Expression and regulation of phospholipase D in the human keratinocyte cell line HaCaT

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Abstract The generation of lipid second messengers via phosphatidylcholine (PC)-specific phospholipase D (PLD) has emerged as an important step leading to transduction of extracellular signals. In the present investigation the expression of human cytosolic PLD isoenzymes in the immortalized human keratinocyte cell line HaCat was determined. At the mRNA level we found the expression of hPLD1b and for the first time in human cells also the expression of hPLD2. For further analysis of enzyme expression at the protein level, hPLD1 peptide fragments were synthesized and specific antibodies were generated (rabbit) to be used for detection of hPLD1 in Western blot experiments. Furthermore, small G-proteins were found to be involved in the regulation of PLD activity in HaCaT cells using the guanine nucleotide analogue GTPγS.

Key words: Phospholipase D; Signal transduction; HaCaT cell; Peptide-specific antibody

1. Introduction

Lipid metabolism plays a crucial role in cell signalling. For example, eikosanoids, diacylglycerol, ceramides, sphingosine-1-phosphate, lysophosphatidic acid and platelet activating factor (PAF) induce and/or mediate cellular responses in many cell types. Phosphatidylcholine-specific phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid and choline. A great variety of stimuli increase PLD activity in a wide range of cell types and the consequent increase of phosphatidic acid, or a metabolite of this lipid, mediates PLD-induced cell signalling (for review see [1,2]). A considerable portion of the protein kinase C activator diacylglycerol arises from PLD-generated phosphatidic acid by the action of phosphatidate phosphohydrolase [3]. Studies on the regulation of PLD suggest that this enzyme may play a role in mitogenic signalling processes controlled by monomeric G-proteins of the ADP-ribosylation factor (ARF) and Rho families, and in intracellular vesicle trafficking [4,5]. Cloning of PLD isoenzymes from plants [6] as well as from yeast [7] and humans [8] provides new insights into the regulation and the structure-function relationships of these enzymes.

In mammals, two different cytoplasmic isoenzymes of PLD have been identified up to now (PLD1 and PLD2). In humans, two splice variants of the PLD1 isoenzyme (PLD1a and PLD1b) were described by Hammond et al. [8,9]. The other isoenzyme, PLD2, was only detected in mice [10] and rats [11], but no occurrence of PLD2 in humans has been published so far. Epidermal growth control is of particular interest, because in many dermatoses a dysregulation of proliferative and differentiating processes was shown. As indicated above, PLD is involved in the generation of mitotic signals like diacylglycerol, therefore we focussed our interest on the characterization of PLD in human keratinocytes. Although PLD activity has been found in human keratinocytes [12–14], no data are available on the expression of the different isoforms in these cells. In the present study, we identified the isoenzymes PLD1b and also PLD2 in the human keratinocyte cell line HaCaT. Furthermore, peptide-specific antibodies were generated to identify PLD1 at the protein level.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine (1.85–2.29 GBq/mmol) was purchased from Amersham Buchler (Braunschweig, Germany), [9,10-^{3}H(N)]myristic acid (518 GBq/mmol) was purchased from NEN DuPont (Bad Homburg, Germany), and cabbage PLD was from Sigma (München, Germany).

2.2. Cell culture

HaCaT cells [15] were grown in 90% Roswell Park Memorial Institute medium (RPMI 1640), 10% heat-inactivated fetal calf serum supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) in 80 cm^2 tissue culture flasks (Nunc, Wiesbaden, Germany). Media and culture reagents were obtained from Seromed-Biochrom (Berlin, Germany).

