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Phospholipase D activation by the mitogens platelet-derived growth factor and 12-*O*-tetradecanoylphorbol 13-acetate in NIH-3T3 cells

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The effect of mitogens on phospholipase D activity was investigated in NIH-3T3 fibroblasts by measuring the accumulation of phosphatidylpropanol, produced by phospholipase D phosphatidyl transferase activity when 1-propanol acts as the phosphatidyl group acceptor. Platelet-derived growth factor (PDGF) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) stimulated phosphatidylpropanol production by the cells. The dose–response relationships for activation of phospholipase D and stimulation of thymidine incorporation by PDGF and TPA were comparable. The possibility that activation of phospholipase D is utilized by mitogens as a trans-membrane pathway for signalling cell growth is discussed.

Cell proliferation; Platelet-derived growth factor; Phospholipase D; Phosphatidic acid; Phosphatidylpropanol; (NIH-3T3 cell)

1. INTRODUCTION

Phospholipase D (EC 3.1.4.4) hydrolyses phospholipids to yield the free polar headgroup and phosphatidic acid. However, phospholipase D activity can best be assessed in vivo by its ability to catalyze a phosphatidyl transfer reaction in which a primary alcohol (e.g. ethanol or 1-propanol) serves as the phosphatidyl group acceptor, producing the phosphatidic acid alkyl ester (e.g. phosphatidylethanol or phosphatidylpropanol) [1,2]. The production of phosphatidylalcohol in intact cells (in the presence of a primary alcohol) appears to be exclusively mediated by phospholipase D [3,4]. In contrast, the natural phospholipase D product phosphatidic acid can also be produced by alternative pathways (such as de novo synthesis and by diacylglycerol kinase); moreover, once produced, phosphatidic acid is likely to be quickly metabolized. We have recently utilized phosphatidylethanol production to demonstrate the activation of phospholipase D by the phorbol ester TPA in NG108-15 cells [5], and by gonadotropin-releasing hormone in ovarian granulosa cells [6]. Agonist-activated production of phosphatidylethanol by phospholipase D was similarly shown in HL-60 cells [3,4,7], hepatocytes [8,9], and platelets [10]. In addition, evidence that cell surface receptors may couple with phospholipase D via a guanine nucleotide-binding protein [8,9,11] suggests that the activation of phospholipase D represents a novel signal transduction pathway. Here we report that the mitogens PDGF and TPA stimulate phosphatidylpropanol accumulation in NIH-3T3 cells.

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Abbreviations: PDGF, platelet-derived growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2.1. *Assay of phospholipase D activity in NIH-3T3 cells*

NIH 3T3 cells were grown in DMEM suplemented with 10% FCS (DMEM/FCS), at 37°C under an atmosphere containing 8% CO_2. At 8–10 weeks interval, fresh stock cultures were routinely initiated from liquid nitrogen storage. Before experiments, cells were subcultured at a concentration of 2×10^5 cells/35 mm dishes in DMEM/FCS. Phospholipase D activity was determined by measuring the transfer of phospholipid phosphatidyl moieties, metabolically labeled with [^{3}H]oleic acid, into phosphatidylpropanol. Routinely, 1 day after plating, medium was replaced with DMEM containing 1 mg/ml of fatty acid-free BSA (DMEM/BSA) and [^{3}H]oleic acid (10 μCi/ml per 35 mm dish). Mitogens were added to the cultured cells 24 h later, usually in the presence of propanol at a final concentration of 1% v/v (134 mM). After 30 min incubations were terminated, cellular lipids were extracted and phosphatidylpropanol was quantitated as previously described for phosphatidylethanol [5,6].

2.2. *[^{3}H]Thymidine incorporation*

One day after plating medium was replaced with DMEM/BSA. Mitogens were added to the cultured cells 24 h later, and the cells were further incubated with the growth factors for 19 h; [^{3}H]thymidine (1 μCi/dish) was added for the last 3 h of the incubation. The incorporation of [^{3}H]thymidine into TCA-insoluble material was measured according to Pandiella et al. [12]. Results are presented as a percentage of net radioactivity incorporated in 10% FCS-treated cells, and were calculated according to the equation: $100 \times$ [cpm (mitogen) − cpm (DMEM/BSA)] / [cpm (FCS) − cpm (DMEM/BSA)].

