Epidermal-growth-factor-induced production of phosphatidylalcohols by HeLa cells and A431 cells through activation of phospholipase D

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In response to epidermal growth factor (EGF), HeLa cells and A431 cells rapidly accumulate substantial amounts of phosphatidic acid (up to 0.16 and 0.2 $\mu\text{g}/10^6$ cells respectively), which represents approx. 0.17% of total phospholipid. Phosphatidic acid may be a potential product of diacylglycerol kinase and/or of phospholipase D. To evaluate the contribution of phospholipase D, the phosphatidyl-transfer reaction to a primary alcohol (mostly butan-1-ol; 0.2%) was measured; this reaction is known to be mediated exclusively by phospholipase D in intact cells. In HeLa and in A431 cells prelabelled with [$1\text{-}^{14}\text{C}$]oleic acid, EGF (10 and 100 nM respectively) caused a 3-fold increase in radioactive phosphatidylbutanol within 5 min at the expense of labelled phosphatidic acid. Dose-response relationships showed 10 nM- and 100 nM-EGF to be maximally effective in HeLa cells and A431 cells respectively. Mass determinations showed that the phosphatidylbutanol formed within 5 min represented only part of the phosphatidic acid. Depletion of protein kinase C by pretreatment of A431 cells for 17 h with the phorbol ester phorbol 12-myristate 13-acetate (0.1 μM) did not impair EGF-induced formation of phosphatidylbutanol, thus indicating that the reaction was independent of this enzyme. Since phosphatidic acid is suggested to exert second-messenger functions as well as to induce biophysical changes in cellular membranes, its formation, including that via the phospholipase D pathway, may represent an important link between extracellular signals and intracellular targets.

INTRODUCTION

Epidermal growth factor (EGF), a polypeptide consisting of 53 amino acid residues, triggers cellular responses by binding to a specific cell-surface receptor which possesses protein tyrosine kinase activity (for recent reviews see [1,2]). Activation of the protein kinase activity is essential for eliciting subsequent cellular responses, including the regulation of cellular proliferation. For a number of epithelial cells, EGF is a potent mitogen; however, in certain cells such as A431 cells EGF can inhibit proliferation over a 1–2-day period [3–5]. In addition, EGF exerts a more immediate effect on the cell cycle; it transiently inhibits the progression from G_2 phase to mitosis in cells carrying intact EGF receptor, including A431 cells and HeLa cells [6]. The mechanism by which EGF acts to regulate cell proliferation is still unclear.

The signal-transduction system utilized by EGF via the EGF receptor involves the generation of second messengers, diacylglycerol and inositol trisphosphate [7–9]. Diacylglycerol stimulates protein kinase C, leading to phosphorylation of a number of proteins [10]. Inositol trisphosphate causes a release of Ca^{2+} from intracellular stores [11–13], thus leading to subsequent activation of Ca^{2+} -dependent enzymes. The mechanism by which these signals are generated appears to involve the stimulation of a phospholipase C, possibly by phosphorylation through the EGF receptor kinase [14–17].

Moreover, on application of EGF, A431 cells as well as HeLa cells rapidly accumulate substantial amounts of phosphatidic acid. Phosphatidic acid, a potential product of diacylglycerol kinase, remains at elevated levels to a larger extent and for a longer period than expected simply from diacylglycerol availability through phosphatidic acid breakdown, thus indicating a

possible involvement of phospholipase D, as proposed by Bocckino *et al.* [18].

Phospholipase D (EC 3.1.4.4) catalyses the hydrolysis of phospholipids to yield the free polar headgroup and phosphatidic acid. In the presence of primary alcohols (e.g. ethanol, butan-1-ol), phospholipase D effects a phosphatidyl-transfer reaction, producing phosphatidylalcohols [19–21]. In intact cells the production of phosphatidylalcohol appears to be mediated exclusively by phospholipase D [22,23], and is therefore a specific marker for the activation of this enzyme. Activation of phospholipase D and phosphatidylalcohol production has been shown to occur in response to several agonists, including platelet-derived growth factor [24–30]. In the present work we demonstrate that A431 cells and HeLa cells in the presence of alcohol produce substantial amounts of phosphatidylalcohol in response to EGF, indicating that activation of phospholipase D is likely to contribute to the generation of phosphatidic acid in these cells.

