Protein Kinase C Regulates Keratinocyte Transglutaminase (TGK) Gene Expression in Cultured Primary Mouse Epidermal Keratinocytes Induced to Terminally Differentiate by Calcium

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During the final stage of epidermal differentiation, activation of keratinocyte transglutaminase results in covalent cross-linking of a variety of proteins to form highly protective cornified cell envelopes. We have studied the regulation of keratinocyte transglutaminase (TGK) gene expression in murine epidermal keratinocytes induced to terminally differentiate in vitro by increasing the level of extracellular Ca++ or treatment with the protein kinase C (PKC) activator 12-O-tetradecanoylphorbol-13-acetate (TPA). Raising extracellular Ca++ induces squamous differentiation of cultured keratinocytes and elicits a concentration-dependent increase in the expression of TGK mRNA; keratinocytes grown for 24 h in 0.12 mM Ca++ medium express ~12 times as much TGK mRNA as basal cells (grown in 0.05 mM Ca++ medium), whereas cultures exposed to 1.4 mM Ca++ express ~17 times as much. TPA induces squamous differentiation and TGK mRNA even in basal keratinocyte cultures grown in 0.05 mM Ca++ medium, suggesting that expression of this differentiation marker is regulated by the PKC signaling pathway.

The barrier function of mammalian skin can be attributed largely to the stratum corneum, a protective layer of terminally differentiated keratinocytes that provides the interface between organism and external environment. The stratum corneum is the most superficial of four cellular compartments in the epidermis, each expressing a unique pattern of keratinocyte differentiation markers. The keratin pairs K5/K14 and K1/K10 are expressed in basal and spinous cells, respectively; loricrin, filaggrin, and involucrin are expressed in granular cells [1,2]. During the final stages of epidermal differentiation, epidermal transglutaminase generates ε-(γ-glutamyl)lysine bonds that cross-link substrates such as loricrin and involucrin to form a highly protective cornified cell envelope (reviewed in [3,4]).

The analysis of keratinocytes isolated from mouse and human epidermis has identified Ca++ as a key regulator of terminal differentiation in vitro [5]. Keratinocytes require medium with a reduced extracellular Ca++ concentration (0.05 mM) to maintain a proliferative, basal cell-like phenotype [6]. Raising Ca++ in the medium to 0.12 mM triggers stepwise changes in gene expression similar to those observed in epidermis: induction of the structural markers K1 and K10 is followed by the appearance of loricin and filaggrin [7]. Epidermal transglutaminase is also activated in 0.12 mM Ca++ medium; however, higher transglutaminase activity is detected when cells are exposed to medium containing 1.4 mM Ca++ [8]. Combined with these in vitro findings, the demonstration of a Ca++ gradient in mouse and human epidermis, with elevated Ca++ levels in the innermost cell layers [9-11], suggests that Ca++ provides a physiologic signal for keratinocyte differentiation.

12-O-tetradecanoylphorabol-13-acetate (TPA) is a potent inducer of epidermal transglutaminase activity and cornified envelopes both in vitro and in vivo [8,12,13], indicating that pharmacologic activation of PKC can trigger the terminal stage of keratinocyte differentiation. Furthermore, cultured keratinocytes grown in the presence of elevated extracellular Ca++ exhibit increased levels of inositol phosphates [14-16], intracellular Ca++ [17], and diacylglycerol [16,18], suggesting that PKC is also involved in Ca++-mediated keratinocyte differentiation. Consistent with this hypothesis, Ca++-dependent accumulation of loricin and filaggrin is blocked in cells where PKC has been inactivated, whereas expression of mRNA encoding these markers is enhanced by PKC activators [19].

In this report, we have used pharmacologic agents combined with changes in extracellular Ca++ to determine the involvement of PKC...

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Abbreviation: TGK, keratinocyte transglutaminase.
in regulating keratinocyte transglutaminase (TGK) gene expression in vitro. Our findings indicate that activation of PKC is both necessary and sufficient to induce TGK gene expression in cultured keratinocytes, strongly supporting the concept that late stages of epidermal differentiation are regulated through Ca++-dependent activation of the PKC pathway.

