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12-O-TETRADECANOYLPHORBOL-13-ACETATE ACTIVATES PHOSPHATIDYLETHANOL AND PHOSPHATIDYLGLYCEROL SYNTHESIS BY PHOSPHOLIPASE D IN CELL LYSATES

Connie S. Tettenborn and Gerald C. Mueller

McArdle Laboratory for Cancer Research
The University of Wisconsin-Madison
Madison, WI 53706

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SUMMARY A cell-free system for the synthesis of phosphatidylalcohols was developed in sonicates of HL-60 cells. With [^{32}P]phosphatidylcholine as the exogenous substrate, both phosphatidylethanol and phosphatidylglycerol were formed through a phospholipase D-catalyzed transphosphatidylation of ethanol and glycerol, respectively. The transphosphatidylation by phospholipase D was stimulated *in vitro* by 12-O-tetradecanoylphorbol-13-acetate (TPA) and required the addition of ATP for an optimal response. GTP-γ-S, an activator of G protein systems, also stimulated the process by an independent mechanism. It is postulated that the stimulation of phospholipid metabolism through phospholipase D activation represents an important mechanism whereby TPA might modulate intracellular signal generating systems or influence the activity of membrane-bound proteins by altering their lipid environment.

Previous studies from this laboratory have demonstrated that phosphatidylethanol (PEt) synthesis is stimulated in ethanol-treated animal cells by phorbol esters and other compounds which activate protein kinase C [1,2]. This unique acidic lipid has also been observed under conditions in which phospholipase D has been activated by a receptor-mediated process in whole cells [3,4] or by fatty acids in rat brain microsomal preparations [5-7]. Recently Pai et al. [3], using exogenous alkyl-[^{32}P]lysophosphatidylcholine to prelabel the cellular phosphatidylcholine pools, showed conclusively that the *in vivo* formation of PEt in response to the chemotactic peptide, N-formyl-Met-Leu-Phe, involved a transphosphatidylation. Presumably, this reaction is mediated by a phospholipase D type of enzyme. To study the reaction mechanism further and to identify the roles of phorbol esters and protein kinase C as activators of this process, we have undertaken the study of PEt synthesis in cell lysates of HL-60 cells. In this report, we show that exogenous [^{32}P]phosphatidylcholine is converted to [^{32}P]PEt in lysates treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) in the presence of ATP. In addition, we have found that GTP-γ-S is able to substitute for the combination of TPA and ATP in the activation of PEt synthesis in much the same manner that GTP-γ-S substitutes for the vasopressin-mediated activation of phospholipase D in hepatocyte fragments [4]. The likelihood that transphosphatidylation by a phospholipase D type of enzyme is regulated by both protein kinase C-mediated phosphorylation and a G protein is discussed.

MATERIALS AND METHODS

Cell cultures

HL-60 cells were maintained in a 5% CO_2 atmosphere with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 30 mg penicillin and 50 mg streptomycin

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per liter of culture medium. Stock cultures were diluted with fresh medium every 6-7 days to maintain a cell concentration between 2 x 10^4 and 1 x 10^6 cells/ml. For experiments, cultures were prepared in fresh medium with 7.5-8.5 x 10^5 cells/ml and incubated 16-18 h with 0.1 or 0.2 μCi [^{3}H]arachidonic acid/ml. Cells were harvested and washed with phosphate-buffered saline containing 0.14 M NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.7\ H_2O$ and 1.5 mM KH_2PO_4 (pH 7.4) prior to sonication as described below.

