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INVESTIGATIVE REPORT

Lysophosphatidylcholine Induces Keratinocyte Differentiation and Upregulation of AP-1- and NF-κB DNA-binding Activity

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Lysophosphatidylcholine (lysoPC) is generated by the action of phospholipase A_2 on membrane phosphatidylcholine, the most abundant cellular phospholipid. *In vitro*, lysoPC has pro-inflammatory properties, as it upregulates the expression of adhesion molecules and is a chemoattractant to monocytes and T lymphocytes. It upregulates the expression of a variety of genes including genes encoding growth factors and cyclooxygenase-2 and modulates other cellular responses like proliferation and differentiation. A role for lysoPC as an intracellular messenger transducing signals from membrane-associated receptors has also been suggested. However, the mechanisms behind the diverse actions of lysoPC are poorly understood. In this study we found that lysoPC in non-toxic concentrations caused increased activator protein-1 (AP-1) DNA-binding activity and transglutaminase-1 expression in cultured human keratinocytes. The effects on transglutaminase-1 and AP-1 were dependent on protein kinase C and mitogen-activated protein kinase kinase. In addition, lysoPC caused a rapid and transient increase in DNA-binding activity of nuclear factor-κB.
Key words: lysophosphatidylcholine; AP-1; NF-κB; EMSA; proliferation; differentiation.

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The differentiation of the human epidermis is a complex process in which protein kinase C (PKC) is an important factor as it links extracellular signals to changes in gene expression (1). Various isoforms of PKC exist. The classical calcium-dependent PKCs (PKC-α, -βI, -βII, -γ) and the new (PKCδ, -ε, -L,-θ) and atypical PKCs (PKC-ζ, -ι), which are calcium unresponsive (2). PKC can be activated by a variety of lipids (3, 4) including lysophosphatidylcholine (lysoPC) (5). LysoPC is a polar phospholipid produced by the action of PLA_2 on membrane phospholipids. The implication of lysoPC in signal transduction is partly due to its effect on PKC, but in addition it can increase the intracellular calcium concentration (6) and the cytosolic level of cyclic adenosine monophosphate (cAMP) (7). Apart from its putative role in signal transduction, this lipid has immunocompetent properties, as it induces the liberation of arachidonic acid in endothelial cells (8) and is a chemoattractant towards human monocytes and T lymphocytes (9, 10). LysoPC also influences differentiation (11) and gene expression (12) and *in vivo* increased concentrations of this lipid have been found in psoriatic skin lesions (13), atherosclerotic plaques (14), and in plasma in patients with acute asthma (15).

When PKC is activated it can either influence gene expression directly or via mitogen-activated protein (MAP) kinases (16). The influence on gene expression is mediated by transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). PKC may in turn phosphorylate these transcription factors and thereby stimulate gene transcription by binding to specific response elements in the promoter region of responsive genes.

In skin the PKC-AP-1 pathway has been suggested as a possible mechanism to regulate differentiation-specific changes (17). The link between AP-1 and differentiation-specific changes can be explained by the fact that several proteins of importance for keratinocyte differentiation, including transglutaminase-1 (TG-1), all have AP-1-binding sites in their promoter region (18).

Both lysoPC and NF-κB have proinflammatory properties and they are both involved in interleukin (IL)-1β production (19). In addition lysoPC has been found to influence NF-κB DNA-binding activity in endothelial cells (20).

In the present study we found that lysoPC caused an increased TG-1 expression and AP-1 DNA-binding activity in cultured human keratinocytes. The PKC isozyme, PKCδ and MAPKK participated in the transmission of the lysoPC-induced effects. Furthermore, lysoPC causes a rapid and transient increase in the DNA-binding activity of NF-κB as determined by electrophoretic mobility shift assay (EMSA).

