

Cardiolipin-Specific Phospholipase D Activity in *Haemophilus parainfluenzae*

YOSHIE ONO AND DAVID C. WHITE

Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40506

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A highly active phospholipase D that is specific for cardiolipin was detected in the gram-negative bacterium *Haemophilus parainfluenzae*. Previously reported phospholipase D preparations have come exclusively from higher plants. The bacterial enzyme hydrolyzed cardiolipin to phosphatidyl glycerol and phosphatidic acid. During the incubation, phosphatidic acid disappeared. Phosphatidyl ethanolamine, methylated phosphatidyl ethanolamines, phosphatidyl choline, and phosphatidyl glycerol were not hydrolyzed when cardiolipin was rapidly hydrolyzed.

Phospholipases A, B, and C have been reported in bacteria (8, 9, 14-16), but phospholipase D has hitherto been reported only in higher plants (17). In this study, a crude cell-free homogenate of *Haemophilus parainfluenzae* was shown to contain a highly active phospholipase D that acts on cardiolipin to liberate phosphatidyl glycerol and phosphatidic acid as initial products. The phosphatidic acid disappears with the formation of both water-soluble and nonextractable components as well as a trace of another lipid.

MATERIALS AND METHODS

Materials. The nonionic detergent Triton X-100 was obtained from Rohm and Haas (Philadelphia, Pa.). Methyl-¹⁴C-phosphatidyl choline and methylated phosphatidyl ethanolamines from yeast were generously supplied by Marion Steiner of this department. Other materials were as described previously (20-22).

Growth of bacteria. *H. parainfluenzae* was grown in 2.5-liter low-form Erlenmeyer flasks containing 1.7 liters of proteose peptone medium (19) to the mid-exponential phase [0.32 mg (dry weight) per ml] and was harvested by centrifugation (20). *Escherichia coli* K-12 was grown in phosphate-deficient medium (13) with 10 mc of H₃³²PO₄ per 250 ml for 15 hr.

Enzyme preparation. *H. parainfluenzae* (1.02 g, dry weight) was suspended in 50 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM MgCl₂. The suspension was treated with sound at the maximal level of an LS-75 Sonifier (Branson Ultrasonic Corp., Stamford, Conn.) for 5 min with the temperature maintained at less than 8°C. The suspension was diluted to 100 ml with the phosphate buffer and was centrifuged at 4,300 × *g* for 10 min at 4°C. The pellet was discarded, and the supernatant fluid (between 4 and 5 mg of protein per ml) was used without further purification.

Substrate preparation. Lipids were extracted from *E. coli* by the procedure of Bligh and Dyer (1), and

the cardiolipin, phosphatidyl glycerol, and phosphatidyl ethanolamine were separated by thin-layer chromatography (22). Silica gel G thin-layer plates 75 mm thick were spotted along one edge with the lipid mixture (17 nmoles per spot). The plates were developed by ascending chromatography with chloroform-methanol-water (70:25:30, v/v) and were covered with Saran wrap. The lipids were demonstrated by radioautography. Bands corresponding to cardiolipin, phosphatidyl ethanolamine, and phosphatidyl glycerol were recovered from the silica gel, and each band was rechromatographed with a solvent system of chloroform-methanol-water-14.5 M ammonium hydroxide (140:50:7:1, v/v). After recovery from the silica gel, each fraction was partitioned between chloroform and aqueous methanol by the addition of methanol and water (final ratios: chloroform-methanol-water, 1:1:0.9, v/v).

A portion of the phosphatidyl ethanolamine was deacylated by mild alkaline methanolysis, and the glycerolphosphoryl ethanolamine (GPE) was chromatographed in two dimensions on amino-cellulose paper (20). The GPE contained 5% glycerol-phosphoryl glycerol (GPG) derived from phosphatidyl glycerol. A portion of the phosphatidyl glycerol was deacylated, and the GPG was chromatographed on amino-cellulose paper. The GPG contained 1% GPE and 99% GPG. A portion of the cardiolipin was deacylated, and the water-soluble ester was chromatographed on amino-cellulose paper. The diglycerol phosphoryl glycerol (GPGPG) derived from cardiolipin contained 5% GPE but no GPG.

Substrate emulsions were prepared by adding 1 ml of a 2% (w/v) Triton X-100 aqueous solution to 5 μmoles of cardiolipin that had been deposited on the bottom of a test tube by evaporation of the solvent in a stream of nitrogen. An emulsion that was stable for at least 2 days was made by agitating the tube on a vortex mixer for 60 min. The reaction was started by the addition of 50 μliters of the lipid emulsion to 1 ml of homogenate from *H. parainfluenzae* incubated at 37°C.

