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Phospholipase D Activity of Gram-Negative Bacteria

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A phospholipase hydrolyzing cardiolipin to phosphatidic acid and phosphatidyl glycerol was characterized in gram-negative bacteria but was absent in preparations of gram-positive bacteria, *Saccharomyces cerevisiae*, and rat liver mitochondria. In cell-free extracts of *Escherichia coli*, *Salmonella typhimurium*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*, this cardiolipin-hydrolyzing enzyme had similar pH and Mg^{2+} requirements and displayed a specificity which excluded phosphatidyl glycerol and phosphatidyl ethanolamine as substrates.

Cardiolipin is a phosphoglyceride commonly found in membranes that carry out respiration and oxidative phosphorylation (11). This phosphatide is present in a wide variety of bacteria and may account for as much as 28% of the membrane lipids, depending on the organism and the conditions of incubation (4, 7, 16-19). Usually, adverse treatments such as those interfering with oxidative phosphorylation or energy metabolism (1, 8) cause cardiolipin to increase at the expense of phosphatidyl glycerol. In starved *Escherichia coli* cells transferred to a medium containing adequate energy supply, there is an adaptation period during which abnormally high levels of cardiolipin fall and normal levels of phosphatidyl glycerol are restored (1).

This intraconvertibility of polyglycerophosphatides is interesting insofar as it appears to be intimately associated with the energy state of the cell and this could be a basic, widely occurring phenomenon in membranes carrying out adenosine 5'-triphosphate formation. However, a key enzyme effecting the changes in polyglycerophosphatide levels, cardiolipin-specific phospholipase D, has been characterized only in two bacteria (1, 14). The present study is a survey of several organisms, as well as rat liver mitochondria, for their ability to hydrolyze cardiolipin to phosphatidic acid and phosphatidyl glycerol.

MATERIALS AND METHODS

The various bacteria, *E. coli* (ATCC 11305), *Salmonella typhimurium* (ATCC 13311), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 12600), *Bacillus subtilis* (ATCC 6051), and *Bacillus cereus* (ATCC 14579), were grown for 6 h in a liter of medium containing 1 g of yeast extract, 15 g of peptone, 5 g of sodium chloride, and 20 g of dextrose. *Saccharomyces cerevisiae* (ATCC 2338) cells were grown for 23 h at 28 C in 1 liter of medium containing 10 g of yeast extract, 10 g of peptone, and 20 g of dextrose. The bacterial cells were harvested by centrifugation, suspended in 0.1 M sodium phosphate buffer, pH 7.0, and sonicated for four 1-min periods in ice. The yeast cells were homogenized in phosphate buffer with a Braun homogenizer. Unbroken cells were removed by centrifugation for 10 min at 3,000 × *g*, and the cell-free extract was diluted with buffer to give a final protein concentration of 4.0 mg/ml. Protein concentrations were estimated by the method of Lowry et al. (10). Rat liver mitochondria were prepared by the method of McMurray and Dawson (12) but in the absence of 0.1 mM ethylenediaminetetraacetate.

Labeled cardiolipin (specific activity, 2.2 Ci/mol), labeled phosphatidyl glycerol (specific activity 0.86 Ci/mol), and labeled phosphatidyl ethanolamine (specific activity 4.65 Ci/mol) were prepared by the growth of *E. coli* cells in a medium containing [^{32}P]orthophosphate, and the extraction and isolation of the coliform lipids were as described previously (6). Lipid phosphorus was determined by the method of Bartlett (2).

The standard incubation mixture contained, in a final volume of 2 ml: 0.1 M sodium phosphate buffer, pH 7.0; 2 to 4 nmol of labeled substrate sonicated in buffer; 2.8 mM adenosine 5'-triphosphate; 10 mM Mg^{2+}; and 0.4 mg of protein from the various organisms tested. When cardiolipin was the substrate, incubations were for 15 min at 37 C and the lower limit of activity detectable was 2 pmol/min per mg of protein. Organisms which did not contain phospholipase D activity were tested with an incubation time of 30 min and protein concentrations of at least 1 mg/ml. Greater protein concentrations and longer incubation times were also used when phosphatidyl glycerol or phosphatidyl ethanolamine were tested as substrates. Reactions were stopped by lipid extraction according to the method described by Bligh and Dyer (3).

