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Nonspecific Phospholipase C of *Listeria monocytogenes*: Activity on Phospholipids in Triton X-100-Mixed Micelles and in Biological Membranes

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Listeria monocytogenes **secretes a phospholipase C (PLC) which has 39% amino acid sequence identity with the broad-specificity PLC from *Bacillus cereus*. Recent work indicates that the *L. monocytogenes* enzyme plays a role during infections of mammalian cells (J.-A. Vazquez-Boland, C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart, Infect. Immun. 60:219–230, 1992). The homogeneous enzyme has a specific activity of 230 μmol/min/mg when phosphatidylcholine (PC) is dispersed in sodium deoxycholate. With phospholipid–Triton X-100 mixed micelles, the enzyme had a broad pH optimum between 5.5 and 8.0, and the rates of lipid hydrolysis were in the following order: PC > phosphatidylethanolamine (PE) > phosphatidylserine > sphingomyelin >> phosphatidylinositol (PI). Activity on PC was stimulated 35% by 0.5 M NaCl and 60% by 0.05 mM $ZnSO_4$. When *Escherichia coli* phospholipids were dispersed in Triton X-100, PE and phosphatidylglycerol, but not cardiolipin, were hydrolyzed. The enzyme was active on all phospholipids of vesiculated human erythrocytes including PI, which was rapidly hydrolyzed at pH 7.0. PI was also hydrolyzed in PI-PC-cholesterol liposomes by the nonspecific PLC from *L. monocytogenes* and by the homologous enzyme from *B. cereus*. The water-soluble hydrolysis product was identified as inositol-1-phosphate. For the hydrolysis of human erythrocyte ghost phospholipids, a broad pH optimum was also observed. ^{32}P-labelled *Clostridium butyricum* protoplasts, which are rich in ether lipids, were treated with PLC. The enzyme hydrolyzed the plasmalogen form of PE, its glycerol acetal, and cardiolipin, in addition to PE. I^-, Cl^-, and F^- stimulated activity on either PC–Triton X-100 mixed micelles or human erythrocyte ghosts, unlike the enzyme from *B. cereus*, which is strongly inhibited by halides. Tris-HCl, phosphate, and calcium nitrate had similar inhibitory effects on the enzymes from *L. monocytogenes* and *B. cereus*.**

Listeria monocytogenes, a gram-positive, non-spore-forming rod, has become increasingly recognized as an important food-borne human pathogen. It is the cause of serious human diseases, including septicemia in pregnant women and meningoencephalitis in neonates and in immunocompromised adults (12). Murine tissue culture models of infection have permitted morphological delineation of the processes of host cell infection which can be subdivided into several temporal stages including (i) host cell invasion, (ii) escape from the phagosome by lysis, (iii) growth in the cytoplasm and nucleation of host cell actin filaments, (iv) actin-based movement through the cell, and (v) cell-to-cell spread by phagocytosis of a bacterium within an induced pseudopod by a neighboring cell, resulting in the formation of a phagocytic vesicle with a double membrane (8, 14, 15, 34, 44). The products of several *L. monocytogenes* genes, which are closely linked, have recently been implicated in host cell infection (reviewed in reference 39). Several of these genes encode proteins that interact with host cell membranes during infection. They include *hly*, the gene for listeriolysin O, a pore-forming cytolysin; *plcA*, the gene for a phosphatidylinositol (PI)-specific phospholipase C (PLC) (6, 26, 32); and *plcB*, the gene for a relatively nonspecific PLC (46). The expression of these genes is coregulated by the product of *prfA*, a positive regulatory protein (13, 27, 33).

PLC is being actively studied as a potential virulence determinant in bacterial infections (36, 42). *L. monocytogenes* strains with a mutation in *plcB* appear to be defective in escape from the double-membrane vacuole resulting from infection of neighboring cells by ingestion of bacteria within pseudopods (46). Thus, PLC may function in lysing the two membranes of this vacuole, which are of opposite phospholipid asymmetries and which represent unique targets for phospholipase activity. *plcB* has been sequenced previously (46), and the protein has been purified to homogeneity (16). The enzyme is a 29-kDa zinc-dependent protein that hydrolyzes diacylphosphoglycerides and sphingomyelin (SM) (16). Its amino acid sequence has homology with those of similar enzymes from *Bacillus cereus* and *Clostridium perfringens* (alpha-toxin). We have carried out quantitative studies on the activity of this enzyme on a variety of diacyl- and alk-1-enyl acylphospholipids in Triton X-100 detergent dispersions and in natural membranes. The effects of salts, pH, and inhibitors on these activities have also been investigated. These studies show distinct differences between the enzyme from *L. monocytogenes* and the well-characterized PLC from *B. cereus*. The properties of the *L. monocytogenes* PLC are consistent with a proposed role for this enzyme in cell-to-cell spread.

MATERIALS AND METHODS

Materials. L-3-Phosphatidyl[*N*-methyl-^3H]choline (81 Ci/mmol), L-3-phosphatidylethanolamine (PE) 1,2-di[1-^{14}C] palmitoyl (117 mCi/mmol), L-3-phosphatidyl[2-^3H]inositol (17.5 Ci/mmol), L-3-phosphatidyl-L-[U-^{14}C]serine 1,2(dioleoyl) (60 mCi/mmol), and $^{32}P_i$ were obtained from Amer-

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sham Corp., Arlington Heights, Ill. Phospholipid carriers and standards, D-*myo*-inositol-1-phosphate, *myo*-inositol-2-monophosphate, DL-*myo*-inositol 1,2-cyclic monophosphate, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, Mo. Molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories, Richmond, Calif. The sources of chromatographic materials are indicated in the text. Other reagents were obtained from a variety of sources and were of reagent grade or better.

