Published in final edited form as:

Biochemistry. 2009 April 28; 48(16): 3578–3592. doi:10.1021/bi802312d.

***Listeria monocytogenes* phosphatidylinositol-specific phospholipase C: Kinetic activation and homing in on different interfaces[†]**

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Abstract

The phosphatidylinositol-specific phospholipase C from *Listeria monocytogenes* forms aggregates with anionic lipids leading to low activity. The specific activity of the enzyme can be enhanced by dilution of the protein, addition of both zwitterionic / neutral amphiphiles (e.g., diheptanoylphosphatidylcholine or Triton X-100) or 0.1–0.2 M inorganic salts. Activation by amphiphiles occurs with both micellar (phosphatidylinositol dispersed in detergents) and monomeric (dibutoylphosphatidylinositol, diC₄PI) phosphotransferase substrates and inositol 1,2-(cyclic)-phosphate (cIP), the phosphodiesterase substrate. The presence of zwitterionic / neutral amphiphiles (to which the protein binds weakly) dilutes the surface concentration of the interfacial anionic substrate and thereby reduces enzyme/phospholipid particle aggregation. Zwitterionic amphiphiles also can bind directly to the protein and enhance catalysis since they enhance both diC₄PI and cIP hydrolysis. In contrast to activation by amphiphiles, the rate enhancement by salt only occurs for the phosphotransferase step of the reaction. Added salt has a synergistic effect with zwitterionic phospholipids, leading to high specific activities for PI cleavage with only moderate dilution of the anionic substrate in the interface. This kinetic activation correlates with weakening of strong PI-PLC hydrophobic interactions with the interface as monitored by a decrease in the maximum monolayer surface pressure for insertion of the protein. Several point mutations of surface hydrophobic residues (W49A, L151A, L235A, and F237W) can dramatically alter the unusual kinetics of this secreted enzyme. The high affinity of PI-PLC for anionic phospholipids along with a strong hydrophobic interaction, which gives rise to the unusual kinetic behavior, is considered in terms of how it might contribute to the role of this phospholipase in *L. monocytogenes* infectivity.

Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) catalyzes the calcium-independent hydrolysis of PI in two steps: (i) an intramolecular phosphotransferase reaction at a phospholipid aggregate surface to produce diacylglycerol (DAG) and water-soluble inositol 1,2-(cyclic)-phosphate (cIP), followed by (ii) a cyclic phosphodiesterase reaction where cIP is hydrolyzed to inositol-1-phosphate. The second reaction occurs with a soluble monomeric substrate and is much slower (with both lower $V_{\max}$ and significant higher K_m) than the first one (1,2). Previous studies of the PI-PLC from *Bacillus* sp. highlight several kinetic properties common to a wide range of phospholipase enzymes. That bacterial PI-PLC

[†]This work was supported by N.I.H. GM60418 (M.F.R.), AI45153 (H.G.) and GM52598 (W.C.).

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exhibits (i) “interfacial activation”, an enhanced $V_{\max}/K_m$, toward aggregated PI compared to monomeric PI (3–5), (ii) “surface dilution inhibition”, a decrease in specific activity as the surface concentration of the substrate is diluted with detergents or other phospholipids while keeping the total substrate concentration constant (2), and (iii) “scooting mode catalysis”, where enzyme completes several rounds of substrate turnover at the substrate interface before dissociating from the particle (5). The *Bacillus* sp. PI-PLC is also active towards GPI linkages (6).

PI-PLC from *Listeria monocytogenes*, a ubiquitous foodborne intracellular pathogen of humans and animals (7,8), plays a role as virulence factor for the organism by aiding escape of the bacterium from the vacuoles in macrophages (9). PI-PLC aids in escape of the single-membrane primary vacuole (9–11) as well as aiding in the disruption of the inner membrane of the double membrane spreading vacuole (12). A broad-range PLC that primarily targets PC also plays a role in infectivity of this organism that is distinct from that of the PI-PLC (13–15). The *L. monocytogenes* PI-PLC appears to share the same general base and acid catalytic mechanism as *Bacillus* sp. PI-PLC (16,17), and the enzyme has been shown to be activated by short-chain phosphatidylcholine (PC) molecules as well (18). There are, however, several major differences of the *L. monocytogenes* PI-PLC from *Bacillus* homologues, namely a high pI (above 9), kinetic activation by salts, and relatively weak activity toward GPI anchors (17, 19).

In this work, water-soluble synthetic short chain phosphatidylinositol and cIP along with PI/detergent mixed micelles and PI/PC vesicles were used as substrates to understand how salts and other phospholipids affect both steps of PI hydrolysis by the *L. monocytogenes* PI-PLC. The results indicate that *L. monocytogenes* PI-PLC binds tightly to anionic phospholipids (e.g., PI, PG) and tends to form aggregated complexes with those anionic lipids. The enzymatic specific activity is much lower when these complexes form. The two types of activators previously shown for this enzyme work by different mechanisms. Neutral amphiphiles, which enhance both steps of catalysis regardless of the aggregation state of the substrate, bind directly, though weakly, to the protein and enhance its catalytic ability (at sufficiently high mole fractions the amphiphiles can prevent the enzyme from forming the aggregated complexes). In contrast, moderate ionic strength (e.g., salts) only affects the phosphotransferase reaction and then is most efficient when an activating interface is present. The latter is likely to be the result of altering the surface electrostatics and reducing the formation of aggregated complexes, and possibly altering the residence time of the enzyme on interfaces. Several point mutations of surface hydrophobic residues show that the unusual kinetic behaviors have a strong hydrophobic component. A model rationalizing the role of both types of activators in modulating the enzyme activity in situ is presented.

MATERIALS AND METHODS

Chemicals

Short chain dibutyroylphosphatidylinositol (diC₄PI) was acquired from Echelon Biochemicals. Other phospholipids, including the long chain lipids POPC, POPS, POPG, DOPMe, PI from bovine liver, and the short chain phospholipids diC₇PC, and diC₆PC were obtained from Avanti Polar Lipids and used without further purification. Crude soybean PI (50%) was used for the enzymatic generation of cIP as described previously (2) by taking the advantage of the fact that the phosphotransferase (PI cleavage) activity is much higher than the phosphodiesterase (cIP hydrolysis) activity for bacterial PI-PLC. Carboxyfluorescein (a mixture of 5- and 6-carboxyfluorescein) was acquired from Eastman Kodak Co. Citraconic anhydride (2-methylmaleic anhydride) was purchased from Pierce. Other reagents including Triton X-100 and D₂O (99.9%) were purchased from Sigma.

L. monocytogenes PI-PLC expression and purification

The IMPACT-CN system (New England Biolabs) was used to construct the plasmid for expression of this PI-PLC protein as a fusion protein with both a chitin binding domain for ease of purification and inducible self-cleavage activity to remove the fusion tag (20,21). The *plcA* gene without the region coding for the signal sequence was inserted into the pTYB11 N-terminal fusion vector between *Sap* I and *EcoR* I restriction sites following the manufacturer's instructions. The entire DNA sequence of the inserted target gene was determined and compared with that of pBS1462 (our original source of the *plcA* gene) to assure no PCR-induced errors were introduced. The recombinant plasmid, IMPACT-Y, was transformed into *E. coli* ER2566 cells for overexpression. An aliquot of the overnight culture was used to inoculate fresh LB medium with 100 µg/ml ampicillin in a rotary shaker (200 rpm) at 37°C. Expression of recombinant PI-PLC was induced by adding IPTG at a final concentration of 0.8 mM when OD₆₀₀ of growing culture was between 0.7 and 0.8. After induction, the culture was incubated at 16°C for 20 h, harvested by centrifugation, and stored at -80°C. If needed, the frozen cell pellets were thawed at room temperature, resuspended in 50 ml ice-cold column buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.4), sonicated on ice, and centrifuged to remove the cell debris. The cell extract supernatant of ER2566 harboring IMPACT-Y had high PI-PLC activity, albeit no obvious band of 88 kDa on SDS-PAGE gel. Recombinant *L. monocytogenes* PI-PLC was then obtained following the purification protocol in the manufacturer's instruction manual. All protein concentrations were determined by Lowry assay. The best yield for recombinant *L. monocytogenes* PI-PLC was about 6 mg/L. The stock solution could be stored at 4°C without activity loss for ~1 month. SDS-PAGE analysis showed that the purity of PI-PLC after the chitin column was more than 85% and that PI-PLC was contaminated with a band around 50 kDa (the intein tag judging from the SDS-PAGE gel of chitin beads after elution of PI-PLC). If needed, a SP Sepharose Fast Flow (Amersham Pharmacia Biotech) column was used to further purify PI-PLC right after dialysis. The dialyzed eluate from the chitin column was applied at 2 ml/min onto the strong cation exchange column (~25 ml) equilibrated with 100 ml starting buffer (50 mM sodium phosphate buffer, pH 7.0). The protein was eluted using a NaCl gradient ranging from 0 to 0.5 M in 50 mM sodium phosphate (pH 7.0) at rate of 2 ml/min, dialyzed to remove the high salts, filtered and concentrated. Only the PI-PLC band was observed on the SDS-PAGE gel after these two columns.

A series of mutant *plcA* genes harboring mutations to code for W49A, L151A, L235A, F237A, F237W, and W49A/F237A were constructed using Quik-Change™ Site-Directed Mutagenesis Kit. All mutant *plcA* genes were sequenced to confirm that the correct mutations were introduced. Each recombinant plasmid IMPACT-Y was transformed into *E. coli* ER2566 competent cells for expression of mutant protein and purification as described above.

Citraconic anhydride modification of PI-PLC

To alter the surface charge of the *L. monocytogenes* PI-PLC, the protein was treated with citraconic anhydride. Chemical modification with this reagent changes the positive charges of lysine residues on the protein surface to negative ones. Sodium phosphate, 0.5 M at pH 8.5, was used to provide adequate buffer capacity. The PI-PLC to be modified was purified with two columns (chitin column and SP Sepharose Fast Flow column), dialyzed against reaction buffer, and concentrated. 200 µl PI-PLC (6.5 mg/ml) was incubated with 50 µl citraconic anhydride (113 mM, diluted with reaction buffer) in 1 ml of reaction buffer at room temperature for 3 h after which the reaction mixture was dialyzed extensively against 20 mM Tris-HCl (pH 7.0) to quench the reaction. The change in pI for modified protein was measured via 2D gel electrophoresis separating the protein by isoelectric point and then mass. The two batches of citraconylated protein showed that the pI changed from the 9–10 region for unmodified (which migrated as a single peak) to between 7–9 (two distinct peaks were observed for modified enzyme).