Preparation and assay of cell sonicates

After washing with phosphate-buffered saline, the cells were resuspended in either 25 mM Hepes (pH 7.5) containing 85.6 g/L sucrose (buffer A) or in a solution containing 10 mM EGTA, 2 mM EDTA, 50 mM KF (pH 7.5) and 20 mg/L leupeptin (buffer B), as indicated, at a cell concentration of approximately 1 x 10^6 cells/ml (1 ml for every 100 ml culture). The suspension was then sonicated on ice using a Sonifier cell disruptor, Heat Systems-Ultrasonics, Inc., fitted with a microtip for a total of 25-45 sec, using short bursts. Cell sonicates were then centrifuged at 250-500 x g for 5 min at 4°C to remove nuclei and large particulates, unless otherwise indicated in the figure legends. The supernatant (200 μl) was used for each assay in a total volume of 0.8-1 ml. Incubations were for 50-60 min at 37°C in the presence of 50 mM Hepes (pH 7.5) and other compounds as indicated in the figure legends. The reaction was stopped by addition of chloroform/methanol (1:1, v/v).

Analysis of lipids

[^{3}H]Arachidonic acid-labeled lipids were extracted from samples with chloroform/methanol/H_2O (2:2:1.6, v/v) as follows. The reaction was stopped by addition of 4 ml of chloroform/methanol (1:1, v/v) and vortexing. Water was then added to give a final volume of 1.6 ml H_2O. After vortexing, the mixture was separated by centrifugation and the organic lower phase was removed. Solvents were evaporated under N_2 and the lipids remaining were dissolved in a small volume of chloroform/methanol (2:1, v/v) and applied to plastic-backed silica gel TLC plates (Kodak Chromagram sheets). Lipids were separated by elution for 15 cm using the organic phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/H_2O (11:5:2:10, v/v), solvent system II of Wrighton, et al. [8]. The chromatograms were then cut into 0.5 cm fractions, and the level of radioactivity was determined by liquid scintillation spectrometry.

Preparation of [^{32}P]phosphatidylcholine and its use as a substrate for PEt synthesis

A culture of HL-60 cells (125 ml at 8.5 x 10^5 cells/ml) was incubated for 17 h with [^{32}P]orthophosphate, 5 μCi/ml, in phosphate-free Eagle's HeLa medium plus 10% heat-inactivated fetal bovine serum. Cells were then harvested and washed once with phosphate-buffered saline. Lipids were extracted using chloroform/methanol/H_2O (2:2:1.6, v/v) and concentrated under N_2. Total ^{32}P-labeled lipids were then applied to a silica gel 60 TLC plate and developed over a distance of 15 cm using chloroform/methanol/acetic acid (50:25:8, v/v), solvent system III of Wrighton et al. [8]. Phosphatidylcholine was located by autoradiography of the TLC plate and this area of the chromatogram was scraped and eluted using chloroform/methanol (2:1, v/v). The powdered silica gel was washed again with methanol and finally rinsed with methanol using a millipore filter. Total organic washes were dried under N_2 and redissolved in a small volume of chloroform/methanol (2:1, v/v) which was aliquoted to 8 assay tubes and used as follows. The aliquots of ^{32}P-labeled phosphatidylcholine were dried onto the sides of a 12 ml centrifuge tube. Acetone (10 μl) or 1.6 x 10^{-5} M TPA in acetone (10 μl), were added along with 20 μl ethanol or 40 μl of glycerol/H_2O (1:1, v/v), or 20 μl H_2O. The tubes were briefly sonicated on ice in a bath-type sonicator. Buffer and salts were added to give the final concentrations as indicated in the figure legends and the sonication was continued for approximately 3 min more. Finally, 200 μl of unlabeled sonicate (250 x g supernatant prepared as described in the figure legend) was added. The tubes were vortexed and incubated for 50 min at 37°C. To terminate the assay, the lipids were extracted with chloroform/methanol/H_2O (2:2:1.6, v/v) and dried under N_2. Total ^{32}P-labeled lipids were then applied to silica gel 60 TLC plates and developed using system III. The location of the labeled lipids was determined by autoradiography.

Materials

The following special reagents were purchased commercially: [^{3}H]arachidonic acid [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(N)] (specific activity, 60-100 Ci/mmol) and [^{32}P]orthophosphate, (carrier free) from New England Nuclear; GTP-γ-S and β,γ-methylene ATP from Sigma; Kodak Chromagram sheets from VWR.