**MATERIALS AND METHODS**

**Cell Culture**  Primary keratinocytes were isolated from skin of newborn BALB/c mice as previously described [6] and cultured in 60-mm tissue-culture dishes (Costar, Cambridge, MA). Cells were grown in Eagle's minimum essential medium (without Ca++ or Mg++) supplemented with 8% fetal calf serum [6] and 0.25% penicillin-streptomycin solution (GIBCO, Grand Island, NY). The Ca++ concentration in the medium was adjusted to specific levels by adding an appropriate volume of 280 mM CaCl₂. Cells grown in medium with 0.05 mM Ca++ exhibited a basal cell-like phenotype; terminal differentiation was induced by exposure to medium with higher extracellular Ca++ concentrations, as described in the figure legends.

**Northern Blot and Nuclear Run-On Analysis**  Total RNA was isolated from primary keratinocytes by lysis in 4 M guanidine isothiocyanate followed by ultracentrifugation through a cesium chloride gradient [20]. In some experiments, poly(A)⁺ RNA was isolated directly from cell lysates as previously described [21]. Transcripts were separated by electrophoresis in a 1% agarose/0.66 M formaldehyde gel, transferred to reinforced nitrocellulose (BA-S NC; Schleicher & Schuell, Keene, NH), and baked at 80°C in a vacuum oven [7]. Conditions for Northern blotting were previously described [22], except that pre-hybridization and hybridization solutions contained 6 × sodium citrate/sodium chloride buffer (SSC), 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml sheared salmon-sperm DNA, and 50% formamide [23]. TGK mRNA was identified by hybridization to rat or human TGK cDNA fragments (−2 kb) [24], kindly provided by Dr. Robert Rice (University of California, Davis, CA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a full-length rat cDNA in pUC18 [25]. Probes were labeled with [32P]-CTP by nick-translation. Filters were routinely washed at a maximum stringency of 0.5 × SSC with 0.2% SDS, at 65°C. Transcript levels were quantified using a scanning laser densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The abundance of TGK mRNA was normalized to GAPDH transcripts.

![Figure 1](image1.png)  **Figure 1.** The steady-state level of TGK mRNA is increased during Ca++-mediated keratinocyte differentiation. A) Primary keratinocytes grown as basal cells (in 0.05 mM Ca++ medium) were exposed to media with the indicated Ca++ concentration for 24 h to induce terminal differentiation. Poly(A)⁺ RNA was analyzed by Northern blotting using TGK and GAPDH cDNA probes, as described in Materials and Methods. The TGK transcript is ~3.3 kb in length based on residual ribosomal RNA as markers for 5 kb and 2 kb. NB indicates RNA isolated from newborn mouse epidermis used as a loading control. Similar results were obtained in two additional experiments. B) Quantitation of Ca++-induced TGK mRNA expression by scanning densitometry. TGK mRNA at each Ca++ concentration was normalized to GAPDH and the fold-induction expressed relative to the level in basal cell cultures. Values represent data from three experiments ± SEM.

![Figure 2](image2.png)  **Figure 2.** TPA induces TGK mRNA expression. A) Keratinocyte cultures grown in 0.05 mM Ca++ medium were exposed to TPA at the indicated concentrations for 4 h. Total RNA was isolated and Northern blot analysis performed as described in Materials and Methods. B) Scanning densitometry of Northern blot in (A). The level of TGK mRNA was normalized to GAPDH and changes in abundance expressed relative to the DMSO-treated control.

**125I-Epidermal Growth Factor (EGF) Binding Assay**  Binding of 125I-labeled EGF to primary keratinocytes was determined as previously described [27], with minor modifications. Following treatment, cells were washed twice with ice-cold binding buffer (Dulbecco's minimum essential medium with 50 mM N,N-bis[2-hydroxyethyl]-2-amino sulfonic acid [pH 7.4] and 1 mg/ml bovine serum albumin), then incubated with 125I-EGF (0.1 μCl/ml/well; NEN) ± 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. Cultures were washed four times with ice-cold binding buffer and cells harvested in two 500 μl volumes of lysis buffer (0.1 M tris, pH 7.4, 0.5% SDS, 1 mM EDTA). Radioactivity was determined by scintillation counting: non-specific binding was <200 cpm in all groups.


