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Activation of phospholipase D by α-thrombin or epidermal growth factor contributes to the formation of phosphatidic acid, but not to observed increases in 1,2-diacylglycerol

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The receptor-mediated activation of a phosphatidylcholine-hydrolysing phospholipase D (PLD) has recently been described. We investigated the effect of α-thrombin and epidermal growth factor (EGF) on cellular PLD activity in order to determine the role of this enzyme in mitogen-induced increases in phosphatidic acid and *sn*-1,2-diacylglycerol. In the presence of ethanol, stimulation of [^{3}H]myristic acid-labelled quiescent IIC9 cells with α-thrombin or EGF resulted in a rapid increase in radiolabelled phosphatidylethanol which reached a plateau at 1 min, indicating the rapid and transient activation of PLD. We observed a concomitant decrease in the mitogen-stimulated increase of radiolabelled phosphatidic acid. In contrast, ethanol did not significantly effect the elevation of *sn*-1,2-diacylglycerol levels stimulated by α-thrombin or EGF as determined by measurement of *sn*-1,2-diacylglycerol mass or the appearance of [^{3}H]1,2-diacylglycerol. A novel lipid, detected by two-dimensional t.l.c. analysis, was generated in [^{3}H]myristic acid-labelled cells stimulated with α-thrombin, but not EGF, in the presence of ethanol. Treatment *in vitro* of cellular lipids isolated from [^{3}H]myristic acid-labelled cultures with PLD in the presence of ethanol also resulted in the generation of this novel lipid species, supporting the role of this enzyme in its production. These data indicate that in quiescent IIC9 cells: (*a*) α-thrombin or EGF rapidly and transiently activates a PLD; (*b*) although this activation is responsible for part of the mitogen-induced increases in phosphatidic acid, it does not contribute to induced increases in *sn*-1,2-diacylglycerol; and (c) activation of this enzyme appears to be involved in the formation of a novel lipid generated in response to α-thrombin, but not EGF, in IIC9 fibroblasts.

INTRODUCTION

It is becoming increasingly evident that the stimulation of cellular lipid metabolism plays a central role in a large number of receptor-mediated responses. Increases in *sn*-1,2-diacylglycerol (1,2-DG), inositol phosphates and phosphatidic acid (PA) have been implicated as important mediators of agonist-induced responses, including mitogenesis [1,2]. The addition of growth factors to quiescent cultures has been shown to stimulate increases in one or more of these metabolites [3,4]. In addition, exogenous cell-permeant analogues of 1,2-DG have been reported to mimic effects of growth factors, such as the stimulation of protein phosphorylation and DNA synthesis [5,6].

In view of the potential importance of 1,2-DG as a second messenger in transducing mitogenic signals, it is important to understand the mechanisms by which this lipid is generated. It is clear that 1,2-DG may be derived from the stimulated hydrolysis of phosphoinositides [1] or phosphatidylcholine (PC) (7). Although agonist-induced hydrolysis of the phosphoinositides occurs via the activation of a phosphatidylinositol (PI)-specific phospholipase C (PI-PLC), the stimulated hydrolysis of PC may occur via the activation of a PC-specific PLC (PC-PLC), or from the action of a phospholipase D (PLD) leading to the formation of PA and choline. The resulting PA may be hydrolysed by a PA phosphohydrolase to yield 1,2-DG (reviewed in [2] and [7]).

Previous studies from our laboratory have focused on the kinetics, sources and molecular species of mitogen-induced 1,2-DG in the Chinese-hamster embryo fibroblast cell line IIC9 [8–12]. The 1,2-DG formed in response to mitogens such as α-thrombin, epidermal growth factor (EGF) or platelet-derived growth factor is derived largely from the breakdown of PC, except for an early (15 s) peak derived primarily from PI in cells treated with high concentrations (500 ng/ml) of α-thrombin [8,10,11]. In previous studies we found that PC was preferentially labelled during short-term (2 h) incubations with [^{3}H]myristic acid [10]. Studies in the present paper take advantage of this preferential labelling and also make use of the known property of PLD to utilize an alcohol (e.g. ethanol) for the phosphate acceptor in lieu of water (transphosphatidylation) [13]. The resulting phosphatidylethanol (PEt) is a relatively stable metabolite and is readily separated from other phospholipids by t.l.c.