PI-PLC size by laser light scattering

The native protein molecular weight of purified *L. monocytogenes* PI-PLC was determined by laser light scattering (LS) (22) at the HHMI Biopolymer Facility and W.M. Keck Foundation Biotechnology Resource Laboratory. Because the protein interacted with the column matrix dramatically, it was analyzed by a micro-batch approach in which the sample from an injector loop was eluted through the system and the UV/LS/refractive index signals monitored while the buffer was continuously delivered from the HPLC system. Conditions (1.1 ml protein sample, 500 μ l sample loop, and 0.3 ml/min for 1 min 20 sec) were established to provide a reading “plateau” (~0.2 ml, free of air and particle) in all three detectors. Transferrin with a comparable extinction coefficient to *L. monocytogenes* PI-PLC was used as a standard and the same calibration constant was applied to analyze the PI-PLC sample. The concentrations of PI-PLC during the reading plateau were measured with the UV absorbance at 280 nm (extinction coefficient = $33710 \text{ M}^{-1}\cdot\text{cm}^{-1}$, 1 cm length). The molecular weight was determined by solving the equation that relates the excess scattered light to the concentration of solute and the weight-average molecular weight by ASTRA calculations (http://info.med.yale.edu/wmkeck/6_16_98/Astra2a.htm#Calculation of MW by).

Vesicle preparation

The appropriate amount of a lipid stock solution in chloroform was placed in a 20 ml glass scintillation vial; the chloroform was removed with a rotary evaporator. The resulting lipid film was dissolved in 5 ml water, frozen on dry ice, lyophilized overnight, and rehydrated with appropriate buffer. Lipids supplied as powder were dissolved in the buffer directly. To prepare small unilamellar vesicles, the chilled phospholipids suspensions were sonicated on ice using a Branson sonifier W-150 ultrasonic cell disruptor with a 1 cm diameter probe until maximum clarity was achieved. Vesicle preparations were usually centrifuged in a bench-top centrifuge (14,000 rpm for 3 min) to remove any large particles.

PI-PLC enzymatic activity

The specific activity of PI-PLC was measured using ^{31}P NMR spectroscopy (202.3 MHz on a Varian Inova 500 spectrometer) and parameters previously reported (1,2). Two types of assays (end-point and continuous time point) were used for phosphotransferase activity (PI as substrate) and phosphodiesterase activity (cIP as substrate) respectively. Except for diC₄PI, experiments were run in duplicate and the average specific activities are reported; the errors in specific activities were typically <15%.

For long chain PI from bovine liver (8 mM), dispersed in diC₇PC or Triton X-100 (TX-100) micelles, or in small unilamellar vesicles (SUVs) in the absence and presence of POPC, in 50 mM HEPES, pH 7.0, containing 0.5 mg/ml BSA, enzyme (covering a range of 0.01 to 7.2 μ g/ml) was added to the 200 μ l assay mixture and incubated at 25°C. The reaction was quenched by the addition of 400 μ l chloroform at appropriate incubation times (from 1 min to a few hours) which were chosen so that less than 20% PI cleavage occurred. The protein concentrations were kept constant within the same series of assays as protein concentration also affects the specific activity of *L. monocytogenes* PI-PLC. The cIP content in the aqueous phase (separated from the organic layer using centrifugation at 14,000 rpm for 6 min) was quantified in the ^{31}P NMR spectrum using added glucose-6-phosphate (1 mM) as an internal standard. Triton X-100 and salt effects were also measured using the water-soluble substrate diC₄PI. In this assay, 100 μ l assay mixtures containing 2 mM diC₄PI and 1.6 μ g/ml enzyme were used.

In the continuous time point assay for cIP hydrolysis, the release of I-1-P product was monitored as a function of time until 20% of the cIP was converted to I-1-P using a ^{31}P NMR experiment where the pre-acquisition delay was arrayed. The reaction was initiated by adding

3.6 or 7.2 $\mu\text{g/ml}$ PI-PLC to 400 μl assay mixture in a NMR tube typically containing 10 mM cIP in the absence and presence of different additives (diC₇PC, POPC, DOPMe, POPS, salts) in 50 mM imidazole buffer (pH 7.0), incubated at 25°C, and monitored for a period of time, typically from at least 30 minutes to a few hours. The rate of cIP hydrolysis was calculated from the slope of I-1-P production as a function of incubation time.

Monolayer penetration assay

Lipid monolayers were formed by spreading 5 to 10 μl of the appropriate lipids (POPC, or DOPMe, or a mixture of 70% POPC and 30% DOPMe) dissolved in ethanol/hexane (1:9, v/v) onto 10 ml of 50 mM HEPES, pH 7.0, with KCl at three different concentrations (0, 0.16, or 0.5 M) contained in a circular Teflon trough (4 cm diameter $\times$ 1 cm deep). The surface pressure (π) of subphase (the solution in the trough) was measured using a Wilhelmy plate attached to a computer-controlled tensiometer as described for the *B. thuringiensis* PI-PLC (23). The subphase was gently mixed with a magnetic stir bar and the monolayer was allowed to equilibrate until a stable surface pressure (defined as the initial surface pressure, π_0) was obtained. Then a protein solution was injected into the subphase through a small hole in the side of the trough without disruption of the monolayer. The change in surface pressure ($\Delta\pi$) was measured as a function of time. Since the value of $\Delta\pi$ depends on the protein concentration in the low concentration range, protein concentration in the subphase was maintained high enough (above 3 $\mu\text{g/ml}$ for PI-PLC) to ensure the observed $\Delta\pi$ represented a maximal value (24). In general, $\Delta\pi$ is inversely proportional to the π_0 of the lipid monolayer and extrapolations of the $\Delta\pi$ versus π_0 plot to $\pi_0=0$ and $\Delta\pi=0$ yields the maximum $\Delta\pi$ obtainable and the critical surface pressure (π_c) that specifies an upper limit of π_0 into which a protein can penetrate respectively.

Fluorescence assays for vesicle leakage

Vesicle leakage promoted by the bound *L. monocytogenes* PI-PLC was monitored by the release of entrapped carboxyfluorescein (25). Vesicles were prepared by sonication of 20 mM of the appropriate lipids (POPC or DOPMe) with a solution of 100 mM carboxyfluorescein in 10 mM HEPES, pH 7.1. The nonencapsulated carboxyfluorescein was removed by rapid filtration of the vesicle solution through a Sephadex G-10 column at room temperature. POPC prepared with 150 mM KCl in the buffer along with the carboxyfluorescein were also prepared. The carboxyfluorescein-loaded vesicles were used immediately after gel filtration. Vesicle integrity in the absence or presence of PI-PLC protein was monitored with a Fluorolog R-3 spectrofluorimeter using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. All the carboxyfluorescein assays were carried out in the dark and a narrow excitation slit width (1 nm). The percent leakage at time t , L_t , was expressed relative to the initial fluorescence F_0 and the maximum fluorescence F_{TX} obtained after complete lysis of the vesicles by the addition of 25 mM Triton X-100: $L_t = (F_t - F_0) / (F_{TX} - F_0) \times 100\%$.

Precipitation and turbidity assays

Aggregation and precipitation of PI-PLC with SUVs was assessed by incubating protein (0.036 mM) with pure POPG, pure POPC, and with POPG/POPC (1:1 or 1:25) SUVs, then separating any precipitate by centrifugation (15,000 rpm for 10 min with a bench-top centrifuge). The relative partitioning of the protein in the precipitate versus what remains in solution was measured by first lyophilizing both precipitate and solution, then examining relative amounts of protein in each phase by SDS-PAGE.

The time course for aggregation of DOPMe (0.86 mM) / POPC (3.1 mM) SUVs (in 50 mM HEPES, pH 7.0) promoted by different concentrations of PI-PLC (0.15, 0.75 and 1.5 $\mu\text{g/ml}$) was monitored by the increase in optical density at 350 nm of the solution after the addition

of protein (26). Monitoring the OD₃₅₀ of corresponding vesicles without protein provided baseline turbidity.

³¹P NMR linewidth assay

Changes in phospholipid ³¹P linewidths were used to monitor the binding of diC₆PC and diC₇PC to *L. monocytogene* PI-PLC (2 mg/ml). The linewidth of the PC resonance was measured at various concentrations (0.1–20 mM) in the absence and presence of enzyme. Linewidth changes upon micellization of both of these short-chain lipids were small, so that the difference in linewidth caused by the presence of enzyme was initially assumed to reflect the amount of ligand bound to the enzyme in fast exchange with free ligand. As discussed previously (27), the difference in linewidth in the presence and absence of ligand at a given ligand concentration, ($\Delta\nu_{\text{obs}} - \Delta\nu_0$), is a function of total concentration of enzyme (E_T) and ligand ($[PC]_T$), the bound linewidth for the E·PC complex ($\Delta\nu_b$), the dissociation constant (K_d), and n , the number of ligands bound per enzyme molecule. If there are multiple, independent binding sites for the PC (to form E·PC_{*n*}) leading to the change in linewidth, and if the free $[PC] \gg [E \cdot PC]$ complex, then the following equation holds:

$$(\Delta\nu_{\text{obs}} - \Delta\nu_0) = \frac{n \times E_T \times [PC]_T^{n-1} \times (\Delta\nu_b - \Delta\nu_0)}{K_d + [PC]_T^n}$$

RESULTS

Dependence of *L. monocytogenes* PI-PLC specific activity on protein concentration

Previously, the pH behavior for the *L. monocytogenes* PI-PLC phosphotransferase reaction was shown to vary slightly with the detergent matrix for the PI. A sharp optimum pH of 7.0 was observed toward PI solubilized in deoxycholate micelles with less than 30% activity at pH 6.5 and pH 7.5 (the suspension of PI in deoxycholate was no longer clear below pH 6.5), while a broader optimum pH range, from 5.5 to 6.5, was observed for enzyme acting on PI in TX-100 micelles (17). For comparison we tested the effect of pH on the phosphodiesterase reaction in the absence of detergent. The enzyme was optimally active towards cIP at pH 7.0, with 67% and 57% maximum activity at pH 6.5 and 7.5, respectively (Figure 1A). Thus, pH 7.0 was used for both phosphotransferase and cyclic phosphodiesterase kinetic assays.

