Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 236-243

GRANULOCYTE PHOSPHOLIPASE D IS ACTIVATED BY A GUANINE NUCLEOTIDE DEPENDENT PROTEIN FACTOR

John C. Anthes, Peng Wang, Marvin I. Siegel,
Robert W. Egan and M. Motasim Billah

Department of Allergy and Immunology
Schering-Plough Research
Bloomfield, New Jersey 07003

Received December 19, 1990

When post-nuclear homogenates from HL-60 granulocytes are incubated in the presence of $CaCl_2$, GTPγS and ethanol, phospholipase D (PLD) metabolizes both exogenous 2-[^{14}C]arachidonyl-phosphatidylcholine and endogenous phosphatidyl[^{3}H]choline to produce 2-[^{14}C]arachidonyl-phosphatidic acid, 2-[^{14}C]arachidonyl-phosphatidylethanol and [^{3}H]choline. Fractionation of the homogenate by ultracentrifugation into cytosolic and membrane fractions results in the loss of PLD activity. However, when these two fractions are combined in the same proportion as found in the unfractionated homogenate, PLD activity is completely restored. This activity is proportional to the concentration of both the cytosol and the particulate fractions. Release of [^{14}C]arachidonate by PLA_2 that occurs under these assay conditions does not require the combined presence of cytosol and membrane fractions. We conclude that, in granulocyte homogenates, PLD activity but not arachidonate release, exhibits an essential requirement for a heat-labile factor whose activity depends on the presence of GTPγS.

Receptor-linked activation of phospholipase D (PLD) has been demonstrated in various cells (see ref. 1 for review) including granulocytes (2-4), hepatocytes (5), endothelial cells (6), neuroblastoma cells (7) and REF52 rat embryo cells (8). PLD acts preferentially on phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline (4,5). The PA is further metabolized by PA phosphohydrolase to form diglyceride (DG)

Abbreviations:

PLD, Phospholipase D; PLA2, phospholipase A2; DG, diglyceride; GTPγS, guanosine 5'-0-(3-thiotriphosphate); PA, phosphatidic acid; PEt, phosphatidylethanol; PC, phosphatidylcholine; Hepes, N-2-hydroxy-ethylpiperazine-N^{1}-2-ethanesulfonic acid; EGTA, [ethylene bis(oxyethylenitrolo)]tetraacetic acid; AA, arachidonic acid.

0006-291X/91 $1.50

(3,4). This PLD pathway serves as the major route to PA and DG formation in neutrophils that are stimulated with C5a or fMet-Leu-Phe (3,4), underscoring the potential importance of PLD in signal transduction. PLD also catalyzes a transfer of the phosphatidyl moiety of PC to primary alcohols to form phosphatidylalcohols (1-4,6,7).
This transphosphatidylation reaction provides a simple means with which to study PLD activity in intact cells and in cell-free preparations.

The mechanism by which PLD is activated during cell stimulation is poorly understood. Studies with cell-free preparations from rat liver, rat brain and endothelial cells have demonstrated that PLD is associated with cell membranes (5,6,9) and that this PLD is activated by GTPγS in liver and endothelial cell systems (5,6). These results suggest that the membrane-bound PLD is linked to surface receptors through GTP-binding regulatory proteins.

Recently, it has been demonstrated that homogenates from HL-60 granulocytes contain a PLD that is stimulated by the combined presence of Ca^{2+} and GTPγS (10,11). We now demonstrate that this Ca^{2+}/GTPγS-stimulated PLD can be detected in neither the cytosol or the membrane fraction alone and that its expression requires the intact homogenate. These studies demonstrate for the first time that PLD is a multicomponent system and that these components are physically separable.

MATERIALS AND METHODS

Materials: 1-stearoyl-2-[^{14}C]arachidonyl-PC (58 mCi/mmol) and [methyl-^{3}H]choline chloride (74-85 Ci/mmol) were purchased from Amersham, Arlington Heights, IL. Unlabeled lipids were from Avanti Polar Lipids, Birmingham, AL. Precoated silica gel K6 plates were purchased from Whatman, Clifton, NJ. GTPγS was purchased from Boehringer-Mannheim, Indianapolis, IN.
Preparation and prelabeling of cells with [^{3}H]choline: Cell culture and differentiation of HL-60 cells were performed as described previously (12). Cells (1-2 x 10^6 cells/ml) in culture media were incubated with [^{3}H]choline (2 μCi/ml) during the entire differentiation period. Under these conditions, 90% of the lipid-associated ^{3}H was recovered in PC (1 uCi/mg protein) with the remainder being associated with lyso-PC and sphingomyelin.
Preparation of HL-60 granulocyte homogenates, cytosol and membranes: Cells were harvested by centrifugation at 500 x g for 5 min and washed twice in a NaCl-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 KCl-Hepes 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 (2.5-10 x 10^7 cells/ ml) was sonicated for 10 s in a sonic