At intervals, tubes were placed in crushed ice, and 2.5 ml of methanol and 1.25 ml of chloroform were added with mixing (Vortex Mixer, Scientific Industries, Inc., Springfield, Mass.). After extraction for 1 hr, 1.25 ml chloroform and 1.25 ml water were added and mixed. The mixture was allowed to stand overnight at 4 C and then was centrifuged at $500 \times g$ for 10 min to separate the layers. Radioactivity was determined in the water and chloroform layers. The chloroform layer was mixed with unlabeled phosphatidyl ethanolamine, phosphatidyl glycerol, and cardiolipin (0.5 μ mole each) derived from *E. coli*, and a portion was separated on silica gel-impregnated paper (Whatman SG-81) as described previously (23). Another portion was deacylated by mild alkaline methanolysis (20), and the glycerol phosphate esters derived from the lipids were applied to amino-cellulose paper (Whatman AE-81) and cellulose thin-layer plates (Eastman 6065). The esters were separated by two-dimensional chromatography on AE-81 paper (20), and the cellulose thin-layer plates were developed by ascending chromatography (2), as described previously.

Radioautograms were made with Kodak no-screen X-ray film, and the radioactivity in each spot was determined with a scintillation spectrometer (20). Protein was determined colorimetrically (12).

RESULTS

Identification of the products. Incubation of 32 P-labeled cardiolipin for 60 min with a homog-

enate of *H. parainfluenzae* resulted in nearly complete hydrolysis (Fig. 1). The products had the chromatographic mobility of phosphatidic acid and phosphatidyl glycerol. The phosphatidyl glycerol that was recovered represented 49% of the 32 P in the cardiolipin added to the homogenate, and the phosphatidic acid represented 25%. The cardiolipin was contaminated by 5% phosphatidyl ethanolamine that was not affected by the homogenate. About 72% of the 32 P that disappeared from the cardiolipin was recovered in the phosphatidic acid and phosphatidyl glycerol after 60 min. The remainder of the 32 P was recovered in the water phase and in the residue of the Bligh and Dyer extraction.

Portions of the lipid extracts used in Fig. 1 were deacylated, and the resulting glycerol phosphate esters were identified by their chromatographic mobility on amino-cellulose paper (Fig. 2). After 60-min hydrolysis, the lipids accounted for 77% of the 32 P present initially. The proportions of the lipids measured after deacylation were as follows: initially, 94.5% GPGPG derived from cardiolipin and 5.5% GPE derived from phosphatidyl ethanolamine; after 60 min, 0.9% glycerol phosphoryl glycerol phosphate, 2% GPGPG, 22% L- α -glycerol phosphate derived from phosphatidic