2.3. *Materials*

FCS was obtained from Biological Industries (Beth HaEmek,

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Israel). [^{3}H]Oleic acid, [^{3}H]thymidine and EN^{3}HANCE spray were from New England Nuclear (Boston, MA). Human recombinant PDGF-BB (>95% purity) was purchased from Genzyme (Boston, MA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

3. RESULTS

In preliminary experiments we have observed that fetal calf serum stimulated production of phosphatidylethanol in exponentially growing NIH-3T3 cells that were serum-deprived for 24 h (unpublished results). These results prompted us to examine the capability of various mitogens to activate phospholipase D in such cells. However, due to the presence of an unidentified lipid that migrates between phosphatidic acid and phosphatidylethanol, in the present study we utilized the production of phosphatidylpropanol as a marker for phospholipase D activation. As shown in fig.1, PDGF and TPA markedly increased the production of phosphatidylpropanol in cells incubated in the presence of 1-propanol; prostaglandin $F_{2\alpha}$ had a weaker effect on phosphatidylpropanol levels. In the absence of propanol, both PDGF and TPA stimulated the production of phosphatidic acid (fig.1). The dose-response relationship for PDGF-induced activation of phosphatidyl propanol accumulation (within a 30 min incubation period) is shown in fig.2. The PDGF concentration required to cause a half-maximal activation of phospholipase D was 5 ng/ml. The effect of TPA on phospholipase D activity was also dose-dependent (fig.3), with a half-maximal activation obtained at a concentration of 12 nM.

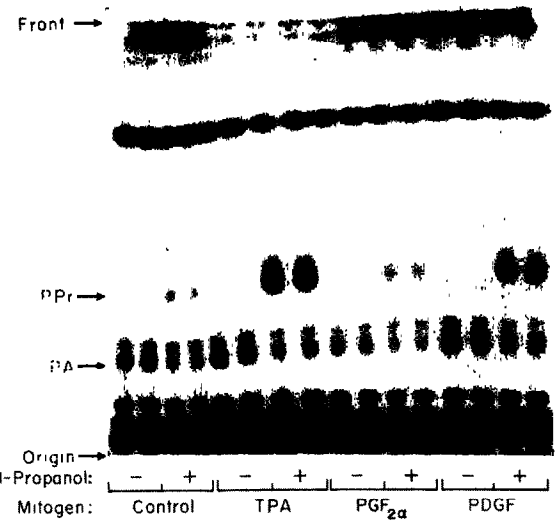

Fig.1. Effect of mitogens on [^{3}H]phosphatidylpropanol formation in NIH-3T3 cells. Cells were prelabeled with [^{3}H]oleic acid, stimulated and extracted as described in section 2. Thin layer chromatography and autoradiography of cellular lipids were carried out as described [5,6]. The presence of 1-propanol (1% by vol., 134 mM) in the incubations is indicated by (+). PDGF, $PGF_{2\alpha}$ and TPA were present at 20 ng/ml, 0.5 μM and 10 nM, respectively. PA, phosphatidic acid; PPr, phosphatidylpropanol.

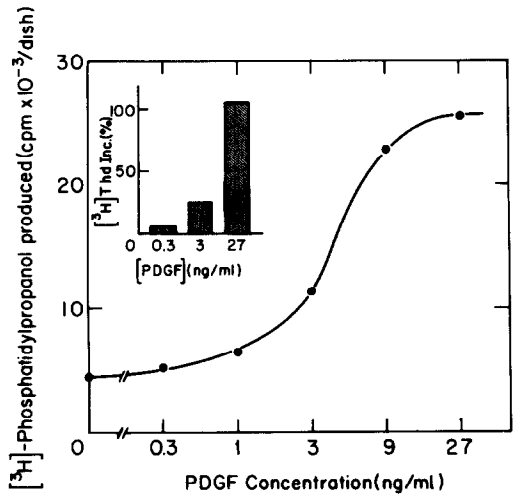

Fig.2. Dependence of [^{3}H]phosphatidylpropanol formation and [^{3}H]thymidine incorporation (inset) on PDGF concentration. [^{3}H]Phosphatidylpropanol determination and [^{3}H]thymidine incorporation were carried out as described in section 2. Results are the mean of duplicate culture dishes in a representative experiment.

The effect of the various mitogens on the incorporation of thymidine into TCA-insoluble material was studied under conditions that were similar to those in which the activation of phospholipase D was observed, i.e. in cells that were serum-deprived for 24 h during the exponential growth phase. PDGF (fig. 2, inset) and TPA (table 1) stimulated [^{3}H]thymidine incorporation into TCA-insoluble material in a dose-dependent manner. In addition, we confirmed previous reports on the potent mitogenic effectiveness of phosphatidic acid, which is comparable to that of FCS and PDGF (table 1).