Cell culture and enzyme purification. *L. monocytogenes* DP-L1553 was used as the enzyme source. It contains an in-frame deletion in *plcA* (7) and was derived from wild-type strain SLCC 5764, which exhibits a hypersecreting phenotype including high-level secretion of listeriolysin, PLC, and PI-PLC. It was grown and maintained for up to 2 months on brain heart infusion agar, by serial passage. For enzyme isolation, cells were subcultured on Trypticase-glucose-yeast broth containing Trypticase peptone (BBL) (30 g), yeast extract (Difco) (20 g), $Na_2HPO_4 \cdot 12H_2O$ (8.3 g), KH_2PO_4 (0.7 g), $ZnSO_4$ (0.1 mM), and deionized distilled water to 1 liter. Activated charcoal (0.2%) (Bio-Rad Laboratories) was present as a suspension (16). The pH was adjusted to 7.5, and 1% glucose was added after sterilization. The cells were grown in 2-liter flasks (300 ml of medium per flask), which were inoculated with a 10% overnight culture, and the cultures were incubated at 37°C on a rotary shaker at 180 rpm for 9 h. After the cells were removed by centrifugation at 10,000 × *g* for 10 min, 0.05% sodium azide was added to the supernatant culture fluid for storage. Protein was concentrated either by addition of solid ammonium sulfate to 90% saturation at 4°C or by ultrafiltration with an Amicon hollow-fiber ultrafilter with a 10-kDa cutoff (16). Subsequent purification was carried out as described elsewhere (16). The peak activity fractions from the second Bio-Gel P100 fractions were subjected to SDS-PAGE (19), and the proteins were stained with either Coomassie blue or silver stain. Fractions containing the homogeneous 29-kDa protein were pooled. During purification, the amount of protein was measured by the dye-binding assay described by Bradford (4).

Enzyme assays. PLC was assayed as described elsewhere (6) with the following modifications. L-3-Phosphatidyl[*N*-methyl-^{3}H]choline, 1,2-dipalmitoyl (0.25 μCi), and phosphatidylcholine (PC; type XI-E, from egg yolk; 288 μg; 0.37 μmol; Sigma) were sonicated in 100 μl of 100 mM morpholinepropanesulfonic acid-morpholineethanesulfonic acid (MOPS-MES) buffer at the indicated pH in 1.84% Triton X-100 per assay. The mixed micelle suspension was diluted to a 200-μl final volume by the addition of salts, water, and enzyme, which was diluted in 0.5% BSA when necessary. Incubations were at 37°C for 0.5 to 2.0 min. At least two time points were taken for each condition to check for the linearity of phospholipid hydrolysis with time. Some assays were conducted with 288 μg of PC (0.25 μCi) suspended in 0.15% sodium deoxycholate. The final assay mixture contained 0.025% BSA, 0.2 M NaCl, and 0.1 mM $ZnSO_4$, which were separately shown to provide optimal activity in this assay (data not shown). Assays for hydrolysis of other phospholipids suspended in Triton X-100 contained 0.37 μmol of carrier plus the following amounts of radioactive lipids: [U-^{14}C-serine]phosphatidylserine (PS), 0.02 to 0.09 μCi; 1,2-[1-^{14}C-palmitoyl]phosphatidylethanolamine (PE), 0.045 μCi; and carrier *Escherichia coli* PE and [^{3}H-inositol]PI, 0.15 μCi. For hydrolysis of PE, diacylglycerol formation was measured as described elsewhere (19). For other phospholipids, the release of radioactive water-soluble phosphate esters was measured (6). At the end of the incubation, 1.6 ml of chloroform-methanol (1:1 [vol/vol]) and 0.8 ml of either 0.1 N HCl or, in the assays with *Clostridium butyricum* protoplasts, 0.05 N NaCl were added. The tube contents were mixed by vortexing and were centrifuged at 1,200 rpm for 7 min in a Sorvall GLC-1 centrifuge. Assays were also performed with nonradioactive phospholipids at the same molar concentrations of phospholipids and Triton X-100. For these assays, the enzyme was exhaustively dialyzed against MOPS buffer (20 mM; pH 7.2) in order to remove P_i. The release of water-soluble phosphate esters was measured in the aqueous phase after lipid extraction by digestion with 0.3 ml of 10 N H_2SO_4 at 160°C overnight, followed by 2 drops of 30% H_2O_2, and heating for a further 2 h. The amount of phosphorus was measured by the method described by Bartlett (2).

Preparation of liposomes. Dried lipids containing 25 μg of PI (0.05 μCi of [^{3}H]PI), 154 μg of PC, and 68 μg of cholesterol were suspended in 0.1 M MOPS-MES (100 μl per assay), first by vigorous vortex mixing and then by sonication with a Heat Systems sonifier (model W-225) with a tapered microtip probe at maximum power for a total of 3 min with 0.5-min bursts, followed by 0.5 min of cooling in a water bath at room temperature. The suspensions were centrifuged at 3,000 × *g* for 10 min to remove metal particles, and the supernatant was used immediately.

Preparation of human erythrocyte ghosts. Venous blood was freshly collected in 3.4 mM EDTA, and leukocytes were removed by centrifugation at 840 × g_{max} at 4°C for 10 min. The buffy coat was removed by aspiration, and the erythrocytes were washed three times by centrifugation in isotonic phosphate buffer (150 mM NaCl in 5 mM sodium phosphate buffer, pH 7.6) (23). Ghosts were then prepared as described elsewhere (24), with careful decanting (11), and were vesiculated by the method described by Steck and Kant (43).

***C. butyricum* protoplast membranes.** ^{32}P-labelled protoplasts prepared from logarithmic-phase cultures of *C. butyricum* ATCC 19398 were treated with PLC as described for experiments previously performed with *B. cereus* PLC (17, 18), and the lipids were extracted by the method described by Bligh and Dyer (3).

***E. coli* lipids.** *E. coli* K-12 was grown at 37°C in a rotary shaker at 180 rpm in Luria-Bertani broth (30). After 4 h, 10 ml of the culture was transferred to a 250-ml flask, and 0.5 mCi of $^{32}P_i$ was added. Incubation with shaking was continued for 3 h, the cells were harvested by centrifugation, and the lipids were extracted (3). The lipids contained 2.3 μCi of ^{32}P. Labelled lipids (5.4 nCi) were diluted with 0.37 μmol of *E. coli* K-12 phospholipids, dried under N_2 gas, and suspended in 100 μl of 100 mM MOPS-MES buffer, pH 5.5 or 7.0, containing 1.84% Triton X-100. The final volume for each time point was 200 μl.

Hydrolysis of vesiculated erythrocyte lipids. Suspensions of vesiculated human erythrocyte ghosts were treated with PLC in either acetate or MOPS-MES buffer at the indicated pH at 37°C. Samples were removed at various times, and the reaction was stopped by the addition of chloroform-methanol (1:1 [vol/vol]) (6). Lipids were extracted as described elsewhere (19).

