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Protein Kinase C-α Coordinately Regulates Cytosolic Phospholipase A_2 Activity and the Expression of Cyclooxygenase-2 through Different Mechanisms in Mouse Keratinocytes

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ABSTRACT

Transgenic mice (K5-PKCα) in which the keratin 5 promoter directs the expression of protein kinase C-α (PKCα) to epidermal keratinocytes display a 10-fold increase in PKCα protein in their epidermis and alterations in phorbol ester-induced cutaneous inflammation [*J Cell Science* 1999;**112:**3497–3506]. In the current study, we have used these K5-PKCα mice to examine the role of PKCα in keratinocyte phospholipid metabolism/eicosanoid production and cutaneous inflammation. Primary keratinocytes from wild-type and transgenic mice were prelabeled in culture with [^{3}H]arachidonic acid (AA) and subsequently treated with TPA. Compared with wild-type keratinocytes, K5-PKCα keratinocytes displayed a 2-fold increase in AA release. TPA treatment resulted in the phosphorylation of cPLA$_2$. PKC inhibitors GF-109203X or H7, but not mitogen-activated protein/extracellular signal-regulated protein kinase (MEK) inhibitor PD 98059, could inhibit phosphorylation and AA release. Topical 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment of K5-PKCα mice resulted in a 5-fold increase in epidermal COX-2 induction and a 2- to 3-fold increase in prostaglandin (PG) E_2 levels above that observed in TPA-treated wild-type mice. PD 98059, GF-109203X, or H7 could block cyclooxygenase-2 (COX-2) induction by TPA. Because C/EBPβ, a basic leucine zipper transcription factor, can be activated via a PKCα/mitogen-activated protein kinase pathway and can influence COX-2 expression, we examined whether C/EBPβ is involved in TPA-induced epidermal COX-2 expression. TPA-induced COX-2 expression was similar in C/EBPβ nullizygous and wild-type mice. In summary, our results indicate that epidermal PKCα coordinately regulates cPLA$_2$ activity and COX-2 expression resulting in increased levels of AA and PGE_2. Furthermore, PKCα-induced AA release and cPLA$_2$ phosphorylation are independent of MEK, whereas PKCα-induced COX-2 expression and PGE_2 production are MEK-dependent and C/EBPβ-independent events.

Elevated levels of eicosanoids (prostaglandins and leukotrienes) have been shown to be associated with a wide array of dermatological disease, such as psoriasis, UV-induced erythema, and contact sensitivity (Ruzicka, 1989). In the mouse skin model of carcinogenesis, elevated levels of eicosanoids have been suggested to be important for tumor promotion (Fischer, 1997), and cyclooxygenase (COX)-1 and COX-2 nullizygous mice are resistant to dimethylbenz(a)anthracene/TPA-induced tumorigenesis (Langenbach et al., 1999). TPA treatment of mouse skin results in a pleiotropic response involving alterations in keratinocyte growth/differentiation and cutaneous inflammation. TPA-induced inflammation is associated with alterations in cytokine production, epidermal COX-2 induction, and the increased production of certain prostaglandins and leukotrienes. These effects are thought to be mediated by protein kinase C (PKC); however, keratinocytes express six isoforms of PKC, and it is not clear which PKC isoforms participate in these various responses induced by TPA.

Recently we have produced transgenic mice in which we used a keratin 5 promoter to direct the expression of PKCα to the epidermis (Wang and Smart, 1999). These mice display a striking inflammatory response characterized by edema and

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ABBREVIATIONS: COX, cyclooxygenase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; cPLA$_2$, cytosolic phospholipase A_2; PKC, protein kinase C; MEK, mitogen-activated protein/extracellular signal-regulated protein kinase; ERK, extracellular signal-regulated protein kinase; AA, arachidonic acid; PG, prostaglandin; C/EBPβ, CCAAT/enhancer-binding protein β; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; EGF, epidermal growth factor; PCR, polymerase chain reaction; EMEM, minimum essential medium with Earle's balanced salt; BSA, bovine serum albumin; FA, fluocinolone acetonide.

extensive epidermal infiltration of neutrophils that form intraepidermal microabscesses in the epidermis after a single topical treatment with the PKC activator, TPA (Wang and Smart, 1999). This exaggerated inflammatory response is accompanied by increased COX-2 induction, and up-regulation of macrophage inflammatory protein-2 and TNFα message levels in the epidermis of TPA-treated K5-PKCα mice compared with TPA-treated wild-type mice (Wang and Smart, 1999). Importantly, transgenic mice in which the expressions of PKCϵ or PKCδ were similarly targeted to the epidermis did not develop an exaggerated inflammatory response to TPA (Reddig et al., 1999, 2000). Collectively these data indicate that PKCα has an important role in cutaneous inflammation.