MATERIALS AND METHODS

Materials

EGF (mouse) was obtained from Boehringer Mannheim, Germany. [^{125}I]-EGF (3.7 MBq/ μg), [$1\text{-}^{14}\text{C}$]oleic acid (2 MBq/ μmol), 1-[$1\text{-}^{14}\text{C}$]palmitoyl-lyso-3-phosphatidylcholine (1.85 MBq/ μmol), *myo*-[2- ^3H]inositol with PT6-271 (370 GBq/ μmol) were obtained from Amersham Buchler, Frankfurt, Germany. Phosphatidylcholine from egg yolk, 1,2-dioleoyl-phosphatidylcholine, 1,2- and 1,3-dioleoylglycerol, 1,2-dioleoyl-phosphatidic acid, phosphatidic acid prepared from egg-yolk phosphatidylcholine and BSA (electrophoretically pure) were purchased from Sigma, Munich, Germany.

Abbreviations used: MEM, minimum essential medium; DMEM, Dulbecco's modified MEM; EGF, epidermal growth factor; PBS, phosphate-buffered saline (containing 8 g of NaCl, 0.2 g of KCl, 1.15 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 0.2 g of KH_2PO_4 per l); PMA, phorbol 12-myristate 13-acetate.

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Preparation of the phosphatidylalcohol standards

Phosphatidylcholine (100 mg) dissolved in 2.5 ml of diethyl ether was incubated with 2.5 ml of crude phospholipase D (prepared from white cabbage as described in [31]) in 3.55 ml of buffer (0.2 M-sodium acetate/0.08 M-CaCl₂, pH 5.6) and 450 μ l of ethanol or butanol (final concn. 5%, v/v) by stirring for 16 h under N₂. Then the diethyl ether was evaporated and 5 ml of chloroform, 6 ml of methanol and 1 ml of 0.5 M-EDTA were added and the mixture was vigorously mixed. After separation of two phases, the lower organic phase was transferred into conical vials and evaporated in a Speed Vac concentrator. The residue was dissolved in 100 μ l of chloroform/methanol (1:1, v/v) and separated on silica-gel G 60 t.l.c. plates (Merck, Darmstadt, Germany) by using the upper phase of ethyl acetate/trimethylpentane/acetic acid/water (13:2:3:10, by vol.) as a solvent system. The progress of the reaction was recorded by t.l.c. For this purpose, samples which contained originally about 1 μ g of phosphatidylcholine were separated on t.l.c. plates and stained with Coomassie Blue as described in detail below. With this method, phosphatidic acid and phosphatidylalcohol (in this case phosphatidylbutanol) could be detected. For isolation of the standards, the lipids were identified by iodine vapour, and the spots were scraped from the t.l.c. plates and dissolved in 5 ml of chloroform/methanol (1:1, v/v). After filtration of the silica gel and evaporation of the solvent, the lipids were distributed in 200 μ l of the chloroform/methanol mixture; 5 μ l samples were used as standard.

Cell cultures

HeLa cells were cultivated as monolayers in minimal essential medium (MEM) containing Earle's salts supplemented with 10% (v/v) calf serum. From binding studies using ¹²⁵I-EGF, an EGF-receptor number of 1.4×10^5 per cell (low- and high-affinity binding sites) could be evaluated in this cell line [6]. The epidermoid carcinoma cell line A431 (generously given by Professor H. zur Hausen) was cultivated in Dulbecco's modified MEM (DMEM) containing 10% calf serum. It contained approx. 10^6 EGF receptors per cell, exhibiting low- and high-affinity binding sites [6]. The phospholipid content of 10^6 HeLa cells (at 1.5×10^5 cells/cm²) was 93 μ g, and that of 10^6 A431 cells (at 0.9×10^5 cells/cm²) 122 μ g.

Experimental procedures with radioactively prelabelled cells

For radioactive prelabelling of phospholipids, 5×10^5 cells were transferred into plastic Petri dishes (Falcon; 3.5 cm diameter). After 24 h the cells were incubated with [1-¹⁴C]oleic acid or 1-[1-¹⁴C]palmitoyl-lyso-3-phosphatidylcholine (each 7.4 kBq/ml, dissolved in medium containing 10% calf serum) for 24 h. Then the medium was replaced by MEM (HeLa cells) or by DMEM (A431 cells) containing 0.5% BSA. After 1 h of equilibration, cells were treated with EGF or phosphate-buffered saline (PBS) as control (final concn. 0.2%) for the periods and with the concentrations indicated. For transphosphatidylolation experiments the cells were preincubated for 20 min with 0.5% ethanol or 0.2% butanol, if not otherwise indicated. The reaction was stopped by removing the medium and adding 2 ml of ice-cold methanol to the cells. The extraction of cellular lipids was performed with chloroform/methanol (2:1, v/v) and 2 ml of 0.2 M-KCl as aqueous phase [32]. The yield of phosphatidic acid could be increased by about 10% when the lipids were extracted with chloroform/methanol (1:1, v/v) and 2 ml of 1 mM-EGTA in 1 M-HCl [33].

