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Overexpression of protein kinase C-α in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2 and TNF-α expression but not tumor promotion

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SUMMARY

Protein kinase Cα (PKCα) is one of six PKC isoforms expressed in keratinocytes of mouse epidermis. To gain an understanding of the role of epidermal PKCα, we have localized its expression to specific cells of normal mouse skin and examined the effect of keratin 5 (K5) promoter directed expression of PKCα in transgenic mice. In normal mouse skin, PKCα was extensively expressed in the outer root sheath (ORS) keratinocytes of the anagen hair follicle and weakly expressed in keratinocytes of interfollicular epidermis. K5-targeted expression of PKCα to epidermal basal keratinocytes and follicular ORS keratinocytes resulted in a tenfold increase in epidermal PKCα. K5-PKCα mice exhibited no abnormalities in keratinocyte growth and differentiation in the epidermis. However, a single topical treatment with the PKC activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) resulted in a striking inflammatory response characterized by edema and extensive epidermal infiltration of neutrophils that formed intraepidermal microabscesses in the epidermis. Compared to TPA-treated wild-type mice, the epidermis of TPA-treated K5-PKCα mice displayed increased expression of cyclooxygenase-2 (COX-2), the neutrophil chemotactic factor macrophage inflammatory protein-2 (MIP-2) mRNA and the proinflammatory cytokine TNFα mRNA but not IL-6 or IL-1α mRNA. To determine if K5-PKCα mice display an altered response to TPA-promotion, 7,12-dimethylbenz[a]anthracene-initiated K5-PKCα mice and wild-type mice were promoted with TPA. No differences in papilloma incidence or multiplicity were observed between K5-PKCα mice and wild-type littermates. These results demonstrate that the overexpression of PKCα in epidermis increases the expression of specific proinflammatory mediators and induces cutaneous inflammation but has little to no effect on epidermal differentiation, proliferation or TPA tumor promotion.

Key words: Neutrophil, Keratinocyte, Cytokine

INTRODUCTION

Epidermis is a stratified squamous epithelium composed of keratinocytes that undergo a highly coordinated program of sequential changes in gene expression during differentiation. Keratinocytes progress from active proliferating basal cells through morphologically distinct spinous and granular cells, ending in the nonviable cornified stratum corneum which serves as a barrier to environmental insult (Fuchs, 1990; Watt, 1989). In addition to providing this important barrier function, keratinocytes respond to environmental stimuli such as ultraviolet radiation, toxicants, irritants, tumor promoters and wounding by producing and/or releasing certain inflammatory mediators, chemotactic factors and adhesion molecules (Bos and Kapsenberg, 1993). Keratinocytes are a rich source of numerous cytokines including IL-1α, IL-6, IL-7, IL-8, GM-CSF and TNF-α as well as various prostaglandins and leukotrienes (Furstenberger, 1990; Matsue et al., 1992; Ruzicka, 1989). Numerous cutaneous inflammatory conditions and skin disease states, including psoriasis and irritant-induced dermatitis are associated with alterations in cytokine production (Enk and Katz, 1992; Kulke et al., 1996).

Protein kinase C (PKC) is a multigene family of serine/threonine kinases that is involved in the transduction of extracellular signals conveyed by growth factors, neurotransmitters, hormones and other biological molecules (Nishizuka, 1992). To date, eleven PKC isoforms have been characterized (Nishizuka, 1992). It has been demonstrated that PKCα, δ, ε, η, μ and ζ are expressed in human (Fisher et al., 1993) and mouse epidermis in vivo (Gschwendt et al., 1992; Leibersperger et al., 1991; Mills et al., 1992; Osada et al., 1993; Wang et al., 1993; Rennecke et al., 1999) and in isolated primary keratinocytes in vitro (Dlugosz et al., 1992). Among these six isoforms expressed in keratinocytes, each isoform is thought to play a specific role in keratinocyte function. PKCs are major cellular receptors for the potent mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Ashendel et al., 1983; Blumberg, 1988; Niedel et al., 1983), and it is thought that

TPA mediates many of its tumor promotional, inflammatory and proliferative/differentiative effects through the activation of one or more PKC isoforms. Alterations in PKC isoform expression and increased levels of the endogenous PKC activator, diacylglyerol (DAG) occur in psoriatic human epidermis and it has been suggested that PKC plays a role in the pathophysiology of this disease (Fisher et al., 1993; Reynolds et al., 1993). Treatment of mouse skin or cultured keratinocytes with TPA produces a pleotropic response. For example, TPA treatment increases the expression of proinflammatory mediators such as TNF-α (Wilmer et al., 1994), IL-1α (Oberyszyn et al., 1993), GM-CSF (Vasunia et al., 1994) and cyclooxygenase-2 (COX-2) (Scholz et al., 1995). TPA treatment also induces hyperplasia, stimulates cornified envelope formation, as well as loricrin and filaggrin expression, but decreases early differentiation proteins keratin 1 (K1) and keratin 10 (K10) (Tofgard et al., 1985; Dlugosz and Yuspa, 1993; Lee et al., 1997). DAGs are intracellular second messengers that are produced by receptor mediated hydrolysis of phospholipids and transmit their signal through the activation of PKC. When applied to skin, DAGs produce alterations in epidermal growth and are also effective tumor promoters (Smart et al., 1988, 1989). Collectively, these results support a function for PKC isoforms signaling pathways in a wide spectra of cutaneous biology, however little is known about the function of individual PKC isoforms in proliferation, differentiation, inflammation and tumor promotion.

Of the PKC isoforms expressed in the epidermis, PKCη is one of the best characterized. PKCη is expressed in the suprabasal layers of the epidermis (Osada et al., 1993) and recent evidence suggests that PKCη is involved in keratinocyte differentiation through the regulation of transglutaminase (Ohba et al., 1998). Transgenic mice that overexpress PKCδ in the basal keratinocytes of the epidermis have been characterized and these mice are resistant to TPA-induced tumor promotion (Reddig et al., 1999a). With respect to PKCα, it has been suggested that PKCα plays a role in keratinocyte differentiation and phorbol-ester induced tumor promotion (Dlugosz et al., 1994; Lee et al., 1997; Mills et al., 1992). However, forced expression of PKCα in primary human keratinocytes has no effect on differentiation or proliferation (Ohba et al., 1998). In Madin-Darby canine kidney cells, PKCα regulates phorbol ester stimulated arachidonate release suggesting a role for PKCα in the regulation of PLA_2 and inflammation (Godson et al., 1993). Nonetheless, the function of PKCα within epidermal keratinocytes as well as the localization of PKCα expression within the epidermis is not well characterized. In order to gain some insight into the possible role of PKCα in cutaneous inflammation, keratinocyte proliferation, differentiation, and phorbol-ester induced tumor promotion, we made transgenic mice in which we used a keratin 5 promoter to target PKCα protein expression to basal keratinocytes of epidermis and outer root sheath keratinocytes of the hair follicle.