Release of arachidonic acid (AA) from membrane phospholipids by cytosolic phospholipase A_2 (cPLA$_2$) is considered the rate-limiting step in the generation of eicosanoids (Irvine, 1982). cPLA$_2$ is subject to complex mechanisms of regulation by phosphorylation and cytosolic calcium concentration (Lin et al., 1993; Glover et al., 1995; Schievella et al., 1995). It has been found that ERK phosphorylates cPLA$_2$ at serine 505, and this phosphorylation is related to its activation and mobility shift on SDS-polyacrylamide gels (Lin et al., 1993). It is also reported that phosphorylation of cPLA$_2$ is independent of ERK but dependent on MAPK p38 or JNK (Nishio et al., 1996; Borsch-Haubold et al., 1997, 1999; Buschbeck et al., 1999). PKC has been suggested to regulate cPLA$_2$ phosphorylation and activation based on observations that TPA-treated cells displayed increased cPLA$_2$ phosphorylation and activity (Nemenoff et al., 1993; Xing and Insel, 1996; Lo et al., 1998). Evidence indicates that PKCα is involved in the regulation of [^{3}H]AA release in canine kidney cells, NIH 3T3 fibroblasts, and cat iris sphincter smooth muscle cells (Finkenzeller et al., 1993; Godson et al., 1993; Husain and Abdel-Latif, 1998).

COXs catalyze the conversion of AA to the biologically active prostaglandins (PGs), such as PGE$_2$, PGF$_{2a,}$ PGI$_2$, PGD$_2$, and thromboxane A$_2$ (Smith and Dewitt, 1996). Two isoforms of COX have been identified with different modes of expression and tissue distributions (Meade et al., 1993). COX-1 is constitutively expressed in cells of most tissues and is considered a housekeeping isoform (Smith and Dewitt, 1996). In contrast, COX-2 is not normally detectable in most tissues, but it can be induced by mitogens, cytokines, and certain inflammatory agents (Herschman, 1994; Ristimaki et al., 1994; Inoue et al., 1995; Mestre et al., 1997). TPA, epidermal growth factor (EGF), and UV light stimulate expression of COX-2 in primary keratinocytes in vitro and in the epidermis in vivo (Loftin and Eling, 1996; Maldve and Fischer, 1996; Buckman et al., 1998). The signal transduction pathways involved in COX-2 expression seem to be cell type- and stimulator-specific (Xie and Herschman, 1995, 1996; Barry et al., 2000; Reddy et al., 2000). PKC has been suggested to be involved in the regulation of COX-2 expression in mouse primary keratinocytes (Maldve and Fischer, 1996) and human keratinocytes (Matsuura et al., 1999); however, the isoforms of PKC responsible for COX-2 induction are not known.

C/EBPβ, a member of the basic leucine zipper family of transcription factors, is involved in the transcriptional regulation of COX-2 promoter in some cell types (Kim and Fischer, 1998; Reddy et al., 2000; Yuan et al., 2000). PKCα/MAPK-signaling pathway has been shown to play a role in C/EBPβ phosphorylation and activation (Trautwein et al., 1993). Consistent with this, our studies with K5-PKCα mice demonstrated much higher COX-2 expression induced by TPA; thus, it is possible that PKCα mediates COX-2 expression through the activation/phosphorylation of C/EBPβ. In the current study, we have used K5-PKCα mice to investigate the role of PKCα in phospholipid metabolism/eicosanoid production and cutaneous inflammation and the signaling pathway by which PKCα mediates cPLA$_2$ activation and COX-2 expression.

Materials and Methods

Identifying K5-PKCα Transgenic Mice and C/EBPβ Knockout Mice by PCR Analysis. K5-PKCα transgenic mice were generated and characterized in our previous paper (Wang and Smart, 1999). C/EBPβ knockout mice were generated by homologous recombination as described previously (Sterneck et al., 1997). Genomic DNA was isolated using QIAGEN DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA), and PCR analysis was carried out using *Taq* DNA Polymerase (QIAGEN). For K5-PKCα transgenic mice, the 5′-primer was a K5 promoter sequence (5′-GCCTATTCGCTGCCAAGAGAT-3′), and the 3′-primer was a PKCα cDNA sequence (5′-AAAC-CCCCAGATGAAGTCGGTG-3′). PCR cycles were 3 min at 94°C, 1 min 30 s at 55°C, and 2 min at 72°C for 1 cycle followed by 1 min 15 s at 94°C, 2 min at 51°C, and 2 min at 72°C for 35 cycles. The amplified 513-bp fragment spanned the junction between the K5 promoter and the PKCα cDNA. C/EBPβ knockout mice were genotyped by PCR using two sets of primers. One set is for C/EBPβ (the 5′-primer is 5′-AGCCCCTACCTGGAGCCCCTCGCG-3′; the 3′ primer is 5′-GCG-CAGGGGGAACGGGAAACCG-3′). The second set of primers is for the neomycin resistance gene (the 5′-primer is 5′-GTGCTCGACGT-TGTCACTGAAGCGG-3′; the 3′-primer is 5′-GATATTCGGCAAG-CAGGCATCG-3′). PCR cycles were 30 s at 95°C, 30 s at 63°C, and 1 min at 72°C for 35 cycles. In wild-type mice, a 294-bp C/EBPβ product is produced, whereas in C/EBPβ knockout mice a 351-bp product is produced. In heterozygous mice, both products are produced.

