Coordinate Changes in Gene Expression Which Mark the Spinous to Granular Cell Transition in Epidermis Are Regulated by Protein Kinase C

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Abstract. The protective function of skin depends on successful completion of a tightly regulated multi-step differentiation program, during which the induction of markers for a specific stage in epidermal differentiation is coupled to repression of markers expressed at the preceding stage. We have explored the role of protein kinase C (PKC) in this process using an in vitro model system, in which cultures of primary mouse epidermal keratinocytes are induced to terminally differentiate by raising the Ca^{2+} concentration in the medium from 0.05 to 0.12 mM. At doses which activate PKC, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol block Ca^{2+}-mediated induction of the spinous cell markers keratins K1 and K10 at both the protein and mRNA level. TPA and 1-oleoyl-2-acetylglycerol also rapidly repress K1 and K10 mRNA expression when added to differentiating keratinocyte cultures already expressing these markers. The inhibition of K1 mRNA expression by TPA is blocked in cells where PKC has been inactivated with bryostatin. TPA-mediated loss of K1 mRNA is also blocked in cells exposed to cycloheximide or actinomycin D implicating a PKC-induced protein factor in this process. The loss of K1 mRNA in TPA-treated cultures is the result of both a selective destabilization of K1 transcripts and a rapid inhibition of K1 gene transcription. In contrast to the dramatic repression of mRNAs typical for spinous cell differentiation, activation of PKC concurrently enhances expression of mRNAs and proteins for the granular cell markers loricrin and filaggrin. This response does not occur in cells pre-treated with bryostatin to inactivate PKC. Our results suggest that PKC is a fundamental regulator of the coordinate changes in keratinocyte gene expression that occur during the spinous to granular cell transition in epidermis.

THE epidermis provides an essential barrier between organism and environment. The protective function of skin is the result of a tightly regulated differentiation program which takes place as keratinocytes leave the basal layer and migrate through the spinous, granular, and cornified layers of the epidermis. The evolution of a basal cell to a terminally differentiated cornified cell is associated with the sequential induction of a family of differentiation-specific proteins, both structural and regulatory, that are unique to keratinocytes. The early markers keratins K1 and K10 are expressed in spinous cells while late differentiation markers such as loricrin and filaggrin appear in the granular layer (Roop et al., 1988; Fuchs, 1990). Ultimately, activation of keratinocyte-specific transglutaminase results in covalent cross-linking of certain proteins (such as loricrin) to form the rigid cornified envelope characteristic of terminally differentiated keratinocytes (reviewed in Polakowska and Goldsmith, 1991). At the mRNA level, expression of epidermal differentiation markers is rigidly compartmentalized with induction of certain genes tightly coupled to repression of others (Stoler et al., 1988; Coulombe et al., 1989; Mehrel et al., 1990). For example, the abundance of mRNAs encoding the basal cell keratins K5 and K14 is markedly reduced in the first spinous layer at a time when induction of K1 and K10 mRNA takes place. Similarly, there is an abrupt loss of K1 and K10 transcripts as cells leaving the spinous layer begin expressing loricrin and filaggrin mRNA in the granular layer. Elucidating the mechanism by which these divergent effects on gene expression are coordinated remains a central problem in understanding epidermal differentiation.

Cell culture studies have been useful in identifying factors influencing keratinocyte differentiation. Comparative analysis of keratinocytes from human and rodent species indicates that terminal differentiation is regulated by specific extracellular Ca^{2+} concentrations (Yuspa, 1985). Cultures established from newborn mouse epidermis and grown in medium with a reduced Ca^{2+} concentration (0.05 mM) yield a proliferating population of undifferentiated, basal cell-like keratinocytes (Hennings et al., 1980). Raising Ca^{2+} in the medium to 0.12 mM triggers terminal differentiation which resembles this process as it occurs in vivo in many respects: the early markers K1 and K10 are detected within 8–24 h;

the late markers loricrin and filaggrin after 24–48 h; and cornified envelopes are assembled as a result of transglutaminase activation (Yuspa et al., 1989). Induction of mRNA for the late markers loricrin and filaggrin is coupled to reduced expression of transcripts for the early markers K1 and K10 (Yuspa et al., 1989), suggesting that Ca^{2+} may signal both positive and negative modulation of keratinocyte-specific gene expression. One change that is not observed in vitro is the down modulation of K14 expression that takes place in differentiating keratinocytes in vivo: this keratin is expressed constitutively in cultured keratinocytes. A relationship between elevated Ca^{2+} and keratinocyte differentiation is observed both in vitro and in vivo, where a Ca^{2+} gradient has been documented in both human (Malmquist et al., 1984; Menon and Elias, 1991) and mouse (Menon et al., 1985) epidermis, with higher Ca^{2+} levels in differentiated cell layers than the undifferentiated basal layer. In light of these findings it appears likely that a Ca^{2+}-responsive signalling pathway is involved in the regulation of keratinocyte differentiation.

There is a large body of evidence linking the phospholipase C-protein kinase C (PKC)[1] signal transduction pathway to keratinocyte differentiation. In cultured mammalian keratinocytes, raising extracellular Ca^{2+} induces phosphatidylinositol-specific phospholipase C γ and δ (K. Punnonen, M. F. Denning, E. Lee, L. Li, S. G. Rhee, and S. H. Yuspa, manuscript in preparation) and increases the level of inositol phosphates (Tang et al., 1988; Jaken and Yuspa, 1988; Lee and Yuspa, 1991), intracellular Ca^{2+} (Kruszewski et al., 1991), and diacylglycerol (Lee and Yuspa, 1991; Ziboh et al., 1984). Ca^{2+} also induces qualitative changes in keratinocyte PKC (Dunn et al., 1985) and alters the subcellular distribution of PKC activity (Isseroff et al., 1989). Furthermore, detection of Fos protein specifically in differentiating keratinocytes in vivo supports the hypothesis that PKC is activated during this process (Fisher et al., 1991). Direct activation of PKC in cultured keratinocytes using 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces epidermal transglutaminase and cornified envelope formation, markers for the final stage of keratinocyte differentiation (Yuspa et al., 1982; Parkinson et al., 1984); in contrast, induction of the early markers K1 and K10 is inhibited by TPA (Roop et al., 1987). A similar uncoupling of differentiation marker expression occurs in vivo: TPA rapidly induces epidermal transglutaminase activity and cornification but blocks expression of the spinous cell markers K1 and K10 (Toftgard et al., 1985; Molloy and Laskin, 1987; Lichti and Yuspa, 1988). The molecular regulation of these seemingly paradoxical effects of TPA on keratinocyte differentiation has not been addressed previously.