dismembrator model 300 (Fisher) with a prechilled intermediate probe set at 60. The sonicate was centrifuged at 500 x g for 7 min, and nuclei and unbroken cells were discarded. This post-nuclear supernatant (termed "homogenate") was centrifuged for 90 min at 100,000 x g (minimum) (4°C). The supernatant (termed "cytosol") was collected and the pellet (termed "membrane") was washed and resuspended in the KCl-Hepes buffer. The pellets obtained from [^{3}H]choline-labeled cells were washed with KCl-Hepes buffer containing 5 mM choline chloride. Protein was determined by the method of Bradford (13) using bovine serum albumin as a standard.

Incubation conditions and lipid extraction: Assays in a total volume of 200 ul were initiated by the addition of 50 ul of either homogenate, cytosol, membrane or cytosol plus membrane to assay mixtures that contained 100 mM KCl, 5 mM NaCl, 50 mM Hepes (pH 7.2), 5 mM $CaCl_2$, 8.5 mM $MgCl_2$, 100 uM GTPγS, 0.5% ethanol and [^{14}C]PC. 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 (10 ul) were added to the assay mixtures immediately before the start of the assay. The reaction was stopped by the addition of chloroform/methanol/acetic acid (100:200:4). Following addition of 10 ug each of standard PA, PEt and AA, the phases were separated by the procedure of Bligh and Dyer (14).

Separation of labeled metabolites by thin-layer chromatography: The lower chloroform phase was dried and spotted on silica gel K6 plates. A double development protocol was employed to allow the separation of PA, PEt and AA. PA (R_f=0.1) and PEt (R_f=0.27) were separated from each other and from other lipids by developing the plates halfway using the upper phase of the solvent system consisting of ethyl acetate/isocotane/acetic acid/water (110:50:20:100, by volume) (2). After drying, the plates were developed again to the top using a solvent system consisting of benzene/diethyl ether/ethyl acetate/acetic acid (80:10:10:2, by volume) (15). This system resolved AA from other neutral lipids without effecting the migration of PA and PEt. The lipids were located by staining with iodine vapor and the appropriate areas were scraped and quantified by liquid scintillation spectrometry.

[^{3}H]Choline was separated from [^{3}H]glycerophosphocholine and [^{3}H]phosphocholine as described (16). Briefly, aliquots of upper aqueous phase was applied to a Bio-Rex 70 cation exchange resin and rinsed with 10 ml of water. [^{3}H]Choline was eluted with 10 mls of 50 mM glycine/500 mM NaCL (pH 3.0). Under these conditions, only [^{3}H]choline was retained by the cation exchange column whereas [^{3}H]GPC and [^{3}H]phosphocholine were eluted in the water rinse.

RESULTS

When post-nuclear homogenates from HL-60 granulocytes were incubated with Ca^{2+}, GTPγS and ethanol, exogenously added [^{14}C]PC was degraded by a PLD activity to produce [^{14}C]PA and [^{14}C]PEt (Table 1).

Table 1

Distribution of PLD Activity in Homogenates of HL-60 Granulocytes

Fraction	[^{14}C]PA	[^{14}C]PEt
	dpm	dpm
Homogenate	1570	3740
Cytosol (160 μg)	270	300
Membrane (40 μg)	280	260
Cytosol (160 μg) + Membrane (40 μg)	1970	3370
Boiled Homogenate	240	160
Boiled Cytosol	190	110
Boiled Membrane	150	170
Cytosol (160 μg) + Boiled Membrane (40 μg)	190	240
Membrane (40 μg) + Boiled Cytosol (160 μg)	370	240

Duplicate assays in a final volume of 200 μl of assay buffer contained 5 mM $CaCl_2$, 100 μM GTPγS, 0.5% ethanol and 20 μM (5 x 10^5 dpm) [^{14}C]PC. Reactions were initiated by adding the indicated amounts of either homogenate, membrane, cytosol, or membrane plus cytosol. Following incubation at 37°C for 30 min, reactions were stopped by adding chloroform/ methanol/ acetic acid. Following extraction, PA and PEt were separated by thin-layer chromatography, and located by autoradiography and by staining with iodine vapor. Appropriate areas were scraped and radioactivity quantified by liquid scintillation spectrometry.