2.3. Reverse transcriptase polymerase chain reaction (RT/PCR)

Total cellular RNA used to prepare cDNA was isolated using

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Abbreviations: ARF1, ADP-ribosylation factor 1; BCA, bicinchoninic acid; BSA, bovine serum albumin; DPPC, dipalmitoylphosphatidylcholine; EGTA, ethylene glycol-bis[β-aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Na-HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] sodium salt; MgATP, adenosine triphosphate magnesium salt; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PIP_2, 1-phosphatidyinositol-4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate

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RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The reverse transcription (RT) reaction was performed at 42°C for 1 h in 20 μl of 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 2 mM dNTPs (0.5 mM each), 200 ng of random hexamers (Life Technologies, Eggenstein, Germany) as primers, 10 mM DTT, 5 μg of total cellular RNA using 200 units SuperScript IIRT (Life Technologies, Eggenstein, Germany). To terminate the reverse transcription samples were incubated at 70°C for 15 min. Remaining RNA was degraded by an additional incubation at 37°C for 20 min with 2 U of *Escherichia coli* RNase H (Life Technologies, Eggenstein, Germany). The resulting cDNA was used directly for PCR or stored at −20°C. Human PLD1-specific PCR primers (GGAGGGATTGACCTGGCCTA and GCCACATCACGAGCCGCCTT) were designed using the MacMolly Tetra program (Softgene GmbH, Berlin, Germany) and were synthesized by TIB Molbiol (Berlin, Germany). PCR was carried out in 50 μl of 50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 0.8 mM dNTPs (200 μM each), 4 μM primer (2 μM each) using 1.25 U of Taq DNA polymerase (Life Technologies, Eggenstein, Germany). 1 μM of cDNA was added as PCR template. For control reactions 1 μl H_2O and 1 μl of 10 pg/μl pBluescript containing the hPLD1b gene, respectively, were used. Thirty-five cycles of amplification were carried out as follows: at 92°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min. 8 μl of PCR products were analyzed by electrophoresis in 2% agarose gels.

2.4. Preparation of peptide specific antibodies

The peptide MSLKNEPRVNTS(C) corresponding to the amino-terminal 12 amino acids of human PLD1, and the peptide (C)TKEAIVPMEVWT corresponding to the carboxy-terminal 12 amino acids of human PLD1 were synthesized and purified using procedures as described earlier [16]. In both peptides a cysteine in the distal position was added as indicated above for coupling to a carrier. This should not alter the antigenic properties of the peptides. Antibodies against these peptides were then prepared by Eurogentec (Seraing, Belgium) using a standard protocol [17]. A mixture of both peptides was used for immunization in order to increase the probability of generating a PLD-specific antiserum. Briefly, the two synthetic peptide fragments of PLD conjugated over the additional cysteine with keyhole limpet hemocyanin (1000 μg each) were injected at several sites into one rabbit, initially with complete Freund's adjuvant, followed by injection of peptide conjugates (500 μg each) in complete Freund's adjuvant every 2 weeks. Ten days after the third booster injection the blood of the animal was taken and sera were obtained by centrifugation at 8000×*g* for 20 min. 50 μl aliquots of the sera were stored at −20°C. This polyclonal antiserum was termed αNChPLD1 indicating that the antibodies were raised against the N- and C-termini of human PLD1.

2.5. Sandwich ELISA

The sandwich ELISA technique was performed essentially as described [16]. 100 μl of antibody αNChPLD1 (1:500 in PBS containing 0.05% Tween 20, further designated PBST) was incubated in 96-well ELISA plates for 1 h at 37°C and fixed with 0.5% glutaraldehyde for 30 min at room temperature. After washing with PBST, the wells were saturated with 200 μl of a solution of 1% BSA in PBS for 1 h at 37°C. Following this blocking step, the wells were incubated with 0.1 μg/ml peptide mixture in PBST (peptide sequences are described in Section 2.4) overnight at 4°C. After washing, the wells were incubated with 100 μl of antibody αNChPLD1 (1:500 in PBST, or different dilutions of antibodies were used as indicated) for 1 h at 37°C. Detection of peptide-bound antibodies were carried out by incubation with a peroxidase-conjugated swine anti-rabbit antibody (1:1000 in PBST) for 1 h at 37°C. After thoroughly washing the wells 100 μl substrate solution (0.8 mg/ml phenylenediamine, 0.015% H_2O_2 in 0.1 M citrate buffer, pH 6.0) was added. After 5 min at 37°C the reaction was stopped with 100 μl 1.25 M sulfuric acid and the absorbance was measured at 492 nm. Control experiments were performed without specific peptide, without antibody αNChPLD1 and with unspecific peptide (12 amino-terminal amino acids of human choline kinase, the peptide was synthesized by Dr. Becker, Schering AG, Berlin, Germany).