MATERIALS AND METHODS

Production and characterization of K5-PKCα transgenic mice

The keratin 5 promoter and its 3′ untranslated region were removed from the bovine KIII/KIV minilocus (Blessing et al., 1993, gift from the Deutsches Krebsforschungzentrum, Heidelberg, Germany) with *Kpn*I, cloned into a pUC19 vector and PKCα cDNA (gift from Dr Koichi Suzuki of Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) was subcloned into *Xho*I and *Sal*I site of downstream of K5 promoter. Transgenic mice were generated by pronuclear microinjection of fertilized C57BL/C3H F2 oocytes with a *Kpn*I linear 9.7 kb DNA fragment of the pK5-PKCα construct. Genomic DNA was isolated from the tails of potential founder mice, digested with *Eco*RI to release 2.7 kb PKCα transgene and subjected to Southern blot analysis to identify transgene positive mice. Hybridization was conducted with ^{32}P-labeled PKCα cDNA probe. For PCR analysis, the 5′-primer was a K5 promoter sequence (5′-GCCTATTCG-CTGCCAAGAGAT-3′), and the 3′-primer was a PKCα cDNA sequence (5′-AAACCCCCAGATGAAGTCGGTG-3′). The amplified 513 bp fragment spanned the junction between the K5 promoter and the PKCα cDNA and was only detected in transgenic mice. The founder mice which were positive in both PCR analysis and Southern blot analysis were backcrossed to C57BL/6. Female C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC). All mice were housed in the Biological Research Facility at North Carolina State University.

Preparation of epidermal or whole skin homogenates and western blot analysis

The hair of the dorsal skin of the mice was clipped with electric clippers at least two days before each experiment. Mice were killed and the dorsal shaved skin was removed. The whole skin was spread on an index card and immediately immersed in liquid nitrogen. The epidermis of the frozen skin was scraped from the dermis with a surgical scalpel, placed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 2 mM PMSF, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 0.05% (v/v) Triton X-100). For whole skin homogenate preparation, the whole skin was diced and placed in homogenization buffer. The samples were homogenized on ice using a Polytron and centrifuged at 10^5 ***g*** for 35 minutes at 4°C. Protein concentration in the supernatants was determined by the method of Lowry using bovine serum albumin as the standard. Equal amount of protein from each sample was separated on 8% Tris-glycine polyacrylamide gel (Novex San Diego, CA) and electrophorectically transferred to an Immobilon P membrane (Millipore Corporation, Bedford, MA). The membrane was incubated with a polyclonal PKCα antibody (1:500) (Oxford Biomedical Research Inc., Oxford, MI) or with a polyclonal COX-2 antibody (1:1000) (Cayman Chemical Company, Ann Arbor, MI). Donkey anti-rabbit IgG conjugated with horseradish peroxidase (1:2500) (Amersham Corp., Arlington Heights, IL) was used as a secondary antibody. Detection was accomplished with a chemiluminescence system and the resulting bands on the exposed films were quantitated by laser densitometry.

Immunohistochemical staining

Dorsal skin was excised and fixed in 10% neutral buffered formalin for 2 hours and 30 minutes, and then kept in phosphate-buffered saline until it was processed. Skin sections (5 μm) were deparaffinized in xylene and rehydrated through graded ethanol followed by an endogenous peroxidase quench with 3% H_2O_2. The sections were blocked with automation buffer (Biomeda Corp, Foster City, CA) containing 1% BSA, 1% nonfat dry milk and 1% normal goat serum for 30 minutes. The sections were incubated with polyclonal anti-PKCα antibody (1:250) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), COX-2 antibody (1:800), K1 antibody (1:2000), K10 antibody (1:2000), K5 antibody (1:2000), involucrin antibody (1:2000) or loricrin antibody (1:2000) at room temperature for 18 hours. K1, K10, K5, involucrin and loricrin rabbit polyclonal antibodies were purchased from Berkeley Antibody Company (Richmond, CA). Slides were washed twice with automation buffer and incubated with biotinylated goat anti-rabbit IgG (1:500) (Boehringer Mannheim, Indianapolis, IN) at room temperature for 1 hour. After washing with automation buffer, the sections were incubated with horseradish peroxidase-conjugated streptavidin (BioGenex Laboratories, San

Ramon, CA) (1:20 dilution) for 30 minutes at room temperature. The avidin/biotin-peroxidase complexes were visualized by incubation with 3, 3′-diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and mounted. All antibodies used for the detection of differentiation markers produced staining in the appropriate epidermal compartment and no staining was observed when the secondary antibody was used without primary antibody.

5-Bromo-2′-deoxyuridine (BrdU) immunostaining

Transgenic mice and wild-type littermates (7-9 weeks of age) were treated with either 200 μl acetone or TPA in 200 μl acetone on shaved dorsal skin. Seventeen hours after the last TPA treatment, mice were injected intraperitoneally with BrdU (100 mg/kg body weight) (Sigma Chemical Corp., St. Louis, MO) in 100 μl phosphate buffered saline. One hour later, mice were killed and the dorsal skin was removed. The skin was fixed in 10% neutral buffered formalin for 20 hours and transferred to 70% ethanol for 20 hours. The skin sections were embedded in paraffin, and 5 μm sections were incubated with anti-BrdU IgG (Becton Dickinson, San Jose, CA) and processed as described by Oh and Smart (1996). At least 1000 interfollicular basal cells were counted per slide.

RNA isolation and northern blot analysis

The dorsal skin was removed and floated, dermis side down, on Hanks' balanced salt solution with 0.25% trypsin for 1 hour at 37°C. Epidermal sheets were peeled from the underlying dermis, and RNA was isolated from epidermal cell suspension by acid guanidnium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Thirty μg of each RNA sample was electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane and hybridized with ^{32}P-labeled IL-1α, TNFα, or IL-6 cDNA (gifts from Dr P. F. Johnson, National Cancer Institute, Frederick, MD) or MIP-2 cDNA (gift from Dr B. Sherry, Laboratory of Cytokine Biology, Picower Institute for Medical Research, Manhasset, NY) at 65°C overnight. Membranes were reprobed with 7S RNA cDNA (gift from Dr A. Balmain, Beatson Institute for Cancer Research, Glasgow, UK) to confirm that equal amounts of RNA were loaded. All cDNAs were radiolabeled by random priming using a random priming kit (Gibco BRL) and [α-^{32}P]dCTP (3000 Ci per mmol, 10 μCi per μl) and cDNAs were purified from unincorporated [α-^{32}P]dCTP using Push Columns (Stratagene, La Jolla, CA).

