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ACTIVATION OF PHOSPHOLIPASE D BY CHEMOTACTIC PEPTIDE IN HL-60 GRANULOCYTES

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Activation of phospholipase D (PLD) has been investigated in dimethylsulfoxide differentiated HL-60 granulocytes labeled in endogenous 1-0-alkyl-2-acyl-sn-glycero-3-phosphocholine (alkyl-PC) by incubation with [^{3}H]alkyl-lysoPC. Stimulation of these labeled cells with the chemotactic peptide, N-formyl-Met-Leu-Phe (fMLP), induces rapid generation of [^{3}H]phosphatidic acid (PA) and slower formation of [^{3}H]diglyceride, suggesting hydrolysis of alkyl-PC by PLD. A unique feature of PLD is its ability to transfer the phosphatidyl moiety of phospholipids to alcohols (transphosphatidylation). This characteristic has been exploited to identify PLD activity. For example, when ethanol is present during stimulation of the HL-60 cells, [^{3}H]phosphatidylethanol (PEt) is formed with a concomitant decrease in [^{3}H]PA. Cells incubated with [^{32}P]orthophosphate to label the terminal phosphate of ATP do not incorporate ^{32}P into PEt, consistent with the [^{3}H]PEt not being synthesized from [^{3}H]diglyceride. In contrast, [^{3}H]PA arises from both PLD and diglyceride kinase activities. Furthermore, PEt synthesis closely parallels PA formation and both are inhibited by an fMLP receptor antagonist, suggesting that both PA and PEt are derived from agonist-stimulated PLD action. These observations are consistent with phospholipase D-catalyzed breakdown of alkyl-PC in fMLP- stimulated granulocytes. © 1988 Academic Press, Inc.

Receptor-mediated phospholipase C activation leads to the breakdown of phosphoinositides to generate inositol phosphates and diacylglycerols (DG) (1,2). Alternatively, DG can be derived from the action of phospholipase C on phosphatidylcholine (3-6) and/or de novo synthesis (7). DG can subsequently be phosphorylated by DG kinase to generate PA (8). In addition to DG kinase action on DG, PA could be formed directly from phospholipids by the action of phospholipase D (PLD) (9,10,11). PLD (E.C. 3.1.4.4) can catalyze hydrolytic cleavage of the terminal diester bond of glycerophosphatides with the formation of PA and appropriate bases. In addition, PLD possesses the unique ability to catalyze a transphosphatidylation reaction in which the phosphatidyl group of phosphatidylcholine or other phospholipids is transferred to

Abbreviations: DG, diglyceride; PA, phosphatidic acid; PLD, phospholipase D; PEt, phosphatidylethanol; BSA, bovine serum albumin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; alkyl-PC, 1-0-alkyl-2-acyl-sn-glycero-3-phosphocholine. Alkyl-lysoPC, 1-0-alkyl-2-lyso-sn-glycero-3-phosphocholine.

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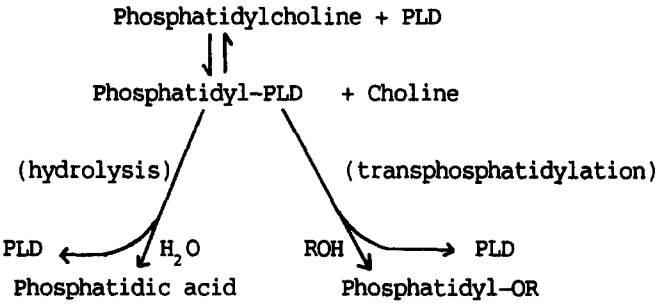
appropriate nucleophiles such as ethanol producing phosphatidylethanol (PEt) (12,13). As shown in the following scheme, both hydrolysis and transphosphatidylation occur via a common phosphatidyl-PLD complex formed by the nucleophilic attack by PLD at the phosphorus of the substrate.



