. Thacher, S. M., and Rice, R. H. (1985) Cell 40, 685–695
57. Kim, S. Y., Kim, I. G., Chung, S. I., and Steinert, P. M. (1994) J. Biol. Chem. 269, 27979–27986
58. Ohba, M., Ishino, K., Kashiwagi, M., Kawabe, S., Chida, K., Huh, N. H., and Kuroki, T. (1998) Mol. Cell. Biol. 18, 5199–5207
59. Ueda, E., Ohno, S., Kuroki, T., Livneh, E., Yamada, K., Yamanishi, K., and Yasuno, H. (1996) J. Biol. Chem. 271, 9790–9794
60. Dlugosz, A. A., and Yuspa, S. H. (1993) J. Cell Biol. 120, 217–225
61. Koizumi, H., Kohno, Y., Osada, S., Ohno, S., Ohkawara, A., and Kuroki, T. (1995) J. Invest. Dermatol. 105, 858–863