Identification and characterization of several forms of phospholipase A_2 in mouse epidermal keratinocytes

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Abstract The tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated the release of arachidonic acid (AA) from mouse keratinocytes. A distinct difference was observed between the fatty acid release profile elicited by TPA and other stimuli. These findings led to the investigation of keratinocyte phospholipase A_2 (PLA_2), which catalyzes the release of *sn*-2 fatty acids from membrane phospholipids and regulates the production of eicosanoids. We characterized and identified several forms of PLA_2 in mouse keratinocytes, a cytosolic or $cPLA_2$ and two secretory or $sPLA_2$s in the membrane. The PLA_2 in keratinocyte cytosol is sensitive to heating and acid treatment, while resistant to reducing reagent. The PLA_2 in keratinocyte membrane is resistant to heating and acid treatment, while sensitive to reducing reagent. These characteristics suggested the presence of a $cPLA_2$ and at least one type of $sPLA_2$. Inhibitor data further confirmed the identities of these PLA_2s. The $cPLA_2$ was activated by TPA, and appeared to be responsible for the majority of the specific release of AA observed in mouse keratinocytes treated with TPA. The calcium ionophore A23187, and 4α-TPA did not elicit the selectivity towards AA observed with TPA. The release of linoleic acid (LA) and oleic acid (OA) from A23187- and 4α-TPA-treated keratinocytes suggests activation of $sPLA_2$. These activities may be due to the existence of both type I and type II $sPLA_2$, as both were identified by polymerase chain reactions. In conclusion, keratinocytes express several forms of phospholipase A_2 that differ in their substrate specificities and mechanisms of activation, resulting in distinct agonist-specific fatty acid release profiles.—**Li-Stiles, B., H-H. Lo, and S. M. Fischer.** Identification and characterization of several forms of phospholipase A_2 in mouse epidermal keratinocytes. *J. Lipid Res.* 1998. **39:** 569–582.

Supplementary key words arachidonic acid • $cPLA_2$ • keratinocytes • PLA_2 • $sPLA_2$ • TPA

Many of the responses of skin to normal or pathological agents are mediated by one or more of the eicosanoids or their precursors. Diverse types of damage to the skin, e.g., ultraviolet irradiation, physical wounding, and chemical irritants including tumor promoters and xenobiotic agents, induce the release of one or more free fatty acids, particularly arachidonic acid (AA) (1–3). Released AA is readily metabolized and these metabolites are involved in such defense mechanisms as inflammation, immune processes, tissue repair, and induced epidermal hyperplasia (4, 5). Other fatty acids can also be hydrolyzed and metabolized, but often have different biological activities than AA-derived eicosanoids. For example, the 13-hydroxyoctadecadienoic acid (13-HODE) metabolite of linoleic acid (LA) in some cases has opposing activities to the 12-hydroxyeicosatetraenoic acid (12(S)-HETE) metabolite of AA (6, 7). As the predominant mechanism for the release of fatty acids from the *sn*-2 position of glycerophospholipids is hydrolysis by the phospholipase A_2 (PLA_2, E. C. 3.1.1.4) enzymes, activation of different forms of PLA_2s may result in the release of different fatty acids and qualitatively or quantitatively alter eicosanoid production and subsequent biological responses.

Several families of PLA_2 have been identified in mammalian tissues: the low-molecular mass (14 kD) types I and II PLA_2, referred to as secretory or $sPLA_2$, and the high-molecular mass (85 kD) cytosolic PLA_2 ($cPLA_2$) (8, 9). The $sPLA_2$s differ from the $cPLA_2$ in several significant ways. $sPLA_2$s are located either in the membrane or extracellularly and have been reported to have a broad range of substrate preferences, with regard to phospholipid and *sn*-2 fatty acids (8). $cPLA_2$, on the other hand, is localized primarily in the cytosol unless it is activated. $cPLA_2$ also shows a substrate preference towards *sn*-2 AA esterified to phosphatidylcho-

Abbreviations: AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; DTT, dithiothreitol; LA, linoleic acid; OA, oleic acid; PCAA, 1-palmitoyl-2-arachidonyl-phosphatidylcholine; PCLA, l-palmitoyl-2-linoleoyl-phosphatidylcholine; PCOA, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PEAA, 1-palmitoyl-2-arachidonyl phosphatidylethanolamine; PKC, protein kinase C; PLA_2, phospholipase A_2; PG, prostaglandin; $sPLA_2$, secretory phospholipase A_2; TPA, 12-O-tetra- decanoylphorbol 13-acetate.

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line. It has been suggested that cell-associated $sPLA_2$ functions primarily in maintaining cell membrane homeostasis, extracellular $sPLA_2$ in inflammatory diseases, while $cPLA_2$ may be involved in the initiation of the inflammatory response. Both $sPLA_2$ and $cPLA_2$ may be differentially regulated to modify the lipid composition of mouse skin during differentiation of epidermal basal cells which move upwards to become corneocytes (10, 11). These changes in lipid composition in the epidermis, especially in the stratum corneum, have been suggested to play important roles in skin barrier function (12–14).

Several other forms of PLA_2 have also been reported. For example, platelets contain a PLA_2 that has recently been identified as the 29 kD zeta-isoform of the 14-3-3 protein (15). A 56 kD cytoplasmic PLA_2 has been isolated from a human monocytic leukemia cell line (16). Enhancing factor protein, found in small intestine, also belongs to the PLA_2 family (17). Calcium-independent forms of PLA_2 ($iPLA_2$) have also been identified in several cell types (18, 19), and the recently cloned types IIc and V PLA_2 were found to exist predominantly in mouse testis (20) and human heart tissues, respectively (21). For most of these, little is known about their function or regulation. The type V $sPLA_2$ has recently been suggested to couple with cyclooxygenase 1 and mediate the early phase prostaglandin synthesis in mast cells as well as in several other cell types (22, 23). The classical type II $sPLA_2$ (IIa) has also been suggested to have similar coupling with the cyclooxygenase enzymes for either the early or the late prostaglandin production depending on the cell type (24–26).

Because of considerable evidence for its preference for AA over other fatty acids, the activation of $cPLA_2$ in particular may be critical for subsequent eicosanoid biosynthesis. Several mechanisms of activation have been demonstrated (27). Growth factor stimulation of cells has been reported to result in increased phosphorylation of $cPLA_2$ on serine residues by either p42 MAP kinase or protein kinase C (PKC) (28–31). Calcium has also been shown to have a role in $cPLA_2$ activation, although it appears to be involved in membrane binding, where the substrate is localized, rather than in increasing catalytic activity (32). Activation of $sPLA_2$ has been observed to occur through at least two mechanisms, one involving elevated cAMP and the other inflammatory cytokines (33). However, little is currently known about the $sPLA_2$s in the epidermis with regard to either substrate preferences, mechanism of activation, or the role of the different forms in specific biological processes.

The purpose of this study is to investigate the role or roles some of these PLA_2s may play during inflammation and tumor promotion. The release of AA in keratinocytes stimulated by the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA), bradykinin, and transforming growth factor-α (TGFα) has been shown to be mediated at least in part by activating $cPLA_2$ (34, 35). This activation resulted in both increased enzyme activity and in the translocation of this enzyme to the membrane (35). The non-tumor promoting analog of TPA, 4α-TPA, has also been shown to stimulate the hydrolysis of AA, however, through a different mechanism (36), possibly by activating a different PLA_2.

We report here the specific hydrolysis of fatty acids stimulated by different agonists using TPA, 4α-TPA, bradykinin, and calcium ionophore A23187. The partial tumor promoter calcium ionophore A23187 has been shown to stimulate the release of incorporated AA from both human and mouse keratinocytes. This apparently is different from TPA which failed to stimulate the hydrolysis of AA from human keratinocytes while significantly stimulating release of incorporated AA from mouse keratinocytes (37). We confirm here the existence of $cPLA_2$ activity as well as mRNA in mouse keratinocytes. We also characterize and demonstrate the presence of both type I and type II $sPLA_2$s in mouse keratinocytes as the presence of $sPLA_2$ in epidermis has only been previously shown with immunohistological staining (38, 39).