PI can be presented to PI-PLC in a range of different matrices – micelles, unilamellar vesicles or water/organic solvent co-mixtures (1,2,4,28,29). Differences in PI-PLC activity toward the different types of interfaces can often provide insights into what factors control the activity of this enzyme. However, *L. monocytogenes* PI-PLC exhibited an added complication in that protein concentration also affected enzyme activity. Since the assay for phosphotransferase activity is not continuous, we monitored the generation of cIP from PI/TX-100 mixed micelles at various time points to avoid the influence of a lag or burst in product formation. As shown in Figure 1B, the phosphotransferase specific activity of *L. monocytogenes* PI-PLC toward PI (8 mM) in TX-100 (32 mM) mixed micelles increased dramatically from 150 to 1200 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ as protein was diluted from 0.4 to 0.01 $\mu\text{g/ml}$. Further dilution of the protein had only a small effect on specific activity. A similar dependence of specific activity on protein concentration was also observed with PI/diC₇PC mixed micelles. To rule out any possible inhibitors from the expression/purification system, purified recombinant *L. monocytogenes* PI-PLC was further purified with cation exchange or gel filtration (with high salt concentration) chromatography, and the concentration dependence of specific activity on enzyme concentration still persisted.

The loss of activity with high enzyme concentrations has been discussed by Wang (30) for an association-dissociation enzyme system. PI-PLC could be aggregating in solution with the monomer more active than oligomer. However, this PI-PLC interacted anomalously with gel filtration resins even in the presence of 0.5 M NaCl, so that another method was needed to assess the size of the protein in solution. Laser light scattering, with some modification from the standard protocol, was used to characterize the native molecular mass of the protein. The average molecular mass for *L. monocytogenes* PI-PLC was determined as 28.2 kDa at a concentration of 0.14 mg/ml – well above that used in the kinetic assays. This is quite close to the predicted molecular weight of 33 kDa determined from the sequence. Thus, this PI-PLC exists as a monomer in solution in the absence of detergent. If the protein aggregation does account for the unusual dependence of specific activity on protein concentration, the oligomerization of PI-PLC must only occur in presence of an interface and/or the anionic substrates.

The phosphodiesterase activity of *L. monocytogenes* PI-PLC toward 5 to 40 mM cIP without detergent was investigated at two different protein concentrations. Because cIP is a poorer substrate than PI, considerably higher enzyme concentrations (3.6 or 7.2 $\mu\text{g/ml}$) were needed. In this high range of enzyme concentration, the activity was low and varied little between these two protein concentrations. However, there was a very significant difference in kinetic parameters extracted for the two different enzyme concentrations (Figure 2). In both cases, the data could be fit to the Michaelis-Menten equation: $V_{\text{max}} = 0.7 \pm 0.1 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_m = 12.5 \pm 5.0 \text{ mM}$ at the lower PI-PLC concentration (3.6 $\mu\text{g/ml}$), and $V_{\text{max}} = 2.0 \pm 1.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ with $K_m = 100 \pm 62 \text{ mM}$ at double the enzyme concentration. Although the errors in V_{max} and K_m are large for the higher protein concentration, the major effect of the higher enzyme concentration appears to be an increase in K_m for water-soluble substrate cIP. Since *L. monocytogenes* PI-PLC exists as monomer in solution as shown by the laser light scattering measurement, the enzyme may aggregate with the anionic substrate present (in this case soluble cIP). Such aggregation would appear to specifically increase the apparent K_m .

Effect of detergent or vesicle interface on PLC activities

The *Bacillus* sp. PI-PLC enzymes exhibit a specific activation of both phosphotransferase and phosphodiesterase steps by interfaces containing phosphatidylcholine (2). It has been suggested that such activation also occurs for the *L. monocytogenes* PI-PLC (18). Therefore, we examined the effect of micelle matrix on the phosphotransferase activity of recombinant *L. monocytogenes* PI-PLC toward PI dispersed in TX-100. The enzyme concentration, as well as detergent, was varied. TX-100 is a nonionic detergent that clarifies PI bilayers at >2:1 TX-100/PI. If the surface concentration of PI is important for *L. monocytogenes* PI-PLC activity, then increasing TX-100 at fixed PI (8 mM) will eventually “inhibit” the enzyme leading to lower specific activity. Such surface dilution inhibition has been documented for the *B. thuringiensis* PI-PLC (2). However, *L. monocytogenes* PI-PLC presented different kinetic patterns depending on the protein concentration (Figure 3A). For 0.4 $\mu\text{g/ml}$ enzyme, the enzyme exhibited no decrease in specific activity up to $X_{\text{det}} = 0.94$ (16:1 TX-100/PI). In fact, the enzyme phosphotransferase specific activity *increased* dramatically as the surface concentration of the detergent increased above 0.90. The same amount of PI-PLC exhibited the same specific activities towards PI presented in diC₇PC micelles suggesting that the diC₇PC and TX-100 detergents were essentially equivalent. A similar trend, but with higher specific activities, was observed when the protein concentration in the assay was decreased two-fold.

At a much more dilute concentration, 0.02 $\mu\text{g/ml}$, a more typical ‘surface dilution curve’ was observed with the maximum phosphotransferase specific activity around $X_{\text{det}} = 0.78$ (3.5:1 TX-100/PI). This detergent/substrate ratio for optimal activity is higher than what is needed to

minimally solubilize PI bilayers (2:1 TX-100/PI). It has been reported that PI in predominantly PC bilayers is partially demixed (31). A similar demixing in mixed micelles could occur unless an excess of detergent is used. Alternatively, this could reflect a distinct activation by the detergent. At the very low concentration of this PI-PLC, the specific activity of *L. monocytogenes* PI-PLC decreased above $X_{\text{det}} = 0.80$ as might be expected for a regime where the surface concentration of substrate influences the enzyme action. The dilute enzyme is very sensitive to the mole fraction PI in the interface. Comparing the activity curves of three protein concentrations in Figure 3A shows that significantly increased TX-100 could compensate for the lower specific activity at high enzyme concentration, since comparable high activities (1155 and $1260 \mu\text{mol min}^{-1}\text{mg}^{-1}$) were achieved for all the protein concentrations examined. If the protein concentration decreased further, a lower optimum TX-100/PI ratio might be expected. However, $0.02 \mu\text{g/ml}$ is the limit of our assay system. Using $[^3\text{H}]\text{PI}$ as substrate, the phosphotransferase activity of PI-PLC was previously measured at $0.003 \mu\text{g/ml}$ enzyme. The optimal *L. monocytogenes* PI-PLC specific activity ($\sim 2900 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) toward 0.08 mM PI micelles dispersed in TX-100 or deoxycholate was reported at a ratio of 10:1 TX-100/PI or 30:1 deoxycholate/PI (17). The higher specific activity than what we observe with low PI-PLC or moderate PI-PLC with high mole fractions of detergent (X_{det}) is likely due to the addition of ammonium sulfate in the previous report.

The activity of *L. monocytogenes* PI-PLC toward a fixed bulk concentration of PI (8 mM) diluted in a POPC vesicle matrix was also studied. Compared with PI/TX-100 micelles, unilamellar vesicles of long-chain PI are poor substrates for PI-PLC from *L. monocytogenes*, necessitating higher protein concentrations for these assays. At a protein concentration of $1.6 \mu\text{g/ml}$, the enzyme phosphotransferase specific activity increased about 2.2-fold as the mole fraction of POPC increased from 0 to 0.2. Once the POPC mole fraction was greater than 0.2 (at fixed PI), the *L. monocytogenes* PI-PLC specific activity stayed constant to a POPC mole fraction of 0.5 and then decreased as the mole fraction increased further to 0.8 (Figure 3B). Similar to PI/TX-100 micelles, higher specific activities toward PI/POPC SUVs were also observed at a lower enzyme concentration ($0.4 \mu\text{g/ml}$); the activation with 0.2 mole fraction in the vesicle was also larger than with the higher concentration of enzyme.

Examining amphiphile effects with monomeric substrate, in this case cIP, is one way of separating activator effects that alter the substrate versus those that alter the enzyme. With cIP at a fixed concentration, we can screen different additives for their effect on *L. monocytogenes* PI-PLC activity towards this monomeric substrate. Ryan and coworkers (18) have shown that diC_6PC at concentrations below its CMC activate this enzyme with respect to non-natural water-soluble PI substrates. In our system, the plot of phosphodiesterase specific activity of *L. monocytogenes* PI-PLC versus diC_6PC concentration was bimodal (Figure 4). Below the CMC of diC_6PC (14 mM), there was ~ 10 -fold increase in activity upon the addition of diC_6PC ; a second increase in specific activity was observed around the CMC of diC_6PC , with PI-PLC activity 20-fold activity higher than for cIP alone. A slightly more hydrophobic amphiphile, diC_7PC , also enhanced the activity of the enzyme toward the natural soluble substrate cIP (Figure 4). The bimodal dependence of specific activity on diC_7PC concentration is consistent with the PC interfaces being more potent activators than monomeric PC at concentrations where it is monomeric in the absence of protein. POPC SUVs also enhanced cIP hydrolysis (Table 1 and Figure 4), although the maximum activity was considerably less than for diC_7PC or TX-100 activation. SUVs composed of anionic non-substrate phospholipids (POPS and DOPMe) strongly inhibited the cIP hydrolysis reaction (Table 1). Vesicles with 0.2 mole fraction POPS disproportionately reduced *L. monocytogenes* PI-PLC activity toward cIP. Therefore, the enzyme must bind to those anionic phospholipids more tightly than it binds to POPC, and this interaction prevents cIP binding and hydrolysis.

