

Characterization of Group X Phospholipase A₂ as the Major Enzyme Secreted by Human Keratinocytes and its Regulation by the Phorbol Ester TPA

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HaCaT as well as human primary keratinocytes constitutively expressed mRNA of the human secreted phospholipase A₂ subtype groups X, V, IIA, and IID. A similar expression pattern was also found in human skin biopsies. Protein analysis showed that under serum-free conditions only group X secreted phospholipase A₂ is secreted into cell culture supernatants of HaCaT as well as human primary keratinocytes, whereas the other secreted phospholipases A₂ were not detectable at protein level. HaCaT keratinocytes constitutively released secreted phospholipase A₂ activity into the cell culture supernatant, being reflected by a constant release of fatty acids. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate, which is a potent inducer of inflammation in skin, drastically reduced the mRNA level of group X

secreted phospholipase A₂ and other secreted phospholipase A₂ subtypes as well as secreted phospholipase A₂ activity in cell culture supernatants. This suggests that inhibition of secreted phospholipase A₂ expression and activity as well as of fatty acid release by 12-O-tetradecanoylphorbol-13-acetate treatment might be a critical step impairing the integrity of the epidermis during phorbol-ester-induced pathologic processes in skin. The results show that group X secreted phospholipase A₂ represents the major secreted phospholipase A₂ subtype in human keratinocytes and thus may indicate a physiologic role for this enzyme in epidermis *in vivo*. **Key words:** epidermal homeostasis/inflammation/phospholipase A₂ inhibitors/skin. *J Invest Dermatol* 116:31–39, 2001

Secreted phospholipases A₂ (sPLA₂s) comprise a subfamily of phospholipases A₂ that are structurally related but display only modest sequence homology (Cupillard *et al*, 1997; Valentin *et al*, 1999). Many studies show that the induction and activity of mammalian sPLA₂ subtypes are involved in proinflammatory processes in several tissues and organs inducing or enhancing arachidonic acid release and prostaglandin formation (Pfeilschifter *et al*, 1993; Hurt-Camejo *et al*, 1997; Jamal *et al*, 1998; Pruzanski *et al*, 1998; Han *et al*, 1999; Murakami *et al*, 1999; Schiering *et al*, 1999; Shoda *et al*, 1999). Recently, the existence of several sPLA₂ subtypes within the same cell and the location to different subcellular sites was described (Bingham *et al*, 1999). In several cell systems treatment with proinflammatory cytokines resulted in the induction of group IIA sPLA₂ at the transcriptional level. Group IIA sPLA₂ protein is present in secretory vesicles (Vervoordeldonk *et al*, 1994; Bingham *et al*, 1999) from which it is released into the extracellular lumen. sPLA₂s are thought to be involved in inflammatory processes, e.g., in the

kidney (Pfeilschifter, 1994), the joints (Jamal *et al*, 1998), or the skin (Andersen *et al*, 1994). Detailed studies concerning the regulation of group IIA sPLA₂ have been performed, e.g., in rat glomerular mesangial cells, which release this enzyme in large amounts after stimulation with inflammatory cytokines such as interleukin-1 β (IL-1 β) or cAMP-elevating agents (for review see Pfeilschifter, 1994, 1995; Pfeilschifter *et al*, 1997).

In contrast, group V sPLA₂ was identified in heart, mast cells, and macrophages and seems to be a major enzyme involved in arachidonic acid release and prostaglandin formation (Chen and Dennis, 1998; Sawada *et al*, 1999; Shinohara *et al*, 1999). The group IIA and the group V sPLA₂s are thought to be functionally redundant (Murakami *et al*, 1998). It has been reported that these enzymes show differences in their substrate specificity concerning the phospholipid head groups, however, suggesting that group V sPLA₂ works better on biologic membranes than group IIA sPLA₂ (Han *et al*, 1999; Janssen *et al*, 1999). Moreover, Bingham *et al* (1999) unraveled the subcellular location of these sPLA₂ subtypes in bone-marrow-derived mast cells, showing that group V is located in perinuclear membranes similarly to enzymes involved in eicosanoid biosynthesis, whereas group IIA sPLA₂ is present in secretory granules for exocytosis. This study implies that these enzymes exert different functions and thus they are not redundant.

The group IIC sPLA₂ was found in testis of rat and mouse and seems to be a nonfunctional pseudogene in humans (Tischfield, 1997). This subtype is thought to be involved in sperm transport

Manuscript received November 8, 1999; revised September 14, 2000; accepted for publication September 18, 2000.

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Abbreviations: Pyr-1, 3-(2,6-dichlorophenylsulfonfylamino)-4-(2-(4-acetamido)pyridyl)-5-(3-(4-fluorophenoxy)benzyl(thio)-(1H)-pyrazole); sPLA₂, secreted phospholipase A₂.

and fertilization in rodent testis (Chen *et al*, 1997). Several novel subtypes identified are the group IID, IIE, IIF, and X sPLA₂s, which are found in different murine tissues (Valentin *et al*, 1999). A group IID and recently also a group IIE were identified in human tissues (Ishizaki *et al*, 1999; Suzuki *et al*, 2000), but a human ortholog has not been found so far for group IIF sPLA₂. The functions of most of these novel sPLA₂ subtypes remain to be elucidated.

A human group X sPLA₂ was found to be expressed in tissues related with inflammatory responses (Cupillard *et al*, 1997) as well as in alveolar epithelial cells of human lung (Hanasaki *et al*, 1999). This sPLA₂ subtype has specific structural features and its chromosomal location is distinct from those of other sPLA₂s (Cupillard *et al*, 1997). Group X sPLA₂ was found to release arachidonic acid more efficiently than other human sPLA₂ groups when added exogenously to mammalian cells, implicating a potential role in eicosanoid biosynthesis (Hanasaki *et al*, 1999; Bezzine *et al*, 2000).

In the skin, however, a function of sPLA₂ distinct from inflammation was described. The catalytic action of sPLA₂ was found to be crucial for epidermal homeostasis, which is important for keeping the membrane permeability barrier intact against excess water loss (Mao-Qiang *et al*, 1995, 1996). This permeability barrier is located within the extracellular, lipid-enriched domain of the stratum corneum (Lampe *et al*, 1983). In this domain sPLA₂ and many other lipid-hydrolyzing enzymes such as sphingomyelinase, triacylglycerol hydrolase, as well as certain proteases were identified in lamellar granules (also called lamellar bodies; Tinois *et al*, 1989). These are secretory organelles responsible for delivering lipids into the intercellular space building up the permeability barrier. The function of sPLA₂ is to hydrolyze free fatty acids from polar lipids to form more nonpolar lipids, which predominate in the stratum corneum. This action of sPLA₂ might be an important process for the maintenance of epidermal integrity but also during wound healing and skin repair. Only little information is yet available, however, as to which sPLA₂ subtypes exist in the human skin and how individual subtypes contribute to the structural and functional integrity of skin. Recent studies describe the localization of the pancreatic group IB sPLA₂ between the stratum corneum and stratum granulosum of human skin, but group IIA sPLA₂ was not found (Maury *et al*, 2000; Mazereeuw-Hautier *et al*, 2000). Other sPLA₂ subtypes were not considered in this study, however.

Using the human keratinocyte cell line HaCaT, human primary keratinocytes, and human skin biopsies we identified mRNAs of the sPLA₂ subtypes IIA, IID, V, and X with group X sPLA₂ being the predominant species. On the protein level we detected only group X sPLA₂ in cell lysates and cell culture supernatants of HaCaT cells and human primary keratinocytes cultured under serum-free conditions, indicating that this is the major enzyme secreted by these cells under these conditions. Moreover, we show that the potent inflammatory phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited the mRNA expression of group X sPLA₂ and also of the other sPLA₂ subtypes.