Because the transphosphatidylation reaction is unique to PLD, the formation of phosphatidylalcohols is a useful indicator of PLD activity in intact cells. In this report, we demonstrate that stimulation of dimethyl-sulfoxide differentiated HL-60 granulocytes with fMLP in the presence of ethanol generates PEt in close parallel with PA formation and that PEt formation is not a consequence of de novo synthesis from DG. These observations demonstrate receptor-linked activation of PLD in HL-60 granulocytes.

MATERIALS & METHODS

Materials: [^{3}H]alkyl-lysoPC [lyso-platelet activating factor, (1-O-[^{3}H]octadecyl), 92 Ci/mmol] and [^{32}P]orthophosphate (carrier-free) were purchased from Amersham, Arlington Heights, IL. Various unlabeled phospholipids were from Avanti Polar Lipids, Birmingham, AL. Precoated silica gel G plates (0.25 mm thick) were purchased from Brinkmann Instruments, Westbury, NY. All other reagents including fatty acid-free BSA, cytochalasin B, fMLP, phospholipase A_2 from Naja Naja, and phospholipase C from Clostridium perfringens were purchased from Sigma, St. Louis, MO.

Chemical synthesis of PEt and lysoPEt: Synthetic PEt was prepared chemically as described (14) with slight modifications. Briefly, [1-palmitoyl-2-oleoyl]-PA (25 mg) was mixed with ethanol (100 ul), pyridine (1.25 ml) and trichloroacetonitrile (0.5 ml). The reaction mixture was refluxed at 60°C for 40 h. Pyridine was removed under vacuum and the residue was extracted. PEt was purified by thin-layer chromatography using solvent system I (see below). The structure of PEt was confirmed by fast atom bombardment spectrometry. The molecular ions, $(M+H)^+$, $(M+Na)^+$ and $(M+K)^+$, were observed with respective mass-to-charge (m/z) ratios of 703, 725 and 741. The major ion fragment was present at m/z of 577 (M-125), corresponding to the loss of $\cdot OPO(OH)OCH_2CH_3$. The fatty acid composition was confirmed by m/z ratios of 313 $(CH_2OHCHOHCH_2OCOC_{15}H_{31})^+$ and 339 $(CH_2OHCHOCOC_{17}H_{33}CH_2OH)^+$.

PEt was treated with phospholipase A_2 as described (15) and the resulting lysoPEt was separated by thin-layer chromatography using solvent system II (see below).

Preparation of HL-60 granulocytes: Human promyelocytic leukemic (HL-60) cells were grown in suspension culture and differentiated by culturing in the presence of 1.3% dimethylsulfoxide as described (15,16).

Labeling of HL-60 granulocytes: Differentiated HL60 cells were washed and resuspended at 2 x 10^7 cells/ml in Hepes-saline-BSA buffer which contained

Hepes (25 mM, pH 7.2), NaCl (125 mM), $MgCl_2$ (0.7 mM), [ethylenebis(oxyethylenitrolo)]tetra-acetic acid (0.5 mM), glucose (10 mM) and fatty acid-free BSA (1 mg/ml). [^{3}H]alkyl-lysoPC was added to the suspension at a final concentration of 5 μCi/ml. [^{32}P]orthophosphate was also added to some incubations at a final concentration of 0.1 mCi/ml. The suspension was incubated for 75 min at 37°C, then the cells were washed twice with Hepes-saline-BSA buffer and finally resuspended in the same buffer at 2 X 10^7 cells/ml. Analysis of the lipids from labeled cells revealed that 90% of the ^{3}H was associated with PC and the remainder with lysoPC (5%) and neutral lipids (5%). Eighty percent of the ^{3}H was retained by the cells. Inositol lipids contained no detectable ^{3}H. In contrast, the ^{32}P incorporated into lipids was primarily in inositol phospholipids and PA. Little or no ^{32}P was incorporated into phosphatidylcholine or other major phospholipids. Under these labeling conditions, the cellular ATP pool also became labeled with ^{32}P (9).