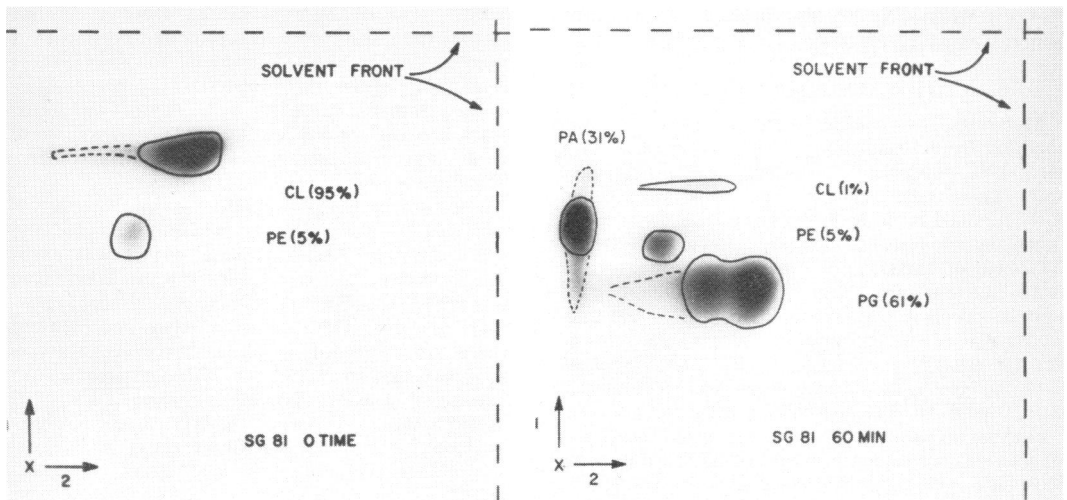


FIG. 1. Radioautogram after chromatographic separation of the phospholipid products of the *H. parainfluenzae* homogenate. The enzyme mixture was composed of 4.2 mg of protein in 1 ml of 50 mM phosphate buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.1% (w/v) Triton X-100, and 250 nmoles of 32 P-labeled cardiolipin (specific activity, 3,000 counts per min per nmole) held at 37 C. The reaction was started by the addition of 0.05 ml of an aqueous suspension of cardiolipin in 2% (w/v) Triton X-100. The reaction was stopped by placing the tubes in crushed ice and adding 2.5 ml of methanol; the lipids were extracted as described in Materials and Methods. The zero-time extract and the 60-min extract were separated on silica gel-impregnated paper (Whatman SG-81) with solvent system of chloroform-methanol-diisobutylketone-acetic acid-water (45:15:30:20:4, v/v) in the first dimension and chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium chloride, pH 10.4 (30:17.5:25:35:6, v/v) in the second dimension (23). CL = cardiolipin; PA = phosphatidic acid; PE = phosphatidyl ethanolamine; PG = phosphatidyl glycerol.

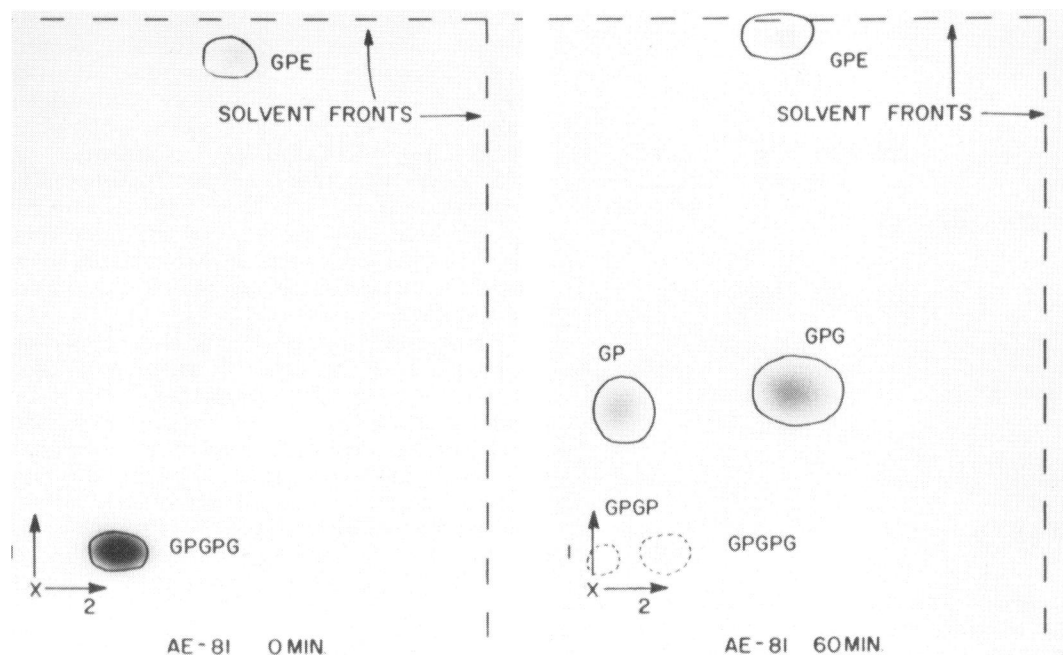


FIG. 2. Radioautogram of the glycerol phosphate esters derived from the lipids after hydrolysis by the *H. parainfluenzae* homogenate. The lipids were deacylated by mild alkaline methanolysis (20) and were separated on acid-washed amino-cellulose paper (Whatman AE-81). The solvent in the first dimension was 3 M formic acid containing 0.4% pyridine. The solvent in the second dimension was 1.15 M ammonium acetate containing 11.8 mM EDTA made to pH 5.0 with acetic acid and diluted 3:7 (v/v) with 95% ethanolic 0.26 M ammonium hydroxide. GP = L- α -glycerol phosphate; GPE = glycerolphosphoryl ethanolamine; GPG = glycerolphosphoryl glycerol; GPGP = glycerolphosphoryl glycerol phosphate; GPGPG = di-glycerolphosphoryl glycerol.

acid, 47% GPG derived from phosphatidyl glycerol, and 5.5% GPE. Another portion of the glycerol phosphate esters derived from the lipids was separated on cellulose thin-layer plates (Fig. 3). Esters with the chromatographic mobility of GPG and GP were detected after hydrolysis. These results suggested that the enzyme activity in the homogenate of *H. parainfluenzae* was phospholipase D.