Separation of lipids by t.l.c.

Diacylglycerols were separated on silica-gel G 60 t.l.c. plates

with benzene/chloroform/methanol (16:3:1, by vol.) as a solvent system with 1,2- and 1,3-dioleoylglycerol as standards. Phosphatidic acid and phosphatidylalcohols were separated on t.l.c. plates impregnated with 1% potassium oxalate and developed in the organic phase of ethyl acetate/trimethylpentane/acetic acid/water (13:2:3:10, by vol.), with phosphatidic acid from egg phosphatidylcholine and the prepared phosphatidylalcohols as standards. Radioactivity was measured with a Linear Analyzer (Berthold, Wildbad, Germany).

Measurement of the total amounts of phospholipid metabolites

For determination of the amounts of diacylglycerol, 5×10^5 HeLa or A431 cells were cultivated in 3.5 cm dishes for 48 h and were treated with EGF or PBS as described above. For determination of the amounts of phosphatidic acid, 1.6×10^6 cells were cultivated in 5 cm plastic Petri dishes for 48 h. Lipid extraction and separation of diacylglycerol were performed as described above. Phosphatidic acid was separated in the solvent system described above, and additionally by two-dimensional t.l.c. chloroform/methanol/25% (w/v) NH₃ (65:35:4, by vol.) in the first dimension and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.) in the second dimension. A standard curve was obtained in each experiment by using 1,3-dioleoylglycerol or phosphatidic acid from egg phosphatidylcholine in the linear range (0.4–1.2 μ g) chromatographed on the same plates. The developed plates were dried for 30 min and then stained for 30 min in a solution of 0.03% Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in 30% methanol/100 mM-NaCl in accordance with [34]. The plates were then destained for 5 min in 30% methanol/100 mM-NaCl. The density of each spot co-migrating with the standards was measured at 633 nm [35] with a chromatogram-spectral-photometer (Zeiss) equipped with an integrator or with a Video-densitometer Bio-Profil (Vilber Lourmat/Fröbel, Lindau, Germany). The staining procedure has also been used for recording phosphatidylalcohol production. For analysis of cellular phosphatidylbutanol, lipid extracts from $(6-8) \times 10^6$ cells were required. A standard curve with phosphatidylbutanol was obtained by phosphorus determination [36] or phosphatidylbutanol prepared from egg phosphatidylcholine and dioleoyl-phosphatidylcholine. In the range 8–20 nmol, identical amounts of both phosphatidylbutanol standards resulted in a comparable staining of the spots. Evaluation of amounts of cellular phosphatidylbutanol was performed by using the molecular mass of dioleoyl-phosphatidylbutanol (757.12 g/mol) as the basis.

Analysis of total radiolabelled inositol phosphates

HeLa- or A431-cell cultures (5×10^5 cells plated in 3.5 cm dishes) established for a period of 17 h were prelabelled with *myo*-[2-³H]inositol (74 kBq/ml) for 24 h. Then the medium was replaced by MEM (HeLa cells) or DMEM (A431 cells) containing 0.5% BSA and 10 mM-LiCl in order to prevent inositol phosphate breakdown. After 2 h, EGF or PBS was added for various periods. The reaction was stopped with 1 ml of ice-cold 10% trichloroacetic acid. After centrifugation of the cell precipitates, the supernatants containing the inositol phosphates were extracted with 5×2 ml of water-saturated diethyl ether to remove trichloroacetic acid. The samples were neutralized with 1 M-KHCO₃ to pH 7.5, diluted with 4 ml of water and separated on SAX 100 mg Amprep mini-columns (Amersham Buchler) in combination with a vacuum manifold system from Amersham Buchler. Inositol phosphates were eluted together with 0.25 M-KHCO₃. Radioactivity was measured in a liquid-scintillation counter.