METHODS

Cells

Both primary keratinocytes obtained from SSIN (SENCAR) mice (University of Texas M.D. Anderson Veterinary Division, Bastrop, TX) and the murine keratinocyte cell lines: HEL-30 (34), MT1/2 (40), CH72 (40), JWF2 (40) were used in this study. Primary cultures of keratinocytes isolated from newborn mice by trypsinization were plated at 37°C and 5% CO_2 in an enriched Waymouth's medium containing 10% fetal bovine serum, and switched to the completely defined, serum-free SPRD-111 medium after overnight attachment (41). HEL-30 cells were grown at 37°C and 5% CO_2 in a modified Hank's buffered salt solution with 5% fetal bovine serum. For RNA isolation used in polymerase chain reaction, all cell lines were grown at 37°C and 5% CO_2 in EMEM containing 8% serum and 1.2 mm calcium chloride.

Membrane and cytosolic extracts

Cells were washed with PBS and once with homogenization buffer (50 mm Tris HCl, pH 8.5) (34, 35). The homogenization buffer also contained 1 mm EDTA, 1 mm EGTA, 150 mm NaCl, 1 mm Na_3VO_4, 1 mm PMSF,

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and 10 μg/ml leupeptin. The cells were scraped into the homogenization buffer and homogenized on ice by pulse sonication for 10 sec. Homogenates were centrifuged at 560,000 *g* for 10 min at 4°C using a Beckman TL-100 ultracentrifuge. Cytosols for $cPLA_2$ assay were used in the assay immediately. The protein concentrations of the cytosols were determined by the Coomassie blue reaction (Bio-Rad).

For membrane preparations, cells were washed with PBS and collected in sterile water. The cells were homogenized on ice for 20 sec using a pulse sonicator. Membrane preparations were then prepared by treatment with 0.36 N H_2SO_4 in 2 m NaCl overnight (pH 1.6) followed by dialysis against 10 mm acetic acid–sodium acetate buffer (pH 4.4) (42). Membranes for PLA_2 assay were stored at −20°C until use. Protein concentration of the membrane preparations was determined by the Coomassie blue reaction (Bio-Rad).

Cell-free $cPLA_2$ activity

The activity of PLA_2 was measured using either the micelle assay or radiolabeled *E. coli* assay. Except where noted, the micelle assay measured the hydrolysis of [1-^{14}C]arachidonic acid (AA) from 1-α-1-palmityl-2-[1-^{14}C]arachidonyl-phosphatidylcholine (PCAA) (53 mCi/mmol, 50 μCi/ml, NEN), as described by Kast, Fürstenberger, and Marks (34, 35). In the study of substrate specificity, 1-α-1-palmitoyl-2-[1-^{14}C]linoleoyl-phosphatidylcholine (PCLA) (50 mCi/mmol, 50 μCi/ml, NEN), 1-α-1-palmitoyl-2-[1-^{14}C] oleoyl-phosphatidylcholine (PCOA) (54 mCi/mmol, 50 μCi/ml, Amersham Corp.), and 1-α-1-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylethanolamine (PEAA) (53 mCi/mmol, 50 μCi/ml, NEN) were used as substrates. The phospholipids were dried under N_2, resuspended in dimethyl sulfoxide by vortexing, then mixed with $cPLA_2$ homogenization buffer containing $CaCl_2$ and dithiothreitol (DTT) to give a final substrate concentration of 4 mm and 10 mm, respectively. The assay was initiated by addition of 30 μl of cytosol (0.3–1 mg/ml protein) to 10 μl substrate mix to give a final substrate concentration of 0.375 μm. The assay was carried out in a 37°C water bath for 30 min. The reaction was stopped by the addition of 100 μl of ethanol containing 20 mm HCl and 0.2 ng/μl cold AA. Fifty microliters of the reaction was spotted onto a silica gel thin-layer chromatography aluminum-backed sheet (EM Separations), and the lipids separated in the organic phase of iso-octane–ethyl acetate–acetic acid–water (50:110:20: 100 (by volume). Areas containing free fatty acid or lysophospholipids and the origins were identified using iodine vapor, cut out, and the radioactivity of each spot was counted. In some experiments, the $cPLA_2$ inhibitor, AA-derived trifluromethane ($AACOCF_3$; Biomol), or the $sPLA_2$ inhibitor, 1-palmitylthio-2-palmitoylamino-1,2-dioxy-*sn*-glycero-3-phosphocholine (thioetheramide PC; Biomol), were used; cytosols were incubated with various concentrations of inhibitors at 37°C for 10 min before the addition of substrate. In some cases, cytosol were heated to 57°C for 5 min before the addition to the reaction mix to determine thermal sensitivity. Assays were also performed with and without DTT to determine the effects of reducing agents on the activity of keratinocyte $cPLA_2$.

Preparation of radiolabeled *E. coli* membrane suspension

Radiolabeled *E. coli* were prepared by the method of Elsbach and Weiss (43). An overnight culture of JM109 *E. coli* was diluted 1:20 in LB broth and incubated at 37°C for 3 h in the presence of 1 mCi/ml [^{14}C]oleic acid (OA; NEN). Radiolabeled *E. coli* were then washed by incubating in fresh LB broth for 30 min followed by washing with 1% bovine serum albumin (BSA) to remove unincorporated radiolabel. The washed *E. coli* were autoclaved and resuspended in the appropriate amount of 0.85% saline to achieve 5,000 cpm/ul. Radiolabeled *E. coli* membrane suspensions were stored at −20°C until use. Typically, between 50–90% of the added [^{14}C]OA was incorporated into *E. coli.*

In vitro $sPLA_2$ assay

The $sPLA_2$ activity was measured using radiolabeled [^{14}C]OA *E. coli* membrane suspension as substrate. In the substrate specificity studies, [^{14}C]AA *E. coli* and micellar substrates such as PCAA, PCLA, PCOA, and PEAA were also used. $sPLA_2$ assay was performed with 10 μg protein from keratinocyte membrane preparation. The reaction mix contained 5 mm calcium chloride, 125 mm Tris HCl (pH 8.5), 2.5 mg/ml BSA, and 50,000 cpm [^{14}C]OA-labeled *E. coli* membrane suspension. The reaction was carried out in a 37°C water bath for 30 min and was stopped by the addition of 100 μl 2 N HCl and 100 μl 20 mg/ml BSA. The reaction mix was microfuged and 250 μl supernatant was counted to monitor the [^{14}C]OA release from the *E. coli* membrane. Total counts were also obtained by counting pellets as well as supernatant. In some experiments, the $cPLA_2$ inhibitor $AACOCF_3$, the $sPLA_2$ inhibitor, thioetheramide PC, or the $iPLA_2$ inhibitor, bromoenol lactone E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS; Biomol) were used to determine the specificity of the PLA_2. Membrane preparations were heated at 57°C for 5 min, or 10 mm DTT was added in the assay in some cases to characterize the PLA_2 in keratinocyte membranes.

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PLA_2 activity in intact cells

Confluent cultures of primary keratinocytes or HEL-30 cells were labeled with [1-^{14}C]arachidonic acid (57 mCi/mmol, 0.1 mCi/ml, NEN), [1-^{14}C]linoleic acid (53 mCi/mmol, 0.1 mCi/ml, NEN) or [1-^{14}C]oleic acid (50 mCi/mmol, 0.1 mCi/ml, NEN) at a concentration of 0.3 μCi/ml medium for 16 h (28). Dishes were then washed twice with PBS and fed with fresh medium containing 1.6 μm TPA, 16 μm 4α-TPA, 0.1 μm calcium ionophore A23187, 10 μm bradykinin or vehicle. At appropriate times, aliquots of medium were counted by liquid scintillation counting. Triplicates were used at each time point.