Release of [^{3}H]Arachidonic Acid from Primary Mouse Keratinocytes. Primary keratinocytes were isolated from 2- to 3-day-old newborn mice by trypsinization overnight at 4°C. Isolated epidermal cells were plated at 3×10^6 cells per 35-mm dish in EMEM supplemented with 10% fetal bovine serum and 4 ng/ml of epidermal growth factor for 4 h 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, cells were labeled with 0.60 μCi/ml [^{3}H]arachidonic acid (0.1 mCi/ml, PerkinElmer Life Sciences, Boston, MA) for 20 h. After gently washing three times with warm EMEM containing 1 mg/ml bovine serum albumin (BSA) and 20 mM HEPES, pH 7.4, cells were treated with TPA at 1 μg/ml in EMEM with 1 mg/ml BSA and 20 mM HEPES, pH 7.4. For inhibition studies, PKC inhibitor GF-109203X (Alexis Corp., San Diego, CA), H7 or inactive control compounds bisindolylmaleimide V and HA-1004 (LC Laboratories, Woburn, MA), or MEK inhibitor PD 98059 (Calbiochem-Novabiochem Corp., La Jolla, CA) was added with 1 μg/ml TPA. PD 98059 was initially tested at 10, 30, and 50 μM for its ability to inhibit TPA-induced COX-2 induction. We found that 30 and 50 μM produced the same response, so we used 30 μM concentration in all of our studies. At different times, 250 μl of medium was collected and centrifuged at 12,000 rpm for 5 min to eliminate cell debris, and radioactivity in 200 μl of supernatant was measured by scintillation

counting. After 2 h stimulation, 250 μl of PBS containing 1% SDS was added into each well, the cells were scraped, and the total cellular radioactivity was determined.

Preparation of Primary Keratinocyte Homogenates and Western Analysis of cPLA$_2$ Phosphorylation. Primary keratinocytes were isolated from newborn mouse skin and cultured for 5 days, and then cells were starved in EMEM without serum or EGF for 20 h. Keratinocytes were stimulated with TPA, and at various time cells were scraped and placed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 50 mM sodium fluoride, and 200 μM sodium orthovanadate). The cells were sonicated three times for 10 s on ice and centrifuged at 12,000 rpm for 25 min at 4°C. Protein concentration in the supernatant 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). To observe the mobility shift of phosphorylated cPLA$_2$, the 8% Tris-glycine polyacrylamide gel was electrophoresed at 130 V for 3 h and 30 min. Proteins were electrophoretically transferred to an Immobilon P membrane (Millipore Corporation, Bedford, MA). The membrane was blocked in PBS containing 5% nonfat dry milk and 1% BSA for 1 h, and then the membrane was incubated overnight at 4°C with a rabbit polyclonal cPLA$_2$ antibody (1:1000), which is raised against amino-terminal domain amino acids 1 to 216 of human cPLA$_2$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 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 Kodak Image Station 440CF (Eastman Kodak, Rochester, NY).

Preparation of Epidermal Homogenates and Western Analysis of COX-2 Expression. Both male and female mice at 10 to 12 weeks of age were used for the studies. The hair of the dorsal skin of the mice was clipped with an electric clipper at least 2 days before each experiment. Only mice in the telogen phase of the hair follicle cycle were used. 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, pH 7.5, 10 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 100 μg/ml leupeptin, and 0.05% (v/v) Triton X-100]. The samples were homogenized on ice using Polytron and centrifuged at $10^5 g$ for 35 min at 4°C. Protein concentration in the supernatants was determined by the method of Lowry using bovine serum albumin as the standard. Equal amounts of protein from each sample were separated on 8% Tris-glycine polyacrylamide gel (Novex) and electrophoretically transferred to an Immobilon P membrane (Millipore Corporation). The membrane was incubated with a polyclonal COX-2 antibody (1:1000) (Cayman Chemical Company, Ann Arbor, MI).

Preparation of Whole Skin Homogenates and Measurement of PGE$_2$ Level. Both male and female mice at 10 to 12 weeks of age were used for the study. The hair of the dorsal skin of the mice was clipped with an electric clipper at least 2 days before each experiment. Only mice in the telogen phase of the hair follicle cycle were used. TPA (5 nmol) in 200 μl of acetone or 200 μl of acetone alone was applied to dorsal shaved skin area. Eight hours later, mice were killed and the dorsal treated skin was removed and snap frozen in liquid nitrogen. The frozen skin samples were weighed (100 mg) and ground with a mortar and pestle under liquid nitrogen on dry ice, placed in 1 ml of PBS containing 100 μM indomethacin (Sigma, St. Louis, MO). The samples were homogenized on ice using a Polytron and centrifuged at 12,000 rpm for 35 min at 4°C. For methyl-oximation of PGE$_2$, each sample was mixed with equal amount of methyl oximate reagent, incubated at 60°C for 1 h, and then the samples were stored at −80°C. PGE$_2$ production was measured using a competitive radioimmunoassay (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

TPA-Treated K5-PKCα Primary Keratinocytes Display Increased [^{3}H]Arachidonic Acid Release. Primary keratinocytes were isolated from wild-type and K5-PKCα newborn mice and cultured in medium containing low calcium (0.05 mM). In low calcium medium, primary keratinocytes resemble the basal keratinocytes of the epidermis. After 5 days in culture, primary keratinocytes were prelabeled with [^{3}H]AA for 20 h and then treated with TPA. As shown in Fig. 1, TPA stimulated a rapid time-dependent release of [^{3}H]AA in both wild-type and K5-PKCα keratinocytes; however, K5-PKCα keratinocytes released nearly twice as much [^{3}H]AA as did wild-type keratinocytes. These results indicate that PKCα can influence AA release and suggest that PKCα has a role in the regulation of cPLA$_2$ activation in keratinocytes.