In light of the in vitro and in vivo observations indicating that Ca^{2+} signals keratinocyte differentiation, the demonstration of Ca^{2+}-mediated changes in the phospholipase C-PKC pathway in cultured keratinocytes, and induction of certain late keratinocyte markers by direct activation of PKC with phorbol esters, we have proposed that the PKC signaling pathway plays a central role in regulating keratinocyte differentiation (Yuspa et al., 1990). We have now tested this hypothesis in vitro by examining the molecular regulation of early and late differentiation markers in cultured mouse epidermal keratinocytes. PKC activation rapidly alters keratinocyte gene expression at transcriptional and posttranscriptional levels resulting in enhanced expression of late-stage differentiation markers coupled to repression of early markers. Our findings suggest that PKC is a fundamental regulator of the coordinate changes in gene expression, both positive and negative, which occur during keratinocyte differentiation.

1. *Abbreviations used in this paper*: GAPDH, glyceraldehyde phosphate dehydrogenase; OAG, 1-oleoyl-2-acetylglycerol; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Materials and Methods

Reagents

TPA was obtained from LC Services (Woburn, MA); OAG from Molecular Probes (Eugene, OR) and Avanti Polar Lipids (Alabaster, AL); actinomycin D from Fluka (Ronkonkoma, NY); and *N,N*-bis-(2-hydroxyethyl)-2-aminosulfonic acid from Calbiochem-Behring Corp. (San Diego, CA). Bryostatin (bryostatin 1) was kindly provided by Dr. G. Pettit (Arizona State University, Tempe, AZ), and GF 109203X was a gift from Dr. J. Kirilovsky (Glaxo Laboratories, Les Ulis, France). Bryostatin stock was prepared in absolute ethanol; other reagents were dissolved in DMSO and stored at −20°C. Reagents were added to media just before use. To facilitate dispersal of OAG, medium was vortexed at the time of OAG addition.

Cell Culture

Primary epidermal keratinocytes were obtained from newborn BALB/c mouse skin as described (Hennings et al., 1980). Medium was prepared with Eagle's minimum essential medium (without Ca^{2+} and Mg^{2+}) supplemented with 8% FCS (Ca^{2+} depleted using chelex resin [Bio-Rad Laboratories, Richmond, CA]) and 0.25% penicillin-streptomycin solution (Gibco Laboratories, Grand Island, NY). The concentration of Ca^{2+} in the medium was adjusted by addition of 0.3 M $CaCl_2$ stock. Cells were cultured in medium with 0.05 mM Ca^{2+} to maintain a proliferating population of basal cells; terminal differentiation was induced by exposing cultures to medium containing 0.12 mM Ca^{2+} for 20–36 h (Yuspa et al., 1989).

Western Blot Analysis

For analysis of differentiation markers, cultures were washed once with PBS (Ca^{2+}- and Mg^{2+}-free) and total cell lysates prepared using ~10 μl/cm^2 of lysis buffer (5% SDS, 20% β-mercaptoethanol in 0.25 M Tris, pH 6.8) (Yuspa et al., 1989). Proteins were separated using an 8.5% polyacrylamide gel and transferred to reinforced nitrocellulose membrane (0.2 μm BA-S NC; Schleicher & Schuell, Keene, NH). Filters were incubated with blocking buffer (TBS, pH 7.4, with 5% milk, 0.1% Tween 20) for 30 min, primary antibody for 1–2 h, and secondary antibody for 45 min with several washes (TBS, 0.2% Tween 20) between incubations. Antibodies directed against keratinocyte differentiation markers (Yuspa et al., 1989) were diluted in 1% gelatin in TBS, secondary antibodies were IgGs conjugated to HRP used at a 1:5,000 dilution (Bio-Rad Laboratories), diluted in 2% milk in TBS. Immunoreactive proteins were visualized by enhanced chemiluminescence using a commercial kit (Amersham Corp., Arlington Heights, IL).

RNA Isolation and Analysis

Total RNA was isolated by centrifugation through 5.7 M cesium chloride (Chirgwin et al., 1979) and separated in a 1% agarose gel containing 0.6 M formaldehyde (Davis et al., 1986). Northern blot analysis was performed essentially as described (Dlugosz and Yuspa, 1991). To analyze effects on mRNA stability, differentiation was induced by a 20–24 h exposure to 0.12 mM Ca^{2+} medium. Reagents were then added directly to cultures and cells harvested after an additional 2, 4, and 8 h. Actinomycin D stock solution was prepared in DMSO: when used at a final concentration of 0.1 μg/ml, incorporation of [^{3}H] uridine into TCA-insoluble material was blocked by 95% in primary mouse keratinocyte cultures (Yuspa et al., 1980). Transcripts were identified using the following cDNAs either as purified inserts or in plasmid vectors, after labeling with ^{32}P by nick translation: K1, K10, K14, and loricrin:3′ non-coding fragments (200–450 bp) (Roop et al., 1983; Mehrel et al., 1990); filaggrin, 300-bp fragment of coding sequence (Yuspa

et al., 1989); c-*myc*, 1.4-kb fragment spanning the entire mouse c-*myc* coding sequence (Stanton et al., 1983); GAPDH, full-length rat cDNA (Fort et al., 1985). Transcript levels were quantified using a computing laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Nuclear Run-On Analysis

Gene transcription was examined by in vitro elongation of nascent transcripts as previously described (Greenberg and Ziff, 1984), with minor modifications. Briefly, nuclei were isolated from primary keratinocytes by lysis in buffer containing 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40. Nuclear preparations were frozen in storage buffer (50 mM Tris, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol) and stored at −70° until use. Nuclei were thawed on ice and incubated with an equal volume of 2× reaction buffer (10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 200 U/ml RNAsin) (Promega, Madison, WI); 500 μM ATP, CTP, GTP, 10 μM UTP, and 200 μCi ^{32}P-UTP (800 Ci/mmol, New England Nuclear, Boston, MA), at 30° for 30 min. Labeled transcripts were isolated by ultracentrifugation through cesium chloride (Chirgwin et al., 1979) and an equal number of counts per minute was hybridized for 1.5–4 d at 42°C to nitrocellulose-immobilized plasmids, described above for Northern analysis.