MATERIALS AND METHODS

Materials

The 25 cm^2 culture flasks, 96-well flat-bottomed microplates and rubber policemen were purchased from Nunc (Roskilde, Denmark). Keratinocyte growth medium, Dulbecco's

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phosphate-buffered saline (PBS), trypsin and gentamicin were obtained from Life Technologies (Roskilde, Denmark). Charcoal-filtered fetal calf serum was from In vitro (Fredensborg, Denmark). Igepal was purchased at ICN Biomedicals (Ohio, USA), Bradford reagent was from Biorad (Copenhagen, Denmark). GF 109203X, PD 98059 and Rottlerin were purchased from Calbiochem (Baden-Soden, Germany). WEB 2170 was from Boehringer-Ingelheim (Munich, Germany). Lysophosphatidylcholine;1-palmitoyl (99% pure), phosphatidylcholine, bovine serum albumin, phosphate-citrate buffer with urea hydrogen peroxide tablets, O-phenylenediamine dihydrochloride tablets, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tween 20, ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis (β aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), DL-dithiothreitol (DTT), (N-[2-hydroxyethyl] piperazine-N′-2-ethane sulfonic acid) (HEPES), phenylmethyl sulfonyl flouride (PMSF), glycerol, poly-(dI-dC), Tris-base, Tris-HCl and boric acid were from Sigma Chemical Co. (St Louis, MO, USA). Diacylglycerol (DAG) was obtained from Fluka (Deisenhofen, Germany). Acetone, ethanol, sulfuric acid, KCl, dimethylsulfoxide (DMSO) and $CaCl_2$ were purchased from Merck (Darmstadt, Germany). Anti-TG monoclonal antibody (mAB) was purchased from Campro Scientific (Veenendaal, The Netherlands). Goat-antimouse HRP-conjugated AB, AP-1 consensus oligo (5′-CGCTT-GATGAGTCAGCCGGAA-3′), NF-κB oligo (5′-AGTT-GAGGGGACTTTCCCAGGC-3′), SP-1 oligo (5′-ATT-CGATCGGGGCGGGGCGAGC-3′), gel shift binding 5x buffer, T4 polynucleotide kinase, T4 polynucleotide kinase 10x buffer were purchased from Promega (Madison, WI, USA). [γ-^{32}P]ATP was from Amersham (Birkeroed, Denmark). Nick spin columns Sephadex G-50 fine DNA-grade were obtained from Pharmacia Biotech (Alleroed, Denmark).

Cell culture

Normal human keratinocytes were obtained by trypsinization of keratome biopsies from skin samples of adults undergoing plastic surgery (21). Immediately after removal from the body the skin samples were put on ice. First-passage keratinocytes were grown in serum-free medium supplemented with 5 ng/ml human recombinant epidermal growth factor, 50 μg/ml bovine pituitary extract and 5 μg/ml gentamicin. The cells were grown at 37°C and 5% CO_2 in an incubator.

The keratinocytes which should be used for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and transglutaminase (TG) assay were grown to 90% confluence, trypsinated and suspended at a cell concentration of 2×10^4 cells/ml and plated at 4000 cells per well in preheated 96-well microplates. The plates were placed in an incubator and the serum-free medium was changed after 24 h. At 30% confluence as judged by light microscopy the different stimuli were added. The effect of lysoPC was assessed with or without 3% charcoal-stripped fetal calf serum and at a calcium concentration of 0.17 mM. 1.2 mM calcium served as a positive control.

In those experiments where stimulation was carried out for >24 h medium was changed every day. When AP-1- or NF-κB-binding activity was determined keratinocytes were cultured in 25 cm^2 culture flasks. The cells were stimulated with lysoPC (10^{-9} M) for 0.5, 1, 2, 6, 12, 24, 48 or 72 h and with DAG (10^{-6} M) or phosphatidylcholine (10^{-6} M) for 0.5 h or 24 h.

The toxicity of the different lysoPC concentrations, the vehicle and the inhibitors was assessed by the trypan blue exclusion test.

Addition of inhibitors

To determine whether PKC activation was involved in the lysoPC-induced effects the staurosporine analogue GF 109203X (22) was added to the keratinocyte cultures. It was used at the following concentrations 10 nM, 20 nM, 132 nM and 250 nM. These concentrations inhibit different isoforms. An inhibitor concentration of 10 nM reduces the activity of PKCα, at 20 nM PKCβ also becomes inhibited, and an inhibitor concentration of 132 nM inhibits the activity of PKCε. At concentrations above 210 nM the activity of PKCδ also becomes markedly inhibited ($>50\%$ of the enzyme activity). The PKC inhibitor Rottlerin was used at a concentration of 6 μM in order to inhibit the PKCδ isoform (23).

