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PHOSPHOLIPASE D IN HOMOGENATES FROM HL-60 GRANULOCYTES: IMPLICATIONS OF CALCIUM AND G PROTEIN CONTROL

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Occupancy of chemotactic peptide receptors leads to rapid initiation of phospholipase D (PLD) activity in intact dimethylsulfoxide-differentiated HL-60 granulocytes (Pai, J.-K, Siegel, M.I., Egan, R.W., and Billah, M.M. (1988) J. Biol. Chem. 263, 12472). To gain further insight into the activation mechanisms, PLD has been studied in cell lysates from HL-60 granulocytes, using 1-O-alkyl-2-oleoyl-[^{32}P]phosphatidylcholine (alkyl-[^{32}P]PC), 1-O-[^{3}H]alkyl-2-oleoyl-phosphatidylcholine ([^{3}H]alkyl-PC) and [^{14}C]arachidonyl-phospholipids as substrates. In the presence of Ca^{2+} and GTPγS, post-nuclear homogenates degrade alkyl-[^{32}P]PC to produce 1-O-alkyl-[^{32}P]phosphatidic acid (alkyl-[^{32}P]-PA), and in the presence of ethanol, also 1-O-alkyl-[^{32}P]phosphatidylethanol (alkyl-[^{32}P]PEt). By comparing the $^{3}H/^{32}P$ ratios of PA and PEt to that of PC, it is concluded that PA and PEt are formed exclusively by a PLD that catalyzes both hydrolysis and transphosphatidylation between PC and ethanol. Furthermore, PC containing either ester- or ether-linkage at the sn-1 position is degraded in preference to phosphatidylethanolamine and phosphatidylinositol by PLD in HL-60 cell homogenates. It is concluded that HL-60 granulocytes contain a PC-specific PLD that requires both Ca^{2+} and GTP for activation.

Recent studies indicate that PLD (1), like phospholipases A_2 and C, is activated in intact cells in response to specific stimulation (2-11). For instance, the chemotactic peptide, fMet-Leu-Phe, induces PLD in dimethylsulfoxide-differentiated HL-60 granulocytes (2,3). In those experiments (3), HL-60 granulocytes have been labeled selectively with ^{32}P in endogenous alkyl-PC in the absence of [^{32}P]ATP. Upon stimulation with fMLP, they produce [^{32}P]PA and [^{32}P]PEt as a result of PLD-catalyzed hydrolysis and transphosphatidylation, respectively. This PLD activation requires extracellular Ca^{2+} and is inhibited by pertussis toxin pretreatment. Studies using cell-free preparations from rat hepatocytes and undifferentiated HL-60 cells suggest possible involvement of GTP-binding proteins in PLD activation (4,12).

ABBREVIATIONS

PLD, phospholipase D; alkyl-PC, 1-O-alkyl-2-oleoyl-sn-glycero-3-phosphocholine; GTPγS, guanosine 5'-O-(3-thiotriphosphate); PA, phosphatidic acid; PEt, phosphatidylethanol; PC, phosphatidylcholine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, [ethylene bis(oxyethylenitrolo)]tetraacetic acid; GppNHp, guanosine 5'-(β,γ-imidotriphosphate).

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However, no information regarding the interactions between Ca^{2+} and putative GTP-bindng proteins in the activation of granulocyte PLD is available.

To gain further insight into the mechanism of PLD activation in HL-60 granulocytes and to determine its substrate specificity, we have developed a PLD assay in cell homogenates. Using exogenous alkyl-[^{32}P]PC and [^{3}H]alkyl-PC prepared synthetically in a novel series of enzymatic and chemical steps, we conclusively demonstrate in this report that cell lysates of HL-60 granulocytes produce PA and PEt by PLD and that the PLD activity, which exclusively utilizes PC as substrate, requires both Ca^{2+} and GTPγS.