The data in this report indicate that α-thrombin and EGF stimulate a rapid and transient increase in a PLD activity. Activation of this enzyme is responsible for a portion of the growth-factor-induced increase in PA. In contrast, our data indicate that this enzyme is not involved in mitogen-induced increase in 1,2-DG in IIC9 fibroblasts. However, PLD is apparently involved in the formation of a novel lipid, Lipid Th, induced by α-thrombin, but not EGF, in quiescent IIC9 cells. The potential physiological role of the mitogen-induced activation of PLD is discussed.

MATERIALS AND METHODS

Materials

Media components were obtained from GIBCO. Plastic culture dishes were purchased from Falcon Labware (Oxnard, CA, U.S.A.). BSA (radioimmunoassay grade, fraction V), PLD

Abbreviations used: 1,2-DG, *sn*-1,2-diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; EGF, epidermal growth factor; PEt, phosphatidylethanol.

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(cabbage, Type V) and highly purified human α-thrombin (approx. 4000 NIH units/ml) were obtained from Sigma. Hydrofluor scintillation-counting mixture was obtained from National Diagnostics (Somerville, NJ, U.S.A.). Human transferrin was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Octyl β-D-glucoside was obtained from Calbiochem (San Diego, CA, U.S.A.). [^{3}H]Myristic acid (39.3 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Du Pont–New England Nuclear. Bovine cardiolipin and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). *Escherichia coli* diacylglycerol kinase was purchased from Lipidex (Piscataway, NJ, U.S.A.). Aluminium-backed silica gel 60 t.l.c. plates with a pre-concentration zone were purchased from EM Diagnostics (Gibbstown, NJ, U.S.A.), Anasil O plates were purchased from Analabs (Norwalk, CT, U.S.A.), and silica gel HL plates were obtained from Analtech (Newark, DE, U.S.A.). All other reagents were of reagent grade or higher.

Cells and cell culture

IIC9 cells, a subclone of Chinese-hamster embryo fibroblasts [14,15], were grown, maintained, and serum-starved as previously described [8]. Briefly, cultures were grown and maintained in α-Minimal Essential Medium/Ham's F-12 (1:1, v/v) containing 5% (v/v) fetal-calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 2 mM-L-glutamine. Sub-confluent cultures were serum-starved by washing three times with Dulbecco's modified Eagle's medium containing 1 mg of radioimmunoassay-grade BSA/ml, 100 units of penicillin/ml, 100 μg of stremptomycin/ml, 2 mM-L-glutamine and 20 mM-NaHepes, pH 7.4. The cultures were then fed with this medium supplemented with 5 μg of human transferrin/ml (serum-free medium) and incubated for 2 days at 37 °C.

Assay for activation of PLD

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h at 37 °C. The cells were then pretreated with fresh serum-free medium in the presence or absence of ethanol (100 mM) for 10 min, followed by incubation in the same medium in the presence or absence of α-thrombin (500 ng/ml) or EGF (50 ng/ml) at 37 °C as indicated in the Figure legends. Incubations were terminated and lipids were extracted as previously described, except that water was used instead of 1 M-NaCl to cause phase separation [8]. Samples of the labelled lipid products were separated by t.l.c. on silica-gel HL plates in the solvent system chloroform/methanol/acetic acid (13:3:1, by vol.). After drying, the t.l.c. plates were sprayed with EN^{3}HANCE (NEN) followed by autoradiography at −70 °C. The labelled PEt was identified by comparison with a non-labelled standard made visible by iodine staining. The area corresponding to PEt was scraped into a vial and quantified by liquid-scintillation counting.

Assay for PA formation

Serum-starved cultures were labelled with [^{3}H]myristic acid, pretreated with medium containing ethanol (100 mM) or control medium for 10 min, and incubated with fresh medium with and without ethanol in the presence or absence of α-thrombin or EGF as described above. Incubations were terminated and total cellular lipids were extracted as described above. After extraction, cellular lipids were analysed by two-dimensional t.l.c. on Anasil O plates. The first solvent system (solvent system 1) was chloroform/methanol/30% NH_3/water (60:40:5:2, by vol.) and the second (solvent system 2) was chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.). The region of the plate containing PA, identified by comparison with a known standard, was quantified by liquid-scintillation counting as previously described [10].