Incubation conditions: Cytochalasin B and fMLP were initially dissolved in dimethylsulfoxide and then diluted with buffer to appropriate concentrations. The final concentration of dimethylsulfoxide in assays did not exceed 0.1%, which had no discernible effects on the parameters being measured. The assay mixtures containing 10^7 prelabeled cells, 1.5 mM $CaCl_2$ and 5 μM cytochalasin B in a total volume of 950 μl Hepes- saline-BSA buffer were incubated at 37°C for 5 min. before initiating the reaction by adding buffer or fMLP with or without 0.5% ethanol, in a volume of 50 μl. After appropriate times, the reaction was stopped by adding chloroform/methanol/acetic acid (100:200:4, by volume) and standard samples (10 μg each) of PA and PEt were added.

Lipid extraction and thin-layer chromatography: The phases were separated by the procedure of Bligh and Dyer (17). The lower chloroform phase was dried and spotted on silica gel G plates. The plates were developed twice using the organic phase of isooctane/ethyl acetate/acetic acid/water (110:50:20:100, by volume) (solvent system I). In this system, PA (R_f = 0.1) and PEt (R_f = 0.33) were separated from each other and from neutral lipids (R_f = 0.7-0.95) and major phospholipids (R_f = 0). However, triglycerides, diglyceride and fatty acids were not separated sufficiently to allow quantitation (Fig. 1). In some experiments, a different solvent system consisting of chloroform/methanol/acetic acid (65:15:5, by volume) (solvent system II) was used to separate PEt (R_f = 0.65) from PA (R_f = 0.45) and other phospholipids (R_f = 0-0.2). DG (R_f = 0.27) was separated from monoglyceride (R_f = 0.1) and triglyceride (R_f = 0.85) using hexane/ diethyl ether/methanol/acetic acid (90:20:3:2, by volume) (solvent system III). The lipids were located by staining with iodine vapor and the silica gel areas containing PA and PEt were quantitated by liquid scintillation spectrometry.

Data presentation: Assays were performed at least in duplicate. Each set of experiments was performed at least three times. The data presented are from representative experiments.

RESULTS

Formation of [^{3}H]PA and [^{3}H]PEt by stimulated granulocytes: In order to detect the possible activation of PLD, HL-60 granulocytes were labeled in alkyl-PC by incubating with [^{3}H]alkyl-lysoPC (16). These labeled cells were then stimulated with fMLP and cytochalasin B (18) in the absence or the presence of 0.5% ethanol. In the absence of ethanol, stimulated cells produced [^{3}H]PA while in the presence of ethanol an additional ^{3}H-labeled product appeared (Fig. 1). This ^{3}H-labeled product comigrated with synthetic PEt in solvent system I (Fig. 1) as well as in solvent system II (data not shown). The putative [^{3}H]PEt was eluted from the silica (19) and treated with either phospholipase A_2 or phospholipase C, which hydrolyzed this product to compounds that comigrated with synthetic lysoPEt (R_f = 0.25 in solvent system