TPA Treatment Results in the Phosphorylation of cPLA$_2$, and Both cPLA2 Phosphorylation and Arachidonic Acid Release Are Inhibited by PKC Inhibitor GF-109203X but Not the MEK Inhibitor PD 98059. Because cPLA$_2$ activity has been shown to be regulated by phosphorylation via several different pathways including a MEK pathway involving ERK1/ERK2 (Lin et al., 1993; Gordon et al., 1996) as well as a PKC pathway (Nemenoff et al., 1993; Xing and Insel, 1996), we examined the effect of the MEK inhibitor PD 98059 and the PKC inhibitors GF-109203X and H7 on AA release. As shown in Fig. 2A, TPA treatment of primary keratinocytes prelabeled with [^{3}H]AA resulted in the release of [^{3}H]AA, and this release could be

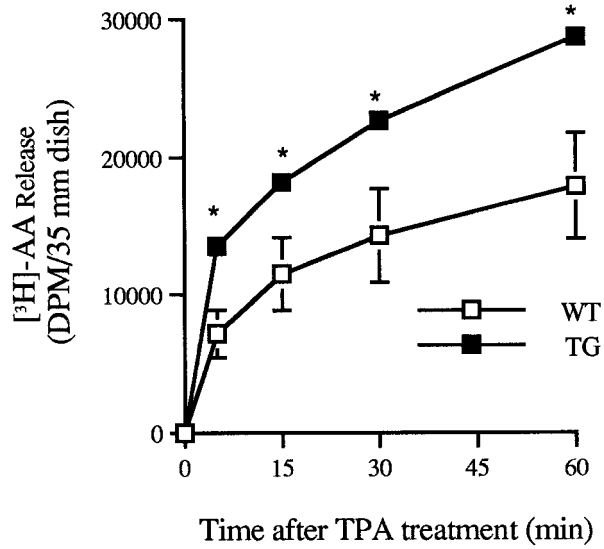

Fig. 1. K5-PKCα primary keratinocytes display increased [^{3}H]AA release after TPA treatment compared with wild-type primary keratinocytes. Both wild-type and K5-PKCα transgenic primary keratinocytes were isolated from newborn mouse skin and cultured for 5 days. Keratinocytes were labeled for 20 h with [^{3}H]AA in EMEM containing 4% chelexed FBS, 10 ng/ml EGF, and 0.05 mM Ca^{2+}. Cells were washed with EMEM containing 1 mg/ml BSA, 20 mM HEPES, and 0.05 mM Ca^{2+}, pH 7.4, three times, and then the same medium containing either dimethyl sulfoxide or 1 μg/ml TPA was added. Aliquots were removed at the designated times and counted. Values represent the mean ± S.E.M. of three experiments each done in triplicate. *, significantly different from TPA-treated wild-type mice ($p < 0.05$) as determined by Student's t test.

blocked by the PKC inhibitors GF-109203X and H7 but not the MEK inhibitor PD 98059. Bisindolylmaleimide V, an inactive control compound for GF-109203X, did not inhibit TPA-induced AA release. To determine whether AA release is accompanied by cPLA$_2$ phosphorylation, which is associated with its activation, primary keratinocytes were treated with TPA and cell lysates were subjected to Western analysis. As shown in Fig. 2B, TPA treatment resulted in an upward shift in the mobility of cPLA$_2$. This upward shift is consistent with the phosphorylation of cPLA$_2$ (Lin et al., 1993; Gordon et al., 1996). cPLA$_2$ phosphorylation could be inhibited by GF-109203X but not by the MEK inhibitor PD 98059. Similar results were obtained in K5-PKCα transgenic keratinocytes (data not shown). Collectively these results indicate that in keratinocytes a PKCα pathway modulates cPLA$_2$ phosphorylation and AA release independent of the MEK pathway. In addition, we found that the p38 inhibitor SB 203580, at concentrations of 15 and 30 μM, had no effect on the TPA-induced cPLA$_2$ mobility shift, suggesting that p38 is not responsible (data not shown).