Northern analyses

Total RNA or poly A$^+$ RNA was isolated using GITC reagent from cultured mouse keratinocytes (100 mm^2 tissue culture plates) treated with various stimuli for various time periods. Total RNA was isolated with water-saturated phenol and poly A$^+$ RNA was isolated using QIAquick mRNA isolation kit (Qiagen). RNAs were separated on a 1% agarose–formaldehyde gel and transferred to nylon membranes. The membranes were cross-linked using a UV-cross linker (Stratagene), and hybridized with radiolabeled $cPLA_2$ or $sPLA_2$ cDNA probes. Hybridization was performed at 68°C for $cPLA_2$ and 65°C for $sPLA_2$. The washing conditions were two 15-min room temperature washes with 2 × SSC and one 30-min wash with 0.1 × SSC at 65°C for $cPLA_2$ and 55°C for $sPLA_2$. $cPLA_2$ cDNA was a generous gift from Dr. Lin from the Genetics Institute (Cambridge, MA), and rat type I and type II $sPLA_2$ cDNAs were kindly provided by the Shionogi Research Laboratory, Osaka, Japan.

Reverse transcription-polymerase chain reaction (RT-PCR)

The primers used for type I $sPLA_2$ were 5′CAACAA CTACGGCTGCTACTGTGGC3′ (SP4, forward) and 5′CGGTCACAGTTGCAGATGAAGCTCTC3′ (SP5, reverse). The primers used for type II $sPLA_2$ were 5′GGCAGAGGATCCCCCAAGGATGCCAC3′ (SP1, forward) and 5′CCCTTTGCAAAACATGTTGGGG3′ (SP3, reverse). First strand cDNA was synthesized using reverse transcription (RT) reaction with the Superscript™ kit provided by Gibco BRL. Total RNA were isolated from different sources including primary mouse keratinocytes, HEL-30 papilloma cell line from NMRI mouse (34), MT1/2 papilloma cell line from SENCAR mouse (40), CH72 squamous cell carcinoma cell line from SENCAR mouse (40), JWF2 squamous cell carcinoma cell line from SENCAR mouse (40), pancreatic cells (as a positive control for type I $sPLA_2$), and primary small intestinal cells (as a positive control for type II $sPLA_2$) and 1 μg of each was used as templates for the RT reaction. Oligo $dT_{(12–18)}$ was used as a primer for the RT reaction. The cDNA templates generated from the RT reactions were used in PCR reaction with SP1–SP3 primers or SP4–SP5 primers (Genosys) and Taq DNA polymerase (Roche). The conditions for PCR reaction were: 63°C for annealing, 72°C for elongation, and 94°C for denaturing, and the reaction was performed for 45 cycles. The products from the PCR reaction were visualized on a 2% agarose gel and transferred to nylon membranes for Southern analysis. The PCR products were then subjected to restriction analysis to partially confirm their identity. The confirmed PCR products were subcloned into an Invitrogen cloning vector, pCR™II (3.9 kb) using a TA cloning kit (Invitrogen) and used to transform DH5α™ competent *E. coli* (maximum efficiency, Gibco BRL) before sequencing. Sequencing was performed by the Molecular Biology Service Core at Science Park, Smithville, TX. Sequences of the keratinocyte PCR products were compared with those of the pancreatic and the small intestinal PCR products as well as the published rat (44) and mouse (45) type I and type II $sPLA_2$ sequences, respectively.

Southern analysis

The nylon membranes with PCR products were cross-linked using a UV-cross linker (Stratagene), and hybridized with radiolabeled type I or type II $sPLA_2$ cDNA probes. The probed membranes were then washed and exposed to X-ray film (Eastman Kodak, Rochester, NY) for detection.

Statistical analyses

Results are expressed as means ± standard errors of triplicate data; all data were subjected to one-way analysis of variance (ANOVA) test. When significant differences were detected, Tukey's honest significant difference test was used for multiple comparisons among the groups. Whenever applicable, Student's *t* test was used to determine the differences between two samples. $P \leq 0.05$ was considered significant.

RESULTS

Differential activation of $cPLA_2$ and $sPLA_2$ in keratinocytes

The level of phospholipase activity in cultured keratinocytes was determined by the release of incorporated fatty acid from [^{14}C]AA-, LA-, or OA-prelabeled cells in the presence or absence of either TPA (1.6 μm) 4α-TPA

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(16 μm), calcium ionophore A23187 (0.1 μm), or bradykinin (10 μm). Incorporation of radiolabeled fatty acids was equal in all cell lines and cultures and equilibrium was reached at around 8–9 h (37). The incorporation of AA into PC was not greater than incorporation of LA or OA (data not shown). The time course of the release of the incorporated fatty acids from keratinocytes is shown in **Fig. 1**. The release of incorporated AA from both HEL-30 cells (data not shown) and cultured primary keratinocytes occurred in a time-dependent manner and reached a maximum at approximately 3 h with or without treatment with TPA. Compared to vehicle control, TPA enhanced the release of incorporated AA from cultured keratinocytes by about 3- to 3.5-fold (Fig. 1A). Compared to the release of AA, the release of incorporated LA and OA was significantly reduced. An initial increase in LA and OA release was observed at 30 min, possibly due to changing the medium, with no further increase. The maximum release of LA and OA at 3 h was only one-third