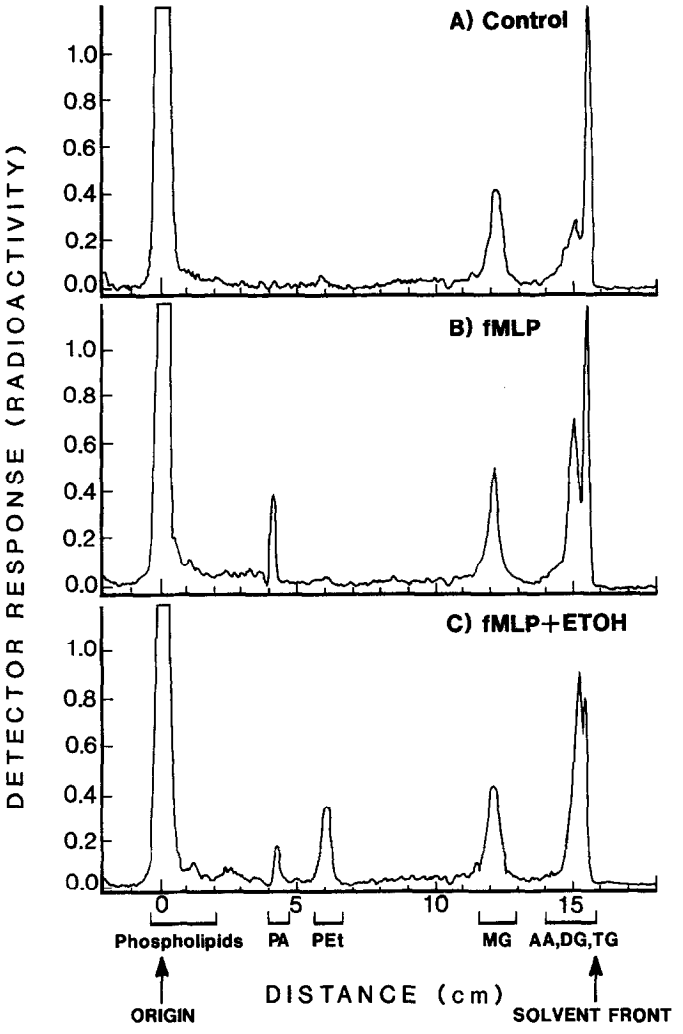


Fig. 1. Formation of [^{3}H]PA and [^{3}H]PEt by fMLP-stimulated HL-60 granulocytes: Samples (1 ml) containing 10^7 ^{3}H-labeled HL-60 granulocytes, 1.5 mM $CaCl_2$ and 5 μM cytochalasin B were preincubated for 5 min before adding 0.5% ethanol only (panel A) or fMLP (100 nM) in the absence (panel B) or the presence (panel C) of ethanol (0.5%). After 30 s, the reaction was stopped and standard lipids were added. Following extraction, lipids were separated by thin-layer chromatography using solvent system I. Chromatograms were scanned for ^{3}H radioactivity using a plate scanner (1M3000,RITA) and standard lipids were located by exposure to iodine vapor. MG, monoglyceride; TG, triglyceride; AA, arachidonic acid. For other details, see "Experimental Procedures".

II) and DG, (R_f = 0.27 in solvent system III), respectively. These data are consistent with the ^{3}H-labeled product being PEt.

[^{3}H]PEt formation was directly dependent on ethanol concentration (data not shown). Negligible amounts were detected in the absence of ethanol. Maximum PEt formation occurred at 0.5% ethanol. When ethanol was replaced by methanol, propanol or butanol, the corresponding phosphatidylalcohols were

formed, as indicated by their chromatographic mobilities that increased with increasing chain length of the alcohol. In solvent system I, the R_f values for various phosphatidylalcohols formed by fMLP-stimulated cells in the presence of methanol, ethanol, propanol and butanol were 0.26, 0.31, 0.36 and 0.41, respectively. These results indicate that upon stimulation of granulocytes with fMLP in the presence of an alcohol, phosphatidylalcohols are generated, presumably as a result of PLD-mediated transphosphatidylation.

Kinetics of formation of [^{3}H]PA and [^{3}H]PEt and inhibition by t-Boc-Met-Leu-Phe: Illustrated in Fig. 2 are the time-courses for the formation of [^{3}H]PA and [^{3}H]PEt in the absence and the presence of ethanol. In the absence of ethanol, PA was formed rapidly, reaching a maximum within 30 s and decreasing rapidly thereafter (upper panel). As found previously, (10,11), DG was formed less rapidly. In the presence of ethanol, [^{3}H]PEt was also formed and its appearance closely paralleled [^{3}H]PA formation (lower panel). However, unlike [^{3}H]PA, the [^{3}H]PEt levels remained virtually unchanged for 5 min. No change in the [^{3}H]alkyl-PC was detected during the course of the incubation (data not shown) because even 30 s after stimulation, [^{3}H]PA (15,000 dpm/assay) accounted for only about 2% of the [^{3}H]alkyl-PC (820,000 dpm/assay). As shown in Fig. 3, the fMLP dose-response for [^{3}H]PA was very similar to that for [^{3}H]PEt. Furthermore, both products were similarly inhibited by the specific fMLP antagonist, t-Boc-Met-Leu-Phe (Fig. 4). These results suggest that PLD is linked to receptor. In addition, [^{3}H]PA formed in the presence of ethanol was substantially less than that formed in the absence of ethanol (Fig. 2 and Fig. 3), suggesting that PLD is responsible for forming a substantial percentage of the [^{3}H]PA.