MATERIALS AND METHODS

Cell culture HaCaT cells (Boukamp *et al*, 1988) were cultured routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units per ml), and streptomycin (100 µg per ml). For experiments, cells were transferred to plastic Petri dishes (Falcon, 3.5 cm with 1 ml medium or 10 cm diameter with 5 ml medium) and cultured for 72 h in DMEM containing 2.5% FBS to subconfluency. Twenty-four hours prior to the experiment the serum concentration was further reduced to 0.25% FBS. During the time course of the experiments cells were kept in DMEM without serum. For analysis of fatty acid release HaCaT cells were kept in DMEM plus 0.1% fatty-acid-free bovine serum albumin (BSA).

Human primary keratinocytes were cultured in serum-free keratinocyte medium KBM2 (Clonetics) using standard protocols as described earlier (Stein *et al*, 1997).

Analysis of sPLA₂ mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR) HaCaT as well as human

primary keratinocyte cell cultures were washed twice with phosphate-buffered saline (PBS) and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium isothiocyanate-phenol-chloroform method (Sambrook *et al*, 1989).

Human skin samples, which were frozen in liquid nitrogen immediately after surgery, were homogenized in guanidinium isothiocyanate with an ultra-turrax-homogenizer and RNA was extracted as described above. For excluding contamination with DNA, treatment of RNA preparations with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) was performed before RT-PCR.

Expression of mRNA for the different sPLA₂ subtypes was investigated by RT-PCR using a total of 5 µg of RNA. First strand cDNA was transcribed with superscript II RNase H-RT obtained from Gibco BRL and OligodT 15-Primer (Promega). From the reverse transcriptase product (cDNA) aliquots of 500 ng were taken for PCR with the primers to be tested. From poly-A⁺ mRNA of lung 0.1 µg were used for the reverse transcription and an aliquot corresponding to 20 ng of cDNA was taken for PCR. PCR was performed on a Perkin Elmer Thermal Cycler with specific primers for each subtype and REDTaq DNA polymerase (Sigma, Munich, Germany): group IB, sense, 5'-aactctgtgctgactgtgct-3'; antisense, 5'-ctcttggtgtccaggtct, amplified product 428 bp; group IIA, sense, 5'-gactgttctcaacaactgtggag-3'; antisense, 5'-aggagagcagtagaggctggaaa-3', amplified product 437 bp; group IID, sense, 5'-ggcaagcagtgactggaaaatg-3'; antisense, 5'-agaaacgcagtcgctcttggtaggt-3', amplified product 311 bp; group V, sense, 5'-acattcgacacagctctac-3'; antisense, 5'-tgattggaggagactctt-3', amplified product 452 bp; group X, sense, 5'-ccagatattacgtgtgac-3'; antisense, 5'-gtttgggctaagcagttagc-3', amplified product 327 bp; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense, 5'-accagctccatgccatcac-3'; antisense, 5'-tcaccacctgtgtctgta-3', amplified product 452 bp.

Semi-quantitative RT-PCR For semiquantitative RT-PCR the number of amplification steps was tested first for linearity with different dilutions of reverse transcriptase product. Then GAPDH was performed for an equivalent quantity of the reverse transcriptase reaction. The different cDNA probes were amplified in a prepared mastermix containing dNTPs, specific primers, and Red Taq-polymerase (Sigma) in the corresponding PCR buffer.

For the PCR reactions the cycling parameters were optimized as follows: group IIA, 94°C for 1 min (one cycle) followed immediately by 94°C for 1 min, 60°C for 1 min 30 s, and 72°C for 3 min (32 cycles); group IID, 94°C for 1 min followed immediately by 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s (36 cycles); group V, 94°C for 1 min (one cycle) followed immediately by 94°C for 30 s, 54°C for 45 s, and 72°C for 2 min (33 cycles); group X, 94°C for 1 min (one cycle) followed immediately by 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s (34 cycles); GAPDH, 94°C for 1 min (one cycle) followed immediately by 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min (23 cycles).

Final extension phase for the PCRs of all subtypes was 10 min at 72°C. PCR products were separated on 2% agarose gels containing 0.5 µg per ml ethidium bromide and were visualized with an ultraviolet-gel camera (Biorad, Munich, Germany).

Northern blot analysis Samples of 30 µg of RNA were separated on 1.4% agarose/formaldehyde gels and transferred to a Gene Screen membrane. After ultraviolet crosslinking and prehybridization in Ultrahyb buffer (Ambion, Austin, TX) for 1 h the filters were hybridized in Ultrahyb buffer for 16 h at 68°C to [α -³²P]dCTP-labeled cDNA inserts from groups IIA, V, and X sPLA₂. DNA probes were radioactively labeled with [α -³²P]dCTP by random priming. Finally, the filters were washed twice with 2 × sodium citrate/chloride buffer (SSC)/0.1% sodium dodecyl sulfate (SDS) for 2 × 10 min and several times at 68°C with 0.1 × SSC/0.1% SDS. The signal was detected and quantified with a phosphorimager BAS 1500 from Fuji (Raytest, Straubenhardt, Germany). To correct for variations in RNA loading the respective cDNA probes were stripped and the blots were hybridized for 16 h with Ultrahyb buffer at 42°C to the [α -³²P]dCTP-labeled cDNA insert for GAPDH. The numbers at the top of the northern blot represent the corrected density expressed as the percentage of group X sPLA₂ mRNA found in cells at time point zero.

sPLA₂ activity assay sPLA₂ activity in the supernatant of HaCaT cells was determined with [14 C]-oleate-labeled membranes from *Escherichia coli* as a substrate as described previously (Scholz *et al*, 1999). Assay mixtures (750 µl) contained 100 mM Tris/HCl (pH 7.0), 1 mM CaCl₂, [14 C]-oleate-labeled *E. coli* membranes (approximately 5000 cpm), and the enzyme-containing supernatants of the cell cultures. During incubation less than 5% of substrate was hydrolyzed. The reaction mixture was incubated for 1 h at 37°C in a thermomixer. The reaction was stopped by the addition

of 50 μ l 1 mM ethyleneglycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)/1 N HCl and 800 μ l ethyl acetate. After extraction of the lipids the organic phase was dried in a vacuum concentrator. Then the lipids were dissolved in 50 μ l ethyl acetate and separated by thin layer chromatography on silica gel G 60 plates using the organic phase of ethyl acetate:isooctane:acetic acid:water (110:50:20:100 by volume) as a solvent system. Detection and quantification of the released fatty acid were performed with a linear analyzer (Berthold, Wildbad, Germany). In parallel experiments extraction efficiency was determined to be greater than 95%.

Measurement of fatty acid release For measuring fatty acid release HaCaT cells were cultured in 3.5 cm dishes to subconfluency (about 10⁶ cells per dish) in 2 ml cell culture medium. After medium change cells were prelabeled for 24 h with [1-¹⁴C]-linoleic acid or [1-¹⁴C]-oleic acid (0.3 μ Ci per ml) in 1 ml DMEM plus 1 mg per ml fatty-acid-free BSA. After washing the cell cultures twice with PBS/BSA (1 mg per ml) and adding 1 ml of fresh medium the supernatants were collected at different time points as indicated. Supernatants were measured in a β -counter to detect the amounts of released [1-¹⁴C]-labeled fatty acids. The incorporation rate of the different fatty acids into keratinocytes was evaluated by extracting the cells with chloroform:methanol:1 N HCl (4:2:2 by volume) and measuring the radioactivity of the organic phase in a β -counter. For all three fatty acids the incorporation rate after 24 h was about 90%. The amounts of labeled fatty acids in the supernatants represent about 0.2% of total labeled lipids. The amount of lipid peroxidation products derived nonenzymatically or enzymatically from linoleic acid under the conditions of prelabeling did not exceed 5% (unpublished observation).