^{125}I-EGF Binding Assay

Binding of ^{125}I-labeled EGF was determined as previously described (Strickland et al., 1984), with minor modifications. Briefly, primary keratinocytes were grown in 12-well cluster dishes (Costar, Cambridge, MA) at an initial density of 5 × 10^5 cells/ml/well. After treatment for 1 h, cells were washed twice with binding buffer (DME with 50 mM *N,N*-bis-[2-hydroxyethyl]-2-aminosulfonic acid, pH 7.4, and 1 mg/ml BSA) at 4°C, and then incubated with ^{125}I-EGF (0.1 μCi/ml/well; New England Nuclear) ± excess-unlabeled EGF (1 μg/ml, receptor grade; Collaborative Research, Bedford,MA) in 1 ml of binding buffer for 5 h on a bed of ice. Binding buffer containing radiolabelled EGF was removed, cultures washed four times with ice-cold binding buffer and cells harvested in two 500-μl vol of lysis buffer (0.1 M Tris, pH 7.4, 0.5% SDS, 1 mM EDTA). Radioactivity was determined by scintillation counting; correction for non-specific binding (usually less than 5% of total counts in control wells) was based on counts from cultures incubated with excess-unlabeled EGF (Strickland et al., 1984).

Results

TPA Blocks Ca^{2+}-mediated Induction of the Early Differentiation Markers K1 and K10

To explore the role of PKC in keratinocyte differentiation we used activators and inhibitors of PKC in conjunction with changes in extracellular Ca^{2+} (to trigger terminal differentiation). We first studied the effect of TPA on Ca^{2+}-mediated induction of K1 and K10 protein: these are the first differentiation markers to appear after cells leave the basal layer, and are expressed throughout the spinous layer of the epidermis. TPA blocks Ca^{2+}-dependent accumulation of both K1 and K10 in primary keratinocyte cultures but has little effect on the level of K14, a basal-cell keratin constitutively expressed in vitro (Fig. 1). The ability of TPA to block expression of the early markers K1 and K10 is in striking contrast to its potency at inducing two markers for a late stage of keratinocyte differentiation, transglutaminase activity and cornified envelopes (Yuspa et al., 1980, 1982).

PKC Activation Inhibits Ca^{2+}-mediated Induction of K1 mRNA

K1 was selected to further illustrate TPA's inhibitory effect on spinous-cell marker expression at the molecular level. Two processes were examined in vitro: Ca^{2+}-mediated accumulation of K1 mRNA, which occurs in vivo during the transition from a basal cell to spinous cell phenotype; and Ca^{2+}-dependent maintenance (Yuspa et al., 1989) of K1 mRNA expression, characteristic of all spinous cells. Based on Northern blot analysis, TPA blocks Ca^{2+}-mediated accumulation of K1 mRNA in a dose-dependent manner. Nearly complete suppression of K1 mRNA occurs in the presence of 10 nM TPA, with undetectable levels at 100 nM TPA and higher (Fig. 2 *A*). Similar results were obtained for K10 (data not shown). Ca^{2+}-mediated accumulation of K1 mRNA is also blocked by the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG; Fig. 2 *B*) suggesting that this response to TPA is the result of PKC activation. The absence of K1 mRNA does not reflect a general inhibition of gene expression since the level of K14 and GAPDH transcripts is relatively unaffected by TPA (Fig. 2).

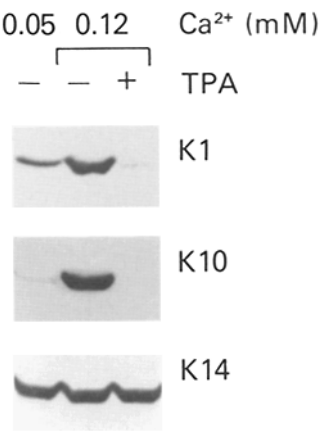

Figure 1. TPA blocks Ca^{2+}-mediated induction of the spinous cell differentiation markers K1 and K10. Basal cell keratinocyte cultures were grown in medium with 0.05 mM Ca^{2+}; terminal differentiation was induced by exposure to 0.12 mM Ca^{2+} medium, ±50 nM TPA, for 36 h. Western blot analysis was performed on total cell lysates using antibodies to the differentiation-specific keratins K1 and K10 and the basal cell keratin K14. Similar results were obtained in two independent experiments.

To further substantiate the relationship between PKC activation and inhibition of K1 mRNA expression, we compared TPA dose-response profiles for both of these responses in cultured keratinocytes. Since TPA rapidly decreases the affinity of EGF for its receptor (Schlessinger, 1986), quantitation of ^{125}I-EGF binding provides a measure of PKC activation in intact cells. TPA activates PKC in cultured keratinocytes in a dose-dependent manner, reducing ^{125}I-EGF binding by ~30% at a dose of 1 nM and >75% at doses of 10 nM or higher (Fig. 3 *A*). In the same experiment 125 μM OAG reduced ^{125}I-EGF binding by 55% (Fig. 3 *A*), confirming this agent's ability to activate PKC in cultured keratinocytes. The dose response for TPA-mediated inhibition of ^{125}I-EGF binding and inhibition of K1 mRNA expression are similar (Figs. 2 *A* and 3 *A*), consistent with the hypothesis that K1 mRNA is down modulated as a result of PKC activation.