TLC and paper chromatography. In assays for hydrolysis of *E. coli* lipids, the chloroform phase was dried and chromatographed in chloroform-methanol-acetic acid (65:25:8 [vol/vol/vol]) on Silica Gel 60 thin-layer plates (E. Merck). The dried plates were exposed to Kodak X-Omat AR film

overnight, and the radioactive lipids were located and scraped into scintillation vials. All scintillation counting was done in Ecosint (National Diagnostics, Manville, N.J.). After enzyme treatment, *C. butyricum* protoplasts were extracted as described above and separated by two-dimensional thin-layer chromatography (TLC) on Silica Gel 60. The plates were exposed to HCl fumes for 20 s between the first and second dimensions to hydrolyze the plasmalogens, permitting separation of the resulting lyso-phospholipids in the second dimension (17, 18). Duplicate aliquots of the extracted erythrocyte lipids were chromatographed on Silica Gel 60 plates with a preconcentrating zone in chloroform-petroleum ether-methanol-acetic acid (5:3:1.6:1 [vol/vol/vol/vol]) (37). The lipids were stained with iodine vapor and marked. The plates were then sprayed with water, and the lipid-containing areas were scraped from the plates into acid-washed tubes for determination of the amount of phosphate as described above, except that the volume of H_2O_2 was increased to 0.3 ml.

The water-soluble product of PI hydrolysis was obtained from the aqueous phase after the chloroform-methanol extract was partitioned with water. It was chromatographed along with carrier inositol-1-monophosphate and inositol-1,2-cyclic monophosphate on cellulose thin-layer plates (E. Merck) in *n*-propanol-NH_4OH-H_2O (5:4:1 [vol/vol/vol]; solvent A) and 95% ethanol–13.5 M NH_4OH (3:2 [vol/vol]; solvent B). It was also chromatographed on Whatman paper (no. 1) in solvent B along with carrier inositol-1-P and inositol-2-P (25). TLC plates and the paper chromatogram were scanned with an Automatic TLC-Linear Analyzer (Berthold, Wildbad, Germany). [^{3}H]PI was also incubated without enzyme, and the water-soluble radioactive impurities detected by scanning were subtracted from the product of enzyme activity. Phosphate esters were detected with a spray containing the Hanes-Isherwood stain (9) and then exposed to a strong UV light (Mineralight model R52G; UVP, Inc., San Gabriel, Calif.).

RESULTS

Triton X-100–phospholipid mixed micelles. (i) Concentration of detergent. Studies of PLC specificity have been carried out on PLC from *B. cereus* with the Triton X-100–phospholipid mixed micelle system (41), and the advantages of this system have been reviewed previously (40). They include a well-defined inert surface matrix which is not altered when small amounts of phospholipid are inserted (41). The addition of increasing concentrations of Triton X-100 at a fixed concentration of PC resulted in strong activation of *L. monocytogenes* PLC activity at up to 0.4% (6.4 mM) detergent, but higher concentrations of up to 0.95% (15.2 mM) had little further effect (Fig. 1). At 0.95% Triton X-100 and 1.85 mM PC, the detergent-phospholipid molar ratio is 8:1, which was found to provide a useful compromise between surface dilution and possible phospholipid intermolecular interactions (41).

(ii) Effects of added protein, NaCl, and $ZnSO_4$. Addition of 30 to 50 μg of BSA stimulated PC hydrolysis 34%. Higher concentrations of BSA (up to 1 mg/ml) were slightly less effective, with an average stimulation of 22%. In the absence of NaCl, 0.05 mM $ZnSO_4$ increased the rate of PC hydrolysis 60%. This stimulation was progressively lost at higher $ZnSO_4$ concentrations (data not shown). Addition of 1 mM $ZnSO_4$ in the absence of NaCl resulted in 35% inhibition (Fig. 2). In the presence of 1 mM $ZnSO_4$, NaCl addition had very little effect on PC hydrolysis. When NaCl was added in the absence of $ZnSO_4$, the effect of the salt concentration on enzyme activity was complex. Activity was inhibited 55% at 25 mM NaCl, returned to its initial value at 100 mM NaCl, and was moderately stimulated at higher salt concentrations up to 0.5 M (Fig. 2).

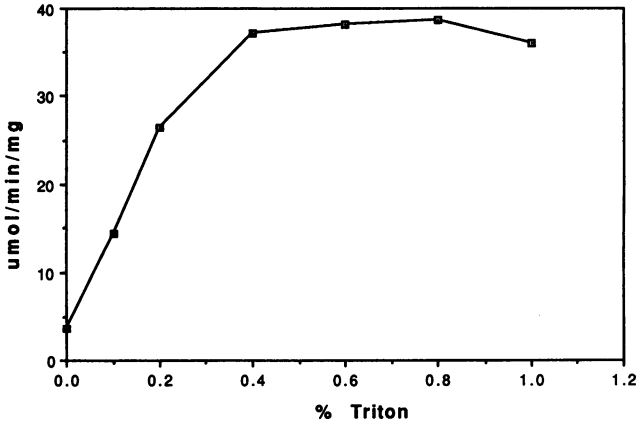

FIG. 1. Effects of Triton X-100 concentration on the hydrolysis of PC. The reactions were carried out in the presence of 50 mM MOPS-MES buffer, pH 7.0, 0.2 M NaCl, 0.025% BSA, 1.85 mM PC (0.25 μCi), and 0.25 μg of enzyme protein per assay mixture at the indicated Triton X-100 concentrations in a final volume of 0.2 ml. Incubations were carried out in separate tubes for 1 and 2 min for each concentration of Triton X-100.

(iii) Effect of pH. Enzyme activity with PC–Triton X-100 mixed micelles in acetate and MOPS-MES buffers from pH 3.8 to 8.0 was measured. The rate of PC hydrolysis was relatively flat between pHs 5.5 and 8.0 (Fig. 3A). Although acetate buffer permitted a somewhat higher rate of hydrolysis at pH 5.3 compared with that in MOPS-MES at pH 5.5, there was a gradual decline in activity at lower pHs.

Substrate specificity. The rates of hydrolysis of various phospholipids in Triton X-100 mixed micelles are given in Table 1. These were studied at neutrality and at pH 5.5 in order to assess the capacity of the enzyme to function in the acidic environment of the phagocytic vesicle. At pH 7.0, PE

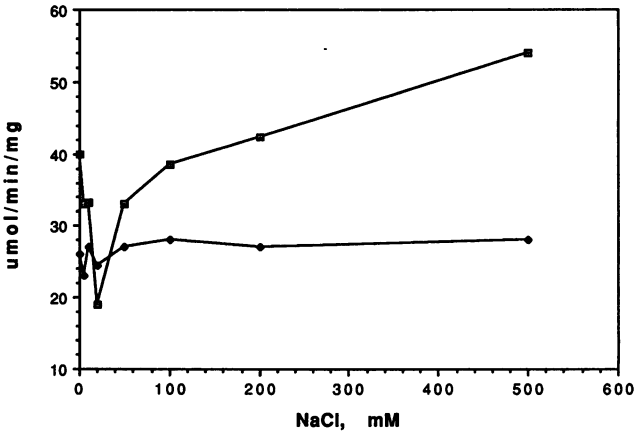

FIG. 2. Effects of NaCl and $ZnSO_4$ on the hydrolysis of PC in Triton X-100 mixed micelles. The reactions were carried out in the presence of 50 mM MOPS-MES buffer, pH 7.0, 0.025% BSA, 0.92% Triton X-100, 1.85 mM PC (0.25 μCi), and 0.25 μg of enzyme protein per assay mixture. Incubations were for 2 min. □, no $ZnSO_4$; ◆, 1 mM $ZnSO_4$.