Isolation and culture of primary keratinocytes

Primary keratinocytes were isolated from 2-3 day old newborn wild-type littermates and transgenic mice by trypsinization overnight at 4°C. Isolated epidermal cells were plated at 6×10^6 cells per 60 mm plate in Ca^{2+} free EMEM supplemented with 10% fetal bovine serum and 4 ng/ml epidermal growth factor for 4 hours to enhance keratinocyte attachment. Cultures were then gently washed with Mg^{2+} and Ca^{2+} free PBS to remove any remaining calcium and unattached cells, and then refed with low calcium medium (Ca^{2+} free EMEM supplemented with 4% Chelex-treated fetal bovine serum, 10 ng/ml hEGF, 100 u/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml Fungizone, with added calcium chloride to a final concentration of 0.05 mM). Medium was changed daily. After 5 days in culture, both wild-type and K5-PKCα primary keratinocytes were treated with either DMSO alone or 100 nM TPA for 5 hours, RNA was isolated from treated keratinocytes by acid guanidnium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987).

Tumor studies on mouse skin

The dorsal skin of female K5-PKCα transgenic mice and wild-type littermates at 7-10 weeks of age were clipped with electric clippers one week before initiation. All mice were initiated with a topical application of 200 nmol DMBA in 200 μl acetone under yellow light. The first initiation-promotion experiment was carried out using F3 generation of wild-type (n=12) and K5-PKCα transgenic (n=14) mice. One week after initiation, mice were treated topically three times a week with 5 nmol TPA in 200 μl acetone. An additional initiation-promotion experiment was conducted on F4 generation of wild-type (n=40) and K5-PKCα (n=18) transgenic mice. One week after initiation with a topical application of 200 nmol DMBA in 200 μl acetone, mice were treated topically three times a week with 3 nmol TPA in 200 μl acetone. The number of papilloma and the percentage of tumor bearing mice were determined every week.

RESULTS

PKCα protein is present in extracts prepared from mouse epidermis and from primary newborn mouse keratinocytes (Dlugosz et al., 1992; Mills et al., 1992). However, the actual location of PKCα within specific cells of the epidermis and its appendages (hair follicle and sebaceous gland) is not known.

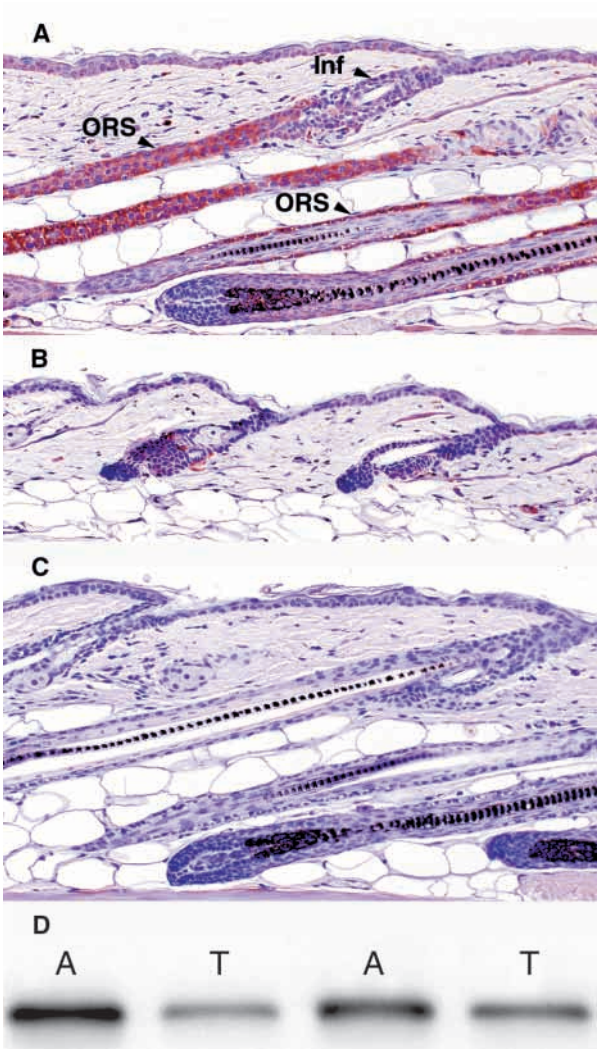

Fig. 1. Expression of PKCα in epidermis and outer root sheath. PKCα immunostaining of C57BL/6 mouse skin; (A) anagen hair follicle, ORS=outer root sheath, Inf=infundibulum; (B) telogen hair follicle; (C) pre-incubation of PKCα antibody with the PKCα antigen, and subsequent use for immunohistochemical staining; (D) western blot analysis for PKCα in skin with anagen (A) hair follicles and skin with telogen (T) hair follicles. Skin containing anagen hair follicles was identified by the presence (anagen-10 weeks of age) or absence (telogen- 6 weeks of age) of pigmented skin.