Unlike what was observed for *B. thuringiensis* PI-PLC, TX-100 was also an effective activator of cIP hydrolysis by *L. monocytogenes* PI-PLC. Comparable activities were obtained with 3 mM TX-100 or 5 mM diC₇PC (Table 1). Since the substrate cIP is monomeric, the amphiphile must activate *L. monocytogenes* by a direct interaction. However, whether in doing so it disfavors anionic ligand-induced protein aggregation that may be occurring or promotes a more active form of the enzyme is not clear.

Effect of salts

Salt activation of PI cleavage by *L. monocytogenes* PI-PLC has been documented previously (17). Although no explanation was proposed, it was noted that in the absence of high salt, *L. monocytogenes* PI-PLC in solution behaved as a large aggregate on some gel filtration columns, but that 1 M (NH₄)₂SO₄ attenuated this behavior. Salts can enhance hydrophobic interactions (e.g., promote insertion of protein hydrophobic side chains into bilayers) as well as weaken electrostatic interactions that hold complexes together. With this in mind, we examined the effect of several different salts ((NH₄)₂SO₄, NH₄Cl, K₂SO₄, and KCl) on *L. monocytogenes* PI-PLC cleavage of PI in TX-100 or diC₇PC mixed micelles and hydrolysis of cIP. As shown in Table 2, the ionic strength, and not the salt identity, was important for *L. monocytogenes* PI-PLC activation towards PI in micelles. At fixed enzyme concentration and 8 mM PI (solubilized in 32 mM TX-100), *L. monocytogenes* PI-PLC activity displayed a hyperbolic dependence on concentration of (NH₄)₂SO₄. For 0.2 µg/ml *L. monocytogenes* PI-PLC, the apparent $V_{\max}$ was $2022 \pm 202 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ with a $K_{0.5}$ (concentration of salt for half the maximum specific activity) of $38 \pm 16 \text{ mM}$; for 1.6 µg/ml *L. monocytogenes* PI-PLC, the apparent $V_{\max}$ decreased somewhat to $1355 \pm 256 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ with a comparable salt $K_{0.5}$ ($42 \pm 20 \text{ mM}$). The lower $V_{\max}$ extrapolated from the salt dependence of activity at an 8-fold higher *L. monocytogenes* PI-PLC concentration indicates that the presence of salt does not completely eliminate the unfavorable factors associated with high enzyme concentration. The ratio of specific activity for 0.21 µg/ml PI-PLC compared to 1.6 µg/ml at 0, 20, and 100 mM (NH₄)₂SO₄ is 3.7, 1.6, and 1.5, respectively. These decreases suggesting that (NH₄)₂SO₄ does partially compensate for the negative effects of high enzyme concentration.

PI-PLC cleavage of PI presented in SUVs was also activated by salt (Table 2). With KCl, the effect leveled off around 0.3 M of the salt. With PC in the SUV along with the PI, the addition of salt also led to a much larger increase in specific activity (Table 2).

In contrast to its activation of PI cleavage by *L. monocytogenes* PI-PLC, (NH₄)₂SO₄ (as well as other salts) inhibited cIP hydrolysis whether diC₇PC was present or not (Table 1). These results make it unlikely that salt biases the enzyme toward a more active conformation, otherwise, it should have enhanced cIP hydrolysis as well as PI cleavage. The added salt could alter the properties of substrate interfaces making them more susceptible to *L. monocytogenes* PI-PLC or it could alter the interaction of the enzyme with the interface. One way of testing these explanations is to examine the effect of salts and amphiphiles on cleavage of diC₄PI, a soluble phosphodiesterase substrate with no tendency to partition into aggregates as long as the substrate concentration is low (the CMC for this lipid is likely to be >150 mM since diC₄PC has a CMC of 250 mM (32) and other short-chain PI species exhibit CMC values comparable to the same chain length PC compound (33)). As a way of ruling out any aggregation of the diC₄PI induced by the amphiphile or salt, the ³¹P linewidth of 2 mM diC₄PI was measured in buffer in the absence and presence of 100 mM KCl, 100 mM TX-100, and then both components. The linewidth, $1.6 \pm 0.1 \text{ Hz}$, was unchanged with the additives.

Keeping the diC₄PI concentration at 2 mM, we measured the effect of KCl and TX-100 on *L. monocytogenes* PI-PLC activity towards the soluble substrate (Table 2). With either 75 mM KCl or 32 mM TX-100 only, the specific activity of *L. monocytogenes* PI-PLC toward diC₄PI increased slightly (about 1.6-fold). However, if both salt and TX-100 were present, there was

~14-fold increase in *L. monocytogenes* PI-PLC toward diC₄PI. A similar synergistic effect was observed with monomeric diC₆PC added – a much larger activation was seen with *both* KCl and amphiphile present. This synergistic effect cannot reflect altering the substrate interface because the substrate is monomeric. This suggests that for the phosphotransferase reaction, the presence of an amphiphile is important for the kinetic activation observed with moderate ionic strength. KCl (0.1 M) could lower the CMC of diC₆PC slightly, but the effect in the absence of enzyme is not significant (34). However, the slight decrease in specific activity for *L. monocytogenes* PI-PLC acting on cIP with monomeric diC₆PC approaching the CMC (Figure 4) may suggest that the enzyme itself could nucleate ‘mini’-micelle formation when it binds a diC₆PC molecule. Studies of *B. thuringiensis* PI-PLC showed that diC₆PC added at a monomeric concentration can bind to the protein, at a site distinct from the catalytic site, and activate the enzyme (27). DiC₄PI is a poor substrate for *L. monocytogenes* PI-PLC, necessitating long incubation times (2 to 5 h) if the same enzyme concentration is to be used in the absence and presence of salts and amphiphiles. With TX-100 present, final products included both cIP and I-1-P for incubation times longer than 1 h. However, if diC₆PC was added as the amphiphile, cIP was the only product even after 2 h. There is a difference when the *L. monocytogenes* PI-PLC interacts with TX-100 versus a short-chain PC molecule such that binding of diC₆PC to the protein promoted cIP release from the active site, while with TX-100 as the amphiphile both products were detected. Nonetheless, both amphiphiles effectively enhanced the enzyme activity.

Binding PI-PLC to interfaces

Short-chain PC molecules activate the *L. monocytogenes* PI-PLC toward cIP in the absence of salt, so there must be a direct interaction of the enzyme with the PC molecules. This can be probed by P NMR linewidth studies (27). As shown in Figure 5, a threshold of diC₇PC (≥ 1 mM) was needed before significant broadening of the phospholipid resonance (0.8 Hz maximum under the conditions used) was detected. As more PC was added, the linewidth difference in the presence and absence of *L. monocytogenes* PI-PLC decreased. This type of profile is typical for multiple PC molecules interacting with the enzyme (and occupation of all these sites needed before the measured spectral change occurs). As shown in Figure 5B, the linewidth difference for diC₇PC caused by the presence of *L. monocytogenes* PI-PLC (2 mg/ml) was consistent with an ‘n’ value of 3–4, ($\Delta\nu_b - \Delta\nu_0$) = 5.5 ± 0.6 Hz, and apparent K_d = 1.5 ± 0.6 mM. The analysis with diC₆PC binding to *L. monocytogenes* PI-PLC yielded the same ‘n’, ($\Delta\nu_b - \Delta\nu_0$) = 3.3 ± 0.8 Hz, and K_d = 17 ± 10 mM (data not shown). In both cases, the ‘ K_d ’ describing the interaction was essentially at the CMC of the short-chain PC. The linewidth for bound PC would suggest significant mobility consistent with a small aggregate or mini-micelle with the protein.

L. monocytogenes PI-PLC binding to SUVs of POPC or anionic vesicles (DOPMe, POPG, etc.) was also examined using a filtration / centrifugation binding assay (23). *L. monocytogenes* PI-PLC (30 μ g/ml or 0.9 μ M) had weak affinity for POPC SUVs – essentially >90% of the protein was eluted through the filter upon centrifugation even after incubation with 1 mM POPC SUVs. In contrast, this bacterial PI-PLC bound very tightly to anionic phospholipid SUVs regardless of headgroup. For DOPMe concentrations >10 μ M, essentially all the protein was bound to the phospholipids and none passed through the filter upon centrifugation. The dramatically slower rate for the solution to pass through the filter suggested that large aggregates of the proteins and vesicles might be forming. To test this, we incubated 36 μ M PI-PLC (a considerably higher concentration) with different concentrations of POPG, POPC or mixed POPG/POPC SUVs (Figure 6A). There was a significant anionic lipid-induced precipitation of protein (Figure 6A). With 0.2 mM POPG and 0.036 mM protein, most of the protein ($64 \pm 10\%$) was associated with the precipitate. With the POPG reduced to 0.02 mM, a moderate amount of the protein ($42 \pm 5\%$) was still associated with the precipitate. When that

amount of POPG was presented in 0.5 mM POPC, the amount of protein in the precipitate was significantly reduced ($16 \pm 3\%$). Mixing PI-PLC with pure POPC SUVs led to only $5 \pm 3\%$ of the protein in a precipitate, much of which could be due to a small fraction of larger vesicles with some PLC bound. If micelles as opposed to vesicles were examined, more PG was needed to precipitate the protein (data not shown). The amount of each lipid in the precipitate (extracted with methanol/chloroform) was measured with ^{31}P NMR, and the lipid composition in the precipitate for POPG/PC SUVs or micelles was very similar to that in solution. Therefore, increasing PI-PLC specific activity as detergent or PC is increased in the SUV assay system is likely to reflect (at least in part) disruption of a large, nonproductive aggregated complex of the enzyme with several vesicles that sequesters it from the substrate in other vesicles.

The progress of aggregation / precipitation was monitored by looking at OD_{350} with continuous stirring (Figure 6B). After adding different concentrations of *L. monocytogenes* PI-PLC to DOPMe/POPC (1:3.6) SUVs, an instant and very dramatic increase in optical density, corresponding to the formation of the precipitate, was observed. The optical density decreased over time, but did not settle back to the initial value; the limiting OD_{350} was highest for the highest concentration of protein. Similar behavior was also observed for incubating this PI-PLC with POPG/POPC SUVs, but not for pure PC vesicles. Since the precipitate could be dispersed by adding salt at concentrations (0.1–0.3 M) comparable to those used in the kinetics), it does not reflect vesicle fusion.