One of the signalling pathways downstream of PKC is the MAP kinase pathway, a family of serine/threonine protein kinases. To determine whether the MAP kinase pathway was involved in the lysoPC-induced effects, we used the MAPKK inhibitor PD 98059 (24). The MAPKK inhibitor PD 98059 and PAF antagonist WEB 2170 (25, 26) were used at concentrations of 50 μM and 1 μM, respectively.

When the PKC inhibitors (GF 109203X, Rottlerin), the MAPKK inhibitor (PD 98059) and the PAF antagonist WEB 2170 were added this was done 30 min before addition of lysoPC. All inhibitors were dissolved in DMSO and the maximal DMSO concentration of the medium was 1‰. Vehicle was added to the control wells.

Determination of proliferation and differentiation

Microplate assays for cell viability (27) and human TG-I were combined in order to measure keratinocyte proliferation and differentiation in the same monolayer (28). The combination of the methods resulted in improved sensitivity and reproducibility. After incubation with the different stimuli, 25 μl of 5 mg/ml MTT in phosphate-buffered saline (PBS without calcium and magnesium) were added to the wells. The plates were placed in an incubator until the formazan crystals penetrated the cell membrane. In living cells the mitochondrial dehydrogenase enzyme converts the tetrazolium salt to the insoluble MTT-formazan after approximately 2 h. The medium was removed and the plates were subjected to freeze-thaw cycles twice before formazan crystals were dissolved in ethanol:acetone (60:40 w/w) by gentle shaking for 30 min at 4°C. The amount of formazan was quantified in an ELISA reader at 540 nm, subtracting the background values at 650 nm.

After two additional freeze-thaw cycles, an ELISA assay with primary antibody against TG-I, a terminal differentiation marker, was performed.

Non-specific absorption of antibodies on the plates was blocked with 1% bovine serum albumin in PBS without calcium and magnesium; 200 μl were added to each well and the plates were allowed to incubate at 37°C for 1 h. For the next hour the plates were incubated with 100 μl of TG-I antibody diluted 1:1000 in calcium- and magnesium-free PBS with 1% bovine serum albumin. The plates were washed three times in PBS containing 0.05% Tween 20 and then incubated for 1 h with 100 μl peroxidase conjugated goat anti-mouse antibody diluted 1:2500 in PBS with 1% bovine serum albumin. The microplates were washed three times in PBS with 0.05% Tween 20 and once in PBS. The buffer (100 μl) containing the O-phenylenediamine dihydrochloride and the urea hydrogen peroxide tablet was added and after the reactions had proceeded in darkness for approximately 20 min they were stopped with 100 μl of 2 N sulphuric acid. The amount of TG-I was measured by quantifying the

O-phenylenediamine dihydrochloride reaction with an ELISA reader at 490 nm and subtracting the background at 650 nm. In order to compensate for variations in cell number the TG-I data were divided by the proliferation data.

Isolation of the nuclear fraction

Nuclear extracts were prepared as described earlier (29): 950 μl of hypoton buffer (10 mM HEPES, 10 mM KCl, 0.2 mM, EDTA, 0.1 mM EGTA, 1 mM DTT, pH 7.9) containing protease inhibitors were added to the cells and they were scraped off the culture flasks with a rubber policeman. The cells were allowed to swell on ice for 15 min and 4.8 μl of Igepal were added. The cell suspension was passed through a 27-gauge needle six times and then centrifuged at 10,000 *g* for 1.5 min.

The pellet was resuspended in a hyperton buffer (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT) containing protease inhibitors, and rotated at 4°C for 30 min before centrifugation at 10,000 *g* for 5 min. The supernatant obtained from this centrifugation was the nuclear extract. Protein concentration was determined by the Bradford reagent with bovine albumin as a standard.