2.6. Preparation of cell homogenates

Cells were harvested using a cell scraper in 0.5–1 ml of 50 mM Na-HEPES (pH 7.5), 3 mM EGTA, 80 mM KCl, 1 mM 1,4-dithiothreitol, 3 mM $MgCl_2$ and 2 mM $CaCl_2$ (this buffer is further designated assay buffer) containing additionally 1 mM PMSF and 10 μM leupeptin. After Dounce homogenization, the homogenate was centrifuged for 10 min at 400×*g* to get rid of cell debris. The homogenate was stored at −20°C. For SDS-PAGE the homogenate was supplemented with detergents to obtain the following concentrations: 0.1% (w/v) SDS, 1% (v/v) Triton X-100 and 0.5% (w/v) sodium deoxycholate prior to centrifugation. Protein was measured in samples diluted 1:20 in H_2O by the BCA assay [18], with BSA as standard.

2.7. Immunoblotting

Cell homogenates, containing 1 and 10 μg of protein, respectively, were mixed with SDS sample buffer [19]. 0.1 M 1,4-dithiothreitol was used instead of β-mercaptoethanol. Samples were separated by 7.5% SDS-PAGE. After transfer to nitrocellulose membrane the blot was incubated with 10% non-fat dry milk in PBS/0.1% Tween 20 overnight at 4°C to block non-specific binding. The membrane was then incubated with the αNChPLD1 antiserum 1:1000 in PBS/0.1% Tween 20/0.5% BSA for 1 h at room temperature. Bound antibodies were detected after washing using horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibodies (1:5000) and the ECL chemiluminescent detection reagents according to the manufacturer's instructions (Amersham Buchler, Braunschweig, Germany).

2.8. Measurement of phospholipase D activity in permeabilized cells

PLD activity was determined by measuring the transfer of phospholipid phosphatidyl moieties, metabolically prelabeled with [^{3}H]myristic acid, in the presence of ethanol, into phosphatidylethanol (phosphatidyltransferase activity) as described [20] with some modifications. Cell lipids were radioisotopically labeled by incubation of 10^6 HaCaT cells (80% confluence in 6-well plates) in culture medium supplemented with 0.111 MBq of [9,10-^{3}H(N)]myristic acid at 37°C for 24 h. Following the incubation, the cells were washed twice with PBS. An equilibration at 37°C for 5 h in keratinocyte basal medium containing 10 mM HEPES, 0.56 g/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.3 g/l fatty acid free BSA was performed. After washing twice with PBS, cells were treated at 37°C for 30 min with 1 ml of prewarmed permeabilization buffer (permeabilization is necessary for penetration of ATPγS into the cell) consisting of 120 mM potassium glutamate, 20 mM HEPES (pH 7.4), 20 mM potassium acetate, 3 mM magnesium chloride, 1 mM EGTA, supplemented with 0.5 mM calcium chloride, 20 μg/ml digitonin, 1 mg/ml BSA and 1% (v/v) ethanol, 500 μM MgATP and different concentrations of GTPγS. The permeabilization is necessary to measure the influence of GTPγS. The enzymatic reaction was stopped by the addition of 1.2 ml ice-cold 2% acetic acid in methanol. Cells were harvested using a cell scraper and lipids were extracted as described [21]. Organic phase was dried under a stream of nitrogen and lipids were resuspended in 50 μl of chloroform/methanol (1:1), and 15 μl were applied to high-performance thin-layer chromatography plates. Separation was carried out in chloroform/methanol/30% aq.NH_3. (65:35:3, v/v). Radioactivity was quantified by radioscanning using a Berthold LB 2821 HR thin-layer chromatography scanner (Berthold, Wildbad, Germany). Phosphatidylethanol in the samples was identified by comigration of an internal standard prepared with cabbage PLD.