Western blot analysis sPLA₂ protein secretion by HaCaT cells was assayed by precipitating 5 ml of the culture supernatants with 2 ml of 20% trichloroacetic acid. SDS polyacrylamide gel electrophoresis (PAGE) using a 15% polyacrylamide gel was performed under nonreducing conditions according to Laemmli (1970). Cell lysates were obtained after removal of the medium by incubating the cell layer on ice for 10 min in 500 μ l per 10 cm dish of 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μ g per ml leupeptin, 0.5% Triton X-100. One milligram of cellular protein was diluted in 150 μ l Laemmli sample buffer and was separated on a 14 cm \times 14 cm polyacrylamide gel with 8 mm slots. As controls 500 ng of human recombinant groups IIA and X sPLA₂ protein were also loaded on the gel. For western blotting proteins were transferred to PVDF membranes for 30 min at 0.8 mA per cm². Non-specific binding was blocked with 2% (wt/vol) BSA in PBS-Tween 20 for 1 h at room temperature followed by incubation with a 1:100 dilution of a primary antibody against the human group X sPLA₂. The antiserum was generated in rabbits against the peptide PKTERYSWQC (amino acid sequence 101–111; Cupillard *et al.*, 1997), conjugated to keyhole limpet hemocyanin. Then the blot was incubated with horseradish-peroxidase-conjugated goat antirabbit IgG. After washing, peroxidase activity was detected by developing the blots by the enhanced chemiluminescence method (Amersham, Freiburg, Germany). The minimum amount of group X sPLA₂ protein recognized by the serum at 1:100 dilution is 300–400 ng. Calculation of the amounts of group X sPLA₂ protein on the western blots was performed by densitometric analysis using human recombinant group X protein as standard in a range of 500 ng to 1 μ g.

Cross-reactivity of this antiserum with other human and murine sPLA₂ subtypes was tested by loading 500 ng of the respective recombinant, refolded proteins (human groups IB, IIA, IID, V, X) on a gel and performing PAGE and western blot analysis with a 1:100 dilution of the human group X antiserum under nonreducing conditions. The source of all these enzymes will be given elsewhere. The structures of the proteins were confirmed by electrospray mass spectrometry to show that all disulfides were intact.

Enzyme-linked immunosorbent assay (ELISA) for analysis of sPLA₂ protein In order to detect group IIA sPLA₂ in the cell culture supernatants of HaCaT cells an ELISA from Boehringer Mannheim (Mannheim, Germany) designed against the human group IIA sPLA₂ was performed according to the manufacturer's instructions. This ELISA detects purified human group IIA sPLA₂ in a range of 1–500 ng per ml. To analyze a cross-reaction of the sPLA₂ antibody used in this ELISA with other sPLA₂ subtypes, up to 500 ng of purified human recombinant protein of the group V sPLA₂, kindly provided by Dr. Wonhwa Cho (Department of Chemistry, University of Illinois, Chicago, IL), and also 500 ng of the group X were used as positive controls. We found that the antibody against the human group IIA used as standard in this ELISA cross-reacted with human group V sPLA₂. In the standard curve both enzymes were detected

with an equal sensitivity, whereas group X sPLA₂ was not detected (data not shown).

Statistical analysis Data are presented as mean \pm SEM resulting from three independent experiments performed in triplicate ($n=3$), or mean \pm SD when showing one representative experiment out of three performed in triplicate.

Statistical analysis was performed by Student's *t* test to determine significant differences among two groups. *, #, + $p < 0.05$. For comparing all LY-311727 concentrations with the control group statistical analysis was performed by repeated measures ANOVA followed by Dunnett's test. A probability less than 0.05 was defined as significant.

Materials [1-¹⁴C]-oleic acid and [1-¹⁴C]-linoleic acid (specific activity 50 mCi per mmol) were from Biotrend, Köln, Germany, and [α -³²P]dCTP (110 TBq per mmol) was from Amersham. Methyl arachidonyl fluorophosphate was obtained from Calbiochem, Bad Soden/Ts, Germany. LY-311727 was kindly provided by Lilly Research Laboratories (Indianapolis, IN).

The primers for human GAPDH for semiquantitative RT-PCR were kindly provided by Dr. Heiko Mühl, Center of Pharmacology, University Hospital Frankfurt, Germany. The ELISA for detection of human group IIA sPLA₂ was a generous gift of Professor Dr. Ulrich Tibes, Roche Diagnostics, Department of Molecular Pharmacology, Mannheim, Germany. Poly A⁺ RNA from human lung was obtained from Clontech (Heidelberg, Germany).

Immobilon-PVDF membranes were from Millipore (Eschborn, Germany) and Nylon membranes (Gene Screen) were purchased from NEN Life Science (Köln, Germany). All cell culture media and nutrients were from Gibco BRL (Eggenstein, Germany), and all other chemicals used were from Merck (Darmstadt, Germany), Sigma (Munich, Germany), or Fluka (Deisenhofen, Germany).

RESULTS

mRNA expression of sPLA₂ subtypes in HaCaT keratinocytes, in human keratinocytes, and in human skin mRNA was extracted from subconfluent HaCaT cell cultures grown under serum-free conditions, and RT-PCRs were performed under the conditions described in *Methods* with a set of primers specific for the human groups IB, IIA, IID, V, and X sPLA₂s. The identity of the PCR products was confirmed by sequence analysis.

The qualitative analysis shown in **Fig 1(a)** indicates that under serum-free conditions HaCaT cells constitutively express groups IIA, IID, V, and X sPLA₂ mRNA. Southern blot analysis indicated that group X sPLA₂ is the predominant sPLA₂ subtype expressed in HaCaT cells (data not shown). According to this analysis, sPLA₂ subtypes can be ranked in the following order: group X > group V > group IIA > group IID. As the group IID sPLA₂ mRNA is present only at very low levels (indicated by the arrows in the lanes of group IID sPLA₂) we did not further study the expression of this subtype.

In order to compare the mRNA expression pattern of sPLA₂ subtypes in HaCaT cells with that in human primary keratinocytes and in human skin, total RNA from cell cultures of human primary keratinocytes and from human biopsies was extracted and RT-PCR was performed with primer sets specific for the sPLA₂ groups IB, IIA, IID, V, and X. The data in **Fig 1(a)** show the same expression pattern as for HaCaT cells: groups IIA, IID, V, and X sPLA₂ mRNA are constitutively expressed both in human primary keratinocytes and in the human skin biopsies.

With the primers and under the experimental conditions used group IB sPLA₂ mRNA was not detected in HaCaT cells and in human primary keratinocytes and human skin *in vivo* (**Fig 1b**). As positive control for group IB sPLA₂ RT-PCR with the primers was performed with poly A⁺ RNA from human lung, which is known to express this sPLA₂ subtype (Matsuda *et al.*, 1987).

Detection of sPLA₂ protein in HaCaT cells and in human primary keratinocytes by western blot analysis To study the subtypes of sPLA₂ secreted from HaCaT cells and human primary keratinocytes, we performed western blot analysis of protein precipitated from cell culture supernatants and cell lysates from cells cultured under serum-free conditions.