MATERIALS AND METHODS

MATERIALS: [^{3}H]alkyl-lysoPC (90-120 Ci/mmol), 1-stearoyl-2-[^{3}H]arachidonyl-PC (117 Ci/mmol), 1-stearoyl-2-[^{14}C]-arachidonyl-PC (58 mCi/mmol), 1-stearoyl-2-[^{14}C]arachidonyl-phosphatidylethanolamine (58 mCi/mmol) and 1-stearoyl-2-[^{14}C] arachidonyl-phosphatidylinositol (58 mCi/mmol) were purchased from Amersham, Arlington Heights, IL. [γ-^{32}P]ATP (4500 Ci/mmol) was obtained from ICN, Irvine, CA. The unlabeled lipids were from Avanti Polar Lipids, Birmingham, AL. Precoated silica gel G plates were purchased from Brinkman Instruments, Westbury, NY. Precoated silica gel K6 plates were from Whatman. Pyridine, 2,4,6-triisopropylbenzenesulfonyl chloride and 4-(N,N-dimethylamino)-pyridine were from Aldrich, Milwaukee, WI. Oleic anhydride was from NU-Chek Prep, Elysian, MN. Guanine nucleotides were from Boehringer-Mannheim. Choline tosylate was purchased from Synthon, Houston, TX. Synthetic PEt was prepared as described (7). All other reagents were from Sigma, St. Louis, MO.

Preparation of Alkyl-[^{32}P]PC and [^{3}H]Alkyl-PC: Alkyl-[^{32}P]lysoPC was prepared as previously described (3) except that choline acetate was replaced with choline tosylate. Alkyl-PC was synthesized by acylation of alkyl-lysoPC with oleic anhydride. Alkyl-lysoPC containing either ^{3}H or ^{32}P was adjusted to a specific activity of 50 mCi/mmol by adding unlabeled alkyl-lysoPC and dried under a flow of nitrogen. The dried lysophospholipid was mixed with 10 mg of fresh oleic acid anhydride and 3 mg of dry 4-(N,N-dimethylamino)pyridine and the mixture was dissolved in 100 ul of anhydrous $CHCl_3$ (13). The container vial was purged with N_2 and tightly capped. After 3 days in the dark at 25°C, the reaction was stopped by adding 0.75 ml of chloroform/methanol/acetic acid (100:200:4) and 0.25 ml of 1N HCl. A two phase system was obtained by adding 200 μl H_2O and 250 μl $CHCl_3$. The lipids in the lower chloroform phase were separated by TLC using chloroform/ methanol/acetic acid/water (50:30:8:3, by volume). Alkyl-[^{32}P]PC was eluted from the silica, dissolved in toluene/ ethanol (1:1, by volume) and stored at -20°C under N_2. The structure of alkyl-PC thus synthesized was analyzed by fast atom bombardment mass spectrometry and assigned a molecular weight of 746. Conversion and recovery of alkyl-lysoPC to alkyl-PC was typically greater than 80%. Phospholipid phosphorous was quantitated (14) to obtain the specific activities of radiolabeled phospholipids.

Preparation of HL-60 Granulocyte Lysates: Cell culture and differentiation of HL-60 cells was performed as described previously (15). Cells were harvested by centrifugation (4°C) at 500 x g for 5 min and washed twice in a Hepes buffer (25 mM Hepes, pH 7.4, 125 mM NaCl, 2.5 mM KCl, 0.7 mM $MgCl_2$, 0.5 mM EGTA, and 10 mM glucose). Washed cells were resuspended in a buffer consisting of 100 mM KCl, 50 mM Hepes (pH 7.2), 5 mM NaCl, 0.5 mM EGTA, and 3.5 mM $MgCl_2$. The cell suspension (25 X 10^6/ml) was disrupted for 10 s in a sonic dismembrator model 300 (Fisher) with a prechilled intermediate probe set at 60. The sonicate was centrifuged (4°C) at 500 xg for 7 min, and nuclei and unbroken cells were discarded. The resulting homogenate was employed in the assays. Protein was determined by the method of Bradford (16), using BSA as a standard.