Labelling of consensus nucleotides

Four μl of AP-1 or NF-κB consensus oligonucleotides (1.75 pmol/μl), 2 μl of T4 polynucleotide kinase 10x buffer (700 mM Tris-HCl, 100 mM $MgCl_2$ and 50 mM DTT), 4 μl of [γ^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml), 8 μl nuclease-free water and 2 μl T4 polynucleotide kinase were added to an eppendorf tube and allowed to incubate for 10 min at 37°C before the reaction was stopped by adding 1 μl of 0.5 M EDTA. Finally 200 μl of TE buffer (10 mM Tris-HCl, pH-8 and 1 mM EDTA) were added. Labelled and unlabelled oligonucleotides were separated on a Nick spin column at 300 *g* for 5 min.

Gel shift assays

The nuclear samples consisted of 3–5 μg protein which were preincubated for 10 min at room temperature with the gel shift binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 20% glycerol, 0.25 mg/ml poly(dI-dC). In order to obtain the same volume of each sample, sterile water was added. Then 1 μl of ^{32}P-labelled AP-1 or NF-κB probe was added and the samples were incubated for an additional 20 min. After the incubation 1 μl of bromphenol blue was added and the samples were run on a 4% polyacrylamide gel in 0.5x TBE buffer (22.5 mM Tris base, 4 mM boric acid and 1 mM EDTA).

Electrophoresis was performed at a constant voltage of 125 V for 90 min. After the electrophoresis the gels were dried and autoradiographed at −80°C for several days using preflashed films. In competition experiments a specific competitor (unlabelled AP-1 or NF-κB oligo) or a non-specific oligo (unlabelled SP-1 oligo) was added 10 min before addition of labelled AP-1 oligo. The intensity of the bands was quantified using Image-Quant and the relative intensity was derived by dividing by the number in the control band.

Statistical analysis

Data were analysed by a paired t-test, if they showed a parametric distribution. If not, Wilcoxon's test for matched samples was used. A *p* value below 0.05 (two-tailed) was considered significant.

RESULTS

Cell culture

LysoPC produced a dose-dependent upregulation of TG-1 when incubated with the keratinocyte cultures for 24 or 48 h. The dose-response curve was bell-shaped in the interval from 10^{-5} to 10^{-15} M, exhibiting a maximum at 10^{-9} M. Therefore 10^{-9} M was chosen for the experiments involving inhibitors. Addition of 1.2 mM calcium was used as a positive control, which increased the differentiation twofold (Fig. 1) and inhibited proliferation by one third ($n=10$) (data not shown). No effect on proliferation was observed when lysoPC was added to keratinocyte cultures in the concentration range from 10^{-5} M to 10^{-15} M (data not shown).

To investigate if the effect on TG-1 was dependent on PKC, we added the PKC inhibitors GF 109203X and Rottlerin. After both 24 and 48 h GF 109203X (250 nM) and Rottlerin inhibited the increased expression of TG-1 induced by lysoPC (Fig. 1). To determine if the MAP kinase pathway downstream of PKC was involved in the lysoPC-induced upregulation of TG-1, we added the MAPKK inhibitor PD 98059. This inhibitor failed to inhibit the increased expression of TG-1 induced by lysoPC after 24 h, but after 48 h an inhibition was present (Fig. 1). In order to examine if the lysoPC-induced effect on TG-1 was mediated by the PAF receptors, a PAF antagonist was added. This showed only a slight reduction in the lysoPC-induced effect on differentiation (Fig. 1).

AP-1 DNA-binding activity

Because AP-1 is one of the transcription factors regulating TG-1 gene expression we determined the DNA-binding activity of AP-1 after incubation with lysoPC 10^{-9} M for 0.5, 1, 2, 6, 12, 24, 48 and 72 h. The binding activity was significantly increased at 24 h and thereafter gradually decreased (Fig. 2). Therefore, the effect of the PKC inhibitor GF 109203X and the MAPKK inhibitor PD 98059 on the DNA-binding activity of AP-1 was determined at 24 h. In the interval from 20 to 250 nM a dose-dependent inhibition of PKC was seen (Fig. 3). At 250 nM the inhibition was significant ($p<0.05$). When Rottlerin was added before incubation with lysoPC there was no increase in AP-1 DNA-binding activity ($n=3$) (data not shown). The AP-1 DNA-binding activity was also inhibited when the MAPKK inhibitor PD 98059 was added (Fig. 3) indicating that the MAP kinase pathway is of importance for the lysoPC-induced upregulation of AP-1. In contrast to AP-1, the lysoPC-induced upregulation of NF-κB DNA-binding activity occurred after 0.5 h and had declined to control levels at 24 h (Fig. 4). The lysoPC-induced upregulation of AP-1 and NF-κB was only present when keratinocytes were