Lipids were separated on Silica Gel G plates with chloroform-methanol-water (65:25:4, by volume) as solvent. Mild alkaline hydrolyses of isolated lipids or

of total lipid extracts were performed as indicated by Marshall and Kates (13). Water-soluble products were identified by paper chromatography with phenol-water (5:2, wt/wt) and ethanol-1 M ammonium acetate, pH 7.5 (65:35, by volume) (19), as solvents. Components were counted as described previously (13).

RESULTS AND DISCUSSION

The occurrence of a cardiolipin-hydrolyzing phospholipase D in *E. coli* extracts was reported earlier (6). The present study reveals that this enzyme also occurs in other gram-negative bacteria such as *Proteus vulgaris*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. However, the enzyme could not be found in *B. subtilis*, *B. cereus*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and rat liver mitochondria under the standard assay conditions used. In the case of *Staphylococcus aureus*, no hydrolysis of cardiolipin took place also when Mg^{2+}

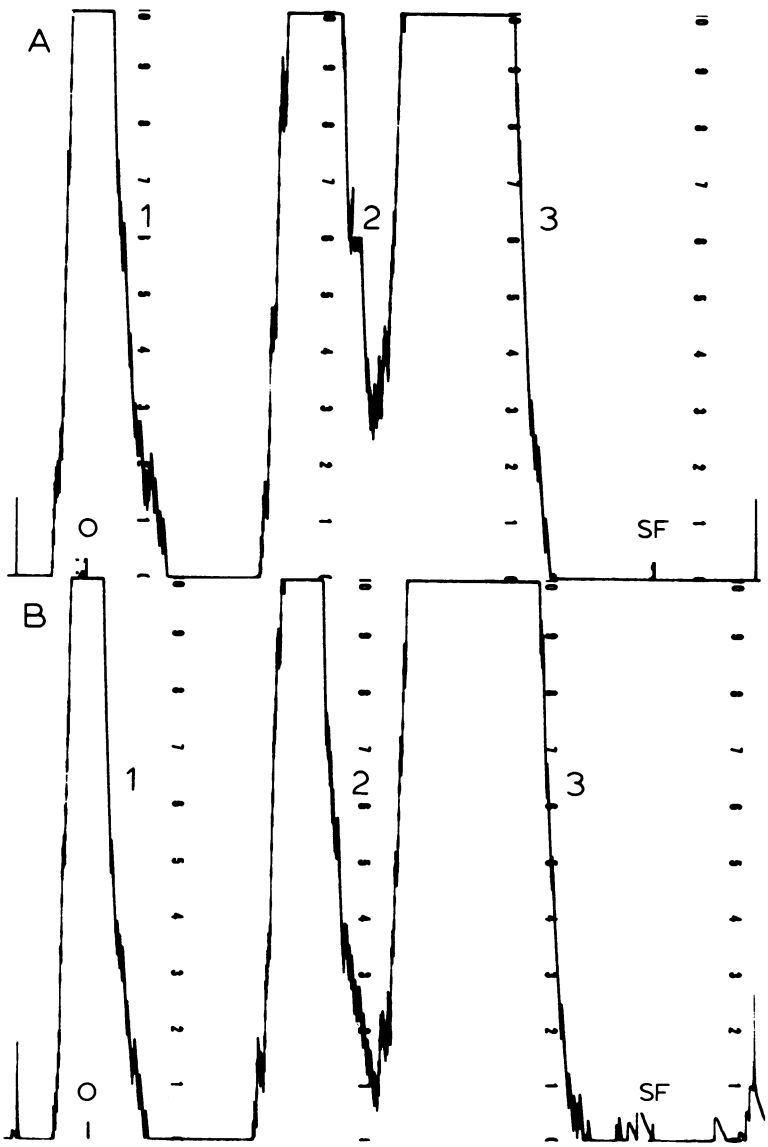

FIG. 1. *Scan of radioactivity of the hydrolysis products obtained by incubating ^{32}P-labeled cardiolipin under standard conditions with cell-free preparations of (A) E. coli, (B) Salmonella typhimurium, (C) Proteus vulgaris, and (D) Pseudomonas aeruginosa. Components 1, 2, and 3 co-chromatographed with phosphatidic acid, phosphatidyl glycerol, and cardiolipin, respectively. Lipids were separated on Silica Gel G plates with chloroform-methanol-water (65:25:4, by volume) as solvent.*

was omitted and the pH was varied from 4.0 to 8.0. These results were surprising, since *Staphylococcus aureus* forms cardiolipin by transphosphatidylation with phosphatidyl glycerol and, much like *Haemophilus parainfluenzae*, responds to inhibitors of oxidative phosphorylation or proton gradient formation by accumulating cardiolipin (18).