2.9. Measurement of phospholipase D activity in cell homogenates

Phospholipid vesicles were used to assay cell homogenates for PLD activity essentially as described [22]. Briefly, vesicles were composed of PE, PIP_2 and DPPC in the molar ratio of 20:1:2. 18.5 kBq of 1-palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine was added as radioactive tracer (final concentration of PC was 8.6 μM). The standard assay was carried out in 30 μl assay buffer (see also Section 2.6) containing 5 μl vesicle mix and 0.67 mg/ml of protein in cell homogenates and different concentrations of GTPγS. The incubation was performed at 37°C for 1 h. Lipids were extracted as described by Bligh and Dyer [21] and separated by high-performance thin-layer chromatography in chloroform/methanol/acetic acid (13:3:1, v/v). Radioactivity was quantified by radioscanning using a Berthold LB 2821 HR thin-layer chromatography scanner (Berthold, Wildbad, Germany). Phosphatidylethanol in the sample was identified by comigration of an internal standard prepared using cabbage PLD.

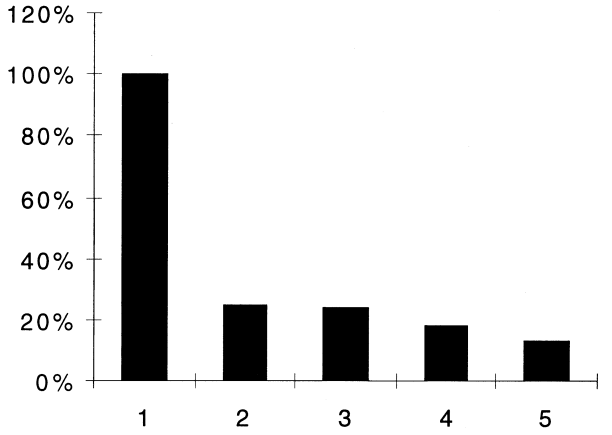

Fig. 1. Characterization of the peptide specifity of phospholipase D antibody αNChPLD1 by sandwich ELISA. The sandwich ELISA was carried out as described in Section 2.5. The signal represented by column 1 was measured in the presence of antibody αNChPLD1 and PLD1 peptides and was set at 100%. Column 2: Signal obtained in the presence of antibody αNChPLD1 but without peptide. Column 3: Signal obtained in the absence of antibody αNChPLD1 but in the presence of peptide. Column 4: Signal obtained in the presence of antibody αNChPLD1 and of the control peptide. Column 5: PBST control. The values represent the mean of two determinations. The experiment was repeated twice and similar results were obtained.

3. Results and discussion

3.1. Preparation and characterization of a peptide-specific antibody against human phospholipase D1

Based on the human PLD1 cDNA sequence [8] we synthesized peptides corresponding to residues 1–12 and 1063–1074 and peptide-specific antibodies were produced in rabbit as an important tool for further experiments. We used the sandwich ELISA technique to determine the peptide specificity of this new antibody. The extinction value obtained in the presence of antibody αNChPLD1 together with the specific peptide mixture was set at 100% (Fig. 1, column 1). Control experiments were carried out with antibody αNChPLD1 but without peptide (column 2, peptide negative control), with antibody and with non-specific control peptide (column 4), without antibody and with peptide (column 3, antibody negative control). As shown in Fig. 1, the extinction values of the control experiments reached at most 25%. Furthermore, the extinction value using non-specific control peptide was below the negative controls represented by columns 2 and 3. In another experiment we used different antibody αNChPLD1 concentrations and found that the dilution 1:500 is optimal for this assay system (data not shown). From the results obtained we concluded that the new peptide-specific antibody against amino- and carboxy-terminal peptides of human PLD1 can be used for further studies.

3.2. Expression of phospholipase D in HaCaT cells

The expression of hPLD1 in the human keratinocyte cell line HaCaT [15] was investigated in RT/PCR and Western blot experiments. For amplification of the hPLD1 cDNA, gene-specific PCR primer pairs were used spanning the alternatively spliced exon of 114 bp [9] in order to discriminate between hPLD1a (estimated product length: 571 bp) and hPLD1b (estimated product length: 457 bp). Fig. 2 shows