RESULTS

Studies of PEt synthesis in sonicates of cells staged *in vivo*

In the initial studies of the pathway for phosphatidylalcohol synthesis, the labeled phosphatidyl groups were provided by the lipids of the cell sonicates. For these experiments, the phospholipid pools were prelabeled by growth of the cells for 15 to 21 h with [^{3}H]arachidonic acid in the presence or absence of TPA as indicated. The data presented in Fig. 1 show the results of such a study using a sonicate prepared from HL-60 cells grown for 15 h in the presence of [^{3}H]arachidonic acid and TPA--but in the absence of an exogenous alcohol. Direct test of this sonicate failed to yield PEt. The addition of ethanol to the sonicate system, however, resulted in a well-resolved peak of PEt. The yield of this product was essentially the same with 2 or 6.5% ethanol in the reaction mixtures.

To test whether or not the pretreatment of the cells with TPA was important, PEt production was measured in sonicates prepared from cultures treated with or without TPA (Table I). The amount of PEt was much greater using the sonicate from a culture pretreated 3.5 h with TPA (16 nM) than a comparable sonicate of a control culture. However, sonicates from control cultures could be stimulated to synthesize more PEt if Ca^{2+} was added to the assay system. Thus, increasing the Ca^{2+} concentration in the sonicate mimicked the effect of TPA in the whole cells. This overlap of effects was also evident in the observation that the largest TPA responses were seen under conditions of no added Ca^{2+} in the assay. Since most phorbol ester effects appear to be mediated by protein kinase C, it was reasonable to ask if the sonicates of TPA pretreated cells might also depend on exogenous ATP. The results in Fig. 2 show that the addition of ATP does indeed support the

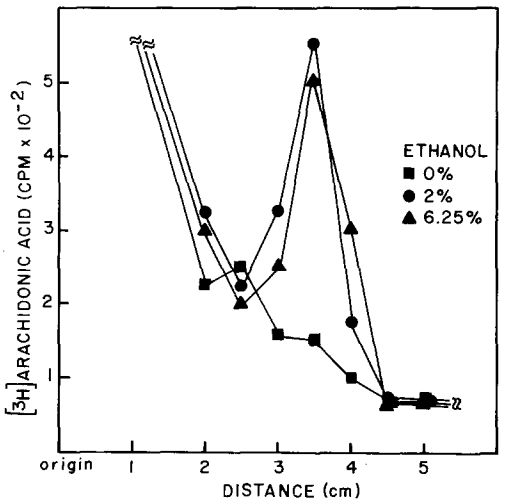


Fig. 1. Synthesis of PEt in a cell lysate. A culture of HL-60 cells (150 ml at 8.5 x 10^5 cells/ml) was incubated for 15 h with [^{3}H]arachidonic acid (0.1 μCi/ml), and 16 nM TPA, both added in acetone (0.2%, final acetone concentration). Cells were harvested, washed, and sonicated in a volume of 1.3 ml of buffer A for 3x15 sec bursts. Aliquots (200 μl) of the 500 x g supernatant were incubated with 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and the indicated concentration of ethanol in a final volume of 0.8 ml for 1 h at 37°C. Lipids were extracted and analyzed by TLC system II as described in **MATERIALS AND METHODS**. (■), 0% ethanol; (●), 2% ethanol; (▲), 6.25% ethanol.