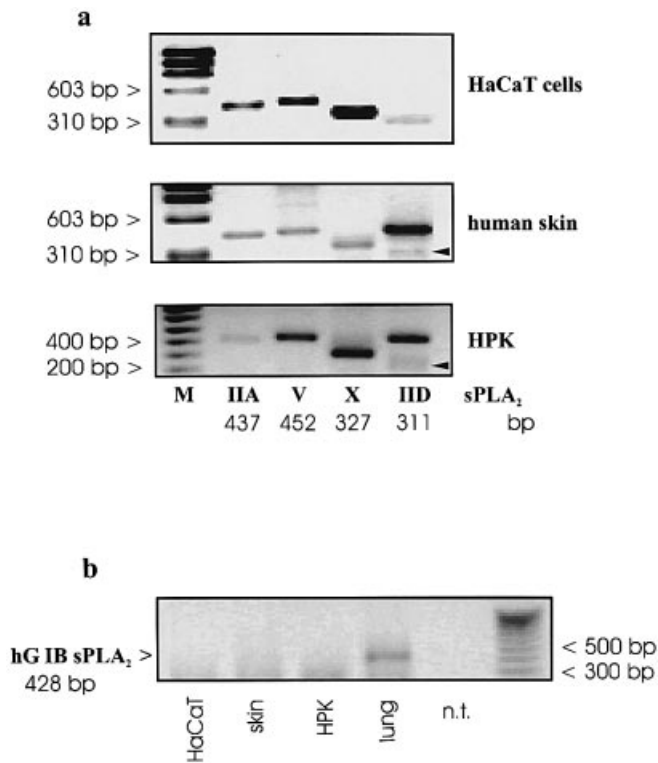


Figure 1. Identification of sPLA₂ subtypes in HaCaT cells, in human primary keratinocytes, and in human skin by RT-PCR. Total RNA was extracted from HaCaT cells, human primary keratinocytes, and human skin samples, and after DNase I treatment of the RNA probes RT-PCR was performed with specific primers for the human groups IIA, IID, V, and X sPLA₂ under the conditions described in *Methods*. The bands for group IID sPLA₂ are indicated by arrows. The upper band in the lane of group IID sPLA₂ derived from unspecific binding as was checked by sequence analysis. This experiment was repeated four times with similar results. RT-PCR for group IB sPLA₂ was performed in the cell cultures as well as in human skin samples, and as a positive control for human group IB sPLA₂ poly A⁺ RNA from human lung was used. Abbreviations: HPK, human primary keratinocytes; n.t., no template.

Using an antiserum raised against a group X sPLA₂-specific peptide, strong immunoreactivity towards purified recombinant human group X sPLA₂, but no cross-reactivity with purified recombinant human groups IB, IIA, IID, or V sPLA₂ proteins, was observed (**Fig 2a**).

With this antiserum group X sPLA₂ protein was detectable in the cell culture supernatants of HaCaT cells as well as human primary keratinocytes (**Fig 2b**). The concentration of group X protein released into the supernatant was determined by densitometric analysis to 200 ng per ml.

In order to analyze whether there is also group IIA or group V sPLA₂ present in cell culture supernatants of HaCaT cells and human primary keratinocytes, we used an ELISA from Boehringer Mannheim, which detects both sPLA₂ subtypes in a range between 1 and 500 ng per ml (see *Methods*). As positive controls for standard curves we used up to 500 ng of both human recombinant group IIA and group V sPLA₂. With this method we did not detect a positive signal in the cell culture supernatants of HaCaT cells or human primary keratinocytes, indicating that group IIA and group V sPLA₂ are not secreted by these cells (data not shown).

Group X sPLA₂ was also detectable in cell lysates of HaCaT cells as well as human primary keratinocytes (**Fig 2c**). From these results we conclude that the group X sPLA₂ is the major enzyme secreted by keratinocytes under serum-free conditions.

Detection of sPLA₂ activity in the supernatants of HaCaT cell cultures As sPLA₂ subtype expression in primary human

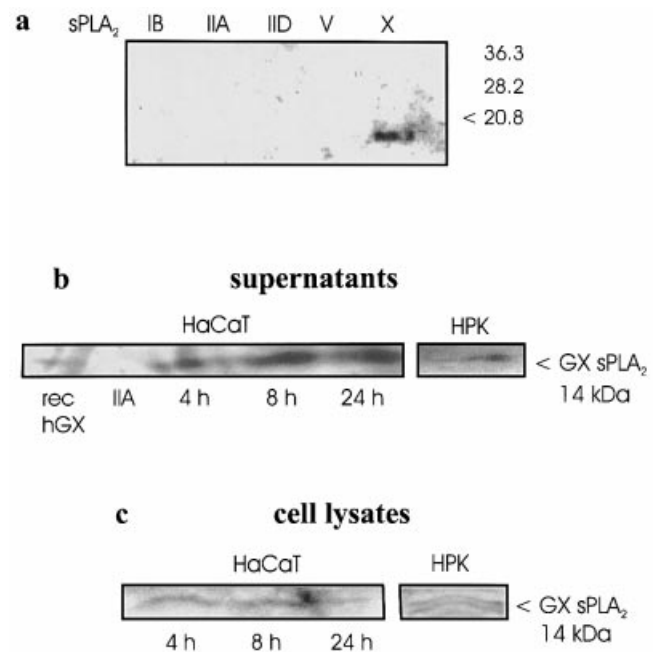


Figure 2. Western blot analysis of group X sPLA₂. HaCaT cells were incubated in serum-free DMEM for the periods indicated. Cell culture supernatants and cellular proteins were collected after the time points indicated. Human primary keratinocytes were cultured as described. Supernatants and cell lysates were harvested without further subcultivation of these cells. Five hundred nanograms of human recombinant groups IB, IIA, IID, V, and X sPLA₂s were blotted and the western blot was incubated with an antiserum created against a peptide sequence specific for group X sPLA₂ (see *Methods*). Proteins from 5 ml of cell culture supernatants of HaCaT cells and human primary keratinocytes were precipitated and separated by SDS-PAGE under nonreducing conditions as described in *Methods*. Western blot analysis was performed using the antiserum against group X sPLA₂ at 1:150 dilution. As positive controls recombinant human groups IIA and X sPLA₂ were also blotted. One milligram protein of cell lysates from HaCaT cells or human primary keratinocytes was separated by SDS-PAGE under nonreducing conditions, and detection of group X sPLA₂ was performed as described above. Representative western blots out of three are shown for supernatants as well as for cell lysates.

keratinocytes was similar to that in HaCaT cells, we used this keratinocyte line for the analysis of sPLA₂ activity. Therefore, supernatants from HaCaT cell cultures were collected at different time points after medium change, and the enzymatic activity was determined using [¹⁴C]-oleic acid-labeled *E. coli* membranes as substrate. sPLA₂ activity increased immediately after medium change, reaching a plateau after 4–8 h (**Fig 3**), and may essentially be attributed to group X sPLA₂, the only subtype detected in the culture supernatant (**Fig 2b**). sPLA₂ activity is expressed as factor of increase in free [¹⁴C]-oleic acid.

Release of free fatty acids from HaCaT cells In order to measure the release of free fatty acids HaCaT cells were prelabeled for 24 h with [1-¹⁴C]-linoleic or [1-¹⁴C]-oleic acid. Fresh culture medium was added and removed after different incubation times, and the amounts of free fatty acids were measured in a β -counter. The values measured in the supernatants at zero time did not exceed background values (about 40 cpm per ml).