Analysis of protein kinase C

This was performed as described by Gschwendt *et al.* [37] in cell lysates. In short, the cells were lysed in Triton X-100 (0.2%) containing buffer [37], and samples of the 105000 g supernatant containing 20 µg of protein were used for the phosphorylation of histone H1 (27.5 µg at 37 °C for 6 min) in the buffer system described in [37] in either the presence or the absence of Ca²⁺ (5 mM), phosphatidylserine (0.3 mg/ml) and phorbol 12-myristate 13-acetate (PMA; 2.3 µM). After the reaction, the assay mixture was spotted on to Whatman P81 paper squares, extracted and analysed by liquid-scintillation counting. To analyse, by immunostaining, the down-regulation of protein kinase C proteins, A431 cells treated with acetone (0.2%; control) or TPA (0.1 µM) for 18 h were washed once with PBS and subsequently lysed in a buffer containing 20 mM-Tris/HCl, pH 7.5, 5 mM-EGTA, 2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride, 50 mM-β-mercaptoethanol, 0.2% Triton X-100 and 10% glycerol. The lysed cells were scraped from the culture dishes and homogenized by sonication. The homogenate was applied to a protamine-agarose column (0.65 ml bed volume), which was pre-equilibrated with 0.5 M-NaCl in column buffer containing 50 mM-Tris/HCl, pH 8.0, 1 mM-EGTA and 20 mM-β-mercaptoethanol. After washing with 2 bed vol. of 0.5 M-NaCl in column buffer, the proteins were eluted with column buffer containing 1.5 M-NaCl. The proteins were separated by SDS/PAGE on a 7.5%-acrylamide gel and transferred to an Immobilon P membrane. The non-specific binding on the membrane was blocked by overnight incubation at 4 °C in 50 mM-Tris/HCl (pH 7.6)/150 mM NaCl (TBS) containing 0.05% Tween 20 (TBS/T) and 10% fetal-calf serum. The membranes were washed three times with TBS/T and then incubated with affinity-purified antibodies against protein kinase C α, β and γ isoenzymes [38] in TBS/10% fetal-calf serum for 60 min at room temperature. The blots were made visible by immunostaining using alkaline phosphatase conjugated to goat anti-rabbit IgG.

RESULTS

In order to establish that phosphatidylinositol metabolism in HeLa and A431 cells reacts to EGF in a typical way [7–9], both products of phospholipase C, inositol phosphates and diacylglycerol, were measured after application of 10 nM- and 100 nM-EGF respectively. For determination of total inositol phosphates, cells were prelabelled with *myo*-[³H]inositol as described in the Materials and methods section. Both cell lines accumulated inositol phosphates in response to EGF in a dose-dependent manner (Fig. 1) as measured in the presence of LiCl for periods up to 10 min, indicating that activation of phosphatidylinositol-specific phospholipase C had occurred.

The second product of phospholipase C, diacylglycerol, represents the precursor for the generation of phosphatidic acid through phosphorylation by diacylglycerol kinase. It may contribute to the observed phosphatidic acid levels in both cell types treated with EGF (see below). Diacylglycerol was determined in absolute amounts, i.e. independent of any radioactive precursor, thus avoiding problems of pool sizes of labelled phospholipids. Results of typical experiments are shown in Figs. 2(a) and 2(b). The increase in diacylglycerol concentration in HeLa cells (about 0.5 µg/10⁶ cells) treated with EGF was approx. 2-fold (Fig. 2a); the diacylglycerol values returned to the level of control cells after 60 min. A431 cells exhibited a smaller increase (approx. 1.3-fold; Fig. 2b) in diacylglycerol content (about 0.15 µg/10⁶ cells). After reaching a maximum at 10 min, the values returned to the control level by 30 min. The smaller increase in the diacylglycerol level and its relatively rapid decrease in EGF-treated A431 cells may result in part from the activation of diacylglycerol kinase by

EGF [39] and subsequent phosphorylation to yield phosphatidic acid.

Phosphatidic acid levels were measured in A431 cells treated with 100 nM-EGF and in HeLa cells treated with 10 nM-EGF after different periods of time. The data shown in Figs. 2(c) and 2(d) demonstrate that HeLa cells contain approximately half of the amount of phosphatidic acid found in A431 cells. On application of EGF, in both cell types the phosphatidic acid rapidly increased 2–3-fold. The amount of phosphatidic acid mobilized within 5 min over the respective control was approx. 0.2 µg/10⁶ A431 cells and 0.16 µg/10⁶ HeLa cells (Figs. 2c and 2d) (in other experiments at least 0.05 µg/10⁶ cells). Since A431

Fig. 1. Dose-response relationship for the formation of total inositol phosphates in HeLa (a) and A431 (b) cells in response to EGF

HeLa and A431 cells prelabelled with *myo*-[2-³H]inositol were treated for 10 min with various concentrations of EGF. Inositol phosphates were isolated and measured as described in the Materials and methods section. Each value represents the mean ± S.D. of 3 cultures.

Fig. 2. Time-dependent quantitative formation of diacylglycerol (a, b) and phosphatidic acid (c, d) in EGF-treated HeLa cells (a, c) and A431 cells (b, d)

HeLa or A431 cells cultivated for 48 h received a change of medium with MEM or DMEM respectively, plus 0.5% BSA. HeLa cells were then treated with PBS (0.2%; □) or 10 nM-EGF (■) and A431 cells with PBS (○) or 100 nM-EGF (●) for the periods indicated. Diacylglycerol and phosphatidic acid were analysed by Coomassie Blue staining as described in the Materials and methods section. Each value represents the mean of two cultures.