![Ethidium Bromide](image)

**Figure 3.** Time-course analysis of TGK mRNA induction in primary keratinocytes exposed to Ca++, TPA, or Ca++ + TPA combined. TPA concentration was 100 nM. Total RNA was isolated at the start of the experiment and after the indicated treatment intervals. Based on densitometric analysis, maximum induction of TGK mRNA in response to Ca++, TPA, and Ca++ + TPA was 8.8-fold (at 10 h), 179-fold (at 6 h) and 201-fold (at 6 h), respectively. Similar results were obtained in an additional experiment with fewer timepoints.

**Reagents**

TPA was obtained from LC Services (Woburn, MA), bryostatin (bryostatin 1) was a gift from Dr. George Pettit (Arizona State University, Tempe, AZ), and the PKC inhibitor GF 109203X was kindly provided by Dr. Jorge Kirilovsky (Glaxo Pharmaceuticals, Les Ulis, France). TPA and GF 109203X stocks were in dimethylsulfoxide; bryostatin was in ethanol.

**RESULTS**

**Ca++ and TPA Induce TGK mRNA in Cultured Keratinocytes**

TGK gene expression was examined in primary mouse epidermal keratinocytes growing as basal cells (in 0.05 mM Ca++ medium) or induced to terminally differentiate in medium containing 0.12 or 1.4 mM Ca++. Although expression of structural differentiation markers is restricted to extracellular Ca++ ranging from 0.1 to 0.3 mM [7], a squamous phenotype is triggered at all Ca++ concentrations ≥ 0.1 mM Ca++ [8]. In primary epidermal keratinocyte cultures, extracellular Ca++ induces TGK mRNA in a concentration-dependent manner. Based on densitometric scanning of Northern blots, keratinocytes grown for 24 h in 0.12 mM Ca++ medium express ~12 times as much TGK mRNA as basal cells (grown in 0.05 mM Ca++ medium), whereas cultures grown in 1.4 mM Ca++ express ~17 times as much (Fig 1). In individual experiments, TGK mRNA expression in 1.4 mM Ca++ medium was always higher (by 37% ± 13% SEM, n = 3) than in 0.12 mM Ca++ medium. 1.4 mM Ca++ medium was used in subsequent experiments to induce terminal differentiation, unless otherwise indicated.

Because PKC activators can substitute for Ca++ to induce epidermal transglutaminase activity [28], we examined the effects of TPA on TGK gene expression in vitro. In this experiment, treatment of basal cell cultures with TPA for 4 h caused a maximal ninefold increase in the level of TGK mRNA with a 50% effective dose (ED50) of ~10 mM (Fig 2). Similar to Ca++, (Fig 1B), the level of TGK mRNA induction by TPA was variable between experiments, probably due to different baseline levels of TGK mRNA in basal cell cultures. In four experiments, induction of TGK mRNA following exposure to 100 nM TPA for 4 h varied from ninefold to 78-fold (average = 46-fold ± 14, SEM). Combined, these results indicate that keratinocyte differentiation induced by either Ca++ or TPA is associated with increased expression of TGK mRNA.

To further explore the regulation of TGK gene expression, keratinocytes were treated with Ca++ or TPA alone or with both agents combined. Total RNA was isolated at the start of the experiment and after 1, 2, 4, 6, 10, and 24 h of treatment. Based on densitometric scanning of Northern blots in this experiment, TGK mRNA was increased a maximum 179-fold in TPA-treated cultures (at 6 h) and 8.8-fold in Ca++-treated cultures (at 10 h). In cultures treated with Ca++ + TPA for 6 h, TGK transcripts were elevated to a maximum level 201 times greater than in basal cell cultures (Fig 3). Although the initial induction of TGK mRNA was detected earlier in response to TPA than Ca++ (2 vs 4 h), TGK mRNA expression was reduced to baseline after 24 h exposure to TPA both in the presence or absence of Ca++ (Fig 3). In contrast, induction of TGK mRNA in response to Ca++ alone remained maximally elevated through 24 h (Fig 3).