Assay for radiolabelled 1,2-DG

Cultures were labelled with [^{3}H]myristic acid, pretreated with serum-free medium containing ethanol (100 mM) or control medium for 10 min, and incubated with fresh medium with and without ethanol in the presence or absence of α-thrombin or EGF as described above. Incubations were terminated and lipids were extracted as described above for the determination of PEt formation. The radiolabelled lipids were separated by t.l.c. on silica-gel HL plates in the solvent system isopropyl ether/acetic acid (24:1, v/v). The region of the plate containing [^{3}H]1,2-DG, identified by comparison with an unlabelled standard detected by iodine staining, was quantified by liquid-scintillation counting as previously described [10].

Mass analysis of 1,2-DG

The mass amounts of cellular 1,2-DG were determined by using *E. coli* diacylglycerol kinase as described by Priess *et al.* [16] as modified in our laboratory [8]. The data are expressed as nmol of 1,2-DG/100 nmol of lipid P (mol %).

Statistical analysis

This was performed by Student's unpaired *t* test by StatWorks.

RESULTS

Activation of PLD

Serum-starved quiescent cultures of IIC9 fibroblasts were labelled with [^{3}H]myristic acid for 2 h at 37 °C, during which time ⩾ 90% of the radiolabel incorporated into phospholipids is esterified to PC, and there is no detectable labelling of PI-glycan [10]. PLD activity was determined by analysing for the presence of PEt as described in the Materials and methods section. Incubation of these cultures with mitogenic concentrations of either α-thrombin (500 ng/ml) or EGF (50 ng/ml) in the presence of ethanol resulted in a rapid increase in PEt. Cellular levels of PEt reached a peak approx. 30–60 s after the addition of either mitogen and then remained constant for at least 10 min (Figs. 1 and 2).

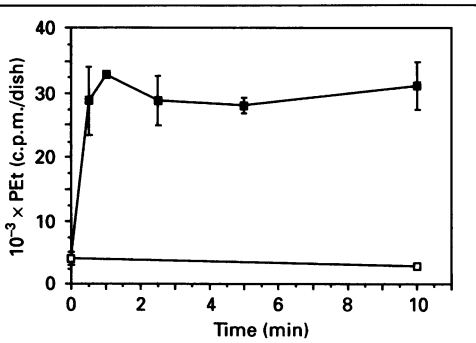


Fig. 1. Time course of α-thrombin-stimulated PEt production

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h. The cells were then pretreated with ethanol (100 mM) for 10 min, followed by incubation at 37 °C in serum-free medium (□) or serum-free medium supplemented with 500 ng of α-thrombin/ml (■). In these experiments and those described in subsequent Figures, ethanol was also added to the medium containing the agonist (or control medium) at the indicated concentrations (for cultures receiving ethanol pretreatment). At the indicated times, incubations were terminated and lipids were extracted and analysed as described in the Materials and methods section. Each point represents the average of duplicate samples. Vertical bars indicate the range of values. Similar results were obtained in two additional experiments.

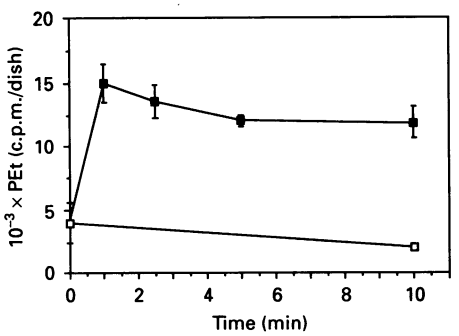


Fig. 2. Time course of EGF-stimulated PEt production

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h. The cells were then pretreated with ethanol (100 mM) for 10 min, followed by incubation at 37 °C in serum-free medium (□) or serum-free medium supplemented with 50 ng of EGF/ml (■). At the indicated times, incubations were terminated and lipids were extracted and analysed as described in the Materials and methods section. Each point represents the average of duplicate samples. Vertical bars represent the range of values. Similar results were obtained in two additional experiments.