Fig. 3. Dose-response relationship for the quantitative formation of phosphatidic acid in HeLa (a) and A431 (b) cells in response to EGF

HeLa and A431 cells cultivated for 48 h received a change of medium with MEM or DMEM respectively, plus 0.5% BSA. Cells were then treated for 5 min with various concentrations of EGF or with PBS for control. Phosphatidic acid was analysed by Coomassie Blue staining as described in the Materials and methods section. Each value represents the mean $\pm$ s.d. of 3 cultures.

cells contain approx. 120 μ g of total phospholipid/10⁶ cells and HeLa cells approx. 90 μ g/10⁶ cells, in both cell lines phosphatidic acid elicited by EGF amounts to approx. 0.17% of total phospholipid. Assuming an average molecular mass for phosphatidic acid of approx. 700 Da and a volume per 10⁶ cells of the order of 5 μ l, an increase in the overall cellular concentration by approx. 50 μ M can be calculated, i.e. a fairly large increase in the phosphatidic acid concentration, which is even larger if the membrane compartment is considered. The dose-response relationships for the formation of phosphatidic acid by both cell lines in response to EGF are shown in Figs. 3(a) and 3(b).

In addition to phosphorylation of diacylglycerol and to synthesis *de novo*, phosphatidic acid may be generated by hydrolysis of glycerophospholipids through phospholipase D. In order to determine the contribution of phospholipase D to the observed phosphatidic acid levels, the transphosphatidyl transfer to primary alcohol, which is a more specific marker for this enzyme [22,23], was measured in intact cells. The alcohols used were ethanol and butan-1-ol at final concentrations of 0.5% and 0.2–0.3% respectively. At these concentrations the growth of both cell lines was not influenced as measured over a period of 3 days, indicating that metabolic responses were measured under

quasi-physiological conditions. HeLa cells were prelabelled with [1-¹⁴C]oleic acid for 24 h in order to measure the production of radioactive phosphatidylethanol and of phosphatidylbutanol (under these conditions 60% of phospholipid-bound radioactivity was found in phosphatidylcholine, 26% in phosphatidylethanolamine and 7% each in phosphatidylserine and phosphatidylinositol). The response to EGF (10 nM) exhibited in both cases an approx. 3-fold increase in the phosphatidylalcohol level at 5 min, followed by a decrease. The levels continued to stay elevated above the control for at least 60 min (Figs. 4a and 4b). The decrease after 5 min may be due to cessation of the production of labelled product and/or increased degradation. The decrease in production of radioactive phosphatidylbutanol was not observed after prelabelling with 1-[1-¹⁴C]palmitoyl-lyso-3-phosphatidylcholine (Fig. 4b), i.e. in the *sn*1 position of the glycerol moiety. Another control experiment carried out with the phorbol ester PMA (0.1 μ M), which is known to activate phospholipase D rapidly in HeLa cells [40], showed a continued increase in production of oleate-labelled phosphatidylbutanol over 60 min (Fig. 4c), indicating that the pool of oleate-labelled substrate for phospholipase D was sufficiently large and that phospholipase D of HeLa cells was capable of working for extended periods of time in the presence of butanol.

Each of the alcohols caused a decrease in the EGF-induced formation of radioactively labelled phosphatidic acid. For butan-1-ol the decrease in phosphatidic acid formation was depressed almost to the level seen in the control group (Fig. 5a), a result which indicated that most of the oleate-labelled phosphatidic acid was formed through the phospholipase D pathway. The decrease in EGF-induced phosphatidic acid formation in the presence of ethanol was less pronounced (Fig. 5b), even though the concentration of ethanol was larger than that of butan-1-ol. A similar observation regarding the efficiency of the phosphatidyl transfer of butan-1-ol in intact cells has been reported by Tettenborn & Mueller [21]. For this reason all further transphosphatidyl transfer experiments were done in the presence of butan-1-ol.

The formation of phosphatidylalcohol in response to EGF was also observed in A431 cells, a cell line carrying a large number of EGF receptors per cell. After prelabelling with [1-¹⁴C]oleic acid, A431 cells formed labelled phosphatidylbutanol on treatment with 100 nM-EGF as shown in Fig. 6(a). The time course exhibits an approx. 3-fold stimulation by 5 min and levels off thereafter. In A431 cells the decrease in oleate-labelled phosphatidic acid formation in the presence of butan-1-ol (Fig.