Mechanisms for PEt formation in granulocytes: [^{3}H]PEt could be synthesized from [^{3}H]DG via de novo mechanisms analogous to those that synthesize phosphatidylethanolamine and phosphatidylinositol. One possible mechanism would involve phosphorylation of ethanol by a kinase to phosphoethanol (20), followed by the formation of CDP-ethanol. PEt could then be synthesized by the transfer of phosphoethanol to DG. The other mechanism would DG phosphorylation of [^{3}H]DG to PA by DG kinase with incorporation of the terminal phosphate of ATP, followed by the formation of CDP-DG. Ethanol could then be added by a phosphatidyl transferase to generate PEt. Both of these mechanisms would result in the incorporation of the terminal phosphate of ATP into PEt.

To examine these possibilities, cells were incubated with [^{3}H]alkyl-lysoPC and [^{32}P]orthophosphate to label alkyl-PC and ATP, respectively. As described under "Experimental Procedures", the terminal phosphate of ATP was labeled with [^{32}P] (9), and the small amounts of ^{32}P incorporated into phospholipids was almost exclusively in inositol phospholipids rather than in PC, where 90% of the ^{3}H label was introduced. In the absence of fMLP, some ^{3}H and ^{32}P was found in PA; however, following fMLP addition, the amount of both