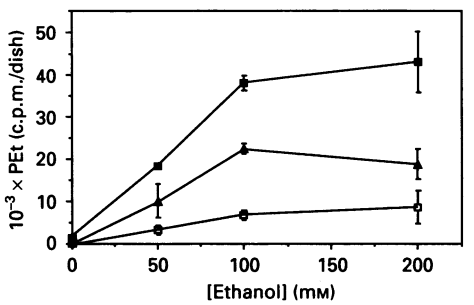


Fig. 3. Concentration-dependence of PEt production

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h. The cells were then pretreated with medium containing various concentrations of ethanol (0–200 mM) for 10 min, followed by incubation in serum-free medium alone (□) or serum-free medium supplemented with 500 ng of α-thrombin/ml (■) or with 50 ng of EGF/ml (▲) at 37 °C. After 5 min, incubations were terminated and lipids were extracted and analysed as described in the legend to Fig. 1. Each point represents the average of duplicate samples. Vertical bars represent the range of values. Similar results were obtained in two additional experiments.

The product of PLD-mediated transphosphatidylation with ethanol (PEt) is a relatively stable metabolite [17]. Our finding that PEt levels stabilized after 1 min indicates that the mitogen-induced activation of PLD is rapid and transient. The transient nature of the observed PEt generation was not due to limiting concentrations of ethanol. As shown in Fig. 3, PEt formation was maximal at 100 mM-ethanol. Incubations in the presence of a higher concentration of ethanol (200 mM) did not result in increased PEt production in response to the mitogens (Fig. 3), nor was there a further increase after longer incubations (results not shown). Furthermore, the amount of PEt formed in response to α-thrombin was approximately twice that induced by EGF, again indicating that ethanol was not a limiting factor.

Effect of ethanol on mitogen-induced 1,2-DG levels

The above data demonstrate that EGF and α-thrombin stimulate a PLD in quiescent IIC9 cells. We have previously demonstrated that these mitogens induce increases in cellular 1,2-DG which are derived from the stimulated hydrolysis of PC in these cells [8–12]. Agonist-induced activation of PLD has been

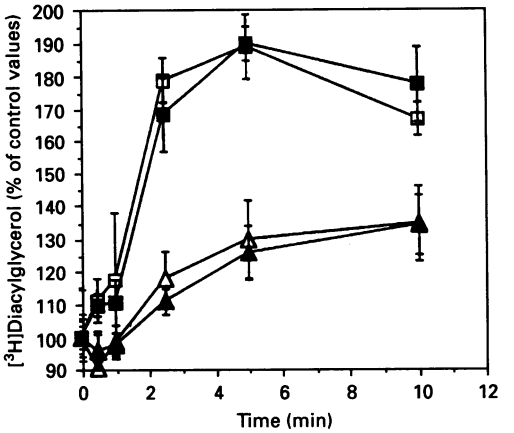


Fig. 4. Effect of ethanol on the formation of [^{3}H]1,2-DG in mitogen-stimulated IIC9 cells

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h. The cells were then preincubated in serum-free medium (■, ▲) or serum-free medium containing 100 mM-ethanol (□, △) for 10 min. After preincubation, the medium was rapidly aspirated and replaced with serum-free medium alone, serum-free medium supplemented with 50 ng of EGF/ml (△, ▲) or with 500 ng of α-thrombin/ml (□, ■), followed by incubation at 37 °C. At the indicated times, incubations were terminated and lipids were extracted and analysed as described in the Materials and methods section. The value for [^{3}H]1,2-DG in control cultures receiving serum-free medium was 51331 ± 9271 c.p.m./dish and for ethanol-treated control cultures was 55617 ± 7035 c.p.m./dish (both means ± S.D., $n = 22$). The data are expressed as percentages of control cultures (with the same pretreatment) without agonist. The data are means ± S.D. of three experiments each performed in duplicate. There were no statistically significant differences between values from cultures treated with or without ethanol, as determined by Student's unpaired t test.

implicated in the generation of PC-derived 1,2-DG in a number of systems [2,18–21]. Therefore it was important to determine whether PLD was involved in the generation of PC-derived 1,2-DG in IIC9 cells in response to α-thrombin or EGF.