Results illustrated in Fig. 1 reveal the hydrolysis products obtained by incubating cell-free homogenates of *E. coli*, *Salmonella typhimurium*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* with ^{32}P-labeled cardiolipin. Components 1, 2, and 3 were identified, respectively, as phosphatidic acid, phosphatidyl glycerol, and cardiolipin by co-chromatography with reference lipids. Also after elution and deacylation, glycerophosphate, glycerophosphoryl-glycerol, and di-(glycerophosphoryl)-glycerol were identified corresponding to components 1, 2, and 3, respectively.

Results in Fig. 2 corroborate those just presented. When whole lipid extracts of active incubation mixture were subjected to mild alkaline hydrolysis, the products obtained were the same for each organism and were identical

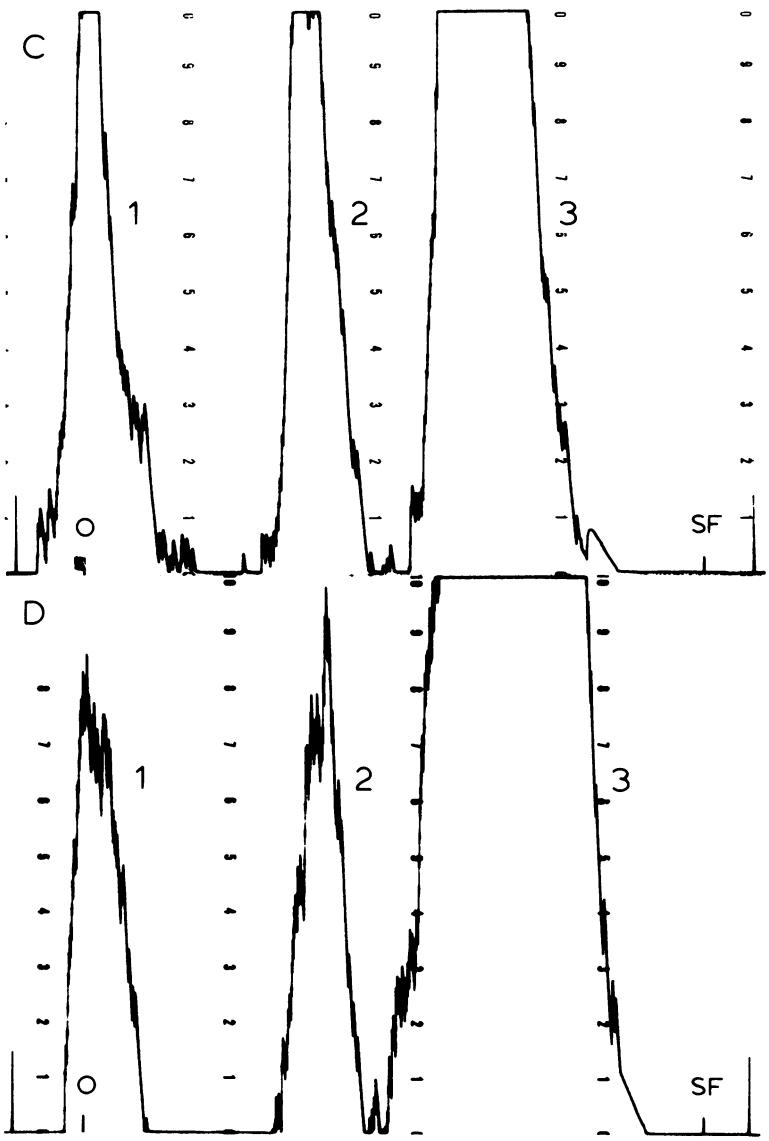

FIG. 1—*Continued*

to glycerophosphate, glycerophosphorylglycerol, and di-(glycerophosphoryl)-glycerol.