To further test the hypothesis that activation of PKC inhibits Ca^{2+}-mediated accumulation of K1 mRNA, keratinocytes were rendered functionally deficient in PKC by exposure to bryostatin, a macrocyclic lactone isolated from the primitive marine organism *Bugula neritina* (Pettit et al., 1982). Bryostatin binds and transiently activates PKC but subsequently functions as a potent PKC antagonist, blocking phorbol ester-induced responses in a variety of cell types including keratinocytes (Sako et al., 1987; Jetten et al., 1989; Gschwendt et al., 1988). In cultures pre-treated with bryostatin for 6 h, TPA is completely ineffective in down regulat-

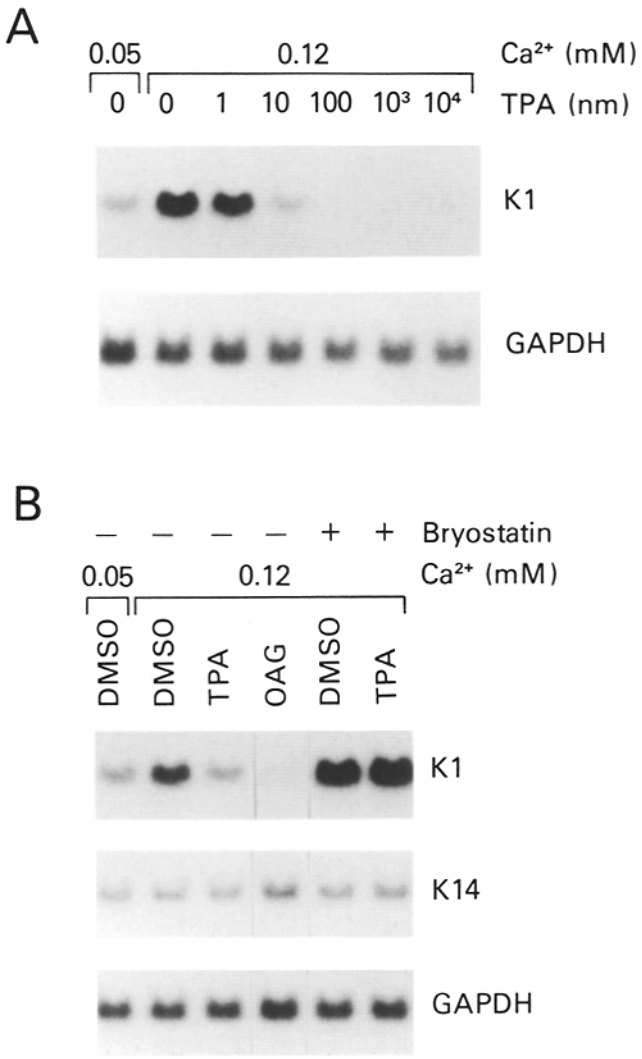

Figure 2. PKC activation blocks Ca^{2+}-mediated accumulation of K1 mRNA. Terminal differentiation was induced by growing cells in 0.12 mM Ca^{2+} medium for 24 h, ± PKC activators. (*A*) Cells were exposed to TPA at the indicated concentrations. (*B*) Cells were treated with 10 nM TPA or 125 μM OAG; bryostatin (60 nM) was added to cultures at the same time as DMSO or TPA. Two-independent experiments yielded similar results.

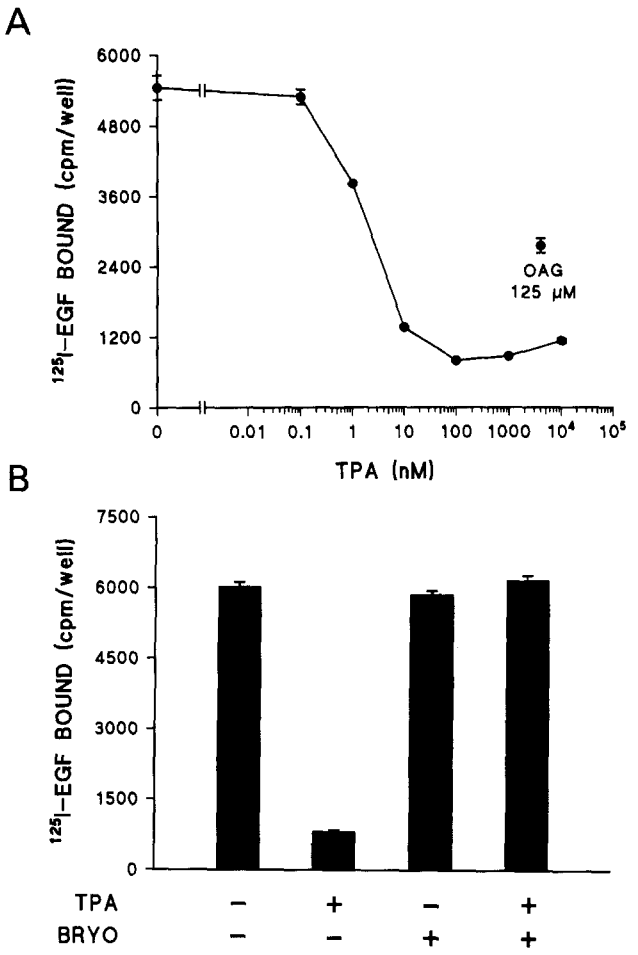

Figure 3. Activation of PKC inhibits ^{125}I-EGF binding in differentiating keratinocytes. (*A*) Primary cultures were exposed to TPA or OAG at the indicated concentrations for 1 h; control cultures received 0.1% DMSO. (*B*) Inactivation of PKC with bryostatin blocks TPA's effect on ^{125}I-EGF binding. Cultures were pre-treated with 60 nM bryostatin for 6 h before the addition of TPA (100 nM). In both experiments, cells were grown in 0.12 mM Ca^{2+} medium for 20 h before treatment with TPA or OAG. Similar results were obtained in additional experiments performed in 0.05 mM Ca^{2+} medium. Data points are means from triplicate dishes, ±SEM.

ing ^{125}I-EGF binding (Fig. 3 *B*). Similarly, bryostatin blocks the inhibitory effect of TPA on K1 mRNA expression (Fig. 2 *B*). Combined, these data suggest that both TPA and OAG inhibit Ca^{2+}-mediated accumulation of K1 mRNA by activating PKC.