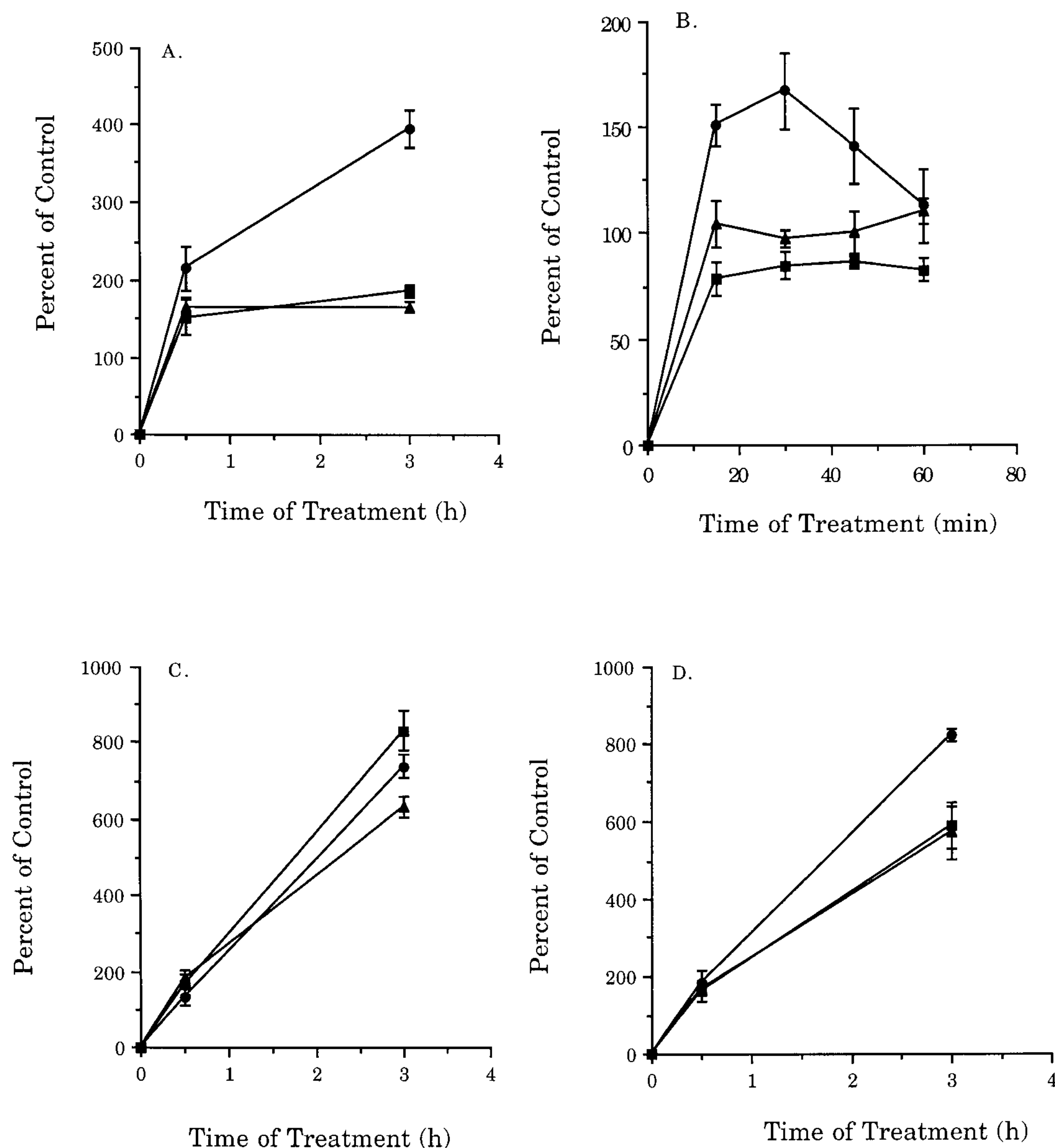

Fig. 1. Release of fatty acids from pre-labeled keratinocytes treated with different stimuli. Primary cultures of keratinocytes were pre-labeled with either 0.3 μCi/ml [^{14}C] arachidonic acid (●), [^{14}C]linoleic acid (▲), or [^{14}C]oleic acid (■) for 16 h. Washed cells were then treated with either A: 1.6 μm TPA; B: 10 μm bradykinin; C: 16 μm 4α-TPA; or D: 0.1 μm calcium ionophore A23187. At indicated time points 100 μl media was taken and the radioactivity was counted. Data points were mean ± SEM, n = 3.

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that of AA (Fig. 1A). A similar trend was also observed with bradykinin, although with a different time course than observed with TPA. The maximum release of incorporated AA was observed at 30 min with bradykinin treatment rather than 3 h with TPA treatment (Fig. 1B). Bradykinin also appeared to reduce the basal level of incorporated OA hydrolysis by approximately 20%. (Fig. 1B)

A different fatty acid release profile was produced when 4α-TPA and calcium ionophore A23187 were used as agonists (Fig. 1C and D). Continuous release was observed for the 3 h duration of the experiment for all three fatty acids incorporated, and no preference was observed for AA or any other fatty acids measured. The differential hydrolysis of AA, LA, and OA with 4α-TPA and calcium ionophore A23187, compared to TPA and bradykinin, was consistent with the involvement of diverse mechanisms, possibly the activation of $cPLA_2$ and $sPLA_2$. To investigate whether these two forms of PLA_2s exist in mouse keratinocytes, the distinctive biochemical and enzymatic properties of the two enzymes were used to characterize the $cPLA_2$ and the $sPLA_2$ in mouse keratinocytes.

PLA_2 activity in cytosol versus membrane

PLA_2 activities were detected in both cytosol and membrane fractions of mouse epidermal keratinocytes (**Fig. 2**). These activities appeared to be dependent on the protein concentrations of the preparations. As shown in **Fig. 3A**, PLA_2 isolated from the cytosolic fraction of keratinocytes exhibited a clear substrate specificity. Keratinocyte $cPLA_2$ preferentially hydrolyzed the release of incorporated AA esterified to the *sn*-2 position of phosphatidylcholine (PC). The release of incorporated LA or OA esterified to PC is less than 50% of that released when PC-AA was used as the substrate (Fig. 3A). Other phospholipids, such as AA esterified to phosphatidylethanolamine, were not readily used as substrates by keratinocyte $cPLA_2$ (Fig. 3A). The PLA_2 activity of keratinocyte cytosol using *E. coli* membrane suspension, a preferred substrate form for $sPLA_2$ (46), is also minimal (1.4% and 0.6%, respectively, for AA- and OA-labeled *E. Coli*). Keratinocyte membrane preparations, however, preferentially catalyzed the release of fatty acids presented in the form of *E. coli* membranes (Fig. 3B). On the other hand, even though the hydrolysis of AA by membranes from keratinocytes is twice that of OA, both AA and OA in the form of *E. coli* membrane suspension were significantly hydrolyzed by keratinocyte membrane preparations and both fatty acids served as substrates for membrane PLA_2 (Fig. 3B). The activity of keratinocyte membrane preparations towards micellar substrates was almost negligible as compared to when *E. coli* membrane suspensions were

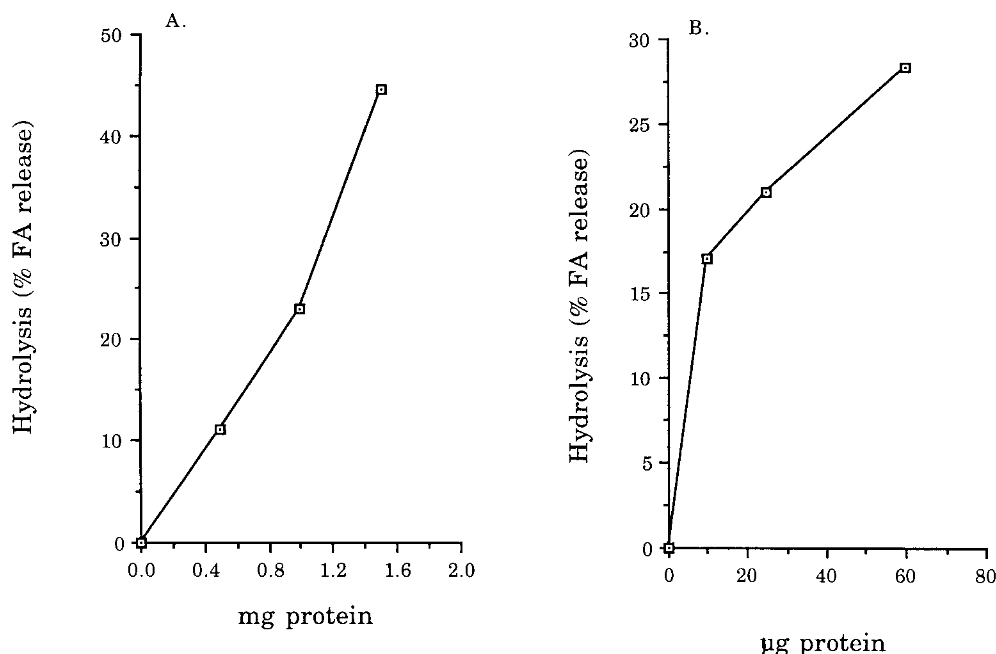

Fig. 2. Protein dependence of PLA_2 activity from keratinocyte membrane and cytosol. Keratinocyte cytosol and membrane cell fractions were extracted as described under Methods. Indicated amounts of protein were used in each assay. $cPLA_2$ and $sPLA_2$ assays were performed, and the activities of these enzymes were expressed as % fatty acid (FA) release. A, keratinocyte cytosol; B, keratinocyte membrane.