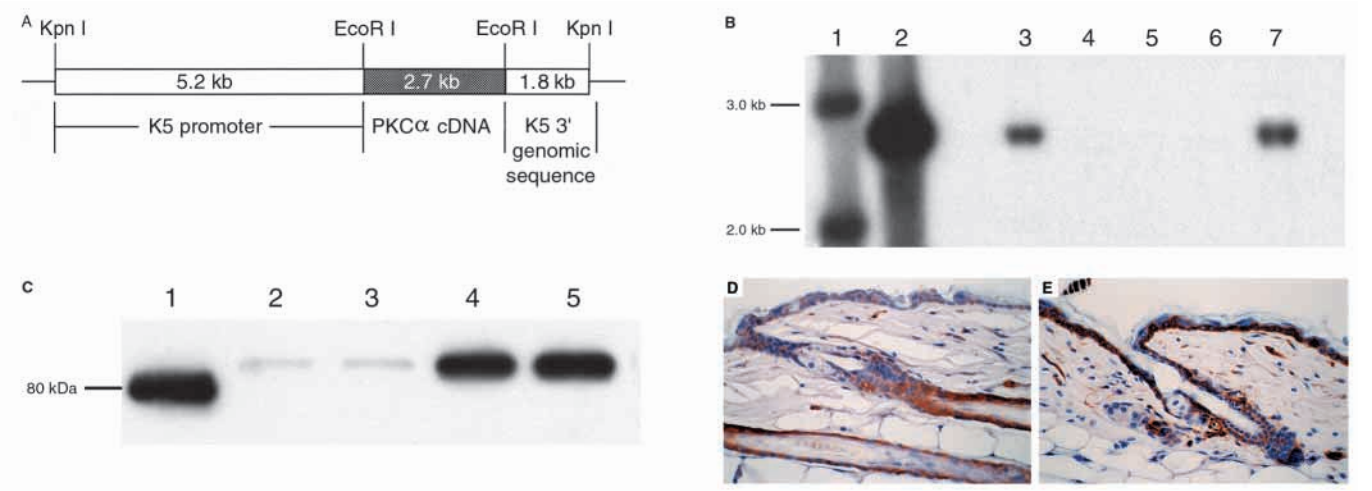

Fig. 2. pK5-PKCα transgene construct and identification of K5-PKCα transgenic mice. (A) Schematic diagram of the pK5-PKCα gene construct. This construct contains a 5.2 kb K5 promoter, a 2.7 kb PKCα cDNA sequence and a 1.8 kb K5 3′ genomic sequence. This linearized 9.7 kb fragment was microinjected into C57BL/C3H fertilized oocytes; (B) Southern blot analysis of *Eco*RI digested mouse tail DNA. Lane 1, 1 kb DNA molecular marker; lane 2, pK5-PKCα construct digested with *Eco*RI; lane 3, transgenic mouse; lane 4-6, wild-type littermates; lane 7, transgenic mouse; (C) western blot analysis of PKCα. Lane 1, PKCα isozyme standard (baculovirus expressed protein is not phosphorylated so its migration is faster than native protein); lane 2 and 3, wild-type littermates; lane 4 and 5, K5-PKCα (line 7) transgenic mice; (D) Immunohistochemical staining of PKCα in wild-type mouse skin; (E) Immunohistochemical staining of PKCα in K5-PKCα (line 7) transgenic mouse skin.

In order to localize the expression of PKCα in mouse skin, immunohistochemical staining using a PKCα isoform specific antibody was performed on mouse skin containing anagen or telogen hair follicles. As shown in Fig. 1A, PKCα is highly expressed in the outer root sheath (ORS) keratinocytes of the lower anagen hair follicle and is weakly expressed in both the basal and suprabasal keratinocytes of epidermis. Within the anagen hair follicle, PKCα is highly expressed in the ORS keratinocytes and is weakly expressed in the inner root sheath cells, cuticle, cortex, medulla or matrix cells. Within the ORS cells of the anagen hair follicle there is a clear demarcation of PKCα expression. As shown in Fig. 1A, ORS keratinocytes below the sebaceous gland abundantly express PKCα while the infundibulum ORS keratinocytes above the sebaceous gland resembles the interfollicular epidermis in that PKCα is weakly expressed in both the basal and suprabasal keratinocytes. Telogen hair follicles display weak staining for PKCα in the outer root sheath of the infundibulum and bulge areas (Fig. 1B). Pre-incubation of PKCα antibody with the PKCα COOH-terminal peptide antigen, and subsequent use for immunohistochemical staining resulted in no detectable staining (Fig. 1C). Western blot analysis of extracts prepared from mouse skin containing predominantly anagen hair follicles demonstrated a 4-fold increase in PKCα levels compared to extracts prepared from mouse skin containing telogen follicles (Fig. 1D). Collectively, these results demonstrate that while PKCα is expressed in the basal and suprabasal keratinocytes of the interfollicular epidermis, it is most abundantly expressed in ORS keratinocytes of the anagen follicle.

To gain an understanding of the role of epidermal PKCα we made transgenic mice in which we targeted the overexpression of PKCα to the epidermal basal keratinocytes and follicular ORS keratinocytes with a keratin 5 (K5) promoter. K5-PKCα transgenic mice were generated by injection of 9.7 kb pK5-PKCα construct (Fig. 2A) into C57BL/C3H fertilized oocytes. The K5 promoter has been previously shown to direct expression of downstream genes to basal keratinocytes of the epidermis and the ORS keratinocytes of hair follicle (Blessing et al., 1993). As shown in Fig. 2B, Southern blot analysis of *Eco*RI digested genomic DNA demonstrated the presence of the transgene in some mice. Seven founder mice were identified and mated with C57BL/6 mice. Founder line 7 expressed highest level of PKCα protein in epidermis and its offspring were used for subsequent studies unless otherwise indicated. As shown in Fig. 2C, western blot analysis demonstrated that epidermal PKCα levels were approximately tenfold greater than that observed in wild-type mice. Immunohistochemical staining of PKCα demonstrated that PKCα transgene was extensively expressed in the basal keratinocytes of the epidermis as well as ORS keratinocytes of the hair follicle (Fig. 2D).

The body size and gross appearance of the skin and hair of K5-PKCα transgenic mice were carefully observed during breeding of the transgenic colony, and no differences were noted when compared to wild-type mice. Immunohistochemical staining for various differentiation markers (keratin 1, keratin 10, involucrin, loricrin) in mouse skin was performed, and no abnormalities in the location or intensity of staining were observed between K5-PKCα transgenic mice and wild-type mice before or after TPA treatment (data not shown). As shown in Table 1, no differences were found between acetone-treated control wild-type and K5-PKCα transgenic mice in epidermal thickness, nucleated cell layers and the number of BrdU S-phase positive keratinocytes, indicating that there were no growth abnormalities in the epidermis of K5-PKCα transgenic mice. However, a single topical treatment with the PKC activator, TPA resulted in a marked increase in epidermal thickness of K5-PKCα transgenic mice compared to the wild-type mice

Table 1. Effects of TPA on epidermal thickness, number of nucleated cell layers and number of BrdU S-phase cells

Mice	Treatment	Epidermal thickness (μm)	Number of nucleated cell layers	BrdU S-phase basal epidermal cells (%)
Wild type	Acetone	16.3±2.5	1-2	4.0±2.6
K5-PKCα	Acetone	17.3±4.6	1-2	4.6±3.1
Wild type	5 nmol TPA	28.2±5.0	2-3	47.7±6.9
K5-PKCα	5 nmol TPA	42.4±11.2*	2-3	26.4±4.3*

Mice (7-9 weeks old; at least 6 animals/group) were treated topically once with either TPA in 200 μl acetone or 200 μl of acetone. Eighteen hours later the dorsal treated area was removed and processed for light microscopy. The epidermal thickness and the number of nucleated cell layers were measured at 10 locations per slide (avoiding areas containing microabscesses) and values averaged. At least 1000 interfollicular basal epidermal cells were counted per skin sample for BrdU S phase cell analysis. Value represents mean ± standard deviation (s.d.). *Significantly different from TPA-treated wild-type mice (P<0.05) as determined by Student's t-test.