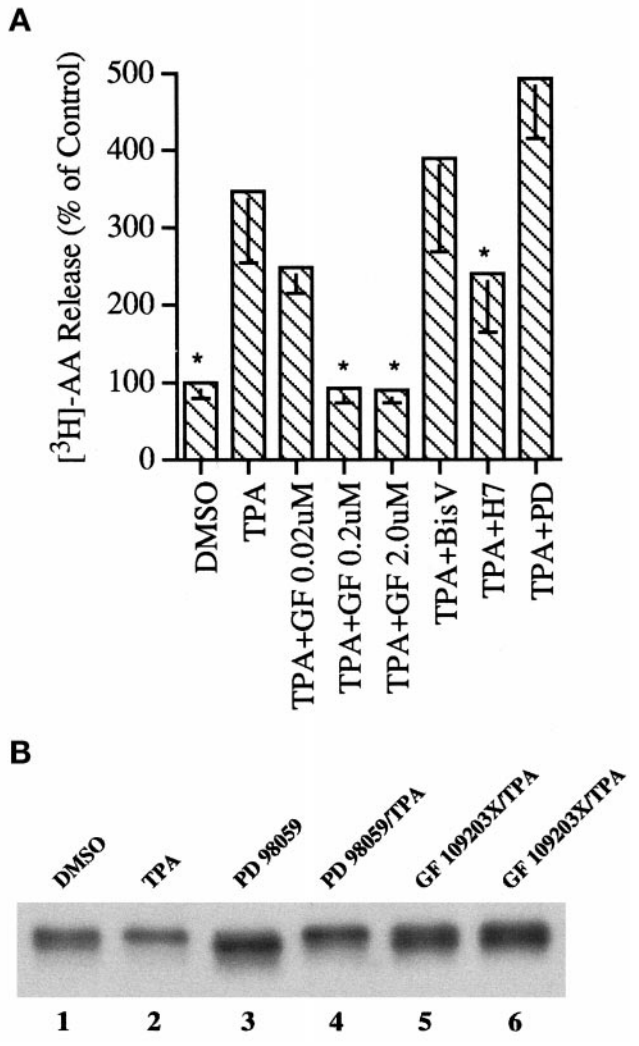

Fig. 2. PKC inhibitor GF-109203X but not MEK inhibitor PD 98059 blocks TPA-induced [^{3}H]AA release and cPLA$_2$ phosphorylation in primary keratinocytes. A, keratinocytes were cultured for 5 days and labeled with [^{3}H]AA for 20 h. Cells were washed three times, and fresh medium containing dimethyl sulfoxide, 1 μg/ml TPA, 0.02 μM GF-109203X + 1 μg/ml TPA, 0.2 μM GF-109203X + 1 μg/ml TPA, 2 μM GF-109203X + 1 μg/ml TPA, 0.2 μM bisindolylmaleimide V + 1 μg/ml TPA, 10 μM H7 + 1 μg/ml TPA, or 30 μM PD 98059 + 1 μg/ml TPA was added into the cells and incubated for 1 h. Aliquots were removed after 1 h of incubation and counted. The results are expressed as the mean ± standard deviation ($n \geq 3$). *, significantly different from TPA-treated keratinocytes ($p < 0.1$) as determined by Student's t test. B, keratinocytes were cultured for 5 days and starved in serum-free medium for 20 h. Cells were pretreated with either 2 μM GF-109203X or 30 μM PD 98059 for 30 min and then stimulated with 1 μg/ml TPA for 10 min. The cell lysates were prepared, and equal amounts of protein (5 μg/ml) were then separated on 8% Tris-glycine polyacrylamide gel for 3 h and 30 min at 130 V, followed by immunoblotting with anti-cPLA$_2$ antibody.

TPA-Treated K5-PKCα Mice Display Increased Expression of Epidermal COX-2 and Elevated Levels of PGE$_2$ in Treated Skin Compared with Similarly Treated Wild-Type Littermates. Previously we have shown that phorbol ester-treated K5-PKCα transgenic mice display increased expression of COX-2 (Wang and Smart, 1999). As shown in Fig. 3, TPA induced COX-2 expression both in wild-type and K5-PKCα mice, and the expression of COX-2 was approximately 5-fold higher in K5-PKCα mice than in wild-type mice. Because glucocorticoids are important therapeutic agents used to treat dermatitis and are known to block COX-2 induction (Herschman, 1994; Crofford, 1997), we wanted to determine whether pretreatment with fluocinolone acetonide (FA) could block TPA-induced COX-2 expression in K5-PKCα mice. Pretreatment of K5-PKCα mice with the glucocorticoid FA greatly reduced TPA-induced COX-2 expression in K5-PKCα mice (Fig. 3). Moreover, FA treatment blocked TPA-induced edema, neutrophil infiltration, and intraepidermal microabscess formation (TPA-treated K5-PKCα mice developed 6.8 ± 2.0 intraepidermal microabscesses per 1 cm of skin; FA/TPA-treated K5-PKCα mice did not develop any intraepidermal microabscesses). These results demonstrate that there is cross-talk between the PKCα pro-inflammatory pathway and the glucocorticoid anti-inflammatory pathway.