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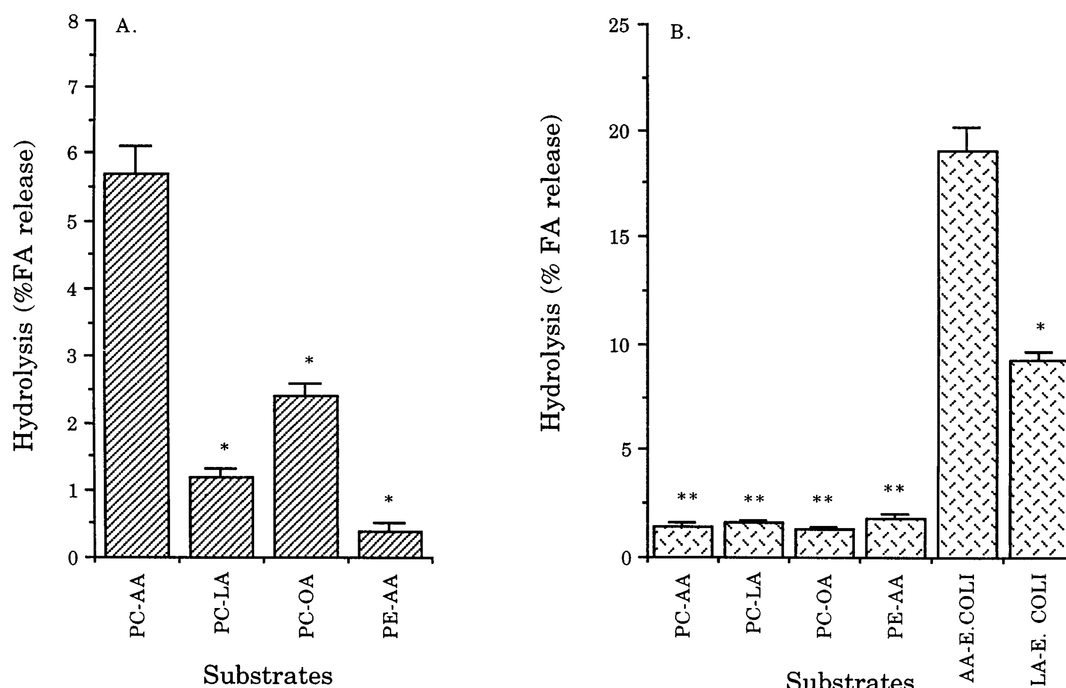

Fig. 3. Substrate specificity of PLA_2s isolated from mouse keratinocytes. Cytosolic and membrane fractions of keratinocytes were assayed with different forms of substrates, including micellar substrates as well as *E. coli* membrane suspensions as substrates. PC-AA, phosphatidylcholine-arachidonic acid; PC-LA, phosphatidylcholine-linoleic acid; PC-OA, phosphatidylcholine-oleic acid; PE-AA, phosphatidylethanolamine-arachidonic acid; AA *E. coli*, arachidonic acid-labeled *E. coli* membrane; OA *E. coli*, oleic acid-labeled *E. coli* membrane. A: keratinocyte cytosol; B: keratinocyte membrane preparations. Data points were mean ± SEM, n = 3. *, Bars with different numbers of asterisks are statistically different from each other ($P < 0.05$).

used as substrates (Fig. 3B). The crude extract of keratinocyte membrane also appears to contain an inhibitor that reduces the activity of a purified pancreatic $sPLA_2$ by half (data not shown). This inhibitor, the identity of which is unknown, was inactivated or removed when acid extraction was performed on the particulate cell fraction.

Effect of calcium, heat, acid, and reducing agents

To further characterize and establish the uniqueness of membrane and cytosolic PLA_2s, enzyme activities in the presence of various calcium concentrations, high heat conditions, or reducing agents were assessed. Calcium is an absolute requirement in the action of both $cPLA_2$ and $sPLA_2$ even though its roles in $sPLA_2$ and $cPLA_2$ activation are different (30, 47, 48). Calcium resides at the catalytic center of $sPLA_2$ and is directly involved in substrate-enzyme interactions (48). For $cPLA_2$ activation, calcium is not required for catalytic activity; rather, it is involved in the translocation of $cPLA_2$ from cytosol to the membrane where the substrates are located (27, 28, 44). When calcium concentrations were varied in the keratinocyte PLA_2 assays, different responses were observed with cytosol and membrane cell preparations (**Fig. 4**). These different responses suggested that these PLA_2s are indeed different forms. Increasing the calcium concentration from 0 to 10 mm dose-dependently enhanced the PLA_2 activity of keratinocyte cytosol, with a plateau at 4 mm (Fig. 4A). This apparent high requirement of keratinocyte cytosolic PLA_2 could be the result of the EDTA and EGTA which are present in the extraction buffer. The membrane PLA_2, however, has an optimum Ca^{2+} requirement of 1 mm (Fig. 4B). Lower and higher Ca^{2+} in the reaction reduced the PLA_2 activity of keratinocyte membrane preparations. However, substantial activity was still observed when no Ca^{2+} was added. Therefore, EDTA was introduced to chelate endogenous calcium. While EDTA significantly reduced the PLA_2 activity of keratinocyte membrane preparations, up to 5 mm EDTA failed to completely inhibit PLA_2 activity in this cell fraction (Fig. 4B), suggesting the possible presence of other mechanisms of fatty acid hydrolysis.

The structural differences between $cPLA_2$ and $sPLA_2$ provide them with distinct biochemical characteristics that can be used to distinguish the two enzymes. The seven disulfide bonds have been reported to provide $sPLA_2$ (14kD) with resistance to heating and acid; however, it is sensitive to reducing reagent (49). The $cPLA_2$ is a much larger protein (85 kD) which lacks those di-

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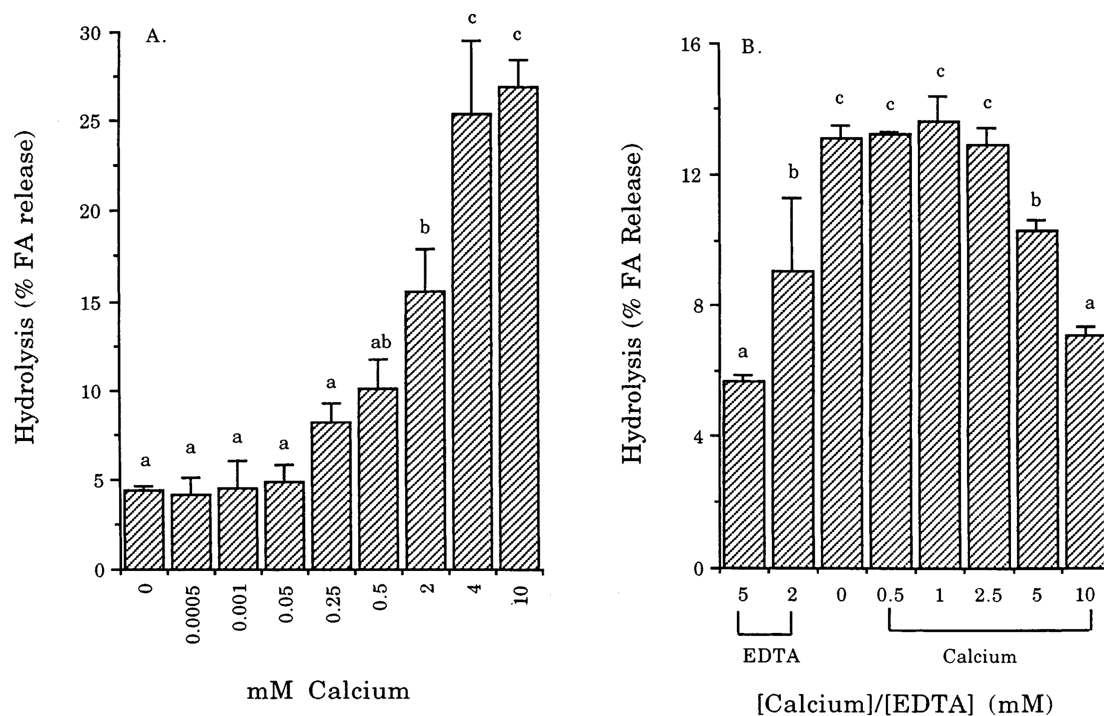

Fig. 4. Effects of calcium concentration on the activities of keratinocyte PLA_2s. Calcium concentration was varied in the assay from 0 to 10 mm. A: keratinocyte cytosol; B: keratinocyte membrane preparation. In panel B, various amount (mm) of calcium chloride or EDTA. Data points were mean ± SEM, n = 3. a,b,c, Bars assigned different letters are significantly different from each other ($P <$ 0.05).