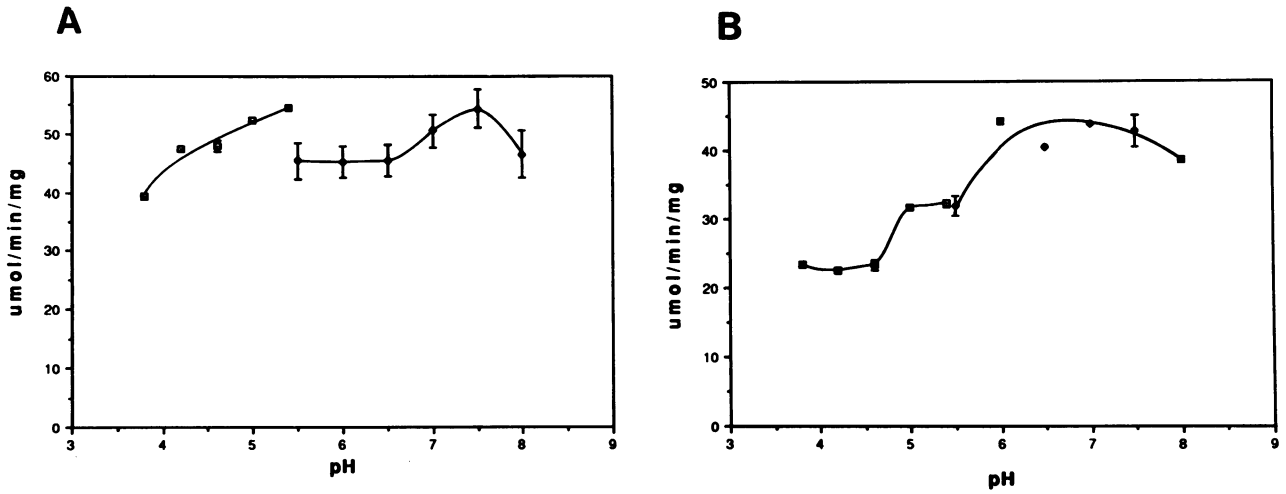

FIG. 3. Effects of pH on the hydrolysis of PC. (A) Triton X-100 mixed micelles. Enzyme activity was measured in the presence of 41 mM acetate (□) or 50 mM MOPS-MES (◆) buffer at the indicated pHs, with 0.1 mM $ZnSO_4$, 0.013% BSA, 1.85 mM PC (0.25 μCi), 0.92% Triton X-100, and 0.25 μg of enzyme protein per assay mixture. Incubations were carried out in separate tubes for 2 and 4 min for each pH. The data are the means and errors of two determinations. (B) Vesiculated erythrocyte ghosts. Enzyme activity was measured in the presence of 50 mM acetate (□) or 50 mM MOPS-MES (◆) buffer at the indicated pHs, with 40 mM NaCl, vesiculated erythrocyte ghosts containing 64 nmol of phospholipid, and 0.45 μg of protein per assay mixture. Duplicate reactions were terminated at 0.5 min for each pH. The release of water-soluble phosphate esters was measured as described under Materials and Methods. The data are the means and errors of two determinations.

was hydrolyzed at almost the same rate as PC. At pH 5.5, the rate of PE hydrolysis was 75% of that of PC, but the difference in these studies was not statistically significant. PS was hydrolyzed at somewhat lower rates than either PE or PC at pH 7.0 and at almost the same rate as PE at pH 5.5. SM was hydrolyzed at about 30% of the rate of PC at both pHs. The hydrolysis of PI in a mixed micelle system was too slow to obtain an accurate measure, but the ability of this enzyme to cleave PI was demonstrated in other studies (see below). In order to eliminate the effects of differences in acyl chains, the rates of hydrolysis of dioleoyl-PE (DOPE) and dioleoyl-PC (DOPC) were compared. At both pHs, the rate of DOPE hydrolysis was about 85% of that of DOPC. Under optimal conditions, the specific activity of the enzyme on PC in 0.95% Triton X-100 was 66.3 ± 13 μmol/min/mg. Assays with PC suspended in 0.15% sodium deoxycholate under optimal conditions yielded a specific activity of 230 ± 35 μmol/min/mg (average specific activities for two independent preparations).

TABLE 1. Substrate specificities of PLC from *L. monocytogenes* with Triton X-100 mixed micelles[a]

Substrate	pH	% of sp act on PC[b] (*n*)
PC	5.5	100[b]
	7.0	100
PE	5.5	75 ± 6 (3)[c]
	7.0	91 ± 23 (4)
DOPE	5.5	85 ± 3 (2)[d]
	7.0	84 ± 5 (2)
PS	5.5	69 ± 3 (2)
	7.0	60 ± 5 (2)
PI	5.5	<2
	7.0	<2
SM	5.5	31 ± 9 (2)
	7.0	27 ± 4 (2)

[a] Assays were performed in the presence of 0.95% Triton X-100, 1.8 μM phospholipid (Triton X-100 to phospholipid, 8:1 molar ratio), 0.1 mM $ZnSO_4$, 0.025% BSA, and from 0.18 to 0.69 μg of enzyme protein. Each substrate was treated with two or three different amounts of enzyme in each assay.

[b] The specific activities for PC were 46.6 ± 10.4 μmol/min/mg (n = 7) at pH 5.5 and 46 ± 8 μmol/min/mg (n = 7) at pH 7.0.

[c] The differences in specific activities for [1-^{14}C-dipalmitoyl]PE dispersed in *E. coli* phospholipids and dipalmitoyl[^{3}H-methylcholine]PC dispersed in egg PC at pH 5.5 (P = 0.1) and for PE at pHs 5.5 and 7.0 (P > 0.1) are not significant by the two-sample t test.