The release of carboxyfluorescein trapped in POPC/POPG vesicles upon the addition of protein was monitored to check the stability of the vesicles in the presence of PI-PLC (Figure 6C). *L. monocytogenes* PI-PLC caused $<25\%$ carboxyfluorescein leakage (compared to total leakage from TX-100) within 2 hours (the reaction time course used for most assays) for three vesicle systems (POPC, POPC with KCl, and DOPMe (as the nonhydrolyzable substrate analogue)). The initial release rate of carboxyfluorescein increased in the order of the affinity of enzyme for the interface (DOPMe $>$ POPC $>$ POPC/KCl). The more tightly the enzyme binds to a given vesicle surface, the longer the enzyme stays on that structure (a nonproductive aggregated complex in the case of the pre DOPMe SUVs) and less chance it will dissociate and bind to another vesicle.

Monolayer studies provide insight into how PC and likely other neutral amphiphiles as well as salt contribute to the kinetic activation of the enzyme. *L. monocytogenes* PI-PLC binds tightly to anionic monolayers of DOPMe (Figure 7A), and π_C , the threshold pressure above that the enzyme cannot insert into the membrane, is high (46 dyne/cm) for this protein under these conditions. The strong interaction with an anionic surface may not be surprising given the high pI for this protein and the potent inhibition of cIP hydrolysis by anionic phospholipid vesicles (Table 2). The addition of KCl at a concentration comparable to its effective $K_{0.5}$ for kinetic activation (0.16 M) had little effect on π_C for the enzyme binding to a DOPMe monolayer, indicating binding and insertion in the anionic interface were not dramatically affected by this concentration of salt (Figure 7B). The slope of the plot decreased suggesting that the portion of protein maximally inserted into a membrane had decreased in the higher ionic salt solution. Binding of *L. monocytogenes* PI-PLC to POPC membranes was characterized by a considerably smaller π_C (31 dyne/cm) that was further reduced in the presence of 0.16 M KCl (to 23 dyne/cm). Binding of *L. monocytogenes* PI-PLC to a mixed monolayer of 70 mol% PC and 30 mol % DOPMe in the absence of KCl resembled the binding curve to a pure DOPMe monolayer (Figure 7A); π_C was unaltered while the maximum $\Delta\pi$ extrapolated decreased reflecting the PC content (24 versus 32 dyne/cm). The addition of 0.16 M KCl to this mixed monolayer had a pronounced effect on *L. monocytogenes* PI-PLC binding. π_C was decreased to 37 dyne/cm while $\Delta\pi$ was the same as that extrapolated from *L. monocytogenes* PI-PLC binding to a PC monolayer. When the KCl in the solution was increased to 0.5 M, the protein showed very minimal binding irrespective of any lipid composition

indicating the driving force for the *L. monocytogenes* PI-PLC to reach the lipid surface has a large electrostatic component. The synergistic effect of PC and KCl in reducing the interaction of *L. monocytogenes* PI-PLC with the monolayer correlates with higher enzymatic activity observed with PI/PC SUVs upon the addition of KCl.

Citraconylation of PI-PLC

The high pI for *L. monocytogenes* PI-PLC coupled with the aggregation and monolayer results suggested that strong interactions with anionic phospholipids can be detrimental to enzyme action. Citraconic anhydride was used to modify a portion of the Lys residues on the protein surface to see if surface charge had a dominant effect on the kinetic behavior. This reagent converts surface Lys residues to amides with a terminal carboxylate. For each Lys modified the charge is decreased by 2. The pI of two separate batches of modified enzyme were checked by 2D gel. Two distinct spots were detected with the pI between pH 7 and 9. Given the 28 Lys in the protein this shift is consistent with ~5–7 Lys modified (estimated by changing several Lys to a Glu and computing the pI). A kinetic analysis of PI cleavage in the TX-100 mixed micelle system (Table 3) showed that the modified enzymes still could be activated to about the same extent by salt and amphiphiles. However, the effect of protein concentration was diminished compared to the control enzyme suggesting that the high cationic character of the protein is a factor in the unusual dependence of specific activity on enzyme concentration. The smaller effects on salt and amphiphile activation indicate that either (i) Arg and/or remaining unmodified Lys residues account for this kinetic activation, or (ii) a hydrophobic interaction with the *L. monocytogenes* PI-PLC is also an important contributor to the activation by salts and amphiphiles.

Site specific mutations of PI-PLC surface hydrophobic residues

Since the unusual dependence of *L. monocytogenes* PI-PLC activity on the enzyme concentration, amphiphiles and salts persisted after citraconylation, hydrophobic interactions of the protein with interfaces may be responsible for the unusual kinetic behavior. For *Bacillus sp.* PI-PLC, several hydrophobic amino acid residues from short α -helix B and some loops, arranged in a semicircle around the active site cleft, form a mobile hydrophobic ridge fully exposed to solvent. Among the exposed residues, two tryptophans, Trp-47 in α -helix B (PIKQVWG) and Trp-242 in one particular loop (SGGTAWN), were shown to be important for the enzyme to bind to both activating zwitterionic and substrate anionic interfaces (35). The structure of *L. monocytogenes* PI-PLC also consists of a short α -helix B and an analogous rim loop (36). Although the sequence similarity is low for these two regions, the structure-based sequence alignment of *L. monocytogenes* versus *B. cereus* PI-PLC did provide several candidates. Both Trp-49 and Leu-51 in α -helix B (ITWTLTKP) and Leu-235 and Phe-237 in the loop (SATSLTF) were chosen as targets for mutagenesis (the location of these residues in the structure is shown in Figure 8A).

Most of the mutant enzymes constructed had reasonable activity and were much more active than wildtype at high protein concentrations (>1 mg/ml). Only F237A showed an increase in specific activity as the protein concentration decreased, behavior like wildtype enzyme (Figure 8B). All the rest either showed little change or a decrease in specific activity as the protein became very dilute. The latter effect could reflect some surface unfolding of a protein under very dilute conditions. W49A and W49/F237A exhibited very low specific activities for cleavage of PI in TX-100 micelles compared to recombinant wildtype enzyme (Figure 8C), consistent with Trp49 having a different role than in the *B. thuringiensis* enzyme. In the *L. monocytogenes* PI-PLC crystal structure, Trp49 is tucked in towards the active site, where it might help align substrate, rather than situated facing the external medium as does Trp47 in the *B. cereus* PI-PLC (36). In the *B. cereus* PI-PLC, Trp47 is critical to anchoring the protein to interfaces. However, in the *L. monocytogenes* PI-PLC, an intact helix B would not orient

Trp49 for insertion into the membrane. The position and proximity of Trp49 to the active site may explain why its replacement with a small residue alters the efficiency of the enzyme. At a concentration of 0.82 $\mu\text{g/ml}$, the specific activity of W49A toward PI was only 2% that of the wildtype enzyme. These two fairly inactive enzymes also exhibited a very distinct decrease in specific activity as the enzyme was diluted in the assay – the opposite of the trends for wildtype enzyme. Incubation of 10 mM cIP and 5 mM TX-100 with 7.2 $\mu\text{g/ml}$ W49A or W49A/F237A for 24 h did not generate any ^{31}P NMR-detectable I-1-P. Clearly, modifying key hydrophobic groups in helix B and the active site loop reduce or remove the unusual concentration dependence of wildtype *L. monocytogenes* PI-PLC.

The surface dilution profile of the more active mutant enzymes (all assayed with fixed PI-PLC of 0.2 $\mu\text{g/ml}$ and 8 mM PI) were also different from wildtype. At fixed total PI, all enzymes exhibited the increase in specific activity as the mole fraction detergent increased from 0.67 to 0.80 (detergent/PI ratio changing from 2:1 to 4:1), suggesting the higher amount of TX-100 was needed to adequately solubilize the PI in this system (emphasized in Figure 9A). However, the further 3-fold increase in specific activity for this concentration of wildtype enzyme as the mole fraction of detergent was increased above 0.9 (Figure 9B) was not observed for any of the mutant enzymes.

We also examined these mutant enzymes for salt effect on the phosphotransferase reaction and amphiphile effects on cIP hydrolysis. Added KCl (0.15 M) still enhanced PI-PLC cleavage of PI in TX-100 micelles for the mutant enzymes (Figure 10A), although KCl effects were much more modest for W49A (1.8-fold increase), L51A (1.6-fold) and L235A (1.4-fold) compared to wildtype, F237A or F237W. In contrast to the salt activation of phosphotransferase activity, all mutant enzymes exhibited a large and similar magnitude increase in the specific activity for cIP hydrolysis with TX-100 micelles present (Figure 10B).

DISCUSSION

Mechanisms for the activation of *L. monocytogenes* PI-PLC

Activation of the *B. thuringiensis* PI-PLC by phosphatidylcholine interfaces has been studied in detail (2,23,27,28,29,35). Binding of *B. thuringiensis* PI-PLC to PC interfaces, which appears to have a strong hydrophobic component (23), alters the enzyme conformation so that it is a better catalyst in both phosphotransferase and cyclic phosphodiesterase reactions. The activation is specific for zwitterionic phospholipids (2). The *L. monocytogenes* PI-PLC has a similar structure to the *B. cereus* enzyme (36), which might suggest very similar catalytic and possibly regulatory behavior. While the catalytic mechanism may be essentially the same (16), the regulation of activity by amphiphiles and ionic strength is quite different for the more cationic protein.