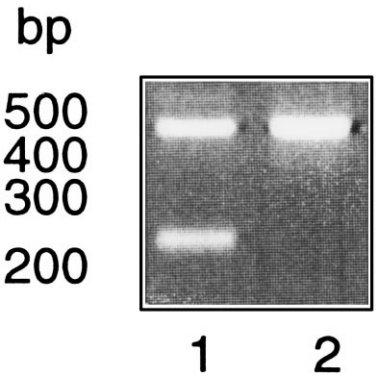

Fig. 2. Expression of phospholipase D isoforms in HaCaT cells as assessed by RT/PCR. An ethidium bromide-stained 2% agarose gel is shown after electrophoretic separation of RT/PCR products. In lane 1 HaCaT cDNA was used as template whereas in lane 2 hPLD1b containing plasmid was used as template DNA. Control experiment in the absence of template DNA is not shown, but no PCR product was detectable. The experiment was repeated and similar results were obtained.

the agarose gels in which RT/PCR products have been electrophoretically separated. cDNA used as template for the PCR experiment shown in lane 1 was prepared using total RNA isolated from HaCaT cells. Plasmid DNA containing the hPLD1b cDNA was used for the control PCR experiment (lane 2). The upper signals seen in both lanes correspond to hPLD1 gene detection. hPLD1b cDNA was detectable in HaCaT cells and in the control PCR as can be seen by the length of the PCR product of 457 bp whereas hPLD1a cDNA was not (no signal corresponding to a PCR product of 571 bp was detectable). The second band of approximately 250 bp seen in lane 1 was unexpected. Therefore, it was isolated from the

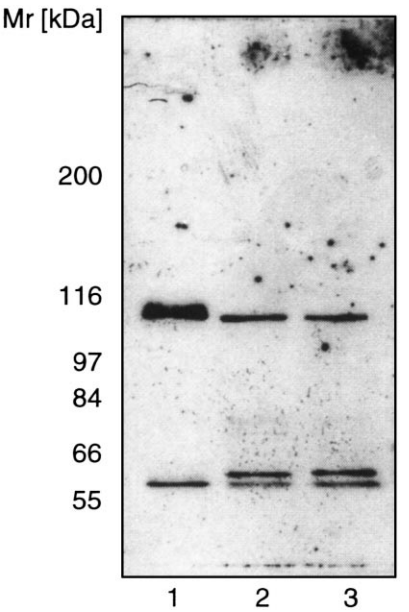

Fig. 3. Western blot analysis of HaCaT cell homogenates. HaCaT cell homogenates containing 10 μg of protein (lanes 2 and 3) were separated on 7.5% SDS-PAGE and subjected to Western blot analysis as described in Section 2. Cell homogenate containing 1 μg of protein obtained from hPLD1b-overexpressing Sf9 cells was used as positive control (lane 1). Positions of molecular weight standards are indicated.