[d] Compared with value for DOPC. The specific activities for DOPC at pHs 5.5 and 7.0 were 42.2 ± 0.4 (n = 2) and 40.3 ± 0.6 (n = 2) μmol/min/mg, respectively.

We also carried out studies on the hydrolysis of ^{32}P-labelled *E. coli* phospholipids dispersed in Triton X-100. These studies showed that PLC from *L. monocytogenes* hydrolyzes PG at rates similar to those observed for PE (Fig. 4). The effects of pH, however, were opposite. At pH 7.0, PE was hydrolyzed more rapidly than PG; the reverse was found at pH 5.5. Although cardiolipin was not hydrolyzed in mixed micelles, studies with *C. butyricum* protoplasts showed that it can serve as a substrate (see below).

Hydrolysis of phospholipids in biological membranes. (i) Human erythrocyte ghosts. The ability of *L. monocytogenes* PLC to hydrolyze the phospholipids in erythrocyte membranes was assessed with vesiculated ghosts (47). As expected from the results of our studies with the Triton X-100–phospholipid mixed micelle system, the enzyme was capable of attacking all of the major erythrocyte lipids (PC, PE, PS, and SM), resulting in rapid and nearly complete hydrolysis (Fig. 5). Control experiments without added enzyme showed no water-soluble phosphate release and only small changes (≤16%) in individual lipids after 3.5 min of incubation at 37°C (data not shown). The specific activity of water-soluble phosphate release from total membrane lipids was the same as that seen with Triton X-100–PC mixed micelles with the same enzyme preparation. When acting on erythrocyte lipids, the enzyme displayed a broad pH optimum from 6.0 to 8.0, with lower activities at pH 5.5 and below. Despite this decline, activity between pHs 3.8 and 4.6

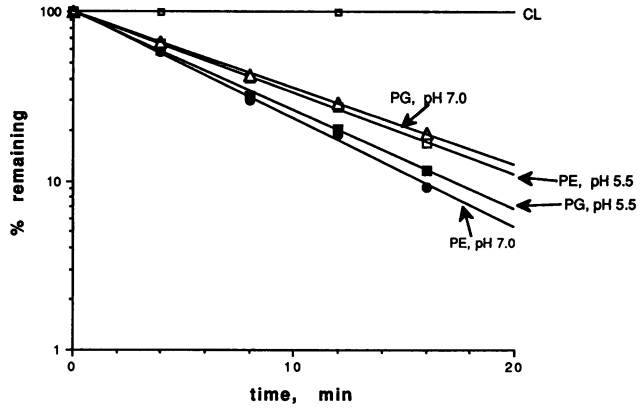

FIG. 4. Hydrolysis of *E. coli* phospholipids in Triton X-100 mixed micelles. ^{32}P-labelled *E. coli* lipids (5.4 nCi) were diluted with 0.37 μmol of unlabelled phospholipids. Assays were carried out under standard conditions in 50 mM MOPS-MES buffers at either pH 5.5 or 7.0, with 0.92% Triton X-100, 0.1 mM $ZnSO_4$, 0.025% BSA, and 0.87 μg of enzyme protein per assay mixture, in a final volume of 0.2 ml for each time point. CL, cardiolipin. The data are from a representative experiment.

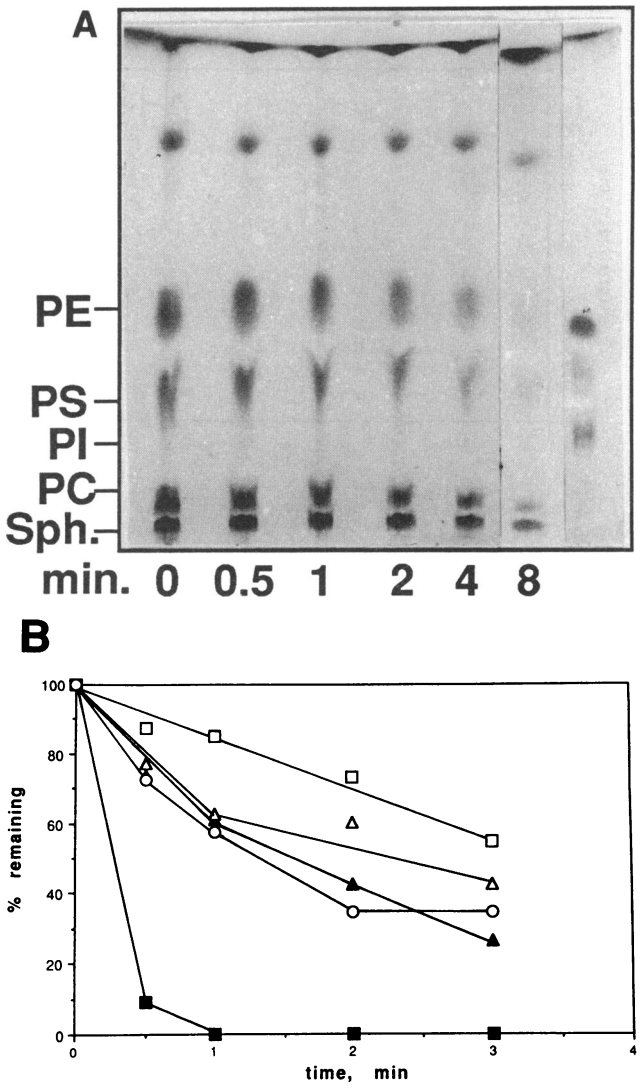

FIG. 5. Hydrolysis of phospholipids in vesiculated human erythrocyte ghosts. Incubation mixtures contained vesiculated human erythrocyte ghosts containing 165 nmol of phospholipid, 50 mM MOPS-MES buffer (pH 7.0), and 1.8 μg of enzyme protein in a final volume of 400 μl per time point. (A) TLC was carried out as described under Materials and Methods. The lipids were stained with molybdenum blue spray reagent (Sigma Chemical Co.) and charred at 120°C. The unknown lipid chromatographing at an R_f of approximately 0.8 is not a phospholipid, since it did not stain with molybdenum blue. Sph., SM. The rightmost lane shows the positions of the PE, PS, and PI standards. (B) Quantitation of hydrolysis of phospholipids. Symbols: □, SM; ▲, PC; ■, PI; △, PS; ○, PE. The data are representative of two separate experiments.

was approximately 50% of that at optimal pHs (Fig. 3B). The effects of salts were also studied with this preparation. NaCl stimulated total phospholipid hydrolysis by 53% at 0.04 M, 23% at 0.06 M, and 11% at 0.15 M. $ZnSO_4$ inhibited by approximately 20% between 0.1 and 1.0 M (data not shown).