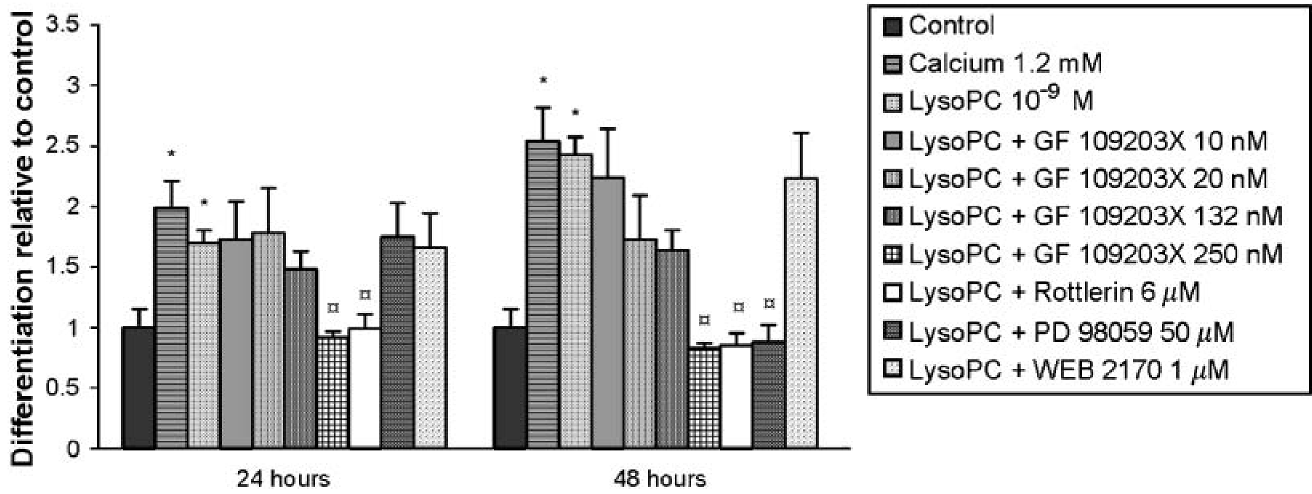

Fig. 1. Transglutaminase assay of cultured human keratinocytes. Second-passage keratinocytes were grown in 96-well microplates in the presence of 3% charcoal-stripped fetal calf serum and 0.17 mM Ca^{2+}. The stimulant was added to cultures of 30% confluence. Details are given in Materials and Methods. Cultures grown in 1.2 mM Ca^{2+} were used as a positive control. Each column represents mean +SEM of 10 experiments performed in triplicate. 10^{-9} M lysophosphatidylcholine (lysoPC) and 1.2 mM Ca^{2+} were compared to vehicle and asterisks (*) represent p values <0.05. ¤ represents p values <0.05 when lysoPC-treated (10^{-9} M) keratinocytes were compared to keratinocytes stimulated with lysoPC (10^{-9} M) and inhibitor. GF Rottlerin, PD and WEB are various inhibitors and antagonists – see M&M for explanation.

grown in medium supplemented with 3% fetal calf serum. Therefore, we stimulated keratinocytes with diacylglycerol or palmitic acid to examine whether lipid components present in serum could induce the same effects as lysoPC. In these experiments we found no stimulatory effect of DAG (10^{-6} M) on the DNA-binding activity of AP-1 examined at 24 h ($n=2$) or on NF-κB examined at 0.5 h ($n=2$) (data not shown). Palmitic acid (10^{-6} M) also failed to induce upregulation of AP-1 and NF-κB at the time points mentioned above ($n=2$) (data not shown).