When assayed under indentical conditions, the cytosolic and particulate fractions obtained by centrifuging the homogenate at 100,000 x g produced [^{14}C]PA and [^{14}C]PEt in amounts that were 10-20% of those formed by the unfractionated homogenates (Table 1). However, when these two fractions were mixed in the same proportion as the homogenate, the formation of [^{14}C]PA and [^{14}C]PEt was completely restored (Table 1), suggesting that both the cytosol and the membrane are required for optimal GTPγS-stimulated PLD activity. Boiling the homogenate destroyed the PLD activity. Furthermore, addition of either boiled membranes to the cytosolic fraction or boiled cytosol to the membrane fraction did not reinstate PLD activity. These results indicate that the factor involved in PLD expression is a protein.

Fig. 1 demonstrates the effects of increasing concentrations of cytosol on the formation of [^{14}C]PA, [^{14}C]PEt and [^{14}C]AA at two membrane concentrations. Cytosol by itself produced [^{14}C]AA but not [^{14}C]PA or [^{14}C]PEt. [^{14}C]AA release was linear with protein concentration. The membrane fraction by itself also produced [^{14}C]AA in amounts comparable

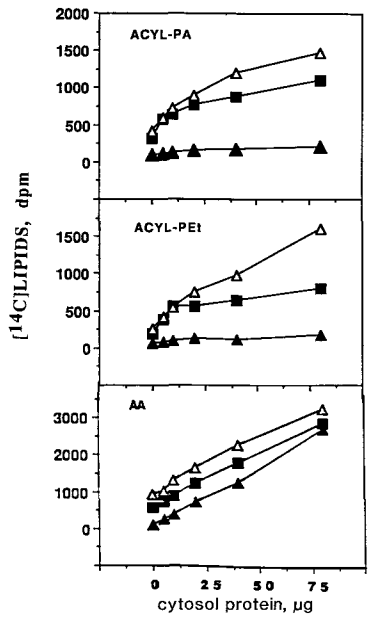

Figure 1. Effects of mixing various proportions of membranes and cytosol on the formation of [^{14}C]PA, [^{14}C]PEt and [^{14}C]AA. Assay mixtures (200 µl) contained 5 mM $CaCl_2$, 100 µM GTPγS, and indicated amounts of cytosol without (▲) or with 20 µg (■) and 40 µg (Δ) of membrane protein. Reactions were initiated by adding 20 µM [^{14}C]PC. After 30 min at 37°C, reactions were stopped and [^{14}C]PA, [^{14}C]PEt and [^{14}C]AA were separated and quantitated as described under "Materials and Methods".

to those by the cytosol. [^{14}C]PA and [^{14}C]PEt were also formed by the membrane fraction, but in small amounts. Addition of the cytosol to the membrane increased PLD activity synergistically. This activity was proportional to the concentration of both membrane and cytosol protein. By contrast, the mixture of cytosol and membrane fractions produced [^{14}C]AA in an additive manner. These results dissociate arachidonate release from PLD activation and suggest that these biochemical processes are under distinct regulatory mechanisms.

In separate experiments, the endogenous PC of HL-60 granulocytes was labeled with [^{3}H]choline. Membranes prepared from these ^{3}H-labeled cells produced [^{3}H]choline when incubated in the presence of Ca^{2+} and GTPγS (Fig. 2). This [^{3}H]choline formation was greatly enhanced by the addition of cytosol that was prepared from unlabeled cells. These results demonstrate that degradation of endogenous PC by PLD also requires the presence of the cytosolic fraction and that synergy between cytosol and membrane in expressing PLD activity is not an artifact of substrate presentation.

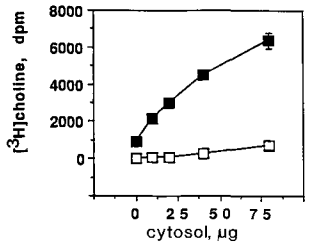

Figure 2. Effect of cytosol on the release of [^{3}H]choline from [^{3}H]choline-labeled membranes of HL-60 granulocytes. Duplicate incubations in a final volume of 200 µl of assay buffer containing 5 mM $CaCl_2$, 100 µM GTPγS, and the indicated amounts of cytosol with 40 µg of unboiled (■) or boiled (□) [^{3}H]choline-labeled membranes were performed for 30 min at 37°C. Reactions were stopped, and, after phase separation, the aqueous phase was analyzed for ^{3}H-labeled products using cation exchange chromatography as described under "Materials and Methods".