(Table 1). While both TPA-treated wild-type and TPA-treated K5-PKCα mice displayed 2-3 nucleated cell layers within the epidermis, the epidermal thickness of TPA-treated K5-PKCα mice increased by 145% compared to only a 75% increase in TPA-treated wild-type mice. This increase in epidermal thickness in the TPA-treated K5-PKCα mice was due to inter- and intracellular epidermal edema. More striking, however, was the dense focal accumulation of neutrophils that formed numerous intraepidermal microabscesses or pustules in the epidermis, infundibulum and sebaceous gland of the TPA-treated K5-PKCα mice (Fig. 3). Microabscesses contained predominantly neutrophils with rare eosinophils and mononuclear leukocytes. The majority of the intraepidermal microabscesses were associated with the follicular infundibulum. As shown in Fig. 3D, some areas of the epidermis displayed extensive neutrophil infiltration. A diffuse dermal inflammation was prominent and similar in both the TPA-treated wild-type and K5-PKCα mice. Neutrophil infiltration into the epidermis and infundibulum could not be detected at 8 hours after TPA application nor was there evidence that TPA was toxic as necrotic or apoptotic keratinocytes were not observed. Neutrophil infiltration appeared at 12 hours, peaked at 18-24 hours, and declined after 48 hours (data not shown). The number of microabscesses formed was increased approximately 7-fold in K5-PKCα mice compared to similarly treated wild-type mice (Table 2). Another transgenic line (line 1) K5-PKCα mice, which expressed 4-fold higher PKCα protein levels in epidermis also displayed a similar accumulation of neutrophils that formed intraepidermal microabscesses, however, the number of microabscesses was intermediate between that observed in line 7 K5-PKCα mice (10-fold increase in PKCα levels) and wild-type mice (Table 2). These data indicate that the enhanced edematous response and the increase in the focal accumulation of neutrophils in the epidermis and infundibulum in the K5-PKCα mice are directly related to the expression and activation of PKCα.

TPA treatment of mouse skin is known to result in the induction of cylooxygenase-2 (COX-2) (Scholz et al., 1995) which metabolizes arachidonic acid to proinflammatory prostaglandins, such as PGE_2 and $PGF_{2\alpha}$, known mediators of vascular dilation and edema. In order to determine whether the overexpression of PKCα in basal keratinocytes influences the expression of COX-2, mice were treated with a single topical dose of TPA. Western blot analysis of epidermal extracts showed that TPA produced a rapid induction of COX-2 in both wild-type and K5-PKCα transgenic mice (Fig. 4A). However, K5-PKCα transgenic mice displayed a striking 10-fold increase in COX-2 induction versus that observed in wild-type mice at 8 hours after TPA application. Immunohistochemical staining for COX-2 in TPA treated mouse skin (8 hours after 5 nmol TPA application) revealed that 48% of the basal keratinocytes expressed COX-2 in K5-PKCα transgenic mice, while COX-2 expression could only be detected in 15% of the basal keratinocytes of wild-type mice (Fig. 4B). These data demonstrate that COX-2 induction is a downstream event of PKCα activation.

Macrophage inflammatory protein (MIP-2) is considered the mouse functional equivalent of human IL-8, and IL-8 is a potent neutrophil chemotactic factor (Baggiolini et al., 1994; Tekamp-Olson et al., 1990; Wolpe et al., 1989). TNFα plays an important role in mediating and amplifying inflammatory responses within the skin and is inducible by TPA treatment in mouse skin (Wilmer et al., 1994). In order to investigate whether PKCα influences the expression of TNFα and MIP-2, both wild-type and K5-PKCα mice were treated with 5 nmol TPA in 200 μl acetone or 200 μl acetone alone. Keratinocytes were isolated from the treated skin area and RNA was extracted and subjected to northern blot analysis. A time course consisting of the following time points 5, 8, 12 and 18 hours revealed that MIP-2 and TNFα were maximally induced at 8 hours (Fig. 5A) and that MIP-2 and TNF-α mRNA levels were 5- and 3-fold higher, respectively, in K5-PKCα mice compared to wild-type littermates. Additional mice were treated with TPA and RNA was extracted at 8 hours after TPA treatment. As shown in Fig. 5B, MIP-2 and TNFα mRNA levels were 3- to 5-fold and 2- to 3-fold higher, respectively, in K5-PKCα

Table 2. Intraepidermal neutrophil microabscess formation after a single application of TPA

Mice	Treatment	Number of the intraepidermal microabscesses/cm length of skin
Wild type	Acetone	0
K5-PKCα transgenic line 7	Acetone	0
K5-PKCα transgenic line 1	Acetone	0
Wild type	TPA 5 nmol	1.0±1.0
K5-PKCα transgenic line 7	TPA 5 nmol	6.8±2.0*
K5-PKCα transgenic line 1	TPA 5 nmol	4.2±1.9*

Mice (7-9 weeks old; at least 4 mice/group) were treated topically once with either TPA in 200 μl acetone or 200 μl of acetone. Eighteen hours later the dorsal treated area was removed and processed for light microscopy. The number of microabscesses were counted and were expressed as the number of microabscesses per cm length of skin section. Value represents the mean ± standard deviation (s.d.). *Significantly different from TPA-treated wild-type mice (P<0.05) as determined by Student's t-test.

Table 3. Effects of multiple TPA application on epidermal thickness, number of nucleated cell layers, number of BrdU S-phase cells, and epidermal and dermal leukocyte infiltration

Mice	Treatment	Epidermal thickness (μm)	Number of nucleated cell layers	BrdU S-phase basal epidermal cells (%)	Number of intraepidermal microabscesses/ cm length of skin	Leukocyte infiltration
Wild type	Acetone	16.3±0.9	1-2	2.8±0.9	0	–
K5-PKCα	Acetone	16.5±1.6	1-2	3.0±1.1	0	–
Wild type	TPA	34.2±2.7	3-4	26.6±3.8	0	+
K5-PKCα	TPA	38.7±3.5	3-4	33.3±6.1	2.5±1.8*	++

Mice (6 animals/group) were treated topically three times a week for four weeks with either 5 nmol TPA in 200 μl acetone or 200 μl of acetone alone. Seventeen hours later, mice were injected intraperitoneally with BrdU (100 mg/kg body weight). One hour later the dorsal treated area was removed and processed for light microscopy. The epidermal thickness and the number of nucleated cell layers were measured at 10 randomly selected locations per slide and the totals averaged. The BrdU S-phase cells was determined by dividing the number of BrdU S-phase positive cell numbers by the total number of basal cells counted (at least 1000 interfollicular basal epidermal cells were counted per skin sample). Leukocyte infiltration slight (+), moderate (++), severe (+++) was characterized by the degree of diffuse infiltration of inflammatory cells in the dermis. Value represents mean ± standard deviation. *Significantly different from wild-type mice ($P<0.05$) as determined by Student's *t*-test.