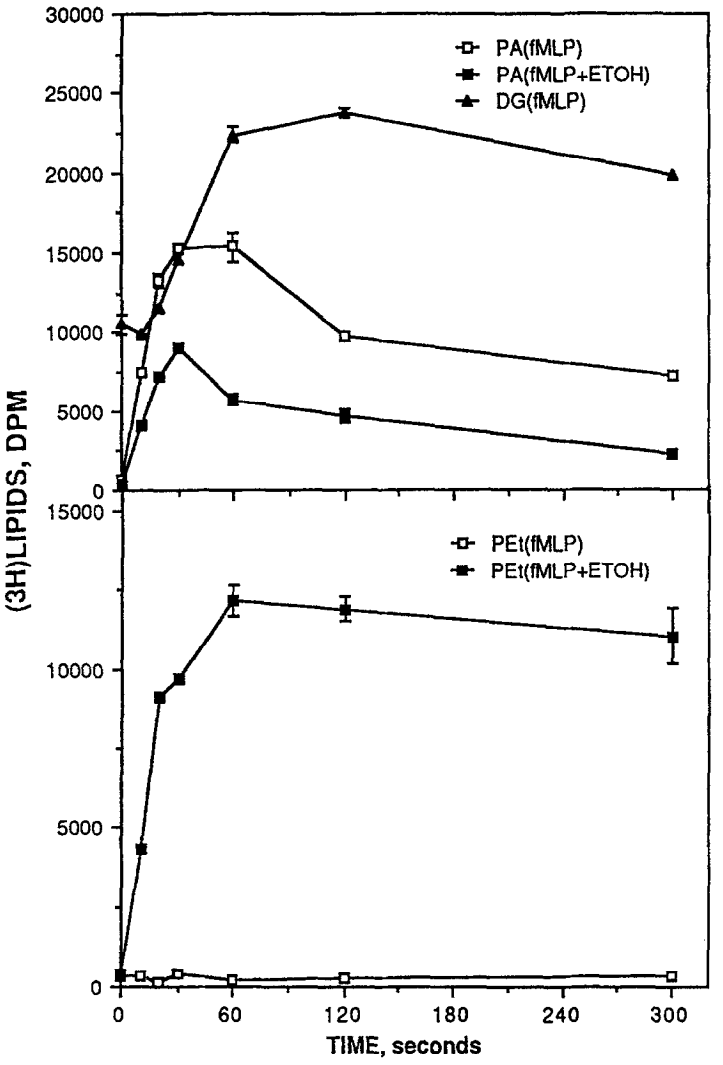


Fig. 2. Time-course of formation of [^{3}H]PA, [^{3}H]PEt and [^{3}H]DG by fMLP-stimulated HL-60 granulocytes: Using the methodology described in Fig. 1, samples were preincubated for 5 min before adding fMLP (100 nM) for various times in the absence or the presence of 0.5% ethanol. [^{3}H]PA and [^{3}H]DG (upper panel) and [^{3}H]PEt (lower panel) were separated by thin-layer chromatography using solvent systems I and III and the radioactivity was quantified by liquid scintillation spectrometry. Control (unstimulated) values for PA, DG and PEt remained unchanged throughout the incubation period. Values are the average of two determinations, which were within ± 5% of the mean.

labels in PA increased substantially (Table 1). The increased ^{32}P incorporation into PA indicates stimulated PA formation via DG kinase and/or de novo mechanisms.

As described in Figure 1, fMLP also stimulated the formation of PEt. Although this PEt contained ^{3}H, there was no significant incorporation of ^{32}P (Table 1), indicating that neither DG kinase nor a putative ethanol kinase was involved in PEt synthesis. Hence, the PEt arose from agonist-induced PLD

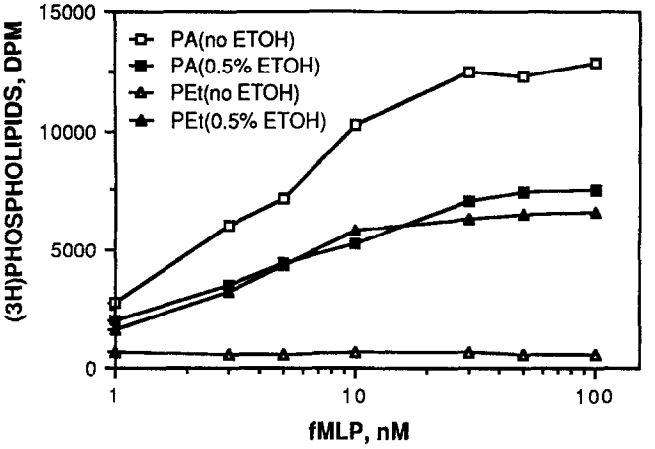


Fig. 3. Dose-response of fMLP-stimulated formation of PA and PEt by HL-60granulocytes: As in Fig. 1, duplicate samples were stimulated for 30 s with fMLP at the indicated concentrations in the absence or presence of 0.5% ethanol and analyzed for PA and PEt as described in Fig. 1 and under "Experimental Procedures".

action. The further observations that fMLP-induced [^{3}H]PA formation decreased in the presence of ethanol while [^{32}P]PA remained constant indicate that much of the PA arose from PLD metabolism of alkyl-PC, while the remainder came from the combined activities of phospholipase C and DG kinase or from de novo synthesis.

DISCUSSION

Phosphorylation of DG by DG kinase as a route to the formation of PA in intact cells has been described extensively. In this pathway, the diglyceride

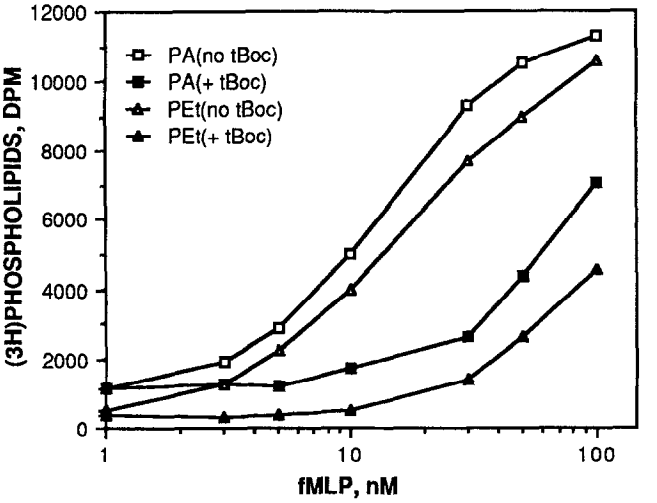


Fig. 4. Inhibition by t-Boc-Met-Leu-Phe of the formation of PA and PEt in fMLP-stimulated HL-60 granulocytes: Using the methodology described in Fig. 1, duplicate samples were pretreated with buffer or with 10 μM t-Boc-Met-Leu-Phe (t-Boc) for 5 min before stimulating with various concentrations of fMLP for 30 s in the presence of 0.5% ethanol.