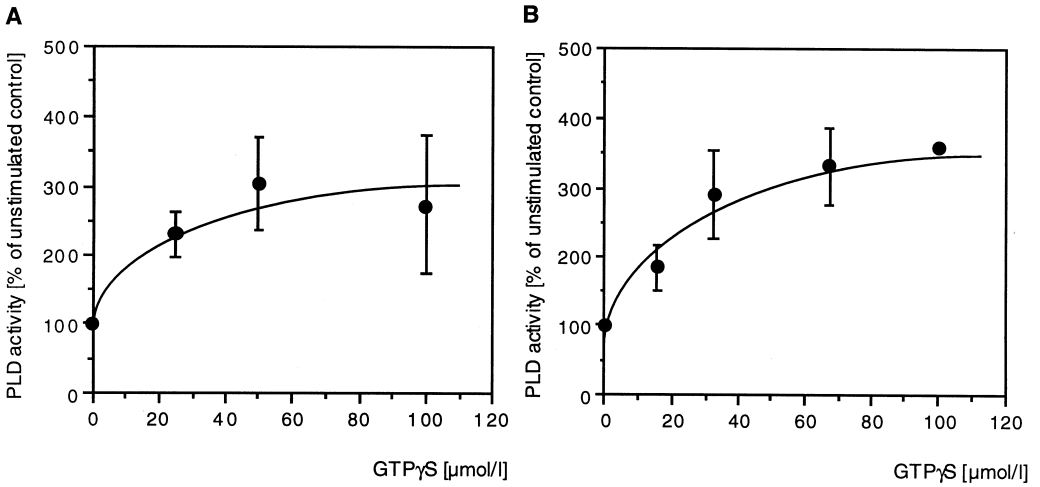

Fig. 4. Regulation of phospholipase D activity by GTPγS. Phospholipase D activity was measured in permeabilized cells (A) and in cell homogenates in vitro (B) in the presence of different concentrations of GTPγS. Assays were performed as described in Section 2. Data points represent the mean of three determinations ± S.D. In one case the standard deviation is within the symbol.

agarose gel and sequenced using the cycle sequencing procedure. Sequence comparison using the NIH nucleic acid data base resulted in 98% identity to the gene for human PLD2 (data base entry by P.M. Steed, K.L. Clark, W.C. Boyar and D.J. Lasala (1997), accession number AF033850). The amplification of hPLD2 cDNA using our hPLD1-specific PCR primers was more random than expected because the selected PCR primers are specific for hPLD1 cDNA. An explanation could be that both PLD genes show high homology and therefore it may be possible that the primers with high specificity for hPLD1 cDNA also anneal with lower stringency to hPLD2 cDNA. However, we conclude that in HaCaT cells the mRNAs for hPLD1b and hPLD2 are present.

Despite the data at the mRNA level we studied human PLD expression at the protein level as well and used the peptide-specific antibody (described in Section 3.1) for Western blot analysis. Fig. 3 shows the Western blot corresponding to SDS-PAGE in which cell homogenates were run and further processed as described in Section 2.7. The signals obtained in all three lanes in the upper part of the blot correspond to a protein with an apparent molecular weight of about 110 kDa. Furthermore, some distinct bands occurred in the lower part of the blot corresponding to polypeptides of about 60 kDa. The calculated molecular weights of hPLD1a and hPLD1b are 124 kDa and 117 kDa, respectively. Since the signal detected in lane 1 corresponds to overexpression of hPLD1b in Sf9 cells we conclude that our peptide-specific antibody is usable for detection of hPLD1 in Western blot analysis. Thus, the detected proteins with an apparent molecular weight of about 110 kDa seen in lanes 2 and 3 represent human phospholipase D isolated from HaCaT cells, although the calculated and the apparent molecular weights of hPLD1 are slightly different.

3.3. Regulation of phospholipase D by GTPγS

Mammalian PLD is known to be regulated by different mechanisms. In HL-60 cells, it was demonstrated that cytosol was required for the stimulatory effect of GTPγS on PLD activity indicating the involvement of small G-proteins [23]. Furthermore, Hammond et al. [8] showed that activation by GTPγS is characteristic for the isoenzyme hPLD1. In order to confirm that the 110 kDa protein is identical with human cytosolic PLD we established the measurement of PLD activity in permeabilized cells and in vitro. Determination of PLD activity was performed using the transphosphatidylation activity of the enzyme generating the non-physiologic product phosphatidylethanol when ethanol was present as a substrate. Cell homogenates and permeabilized HaCaT cells, respectively, were incubated with GTPγS. As shown in Fig. 4, GTPγS caused a concentration-dependent increase of PLD activity in cell culture experiments (Fig. 4A) as well as in vitro (Fig. 4B) indicating the participation of small G-proteins in PLD regulation in HaCaT cells. The concentrations of 15–100 μmol/l for stimulation of PLD were in the same range as reported for PLD in HL60 cells [24].

In conclusion, the present study revealed that the human keratinocyte cell line HaCaT does express cytosolic PLD. At the mRNA level we showed the expression of hPLD1b and, for the first time in human cells, also the expression of hPLD2. For investigating the expression of these enzymes at the protein level peptide-specific antibodies were used for detection in Western blot experiments and PLD1 was detected in HaCaT cells. Furthermore, involvement of small G-proteins in the regulation of PLD activity in HaCaT cells was shown using the guanine nucleotide analogue GTPγS which is characteristic for the isoform hPLD1. Further studies are necessary to elucidate the regulation and function of hPLD in human keratinocytes in more detail.

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