Activation of PKC Blocks K1 mRNA Expression in Differentiating Keratinocytes

In an earlier study from this laboratory, K1 mRNA was rapidly depleted in mouse skin following application of TPA (Toftgard et al., 1985), suggesting that activation of the PKC pathway can influence the maintenance of K1 expression in differentiating spinous cells. To study the molecular basis for this response, differentiation was induced in vitro by growing primary keratinocytes in 0.12 mM Ca^{2+} medium for 20 h. Cultures were then exposed to reagents for an additional 8 h and total RNA isolated for Northern blot analysis. At doses ≥10 nM TPA markedly reduces the steady-state level of K1 mRNA in differentiating keratinocytes; in contrast, there is little change in the level of mRNAs encoding K14 and GAPDH (Fig. 4 *A*). The loss of K1 mRNA is coupled to a similar reduction in K10 mRNA (data not shown). K1 mRNA is also reduced in differentiating keratinocytes exposed to OAG (Fig. 4 *B*), suggesting that this response occurs as a result of PKC activation. Additional support for this hypothesis is provided by the observation that TPA fails to inhibit K1 mRNA expression in differentiating keratinocytes treated with bryostatin to inactivate PKC (Fig. 4 *C*).

Inhibition of K1 mRNA Expression by TPA Requires Protein and RNA Synthesis

Cultures were treated with metabolic inhibitors to further

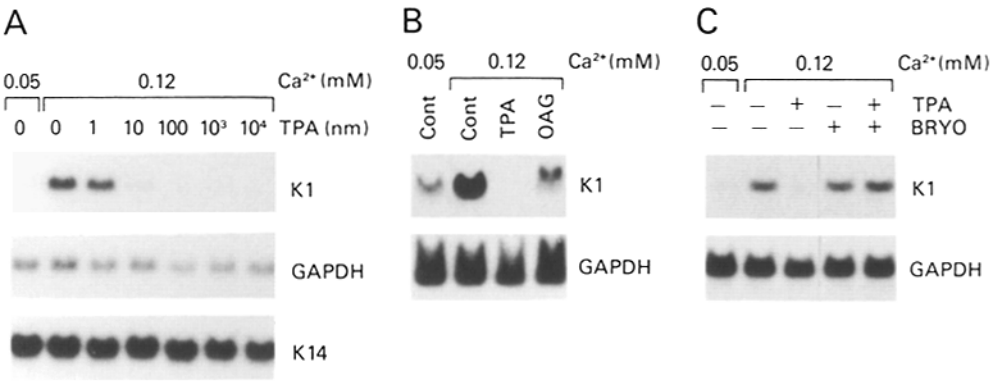

Figure 4. PKC activation selectively represses K1 mRNA in differentiating keratinocytes. Terminal differentiation was induced by growing cultures in medium with 0.12 mM Ca^{2+} for 20–24 h; PKC activators were added for an additional 8 h and total RNA isolated. (*A*) TPA blocks K1 mRNA expression in a dose-dependent manner. (*B*) The PKC activator OAG (125 μM) blocks K1 mRNA expression; TPA was used at a concentration of 50 nM. (The slower mobility of the K1 band in the fourth lane appears to be a running artifact since it was not detected in other northerns from OAG-treated cultures.) (*C*) Inactivation of PKC with bryostatin blocks TPA's effect on K1 expression. Cultures were pretreated with 60 nM bryostatin for 6 h to inactivate PKC; TPA concentration was 100 nM. Similar results were obtained in two independent experiments.

characterize the mechanism by which TPA represses K1 mRNA. Exposure of differentiating keratinocytes to cycloheximide or actinomycin D alone results in reduced steady-state levels of K1 mRNA (Fig. 5), consistent with the proposed requirement for a protein factor in Ca^{2+}-dependent expression of this transcript (Yuspa et al., 1989). In cultures treated with either inhibitor, TPA fails to further reduce the level of K1 mRNA; in contrast, there is a marked reduction of K1 mRNA in control cultures exposed to TPA alone (Fig. 5). These results indicate that TPA-mediated down-modulation of K1 mRNA in differentiating keratinocytes is dependent on protein and RNA synthesis.

Activation of PKC Selectively Reduces K1 mRNA Stability and Gene Transcription

Since transcripts encoding structural proteins generally have a long half-life (Medford et al., 1983) the rapid loss of K1 mRNA in response to TPA suggested an effect on transcript stability. To test this possibility RNA was analyzed from differentiating keratinocytes treated with DMSO, actinomycin D, or TPA for 2, 4, and 8 h. The short-lived c-*myc* transcript was undetectable after a 2-h exposure to actinomycin D (data not shown), indicating that transcription was effectively blocked. The steady-state level of K1, K14, and GAPDH transcripts was slightly reduced in control (DMSO-treated) cultures during the course of this experiment (Fig. 6). In cultures treated with actinomycin D for 8 h, K1 mRNA was reduced by 54% (Fig. 6 *B*). The loss of K1 mRNA was much more rapid in TPA-treated cultures: after a 4-h exposure, transcript abundance was reduced to 18% of the initial level with a further reduction to 8% at 8 h (Fig. 6 *B*). These findings suggest that activation of PKC destabilizes K1 transcripts. Since actinomycin D blocked TPA's inhibitory effect on K1 mRNA expression (Fig. 5), the actual half-life of K1 transcripts could not be determined by concurrent exposure to both agents and may be even shorter than indicated in Fig. 6. The reduction of K1 mRNA stability by TPA appears to be selective: K14 transcript levels were not reduced relative to controls while the effect on GAPDH abundance was modest (Fig. 6 *B*). These findings indicate that the rapid loss of K1 transcripts in TPA-treated keratinocytes occurs at least partly through a reduction of K1 mRNA stability.