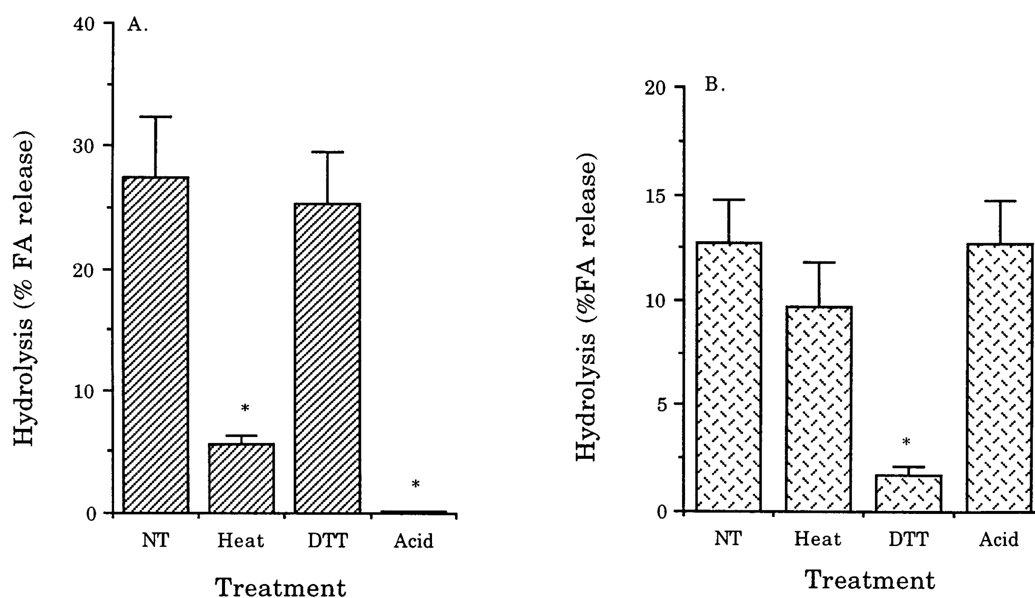

Fig. 5. Effects of heating, reducing reagent, and acid treatment on the activities of keratinocyte PLA_2s. $cPLA_2$ and $sPLA_2$ assays were performed with or without reducing reagent DTT. Keratinocyte cell fractions were either heated at 57°C for 5 min (Heat), with equal volume of 0.36 N H_2SO_4 (Acid), or not treated (NT). A: keratinocyte cytosol; B: keratinocyte membrane preparations. Data points were mean ± SEM, n = 3. *, Bars with asterisks are significantly different from others at $P < 0.05$.

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sulfide bonds (50). Therefore, $cPLA_2$ is much more sensitive to heating and acid treatment and is nonresponsive to reducing reagents. As shown in **Fig. 5A**, the cytosolic fraction has the classic characteristics of $cPLA_2$. It is sensitive to heating and acid treatment, and resistant to the reducing reagent DTT. Incubation of keratinocyte cytosol with 10 mm DTT only marginally reduced PLA_2 activity in this cell fraction, and sometimes it even slightly enhanced this PLA_2 activity (data not shown). However, heating cytosols for 5 min at 57°C significantly reduced PLA_2 activity by 80%, and acid treatment with an equal volume 0.36 N H_2SO_4 completely inhibited $cPLA_2$ activity of keratinocyte cytosols (Fig. 5A). On the other hand, keratinocyte membranes demonstrated just the opposite responses to these treatments. The PLA_2 activity of this cell fraction was sensitive to DTT treatment and resistant to heating and acid treatment. Treatment with 10 mm DTT reduced PLA_2 activity of keratinocyte membrane by 86.6% (Fig. 5B). Neither heating at 57°C for 5 min nor acid treatment altered this PLA_2 activity. In fact, due to the existence of the putative endogenous inhibitor, acid extraction with 0.36 N H_2SO_4 overnight was required in order to obtain an active PLA_2 from keratinocyte membranes.

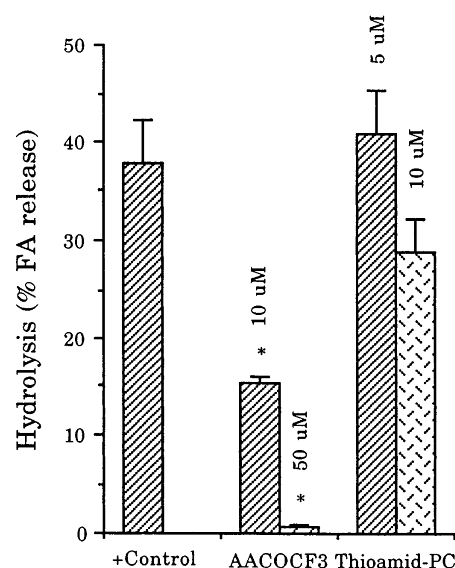

Fig. 6. Effect of PLA_2 inhibitors on keratinocyte cytosolic PLA_2 activity. Indicated amount of $cPLA_2$ inhibitor, $AACOCF_3$ or $sPLA_2$ inhibitor thioetheramid-PC (Thioamide-PC) were used in the assay with keratinocyte cytosol. Data points were mean ± SEM, n = 3. Bars with asterisks are significantly different from others at $P < 0.05$.

Effects of specific inhibitors

To study the role of different PLA_2s in eicosanoid production as well as other functions, considerable effort has been put into developing specific inhibitors for different PLA_2s. Of these inhibitors, the substrate analog, thioetheramide-PC showed specific selectivity towards human recombinant $sPLA_2$ ($IC_{50} = 0.9$ μm) with little or no effect on $cPLA_2$ from monocyte U937 cells (51). An $iPLA_2$ inhibitor, HELSS, has recently been developed that irreversibly inhibits $iPLA_2$. This inhibitor was shown to be specific for $iPLA_2$ with half-maximal inhibition observed at 60 nm after 5 min incubation (52). The $iPLA_2$ was also inhibited by the widely used $cPLA_2$ inhibitor, trifluoromethyl ketone ($AACOCF_3$). The trifluoromethyl ketone is an analog of AA that slowly but tightly binds to $cPLA_2$ and calcium. The formation of the complex Ca^{2+}–$AACOCF_3$–$cPLA_2$ inhibits the enzyme from hydrolyzing AA from phospholipids (53).

To verify the identity of these two PLA_2s in keratinocytes, these inhibitors were used in the assay. The $cPLA_2$-specific inhibitor $AACOCF_3$ (53) dose-dependently inhibited the PLA_2 activity of keratinocyte cytosol while the $sPLA_2$-specific inhibitor thioetheramide PC (51) failed to do so (**Fig. 6**). When 50 μm $AACOCF_3$ was used in the reaction, the PLA_2 activity of keratinocyte cytosol was almost eliminated (Fig. 6). However, when the same amount of $AACOCF_3$ was used with keratinocyte membrane preparations, the PLA_2 in this cell fraction remained active (**Fig. 7A**). Similarly, when 10 μm thioetheramide PC was incubated with keratinocyte cytosol, the PLA_2 activity was only marginally reduced compared to when no inhibitor was added (Fig. 6). This may be due to the cross-reactivity of this inhibitor with $cPLA_2$ at high dose. However, thioetheramide PC dose-dependently inhibited the PLA_2 activity of keratinocyte membrane with an IC_{50} of approximately 1 μm (Fig. 7B). Addition of 50 μm thioetheramide PC in the assay almost abolished the PLA_2 activity of this cell fraction. Addition of up to 500 nM HELSS, an $iPLA_2$ inhibitor (52), was also ineffective (Fig. 7C).