Enzymatic activity. The rate of hydrolysis of cardiolipin (initial concentration, 40 nmoles per ml) was proportional to the concentration of homogenate from *H. parainfluenzae* between 20 and 200 μ g of protein per ml. A plot of the reciprocal of the initial rate of cardiolipin hydrolysis against the reciprocal of the substrate concentration between 30 and 240 nmoles of cardiolipin per ml was linear with an apparent K_m of 0.2 or 0.4 mM and a V_{max} of 20 nmoles of cardiolipin hydrolyzed per min per mg of protein. The pH optimum for cardiolipin hydrolysis was between 7.5 and 8.0 in 50 mM phosphate buffer containing 10 mM $MgCl_2$. At pH 5.5 in 50 mM acetate buffer, the activity was reduced by 50%. There was no activity in the absence of added $MgCl_2$. Maximal activity occurred in the presence

of 10 mM $MgCl_2$. Addition of 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM $MgCl_2$ completely inhibited the enzyme activity. Adding 1 mM $CaCl_2$ and 1 mM $MgCl_2$ did not increase the enzyme activity beyond that in 1 mM $MgCl_2$ alone.

Time course of the reaction of phospholipase D. The substrate and products of the incubation mixture have been identified chromatographically (Fig. 1-3). Figure 3 shows that cardiolipin was hydrolyzed to phosphatidyl glycerol (GPG) and phosphatidic acid (GP). The proportions of these lipids are illustrated in Fig. 4. The phosphatidyl glycerol accounts for half the ^{32}P which disappeared from the cardiolipin. The phosphatidic acid disappeared as the reaction proceeded, with an increase in water-soluble and nonextractable fractions.

Specificity of the phospholipase D. The phosphatidyl ethanolamine contaminating the cardiolipin preparation and the phosphatidyl glycerol resulting from the hydrolysis of cardiolipin apparently were not attacked by the enzyme (Fig. 1-3). Very little hydrolysis of phosphatidyl ethanolamine or phosphatidyl glycerol was detectable. After incubation of 175 nmoles of ^{32}P -

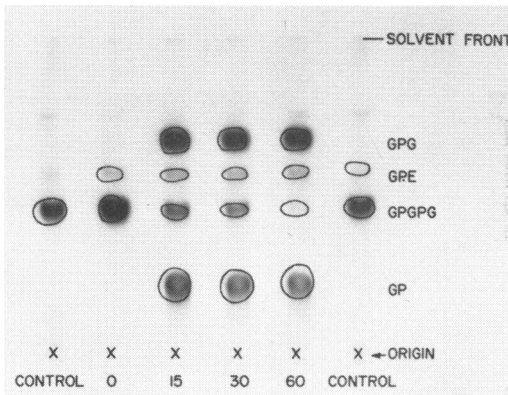


FIG. 3. Radioautogram of the chromatographic separation of the glycerol phosphate esters derived from the lipids after hydrolysis by *H. parainfluenzae* homogenate. The esters were separated on cellulose thin-layer plates (Eastman 6065) by ascending chromatography with a solvent of ethyl alcohol-0.5 M ammonium acetate (pH 7.5), 7:3, v/v (22). Controls without added enzyme were run at each end, as well as samples of 0-, 15-, 30-, and 60-min incubation with the enzyme. R_F values of authentic esters are given on the right. GP = L- α -glycerol phosphate; GPE = glycerolphosphoryl ethanolamine; GPG = glycerolphosphoryl glycerol; GPGPG = di-glycerolphosphoryl glycerol.

labeled phosphatidyl ethanolamine or phosphatidyl glycerol with the enzyme for 90 min, all of both compounds was recovered. Under these conditions, less than 10% of the cardiolipin was recovered after 15 min. A mixture of methyl- ^{14}C -labeled phosphatidyl choline (68%), phosphatidyl dimethyl ethanolamine (19%), phosphatidyl monomethylethanolamine (6%), and ergosterol (7%), containing a total of 28,000 counts/min in 100 nmoles, was incubated with the *H. parainfluenzae* homogenate for 90 min. No ^{14}C could be detected in the water-soluble portion of a Bligh and Dyer extract and all of the ^{14}C was recovered in the chloroform phase.