Fig. 4. Time course of the formation of phosphatidylethanol (a) and phosphatidylbutanol (b, c) in HeLa cells in response to EGF (a, b) or PMA (c)

HeLa cells were cultivated for 24 h and then prelabelled with [1-¹⁴C]oleic acid (○, ●) or 1-[1-¹⁴C]palmitoyl-lyso-3-phosphatidylcholine (△, ▼) for additional 24 h. After change of medium to MEM plus 0.5% BSA, the cells were treated with 10 nM-EGF (●, ▼) or PBS (0.2%; ○, △) in the presence of 0.5% ethanol (a) or 0.3% butan-1-ol (b) for the periods indicated. In (c) the cells were treated with 0.1 μ M-PMA (●) or acetone (0.2%; ○) in the presence of 0.3% butan-1-ol. Analysis of labelled phosphatidylalcohols was performed as described in the Materials and methods section. Each value represents the mean $\pm$ s.d. of 3 cultures.

Fig. 5. Effect of butan-1-ol (a) or ethanol (b) on the EGF-induced formation of phosphatidic acid in HeLa cells

After prelabelling with [1-¹⁴C]oleic acid, the cells were preincubated for 20 min with 0.3 % butan-1-ol (a) or 0.5 % ethanol (b) to reach equilibrium and then treated with 10 nM-EGF or PBS (0.2 %) for the periods indicated. The analysis of phosphatidic acid was performed after one-dimensional separation on t.l.c. plates as described in the Materials and methods section. (a) □, Butan-1-ol/PBS; ●, EGF (10 nM); ■, butan-1-ol/EGF (10 nM). (b) □, Ethanol/PBS; ●, EGF (10 nM); △, ethanol/EGF (10 nM). Each value represents the mean ± s.d. of 3 cultures.

Fig. 6. Time course of the EGF-induced formation of phosphatidylbutanol (a) and phosphatidic acid (b) in A431 cells

The cells were prelabelled for 24 h with [1-¹⁴C]oleic acid. After change of medium to DMEM plus 0.5 % BSA, the cells were pretreated for 20 min with 0.2 % butan-1-ol (□, ■) or water (●) and then incubated with 100 nM-EGF (■, ●) or PBS (0.2 %; □) for the periods indicated. Each value represents the mean ± s.d. of 3 cultures.

Table 1. Phosphatidylalcohol production in EGF-treated HeLa and A431 cells in the presence of different alcohol concentrations

HeLa or A431 cells prelabelled with [1-¹⁴C]oleic acid for 24 h were pretreated for 10 min with various concentrations of butan-1-ol or ethanol and then incubated for 5 min with EGF (10 nM in HeLa cells, 100 nM in A431 cells). Each value represents the mean ± s.d. of 3 dishes; n.d., not detectable.

Cell line (treatment)	Alcohol concn. (%)	Phosphatidic acid (c.p.m./dish)	Phosphatidylbutanol or phosphatidylethanol (c.p.m./dish)
HeLa (10 nM-EGF)	None	125 ± 13	n.d.
	Butan-1-ol	0.05	40 ± 7
		0.1	51 ± 7
		0.3	52 ± 1
	Ethanol	0.25	55 ± 15
		0.5	52 ± 13
A431 (100 nM-EGF)	None	103 ± 5	n.d.
	Butan-1-ol	0.1	76 ± 10
		0.2	60 ± 9
		0.3	66 ± 8
	Ethanol	0.25	77 ± 13
		0.5	77 ± 12

Table 2. EGF-induced increases in phosphatidic acid and phosphatidylbutanol

Analysis (in the absence or presence of 0.2 % butan-1-ol) by Coomassie Blue staining and reflection densitometry in HeLa and A431 cells after a 5 min treatment with EGF. Results are means ± s.d. of 3 determinations.

Cell line	Treatment	Increase (µg/10 ⁶ cells) in :	
		phosphatidic acid	phosphatidylbutanol
HeLa	EGF (10 nM)	0.07 ± 0.002	—
	Butanol/EGF (10 nM)	0.02 ± 0.005	0.056 ± 0.008
A431	EGF (100 nM)	0.13 ± 0.01	—
	Butanol/EGF (100 nM)	0.06 ± 0.02	0.079 ± 0.008

of phosphatidic acid. These data reflect those obtained in the precursor experiments. It can be assumed that in both cell lines only a part of the EGF-induced increase in phosphatidic acid was contributed by phospholipase D.