Because COX-2 metabolizes AA to pro-inflammatory PGs such as PGE$_2$ and PGF$_{2\alpha}$, which are known mediators of vascular dilation and edema, we determined whether PGE$_2$ levels in the skin of TPA-treated K5-PKCα were increased compared with wild-type mice. Wild-type and K5-PKCα mice were treated with a single topical dose of 5 nmol of TPA or acetone as a vehicle control, and 8 h later the skin lysates were prepared for PGE$_2$ quantitation. PGE$_2$ levels were 2.6-fold greater in TPA-treated K5-PKCα mice compared with similarly treated wild-type mice (Fig. 4.). No differences were

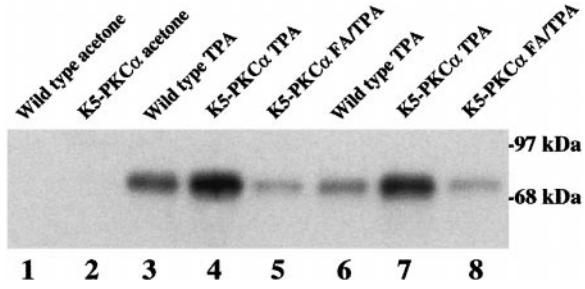

Fig. 3. FA blocks TPA-induced COX-2 expression in K5-PKCα mouse epidermis. Wild-type and K5-PKCα transgenic mice were pretreated with either 2 nmol of FA or acetone vehicle for 30 min, and then mice were treated with 5 nmol of TPA. Eight hours later, the treated skin area was removed and snap frozen in liquid nitrogen. The epidermal homogenates were prepared, and equal amounts of protein (10 μg/ml) were separated on 8% Tris-glycine polyacrylamide gel, followed by immunoblotting with anti-COX-2 antibody.

observed in the basal PGE_2 levels between wild-type and K5-PKCα mouse skin homogenates after acetone treatment, indicating that PKC activation is required (Fig. 4). In addition, FA pretreatment reduced TPA-induced PGE_2 production both in wild-type and K5-PKCα mice by approximately 50% (data not shown). These results demonstrate that PKCα plays an important role in TPA-induced COX-2 expression and PGE_2 production in mouse skin.

TPA-Induced COX-2 Is through a PKCα/MEK Pathway That Does Not Involve the Transcription Factor C/EBPβ. Depending upon the agonist and cell type, COX-2 expression has been reported to be regulated through PKC, MEK(ERK), JNK, MAPK p38, and C/EBPβ or nuclear factor-κB (Yamamoto et al., 1995; Inoue and Tanabe, 1998). To study the regulation of COX-2 expression, primary keratinocytes were isolated from wild-type and K5-PKCα newborn mice and cultured in medium containing low calcium (0.05 mM). After 5 days in culture, primary keratinocytes were treated with TPA at 100 nM or 1 μM. As shown in Fig. 5A, TPA produced a dose-dependent increase in COX-2 expression in primary keratinocytes. TPA-induced COX-2 expression could be blocked by pretreatment with either PKC inhibitor GF-109203X or MEK inhibitor PD 98059 (Fig. 5A). Similar results were obtained in K5-PKCα transgenic keratinocytes (data not shown). As shown in Fig. 5B, lower doses of GF-109203X were also effective. H7 also blocked TPA-induced COX-2 induction, and the inactive control compounds (HA-1004 for H7 and bisindolylmaleimide V for GF-109203X) did not inhibit TPA-induced COX-2 expression. In addition, 4α-TPA, which does not activate PKC, did not induce COX-2 expression. These results indicate that COX-2 induction in keratinocytes by TPA is PKCα- and MEK-dependent. Several groups have reported that C/EBPβ, a basic leucine zipper transcription factor, plays an important role in regulating COX-2 expression (Kim and Fischer, 1998). Because C/EBPβ can be activated via a PKC/MAPK pathway, we examined whether TPA induced epidermal COX-2 through C/EBPβ activation. C/EBPβ nullizygous and wild-type mice were treated with TPA, and at various times the epidermis was collected. Epidermal lysates were subjected to Western analysis and, as shown in Fig. 6, no differences were observed in the induction of COX-2 expression between wild-type littermates and C/EBPβ nullizygous mice at 4, 8, 16, and 24 h after 5 nmol of TPA application. This result indicated that C/EBPβ is not involved in TPA-induced COX-2 expression in mouse skin.