Table 1

Effect of dipalmitoyl phosphatidic acid and TPA on [^{3}H]thymidine incorporation in exponentially growing NIH-3T3 cells

Stimulant	Concentration	[^{3}H]Thymidine incorporation (% of FCS control)
Phosphatidic acid	5 μg/ml	2.0
	10	2.6
	20	37.9
	30	95.0
	40	96.6
	80	88.6
TPA	1 nM	0
	10	6.6
	100	41.7

[^{3}H]Thymidine incorporation was carried out as described in section 2. Results are the mean of duplicate culture dishes in a representative experiment.

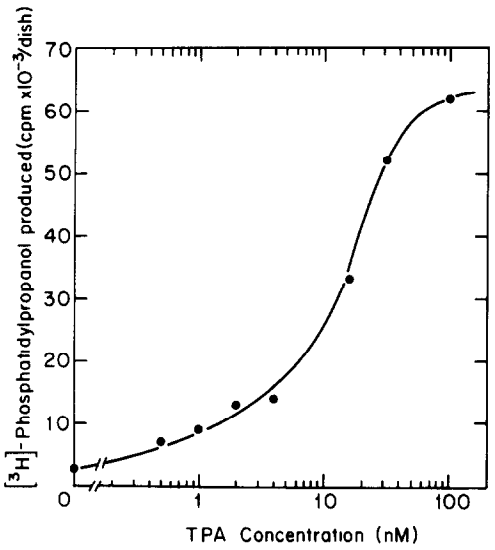

Fig.3. Dependence of [^{3}H]phosphatidylpropanol formation on TPA concentration. [^{3}H]Phosphatidylpropanol was determined as described in section 2. Results are the mean of duplicate culture dishes in a representative experiment.

4. DISCUSSION

The 3T3 family of mouse fibroblast cell lines was often chosen as a model system for studies on cell cycle regulation and on mechanisms of growth factor action. Although the action of various mitogens was extensively investigated in this type of cells, the events leading to DNA synthesis and cell division are still largely obscure. The present results indicate that certain mitogens can activate phospholipase D in NIH-3T3 fibroblasts, suggesting the possibility that phosphatidic acid production by phospholipase D participates in transducing the mitogenic signal.

The effects of TPA and PDGF on phospholipase D activity suggest at least three possible activation mechanisms. TPA was shown to affect phospholipase D activity in several cell types [5–7,11,13], and evidence that its effect is mediated by protein kinase C was presented [5,7,13]. It is possible that phospholipase D is one of the substrates of protein kinase C-catalyzed serine and/or threonine phoshorylation. We have recently shown that, in granulosa cells, TPA and the Ca^{2+}-mobilizing genadotropin-releasing hormone do not regulate phospholipase D activity by the same mechanism [6]. There is evidence that receptor activation of phospholipase D is mediated by a guanine nucleotide-binding protein in hepatocytes and HL-60 cells [8,9,11], as well as in NG108-15 cells (M. Liscovitch, unpublished observations); the effect of PDGF may well involve a similar mechanism. Alternatively, since the PDGF receptor has an intrinsic tyrosine kinase activity [14], the activation of phospholipase D by PDGF could involve a PDGF receptor kinase-catalyzed tyrosine phosphorylation.

Important questions that remain to be examined are whether and how does phospholipase D participate in transducing the mitogenic signal across the cell membrane. The product of phospholipase D, phosphatidic acid, is a known, potent mitogen ([15–17] and table 1). It is unlikely that the effect of phosphatidic acid is secondary to its conversion to diacylglycerol (followed by activation of protein kinase C), since diacylglycerol (as well as TPA) are much less effective, as mitogens, than phosphatidic acid ([15,16], and unpublished observations). While not all tested mitogens activated phospholipase D (unpublished results), other mitogens may elevate phosphatidic acid levels by other mechanisms. Insulin for example was shown to stimulate *de novo* synthesis of phosphatidic acid [18]. Elevation of endogenous phosphatidic acid levels may therefore be a common and essential step in mitogenic signal transduction cascades. The cellular target(s) for phosphatidic acid action remain to be identified; resolution of such protein(s) may provide a better understanding of the role of phospholipase D and phosphatidic acid in control of cell proliferation.

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