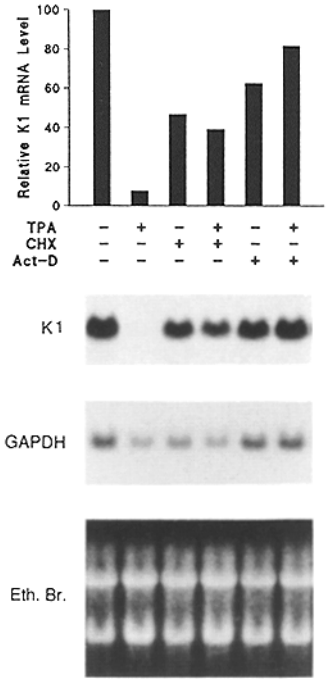

Figure 5. Down-modulation of K1 mRNA by TPA requires protein and RNA synthesis. Differentiating keratinocytes, grown in 0.12 mM Ca^{2+} medium for 24 h, were exposed to the indicated agents for an additional 8 h and total RNA isolated. TPA was used at a concentration of 100 nM; cycloheximide at 20 μg/ml; actinomycin D at 0.1 μg/ml. mRNA was separated in a 1% agarose gel and uniformity of RNA loading confirmed by staining with ethidium bromide. Intensity of the K1 mRNA signals was determined by laser densitometry and values normalized to the DMSO-treated control (100%).

Nuclear run-on analysis was performed to determine whether TPA also influences the transcription rate of the K1 gene. DMSO or TPA was added to cultures of differentiating keratinocytes, nuclei were isolated and nascent transcripts elongated in vitro (Greenberg and Ziff, 1984). A 2-h exposure to TPA resulted in nearly complete inhibition of K1 gene transcription; in contrast, GAPDH and K14 transcription rates were relatively unaffected by TPA (Fig. 7). Combined, the results of these experiments indicate that PKC activation rapidly blocks K1 mRNA expression in differentiating keratinocytes by selectively reducing both K1 gene transcription and K1 mRNA stability.

PKC Mediates Induction of Late Markers of Keratinocyte Differentiation

The loss of K1 and K10 mRNA in cells treated with PKC acti-

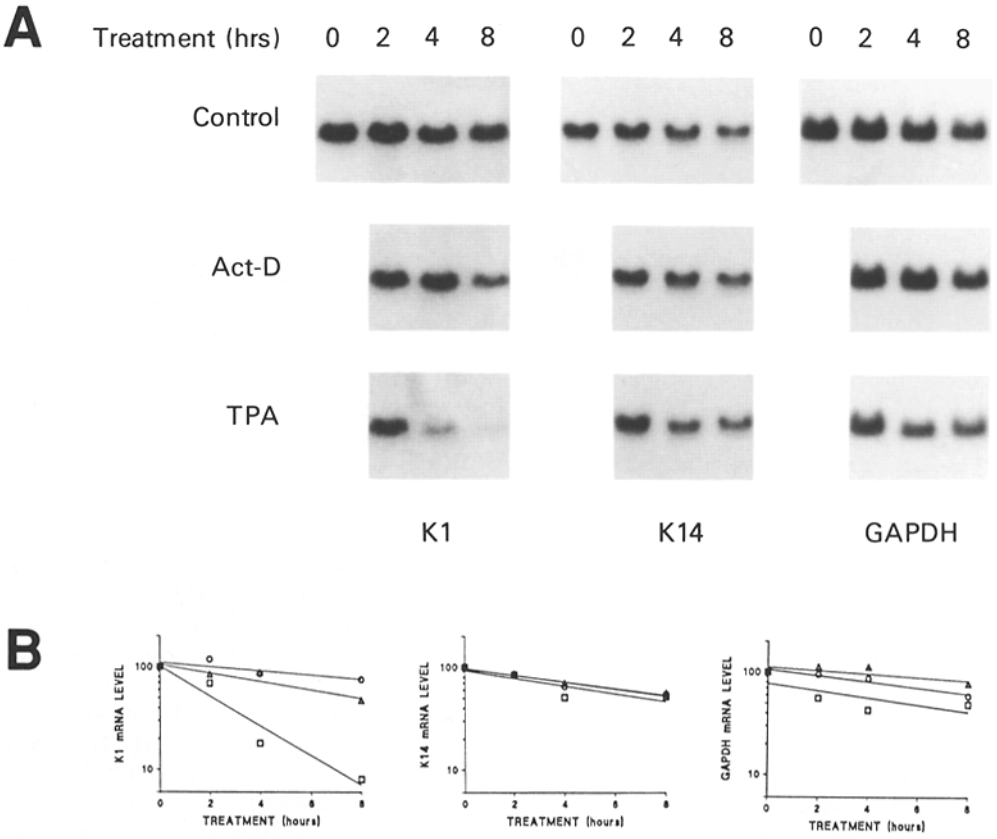

Figure 6. TPA selectively reduces K1 transcript stability. (*A*) Northern blot analysis. (*B*) Transcript levels quantified by densitometric analysis: (○) 0.1% DMSO; (△) 0.5 μg/ml actinomycin-D; (□) 100 nM TPA. Agents were added directly to cultures of differentiating keratinocytes and cells were harvested for RNA isolation at the time of additions (t = 0), and again after 2, 4, and 8 h of treatment. Only 8% of the initial level (t = 0) of K1 mRNA remained after an 8-h exposure to TPA, while 46% remained in cultures treated with actinomycin D. Similar results were obtained in two independent experiments.

vators mimics the changes seen in spinous cell markers during the transition to the granular cell phenotype. The following experiments were designed to determine if the same signal could regulate induction of granular cell markers during this transition. Both loricrin and filaggrin are induced in vitro when keratinocytes are grown in 0.12 mM Ca^{2+} medium for 36 h; exposure to OAG further enhances expression of these markers (Fig. 8). Bryostatin blocks induction of loricrin and filaggrin in the presence or absence of OAG (Fig. 8), and the selective PKC inhibitor GF 109203X (Toullec et al., 1991) also inhibits Ca^{2+}-dependent expression of both proteins (A. A. Dlugosz, A. Dharia, and S. H. Yuspa, manuscript in preparation). These results suggest that PKC is required for Ca^{2+}-mediated induction of loricrin and filaggrin, as well as the enhanced expression of these granular cell markers by OAG. Surprisingly, the expression of loricrin and filaggrin protein was not enhanced in cultures exposed to TPA and at certain doses was inhibited (data not shown). This may be related to extremely efficient induction of transglutaminase and cornified envelopes by TPA with cross-linked proteins being highly insoluble (Mehrel et al., 1990).