Activators for *L. monocytogenes* PI-PLC exist in two classes: (i) neutral amphiphiles (PC and TX-100) and (ii) moderate salt concentrations. The first of these directly bind (albeit weakly) to the enzyme (e.g., diC₇PC binding to *L. monocytogenes* PI-PLC as monitored by ^{31}P linewidth changes) and alter the interaction of the enzyme with its anionic substrates. As with *B. thuringiensis* PI-PLC (4), adding a micellar activator to an assay system with monomer substrates is more effective than adding a monomeric activator. However, micelles of a neutral amphiphile such as TX-100 are just as effective as diC₇PC suggesting the amphiphile binding may not involve a specific site on the enzyme. The affinities of the two PI-PC enzymes for anionic versus zwitterionic interfaces are exact opposites. *L. monocytogenes* PI-PLC binds tightly to surfaces high in anionic phospholipids with much weaker interactions with zwitterionic PC interfaces, while *B. thuringiensis* PI-PLC prefers zwitterionic lipids. Furthermore, the *L. monocytogenes* PI-PLC tends to form large aggregates with vesicles or micelles rich in anionic lipids. As revealed from its crystal structure (36), there are many basic

residues clustered on the bottom side of the *L. monocytogenes* PI-PLC TIM-barrel that are far away from the rather hydrophobic opening to the positively charged active site. With PI dispersed in a variety of aggregates (mixed micelles or vesicles), the electrostatic interaction between the positive residues of enzyme and anionic substrate drives the enzyme to the interface where it will bind tightly. The basic residues on the face of the protein opposite the active site will cause the clustering of nearby negatively charged lipid surfaces (illustrated schematically in Figure 11A). For vesicles this could cause aggregation of PI in the same vesicle or more likely cause aggregation with other highly anionic vesicles, forming a complex in which the enzyme is sequestered from other substrate-containing vesicles. A significantly increased surface concentration of zwitterionic / neutral amphiphiles disperses the anionic substrate, and shields charges on the protein from sparser anionic lipid patches on the same or other particles. Protein-protein interactions must also contribute to this trapping of the enzyme since dramatically reducing the protein concentration also increases enzyme specific activity (Figure 11B).

Increasing the solution ionic strength also relieves formation of the trapped enzyme by reducing the hydrophobic interaction between enzyme and anionic surfaces. That cIP hydrolysis by *L. monocytogenes* PI-PLC is not affected by salt indicates this activator is altering the way the protein interacts with anionic interfaces. The kinetic and biophysical studies presented here show that increased ionic strength alters the way *L. monocytogenes* PI-PLC binds to anionic interfaces. The monolayer studies support the idea that KCl weakens the interaction of the protein with negatively charged surfaces. Increased ionic strength reduces the maximum penetration ($\Delta\pi$) of the enzyme and π_C to a greater extent when PC is present in the interface. These results are critical in explaining the synergistic effects of POPC and KCl on PI-PLC cleavage of PI in vesicles. Clearly, a high π_C and $\Delta\pi$ correlate with lower activity of *L. monocytogenes* PI-PLC. High salt is also likely to affect PI behavior in the membrane by suppressing any demixing of PI and neutral/zwitterionic amphiphiles (31), an effect that may also contribute to enhanced activity.

However, the soluble substrates, diC₄PI for the phosphotransferase reaction and cIP, provide an interesting twist in understanding how the two types of activators work. DiC₄PI cleavage is not dramatically enhanced by *L. monocytogenes* PI-PLC binding to the same concentration of diC₆PC or TX-100 micelles as used in cIP assays. A large increase in the enzyme specific activity is only observed with both the amphiphile and moderate (0.075 M) KCl. The difference between diC₄PI and cIP is that the first has acyl chains, short but with some hydrophobic character. There are multiple cationic sites on the enzyme that could bind this anionic phospholipid leading to inhibited enzyme. cIP may have a much weaker affinity for these secondary sites. Amphiphile alone may not compete well at these secondary sites, so that activation by amphiphile at low ionic strength is small. Increased salt alone (0.1–0.2 M) may not displace these molecules from the protein. However, the presence of an activating amphiphile and added salt might be more effective at weakening electrostatic interactions of diC₄PI with secondary sites and allowing the enzyme with bound substrate to bind to the activating amphiphile surface. This would lead to high specific activity toward diC₄PI only observed in the presence of both amphiphile and salt. In contrast, the enzyme would be adequately activated by amphiphile toward cIP because this substrate molecule does not interact with other sites on the enzyme.

Testing the importance of hydrophobic residues to PI-PLC kinetic behavior

The biophysical studies with wildtype enzyme suggest that hydrophobic interactions as well as electrostatic interactions with negatively charged surfaces contribute to the unusual kinetic behavior of this enzyme. Modifying enzyme surface charge by citraconylation, which should at least partially affect any kinetic effects linked to the high cationic character of this protein,

had little effect on surface dilution or salt effects. The only significant effect was that enzyme with reduced positive charge showed a smaller increase in specific activity as the protein was diluted. The PI-PLC mutants we constructed all modified hydrophobic groups at the barrel rim in features that might be linked to membrane binding. Except for removal of Trp49 (which is the one residue in this series facing inwards towards the active site and possibly involved in orienting or promoting proper access of the substrates), the PI-PLC mutant enzymes we constructed were all quite active, and most importantly lost the strikingly large increase in specific activity with enzyme dilution or with surface dilution of the PI in mixed micelles. Alanine substitutions clearly will reduce hydrophobic interactions, and most of the mutants were not as active as the wildtype if maximum specific activities are compared regardless of protein concentration (Figure 8). The kinetic behavior of these specific mutant enzymes contrasts strongly with that observed for citraconylated enzyme. Both the enzyme concentration dependence and apparent surface dilution inhibition are dramatically reduced for all mutants with altered surface hydrophobic side chains (except W49A which is fairly inactive). Neither trend is abolished when the enzyme surface charge is reduced by modification of the lysines.

The behavior of the mutant enzymes also indicates that salt activation is distinct from the enzyme dilution and surface dilution activation. KCl activation for W49A, L51A and L235A is significantly reduced. In contrast, citraconylated enzyme exhibits a strong KCl activation. If added salt weakens the protein binding to the interface (an observation supported by the monolayer studies), then reducing the binding by removing hydrophobic residues should lessen the salt effect as observed with these mutant enzymes (they are already weaker binders).

Perhaps the most interesting mutation is F237W. Replacement of hydrophobic Phe237 with tryptophan, which prefers interfacial regions, may help to alter the conformation of the protein on a target membrane. F237W PI-PLC, even at high concentrations, has high specific activity comparable to dilute unaltered recombinant PI-PLC. This kinetic behavior suggests that at higher concentrations, F237W does not form the aggregates with anionic lipid-rich vesicles that are disrupted by excess detergent and salt, although it is still activated (to about the same extent as wildtype) by salt. Substitution with alanine generates a mutant that most approaches wildtype PI-PLC in its dependence on enzyme concentration. These results suggest that Phe237 is a key residue in the very tight binding of PI-PLC to membranes containing anionic phospholipids. One could suggest that the high activity of F237W at higher enzyme concentrations is because it still binds to membranes, but the Trp alters the orientation by perhaps not inserting to the same depth as a Phe side chain. The F237W protein may bind more weakly than wildtype. The net result is that the enzyme is not trapped in underproductive precipitates/aggregates, etc. Future studies directed at vesicle binding (with mixtures of anionic phospholipids and PC) of wildtype and mutant proteins under conditions of very low concentrations of proteins should be extremely useful for unraveling how the specific residues mutated contribute to membrane partitioning. The phenylalanine at this particular position may be the key to tightly anchoring the *L. monocytogenes* PI-PLC on the inner leaflet of the plasma membrane of the host cell or onto the inner leaflet of the inner bilayer in the secondary vacuole where it can cleave PI to generate DAG.

Biological relevance of amphiphile regulation of *L. monocytogenes* PI-PLC activity?

Several bacteria, both pathogenic and non-pathogenic, secrete PI-PLC enzymes into the media. The biological role of this enzyme, along with other nonspecific phospholipases, is to aid in survival of the organism, particularly as it infects mammalian cells. Those PI-PLC that cleave GPI-anchored proteins, such as the PI-PLC secreted by *B. thuringiensis*, *B. cereus*, and *Staphylococcus aureus*, are well poised to target those membrane components in the extracellular leaflet of the plasma membrane, which is rich in the zwitterionic lipids PC and

sphingomyelin. The specific PC binding and activation of the *Bacillus* PI-PLC enzymes would help ensure that they bind the target cell membrane where they could catalyze the cleavage of GPI-anchors. However, secreted PI-PLC enzymes whose targets are PI and not GPI-anchors, must have a different role in bacterial survival since PI is not found in the external leaflet of most organisms. In fact, they should not be targeted to PC-rich membranes but should have high affinity for the inner leaflet of the plasma membrane of the target cell.

In *L. monocytogenes* infections, the bacteria are surrounded by two types of vacuolar membranes at different stages and the PI-PLC is involved in both stages (11,37–42). Upon initial infection of the host cell, the bacterium is surrounded by a single-membrane vacuole. In macrophages, both listeriolysin O and *L. monocytogenes* PI-PLC expression are upregulated in this phagosome (43). From the phagosome, *L. monocytogenes* PI-PLC could presumably gain access to host PI by means of phagosomal permeabilization and eventual destruction. Activation of host PKC β by means of DAG generated from intracellular PI and elevated intracellular calcium may play a role in escape from this phagosome (40,42). The inner leaflet of the primary vacuole membrane is presumably like the external leaflet of the plasma membrane – rich in PC or sphingomyelin with a low content of anionic phospholipids. Since the *L. monocytogenes* PI-PLC has weak affinity for PC (and presumably for sphingomyelin and PE as well) under the moderate ionic strengths in cells, it will stay in the vacuolar fluid and be dispersed into the cytoplasm. Once there it will home in on the negatively charged components, including PI, of the target plasma membrane. It should be noted that intracellular concentrations of a typical mammalian cell are 5 to 15 mM NaCl, 140 mM KCl, 0.5 mM Mg²⁺, and 0.1 mM Ca²⁺ (44), an ionic strength similar to what activates *L. monocytogenes* PI-PLC towards mixed PI/PC SUVs. Once at the plasma membrane, the bacterial PI-PLC will generate DAG required to activate host pathways. However, too much PI cleaved would be detrimental to the host and the replication efforts of the bacterium. Moderation of host PI cleavage by this bacterial PI-PLC could occur when the tightly bound PI-PLC is sequestered with other host anionic lipids and can no longer easily access more PI.