TABLE I
EFFECT OF TPA PRETREATMENT ON PEt SYNTHESIS IN A CELL LYSATE

Pretreatment of culture	$[Ca^{2+}]$ in lysate (mM)	[^{3}H]Arachidonic acid in PEt (cpm)
None	---	180 ± 28
"	0.5	638
"	1.0	742
+ TPA	---	1013 ± 48
"	0.5	1398
"	1.0	1737

Two separate HL-60 cell cultures (100 ml each at 7.5 x 10^5 cells/ml) were incubated for 17.5 h at 37°C with [^{3}H]arachidonic acid (0.1 μCi/ml). TPA (16 nM) was then added in acetone to one culture; the other culture received acetone alone (final concentration, 0.1%). After a further 3.5 h incubation at 37°C, the cells were harvested, washed, and sonicated (5x5 sec) in 1 ml of buffer B containing chelators (see **MATERIALS AND METHODS**). Aliquots (200 μl) of the total sonicate were then assayed in the presence of 50 mM Hepes (pH 7.5), 2% ethanol, 10 mM $MgCl_2$, 5 mM ATP, and either no calcium or $CaCl_2$ added to give the final concentrations of free calcium indicated. After incubating for 50 min at 37°C, the lipids were extracted and analyzed as described in **MATERIALS AND METHODS**. The total radioactivity contained in the PEt peak was quantitated. Where shown, the results are the mean ± range of duplicates.

synthesis of phosphatidylethanol *in vitro* using the prelabeled lipids of the cell. A simple explanation for this finding is that the ATP supported the continuing function of protein kinase C that had been activated by treatment of the living cells with TPA. From this result, it would appear that a protein kinase C-mediated phosphorylation of some key component of the PEt-synthesizing system is required for optimal activity.

The activation of PEt synthesis by the direct addition of TPA to the cell lysates

An alternative explanation for the ATP effect, however, was the possibility that this cofactor supported the synthesis of some intermediate substrate for PEt synthesis such as ethyl phosphate or a nucleotide derivative of the alcohol. To explore this situation, a study of the direct activation of sonicates of [^{3}H]arachidonic acid-labeled cells by TPA, ATP, and other cofactors was carried out using HL-60 cells from control (untreated) cultures.

The results presented in Fig. 3 show clearly that ATP by itself had little or no effect on PEt synthesis--whereas the addition of both TPA and ATP to the sonicate reaction mixture yielded a significant amount of PEt. Substituting the non-utilizable analog, β,γ-methylene ATP, for ATP greatly reduced the yield of PEt in the presence of TPA. The addition of TPA alone was essentially ineffective in a series of experiments (data not shown). In an analogous experiment, ethanol was omitted from the reaction mixture. With this omission, no peak corresponding to PEt was produced, thus lending support to the identification of this peak as PEt.

Mechanism of synthesis of PEt

To address the question of whether PEt is synthesized by a phospholipase D-type enzyme, exogenous [^{32}P]phosphatidylcholine was added to a sonicate from control HL-60 cells as a labeled

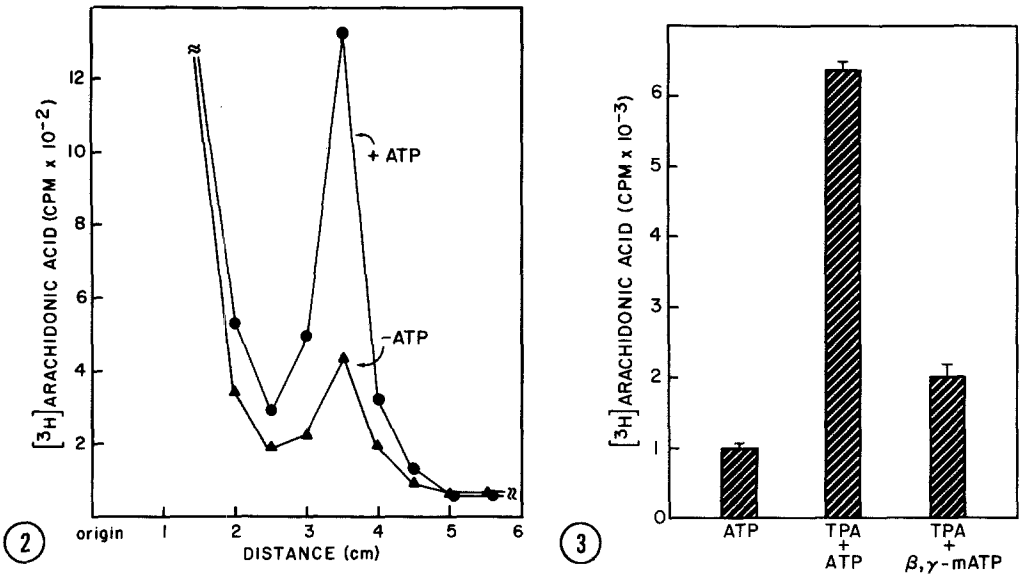
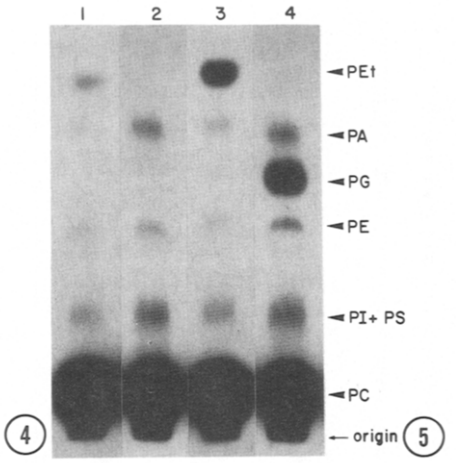