Results in Table 1 indicate that for all four organisms containing phospholipase D activity there were similar pH and Mg^{2+} requirements. In *E. coli* a pH optimum of 7 was found.

In *H. parainfluenzae* and *E. coli* the phospholipase D was found to attack only cardiolipin among the common naturally occurring phosphoglycerides tested. Using extracts from *E. coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*, we were unable to show hydrolysis of phosphatidyl glycerol or phosphatidyl ethanolamine via phospholipase D activity under conditions allowing breakdown of cardiolipin to phosphatidyl glycerol and phosphatidic acid.

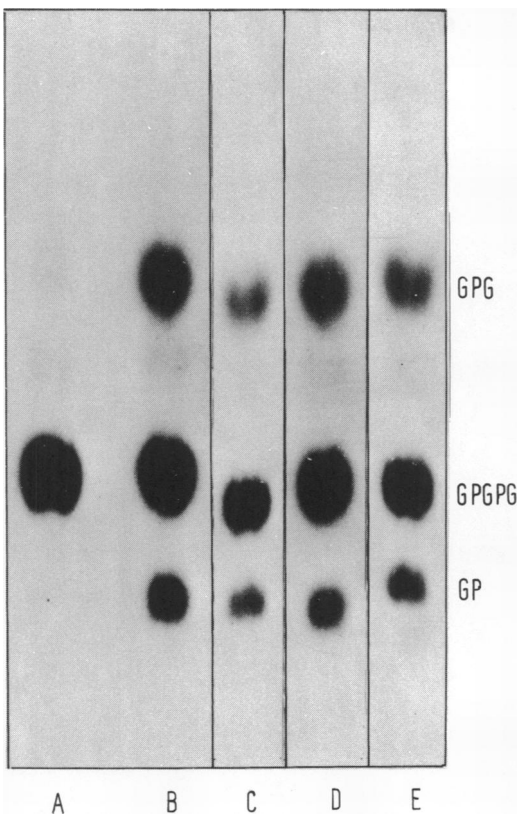

FIG. 2. *Autoradiogram of deacylation products obtained by mild alkaline hydrolysis of lipid extracts from (B) E. coli homogenate, (C) Salmonella typhimurium homogenate, (D) Proteus vulgaris homogenate, and (E) Pseudomonas aeruginosa homogenate incubated with ^{32}P-labeled cardiolipin under standard conditions. In (A) the incubation mixture contained E. coli homogenate and 30 mM ethylenediaminetetraacetate. The deacylation products were separated as indicated on Whatman no. 1 filter paper with ethanol-1 M ammonium acetate, pH 7.5 (65:35, by volume) as solvent (ascending technique). GPG, Glycerolphosphorylglycerol; GPGPG, di-(glycerophosphoryl)-glycerol; GP, glycerophosphate.*

TABLE 1. *Effect of pH and Mg^{2+} on the phospholipase D activity of some gram-negative bacteria*[a]

Bacteria	pmol of cardiolipin hydrolyzed				
	pH 6.2	pH 7.0	pH 8.0	No Mg^{2+}	10 mM Mg^{2+}
Salmonella typhimurium	14	75	7	0	60
Proteus vulgaris	1	65	4	0	63
Pseudomonas aeruginosa	2	42	1	0	36

[a] Conditions were standard except for the pH and Mg^{2+} concentration, which were varied. The control mixtures lacking Mg^{2+} contained 30 mM ethylenediaminetetraacetate. The only two lipid products were identified as phosphatidyl glycerol and phosphatidic acid.

It appears from these results that there is a typical bacterial phospholipase D attacking cardiolipin only and having similar catalytic requirements in all species of gram-negative bacteria tested. In *E. coli* (1) and *H. parainfluenzae* (14), and very likely in other bacteria containing it, this phospholipase D is involved in the turnover of polyglycerophosphatides, which occurs in response to the energy state of the cell. For such organisms it would be important to know eventually how the changes in the structural properties of the membrane brought about by this turnover affect biological functions such as respiration and oxidative phosphorylation.

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