PI-PLC also plays an important role in escape from a secondary vacuole for cell-to-cell spreading of these bacteria (12). In this compartment the bacterium is in a double membrane where the inner bilayer is derived from the primary cell and the outer bilayer from the secondary cell. The inner leaflet of the inner membrane will contain PE, PS and PI, the last of these the substrate for the PI-PLC. Thus, the PI-PLC enzyme secreted into the secondary vacuolar fluid will aid in dissolving this inner bilayer by hydrolysis of the PI and likely disruption and fusion of the bilayer after sufficient DAG is produced. The activity of the non-specific phospholipase/sphingomyelinase (PC-PLC) will generate even larger amounts of DAG plus ceramide, potentially leading to membrane fusion (44), and also cause complete disruption of the inner membrane (12). The high affinity of the wildtype enzyme for anionic phospholipids will insure that it localizes on this membrane surface. Once the inner membrane is dissolved, PI-PLC faces the outer leaflet of the outer membrane – rich in PC and SPM – and will not bind tightly. In escaping the secondary vacuole, there is fusion with early endosomes and a decrease in pH. While this may activate the non-specific PC-PLC and listeriolysin, the fairly narrow pH range of PI-PLC suggests that it would not be very active if the pH dropped much. Therefore, it must contribute to disruption of the inner membrane prior to a more extreme pH drop (to ~5.5) and activation of LLO. Several of the mutant enzymes we have constructed may help sort out the relevance of the PI-PLC at different stages of infection by *L. monocytogenes*.

Abbreviations

cIP	inositol 1,2-(cyclic)-phosphate
CMC	critical micelle concentration

DAG	diacylglycerol
diC ₄ PC	dibutyroylphosphatidylcholine
diC ₆ PC	dihexanoylphosphatidylcholine
diC ₇ PC	diheptanoylphosphatidylcholine
DOPMe	1,2-dioleoylphosphatidylmethanol
GPI	glycosylphosphatidylinositol
I-1-P	D-myo-inositol-1-phosphate
PC	phosphatidylcholine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PI-PLC	phosphatidylinositol-specific phospholipase C
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
POPG	1-palmitoyl-2-oleoyl-phosphatidylglycerol
POPS	1-palmitoyl-2-oleoyl-phosphatidylserine
SUV	small unilamellar vesicle
TIM-barrel	triose phosphate isomerase
TX-100	Triton X-100

REFERENCES

1. Volwerk JJ, Shashidhar MS, Kuppe A, Griffith OH. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* combines intrinsic phosphotransferase and cyclic phosphodiesterase activities: a ³¹P NMR study. *Biochemistry* 1990;29:8056–8062. [PubMed: 2175645]
2. Zhou C, Wu Y, Roberts MF. Activation of phosphatidylinositol-specific phospholipase C towards inositol 1,2-(cyclic)-phosphate. *Biochemistry* 1997;36:347–355. [PubMed: 9003187]
3. Hendrickson HS, Hendrickson EK, Johnson JL, Khan TH, Chial HJ. Kinetics of *Bacillus cereus* phosphatidylinositol-specific phospholipase C with thiophosphate and fluorescent analogs of phosphatidylinositol. *Biochemistry* 1992;31:12169–12172. [PubMed: 1333794]
4. Lewis KA, Garigapati VR, Zhou C, Roberts MF. Substrate requirements of bacterial phosphatidylinositol-specific phospholipase C. *Biochemistry* 1993;32:8836–8841. [PubMed: 8395883]
5. Volwerk JJ, Filthuth E, Griffith OH, Jain MK. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* at the lipid-water interface: interfacial binding, catalysis, and activation. *Biochemistry* 1994;33:3464–3474. [PubMed: 8142343]
6. Low MG, Stiernberg J, Waneck GL, Flavell RA, Kincade PW. Cell-specific heterogeneity in sensitivity of phosphatidylinositol-anchored membrane antigens to release by phospholipase C. *J. Immunol. Methods* 1988;113:101–111. [PubMed: 2459250]
7. Hamon M, Bierre H, Cossart P. *Listeria monocytogenes*: a multifaceted model. *Nat. Rev. Microbiol* 2006;4:423–434. [PubMed: 16710323]
8. Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MS, Djarsana KS, Prasad SP, Vijila HM. *Listeria* – review of epidemiology and pathogenesis. *J. Microbiol. Immunol. Infect* 2007;40:4–13. [PubMed: 17332901]
9. Camilli A, Tilney LG, Portnoy DA. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol* 1993;8:143–157. [PubMed: 8388529]

10. Gaillaird JL, Berche P, Mounier J, Richard S, Sansonetti P. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect. Immun* 1987;55:2822–2829. [PubMed: 3117693]
11. Smith GA, Marquis H, Jones S, Johnston NC, Portnoy DA, Goldfine H. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. *Infect. Immun* 1995;63:4231–4237. [PubMed: 7591052]
12. Alberti-Segui C, Goeden KR, Higgins DE. Differential function of *Listeria monocytogenes* listeriolysin O and phospholipases C in vacuolar dissolution following cell-to-cell spread. *Cell. Microbiol* 2007;9:179–195. [PubMed: 17222191]
13. Gründling A, Gonzalez MD, Higgins DE. Requirement of the *Listeria monocytogenes* broad-range phospholipase PC-PLC during infection of human epithelial cells. *J. Bacteriol* 2003;185:6295–6307. [PubMed: 14563864]
14. Orgun NN, Way SS. A critical role for phospholipase C in protective immunity conferred by listeriolysin O-deficient *Listeria monocytogenes*. *Microb. Pathog* 2008;44:159–163. [PubMed: 17888620]
15. Yeung PS, Zagorski N, Marquis H. The metalloprotease of *Listeria monocytogenes* controls cell wall translocation of the broad-range phospholipase C. *J. Bacteriol* 2005;187:2601–2608. [PubMed: 15805506]
16. Bannam T, Goldfine H. Mutagenesis of active-site histidines of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C: effects on enzyme activity and biological function. *Infect. Immun* 1999;67:182–186. [PubMed: 9864213]
17. Goldfine H, Knob C. Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. *Infect. Immun* 1992;60:4059–4067. [PubMed: 1398918]
18. Ryan M, Zaikova TO, Keana JF, Goldfine H, Griffith OH. *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C: activation and allostery. *Biophys. Chem* 2002;101–102:347–358.
19. Gandhi A, Perussia B, Goldfine H. The phosphatidylinositol (PI)-specific phospholipase C of *Listeria monocytogenes* has weak activity on glycosyl-PI-anchored proteins. *J. Bacteriol* 1993;175:8014–8017. [PubMed: 8253689]
20. Szweda P, Pladzyk R, Kotlowski R, Kur J. Cloning, expression, and purification of the *Staphylococcus simulans* lysostaphin using the intein-chitin-binding domain (CBD) system. *Protein Expr. Purif* 2001;22:467–471. [PubMed: 11483010]
21. Massimelli MJ, Beassoni PR, Forrellad MA, Barra JL, Garrido MN, Domenech CE, Lisa AT. Identification, cloning, and expression of *Pseudomonas aeruginosa* phosphorylcholine phosphatase gene. *Curr. Microbiol* 2005;50:251–256. [PubMed: 15886911]
22. Foltá-Stogniew E. Oligomeric states of proteins determined by size-exclusion chromatography coupled with light scattering, absorbance, and refractive index detectors. *Methods Mol. Biol* 2006;328:97–112. [PubMed: 16785643]
23. Wehbi H, Feng J, Kolbeck J, Ananthanarayanan B, Cho W, Roberts MF. Investigating the interfacial binding of bacterial phosphatidylinositol-specific phospholipase. *Biochemistry* 2003b;42:9374–9382. [PubMed: 12899624]
24. Medkova M, Cho W. Differential membrane-binding and activation mechanisms of protein kinase C- α and - ϵ . *Biochemistry* 1998;37:17544–17552.
25. Weinstein JN, Yoshikami S, Henkart P, Blumenthal R, Hagins WA. Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker. *Science* 1977;195:489–492. [PubMed: 835007]
26. Stieglitz KA, Seaton BA, Roberts MF. Binding of proteolytically processed phospholipase D from *Streptomyces chromofuscus* to phosphatidylcholine membranes facilitates vesicle aggregation and fusion. *Biochemistry* 2001;40:13954–13963. [PubMed: 11705386]
27. Zhou C, Qian X, Roberts MF. Allosteric activation of phosphatidylinositol-specific phospholipase C: phospholipid binding anchors the enzyme to the interface. *Biochemistry* 1997;36:10089–10097. [PubMed: 9254604]

28. Wehbi H, Feng J, Roberts MF. Water-miscible organic cosolvents enhance phosphatidylinositol-specific phospholipase C phosphotransferase as well as phosphodiesterase activity. *Biochim. Biophys. Acta* 2003;1613:15–27. [PubMed: 12832083]
29. Zhou C, Roberts MF. Non-essential activation and competitive inhibition of bacterial phosphatidylinositol-specific phospholipase C by short-chain phospholipids and analogs. *Biochemistry* 1998;37:16430–16439. [PubMed: 9819236]
30. Wang ZX. Kinetic study on the dimer-tetramer interconversion of glycogen phosphorylase a. *Eur. J. Biochem* 1999;259:609–617. [PubMed: 10092844]
31. Redfern DA, Gericke A. Domain formation in phosphatidylinositol monophosphate/phosphatidylcholine mixed vesicles. *Biophys. J* 2004;86:2980–2992. [PubMed: 15111413]
32. Bian J, Roberts MF. Comparison of surface properties and thermodynamic behavior of lyso- and diacylphosphatidylcholines. *J. Coll. Int. Sci* 1992;153:420–428.
33. Garigapati VR, Roberts MF. Synthesis of short chain phosphatidylinositols. *Tetrahedron Lett* 1993;34:769–772.
34. Lin TL, Chen SH, Gabriel NE, Roberts MF. Use of small angle neutron scattering to determine the structure and interaction of dihexanoylphosphatidylcholine micelles. *J. Am. Chem. Soc* 1986;108:3499–3507.
35. Feng J, Hania W, Roberts MF. Role of tryptophan residues in interfacial binding of phosphatidylinositol-specific phospholipase C. *J. Biol. Chem* 2002;277:19867–19875. [PubMed: 11912206]
36. Moser J, Gerstel B, Meyer JE, Chakraborty T, Wehland J, Heinz DW. Crystal structure of the phosphatidylinositol-specific phospholipase C from the human pathogen *Listeria monocytogenes*. *J. Mol. Biol* 1997;273(1):269–282. [PubMed: 9367761]
37. Sibelius U, Rose F, Chakraborty T, Darji A, Wehland J, Weiss S, Seeger W, Grimminger F. Listeriolysin is a potent inducer of the phosphatidylinositol response and lipid mediator generation in human endothelial cells. *Infect. Immun* 1996;64:674–676. [PubMed: 8550226]
38. Sibelius U, Schulz EC, Rose F, Hattar K, Jacobs T, Weiss S, Chakraborty T, Seeger W, Grimminger F. Role of *Listeria monocytogenes* exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C in activation of human neutrophils. *Infect. Immun* 1999;67:1125–1130. [PubMed: 10024552]
39. Sibelius U, Chakraborty T, Krögel B, Wolf J, Rose F, Schmidt R, Wehland J, Seeger W, Grimminger F. The listerial exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C synergize to elicit endothelial cell phosphoinositide metabolism. *J. Immunol* 1996;157:4055–4060. [PubMed: 8892639]
40. Wadsworth SJ, Goldfine H. *Listeria monocytogenes* phospholipase C-dependent calcium signaling modulates bacterial entry into J774 macrophage-like cells. *Infect. Immun* 1999;67:1770–1778. [PubMed: 10085017]
41. Goldfine H, Wadsworth SJ, Johnston NC. Activation of host phospholipases C and D in macrophages after infection with *Listeria monocytogenes*. *Infect. Immun* 2000;68:5735–5741. [PubMed: 10992479]
42. Wadsworth SJ, Goldfine H. Mobilization of protein kinase C in macrophages induced by *Listeria monocytogenes* affects its internalization and escape from the phagosome. *Infect. Immun* 2002;70:4650–4660. [PubMed: 12117979]
43. Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, Gonzalez-Zorn B, Wehland J, Kreft J. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev* 2001;14:584–640. [PubMed: 11432815]
44. Montes LR, Goñi FM, Johnston NC, Goldfine H, Alonso A. Membrane fusion induced by the catalytic activity of a phospholipase C/sphingomyelinase from *Listeria monocytogenes*. *Biochemistry* 2004;43:3688–3695. [PubMed: 15035639]