Incubation Conditions and Lipid Extraction: Assays in a total volume of 200 μl were initiated by the addition of 50 μl of homogenate (200 μg protein) to tubes containing assay buffer consisting of 100 mM KCl, 5 mM NaCl, 50 mM Hepes

(pH 7.2), 5 mM $CaCl_2$, 8.5 mM $MgCl_2$ and various other additions as appropriate. Substrate was prepared by evaporating the solvent under a flow of N_2, and dissolving in 100% ethanol by vigorous vortexing for 30 s. This suspension was diluted with an appropriate volume of distilled water and aliquots of 10 μl were added immediately before the start of the assay. In experiments where no ethanol was added, substrate in solvent was added to assay tubes and dried under a stream of nitrogen. Substrate was resuspended by addition of assay buffer and vortexing. The reaction was stopped by addition of chloroform/methanol/acetic acid (100:200:4, by volume). Following addition of 10 μg each of standard PA, PEt, and 1,2-diacylglycerol, the phases were separated by the procedure of Bligh and Dyer (17). Extraction efficiencies were > 80%.

Lipid Analysis by Thin-Layer Chromatography: The lower chloroform phase was dried and spotted on silica gel K6 plates (Whatman). PA and PEt were separated employing a solvent system consisting of the organic phase of ethyl acetate/isooctane/acetic acid/water (110:50:20:100, by volume). The lipids were located by staining with iodine vapor and the appropriate areas were scraped and quantified by liquid scintillation spectrometry. Counting efficiencies for ^3H and ^{32}P were 42% and 90%, respectively.

Data Presentation: Assays were done in duplicate. Each set of experiments was performed at least three times. The data presented are from representative experiments, where the average of duplicates varied less than 10% from the mean.

RESULTS

PLD Activity in HL-60 Granulocyte Homogenates: When post-nuclear homogenates prepared from dimethylsulfoxide-differentiated HL-60 granulocytes were incubated at 37°C with synthetic alkyl-[^{32}P]PC in the presence of Ca^{2+}, little or no formation of alkyl-[^{32}P]PA was observed. However, when the incubation

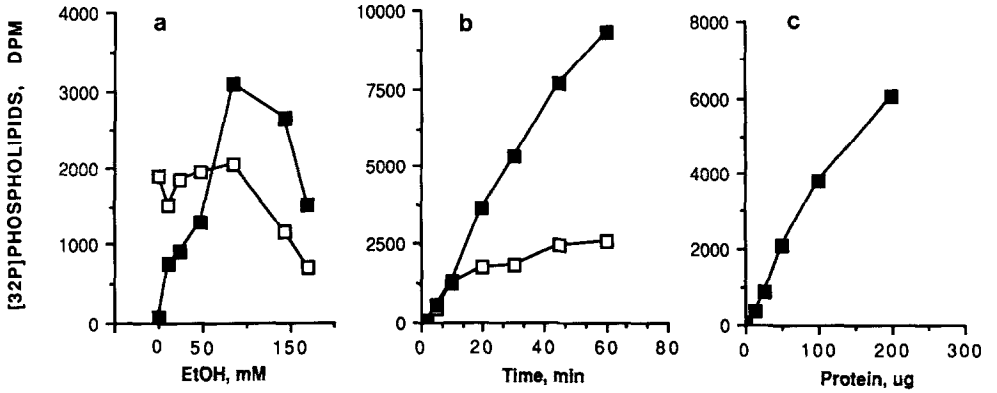

Figure 1. Formation of Alkyl-[^{32}P]PA and Alkyl-[^{32}P]PEt by Homogenates from HL-60 Granulocytes. Duplicate assays in a final volume of 200 μl of assay buffer contained 100 μM GTPγS. The assay mixtures also contained ethanol (EtOH) at various concentrations (a) or at a fixed concentration of 86 mM (b,c), postnuclear homogenate at various amounts (c) or at a fixed amount of 200 μg protein (a,b) and alkyl-[^{32}P]PC at either 50 μM (4 x 10^5 dpm) (a) or 90 μM (5.3 x 10^5 dpm) (b,c). The reaction was initiated by adding homogenates. Following incubation at 37°C for 30 min (a,c) or for various times (b), the reaction was stopped by adding chloroform/methanol/acetic acid and standards of PA and PEt. Following extraction, alkyl-[^{32}P]PA (□), and alkyl-[^{32}P]PEt (■) were located by autoradiography. Standards were visualized by staining with iodine vapor. Appropriate areas were scraped and the radioactivity was quantified by liquid scintillation spectrometry.