The data in **Fig 4** show an increasing release of the different fatty acids from HaCaT cells with time. The time course correlates with the increase in sPLA₂ activity in the supernatants (see **Fig 3**).

Inhibition of sPLA₂ activity and fatty acid release by LY-311727 and Pyr-1 In order to confirm that an sPLA₂ participates in the release of fatty acids, we first incubated HaCaT cells in the presence of LY-311727, known as a potent inhibitor of groups IIA and V sPLA₂ (Balsinde *et al*, 1999) and group X sPLA₂ (Bezzine *et al*

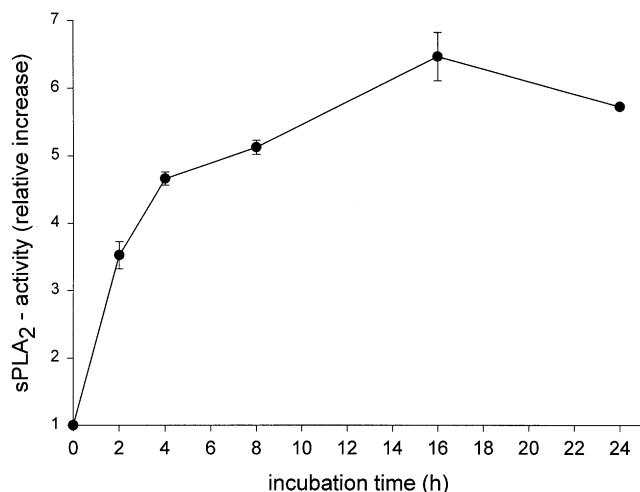


Figure 3. Detection of sPLA₂ activity in HaCaT cell culture supernatants. HaCaT cells were cultured for 72 h in 10% FBS and then incubated for a further 16 h in 0.25% FBS. After medium change to serum-free DMEM cell culture supernatants were collected at once or at different time points. sPLA₂ activity was determined in the cell culture supernatants using [¹⁴C]-oleic-acid-labeled *E. coli* membranes as described in *Methods*. Each value represents the mean of three independent experiments \pm SEM ($n = 3$).

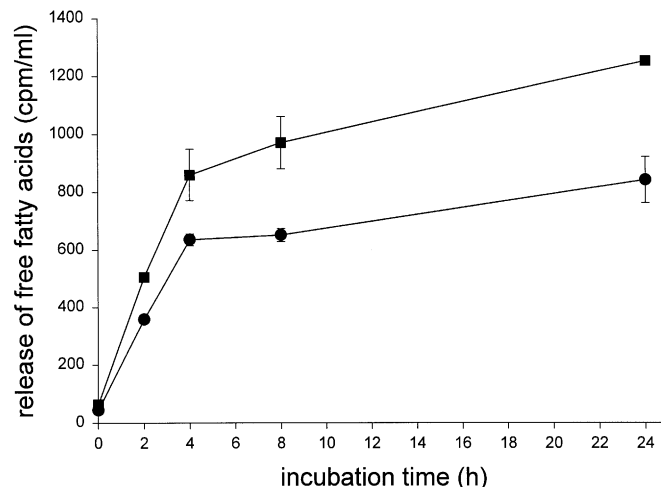


Figure 4. Release of different fatty acids into the cell culture supernatants of HaCaT cells. HaCaT cells were pre-labeled for 24 h with [¹⁴C]-oleic acid (■) or [¹⁴C]-linoleic acid (●; each 0.3 μ Ci per 10⁶ cells). After thorough washing with PBS/BSA and incubation in DMEM plus 1 mg per ml fatty-acid-free BSA cells were incubated for the indicated periods. Then the cell culture supernatants were collected and measured in a β -counter. The release of fatty acids is expressed as cpm per ml. Each value represents the mean of three parallel onsets \pm SD. The data are representative for three different experiments with similar results.

2000), but not of the pancreatic-type group IB sPLA₂ or cytosolic PLA₂ (Schevitz *et al.*, 1995). sPLA₂ activity in the cell culture supernatants was determined after an 8 h incubation of the cells in the presence of the inhibitor. **Figure 5 (a)** shows that sPLA₂ activity was significantly reduced by about 50%–70% with LY-311727 at a concentration of 50 μ M. Fatty acid release (**Fig 5b**) from HaCaT cells pre-labeled with [¹⁴C]-oleic or [¹⁴C]-linoleic acid was inhibited by about 30%–50% in the presence of LY-311727 (50 μ M). These data indicate that at least a part of the released fatty acids can be attributed to group X sPLA₂, as group IIA and group V sPLA₂ were not detected in these cells.

Accordingly, Pyr-1 [3-(2,6-dichlorophenylsulfonylamino)-4-(2-(4-acetamido)pyridyl)-5-(3-(4-fluorophenoxy)benzyl(thio)-(1H)-pyrazole)], a compound that was found to inhibit human group IIA sPLA₂ *in vitro* with a 120-fold selectivity compared with human group X sPLA₂ (Bezzine *et al.*, 2000), had no effect on the enzyme activity or on the fatty acid release measured 8 h after medium change, when given in concentrations up to 30 μ M (data not shown).

Effect of phorbol ester TPA on sPLA₂ activity and fatty acid release in HaCaT cell culture supernatants Phorbol esters such as TPA are known to induce arachidonic acid release and prostaglandin synthesis in mouse keratinocytes *in vitro* and epidermis *in vivo*. This is predominantly due to the activation of cytosolic PLA₂ (Kast *et al.*, 1993; Lo *et al.*, 1998). Nothing is known about the effects of phorbol esters on fatty acid metabolism and regulation of sPLA₂s in human keratinocytes, however. We therefore asked whether TPA can modulate sPLA₂ expression and activity as well as release of fatty acids. After prelabeling with [¹⁴C]-oleic or [¹⁴C]-linoleic acid for 24 h and subsequent medium change, HaCaT cells were treated for 8 h with TPA (1 μ M) or dimethylsulfoxide as control (0.05%), and the release of labeled fatty acids into the cell culture supernatant was analyzed. In similar experiments cell culture supernatants were collected for analysis of sPLA₂ activity.

The data in **Fig 6(a)** show that TPA inhibited sPLA₂ activity in the supernatant by about 50%. TPA treatment also reduced the levels of oleic as well as linoleic acid in the cell culture supernatants from pre-labeled cells by about 40% (**Fig 6b**).

Influence of TPA on mRNA expression of groups IIA, V, and X sPLA₂ We next asked whether the mRNA expression of individual sPLA₂ subtypes is affected by TPA. Recently we reported that TPA downregulates group IIA sPLA₂ mRNA induction in rat glomerular mesangial cells through a protein-kinase-C-mediated mechanism (Scholz *et al.*, 1999).

Performing semiquantitative RT-PCR we observed that upon medium change the mRNA level of group X sPLA₂ increased after 4 h, reaching a plateau after 8 h in untreated cells (**Fig 7**). This time course corresponds to the sPLA₂ activity shown in **Fig 3**. In addition, mRNA levels of group V and group IIA sPLA₂ also slightly increased with time up to 24 h.

In contrast, TPA led to a marked decrease of the mRNA levels of group X sPLA₂ starting at 4–8 h and being maximally reduced at 24 h after treatment. This inhibition of mRNA expression correlated with the reduced sPLA₂ activity and fatty acid release shown in **Fig 6**. The mRNA levels of group IIA and group V sPLA₂ were also clearly reduced 24 h after TPA treatment compared with controls. These data suggest that the three different sPLA₂ subtypes are downregulated upon TPA application, the most prominent effect being observed for group X sPLA₂.