As described above, short-term (2 h) labelling of quiescent IIC9 cells with [^{3}H]myristic acid results in the preferential labelling of PC. Addition of α-thrombin (500 ng/ml) or EGF (50 ng/ml) to these radiolabelled quiescent cultures of IIC9 cells resulted in an increase in [^{3}H]1,2-DG. However, as shown in Fig. 4, there was no significant decrease in the levels of [^{3}H]1,2-DG stimulated by α-thrombin or EGF in cultures treated with ethanol. Similarly, ethanol did not affect the generation of mitogen-induced 1,2-DG mass levels (Fig. 5). These data indicate that activation of a PLD is not involved in the α-thrombin or EGF stimulation of PC-derived 1,2-DG in IIC9 cells.

Effect of ethanol on PA levels

In the absence of short-chain alcohols, the activation of PLD is coupled to the generation of PA. In view of the above data, it was important to determine the effect of ethanol on the mitogen-stimulated generation of PA. EGF (50 ng/ml) or α-thrombin (500 ng/ml) was added to quiescent IIC9 cells which had been labelled with [^{3}H]myristic acid for 2 h as previously described [10]. This resulted in elevated levels of radiolabelled PA, approx. 4-fold over control unstimulated values, determined by two-dimensional t.l.c. (see the Materials and methods section). In the presence of ethanol (100 mM), these levels were decreased by approx. 50% at 1 and 5 min after the addition of either mitogen (Table 1). These data indicate that at least 50% of the mitogen-stimulated generation of PA is due to the transient activation of a PLD.

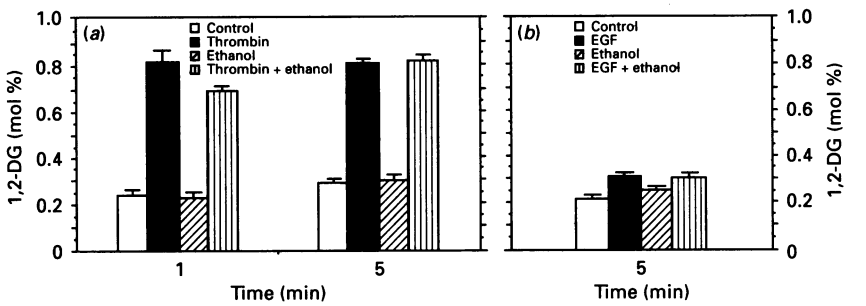


Fig. 5. Effect of ethanol on 1,2-DG mass levels in cells treated with α-thrombin or EGF

Serum-starved cultures were pretreated for 10 min with serum-free medium alone or serum-free medium containing 100 mM-ethanol. The medium was then removed and fresh medium containing 500 ng of α-thrombin/ml (*a*) or 50 ng of EGF/ml (*b*) or without addition (control) was added. The dishes were incubated for 1 or 5 min in the α-thrombin experiments or for 5 min in the EGF experiments, followed by termination and lipid extraction. Cellular mass levels of 1,2-DG were determined by using *E. coli* diacylglycerol kinase (see the Materials and methods section). Data are expressed as nmol of 1,2-DG/100 nmol of lipid P (mol%). Each point represents the average of duplicate samples. Vertical bars represent the range of values. Similar results were obtained in two additional experiments.

Formation of a PLD-dependent novel lipid

Serum-starved quiescent cultures of IIC9 cells which had been labelled with [^{3}H]myristic acid (2 h, 37 °C) and pretreated with or without ethanol (100 mM) were incubated in the presence or absence of α-thrombin for 1–5 min at 37 °C. Lipids were then extracted and analysed by two-dimensional t.l.c. as described above for the measurement of [^{3}H]PA. A novel radiolabelled lipid (Lipid Th), which migrated with $R_F = 0.95$ in solvent system 1 and $R_F = 0.0$ in solvent system 2, was detected (Fig. 6). Lipid Th only appeared when the cultures were incubated with α-thrombin and ethanol (Figs. 6 and 7). It was not present when the cultures were incubated with α-thrombin or ethanol alone. Interestingly, this lipid was not generated in response to EGF (Fig. 7). These data suggested that Lipid Th was formed in response to activation of a PLD in α-thrombin-treated cultures and was the product of a transphosphatidylation reaction. In support of this hypothesis, incubation of cellular lipids isolated from [^{3}H]myristic acid-labelled cultures with partially purified