mixtures contained both Ca^{2+} and GTPγS (see below), alkyl-[^{32}P]PA was formed in substantial amounts (Fig. 1a). Because [^{32}P]PA could only be formed from [^{32}P]PC by the hydrolytic action of PLD, these results conclusively demonstrate the presence of PLD activity in this cell-free preparation. When ethanol was included in the incubation mixtures containing Ca^{2+} and GTPγS, an additional [^{32}P]phospholipid was formed (Fig. 1a). This lipid was identified as alkyl-[^{32}P]PEt by chromatographic mobilities and enzymatic and chemical modifications (3). Formation of [^{32}P]PEt was dependent on ethanol concentration up to a maximum at 86 mM (0.5%). At higher concentrations of ethanol (e.g. 175 mM), homogenates produced less of both [^{32}P]PA and [^{32}P]PEt. These data suggest that PLD-catalyzed transphosphatidylation between PC and ethanol produced PEt. Under standard conditions (100 μM GTPγS, 5 mM Ca^{2+}, 86 mM ethanol, 200 μg of protein), [^{32}P]PA increased for 10 min and then plateaued (Fig 1b). In contrast, the formation of PEt, which is not metabolized substantially, continued linearly up to 60 min (Fig. 1b). PEt formation by homogenates was proportional to protein concentration between 10 and 200 μg (Fig. 1c).

PA is Formed Exclusively by PLD in HL-60 Granulocyte Homogenates: When both [^{3}H]alkyl-PC and alkyl-[^{32}P]PC were added simultaneously to the standard incubation mixtures containing Ca^{2+}, GTPγS and ethanol, homogenates generated PA and PEt, containing both ^{3}H and ^{32}P. Although [^{32}P]PA would be formed exclusively by PLD, [^{3}H]PA could also have been formed by diglyceride kinase-catalyzed phosphorylation of the [^{3}H]diglyceride produced by phospholipase C action on [^{3}H]PC. Thus, a ^{3}H/^{32}P ratio in PA higher than that in PC would indicate a contribution of the phospholipase C/diglyceride kinase pathway to PA formation. As shown in Table I, the ^{3}H/^{32}P ratios of PA and PEt were similar to each other and to that of PC, demonstrating that radiolabeled PA and PEt were formed exclusively by PLD action on PC.

Table I ^{3}H/^{32}P Ratios in Alkyl-PC, Alkyl-PA and Alkyl-PEt Formed by Homogenates of HL-60 Granulocytes

Phospholipids	^{3}H	^{32}P	^{3}H/^{32}P
	(dpm x 10^{-3})		
Alkyl-PC	439 ± 35	186 ± 13	2.4
Alkyl-PA	3.8 ± 0.04	1.8 ± 0.2	2.1
Alkyl-PEt	4.1 ± 0.4	1.9 ± 0.2	2.2

Samples containing homogenate (200 μg protein), 5 mM $CaCl_2$, 100 μM GTPγS, 86 mM ethanol and a mixture of [^{3}H]alkyl-PC and alkyl-[^{32}P]PC were incubated for 30 min. Following separation by TLC, lipids were extracted from silica and quantified by liquid scintillation spectrometry. Values for alkyl-PC were obtained from zero time control samples. Values presented are the mean ± SE of five determinations.