Expression of $cPLA_2$ and $sPLA_2$ mRNA in keratinocytes

The enhanced hydrolysis of AA but not LA or OA after TPA treatment of keratinocytes suggests that TPA activates $cPLA_2$ but not $sPLA_2$. In agreement with the work of Kast et al. (34) with HEL-30 cells, we also found that $cPLA_2$ activity of HEL-30 cells was elevated by 50% 10–30 min after treatment of the cells with 1 μg/ml TPA (data not shown). However, no change was detected at the transcription level using Northern analysis of RNA isolated after TPA treatment of HEL-30 cells (data not shown).

To determine whether the $sPLA_2$ was type I or type II, total RNA (30 μg) or poly A^+ RNA (from two 100 mm^2

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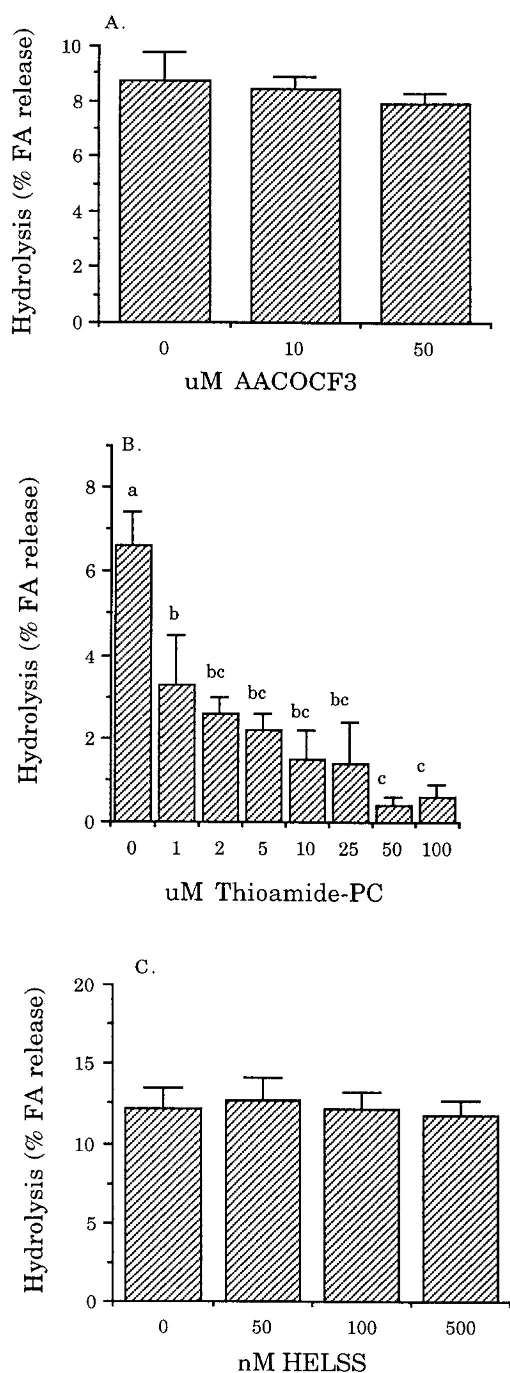

Fig. 7. Effect of PLA_2 inhibitors on keratinocyte membrane PLA_2 activity. Indicated amount of $cPLA_2$ inhibitor, $AACOCF_3$ (A); $sPLA_2$ inhibitor, thioetheramide-PC (Thioamide-PC) (B); or calcium independent PLA_2 inhibitor, HELSS (C) were used in the assay with keratinocyte membranes. Data points were mean ± SEM, n = 3. Bars with different letters are significantly different from each other at $P < 0.05$.

dishes), respectively, were isolated from treated cultured keratinocytes. Total RNA (5 μg) isolated from pancreas and small intestine were also loaded on the gels as positive controls for type I and type II $sPLA_2$, respectively (54, 55). Type I and type II $sPLA_2$ cDNA probes produced the expected signal at 0.85 kb in pancreas and small intestine after overnight exposure to the film. However, no signals were detected in mouse keratinocytes even after treatment with TPA or calcium ionophore A23187 and an exposure time of 1 week (data not shown).

To determine whether $sPLA_2$s were expressed at low levels in keratinocytes, reverse transcriptase-PCR was used to detect type I and type II $sPLA_2$ in primary keratinocytes as well as established cell lines. Both no template and RNA only were included as negative controls for the possible presence of contaminated cDNA. Bands corresponding to the predicted sizes (234 bp for type I $sPLA_2$ and 269 bp for type II $sPLA_2$) were observed in almost all cell types (**Fig. 8**). Sequence comparison demonstrated that the PCR products from primary keratinocytes and pancreatic cells using SP4-SP5 primers shared 93.5% homology, and they shared 92.4% and 89.5% homology with the partial sequence of rat pancreatic type I $sPLA_2$ (44), respectively. The type II $sPLA_2$ PCR products from primary keratinocytes and small intestine have the same sequence and they shared 99.6% homology with the reported type II $sPLA_2$ sequence for mouse small intestine (45). The type I $sPLA_2$ appeared to be expressed in slightly higher levels in the less transformed cells (primaries and HEL-30 cells) than in the more transformed cell types (MT1/2, CH72, and JWF2 cells) (Fig. 8A). On the other hand, the type II $sPLA_2$ were expressed at a similar level in all cell types except the JWF2 cells, where it was not expressed (Fig. 8B). Southern analysis of the PCR products verified the identity of these products to be type I and type II $sPLA_2$ (Fig. 8C and D). Interestingly, the primers for type II $sPLA_2$ also picked up two additional bands in the transformed cell lines (HEL-30, MT1/2, and CH72 cells). These two bands were not present in the primary keratinocytes (Fig. 8B). The identity of these products is not clear. However, analysis of their sequence shows that they do share homology with the mouse type II $sPLA_2$ cDNA.

DISCUSSION

The specificity for AA release in keratinocytes activated by TPA and bradykinin is shown in this paper. Calcium ionophore A23187 and 4α-TPA, on the other hand, do not show this fatty acid specificity. These observations suggest that different mechanisms might exist in mouse keratinocytes for the hydrolysis of fatty acids. The most direct mechanism is through PLA_2,

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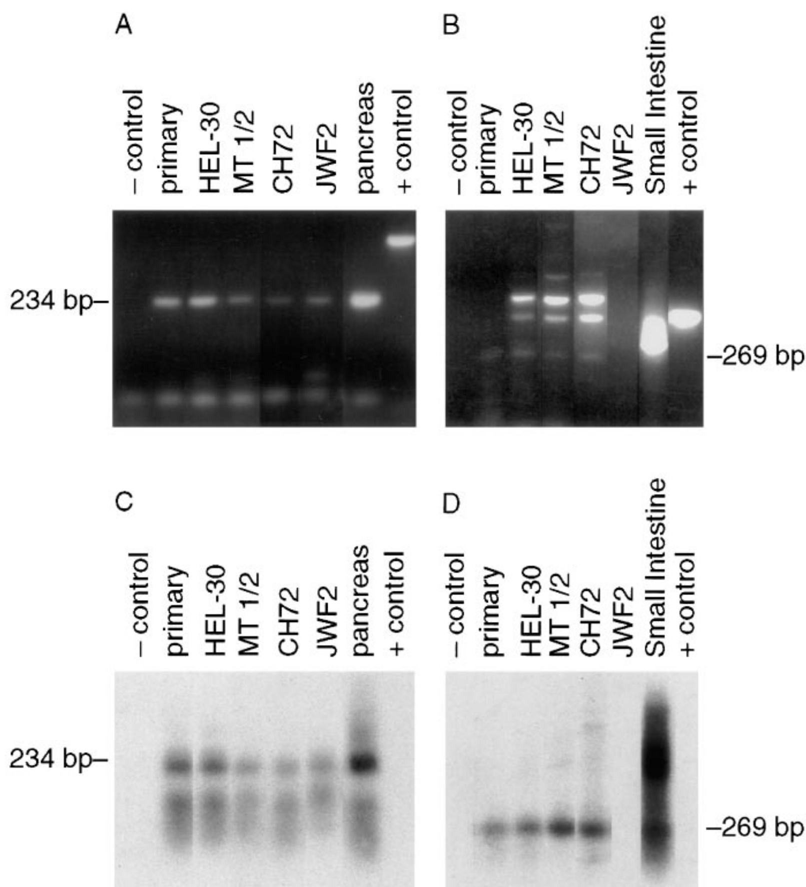