As was found with Triton X-100 mixed micelles at pH 7.0, PC and PE were hydrolyzed most rapidly in erythrocyte ghosts, followed by PS and SM. Surprisingly, PLC hydrolyzed PI rapidly and completely in the erythrocyte membrane (Fig. 3B). Similar results were obtained at pH 5.5; however, the rates of hydrolysis of individual lipids were lower, as was found when total lipid hydrolysis was measured.

(ii) *C. butyricum* protoplasts. PLC from *B. cereus* was previously used to probe the asymmetry of *C. butyricum* protoplasts (17, 18). This preparation enabled us to examine the ability of PLC from *L. monocytogenes* to hydrolyze 1-alk-1'-enyl-2-acyl *sn*-3-glycerophospholipids (plasmalogens) and the unique glycerol acetals of the plasmalogens found in some clostridia. To preserve protoplast integrity, these studies were carried out in the presence of sucrose-phosphate-$MgCl_2$ buffer, pH 7.5 (18). PLC from *L. monocytogenes*, like that from *B. cereus*, cleaved plasmalogens and the glycerol acetal of plasmenylethanolamine at rates comparable to those seen with the diacyl phospholipids (Fig. 6). Both cardiolipin and the plasmalogen form of this lipid were also hydrolyzed, the latter after a 2-min lag, which was presumably related to an inner monolayer location of the majority of this lipid class (17, 18).

Hydrolysis of PI and PC in liposomes. PI-PC-cholesterol liposomes were incubated with enzyme which had been preincubated for 0.5 h at room temperature with 2 mM EDTA, 0.32 mM *o*-phenanthroline, or no inhibitor (Fig. 7). Hydrolysis of PI was nearly linear with time for 1 h (0.018 nmol/min/mg of protein). PC hydrolysis was not linear with time but was at least 40 times faster. EDTA and *o*-phenanthroline inhibited both PI and PC hydrolyses; the latter inhibited PC hydrolysis more strongly. EDTA (2 mM) inhibited PC hydrolysis more strongly in PC–Triton X-100 mixed micelles (data not shown). The water-soluble product(s) of PI hydrolysis by the enzyme from *L. monocytogenes* and by the homologous broad-specificity enzyme from *B. cereus* was examined by chromatography on cellulose thin layers. The R_f of the radioactive product corresponded to that of D-*myo*-inositol-1-P (0.26 and 0.19) and not that of DL-*myo*-inositol-1,2-cyclic monophosphate (0.43 and 0.56) in solvents A and B, respectively. On paper chromatography in solvent B, which separates inositol-1-P from inositol-2-P, the product of hydrolysis by both PLCs comigrated with carrier inositol-1-P (data not shown).

Effects of inhibitors. *B. cereus* PLC acting on erythrocyte

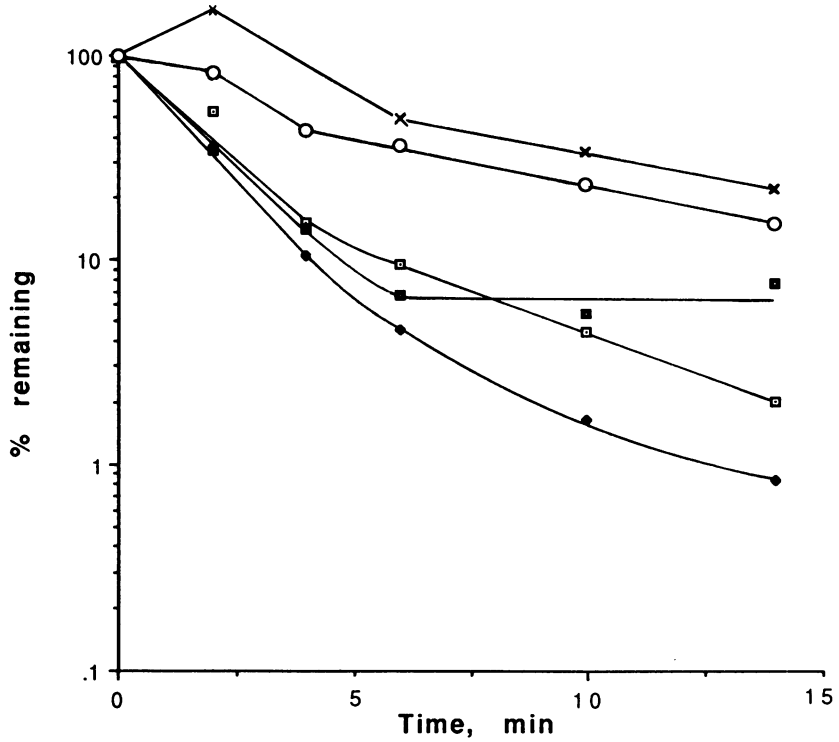

FIG. 6. Hydrolysis of phospholipids in *C. butyricum* protoplasts by PLC. The cells were labelled with ^{32}P by growth in a 50-ml culture with 125 μCi of labelled P_i as described elsewhere (18). Protoplasts were suspended in 1.1 ml of protoplasting buffer and treated with lysozyme (18), and 0.09-ml aliquots of the cell suspension were treated with 0.86 μg of PC-PLC for the times indicated in the presence of 0.09 mM $ZnSO_4$. The symbols for the compounds and their initial percentages are as follows: ■, PE (5.4%); ◆, plasmenylethanolamine (26.5%); ○, cardiolipin (22%); X, plasmalogen form of cardiolipin (15.3%); □, glycerol acetal of plasmenylethanolamine (22%). The data are from a representative experiment.

ghosts is strongly inhibited by halide ions; the effects increase with the size of the anion, with I^- being the most effective. NO_3^- salts, Tris-HCl buffer (1), and P_i (20) are also inhibitory, while acetate and formate inhibit weakly (1). The effects of various salts on the activity of *L. monocytogenes* PLC were tested on vesiculated erythrocyte ghosts and on Triton X-100–phospholipid mixed micelles. With erythrocytes, the strongest inhibition was seen with $Ca(NO_3)_2$. Tris-HCl inhibited less, and unlike the effects seen with the enzyme from *B. cereus*, KI, NaF, and NaCl were stimulatory (Table 2). With PC–Triton X-100 mixed micelles, phosphate, which could not be tested with erythrocyte ghosts, was the strongest inhibitor, and higher concentrations of NaCl were required for stimulation (Fig. 2).