A similar kinetic of group X sPLA₂ mRNA expression and downregulation by TPA was also detectable by northern blot analysis (**Fig 8**). Quantitative analysis of group X sPLA₂ mRNA expression showed that mRNA levels were reduced by about 40% at 4–16 h after TPA treatment concomitantly with the decrease in sPLA₂ activity and fatty acid release (**Fig 6**).

The other sPLA₂ subtypes were not detectable by this method, probably due to the low mRNA expression levels.

DISCUSSION

Several studies report on a role of sPLA₂ as a proinflammatory enzyme in skin of humans and animals. Overexpression of human group IIA sPLA₂ in the skin of transgenic mice resulted in hyperkeratosis, epidermal hyperplasia, and adnexal hyperplasia, which reflect the symptoms observed in skin disorders including human psoriasis (Grass *et al.*, 1996). Other groups have examined the effects of intradermal injection of sPLA₂ from snake venom and of the pancreatic-type group IB sPLA₂ in rabbit and swine (Pruzanski *et al.*, 1986; Nair *et al.*, 1993) causing the induction of a

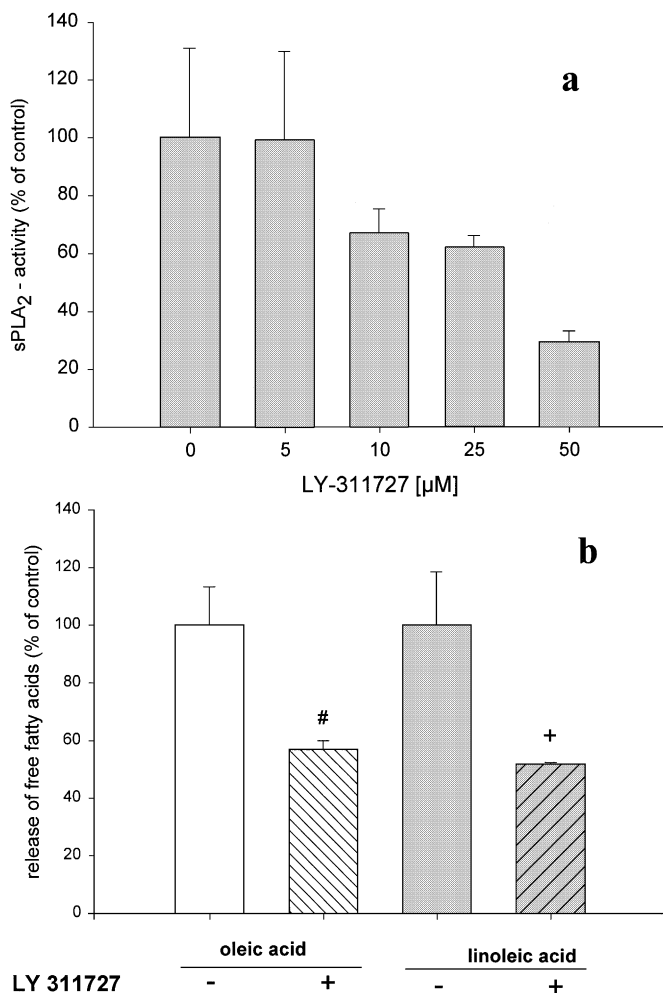


Figure 5. Inhibition of sPLA₂ activity and fatty acid release in HaCaT cells by LY-311727. (a) sPLA₂ activity; (b) fatty acid release. HaCaT cells were treated for 8 h with the indicated concentrations of LY-311727 and then sPLA₂ activity was determined as described in *Methods*. Each value represents the mean of three independent experiments \pm SEM ($n=3$). * $p < 0.05$, ANOVA followed by Dunnett's test. Cells were prelabeled for 24 h with [$1\text{-}^{14}\text{C}$]-oleic acid or [$1\text{-}^{14}\text{C}$]-linoleic acid (each 0.3 μCi per ml). After medium change they were incubated for 8 h in the absence or presence of LY-311727 (50 μM). Then the release of fatty acids was analyzed as described above. Each value represents the mean of three independent experiments \pm SEM ($n=3$). # $p < 0.05$ (oleic acid), + $p < 0.05$ (linoleic acid), Student's *t* test.

transient erythema as well as inflammatory cell infiltration and epidermal hyperplasia. These studies show that high levels of sPLA₂ in the skin induce marked changes of skin phenotype such as epidermal hyperplasia. The acute inflammatory reaction upon intradermal injection may critically depend on high local sPLA₂ concentrations as Grass *et al* (1996) did not observe such effects in transgenic mice overexpressing group IIA sPLA₂ in the skin.

The strong effects of sPLA₂s upon local application or ectopic expression led us to analyze the subtype spectrum of this enzyme family expressed in keratinocytes in culture and *in vivo*. Semi-quantitative PCR and southern blot analysis indicate that HaCaT cells, human primary keratinocytes, and human skin express the four different sPLA₂ subtypes IIA, IID, V, and X. The novel human group IIE sPLA₂ (Suzuki *et al*, 2000) was not detectable (data not shown).

In addition, the mRNA steady-state levels in HaCaT cells coding for the different sPLA₂s were strikingly different in the following order: group X > group V > group IIA > group IID. The

mRNA levels of all sPLA₂ subtypes increased during the time course of the experiment probably due to a stimulatory effect of the medium change. Group IB sPLA₂ mRNA was not detectable in our hands in HaCaT cells, in human primary keratinocytes, or in human skin, using primers generating a positive signal with lung mRNA. Recently, group IB sPLA₂ was found by immunohistochemistry studies to be localized to the stratum corneum of human skin (Mazereeuw-Hautier *et al*, 2000) and was also cloned from human epidermis (Maury *et al*, 2000). The authors did not examine the cross-reactivity of the antibody used with other sPLA₂ subtypes, however. The discrepancy between our results and the observations by Maury and coworkers (2000) might be explained by a weaker expression of group IB sPLA₂ in skin than in lung. Moreover, there might be a strong dilution of group IB sPLA₂ mRNA in RNA extracts from total skin biopsies, which also include some RNA from the dermis where this enzyme is not expressed (Maury *et al*, 2000).

The antibody generated against the group X sPLA₂ did not cross-react with any other sPLA₂ subtypes investigated in this study and was able to detect about 200 ng per ml of group X sPLA₂ in supernatants of HaCaT as well as human primary keratinocytes. We also detected group X sPLA₂ protein in cell lysates indicating that the early increase of extracellular sPLA₂ activity was most probably due to secretion of the enzyme from intracellular stores. The action of an exogenous sPLA₂ on the outer membrane of cells is an important prerequisite for a functional role in hydrolysing phospholipids for maintaining the epidermal permeability barrier. This enzyme might contribute to the hydrolysis of polar lipids from suprabasal layers of the skin providing free fatty acids that are necessary for the synthesis of complex lipids forming the permeability barrier (Redoules *et al*, 1998; Ziboh *et al*, 2000). This view has to be supported by immunohistochemistry determining the localization of group X sPLA₂ in human epidermis in detail. Group IIA as well as group V sPLA₂ protein would not be detected in keratinocytes *in vivo* and *in vitro* despite a modest increase of the corresponding mRNA species.

Incubation of keratinocytes with heparin or 1 M NaCl did not result in an enhanced extracellular sPLA₂ activity (data not shown) indicating the absence of membrane-associated sPLA₂, including group IIA and group V sPLA₂, which are known to bind tightly to cell surface proteoglycans (Cupillard *et al*, 1997). Interestingly, Murakami *et al* (1999) showed that group X sPLA₂ does not bind to a heparin-Sepharose column, suggesting that this subtype does not associate with cell surface proteoglycans.