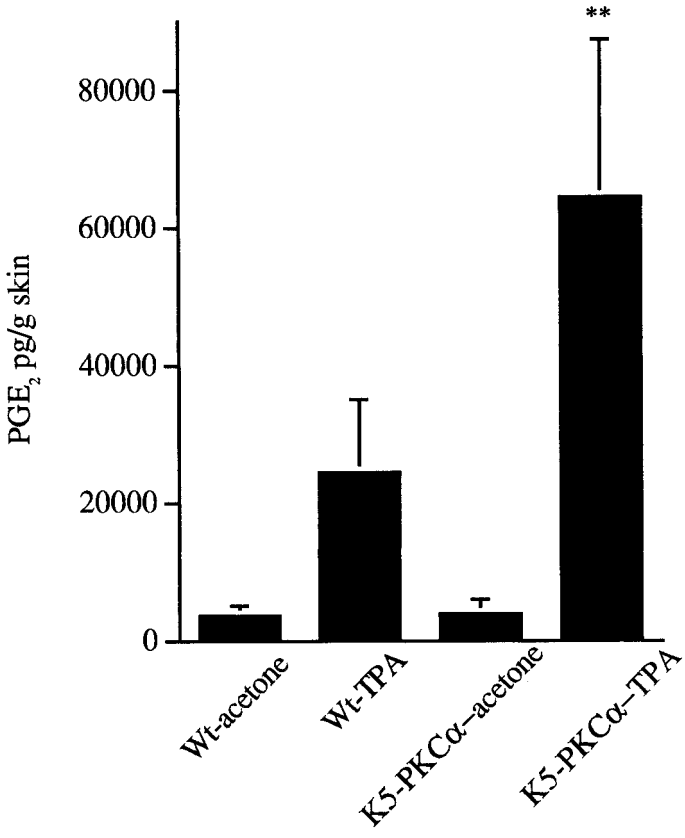

Fig. 4. The levels of PGE_2 are elevated in K5-PKCα mouse skin. Both wild-type and K5-PKCα mice were treated topically once with either 5 nmol of TPA in 200 μl of acetone or 200 μl of acetone alone. Eight hours later, the dorsal treated area was removed and snap frozen in liquid nitrogen. The whole skin homogenates were prepared for measurement of PGE_2 as described under *Materials and Methods*. PGE_2 was measured using a competitive radioimmunoassay. Values represent the mean ± S.D. (n = 4). **, significantly different from TPA-treated wild-type mice ($p < 0.01$) as determined by Student's t-test.

Discussion

In the current study, we have demonstrated that treatment of K5-PKCα transgenic keratinocytes or skin with TPA results in a 2-, 5-, and 2.6-fold increase in [^{3}H]AA release,

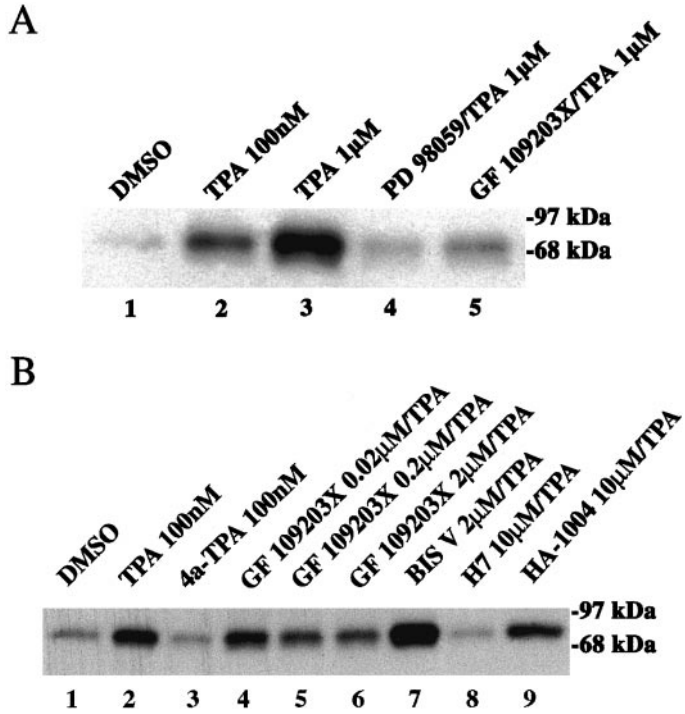

Fig. 5. PKC inhibitor GF-109203X and MEK inhibitor PD 98059 block TPA-induced COX-2 expression in primary keratinocytes. Primary keratinocytes were isolated from newborn mouse skin and cultured for 5 days. A, after 20 h of starvation in serum-free medium, keratinocytes were pretreated with 2 μM GF-109203X or 30 μM PD 98059 for 30 min and then stimulated with 100 nM or 1 μM TPA for 5 h. B, after 20 h of starvation in serum-free medium, keratinocytes were pretreated with 0.02 to 2 μM GF-109203X, 10 μM H7, 2 μM bisindolylmaleimide V, or 10 μM HA-1004 for 30 min and then stimulated with 100 nM TPA for 5 h. The cell lysates were prepared, and equal amounts of protein (5 μg/ml) were separated on 8% Tris-glycine polyacrylamide gel, followed by immunoblotting with anti-COX-2 antibody.

COX-2 expression, and PGE_2 production, respectively, compared with similarly treated wild-type keratinocytes or skin. Moreover, we found that PKC inhibitor GF-109203X blocks TPA-induced [^{3}H]AA release, cPLA$_2$ phosphorylation, and COX-2 induction in primary keratinocytes. The inhibitory effects of GF-109203X are probably mediated through PKCα because GF-109203X is a selective inhibitor of the conventional forms of PKC and PKCα is the only conventional isoform of PKC expressed in keratinocytes. Although keratinocytes express PKCα, -δ, -ϵ, -ζ, -η, and -μ, our results indicate that PKCα plays a major role in regulation of phospholipid metabolism/eicosanoid production and cutaneous inflammation. We have observed that the activation of PKCα in keratinocytes results in the coordinate regulation of cPLA$_2$ phosphorylation/AA release and COX-2 induction/PGE_2 production.