**TGK mRNA Expression is Regulated at the Transcriptional Level**

Nuclear run-on analysis was performed to determine whether Ca++ and TPA affect TGK gene expression at the transcriptional level. For these experiments, cultures were induced to differentiate in medium with 0.12 mM Ca++ rather than 1.4 mM Ca++ because of difficulties in the isolation of nuclei from keratinocytes grown at high extracellular Ca++ concentrations. The relative transcription rate of the TGK gene is increased during 0.12 mM Ca++-induced keratinocyte differentiation (Fig 4A). In differentiating keratinocytes exposed to TPA for 3 h, TGK transcription is induced to a greater extent (Fig 4B). These findings suggest that accumulation of TGK mRNA in response to Ca++ or TPA is due at least in part to increased transcription of the TGK gene.

**TGK mRNA Induction is Dependent on Protein Synthesis**

To further characterize the molecular regulation of TGK gene expression, primary keratinocytes were exposed for 6 h to TPA or solvent, ± Ca++, in the presence or absence of 20 μg/ml cycloheximide. This concentration of cycloheximide has previously been shown to block protein synthesis by > 95% in cultured mouse epidermal keratinocytes [28]. Accumulation of TGK mRNA is blocked by cycloheximide in cultures treated with TPA, Ca++, or Ca++ + TPA (Fig 5), suggesting that a protein factor is required for the induction of TGK gene expression by both stimuli. GAPDH mRNA levels do not appear to be influenced by cycloheximide (Fig 5).

**Induction of TGK mRNA by Ca++ or TPA Requires PKC**

The induction of TGK mRNA by TPA even in basal cell keratinocyte cultures suggested that expression of this marker in response to Ca++ occurs through activation of the PKC signaling pathway. To test this hypothesis, TGK mRNA expression was examined in cultures treated with the selective PKC inhibitor GF
109203X [29]. The effect of this compound on inhibition of 125I-EGF binding by TPA [30] was examined as a measure of its ability to block PKC-mediated events in cultured keratinocytes. GF 109203X blocked this response to TPA in a dose-dependent manner, with control levels of 125I-EGF binding achieved at 0.1 µM GF 109203X (Fig 6A), suggesting that this agent is an effective PKC inhibitor in cultured keratinocytes. GF 109203X also inhibited TPA- and Ca++-mediated accumulation of TGK mRNA (Fig 6B,C). Based on results of scanning densitometry, the approximate IC50 for blocking TGK mRNA induced by either Ca++ or TPA are within the same order of magnitude.

As an additional approach to assess PKC's involvement in TGK gene expression, keratinocytes were pre-treated with 60 nM bryostatin to functionally inactivate PKC. This agent selectively blocks a variety of PKC-mediated responses in cultured keratinocytes [31–33]. Both TPA- and Ca++-mediated induction of TGK mRNA is blocked in keratinocytes pre-treated with bryostatin (Fig 7). Similar to the results with GF 109203X (Fig 6), the approximate IC50 for blocking TGK mRNA expression in TPA- or Ca++-treated cultures are within the same order of magnitude, based on densitometric analysis. Combined, the results of these experiments suggest that activation of the PKC pathway is required for the induction of TGK gene expression in response to either TPA or Ca++.

**DISCUSSION**

Keratinocyte differentiation can be induced in vitro by raising extracellular Ca++ from 0.05 to ≥0.1 mM, and elevated Ca++ levels have been demonstrated in vitro in differentiating layers of epidermis, suggesting that this ion provides a physiologic signal regulating epidermal differentiation. Based on analysis of TGK gene expression as a marker for keratinocyte differentiation, the results of this study suggest that PKC mediates the Ca++ signal for this aspect of kerati-
PKC REGULATES TGK GENE EXPRESSION