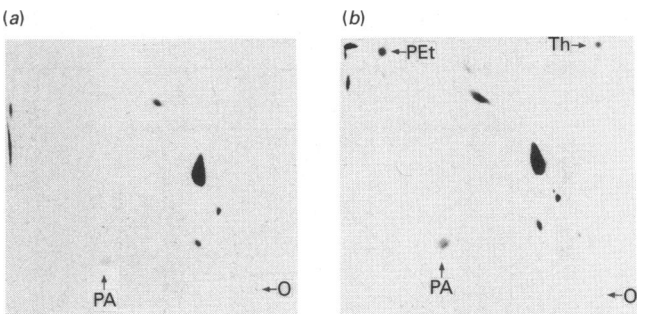


Fig. 6. Autoradiography of phospholipids from [^{3}H]myristic acid-labelled cells

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h and pretreated with 100 mM-ethanol for 10 min followed by incubation in serum-free medium (*a*) or in serum-free medium supplemented with 500 ng of α-thrombin/ml (*b*). Cellular lipids were extracted and resolved by two-dimensional t.l.c. as described in the Materials and methods section. Indicated are the locations of the origin (O), PA, PEt and a novel lipid species (Th).

Table 1. Effect of ethanol on PA formation

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h. The cells were then pre-treated with serum-free medium alone or with serum-free medium containing 100 mM-ethanol for 10 min. After preincubation, the medium was replaced with serum-free medium without addition, or medium containing α-thrombin (500 ng/ml) or EGF (50 ng/ml). At the indicated time, the medium was aspirated and the cellular lipids were extracted and analysed as described in the Materials and methods section. The results are presented as the percentage increase in [^{3}H]PA in stimulated cultures above levels in control cultures with the same pretreatment, and represent means ± S.D. from two experiments each performed in duplicate. Shown also is the '% Inhibition' (and statistical significance) of PA formation in cultures treated with agonist in the presence of ethanol as compared with cultures stimulated with agonist in the absence of ethanol.

Treatment (time)	Increase in PA (%)	% Inhibition
Thrombin (1 min)	400 ± 51	47 ($P < 0.01$)
Thrombin + ethanol (1 min)	213 ± 74	
Thrombin (5 min)	181 ± 42	45 ($P < 0.01$)
Thrombin + ethanol (5 min)	100 ± 15	
EGF (1 min)	103 ± 16	51 ($P < 0.01$)
EGF + ethanol (1 min)	51 ± 25	
EGF (5 min)	63 ± 22	64 ($P < 0.03$)
EGF + ethanol (5 min)	23 ± 17	

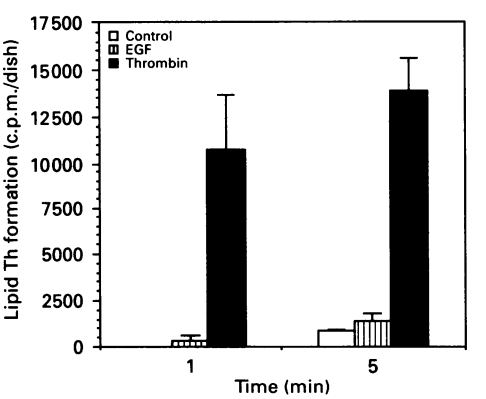


Fig. 7. PLD-mediated formation of a novel lipid species

Serum-starved cultures of IIC9 cells were labelled with [^{3}H]myristic acid (5 μCi/ml) for 2 h. The cultures were then preincubated for 10 min in serum-free medium containing ethanol (100 mM) followed by treatment with 500 ng of α-thrombin/ml, 50 ng of EGF/ml or serum-free medium without agonist (all media contained 100 mM-ethanol). At the indicated times, the incubations were terminated and cellular lipids were extracted and resolved by two-dimensional t.l.c. After autoradiography, the area of plates corresponding to 'Lipid Th' was scraped into vials and quantified by liquid-scintillation counting. The data represent the means ± S.D. of two experiments, each performed in duplicate.