On application of different concentrations of EGF to both human cells lines prelabelled with [1-¹⁴C]oleic acid, a dose-response of the formation of labelled phosphatidylbutanol was observed in HeLa cells and in A431 cells, as shown in Figs. 7(a) and 7(b). Half-maximal phosphatidylbutanol formation by HeLa cells was observed in response to EGF between 1 and 10 nM, and in A431 cells at approx. 10 nM-EGF. The dose-response for the formation of oleate-labelled phosphatidic acid was measured in the absence as well as in the presence of butan-1-ol in both cell lines. From these experiments it was evident that the formation of radioactive phosphatidic acid in the presence of butan-1-ol was again less decreased in A431 cells than in HeLa cells (results not shown).

6b) did not reach the extent observed in Hela cells (Fig. 5a). These data indicated that at least part of the phosphatidic acid formation was catalysed through the phospholipase D mechanism. The degree of formation of labelled phosphatidylbutanol was smaller than in HeLa cells. This view is reinforced by analysis of the production of phosphatidic acid and of phosphatidylalcohol by both cell types treated with EGF (10 nM in HeLa cells and 100 nM in A431 cells) for 5 min in the presence of different concentrations of butan-1-ol or ethanol. The results given in Table 1 demonstrate that the phosphatidic acid generation was decreased to a comparable extent by different alcohol concentrations, i.e. in this range almost independently of it. The decrease in phosphatidic acid in the presence of alcohol was again larger in HeLa cells than in A431 cells.

The production of phosphatidylbutanol was also determined by Coomassie Blue staining and densitometry with dioleoylphosphatidylbutanol as the standard. In the experiment shown in Table 2, HeLa cells and A431 cells were treated with EGF in the absence and presence of butan-1-ol. By 5 min the formation of phosphatidylbutanol occurred in both cell lines at the expense

Fig. 7. Dose-response relationship for the formation of phosphatidylbutanol in HeLa (a) and A431 (b) cells in response to EGF

HeLa and A431 cells prelabelled with [$1\text{-}^{14}\text{C}$]oleic acid were pretreated for 20 min with 0.2% butan-1-ol and then incubated for 5 min with various concentrations of EGF. Each value represents the mean $\pm$ s.d. of 3 cultures.

Fig. 8. Effect of PMA treatment on protein kinase C protein levels in A431 cells

Homogenates of A431 cells treated with PMA (0.1 μM ; lane 1) or acetone (0.2%; control, lane 2) for 18 h containing 120 μg of total cellular protein were resolved by immunoblotting as described in the Materials and methods section. Protein kinase C (PKC) was detected by using affinity-purified antibodies against protein kinase C α , β and γ peptides.

Table 3. EGF-induced production of phosphatidylbutanol in A431 cells without and with PMA-induced downregulation of protein kinase C

Analysis (by Coomassie Blue staining and densitometry) of phosphatidylbutanol in A431 cells pretreated for 17 h with 0.2% acetone or 0.1 μM -PMA and then incubated for 10 min with EGF (100 nM) or PBS (0.2%) in the presence of butan-1-ol; two values per group.

Pretreatment	EGF-induced increase in phosphatidylbutanol ($\mu\text{g}/10^6$ cells)
Acetone	0.060, 0.067
PMA	0.062, 0.062

Diacylglycerol formed by cells in response to EGF is thought to exert a feedback control via protein kinase C. To evaluate the role of protein kinase C with respect to EGF-induced activation of phospholipase D, cells were depleted of most of protein kinase C by overnight pretreatment with PMA (10^{-7} M), as shown in the Western blot by the use of antiserum specific for protein kinase C types α , β and γ (Fig. 8); the protein kinase C-activity was decreased from 0.29 pmol of phosphate transferred/min per μg cellular protein in acetone-treated cells to 0.01 pmol/min per μg in PMA-treated cells. For this purpose only A431 cells

were used, since these cells, in contrast with HeLa cells [32], exhibit in our hands under these conditions only small changes in their phospholipid metabolism after treatment with PMA. Control cells were treated for the same period of time with the solvent (0.2% acetone). The cells subsequently received new medium, were equilibrated for 20 min with 0.2% butan-1-ol and then treated with EGF (100 nM) or with PBS for control. For determination of phosphatidylbutanol, extracts were separated by t.l.c. stained with Coomassie Blue and evaluated by densitometry. In A431 cells pretreated with acetone as well as in those pretreated with PMA, EGF caused an increase in phosphatidylbutanol (Table 3). The data imply that depletion of protein kinase C types subject to down-regulation by PMA did not impair EGF-induced activation of phospholipase D.