Table 1. fMLP-induced incorporation of ^{3}H and ^{32}P into PA and PEt
HL60 granulocytes were double labeled with ^{3}H and ^{32}P as described under "Experimental Procedures". All other conditions are as described in Fig. 1.

Additions	PA		PEt	
	^{3}H	^{32}P	^{3}H	^{32}P
	dpm			
None	300	1400	700	300
Ethanol	300	1400	700	300
fMLP	3900	3500	1100	300
fMLP + Ethanol	2200	3500	3800	500

substrate for the kinase reaction could be formed either by de novo synthesis from glycerol or by the action of phospholipase C on phospholipids. Nevertheless, a PLD linked to receptor activation either directly or through intracellular signaling would generate the same products (PA directly and DG through PA phosphohydrolase (21)). This manuscript describes the pathways for PA formation in dimethylsulfoxide-differentiated HL-60 cells and utilizes the formation of PEt to help identify PLD activity in these intact cells.

In HL-60 cells, alkyl-PC constitutes about 20% of the total phospholipids (15). Following fMLP stimulation of these cells labeled in [^{3}H]alkyl-PC, rapid synthesis of PEt (in the presence of 0.5% ethanol) occurs (Figs. 2 and 3). This PEt synthesis does not occur via de novo pathways (Table 1). A role for base exchange in PEt formation is also unlikely, because, in contrast to PLD, the base exchange enzyme does not utilize ethanol as a substrate (22). Thus, in stimulated granulocytes, PEt is formed exclusively by PLD.

Both PA formation and PEt synthesis occur at similar rates (Fig. 2 and 3) and both are inhibited by an fMLP receptor antagonist (Fig. 4). In addition, PEt synthesis is accompanied by reduced accumulation of PA (Fig. 1-3 and Table 1). These observations suggest that both PA and PEt are formed by the action of PLD via a common phosphatidyl-PLD intermediate. The slower rate of [^{3}H]DG appearance is also consistent with PLD activity, where PA is the primary product followed by PA phosphohydrolase-catalyzed formation of DG (21). Although PEt derives exclusively from PLD, PA is also formed by phosphorylation of DG by DG kinase, as indicated by increased incorporation of ^{32}P into PA (Table 1). Additional experiments are in progress to determine the relative contributions of PLD and DG kinase to the net PA accumulation. It remains to be determined whether phospholipids other than alkyl-PC also degraded by PLD.

Although PLD has been studied primarily in higher plant tissues (23-26), several recent reports (10,27-29) have confirmed the existence of PLD in

mammalian systems as well. PLD activities have been studied in rat brain, both in microsomes and in a partially purified preparation (27,28). A PLD activity has also been detected in human eosinophils (29), a cell system related to dimethylsulfoxide-differentiated HL-60 cells. Most recently, PLD activity has been implicated in PA formation by vasopressin-stimulated hepatocytes and guanine nucleotide-sensitive PLD activity has been identified in hepatocyte membranes (11). In addition, PEt is formed in organs from ethanol intoxicated rats (30,31) and phorbol esters, in the presence of ethanol, stimulate PEt synthesis in bovine lymphocytes (19). This report provides the first evidence for receptor- linked activation of PLD in HL-60 cells.

The importance of PLD activity to neutrophil stimulation remains to be explored. It may be simply another route for forming diglycerides. On the other hand, PA possesses potent ionophoretic properties in liposome preparations (32) and releases Ca^{2+} from platelet microsomes (33). The rapidity of PA formation by PLD is consistent with a possible role for PA in Ca^{2+} mobilization in stimulated neutrophils.