transgenic mice compared with wild-type littermates (all samples were normalized to 7S RNA signal to control for loading variation). We also examined IL-6 and IL-1α mRNA levels and found that the mRNA levels for both cytokines were similar in the TPA-treated K5-PKCα and wild-type mice (data not shown). These results demonstrate that PKCα influences TPA-induced MIP-2 and TNFα expression in the epidermis. Since this represents the first demonstration that TPA can increase the levels of MIP-2 mRNA in the epidermis we wanted to determine whether such an increase also occurs in primary keratinocytes in culture. Keratinocytes were isolated from newborn wild-type and K5-PKCα mice and the cells were placed in primary culture. After 5 days in culture, keratinocytes were treated with 100 nM TPA and RNA were isolated 5 hours after TPA treatment. As shown in Fig. 5C, TPA increased the levels of MIP-2 mRNA in both wild-type and K5-PKCα keratinocytes, however K5-PKCα transgenic cells displayed increased MIP-2 mRNA levels.

To determine whether multiple treatments with TPA alters keratinocyte growth, formation of microabscesses and PKCα expression, six K5-PKCα mice and six wild-type mice were treated three times weekly with 5 nmol TPA for four weeks and killed 18 hours after the last treatment. As shown in Table 3 the number of BrdU positive S-phase basal keratinocytes and the number of nucleated cells layers were similar in K5-PKCα and wild-type mice. However, the K5-PKCα mice continued to display an increase in intraepidermal microabscess formation compared to wild-type mice and K5-PKCα mice also demonstrated increased infiltration of leukocytes in the dermis (Table 3). Immunohistochemical staining for PKCα demonstrated prominent staining for PKCα in K5-PKCα epidermis and ORS cells, however the intesity of staining for PKCα in the epidermis and ORS cells of TPA treated wild-type mice was diminished compared to acetone treated wild-type mice.

Finally, to determine whether overexpression of PKCα influences TPA-induced tumor promotion, we conducted an initiation-promotion tumor experiment using wild-type and K5-PKCα transgenic mice. One week after initiation with 200 nmol DMBA, all mice were promoted with 5 nmol TPA three times per week for 28 weeks, no significant differences in papilloma number and tumor incidence were found between wild-type mice and K5-PKCα transgenic mice (Fig. 6). To determine if PKCα was down regulated in the epidermis of these mice, which could account for the tumor response in the K5-PKCα mice, mouse skin from wild-type and transgenic mice were collected 24 hours after the last TPA application of the tumor experiment. Epidermis was collected from the treated area and western blot analysis was conducted. PKCα levels in the epidermis of transgenic mice continued to be overexpressed and the levels were at least 10 times greater than the PKCα levels in the epidermis of wild-type mice (data not shown). While PKCα levels were decreased in the TPA-treated wild-type epidermis compared to untreated wild-type epidermis, a detectable signal was always observed. PKCα levels in TPA-treated wild-type mice were decreased compared to untreated wild-type mice. Since K5-PKCα transgenic mice did not display an altered tumor response to 5 nmol TPA treatment, we repeated the initiation-promotion experiment using a lower dose of TPA in an attempt to detect an increased sensitivity to this lower dose of phorbol ester. One week after initiation with 200 nmol DMBA mice were treated with 3 nmol TPA three times a week for 18 weeks. No differences in papilloma number and tumor incidence were observed between wild-type and K5-PKCα transgenic mice (data not shown). Immunohistochemical staining for PKCα in seven papillomas and surrounding skin from K5-PKCα mice revealed that PKCα levels in the papilloma were decreased by approximately 50% in four papillomas compared to the surrounding skin and only weakly detectable in the other three papillomas. PKCα immunohistochemical staining was not detectable in the five wild-type papillomas examined.

DISCUSSION

Our results demonstrate that the expression of PKCα in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation involving the expression of COX-2, MIP-2, and TNFα and the focal accumulation of neutrophils within the epidermis. While similar alterations occurred in the wild-type littermates, the magnitude of the responses were much lower in the wild-type mice compared to the transgenic mice. Taken together, these results indicate that an important function of PKCα in epidermal keratinocytes involves the regulation of the expression of inflammatory

mediators that produce edema and neutrophil infiltration. While the actual elucidation of signaling pathways within the keratinocytes responsible for the increase in COX-2, TNFα and MIP-2 will require additional experiments, it is evident that these events are downstream of PKCα activation. This is the first evidence for a functional role of epidermal PKCα in cutaneous inflammation.