DISCUSSION

The present study demonstrates that the Ca^{2+}/GTPγS-stimulated PLD of granulocyte homogenate acts upon both endogenous and exogenous PC to produce PA and PEt, and that, with either type of substrate presentation, the combined presence of cytosol and membrane is required for PLD expression. These studies provide the first evidence that activation of granulocyte PLD requires a factor that is inactivated by heat. Although the localization of granulocyte PLD has yet to be established, in several other systems, PLD is associated with the membrane fraction (5,6,9). More significantly, in hepatocytes (5) and in endothelial cells (6), GTPγS stimulates membrane-associated PLD activities. Extending these considerations to granulocyte PLD, it may be suggested that the factor needed for PLD activation in granulocyte homogenates is of cytosolic origin.

Membrane-bound NADPH oxidase of neutrophils is also activated by GTPγS when membranes are incubated in the presence of cytosol (17,18). It is well-established that NADPH oxidase requires several cytosolic factors (19,20), one of which appears to be a G protein (18). The fact that a GTP analog is essential for PLD activity in granulocyte homogenates (10,11) indicates that the PLD-activating factor is a G protein.

Mobilization of AA from PC by cell-free preparations from granulocytes (Fig. 2) can occur by several pathways. A phospholipase A_2 can act directly on PC. Alternatively, PA and DG derived from PC by the PLD pathway may be further deacylated by specific lipases (21,22). That this latter PLD pathway is not involved in AA release is suggested by two lines of evidence. First, AA release occurs in the absence of detectable PLD

activity (Fig. 2) and second, the synergistic increase in PLD activity observed upon mixing cytosol with the membranes is not associated with similar increases in AA release. Thus, under the present assay conditions, it is likely that PC is degraded by phospholipase A_2 to produce AA. Because neither phospholipase A_2 (Fig. 1) nor phosphoinositide-specific phospholipase C (12) requires the combined presence of cytosol and membrane, it follows that the putative cytosolic factor probably acts specifically on PLD. Apparently, in granulocytes, phospholipases of different specificities are activated independently through distinct regulatory mechanisms.

ACKNOWLEDGMENTS

We thank Virginia Citarella and Lisa Ramirez for typing the manuscript.

REFERENCES

1. Billah, M.M, and Anthes, J.C. (1990) Biochem. J. 269, 281-291.
2. Pai, J.-K., Siegel, M.I., Egan, R.W., and Billah, M.M. (1988) J. Biol. Chem. 263, 12472-12477.
3. Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W., and Siegel, M.I. (1989) J. Biol. Chem. 264, 17069-17077.
4. Mullmann, T.J., Siegel, M.I., Egan, R.W., and Billah, M.M. (1990) J. Immunol. 144, 1901-1908.
5. Bocckino, S.P., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987) J. Biol. Chem. 262, 15309-15315.
6. Martin, T.W., and Michaelis, K.M. (1989) J. Biol. Chem. 264, 8847-8856.
7. Liscovitch, M., and Amsterdam, A. (1989) J. Biol. Chem. 264, 11762-11767.
8. Welsh, C.J., Cao, H-T., Chabbott, H., and Cabot, M.C. (1988) Biochem. Biophys. Res. Commun. 152, 565-572.
9. Kobayashi, M., and Kanfer, J.N. (1987) J. Neurochem. 48, 1597-1603.
10. Tettenborn, S.C., and Mueller, G.C. (1988) Biochem. Biophys. Res. Commun. 155, 249-255.
11. Anthes, J.C., Eckel, S., Siegel, M.I., Egan, R.W., and Billah, M.M. (1989) Biochem. Biophys. Res. Commun. 163, 657-664.
12. Anthes, J.C., Billah, M.M., Cali, A., Egan, R.W., and Siegel, M.I. (1987) Biochem. Biophys. Res. Commun. 145, 825-833.
13. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
14. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-919.
15. Storry, J.E., and Tuckley, B. (1967) Lipids 2, 501-502.
16. Martin, T.W., and Michaelis, K.C. (1988) Biochem. Biophys. Acta 962, 282-296.
17. Gabig, T., English, G.D., Akard, L.P., Schell, M.J. (1987) J. Biol. Chem. 262, 1685-1690.

18. Ligeti, E., Tardiff, M., and Vignais, P.V. (1989) Biochemistry 28, 7116-7123.
19. McPhail, L.C., Shirley, P.S., Clayton, C.C., and Snyderman, R. (1984) J. Clin. Invest. 75, 1735-1739.
20. Curnutte, J.T., Scott, P.J., Kuuer, R., and Berkow, R. (1987) J. Biol. Chem. 262, 5563-5569.
21. Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1981) J. Biol. Chem. 256, 5399-5405.
22. Prescott, S.M., and Majerus, P.W. (1983) J. Biol. Chem. 258, 764-769.