Although the role of Ca++ in triggering keratinocyte-specific gene expression is well established, the biochemical pathways regulating this complex process have not been fully elucidated. Induction of epidermal transglutaminase activity and cornified envelope formation by TPA, both in vitro and in vivo [8], suggested that the terminal stage of keratinocyte differentiation is regulated by PKC. The observation that increased extracellular Ca++ is associated with elevated cellular diacylglycerol levels [16,18] provided a biochemical link coupling the Ca++ signal for keratinocyte differentiation to the PKC signaling pathway. The ability to block Ca++-mediated TGK gene expression by inactivating PKC in mouse (Figs 6, 7) as well as human [35] keratinocytes strongly suggests that Ca++ induces this differentiation marker through the PKC signaling pathway. Along with the upregulation of TGK gene expression by PKC, the additional influence of other factors is likely to be important in determining the final level of expression. For example, whereas dexamethasone also induces TGK mRNA in cultured keratinocytes, retinoic acid blocks accumulation of TGK mRNA in response to TPA, Ca++, or dexamethasone [36].

There are several distinctions between TPA- and Ca++-induced TGK gene expression (Fig 3) that may be related to the way these two stimuli influence PKC: 1) TPA induces TGK mRNA more rapidly than Ca++. The slower induction of TGK mRNA in Ca++-treated cells may reflect slow accumulation of endogenous diacylglycerol with a corresponding delay in PKC activation [16]. In contrast, TPA directly activates PKC resulting in expression of certain genes (e.g., fos and jun) within minutes, others (e.g., transin and collagenase) within hours [37]. 2) TGK mRNA is induced to a higher level by TPA than Ca++. Consistent with this result, phospholipids are far more effective activators of PKC than physiologic agents (hormones, growth factors, etc.) that generate endogenous diacylglycerols via hydrolysis of membrane phospholipids. 3) Despite the more rapid appearance and greater induction of TGK mRNA in response to TPA, by 24 h transcript levels return to baseline, whereas in Ca++-treated keratinocytes TGK mRNA remains maximally elevated. Transient induction of TGK mRNA by TPA may be due to PKC downregulation [38] resulting in a limited activation phase in TPA-treated cultures and brief induction of TGK mRNA. Unlike TPA, Ca++ does not downregulate PKC in cultured keratinocytes* [39], permitting prolonged activation of the PKC pathway in response to this signal. Relative to Ca++-treated cultures, the low level of TGK mRNA in cultures exposed for 24 h to Ca++ + TPA supports the notion that PKC is required for prolonged expression of TGK mRNA in response to Ca++.

Inhibition of Ca++- and TPA-mediated TGK mRNA induction by cycloheximide (Fig 5) suggests that TGK gene expression is dependent on a protein factor(s) that is regulated by PKC. This result may explain findings reported in an earlier study in which TPA-mediated transglutaminase activity was blocked by cycloheximide [28]. Recent documentation of an AP-1 site upstream of the human TGK initiation codon [40] suggests that PKC may regulate transcription of the TGK gene via Fos and Jun family members. Supporting this notion, TPA increases luciferase activity in cells transfected with a rabbit TGK promoter reporter construct, and this increase is blocked by bryostatin [41]. Additional studies are needed to determine whether PKC-mediated changes in transcript stability also contribute to the increased levels of TGK mRNA seen in TPA- and Ca++-treated cultures.

Ten PKC isoforms have been described [42]; five of these, PKC \( \alpha, \delta, \epsilon, \zeta, \) and \( \eta \), are expressed in cultured mouse epidermal keratinocytes* [43]. Studies in several cell types indicate that different PKC isoforms have distinct functions [44,45]. The restricted expression of PKC \( \eta \) in granular cells suggests that this isoform is involved in regulating late stages of epidermal differentiation [46]. Experiments are currently underway to directly assess the role of individual PKC isoforms in regulating keratinocyte differentiation in vitro.

The results of this study may have implications for the treatment of a variety of skin disorders. For example, marked thickening of the stratum corneum is frequently a prominent feature in psoriasis. This change may be secondary to the increased TGK mRNA levels and transglutaminase activity that have been reported in lesional skin. Identification of expression suggests that selective inhibitors of this signaling pathway may be useful in treating dermatoses characterized by hyperkeratosis.

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