DISCUSSION

These results demonstrate that *H. parainfluenzae* has a cardiolipin-hydrolyzing enzyme. Phosphatidyl glycerol and phosphatidic acid are the hydrolysis products, indicating that the homogenate has phospholipase D activity. Incubation of the homogenate with other phospholipids suggests that this phospholipase D is remarkably specific for cardiolipin.

In the aerobic exponential phase of growth in *H. parainfluenzae*, the polar portions of the phospholipids turn over more rapidly than the diglyceride glycerols, although the proportions and amounts of each lipid remain constant (22).

Phosphatidyl glycerol is most actively metabolized, half of the radioactivity of the phosphate and unacylated glycerol moieties being lost in one division. Half of the radioactivity of the middle glycerol and the phosphate moieties of cardiolipin is lost in four divisions; radioactivity is not lost from the terminal glycerol moieties. The enzymes responsible for these activities have not as yet been characterized, and the cardiolipin-specific phospholipase D activity appears to be much more active than necessary to account for the turnover.

In the crude homogenate, the *H. parainfluenzae* phospholipase D activity is markedly different from the plant phospholipase D. The plant phospholipase D is activated by organic solvents (11), anionic amphipathic substances (5), calcium ions (3), and sonic dispersion of the substrates (5); it also has a remarkable transferase activity with primary alcohols (4, 7, 24). Plant phospholipase D has a pH optimum between 5 and 6, depending on the anionic amphipathic activator

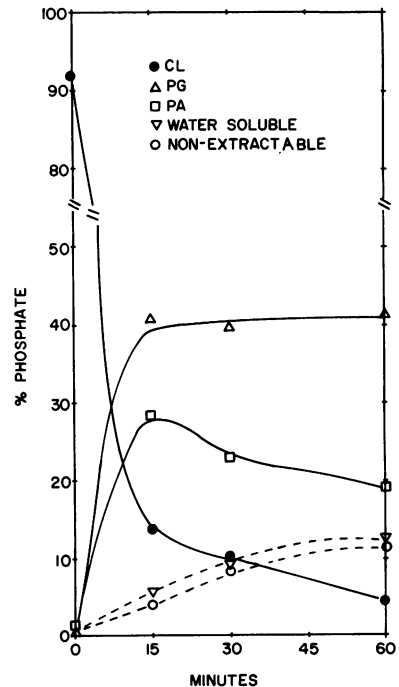


FIG. 4. Time course of the products of cardiolipin hydrolysis by the *H. parainfluenzae* homogenate. The reaction mixture described in Fig. 1 was assayed as in Fig. 3. The percentage of the total lipid phosphate added to the mixture was calculated from the ^{32}P . Symbols: ∇ , ^{32}P remaining in the water; \circ , ^{32}P remaining in the cell residue after extraction of the incubation mixture. CL = cardiolipin; PG = phosphatidyl glycerol; PA = phosphatidic acid.

(18). The *H. parainfluenzae* enzyme is active at pH 7.5 in the absence of added organic solvents, anionic amphipathic activators, or calcium ions. Phosphatidyl choline and phosphatidyl ethanolamine were not attacked at pH 5.5. The anionic amphipathic activator sodium dodecyl sulfate (1 mM) completely inhibited enzyme activity. In the presence of the crude homogenate, the hydrolysis of phosphatidyl ethanolamine or phosphatidyl glycerol by added plant phospholipase D depended on the addition of ether and calcium ions. The *H. parainfluenzae* homogenate was inactive in the absence of added $MgCl_2$. EDTA inhibited the *H. parainfluenzae* enzyme, and $CaCl_2$ had no effect in the presence of $MgCl_2$.

Phospholipase D purified from Brussels sprouts has been reported not to hydrolyze cardiolipin, phosphatidyl glycerol phosphate, acyl diglycerol phosphate, or bis-phosphatidic acid, even with high enzyme concentrations or long incubation periods (5, 6). The enzyme from peanut seeds will attack cardiolipin if diethyl ether is omitted and deoxycholate is added to the reaction mixture (10). The very crude homogenate of *H. parainfluenzae* has about the same enzymatic activity for cardiolipin as the partially purified peanut seed enzyme.

Present work is directed toward the purification of this enzyme and some understanding of its role in the metabolism of the membrane.

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