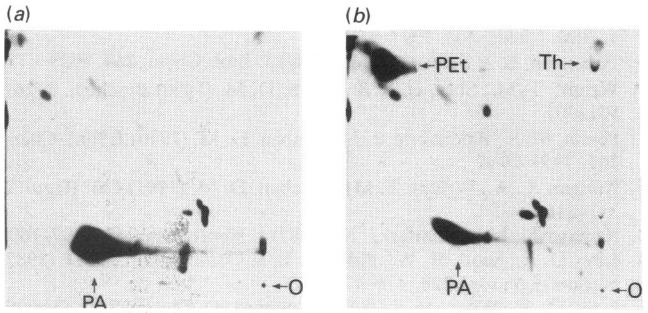


Fig. 8. Treatment of [^{3}H]myristic acid-labelled cellular phospholipids with PLD

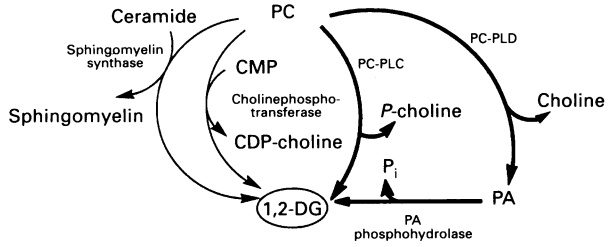
Cellular lipids extracted from serum-starved quiescent cultures labelled with [^{3}H]myristic acid (5 mCi/ml) for 2 h were treated with PLD (cabbage, Type V; Sigma). The labelled cellular lipids were dissolved in diethyl ether (0.4 ml). To each sample was added 0.8 ml of reaction buffer containing 12.5 mM-$CaCl_2$, 0.2 M-sodium acetate, pH 5.6, and PLD (125 units). The samples were vortex-mixed continuously for 4 h, followed by evaporation of the ether under a stream of nitrogen. The aqueous phase was then extracted and the labelled products were analysed by two-dimensional t.l.c. Shown are autoradiographs from PLD reactions carried out without (*a*) and with (*b*) the addition of ethanol (0.15 ml). Indicated are the locations of the origin (O), PA, PEt and 'Lipid Th' (Th).

PLD (cabbage) resulted in the formation of Lipid Th (Fig. 8). The formation of this lipid was dependent on the presence of ethanol (Fig. 8) and PLD (results not shown).

DISCUSSION

It is now well appreciated that the increase in cellular 1,2-DG is an important event in the transduction of mitogenic signals. Although the stimulated hydrolysis of phosphoinositides is responsible for this increase under some conditions, there is recent evidence that the stimulated hydrolysis of PC often constitutes most, if not all, of the increase in 1,2-DG mass in response to many agonists [2,7,8,10,11,22]. In general, mitogen-induced hydrolysis of phosphoinositides is rapid and transient, whereas PC hydrolysis is sustained [2,8–10]. Since in many cases the stimulation of DNA synthesis requires the continued presence of the mitogen for several hours, it is likely that prolonged second-messenger generation such as PC-derived 1,2-DG may play a vital role in the transduction of mitogenic signals.

Thus far two agonist-regulated pathways for the generation of 1,2-DG from PC have been identified (Scheme 1). The first pathway, described by Exton and co-workers in hepatocytes stimulated with P-2 purinergic agonists [23], involves the activation of a PC-specific PLC which results in the generation of phosphocholine and 1,2-DG. The second pathway involves the activation of a PC-hydrolysing PLD, resulting in the generation of PA and free choline. The action of a PA phosphohydrolase then generates 1,2-DG and P_i from PA [20,24]. This second pathway has been described in a variety of cell types [2,18–21,24,25]; however, the relative contribution of this pathway to the generation of 1,2-DG has in many cases not been determined. Two additional pathways for the formation of 1,2-DG from PC are shown in Scheme 1: first, the transfer of phosphocholine to ceramide, catalysed by sphingomyelin synthase (EC 2.7.8.3) [26], resulting in sphingomyelin formation; and second, the transfer of phosphocholine to CMP by cholinephosphotransferase, resulting in the formation of CDP-choline [27]. Although these enzymic pathways are well described, we did not find evidence of their activation (results not shown), nor has their regulation been reported to be agonist-dependent.



Scheme 1. Metabolic pathways from PC to 1,2-DG

Agonist-regulated pathways are indicated by bold arrows.