Fig. 2. Effect of ATP on PEt synthesis. A culture of HL-60 cells (100 ml at 7.7 x 10^5 cells/ml) was incubated 16 h with [^{3}H]arachidonic acid and then 3.5 h with 16 nM TPA. Cells were harvested, washed, and sonicated (4x10 sec) in 1 ml of buffer B. Aliquots (200 µl) of the 500 x g sonicate supernatant were then assayed in the presence of 50 mM Hepes, pH 7.5, 2% ethanol, approximately 1 mM free calcium, 13.4 mM $MgCl_2$ and with or without ATP (5 mM) in a final volume of 1 ml. After incubation for 50 min at 37°C, lipids were extracted and analyzed by TLC in system II, as described in **MATERIALS AND METHODS**. Data represent the average of duplicates which varied less than 10%. ▲ , no nucleotide; ● , 5 mM ATP.

Fig. 3. Addition of TPA directly to a lysate. A culture of HL-60 cells (200 ml at 7.5 x 10^5 cells/ml) was incubated for 17.5 h at 37°C with [^{3}H]arachidonic acid (0.2 µCi/ml). Cells were then washed and sonicated (6x5 sec) in 2 ml of Buffer B. Unlysed cells and nuclei were removed by centrifugation for 5 min at 250 x g. Aliquots (200 µl) of the supernatant were then assayed in the presence of 50 mM Hepes (pH 7.5), 2% ethanol, 10 mM $MgCl_2$, 2 mM $CaCl_2$ (equivalent to the concentration of EGTA), either ATP or β,γ-methylene ATP, both at 5 mM, and 160 nM TPA added in acetone or acetone alone (1%), in a final volume of 1 ml. After incubation for 50 min at 37°C, lipids were extracted and analyzed by TLC in system II as described in **MATERIALS AND METHODS**. The total amount of radioactivity incorporated into phosphatidylethanol was quantitated. Error bars indicate the range of duplicates.

precursor of the phosphatidyl group. [^{32}P]PEt formation was then assayed in the presence of ATP, with or without 160 nM TPA. Ethanol was omitted from one set of assays, and in another set, ethanol was replaced with glycerol. When the lipids were separated and autoradiographed (Fig. 4), several conclusions could readily be made. First, the addition of TPA directly to the sonicate resulted in greater synthesis of a ^{32}P-labeled lipid that co-chromatographed with a [^{3}H]PEt standard (compare lanes 1 and 3). When ethanol was omitted from the reaction mixture, this spot did not appear (lane 2), further demonstrating the identity of the TPA-induced lipid as PEt. Second, when glycerol was added instead of ethanol, a new ^{32}P-labeled spot appeared (lane 4). This material migrated in the chromatograms to the location expected for phosphatidylglycerol.