Table II Substrate Specificity of PLD in HL-60 Granulocyte Homogenates

Addition	Substrate	[^{14}C]PEt
		dpm
EGTA (0.5 mM)	PC	0
	PE	0
	PI	140
Ca^{2+} (5 mM)	PC	100
	PE	0
	PI	120
EGTA (0.5 mM) + GTPγS (100 μM)	PC	323
	PE	0
	PI	86
Ca^{2+} (5 mM) + GTPγS (100 μM)	PC	2520
	PE	120
	PI	130

Duplicate samples containing homogenates (200 μg protein), 86 mM ethanol and 20 μM (5 x 10^5 dpm) of either [^{14}C]diacyl-phosphatidylcholine, [^{14}C]diacyl-phosphatidylethanolamine (PE), or [^{14}C]diacyl-phosphatidylinositol (PI) and indicated additions were incubated at 37°C for 30 min. Reactions were stopped and standard lipids added. [^{14}C]PEt and [^{14}C]PA were separated by TLC as described under "Methods". Lipids were visualized by iodine staining and areas corresponding to authentic PA and PEt were scraped and quantified by liquid scintillation spectrometry. Counting efficiency for ^{14}C was 87%. Appropriate controls with boiled homogenate were performed.

Phosphatidylcholine is the Preferred Substrate for PLD Activity in HL-60 Granulocyte Homogenates: To determine whether phospholipids other than alkyl-PC were also substrates for PLD, diacyl-PC, diacyl-phosphatidylethanolamine and diacyl-phosphatydlyinositol, each containing [^{14}C]arachidonate at the sn-2 position, were incubated separately with the homogenate. Under standard conditions of Ca^{2+}, GTPγS and ethanol, diacyl-PC was effectively utilized as a substrate for PLD as measured by [^{14}C]PEt formation (Table II). These data indicate that PLD utilized not only 1-ether-PC (alkyl-PC), but also 1-ester-PC (diacyl-PC) in a Ca^{2+}- and GTPγS-dependent reaction. On the other hand, phosphatidylethanolamine and phosphatidylinositol were not degraded by PLD.

Role of Ca^{2+} and GTP on PLD Activity by HL-60 Granulocyte Homogenates: Illustrated in Fig. 2 is the effect of Ca^{2+} on PLD activity in homogenates containing GTPγS. In the presence of EGTA and no added Ca^{2+}, neither [^{32}P]PA nor [^{32}P]PEt was formed. However, when Ca^{2+} was added in excess of EGTA, both PA and PEt were formed in the presence but not in the absence of GTPγS. Substantial PLD activity was detected at 100 μM Ca^{2+}, with maximal activity occurring at 1 mM Ca^{2+}.

In the presence of 5 mM Ca^{2+}, nonhydrolyzable guanine nucleotides caused dose-dependent formation of PEt (Fig 3). GTPγS was much more effective than GppNHp. GTP was without effect, probably due to rapid hydrolysis.