In summary, the present study demonstrates receptor-linked activation of PLD in fMLP-stimulated granulocytes. In addition, the data strongly suggest that PEt formation is a valid measure of PLD activity in intact cells. Extensions of these studies to other similar cells and phospholipid substrates are underway.

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REFERENCES

1. Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81-147.
2. Hokin, L.E. (1985) Annu. Rev. Biochem. 54, 205-235.
3. Grove, R.I., and Schimmel, S.D. (1982) Biochim. Biophys. Acta 711, 272-280.
4. Daniel, L.W., Waite, M., and Wykle, R.L. (1986) J. Biol. Chem. 261, 9128-9132.
5. Besterman, J.M., Duronio, V., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. USA 83, 6785-6789.
6. Muir, J.G., and Murray, A.W. (1987) J. Cell. Physiol. 130, 382-391.
7. Farese, R.V., Konda, T.S., Davis, J.S., Standaert, M.L., Pollet, R.J., and Cooper, D.R. (1987) Science 236, 586-589.
8. Raetz, C.R.H. (1978) Microbiol. Rev. 42, 614-659.
9. Cockroft, S. (1984) Biochim. Biophys. Acta 795, 37-46.
10. Cockroft, S., and Allan, D. (1984) Biochem. J. 222, 557-559.
11. Bocckino, S.B., Blackmore, P.F., Wilson, P.B. and Exton, J.H. (1987) J. Biol. Chem. 262, 15309-15315.
12. Yang, S., Freer, S., and Benson, A. (1967) J. Biol. Chem. 242, 477-484.
13. Dawson, R. (1967) Biochem. J. 102, 205-210.
14. Bittman, R., Rosenthal, A.R., and Vargas, L.A. (1984) Chem. Phys. Lipids 34, 201-205.

15. Billah, M.M., Eckel, S., Myers, R.F., and Siegel, M.I. (1986) J. Biol Chem. 261, 5824-5831.
16. Collins, S.J., Ruscelli, F.W., Gallagher, R.E., and Gallo, R.C. (1979) J. Exp. Med. 149, 969-974.
17. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
18. Honeycutt, P., and Niedel, J. (1986) J. Biol. Chem. 261, 15900-15905.
19. Pai, J.-K., Liebl, E.C., Tettenborn, C.S., Ikegwuonu, F.I., and Mueller, G.C. (1987) Carcinogenesis 8, 173-178.
20. Tomaszewski, M., and Buchowicz, J. (1972) Biochem. J. 129, 183-186.
21. Brindley, D.N. (1984) Prog. Lipid Res. 23, 115-133.
22. Kanfer, J.N. (1980) Can. J. Biochem. 58, 1370-1380.
23. Hanahan, D.J., and Chaikoff, I.L. (1947) J. Biol. CHem. 168, 233-240.
24. Ferrari, R.A., and Benson, A.A. (1961) Arch. Biochim. Biophys., 93, 185-192.
25. Sastry, P.S., and Kates, M. (1965) Can. J. Biochem. 43, 1445-1453.
26. Heller, M. (1978) Adv. Lipid Res. 16, 267-326.
27. Chalifour, R., and Kanfer, J.N. (1982) J. Neurochem. 39, 299-305.
28. Taki, T., and Kanfer, J.N. (1979) J. Biol. Chem. 254, 9701-9765.
29. Kater, L.A., Goetzl, E.J., and Austen, K.F. (1976) J. Clin. Invest. 57, 1173-1180.
30. Alling, C., Gustavsson, L., and Anggard, E. (1983) FEBS Lett. 152, 24-28.
31. Alling, C. Gustavsson, L., Mansson, J.-E., Benthin, G., and Anggard, E. (1984) Biochim. Biophys. Acta 793, 119-122.
32. Serhan, C., Anderson, P., Goodman, E., Dunham, P. and Weissman, G. (1981) J. Biol. Chem., 256, 2736-2741.
33. Gerrard, J.M., Butler, A.M., Peterson, S.A., and White, J.G. (1978) Prostaglandins Med. 1, 387-396.