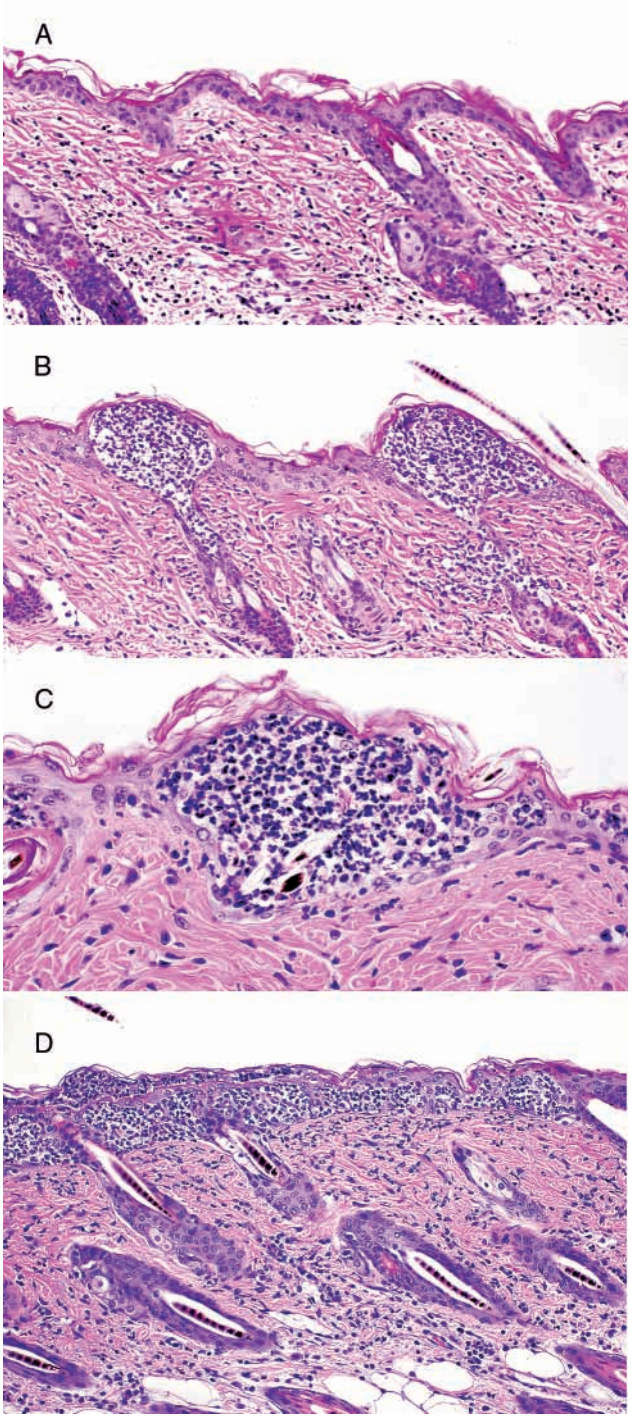

Fig. 3. Intraepidermal neutrophil microabscess formation after a single application of TPA in transgenic mice. Wild-type and transgenic mice were treated with TPA and the skin was removed 18 hours after treatment and fixed in 10% neutral buffered formalin. The hematoxylin-eosin stained sections were prepared for light microscopy. (A) TPA-treated wild-type mouse skin; (B) TPA-treated K5-PKCα mouse skin; (C) Higher magnification of neutrophil microabscess; (D) TPA-treated K5-PKCα mouse skin demonstrating more extensive neutrophil infiltration.

Mouse MIP-2 is a homologue to the human C-X-C chemokine GRO and MIP-2 has been characterized as a potent and major neutrophil chemoattractant, functionally equivalent to human IL-8 (Baggiolini et al., 1994; Tekamp-Olson et al., 1990; Wolpe et al., 1989). To our knowledge, this is the first demonstration that TPA treatment of mouse skin or primary keratinocytes increases epidermal MIP-2 mRNA levels and that PKCα activation is involved. The formation of microabscesses composed of neutrophils is a striking characteristic of the TPA-treated K5-PKCα mice. While we have not shown that MIP-2 is the factor responsible for the accumulation of neutrophils within the epidermis, it highly probable this chemokine is involved. It is interesting that one hallmark of psoriasis in human skin is the focal accumulation of neutrophils to form microabscesses within the upper epidermis (Mehregan et al., 1995). In addition, IL-8 and GRO-α are upregulated in human psoriatic lesions and these chemokines are considered to be critical in the epidermal infiltration of neutrophils (Kulke et al., 1996). IL-8, similar to our findings with MIP-2 in mouse keratinocytes, is induced by TPA treatment of human keratinocytes (Barker et al., 1991). Fisher and coworkers have found that DAGs, the intracellular ligands for PKC, are elevated in psoriatic lesions and this is accompanied by alterations in PKC isoform levels and activity (Fisher et al., 1993; Reynolds et al., 1993). Thus, PKCα may be an important therapeutic target in certain cutaneous inflammatory conditions and K5-PKCα transgenic mice may provide a model to study psoriasis-like inflammation, neutrophil migration and accumulation and the regulation of COX-2, TNF-α and MIP-2 within the epidermis.

It is noteworthy that K5-PKCα transgenic mice did not display any phenotypic differences from wild-type mice in the absence of phorbol ester treatment indicating that PKCα is catalytically inactive within the keratinocyte until stimulated by phorbol ester. While phorbol ester is a pharmacological exogenous activatior of PKC, DAGs are intracellular endogenous second messengers that are produced by receptor

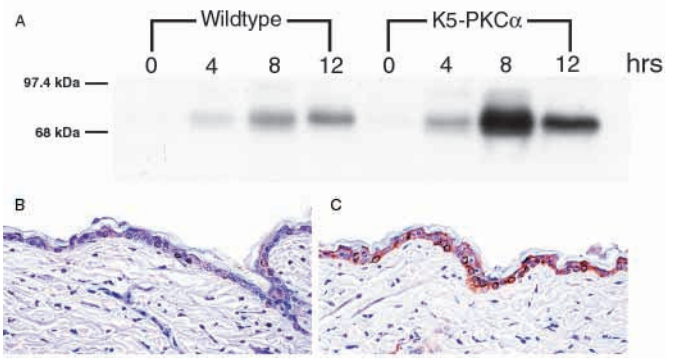

Fig. 4. COX-2 induction in transgenic and wild-type mouse epidermis. (A) Western analysis of COX-2 in epidermis isolated from wild-type and transgenic mice at various times after TPA treatment; (B) Immunohistochemical staining for COX-2 in wild-type mouse skin 8 hours after TPA treatment; (C) Immunohistochemical staining for COX-2 in K5-PKCα mouse skin 8 hours after TPA treatment.