In summary, according to RT-PCR and northern blot and western blot analysis sPLA₂ activity in human keratinocytes is derived from group X sPLA₂. This sPLA₂ subtype efficiently hydrolyzes phosphatidylcholine (Murakami *et al*, 1999), a major phospholipid of the outer leaflet of the plasma membrane. A recent report by Bezzine *et al* (2000) describes an efficient release of arachidonic acid from adherent cell cultures by exogenously added group X sPLA₂ indicating that this enzyme is involved in prostaglandin biosynthesis. This does not exclude, however, that group X sPLA₂ also hydrolyzes other phospholipid species different from phosphatidylcholine (Cupillard *et al*, 1997).

The absence of group IIA or group V sPLA₂ protein despite their mRNA expression suggests that these enzymes are probably not involved in homeostatic processes in the epidermis. This is supported by the fact that group IIA sPLA₂ protein is not detectable in skin under physiologic conditions (Mazereeuw-Hautier *et al*, 2000), and the high levels of this enzyme obtained in human psoriatic skin (Andersen *et al*, 1994) will probably never be reached in intact noninflamed epidermis. In keratinocyte cultures group IIA and/or group V sPLA₂ might appear after stimulation, e.g., by proinflammatory cytokines or growth factors. In this respect indirect evidence was given by Thommesen *et al* (1998) who showed that in tumor-necrosis-factor- α -stimulated HaCaT cells the release of arachidonic acid was reduced by the sPLA₂ inhibitor LY-311727, indicating a participation of an exogenous sPLA₂ in this process. The responsible subtype was not identified in

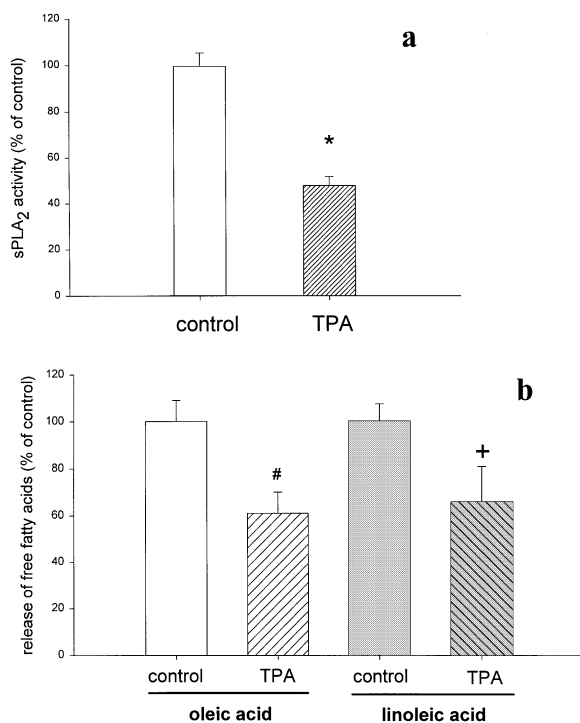


Figure 6. Effect of TPA treatment on sPLA₂ activity and fatty acid release in HaCaT cells. (a) sPLA₂ activity; (b) fatty acid release. Cells were treated for 8 h with TPA (1 μ M). Supernatants were collected and sPLA₂ activity was measured as described in *Methods*. Each value represents the mean of three independent experiments \pm SEM ($n = 3$). * $p < 0.05$, Student's t test. Cells were prelabeled with different [14 C]-labeled fatty acids (each 0.3 μ Ci per ml) as indicated. After medium change cells were further incubated for 8 h in the absence or presence of TPA (1 μ M). Analysis of fatty acids released into the cell culture supernatants was performed as described in *Methods*. Each value represents the mean of three independent experiments \pm SEM ($n = 3$). # $p < 0.05$ (oleic acid), + $p < 0.05$ (linoleic acid), Student's t test.

this study, however. In a recent study induction of group IIA or group V sPLA₂ protein in activated human primary keratinocytes was recently shown by Rys-Sikora *et al* (2000). In their study, however, other sPLA₂ subtypes such as group X sPLA₂ were not investigated. Moreover, one has to consider that it is important whether cells were cultured in serum-containing or in serum-free medium. In our study we tried to prevent additional stimulation by serum factors, which might be the reason why we did not detect group IIA or V sPLA₂ protein.

In this study we also followed the release of oleic and linoleic acid, which are known to be preferentially localized to the sn-2 position of phospholipids. The release of free fatty acids from cells represents a steady-state equilibrium between hydrolysis and the reesterification of the free fatty acids. Thus, the increased release of oleic acid over linoleic acid (Fig 4) may be due to a less effective reincorporation of this fatty acid into phospholipids.

Using the sPLA₂ inhibitor LY-311727, we found a partial inhibition of sPLA₂ activity and fatty acid release from HaCaT cells. This substance is known to inhibit a variety of sPLA₂s (Chen and Dennis, 1998; Bezzine *et al*, 2000), whereas it does not inhibit intracellular PLA₂s or group IB sPLA₂ (Schevitz *et al*, 1995). In cultures of rat mesangial cells that release group IIA sPLA₂ (Pfeilschifter *et al*, 1993), 10 μ M of this compound completely inhibited the enzyme activity (unpublished results). In HaCaT cells, however, a concentration of 50 μ M was required to inhibit sPLA₂ activity in the supernatant by 70%. Murakami *et al* (1999) reported that group X sPLA₂ activity was indeed resistant to 10 μ M of LY-311727, and Bezzine *et al* (2000) showed a 2.5-fold selectivity of LY-311727 *in vitro* in inhibiting human group IIA versus group X, which is only modest and probably not enough to distinguish

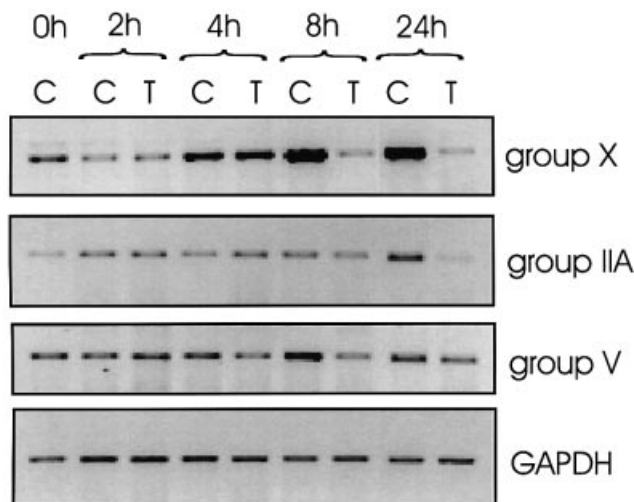


Figure 7. Time course of the mRNA expression of groups X, IIA, and V sPLA₂ and modulation by phorbol ester TPA. Detection of mRNA expression of groups X, IIA, and V sPLA₂ and the effects of TPA on their mRNA levels was performed by a semiquantitative RT-PCR as described in *Methods*. The data are representative for three separate experiments with similar results. Abbreviations: C, control; T, TPA.