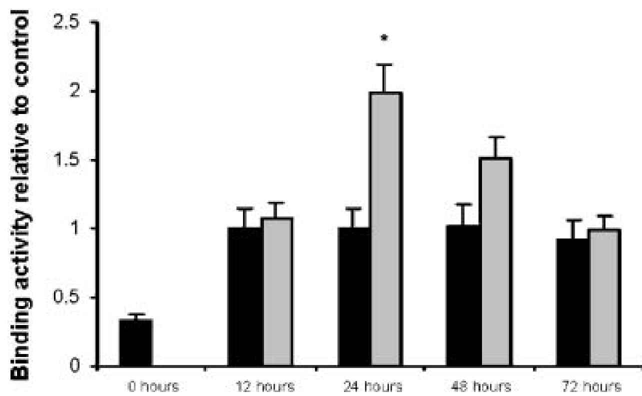

Fig. 2. Activator protein-1 (AP-1) DNA-binding activity of keratinocytes treated with lysophosphatidylcholine (lysoPC). Electrophoretic mobility shift assay of keratinocytes grown in 25 cm^2 culture flasks in the presence of 3% charcoal-stripped fetal calf serum and 0.17 mM Ca^{2+}. The stimulant was added to cultures of 30% confluence and keratinocytes were stimulated for 12–72 h. The nuclear fraction was isolated and the AP-1 DNA-binding activity was determined (see Materials and methods). The columns for 24, 48 and 72 h represent mean + SEM of six experiments performed in duplicate. The column for 12 h represents mean of two experiments performed in duplicate. * represents p values <0.05.

DISCUSSION

LysoPC has previously been implicated in signal transduction. It has been reported to increase the cytosolic concentration of cAMP (7) via G-protein-dependent activation of adenylyl cyclase and to stimulate PKC in the presence (30) or absence of DAG (5). In our study the metabolism of lysoPC to other PKC stimulatory components like DAG (9) or fatty acids cannot be ruled out. Nishizuka suggested that lysoPC plays a role in long-term cellular responses and is generated to maintain PKC activation after the initial activation induced by DAG or calcium (30). In support of our findings keratinocytes in culture express mRNA encoding PKCδ but also PKCα, PKCε, PKCξ

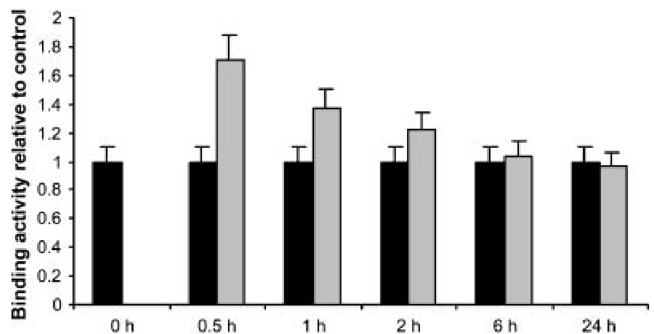

Fig. 3. NF-κB DNA-binding activity of keratinocytes treated with lysophosphatidylcholine (lysoPC). Conditions as in Fig. 2. LysoPC was added to cultures of 30% confluence and the keratinocytes were stimulated for the time periods indicated above. The nuclear fraction was isolated and the NF-κB DNA-binding activity was determined. Densitometric analysis of ^{32}P-bands were carried out and the NF-κB DNA-binding activities of the samples were divided by the control values. Each column represents the mean of two experiments performed in duplicate.