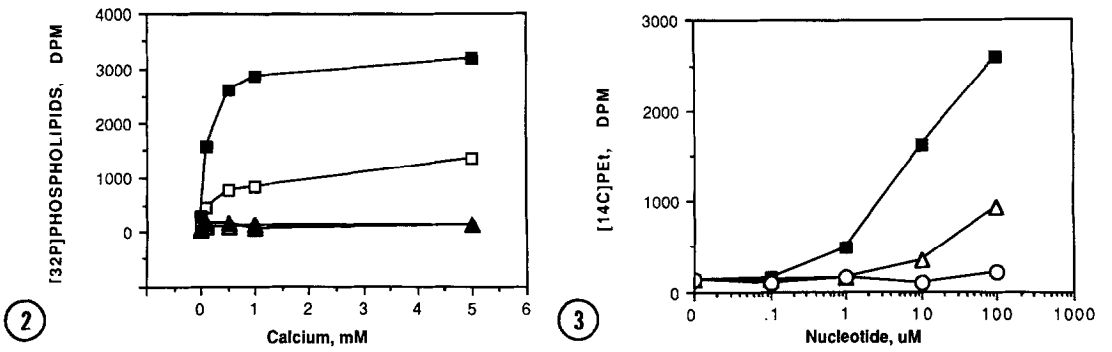

Figure 2. Effect of Ca^{2+} on Alkyl-[^{32}P]PA and Alkyl-[^{32}P]PEt Formation by Homogenates from HL-60 granulocytes. Duplicate samples (200 μl) containing 200 μg of homogenate protein, 50 μM alkyl-[^{32}P]PC (4 X 10^5 dpm), 86 mM ethanol and either EGTA (0.5 mM) and no added $CaCl_2$ or the indicated Ca^{2+} concentrations in excess of EGTA were incubated in the absence or the presence of 100 μM GTPγS for 30 min at 37°C. Alkyl-[^{32}P]PA (□,△) and alkyl-[^{32}P]PEt (■,▲) formed in the absence (△,▲) or the presence (□,■) of GTPγS were quantified as described in Fig. 1.

Figure 3. Effects of Guanine Nucleotides on [^{14}C]PEt Formation by Homogenates from HL-60 Granulocytes. Duplicate samples containing 200 μg of homogenate protein, 5 mM $CaCl_2$, 20 μM (5 x 10^5 dpm) [^{14}C]PC 86 mM ethanol and various concentrations of either GTPγS (■), GppNHp (△) or GTP (○) were incubated for 30 min. [^{14}C]PEt was quantified as described in "Experimental Procedures".

DISCUSSION

In the present study, alkyl-[^{32}P]PC has been synthesized in a novel series of enzymatic and chemical steps. This ^{32}P-labeled phospholipid is degraded by HL-60 cell homogenates to produce [^{32}P]PA and [^{32}P]PEt (Fig. 1), providing conclusive proof for cell-free PLD hydrolysis and transphosphatidylation activities. By several different criteria (time-course, Ca^{2+} concentration, requirement for GTPγS), PA formation is accompanied by PEt generation supporting our previous contention from studies with intact cells (2,3) that both hydrolysis and transphosphatidylation are catalyzed by a common PLD activity. Therefore, PEt is a useful indicator of PLD activity in cell-free preparations as well as intact cells. From studies using alkyl-PC containing ^{3}H and ^{32}P to demonstrate that the ^{3}H/^{32}P ratio of PA and PEt are the same as that of PC (Table I), it is, therefore, concluded that PA and PEt are products of PLD exclusively in granulocyte homogenates.

Previous studies with intact granulocytes have shown that alkyl-PC is hydrolyzed by PLD (2,3). The present experiments with granulocyte homogenates now show that diacyl-PC can also be degraded by PLD (Table II). However, no GTPγS stimulatable PLD activity has been detected against phosphatidylinositol or phosphatidylethanolamine (Table II). That phosphatidylinositol is not a major substrate for PLD is further supported by the previous observation that

in neutrophils, the fatty acid composition of PA formed during stimulation differs from that of phosphatidylinositol (18). Similar substrate specificity for PC is exhibited by GTPγS-stimulated PLD in rat hepatocyte membranes (4). Apparently, it is the phospholipid base and not the nature of the linkage at the sn-1 position that determines substrate selectivity of these PLDs.

The combined presence of GTPγS and Ca^{2+} is obligatory for maximal expression of PLD in these cell-free preparations (Fig. 3). Neither GTPγS nor Ca^{2+} by itself can support the activity. These results are in agreement with our previous findings (3) that in chemotactic peptide-stimulated intact granulocytes, PLD activity requires the presence of extracellular Ca^{2+} and that pertussis toxin inhibits this activation. Granulocyte PLD differs from hepatocyte PLD in that the latter can be activated with GTPγS in the absence of Ca^{2+} (4,5). However, partially purified PLD from rat brain can hydrolyze both PC and phosphatidylethanolamine (1). Furthermore, a recent report suggests that a post-nuclear fraction of A23187 stimulated human neutrophils might degrade phosphatidylinositol via a PLD activity (19).

In conclusion, these data demonstrate that dimethylsulfoxide-differentiated HL-60 granulocyte lysates contain a PLD activity which catalyzes preferential degradation of PC and requires both Ca^{2+} and GTP for activation.

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