This demonstration that ^{32}P-label from exogenous [^{32}P]phosphatidylcholine could be incorporated into PEt or phosphatidylglycerol provides direct evidence for transphosphatidylation by a phospholipase D-type enzyme. When a headgroup, in the form of ethanol or glycerol, is not provided, the [^{32}P]phosphatidylcholine is converted to [^{32}P]phosphatidic acid (lane 2). Note that the labeling of phosphatidic acid is greater in the presence of TPA (compare lane 1 with lane 3).



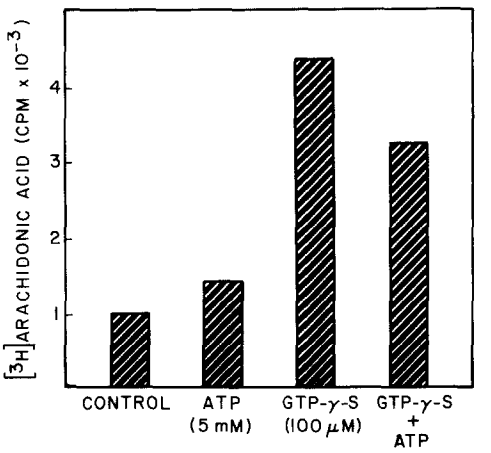


Fig. 4. Addition of ^{32}P-labeled phosphatidylcholine to a lysate. Unlabeled HL-60 cell sonicate was prepared from a culture (200 ml at 7.5 x 10^5 cells/ml) which had been incubated overnight. Cells were washed and sonicated (5x5 sec) in buffer B, 2 ml. After centrifugation for 5 min at 250 x g, aliquots (200 μl) of the supernatant were added to assay tubes cotaining [^{32}P]phosphatidylcholine prepared as described in **MATERIALS AND METHODS**. Assays contained 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 5 mM ATP, 2 mM $CaCl_2$ (equivalent to the EGTA concentration), 2% of either ethanol or glycerol and either 160 nM TPA, added in acetone or acetone alone (1%) in a final volume of 1 ml. Following incubation for 50 min at 37°C, the lipids were extracted and separated via TLC using chloroform/methanol/acetic acid (solvent system III). The chromatograms were autoradiographed to visualize labeled phospholipids. Lane 1, ethanol control (no TPA); lane 2, TPA alone (no ethanol); lane 3, TPA plus ethanol; lane 4, TPA plus glycerol. Standard phospholipids: PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; PEt, phosphatidylethanol.

Fig. 5. Effect of GTP-γ-S on PEt synthesis. A culture of HL-60 cells (200 ml at 7.5 x 10^5 cells/ml) was incubated for 16 h with [^3H]arachidonic acid (0.2 μCi/ml). Cells were then washed and sonicated (6x5 sec) in 2 ml of buffer B. Unlysed cells and nuclei were removed by centrifugation for 5 min at 250 x g. Aliquots (200 μl) of the supernatant were then assayed in the presence of 50 mM Hepes, pH 7.5, 2% ethanol, 10 mM $MgCl_2$, 2 mM $CaCl_2$ (equivalent to the concentration of EGTA) and either no nucleotide, 100 μM GTP-γ-S, and/or 5 mM ATP. After incubating for 50 min at 37°C, the lipids were extracted and analyzed by TLC in system II as described in **MATERIALS AND METHODS**. The total amount of radioactivity incorporated into phosphatidylethanol was quantitated. Data represent the average of duplicates which varied less than 6% from the mean.

[^{32}P]Phosphatidylcholine was also converted to phosphatidylethanolamine and to a product that is probably phosphatidylserine by this total cell homogenate. This conversion, however, was the same with or without the addition of TPA and is thus likely to reflect an endogenous base exchange activity in the HL-60 cell line that works with these headgroups. The base exchange enzymes appear to be ruled out in the TPA-induced synthesis of phosphatidylglycerol by the report of Kanfer [9] that glycerol is not a substrate for such enzymes.