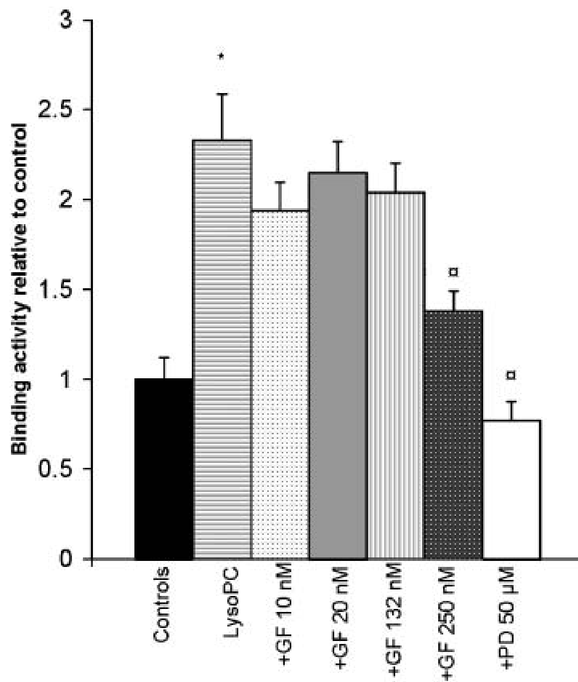

Fig. 4. Activator protein-1 (AP-1) DNA-binding activity of keratinocytes treated with lysophosphatidylcholine (lysoPC) and inhibitors. Mobility shift assay of keratinocytes grown as in Fig. 2. The keratinocytes were stimulated for 24 h. Each column represents the mean + SEM of eight experiments performed in duplicate. * represents p values <0.05 when keratinocytes treated with lysoPC (10^{-9} M) or Ca^{2+} (1.2 mM) were compared to keratinocytes incubated with vehicle. ¤ represents p values <0.05 when lysoPC-treated keratinocytes were compared to lysoPC- (10^{-9} M) and inhibitor-treated keratinocytes.

and PKCε, whereas PKCβ and PKCγ were absent (31). In the skin PKCδ was found in all epidermal layers (32) and PKCα and PKCδ were found in mouse skin (1). Apparently PKCβ is expressed by immunocompetent cells (33).

Regarding the pathways downstream of PKC, incubation with the MAPKK inhibitor PD 98059 had no effect on the lysoPC-induced TG-1 expression after 24 h. However, after 48 h the lysoPC-induced TG-1 expression and increased AP-1 DNA-binding activity were completely abolished. Whether it is the proliferation or differentiation that is affected seems to depend on the cell type. LysoPC has been reported to promote differentiation in monocytes (11) and to increase proliferation in smooth muscle cells (34). The active concentration of lysoPC (10^{-9} M) is lower than the concentration at which this lipid normally exerts its biological actions (9, 10). A possible explanation is an increased sensitivity of the keratinocytes to this lipid; another that lysoPC potentiates the differentiation induced by factors present in fetal calf serum, as the effects of lysoPC could only be seen when fetal calf serum was present in the medium (data not shown). Although we have not identified the component in serum responsible for this effect our results show that the effect of lysoPC on the upregulation of AP-1 and NF-κB is specific, as other lipid components like DAG and palmitic acid failed to produce a response. In keratinocytes the lysoPC stimulation culminates in upregulation of AP-1 DNA-binding activity at 24 h preceeding the maximal TG-1 expression at 48 h. This time-frame suggests that the AP-1 upregulation could cause the increased TG-1 expression as the TG-1 gene possesses AP-1-binding sites (18). LysoPC-induced upregulation of AP-1 has previously been reported in human umbilical vein endothelial cells (20) but here the time required to upregulate AP-1 was much shorter (4 h). In our study there was no upregulation of AP-1 prior to 24 h (0.5, 1, 2, 6 and 12 h). In keratinocytes we observed an upregulation of NF-κB at 0.5 h, which is earlier than the response observed in endothelial cells where the upregulation of NF-κB was maximal at 1–2 h (20). These differences between AP-1 and NF-κB activation can be explained by the fact that NF-κB is constitutively expressed in the keratinocytes and therefore can be activated by immediate responses, whereas activation of AP-1 is regulated predominantly at the transcriptional level with induction of Jun and Fos proteins (35).

In conclusion, lysoPC can upregulate AP-1 and TG-1 via pathways involving PKCδ and MAPKK. To get a more complete picture of the effects of lysoPC in keratinocytes it would be of interest to examine other signal transduction pathways like cAMP/PKA and to determine the events proximal and distal to PKC activation, including a study of which members of the Fos and Jun families participate in these events.

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