The pathway through which PKCα mediates the activation of cPLA$_2$ in keratinocytes is not known; however, our results suggest that these events are MEK/ERK-independent because the MEK inhibitor PD 98059 did not inhibit cPLA$_2$ phosphorylation or AA release. cPLA$_2$ can be phosphorylated and activated through numerous pathways depending upon the cell type and the activator and can involve MEK/ERK-dependent or MEK/ERK-independent pathways (Lin et al., 1993; Gordon et al., 1996; Nishio et al., 1996; Borsch-Haubold et al., 1997). For example, in human neutrophils lipopolysaccharide or TNFα stimulation results in cPLA$_2$ phosphorylation and AA release independent of ERK (Fouda et al., 1995; Waterman and Sha'afi, 1995). TNFα stimulation of human neutrophils results in an increase in MAPK p38, and that MAPK p38-specific inhibitor SB 203580 blocked TNFα-induced cPLA$_2$ phosphorylation and activation (Waterman et al., 1996). However, we found that SB 203580 was not effective in blocking TPA-induced cPLA2 mobility shift in mouse keratinocytes. In human platelets, collagen or thrombin also induces cPLA$_2$ phosphorylation via MAPK p38 (Borsch-Haubold et al., 1997). In addition, JNK has been suggested to be involved in AA release in rabbit aortic smooth muscle cells (Nishio et al., 1996) and human platelets (Borsch-Haubold et al., 1999; Buschbeck et al., 1999). Therefore, the JNK signaling pathway is a candidate through which PKCα regulates cPLA$_2$ phosphorylation and activation in keratinocytes.

TPA treatment of K5-PKCα mice results in a 5-fold increase in epidermal COX-2 protein over that observed in similarly treated wild-type mice. In addition, pretreatment of primary keratinocytes with GF-109203X or PD 98059 blocked TPA-induced COX-2 expression, indicating COX-2 expression is mediated through a PKCα pathway involving MEK/ERK. Our results are consistent with earlier studies in primary keratinocytes demonstrating that the down-regulation of PKC or pretreatment with PKC inhibitor H7 blocked TPA-induced COX-2 expression (Maldve and Fischer, 1996). Because PKCα can phosphorylate and activate Raf-1 directly (Kolch et al., 1993), it is possible that a PKCα-RAF-MEK-ERK signaling pathway plays an important role in TPA-induced COX-2 expression in keratinocytes. In support of such a notion are studies in NIH 3T3 and mast cells wherein COX-2 expression is mediated through a RAS/RAF-1/MEK/ERK pathway (Xie and Herschman, 1996; Reddy et al., 2000).

Downstream effectors of ERK activation important in COX-induction are not known; however, numerous studies have demonstrated that the basic leucine zipper transcription factor C/EBPβ is involved in the transcriptional regulation of COX-2 (Kim and Fischer, 1998; Reddy et al., 2000; Yuan et al., 2000). Because C/EBPβ is abundantly expressed in mouse epidermal keratinocytes (Oh and Smart, 1998) and its transcriptional activity can be regulated by phosphorylation via a PKCα pathway involving MEK/ERK (Trautwein et al., 1993), we examined the possible role of this transcription factor in TPA-induced COX-2 expression. We found that TPA induced epidermal COX-2 protein levels in C/EBPβ knockout mice and wild-type littermates to a similar extent, indicating that TPA-induced COX-2 expression is independent of C/EBPβ. Additional studies are required to determine the downstream transcription factors of ERK activation that are important in TPA-induced COX-2 expression.

Previous studies from our laboratory demonstrated that the expression of PKCα in the epidermis of K5-PKCα transgenic mice results in striking alterations in phorbol ester-induced inflammation involving the expression of COX-2, macrophage inflammatory protein-2, and TNFα and the focal accumulation of neutrophils within the epidermis (Wang and Smart, 1999). Our current study provides additional support for a role of PKCα in cutaneous inflammation involving regulation of phospholipid metabolism/eicosanoid production. Notably, other transgenic mice that overexpress PKCδ and -ϵ do not display alterations in phorbol ester-induced inflammation (Reddig et al., 1999, 2000). These studies support our conclusions that specific PKC isoforms have specific functions in keratinocytes and that a principal function of PKCα in epidermal keratinocytes involves the regulation of the expression of inflammatory mediators that produce edema and neutrophil infiltration. PKCα may represent a therapeutic target for cutaneous diseases involving inflammation because it has a potent influence on the inflammatory process but has little to no effect on epidermal growth and differentiation.

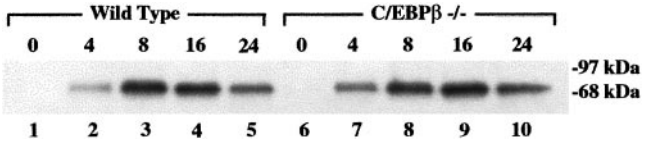

Fig. 6. Western analysis of COX-2 expression in epidermis isolated from wild-type and C/EBPβ knockout mice. Wild-type and C/EBPβ knockout (C/EBPβ $-/-$) mice were treated with 5 nmol of TPA. At 0, 4, 8, 16, and 24 h after TPA treatment, the treated skin area was removed and snap frozen in liquid nitrogen. The epidermal homogenates were prepared, and equal amounts of protein (10 μg) were separated on 8% Tris-glycine polyacrylamide gel, followed by immunoblotting with anti-COX-2 antibody.

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