Northern blot analysis was performed to examine the effects of OAG and TPA on granular cell markers at the

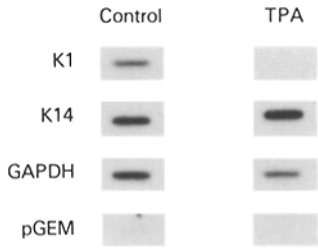

Figure 7. TPA selectively represses K1 gene transcription. Nuclei were isolated from differentiating keratinocytes, grown in 0.12 mM Ca^{2+} medium for 20 h, that had been exposed to 0.1% DMSO (control) or 100 nM TPA for an additional 2 h. Nascent transcripts were elongated in vitro and hybridized for 2.5 d at 42°C to the indicated cDNAs immobilized on nitrocellulose. pGEM represents pGEM 7Zf(+) plasmid DNA used as a negative control. Final wash stringency was 0.2× SSC, 0.1% SDS at 65°C followed by treatment with 5 μg/ml RNAse A in 2× SSC, (37°C, 45 min) before autoradiography. Similar results were obtained after TPA exposures of three and four hours in two additional experiments.

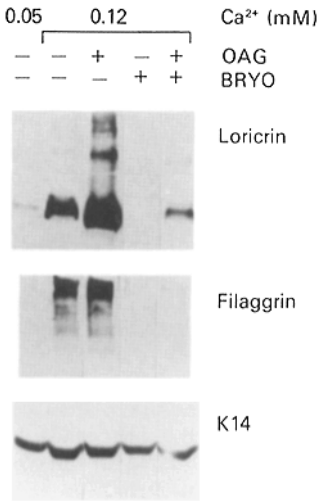

Figure 8. Ca^{2+}-mediated induction of loricrin and filaggrin is PKC dependent. Primary keratinocytes were grown in 0.12 mM Ca^{2+} medium for 36 h with the indicated additions and total cell lysates analyzed by western blotting. Non-adjacent lanes from a single blot were combined to produce this figure. The concentration of OAG was 125 μM; bryostatin 60 nM. Ca^{2+}-mediated expression of loricrin and filaggrin was blocked by bryostatin and enhanced by OAG in two independent experiments.

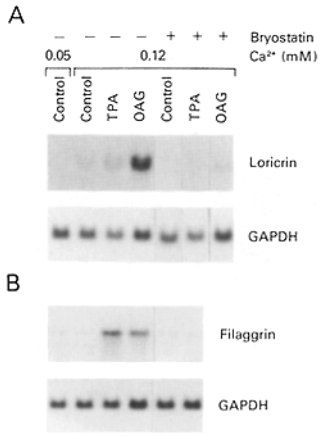

Figure 9. PKC activation enhances expression of mRNA encoding the granular cell differentiation markers loricrin and filaggrin. Primary keratinocytes were cultured in 0.12 mM Ca^{2+} medium for 24 h with the indicated agents. TPA concentration was 10 nM, OAG 125 μM, and bryostatin 60 nM. Control cultures contain low levels of mRNA for both markers at this relatively early time point following Ca^{2+} shift. Expression of loricrin and/or filaggrin mRNA was enhanced by OAG or TPA in two separate experiments.

mRNA level. In cells exposed to 0.12 mM Ca^{2+} for 24 h, OAG caused a striking increase in loricrin mRNA, while 10 nM TPA induced a modest enhancement relative to controls (Fig. 9 *A*). Expression of filaggrin transcripts was also enhanced by activation of PKC, and in this case OAG and TPA elicited similar increases (Fig. 9 *B*). Bryostatin blocked the enhanced expression of both granular cell mRNAs (Fig. 9). Along with our findings at the protein level (Fig. 8), these results strongly implicate PKC activation in the upregulation of loricrin and filaggrin gene expression. In summary, our data indicate that activation of PKC can coordinately repress the spinous cell markers K1 and K10 while at the same time inducing the granular cell markers loricrin and filaggrin.

Discussion

Keratinocyte differentiation is induced in vitro by exposure to specific extracellular Ca^{2+} concentrations (Yuspa et al., 1989), and the correlation between differentiation and elevated Ca^{2+} in mammalian epidermis (Menon and Elias, 1991; Menon et al., 1985; Malmquist et al., 1984) suggests that this ion also regulates keratinocyte differentiation in vivo. The recent finding that raising extracellular Ca^{2+} induces graded increases in phosphatidylinositol metabolism and diacylglycerol levels in cultured keratinocytes (Lee and Yuspa, 1991) suggested that PKC was activated during Ca^{2+}-mediated differentiation. In the present study we have explored the potential role of PKC as a regulator of keratinocyte differentiation using an in vitro model system. Activation of PKC in cultured epidermal keratinocytes results in repression of transcripts for the early differentiation markers K1 and K10 coupled to induction of mRNAs encoding the late markers loricrin and filaggrin, a phenotype expressed by granular cells in vivo. These responses reflect PKC-mediated changes at transcriptional and posttranscriptional levels. Our findings identify PKC as a fundamental regulator of keratinocyte differentiation coordinating both positive and negative changes in gene expression which occur at a specific stage in this process.

In previous studies TPA was reported to have opposite effects on different markers of keratinocyte differentiation: transglutaminase activity and cornification were induced (Yuspa et al., 1982; Parkinson et al., 1984) whereas K1 and K10 expression were blocked (Roop et al., 1987; Molloy and Laskin, 1987; Toftgard et al., 1985). These observations are entirely consistent with the hypothesis, based on our present findings, that PKC selectively triggers a late stage of keratinocyte differentiation in which early marker expression is actively repressed. The rapid down-modulation of K1 mRNA by TPA is the result of a selective reduction in K1 mRNA stability (Fig. 6) and transcription (Fig. 7). Our results strongly support previous work indicating that expression of certain keratins is regulated at the posttranscriptional level (Tyner and Fuchs, 1986). Transcript destabilization may be required for the rapid disappearance of K1 transcripts in cells entering the granular compartment of epidermis (Roop et al., 1988; Stoler et al., 1988). TPA influences mRNA stability in several other systems, either increasing (Weber et al., 1989; Wager and Assoian, 1990) or decreasing (Saceda et al., 1991; Brooks et al., 1991; Choi et al., 1991; Zhu et al., 1991) transcript half-life. As in this report (Fig. 5) a protein factor appears to be involved in several cases. These observations raise the possibility that susceptibility to TPA-mediated degradation (or stabilization) defines a set of transcripts the same way that transcriptional activation by TPA defines a set of genes. As described for other modulators of transcript stability (reviewed in Cleveland and Yen, 1989), mRNAs may contain recognition sequences analogous to TPA-response elements of DNA, with distinct protein factors influencing transcript stability. Expression of mRNAs encoding functionally linked proteins could thus be coordinately regulated at the posttranscriptional level. Cultured murine epidermal keratinocytes may provide a useful model for testing this hypothesis.