DISCUSSION

Our studies have determined the activities of *L. monocytogenes* PLC on phospholipids in mixed micelles with Triton X-100 and on phospholipids in biological membranes. They show that this nonspecific PLC hydrolyzes all phospholipids tested, including PC, PS, PE, and SM, as previously shown (16), PI, cardiolipin, PG, plasmalogens, and the glycerol acetal of plasmenylethanolamine. With both phospholipid–Triton X-100 mixed micelles and vesiculated erythrocyte ghosts, the pH range is broad. The decrease in activity at lower pH observed with erythrocyte ghosts may result from an effect on the organization of these membranes. We found that this enzyme is slightly stimulated by KI and NaCl, in contrast to the strong inhibition by halides observed with the homologous broad-specificity PLC of *B. cereus* (1).

In Triton X-100 mixed micelles, the specific activity of recently isolated, homogeneous *L. monocytogenes* PLC on PC was 66 μmol/min/mg at pH 7.0. In sodium deoxycholate dispersions, the specific activity was 230 μmol/min/mg. These specific activities are considerably lower than the 1,500 μmol/min/mg reported for highly purified protein from *B. cereus* (28) or the 2,900 μmol/min/mg reported for the affinity chromatography-purified enzyme (29). This suggests that structural differences outside the highly conserved active site may affect the rate of catalysis. However, tyrosine 56 in the active-site region of *B. cereus* PLC is replaced by histidine in *L. monocytogenes*. This residue is believed to be involved in docking of the choline head group (5, 22). We cannot rule out the possibility that some of the enzyme has been inactivated, but multiple preparations gave similar activities on PC–Triton X-100 mixed micelles. Other prokaryotic PLCs have been found to have specific activities similar to that of the PLC of *L. monocytogenes* (45).

When phospholipids were dispersed in Triton X-100, the rates of cleavage were in the following order: PC > PE > PS > SM >> PI. These findings are consistent with the qualitative results obtained with sodium cholate dispersions (16). With the exception of PE, the rates of hydrolysis of the phospholipids were identical at pHs 5.5 and 7.0, and this single difference was not statistically significant. A similar decrease in the rate of PE hydrolysis at pH 5.5 was seen in experiments with *E. coli* lipids (Fig. 4) but not with DOPE

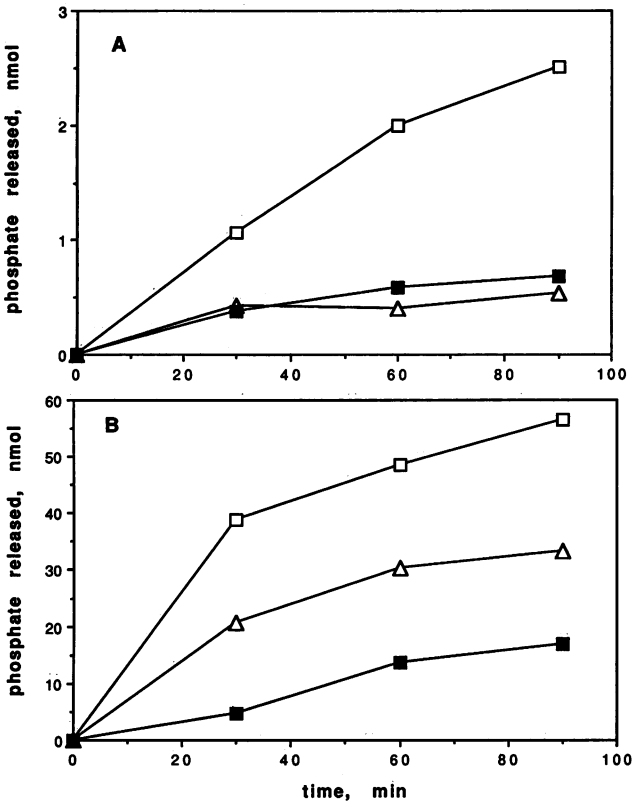

FIG. 7. Hydrolysis of PI and PC in liposomes. PI-PC-cholesterol liposomes were prepared by sonication in 0.1 M MOPS-MES buffer, pH 7.0, as described in Materials and Methods. In addition to the lipid suspension, incubation mixtures contained 50 μg of BSA and 1.9 μg of dialyzed enzyme in a final volume of 0.2 ml. The final buffer concentration was 50 mM. The enzyme had been preincubated for 30 min at room temperature alone (□), with 2 mM EDTA (◆), or with 0.32 mM *o*-phenanthroline (■). (A) PI hydrolysis was measured by release of [^{3}H]inositol-P into the aqueous phase after partition against chloroform-methanol (see standard assay). (B) PC hydrolysis was measured chemically by release of phosphorus into the aqueous phase. The amount of PI hydrolyzed at each time point was subtracted from the total amount of phosphorus released to determine PC hydrolysis. These results are representative of two identical experiments.

dispersed in Triton X-100 (Table 1). With *E. coli* lipids, pH had the opposite effect on the rate of PG hydrolysis (Fig. 4). In this system, cardiolipin was not appreciably hydrolyzed.

The specificity of the enzyme broadens with natural membranes. Hydrolysis of cardiolipin and PI was evident in *C. butyricum* protoplasts (Fig. 6) and in human erythrocytes (Fig. 5), respectively. The rate of cardiolipin hydrolysis in protoplasts was relatively low, as observed with PLC from *B. cereus* (31, 38), but the rate of PI hydrolysis in erythrocyte ghosts was rapid, even when its relatively low concentration in the cell membrane was taken into account. Thus, conclusions on the prospective hydrolysis of a given lipid class by bacterial PLCs cannot be drawn from studies of lipids dispersed in detergents alone, as has previously been the experience with the broad-specificity PLCs from *B. cereus* (28) and *C. perfringens* (10). Our experiments with *C. butyricum* protoplasts also demonstrated the ability of *L. monocytogenes* PLC to hydrolyze plasmenylethanolamine and its glycerol acetal at rates comparable to that of the

TABLE 2. Effects of inhibitors on PLC activity[a]

Addition and concn (M)	Enzyme activity (% of control)	
	Erythrocyte lipids	PC–Triton X-100 mixed micelles
None	100	100
KI (0.1)	120	110
NaCl (0.1)	120[b]	95
NaF (0.1)	140	130
Tris-HCl (0.1)	53	42
KPO_4 (0.08)	ND[c]	25
$Ca(NO_3)_2$ (0.05)	35	45

[a] Assays were carried out under standard conditions at pH 7.0 without $ZnSO_4$ for Triton X-100 mixed micelles and as described in the legend to Fig. 5 for erythrocyte ghosts.

[b] Determined by extrapolation.