DISCUSSION

HeLa and A431 cells were shown to respond to EGF with the well-known stimulation of inositol-phospholipid turnover through activation of phospholipase C, as evidenced by increases in inositol phosphates and diacylglycerol. In these cells EGF also caused an increase in phosphatidic acid. The relatively low level of diacylglycerol found in A431 cells after treatment with EGF in the present study, as well as by others [8], and the known possibility of an EGF-induced activation of diacylglycerol kinase [39] indicated that part of the observed phosphatidic acid might have been formed through phosphorylation of diacylglycerol. Part of the increase in phosphatidic acid, however, could be due to an additional activation of a phospholipase D. In the presence of primary alcohols, EGF induced in HeLa and A431 cells the production of phosphatidylalcohol, a specific marker for the activation of phospholipase D. Since in the presence of a primary alcohol the phospholipase D-catalysed phosphatidyl transfer is predominantly aimed towards the alcohol, major amounts of phosphatidic acid appearing in parallel are most probably generated via different pathways. In the presence of alcohol, in both cell lines the formation of phosphatidylalcohol is increased at the expense of phosphatidic acid formation, but not to the extent expected if a phospholipase D were solely responsible. The interference by alcohol with phosphatidic acid formation was more pronounced in HeLa cells than in A431 cells, indicating that in HeLa cells the phospholipase D pathway contributed to the phosphatidate level to a larger extent. The observation that the phorbol ester PMA activated phosphatidylalcohol formation in HeLa cells more than 5-fold within a short period and led to an increase in phosphatidylalcohol formation of the same order indicated that only part of the cellular phospholipase D became activated through the action of EGF.

The way in which EGF induces the activation of phospholipase D is not known. The phospholipase D activity observed in EGF-treated A431 cells after down-regulation of most of the protein kinase C activity excludes a direct involvement of those types of protein kinase C which are subject to PMA-induced disappearance. The results reported here support the notion that protein kinase C-dependent and -independent pathways exist to activate phospholipase D, the latter being particularly involved in receptor-mediated processes [28,33]. A feedback modulation after PMA pretreatment, as in the case of phosphatidylinositol hydrolysis [41], seems to be unlikely for phospholipase D. The possibility of a direct activation of phospholipase D, e.g. via phosphorylation through EGF receptor kinase, can only be tested after elucidation of the molecular structure and the regulation of phospholipase D, which is at present not fully understood. As a second possibility, EGF may induce activation of phospholipase D through an increase in intracellular Ca^{2+} concentration via inositol trisphosphate generated through ac-

tivation of phosphatidylinositol-specific phospholipase C [7–9]. A Ca^{2+} -induced activation of phospholipase D has been proposed for the action of the Ca^{2+} ionophore A23187 [42].

With respect to the possible role of phospholipase D activation and the role of phosphatidic acid in signal transduction, the amounts formed may point to the possible function of phosphatidic acid. It has previously been suggested that phosphatidic acid, in addition to diacylglycerol and inositol trisphosphate, may play a crucial role in signal transduction and cellular proliferation [43–46]. The amount of phosphatidic acid formed in response to treatment of both cell lines with EGF is in the order of approx. 0.1 mM. In view of this high concentration and the 2–3-fold increase, phosphatidic acid, if it serves a second-messenger function at all, does not do so exclusively, but probably acts by physical means (for review see [43]), e.g. by altering membrane properties and thereby by modulating the biological activity of membrane proteins, including receptors and enzymes specific for a given cellular phenotype. Cellular effects observed by application of exogenous phosphatidic acid (e.g. [44]) may not necessarily mimic increases in intracellular phosphatidic acid.

The increase in phosphatidic acid through activation of phospholipase D is not restricted to the action of EGF, but appears to represent a general part of the concerted cellular response to several ligands. Increases in cellular phosphatidic acid have been observed as part of the cellular response to agonists such as angiotensin II, bradykinin, *N*-formylmethionyl-leucylphenylalanine (for review see [47]), i.e. agonists which control cellular reactions other than proliferation. Therefore it is possible that the increase in cellular phosphatidic acid content represents in a way a 'reset signal', which accompanies more specific signal pathways triggered by a given agonist and causes an alteration of the membrane make-up as a prerequisite enabling a cell to change from one biological state to another; for EGF-induced growth stimulation from the resting to the proliferative state, or, as for the EGF effects in HeLa cells and A431 cells, from the proliferative state to a more or less inhibited state [48].

After completion of the experimental work, we became aware of meeting abstracts which report on EGF-induced production of phosphatidylalcohol in other cellular systems [49–51], thus supporting the idea that activation of phospholipase D in cells treated with EGF may represent a general mechanism.

We thank Dr. J. Reed for help with the English and Mrs. A. Lampe-Gegenheimer for expert secretarial assistance. The work was supported by the Deutsche Forschungsgemeinschaft.

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