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Phorbol esters stimulate a phospholipase D-catalysed reaction with both ester- and ether-linked phospholipids in HeLa cells

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The phorbol ester, 12-tetradecanoylphorbol-13-acetate (TPA), stimulated the accumulation of labelled phosphatidylethanol (PEth) in HeLa cells prelabelled with [^{3}H]hexadecanol and incubated in the presence of ethanol. PEth formation involves a phospholipase D-catalysed phosphatidyl transferase reaction. [^{3}H]Hexadecanol labelled both ester- and ether-derivatives of HeLa cell phospholipids and TPA stimulated the conversion of both classes of lipid to PEth.

Phorbol ester; Phospholipase D; Ether phospholipid

1. INTRODUCTION

It is well established that phorbol esters [1–4] and growth factors such as platelet-derived growth factor [5] and bombesin [6] can lead to altered metabolism of PC and to the accumulation of PC-derived DAG. Apart from providing an alternate source of DAG other than the inositol phospholipids, this phenomenon is of interest as both PC and PE are known to exist as ester- and ether-linked forms in most bacterial and mammalian cells, and the relative proportions of each vary widely [7]. Hydrolysis of PC and PE may therefore lead to accumulation of a mixture of DAG and ether-linked diglycerides. Whereas DAG is an activator of PK-C activity, the latter derivatives have been reported to inhibit the kinase [8].

Although PK-C has been implicated in regulating PC hydrolysis [4–6], the initiating event in diglyceride production is unclear. In some cell types the direct production of diglyceride by phospholipase C action may occur [4–6], while in others there is evidence that phospholipase D activity leads to the production of phosphatidic acid which is then dephosphorylated to diglyceride [9–11]. The most direct evidence for a phospholipase D involvement comes from the report that, in the presence of ethanol, TPA stimulates the accumulation of PEth in NG 108-15 cells [12]. PEth is formed as a result of the phosphatidyl transferase activity of phospholipase D, and its accumulation is therefore a convenient index of enzyme activity. A similar accumulation of PEth has been observed in hepatocytes exposed to the Ca^{2+}-mobilizing hormones vasopressin, angiotensin II and epinephrine [13].

In the present paper we report that TPA stimulates the accumulation of both ester and ether forms of PEth in HeLa cells prelabelled with [^{3}H]hexadecanol and incubated in the presence of ethanol.

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Abbreviations: DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEth, phosphatidylethanol; PK-C, protein kinase C; TPA, 12-tetradecanoylphorbol-13-acetate

2. MATERIALS AND METHODS

2.1. *Materials*

[9,10(*n*)-^{3}H]Palmitic acid (54 Ci/mmol) was from Amersham (England) and [^{3}H]hexadecanol was prepared by reduction of [^{3}H]palmitic acid [14]. TPA was from PL-Biochemicals, Milwaukee. Phospholipase D (Cabbage) was from Sigma Chemical Co., St. Louis and used to prepare PEth as described [14].

2.2. *Cell culture*

HeLa cells were grown in Dulbecco's minimal essential medium in either 35 or 100 mm dishes. Cultures were labelled with [^{3}H]hexadecanol (0.8 μCi/ml) for 24 h. Ethanol (51 mM), TPA (0–1 μM) and/or dimethyl sulfoxide (0.1%) were added as appropriate and cultures incubated for various times.

2.3. *Lipid extraction and fractionation*

After incubation, cells were washed with cold phosphate-buffered saline, lipids extracted [15] and PEth isolated by thin-layer chromatography [12]. For the analysis of ether-linked PEth, samples were eluted with chloroform/methanol (2:1, v/v) containing 50 μg/ml butylated hydroxytoluene, and subjected to two mild alkaline methanolysis treatments to cleave all the ester bonds [16]. [^{3}H]Ether-linked lyso-PEth and [^{3}H]palmitic acid (released from ester-linkage to phospholipid) in the final organic phase were separated using a solvent containing hexane/ether/acetic acid/H_2O (35:65:0.5:0.5, v/v). Radioactivity was determined by scintillation counting.

3. RESULTS AND DISCUSSION

TPA stimulated the formation of PEth when HeLa cells were incubated in the presence of ethanol (figs 1

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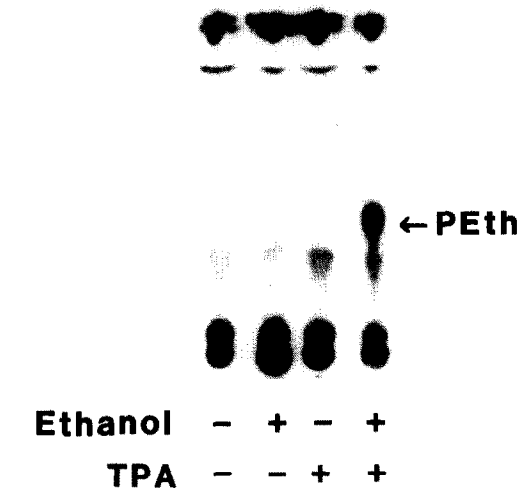

Fig.1. Accumulation of PEth in HeLa cells. Cells were prelabelled with [^{3}H]hexadecanol and then incubated with ethanol (85 mM), TPA (100 nM) or ethanol plus TPA for 2 h. After chromatography of lipid extracts as described in section 2, the plate was sprayed with 21% PPO in acetone and used for autofluorography.