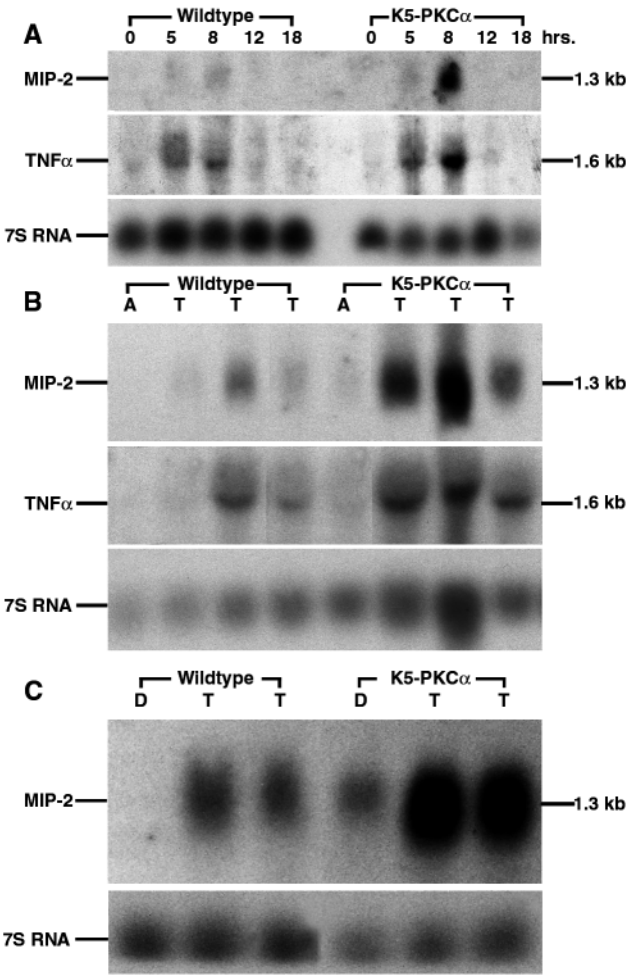

Fig. 5. MIP-2 and TNFα mRNA levels in wild-type and K5-PKCα transgenic mouse epidermis. (A) Epidermal MIP-2 and TNFα mRNA levels at various times after TPA treatment; (B) MIP-2 and TNFα epidermal mRNA levels at 8 hours after TPA application. Four wild-type and four transgenic mice were treated with either acetone (A) or 5 nmol TPA (T) and epidermal RNA was isolated 8 hours later; (C) MIP-2 mRNA induction by TPA in cultured primary keratinocytes. Primary keratinocytes from wild-type and K5-PKCα newborn mice were cultured for 5 days and treated with DMSO or 100 nM TPA and RNA was isolated 5 hour later. Thirty μg and 16 μg of total RNA were loaded from wild-type and K5-PKCα keratinocytes respectively. Membranes were reprobed with 7S RNA cDNA to control for the loading of RNA.

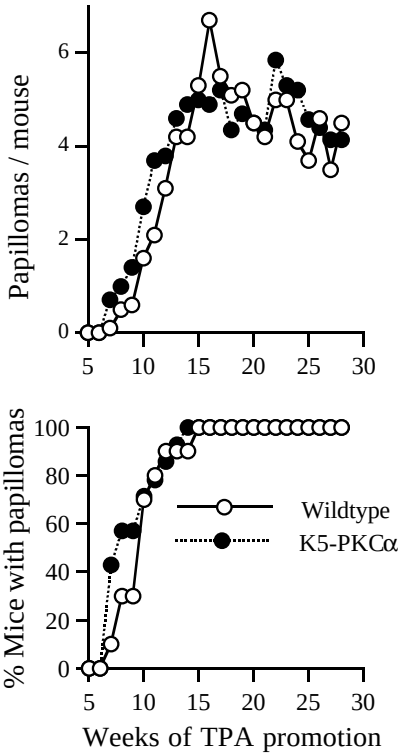

Fig. 6. Initiation promotion study in K5-PKCα transgenic mice and wild-type littermates. Female mice (12-14 /group) were initiated at 7-10 weeks of age with a single topical application of 200 nmol DMBA. Beginning 1 week later, mice were treated topically three times a week with 5 nmol TPA for 28 weeks.

mediated hydrolysis of phospholipids and transmit their signal through activation of PKC. Therefore, it is likely that signals such as certain cytokines and biological molecules that mediate their responses through phospholipid hydrolysis, resulting the production of diacylglycerols would result in the activation of PKCα and cutaneous inflammation.

Our in vivo results indicate that PKCα expression has little to no influence on keratinocyte growth as the number of BrdU positive S-phase epidermal cells were similar in untreated or TPA-treated K5-PKCα mice compared to similarly treated wild-type mice. The only time any difference was noted was after a single application of TPA, in this case we observed a decrease in the number of S-phase cells in the K5-PKCα mice compared to the wild-type mice. This decrease may be related to the robust inflammatory response observed in these mice. Our in vivo results also indicate that the expression of PKCα in the epidermis has no effect on the intensity or location of expression of K1, K10, loricrin or involucrin in untreated or TPA-treated K5-PKCα mice compared to similarly treated wild-type mice. These results indicate that the overexpression of PKCα does not have a major influence on epidermal differentiation and proliferation and are in agreement with recent findings that the overexpression of PKCα in primary human keratinocytes has no effect on differentiation or proliferation (Ohba et al., 1998). However, it is possible that the overexpression of PKCα may alter the expression of other PKC isoforms which may mask an effect of PKCα. PKCα has been proposed to be important in the regulation of the spinous to granular transition and to supress K1 and K10 expression based on studies conducted with newborn primary mouse keratinocytes (Dlugosz and Yuspa, 1993, Lee et al., 1997). Primary cultures of newborn mouse keratinocytes would likely contain epidermal and follicular ORS keratinocytes. Based on our findings that PKCα is extensively expressed in the ORS keratinocytes below the sebaceous gland where K1 and K10 are not expressed, it is possible that the activation of PKCα in this population of cultured newborn keratinocytes may suppress K1 and K10 expression.

Recently, transgenic mice that overexpress PKCδ or PKCε in the basal keratinocytes of their epidermis have been characterized and these mice are resistant to TPA-induced

tumor promotion (Reddig et al., 1999a,b). It was most unexpected that K5-PKCα mice did not display an altered sensitivity to TPA-induced tumor promotion. However, when our observations that the expression of PKCα has little influence on epidermal keratinocyte growth and differentiation are considered, these tumor promotion results are somewhat less surprising. Nevertheless, K5-PKCα mice do demonstrate an enhanced inflammatory response and inflammation is considered to contribute to tumor formation in mouse skin. We have not evaluated the inflammatory response of these mice throughout the tumor experiment, however, we did evaluate the effect of thrice weekly application of TPA for four weeks on PKCα levels and microabscesss formation. While we did observe a significant diminution in the number of microabscesses, epidermal neutrophil infiltration remained increased in the K5-PKCα mice compared to the wild-type mice. In addition, epidermal PKCα continued to be highly expressed in the transgenic mice treated with 5 nmol TPA three times a week for 4 weeks or for 28 weeks. This result indicates the lack of anticipated increased papilloma formation in the K5-PKCα mice is not due to the down regulation of PKCα in the epidermis. However, PKCα levels were diminished in the papillomas of K5-PKCα mice compared to surrounding epidermis and it is possible that the down-regulation of PKCα is permissive for papilloma growth as previously suggested (Hansen et al., 1990; Mills et al., 1992; Wang et al., 1994). Our results indicate that PKCα overexpression does not influence tumor promotion in C57BL/6 mice when targeted to the basal keratincoytes and suggests that other PKC isoforms, such as PKC δ, ε, η and μ may be the critical targets in TPA-induced tumor promotion. Alternatively it is possible that the level of PKCα in the wild-type mice is sufficient to contribute to the promotional response and the overexpression of PKCα has no additional effect. Experiments utilizing conditional PKCα knockout mice may address this question.

In conclusion, our studies provide new fundamental insight into the role of PKCα in the epidermis and indicate that a principal function of this PKC isoform in epidermal keratinocytes involves the regulation of the expression of inflammatory mediators that produce edema and neutrophil infiltration. PKCα may be an especially good therapeutic target for cutaneous diseases involving inflammation since it has a potent influence on the inflammatory process but has little to no effect on epidermal growth and differentiation.

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