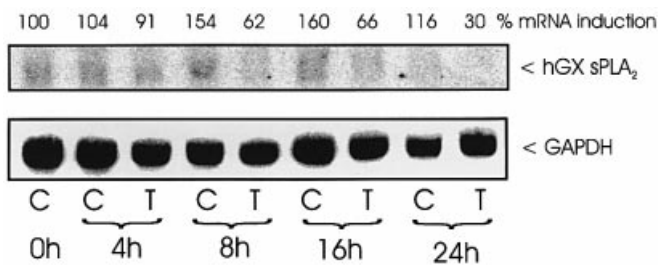


Figure 8. Northern blot analysis of the effect of TPA on group X sPLA₂ mRNA expression. Northern blot analysis was performed as described in *Methods*. The numbers at the top of the northern blot represent the corrected density expressed as the percentage of group X sPLA₂ mRNA found in cells at time point zero.

between these two enzymes in cell studies. Therefore, LY-311727 can only be considered as a general inhibitor of sPLA₂ activity with a broad specificity for the different sPLA₂ subtypes. Pyr-1, another sPLA₂ inhibitor that has a 120-fold selectivity for human group IIA compared with human group X sPLA₂ *in vitro* (Bezzine *et al*, 2000), had no inhibitory effect on the sPLA₂ activity and fatty acid release in HaCaT cells (data not shown), strongly suggesting that group IIA sPLA₂ activity does not contribute to these processes. Up to now, no specific inhibitor of the group X sPLA₂ is available. It would be very interesting to study the effects of such a specific inhibitor on epidermis *in vivo*, as suppression of sPLA₂ activity in general has been shown to disrupt the permeability barrier and to disturb epidermal homeostasis (Mao-Qiang *et al*, 1995). According to the selective expression and the characteristics of enzymatic activity (see above) group X sPLA₂ appears to be the PLA₂ subtype involved in the maintenance of epidermal integrity and function.

We cannot exclude that cytosolic PLA₂s also contribute to fatty acid release from HaCaT cells. Treatment of the cells with methyl arachidonyl fluorophosphonate, a potent inhibitor of this enzyme and, with a lower potency, of a Ca²⁺-independent cytosolic PLA₂ (group VI PLA₂; Balsinde *et al*, 1999), inhibited the release of linoleic acid by 20%–30%, but not that of oleic acid, from HaCaT cells. Selectivity for the hydrolysis of polyunsaturated fatty acids is known to be a characteristic property of group IV cytosolic PLA₂. The fact that all fatty acids were at least partially reduced by LY-311727, however, supports the involvement of an sPLA₂ in this process.

A disruption of the epidermal layers occurs during inflammatory processes in the skin. In this respect the phorbol ester TPA, which is used as a tumor promoter in the mouse multistage skin carcinogenesis model (for review, see Marks and Fürstenberger, 1990), is *per se* a potent inflammatory substance leading to hyperplasia and hyperkeratosis. Moreover, TPA is a strong activator of cytosolic PLA₂ in mouse keratinocytes resulting in increased release of arachidonic acid and subsequent eicosanoid formation (Kast *et al*, 1993; Lo *et al*, 1998) through induction of cyclooxygenase 2 (Scholz *et al*, 1995; Müller-Decker *et al*, 1998) and lipoxygenase gene expression (Krieg *et al*, 1998). Incubation of HaCaT cells as a human keratinocyte model with TPA reduced sPLA₂ activity by about 50% and fatty acid release by about 40%. We reported here that all the sPLA₂ subtypes IIA, V, and X were downregulated by TPA at the transcriptional level in HaCaT cells. A clear decrease of sPLA₂ enzyme activity and fatty acid release was observed in parallel. TPA was also found to inhibit the cytokine-induced mRNA expression and activity of group IIA sPLA₂ in rat mesangial cells (Scholz *et al*, 1999). Whether or not TPA elicits similar effects in skin *in vivo* remains to be elucidated. A hallmark of TPA action both in mouse and in human skin is the induction of inflammatory processes (Marks and Fürstenberger, 1990). In fact, certain sPLA₂ subtypes, which are expressed at very low levels in healthy skin, are upregulated under inflammatory conditions on mRNA and protein level and might contribute to proinflammatory eicosanoid formation such as group IIA sPLA₂ in activated keratinocytes (Rys-Sikora *et al*, 2000) and psoriatic tissue (Andersen *et al*, 1994), or group V in mouse macrophages or mast cells (Balboa *et al*, 1996; Reddy *et al*, 1997; Bingham *et al*, 1999).

A role of group X sPLA₂ in the TPA-stimulated early release of arachidonic acid from HaCaT cells might be hypothesized, as it was shown that human group X sPLA₂ releases arachidonic acid for cyclooxygenase-2-dependent prostaglandin E₂ formation when added exogenously to adherent mammalian cells (Bezzine *et al*, 2000). From the time course of downregulation of group X sPLA₂ starting between 4 and 8 h, however, and that of phorbol-ester-induced prostaglandin formation starting after 6–8 h, we suggest that group X sPLA₂-catalyzed arachidonic acid release is not a prerequisite for the initiation of prostaglandin synthesis induced by TPA. This was supported by the observation that treatment of the cells with TPA in the presence of 50 μM of LY-311727, which significantly reduced fatty acid release, did not result in reduced prostaglandin E₂ formation (unpublished observation).

The mechanism by which TPA downregulates the different sPLA₂ subtypes in HaCaT cells is unknown. TPA was shown to activate different transcription factors such as AP-1 and ets via protein kinase C (Dong *et al*, 1997; Sark *et al*, 1998), which may result in inhibition of sPLA₂ gene expression. Another possibility is the modulation of the cytokine-induced signaling pathway upstream of the transcription. In this respect we found that TPA-activated protein kinase C inhibits IL-1β-stimulated activation of a neutral sphingomyelinase during the first 10 min after treatment of mesangial cells (Kaszkın *et al*, 1998), indicating that TPA interferes with an IL-1-receptor-associated signaling molecule such as the IL-1-receptor-associated kinase or other IL-1β-activated downstream kinases (for review, see Auron, 1998).

On the other hand this raised the question: what triggers the permanent release of group X sPLA₂ into the supernatant of proliferating keratinocytes? These cells are known to spontaneously release growth factors such as vascular endothelial growth factor (VEGF) (Weninger *et al*, 1998; Charvat *et al*, 1999) and cytokines such as IL-6 (Aragane *et al*, 1996). It might be speculated that such cytokines maintain a constant secretion of group X sPLA₂ in an autocrine manner. In skin repair processes these cytokines have important functions in stimulating mechanisms crucial for wound healing such as induction of VEGF expression or formation of nitric oxide (Frank *et al*, 1995, 1998). These factors are necessary for angiogenesis, wound closing, and epidermal reorganization. It seems reasonable to propose also a physiologic role of group X sPLA₂ in keratinocytes in these processes, which has to be

investigated in future studies. TPA might interfere with group X sPLA₂ secretion by inhibiting the cytokine-mediated signal transduction. Future studies will further elucidate the mechanisms of the negative modulation of sPLA₂ induced by TPA.

We thank Dr. Wonhwa Cho for the human recombinant group V sPLA₂ protein, and Dr. Heiko Mühl for help in performing the semiquantitative PCR. We thank Dr. Karin Müller-Decker from the Cancer Research Center, FSP Tumor Cell Regulation, for helpful discussion and Silke Spitzer for excellent technical assistance. The HaCaT cells were kindly provided by Dr. Petra Boukamp from the German Cancer Research Center, FSP Tumor Cell Regulation. Human primary keratinocytes were a generous gift from Dr. A. Bernd, Institute of Dermatology and Venerology, University Hospital Frankfurt, Germany. Human skin biopsies were a generous gift from Dr. J. Schweizer (German Cancer Research Center, Heidelberg, Germany). This work was supported by the Deutsche Krebshilfe/Dr. Mildred Scheel-Stiftung and by the Paul and Cilly Weill-Stiftung.

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