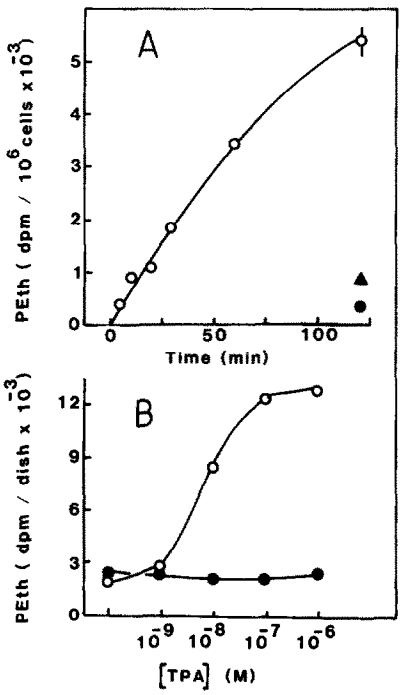

Fig.2. Dependence of [^{3}H]PEth formation on time of incubation and TPA concentration. (Panel A) Prelabelled cells were incubated with ethanol (51 mM) and TPA (100 nM) for varying times before isolating [^{3}H]PEth. (●) Incubations with TPA alone; (▲) incubations with ethanol alone. (Panel B) Prelabelled cells were incubated with 51 mM ethanol and varying concentrations of TPA for 2 h before isolation of [^{3}H]PEth (○). Additional cultures were incubated with 300 nM TPA during the prelabelling period, washed, and then incubated with 51 mM ethanol and TPA for 2 h before isolation of [^{3}H]PEth (●). All results are the means ± SE of triplicate determinations.

and 2). PEth accumulation could be detected after 5 min, continued for at least 2 h and was dependent on the presence of both TPA and ethanol. A half-maximal response was obtained at a TPA concentration of about 10^{-8} M (fig.2) and 30 mM ethanol (data not shown). In addition, no accumulation of PEth was observed in HeLa cells which had been incubated with 300 nM TPA during the labelling period with [^{3}H]hexadecanol (fig.2, panel B). We have previously shown that preincubation with TPA leads to an essentially total loss of immunologically detectable PK-C (unpublished data). Consequently, this result is consistent with an involvement of PK-C in activating PEth formation. Other studies [12] have concluded that PEth accumulation is the result of *trans*-phosphatidylation catalysed by phospholipase D. The serine and ethanolamine base-exchange enzyme(s) does not catalyse the formation of PEth [17], and the specificity of the phosphatidyl transferases makes it unlikely that these enzymes would catalyse the formation of PEth from CDP-diacylglycerol [18].

During the preincubation period with [^{3}H]hexadecanol, radioactivity was incorporated into ester and ether-linked phospholipid in the ratio of 8:1. Incorporation into the ester-derivatives is presumably a consequence of the oxidation of [^{3}H]hexadecanol to [^{3}H]palmitic acid. As shown in table 1, TPA stimulated the accumulation of both ether- and ester-linked PEth, indicating that both forms of phospholipid are substrates for phospholipase D. The data do not allow an accurate estimation of the relative proportions of the two forms of PEth produced, as ester-linked [^{3}H]palmitic acid can be derived from both di-ester and ether-ester derivatives of phospholipids. Although the ratios of ester:ether PEth at 30 min and 2 h (6.4:1 and 5.4:1, respectively) were somewhat lower than the original phospholipid (8:1), there does not seem to have been a major preference of either form for phospholipase D attack.

These results indicate that endogenous ether

Table 1

Effect of TPA on the accumulation of ester- and ether-linked PEth

	Ether-linked PEth (dpm/dish)		Ester-linked PEth (dpm/dish)	
	30 min	2 h	30 min	2 h
Control	ND	73 ± 66	279 ± 58	351 ± 29
TPA	1175 ± 49	2992 ± 98 ($P < 0.001$)	7560 ± 248 ($P < 0.001$)	16087 ± 425 ($P < 0.001$)

HeLa cell cultures were prelabelled with [^{3}H]hexadecanol and incubated in the presence of ethanol (51 mM) and either dimethyl sulfoxide (0.1%) or TPA (100 nM) for 30 min or 2 h. PEth was isolated and radioactivity associated with palmitic acid (ester-linked) and lyso-PEth (ether-linked) determined as described in section 2. Results are the means ± SE of triplicate determinations. ND, not detectable. Total phospholipids after prelabelling contained 418703 ± 6600 dpm/dish as ester and 51416 ± 1225 dpm/dish as ether phospholipid

phospholipids are potential sources of regulatory molecules such as PA and diglyceride.

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