In addition to its effect on K1 mRNA stability, TPA rapidly inhibits transcription of the K1 gene (Fig. 7). Potential mechanisms include TPA-mediated inhibition of a transcriptional activator or induction of a transcriptional repressor. Consistent with the first possibility, phosphorylation of myogenin by PKC blocks binding to muscle-specific enhancer elements (Eric Olson, University of Texas M.D. Anderson Medical Center; personal communication) and may be responsible for TPA's ability to inhibit marker expression in cultured myotubes (Choi et al., 1991; Zhu et al., 1991). However, master regulators of keratinocyte differentiation analogous to those described for muscle cells have yet to be described. Binding of positive-acting factors to target sequences may also be blocked by complex formation with other regulatory proteins, as has been reported for AP-1 and the activated glucocorticoid or retinoic acid receptors (Angel and Karin, 1991). Furthermore, protein-protein interactions may inhibit transactivation without influencing DNA-binding: this is the proposed mechanism by which the adenovirus E1A protein blocks AP-1-mediated transcription (Offringa et al., 1990). Investigation of these possibilities awaits characterization of positive-acting factors involved in K1 expression.

An alternative mechanism by which TPA could block K1 transcription is through induction of a transcriptional repressor. TPA increases c-*fos* and c-*jun* mRNA in cultured keratinocytes (Dotto et al., 1986) and may elevate the level of AP-1, which can function as a transcriptional repressor (Takimoto et al., 1989; Distel et al., 1987) in addition to its well-characterized role as a transcriptional activator (Angel

and Karin, 1991). Furthermore, Fos can repress transcription independent of AP-1 by binding to CArG elements (Gius et al., 1990). Analysis of regulatory sequences located 3′ to the human K1 gene has revealed an AP-1 site at nucleotides 224-230 (C. A. Huff, S. H. Yuspa, and D. Rosenthal, manuscript in press) but the functional significance of this element has not been established. A role for AP-1 as a positive regulator of keratin expression has, however, been reported: the first intron of the human K18 gene contains a functional AP-1 site (Oshima et al., 1990).

AP-2 binding sites have been reported upstream of the human K5, K14, K1, and K6b genes, and in the case of K14 this element appears to be necessary, although not sufficient, for gene expression (Leask et al., 1990, 1991). Factors which bind to these sites have been detected in a restricted number of tissues and cell types, including mouse, human, and *Xenopus* keratinocytes (Leask et al., 1991; Snape et al., 1991). AP-2 elements may provide additional targets for regulation of keratin gene expression by PKC, since AP-2 activity is elevated in cells treated with TPA, cyclic AMP, or retinoic acid (Luscher et al., 1989; Imagawa et al., 1987). Understanding the role of PKC in regulating keratin gene transcription awaits further analysis of relevant *cis*-acting elements and protein factors with which they interact.

In contrast to the inhibitory effect on K1 and K10, activation of PKC enhances expression of loricrin and filaggrin at both the mRNA and protein level. Treatment with bryostatin blocks the increased abundance of these transcripts in response to TPA or OAG as well as their baseline induction by Ca^{2+}, suggesting that expression of granular cell markers is a PKC-dependent process. In preliminary experiments, TPA did not induce filaggrin mRNA in basal cell keratinocyte cultures (grown in medium with 0.05 mM Ca^{2+}), suggesting that both Ca^{2+} and PKC activation are required for expression of this granular cell marker. Evidence for an obligatory role for Ca^{2+} was obtained using the intracellular calcium chelator BAPTA, which completely blocks expression of differentiation markers in cultured murine epidermal keratinocytes (L. Li and S. H. Yuspa, unpublished data). Furthermore, Ca^{2+}-sensitive regulatory sequences controlling expression of the human K1 gene have recently been described (C. A. Huff, S. H. Yuspa, and D. Rosenthal, manuscript in press). Noteworthy in light of a proposed corequirement for Ca^{2+} and PKC in regulating transcription, a VL30 enhancer element has recently been described which is dependent on Ca^{2+} for its responsiveness to TPA or EGF (Lenormand et al., 1992).

TPA is a less potent inducer of loricrin mRNA than OAG while these two agents are equally effective at inducing filaggrin transcripts (Fig. 9). These results suggest subtle differences in the regulation of individual late differentiation markers by PKC. TPA and OAG have previously been reported to elicit different responses, both qualitatively and quantitatively, in other cell types (Komorowski and Tsang, 1990) but the basis for these differences has not been determined. Since PKC consists of a family of isozymes (Stabel and Parker, 1991) which appear to have distinct functions (Otte and Moon, 1992; Gusovsky and Gutkind, 1991), one potential explanation is selective (or preferential) activation of specific PKC isozymes by different activators. Consistent with this possibility, interferon α increases diacylglycerol levels in Daudi cells and activates PKC ϵ but not PKC α, whereas both isoforms are activated by TPA (Pfeffer et al., 1991). We have recently reported expression of mRNA encoding PKC α, δ, ϵ, ζ, and η in cultured keratinocytes (Dlugosz et al., 1992) and the presence of each of these isoforms has been confirmed at the protein level, with the exception of PKC ϵ (unpublished data). Experiments aimed at defining the role of individual PKC isozymes in keratinocyte differentiation are currently underway.

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