Fig. 8. Expression of type I and type II $sPLA_2$ mRNA in cultured mouse keratinocytes as detected by RT-PCR. One μg total RNA was used for each RT-PCR reaction. RT-PCR was performed as described under Methods. One μg total RNA from pancreas and small intestine was also amplified as positive controls for type I and type II $sPLA_2$, respectively. The predicted bands corresponding to type I $sPLA_2$ (234 bp) and type II $sPLA_2$ (269 bp) were detected in both cell lines and positive controls. A: amplification of type I $sPLA_2$; B: amplification of type II $sPLA_2$; C: Southern analysis of the blot in panel A; D: Southern analysis of the blot in panel B. Lines point to the size of $sPLA_2$ signals.

which hydrolyzes the hydrolysis of *sn*-2 fatty acids from membrane phospholipids. Others include the phospholipase C and phospholipase D pathways with an additional step catalyzed by diacylglycerol lipase. The phospholipase C or D pathway does not appear to play a major role in the diversity of fatty acid release observed in mouse keratinocytes as no differences in radiolabeled phosphatidic acid were found with treatments using different agonists (data not shown). Therefore, activation of different members of the PLA_2 family might be responsible for the differential hydrolysis profile stimulated with different agonists.

The two classic types of PLA_2s are $sPLA_2$ and $cPLA_2$. $cPLA_2$ has been reported to preferentially hydrolyze AA from the *sn*-2 position of phosphatidylcholine (PC) in a variety of cell types, including macrophages and platelets (7, 8). We show here that PLA_2 from the cytosolic fraction of keratinocytes exhibited similar substrate specificity. This PLA_2 also showed the classic biochemical characteristics of $cPLA_2$ with the exception of the requirement of calcium. The divergence of calcium requirement we observed in this paper, as compared to that of previous ones, is not clear. The presence of EDTA and EGTA in the extraction buffer may be a contributing factor to the increase of calcium requirement in the in vitro assay. However, it had also been observed that under in vitro conditions, the calcium requirement of $cPLA_2$ differs from that of in vivo conditions (56). A biphasic response curve has also been observed for calcium in human keratinocytes (56). A $cPLA_2$ inhibitor $AACOCF_3$ was also shown to readily inhibit the PLA_2 activity of keratinocyte cytosol. While this inhibitor may also inhibit $iPLA_2$, the requirement of calcium for enzyme activity clearly demonstrated that this PLA_2

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activity indeed is from $cPLA_2$. Therefore, in agreement with the work of Kast et al. (34, 35) in keratinocytes, we conclude that mouse keratinocyte cytosol possesses a classic $cPLA_2$.

The effect of TPA on AA hydrolysis was at least partially mediated by $cPLA_2$ as data from our laboratory as well as from Kast et al. (35) demonstrated that TPA stimulated the activation of $cPLA_2$ at 15 and 30 min. The lack of concordance in the time course of AA release and $cPLA_2$ activation suggests the involvement of additional mechanisms in the hydrolysis of AA. The initial release of AA mediated by $cPLA_2$ could signal the subsequent activation of a variety of other pathways which may include other PLA_2s, such as $sPLA_2$. Several recent reports have suggested that stimulation of one PLA_2 and the subsequent production of eicosanoids as well as platelet activating factor (PAF) may lead to the activation of one or more other PLA_2s (22–26).

Unlike $cPLA_2$, $sPLA_2$ has no preference for the type of fatty acid at the *sn*-2 position. It hydrolyzes OA as well as AA as long as the fatty acid is esterified to the *sn*-2 position of the phospholipids. We show here that PLA_2 from keratinocyte membrane preparations hydrolyzed both AA- and OA-labeled *E. coli* membrane. In keratinocytes, the particular form of $sPLA_2$ had not been previously identified although the existence of a $sPLA_2$ in human keratinocytes has been suggested (38). However, this $sPLA_2$ was only detected in the more differentiated epidermis using immunofluorescence staining with antibodies against human recombinant type II $sPLA_2$ (38). In this paper, we provide evidence that mouse keratinocytes possess a form (or forms) of phospholipase with the established characteristics of $sPLA_2$. These characteristics are in keeping with the importance of disulfide bonds in stabilizing the structure of $sPLA_2$, and with the dependency of catalytic activity of $sPLA_2$ on calcium. Therefore, we suggest that one or more forms of $sPLA_2$ are expressed in mouse keratinocyte membrane. The existence of a $sPLA_2$ in mouse keratinocytes is confirmed by a recent report suggesting $sPLA_2$ is required for maintaining permeability barrier homeostasis in mouse epidermis (39). Using RT-PCR with primers based on the rat pancreatic type I and mouse small intestinal type II $sPLA_2$ sequence, the predicted products were detected in mouse keratinocytes with homologies to the type I and type II $sPLA_2$, respectively. These results suggested that both type I and type II $sPLA_2$ exist in mouse keratinocytes and that they are expressed in low abundancy.

Using antisense transfections, we have shown that $cPLA_2$ activation is partially responsible for the specificity of AA release observed in keratinocytes treated with TPA (unpublished data). The activation of $cPLA_2$ by TPA may be a direct or indirect result of activated PKC as an unequivocal effect of TPA is to activate PKC (57). The hydrolysis of LA and OA stimulated by calcium ionophore A23187 and 4α-TPA is likely to be the result of activation of $sPLA_2$ although direct evidence for activation of $sPLA_2$ by calcium ionophore A23187 and 4α-TPA has not yet been demonstrated. The mechanisms by which $sPLA_2$ may be activated are also not clear. Some cytokines such as interleukin (IL)-6, IL-1 and tumor necrosis factor have been shown to transcriptionally up-regulate type II $sPLA_2$ in immune cells as well as in astrocytes and mesangial cells (58–60). Post-translational activation by protease has also been suggested for two forms of $sPLA_2$ (20, 61). The signaling pathways involved in the activation of $sPLA_2$ have also not yet been defined, but the possible involvement of one or multiple forms of G protein has been suggested (62, 63). The inhibition of OA hydrolysis by bradykinin might be a result of activation of a G protein (34) and subsequent inhibition of $sPLA_2$.

In conclusion, we have identified and characterized three forms of PLA_2 in keratinocytes, $cPLA_2$ in cytosol, and a type I and a type II $sPLA_2$ in the membrane. These PLA_2s differ in their substrate specificities and thus may produce the agonist-specific fatty acid release observed with different stimuli. Keratinocyte $cPLA_2$ is at least partially responsible for the specific hydrolysis of AA from mouse keratinocytes. The type I and II $sPLA_2$ may be responsible for the non-specific hydrolysis of other fatty acids from mouse keratinocytes through unknown mechanisms.

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