[c] ND, not determined. The assay for erythrocyte ghost hydrolysis by release of water-soluble phosphate could not be performed in the presence of P_i.

hydrolysis of PE. There appears to be no requirement for an ester bond at the *sn*-1 position of phosphoglycerides. Plasmalogens are widely distributed in mammalian membranes. Heart, skeletal muscle, and brain cells, as well as other cells of the central nervous system, contain the highest proportions of these lipids (21). In rabbit alveolar and guinea pig peritoneal macrophages, ethanolamine glycerophospholipids constitute 22 and 27% of total phospholipids, respectively, and the plasmalogen form constitutes from 41 to 61% of the ethanolamine lipids in these cells and in rat alveolar macrophages (35). Thus, significant host cells for *L. monocytogenes* infections can be assumed to contain both types of common glycerolipids.

The ability of PLC to hydrolyze PI in liposomes was assessed to ensure that the hydrolysis seen in erythrocyte ghosts was not caused by an endogenous activity, which may have been activated by the hydrolysis of the other phospholipids in the membrane, and to demonstrate that there was no PI-PLC in the enzyme preparation obtained from a *plcA* deletion mutant (PI-PLC negative). The ability of the broad-specificity PLC to hydrolyze PI both in erythrocyte ghosts (Fig. 5) and in liposomes (Fig. 7) was clearly demonstrated, whereas hydrolysis was not seen in cholate dispersions (16) and was difficult to measure in Triton X-100 dispersions (Table 1). Inhibition of PI hydrolysis in liposomes by EDTA and *o*-phenanthroline showed that the activity was not caused by PI-PLC, since that enzyme is not inhibited by these chelators (19, 32), which presumably remove Zn^{2+} from the active site of the broad-specificity enzyme (16). Antibody directed to the PI-PLC isolated from *L. monocytogenes* (19) produced no signal when tested against purified broad-specificity PLC by Western blot analysis (data not shown). Lastly, the water-soluble product of PI hydrolysis by both *L. monocytogenes* and *B. cereus* PLCs migrated with inositol-1-monophosphate in two solvent systems on cellulose TLC and on paper chromatography. In contrast, PI-PLCs from both of these organisms yield inositol-1,2-cyclic monophosphate (6, 23). Thus, the mechanism of hydrolysis of PI by the broad-specificity PLCs differs from that of bacterial PI-PLCs.

Although hydrolysis of PI by PLC from *B. cereus* has previously been reported (48), this work was done before the characterization of a distinct PLC specific for PI, which is also secreted by this organism (23). Our work shows that the broad-specificity enzymes from both *L. monocytogenes* and

B. cereus are capable of cleaving PI; however, the PI-PLCs are much more active on PI than the broad-specificity enzymes (19, 23).

PLC from *L. monocytogenes* is active over a broad pH range, with optimal activities on PC–Triton X-100 mixed micelles from pH 5.5 to 8.0 and on human erythrocyte phospholipids from pH 6.0 to 8.0. Considerable activity was observed with both systems to pH 3.8 (Fig. 3). Previous studies with sodium cholate dispersions and on PC-cholesterol liposomes did not extend below pH 5.0; however, at pH 5.0, activity with PC-cholesterol liposomes was 72% of that at pH 7.0, in agreement with our results with erythrocyte ghosts.

L. monocytogenes PLC has 39% amino acid sequence identity with the enzyme from *B. cereus* (46). Sequence homology is strongly conserved for amino acids that bind zinc in the active site of the *B. cereus* enzyme. Therefore, it is reasonable to conclude that *L. monocytogenes* PLC also contains zinc (46). Consistent with this, enzyme activity was inhibited by EDTA and *o*-phenanthroline, and little activity was observed in culture fluids when the cells were grown in the absence of $ZnSO_4$ (16). When purified in the absence of Zn^{2+}, the enzyme retains activity and is stimulated only by 60% by the addition of 0.05 M $ZnSO_4$. For unknown reasons, concentrations of $ZnSO_4$ above 0.5 mM are somewhat inhibitory.

Unlike the *B. cereus* enzyme, *L. monocytogenes* PLC is not inhibited by halides including iodide, chloride, and fluoride. Thus, the enzyme is active in isotonic saline with erythrocyte ghosts and in 500 mM NaCl with Triton X-100 dispersions, which suggests that it could act both in the extracellular space and in the phagocytic vesicles of infected cells. Recent X-ray crystallographic studies on iodide-treated PLC crystals from *B. cereus* indicate the presence of nine iodide ions. The one with the highest occupancy is located in the entrance of the active-site cleft of the enzyme (22). It is thought that this iodide inhibits by blocking entrance of substrate to the active-site cleft rather than by acting directly on the zinc ions. Tris-HCl, which inhibits both PLCs, has also been shown to lie in the cleft of the *B. cereus* enzyme, but closer to one of the active-site zinc ions than iodide (22). Phosphate, an inhibitor of both enzymes, binds to all three metal ions in the active site of the *B. cereus* enzyme, suggesting that they are involved in catalysis (20). Comparative studies of the structure of the PLC from *L. monocytogenes* with the known structure of the enzyme from *B. cereus* will eventually aid in understanding the relationships between the structures and functions of these two enzymes.

Understanding the role of PLC in the pathogenesis of *L. monocytogenes* and differentiating its function from that of a PI-specific PLC produced by this organism (6, 26, 32) in the infection of the host cell are important goals of our studies. Studies of an insertion mutant defective in PLC showed that it was defective in cell-to-cell spread (46). Cells were able to escape from the primary phagosome in monolayers of the J774 macrophage cell line but appeared to accumulate in double-membrane vacuoles, which were presumably formed after ingestion by an adjacent macrophage of bacteria contained within a cellular process of the initially infected cell (44). A mutant containing an in-frame deletion in *plcA*, on the other hand, was found to be slowed during escape from the primary phagocytic vesicle (7). Thus, it appears that the two PLCs make their major contributions during different stages in cellular infections. Loss of these enzymes through in-frame deletions, which do not produce polar effects, results in less than a 1-log change in the 50% lethal dose (LD_{50}) for a *plcA* mutant (7) and an approximately 1-log increase in the LD_{50} for a *plcB* mutant. It appears that these enzymes are involved in the infectious process but are not essential. However, a double *plcA plcB* mutant suffers a 3-log increase in the LD_{50} (42a). The finding that *L. monocytogenes* has two distinct PLCs with different specificities renews the question of what their targets during the infectious process are. We are currently studying the timing of expression of these enzymes during host cell infection and their potential effects on host cell lipids.

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