Our previous studies demonstrated that EGF and α-thrombin stimulate the hydrolysis of PC in quiescent IIC9 cells and that this hydrolysis is temporally associated with the formation of 1,2-DG and the commitment of cells to DNA synthesis [8,12]. In our present studies, therefore, we sought to determine which pathway(s) contributed to the formation of 1,2-DG in response to α-thrombin and EGF, and whether PA was also generated. Interestingly, whereas the addition of α-thrombin or EGF to quiescent IIC9 cells resulted in the activation of a PLD, this enzyme was not involved in the generation of the mitogen-induced 1,2-DG. As shown in Table 1, ethanol decreased the level of mitogen-induced PA to approx. 50% of control levels at 5 min without significantly affecting the level of mitogen-induced 1,2-DG (Figs. 4 and 5). Furthermore, whereas the activation of PLD was transient, being complete after 30–60 s, the generation of 1,2-DG was sustained (Fig. 4, and refs. [8] and [9]). In fact a peak of 1,2-DG mass occurs 5 min after stimulation with either mitogen [8,9], at which time PLD activity was no longer detectable. These results are consistent with our previous findings that the addition of EGF to quiescent cells resulted in a rapid increase in intracellular choline, which preceded the increase in intracellular phosphocholine [10]. Similar results were observed in response to α-thrombin (T. M. Wright & D. M. Raben, unpublished work). The data in the present paper, in addition to our previous results [8–12], indicate that the addition of α-thrombin or EGF to quiescent IIC9 fibroblasts results in the activation of PLC as well as PLD-mediated hydrolysis of PC, but that only the PC-PLC is involved in the mitogen-induced formation of 1,2-DG.

Our observation that the hydrolysis of PC by PLD, although responsible for a component of the stimulated PA formation, does not contribute significantly to the mitogen-stimulated formation of 1,2-DG, stands in contrast with the reported role of PLD in the generation of 1,2-DG in a variety of other systems, including hepatocytes [20] and REF52 fibroblasts [19] stimulated with vasopressin, neutrophils treated with the combination of fMet-Leu-Phe and cytochalasin B [28], muscarinic receptor activation in 1321N1 astrocytoma cells [29], and mast cells activated through the IgE receptor [30]. Consistent with our findings, however, Fisher *et al.* [31] recently reported that the treatment of EGF-stimulated dermal fibroblasts with ethanol did not diminish the observed formation of 1,2-DG at 5 min and 2 h. The apparent discrepancy between these reports is likely to be the result of differences in cell types and the agonists examined. At the biochemical level, these differences must represent variations in the metabolism of the PA formed and may possibly result from differences in the intracellular distribution of the PLD.

The possibility that the activity responsible for the increase in PEt formation resides in a base-exchange enzyme is unlikely. Base exchange does not involve the formation of PA, whereas the hydrolysis of phospholipids via PLD does result in the generation

of this lipid. The ability of ethanol to inhibit at least 50% of the mitogen-induced generation of PA in [^{3}H]myristic acid-labelled cells suggests that the generation of mitogen-induced PEt, and this portion of the induced PA, is formed via a PLD-mediated hydrolysis of PC and not base exchange.

The physiological role of PA has not been established. Recently much attention has been focused on the role of PA in the transduction of mitogenic signals [32–34]. Growth-factor-induced increases in PA have been reported in a variety of systems [2]. There is evidence that PA may serve to activate the hydrolysis of phosphoinositides in some cells, thereby acting as an intermediate in the signal-transduction cascade [33]. The addition of PA or purified PLD (*Streptomyces chromofuscus*) to A431 cells resulted in the induction of *c-fos* and *c-myc* proto-oncogenes [33] and DNA synthesis [33,34]. Recently, PA has been implicated in the regulation of certain cellular kinases [35] and GAP (GTPase-Activating Protein) [36,37]. Together with our observations, these studies lend support to the hypothesis that PA is likely to play an important role in the transduction of a number of agonist-induced events, including the transduction of EGF- and α-thrombin-induced mitogenic signals.

In the course of our experiments, we have detected a novel lipid species, Lipid Th, which is generated in response to α-thrombin. This lipid was detected only in α-thrombin-stimulated cells in the presence of ethanol. In addition, it is generated via PLD-mediated hydrolysis of [^{3}H]myristic acid-labelled lipids isolated from IIC9 cells (Fig. 8). These data suggest that the parent lipid is transphosphatidylated via the α-thrombin-activated PLD. Interestingly, this lipid is not generated in response to EGF. The biochemical characterization of this lipid and the determination of its physiological role are necessary.

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