The activation of PEt synthesis by addition of GTP-γ-S *in vitro*

The above results point to an activation of phospholipase D by TPA. Bocckino et al. [10], however, have shown that this enzyme is activated in rat hepatocytes by vasopressin which mediates its effects through a specific receptor system located in the cell's surface membranes.

They further demonstrated that phospholipase D was stimulated by GTP-γ-S in membrane preparations from such cells. This observation suggests that phospholipase D also exists as a G protein complex. To test this possibility we, therefore, looked for an effect of GTP-γ-S on PEt synthesis in HL-60 cell sonicates. This non-hydrolyzable GTP analog stimulated PEt synthesis approximately 4-fold (Fig. 5). This stimulation, however, did not require either ATP or TPA. In fact, the addition of ATP slightly inhibited the GTP-γ-S-stimulated PEt accumulation. This result, while preliminary, is in accord with the findings of Bocckino et al. [10] and further suggests that the enzyme responsible for PEt synthesis is also regulated by a guanine nucleotide binding protein.

DISCUSSION

Using sonicates derived from cultures of HL-60 cells, we have provided evidence for the direct activation of phosphatidylalcohol synthesis in a cell-free system by TPA. Since the response also depended on the addition of ATP, it would appear that the activation of the system depended on a protein kinase C-mediated event. The observation that PEt could be labeled with $^{32}PO_4$ derived from exogenous [^{32}P]phosphatidylcholine added to the cell sonicate demonstrated that PEt was synthesized by a transphosphatidylation reaction catalyzed by a phospholipase D type of enzyme. Whether the enzyme is the direct target of protein kinase C remains to be established. Notably, PEt synthesis was also stimulated by high Ca^{2+} in the control sonicates. This result could be due either to a direct activation of phospholipase D by Ca^{2+}, as was reported for rat brain phospholipase D [11], or the facilitated action of endogenous protein kinase C on the enzyme. The observation that the system was also activated by the direct addition of GTP-γ-S implies a role of GTP-binding proteins in its regulation as well. This mechanism of regulation appears separate from that of the TPA-induced response. Thus the regulation of this enzyme by two different mechanisms could provide another interesting example of the interplay between different second messenger systems.

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REFERENCES

1. Pai, J.-K., Liebl, E. C., Tettenborn, C. S., Ikegwuonu, F. I. and Mueller, G. C. (1987) Carcinogenesis 8, 173-178.
2. Tettenborn, C. S. and Mueller, G. C. (1987) Biochim. Biophys. Acta 931, 242-250.
3. Pai, J.-K., Siegel, M. I., Egan, R. W. and Billah, M. M. (1988) Biochem. Biophys. Res. Commun. 150, 355-364.
4. Bocckino, S. B., Wilson, P. B. and Exton, J. H. (1987) FEBS Lett. 225, 201- 204.
5. Gustavsson, L. and Alling, C. (1987) Biochem. Biophys. Res. Commun. 142, 958-963.
6. Kobayashi, M. and Kanfer, J. N. (1987) J. of Neurochem. 48, 1597-1603.
7. Kanfer, J. N. and Hattori, H. (1986) In Enzymes of Lipid Metabolism II, (Freysz, L., Dreyfus, H., Massarelli, R. and Gatt, S., eds.), pp. 665-679, Plenum Publishing Corp.
8. Wrighton, S. A., Pai, J.-K., and Mueller, G. C. (1983) Carcinogenesis 4, 1247-1251.
9. Kanfer, J. N. (1980) Can. J. Biochem. 58, 1370-1380.
10. Bocckino, S. B., Blackmore, P. F., Wilson, P. B. and Exton J. H. (1987) J. Biol. Chem. 262, 15309-15315.
11. Taki, T. and Kanfer, J. N. (1979) J. Biol. Chem. 254, 9761-9765.