Figure 1.

(A) Dependence of cIP hydrolysis by *L. monocytogenes* PI-PLC (3.6 $\mu\text{g/ml}$) on the pH of the solution. (B) Dependence of *L. monocytogenes* PI-PLC phosphotransferase specific activity on enzyme concentration ($\mu\text{g/ml}$): PI (8 mM) solubilized in either 32 mM TX-100 (open circle) or 32 mM diC₇PC (filled square).

Figure 2. Effect of cIP concentration on *L. monocytogenes* PI-PLC phosphodiesterase specific activity: (filled circle) 3.6 $\mu\text{g/ml}$, and (open circle) 7.2 $\mu\text{g/ml}$ PI-PLC. Assay conditions included 50 mM imidazole, pH 7.0, and variable cIP concentrations.

Figure 3.

(A) Dependence of *L. monocytogenes* PI-PLC phosphotransferase specific activity on mole fraction of detergent: 0.4 $\mu\text{g/ml}$ PI-PLC with 8 mM PI solubilized in TX-100 (open square) or diC₇PC (X); 0.02 $\mu\text{g/ml}$ (filled circle) or 0.2 $\mu\text{g/ml}$ (open circle) PI-PLC with the PI solubilized in TX-100. (B) Dependence of phosphotransferase specific activity on mole fraction of POPC in PI (8 mM)/POPC SUVs with 0.4 $\mu\text{g/ml}$ (filled circle) or 1.6 $\mu\text{g/ml}$ (open circle) enzyme.

Figure 4.
Effect of diC₆PC (filled circle), diC₇PC (open circle), or POPC SUVs (filled triangle) on cIP (10 mM) hydrolysis by 3.6 μg/ml *L. monocytogenes* PI-PLC.

Figure 5.

(A) ³¹P linewidth (Hz) of diC₇PC in the absence (filled square) and presence (filled circle) of *L. monocytogenes* PI-PLC. The dotted line indicates the CMC of pure diC₇PC. (B) Increase in linewidth (Hz) for diC₇PC induced by the inclusion of 2 mg/ml PI-PLC (differences in linewidth in (A)). The line represents the optimized fit to $(\Delta v_{\text{obs}} - \Delta v_0) = nET[\text{PC}]T^{n-1}((\Delta v_b - \Delta v_0)/\{K_d + [\text{PC}]T^n\})$ as described in the methods section.

Figure 6.

(A) Binding of *L. monocytogenes* PI-PLC (1.2 mg/ml, 36 μ M) to SUVs can cause precipitation of the vesicles depending on composition. The numbers below each lane indicate mM concentration of PG or PC in SUVs mixed with the protein. (B) Turbidity changes measured at 350 nm (OD₃₅₀) of 0.86 mM DOPMe / 3.1 mM POPC co-sonicated SUVs upon the addition (at 10 min) of 0.15 mg/ml (filled square), 0.75 mg/ml (open circle), and 1.5 mg/ml (filled circle) PI-PLC. For comparison, the effect of PI-PLC added to pure POPC SUVs is shown (X). (C) Carboxyfluorescein released (% of total entrapped) from 20 mM POPC (open circle), 20 mM POPC with 150 mM KCl (filled circle), and 20 mM DOPMe (X) vesicles mediated by *L.*

monocytogenes PI-PLC (0.5 mg/ml). TX-100 (20 mM) was used to release all entrapped carboxyfluorescein.

Figure 7. Effect of *L. monocytogenes* PI-PLC on the surface pressure of DOPMe (open circle), POPC (filled circle), and POPC/DOPMe (7:3) (filled square) monolayers in the (A) absence and (B) presence of 0.16 M KCl.

Figure 8.

(A) Ribbon diagram of the *L. monocytogenes* PI-PLC showing the surface hydrophobic groups mutated (the two aromatic residues in red and the two Leu in blue; myo-inositol in yellow is shown in the active site). (B) Dependence of mutant enzyme phosphotransferase specific activity (8 mM PI/32 mM TX-100 corresponding to $X_{det} = 0.80$) on enzyme concentrations: wildtype (WT) (filled circle), L51A (filled triangle), L235A (open triangle), F237A (open square), and F237W (filled square). (C) Effect of protein concentration on PI (8 mM PI, $X_{det} = 0.8$) cleavage by W49A (open circle) and W49A/F237A (X). For (B) the errors in activities are less than 20% for each specific activity; for (C) errors were as high as 30% for the lowest concentrations of enzyme used.

Figure 9.

Effect of detergent (Triton X-100) mole fraction, X_{det} , on mutated PI-PLC specific activities with fixed PI (8 mM) and enzyme concentration (0.2 μ g/ml): WT (filled circle) with only data for $X_{det} \geq 0.8$ shown, L51A (filled triangle), L235A (open triangle), F237A (open square), and F237W (filled square). The errors in activities are less than 20% for each specific activity. In (A) the data from $X_{det} = 0.67$ (16 mM TX-100) to 0.92 (80 mM TX-100) are presented, but with specific activity on a semi-log scale to emphasize the similar behavior for the mutant proteins; in (B) the data for the region from $X_{det} = 0.80$ and higher is presented to emphasize that none of the mutant proteins show a significant increase above $X_{det} = 0.9$ as seen for the wildtype enzyme at the same concentration.

Figure 10.

(A) Effect of 0.15 M KCl on cleavage of PI (8 mM) in TX-100 (32 mM) mixed micelles with 0.2 μ g/ml of wildtype or different interfacial mutant enzymes. (B) Effect of 3 mM TX-100 on the hydrolysis of cIP by *L. monocytogenes* wildtype and mutant enzymes. In each panel the specific activity is shown in the absence (open bars) and present (grey bars) of the additive. Errors in activities are less than 20% for each specific activity.

Figure 11.

Schematic representation of the interactions of *L. monocytogenes* PI-PLC with mixed phospholipid interfaces (red is an anionic phospholipid such as PI substrate, grey is a neutral detergent or lipid). In (A) protein oriented at the interface can interact with the anionic phospholipids in a separate particle (as well as the anionic lipids within a given particle), trapping the enzyme in aggregates that can not easily exchange product for substrate in other regions of a particle or even other aggregates. This leads to low specific activity. If sufficient amphiphiles (or high salt) are added, these nonproductive interactions are suppressed and high specific activity is obtained. In (B) the high concentrations of protein together with the moderate surface concentration of anionic substrate form aggregates that again sequester the

enzyme in complexes leading to low specific activity. Decreasing the enzyme concentration as well as adding salts suppresses nonproductive complex formation.

Table 1Effect of different additives on cIP (10 mM) hydrolysis catalyzed by *L. monocytogenes* PI-PLC.^a

Assay system	PI-PLC ($\mu\text{g/ml}$)	Additive	(mM)	Relative activity ^a
cIP	7.2	TX-100	3	14.8
cIP	7.2	diC ₇ PC	5	15.1
cIP	7.2	POPC	2	4.38
cIP	7.2	DOPMe	1	<0.1
cIP	7.2	POPS	1	0.43
cIP	3.6	POPC	2	7.94
cIP + 2 mM POPC	3.6	POPS	0.1	0.31
cIP	7.2	(NH ₄) ₂ SO ₄	150	0.65
cIP + 5 mM diC ₇ PC	7.2	(NH ₄) ₂ SO ₄	100	0.83

^a Assays measured in 50 mM HEPES, pH 7.0, 25°C. Relative activity is the ratio of the specific activity measured with the additive present compared to the activity in the absence of that additive (salts or lipids). For 10 mM cIP without additives, the enzyme specific activity was $0.29 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ for 3.6 $\mu\text{g/ml}$ protein or $0.16 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ for 7.2 $\mu\text{g/ml}$ protein.

Table 2

Effect of inorganic salts on the specific activity of *L. monocytogenes* PI-PLC toward PI substrate presented in TX-100 mixed micelles, SUVs in the absence or presence of POPC, or as monomers with added amphiphiles.

Substrate	Amphiphile	Salt (M)	Specific Activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
PI ^a	TX-100	-	274
	TX-100	(NH ₄) ₂ SO ₄ (0.10)	1430
	TX-100	NH ₄ Cl (0.15)	936
	TX-100	K ₂ SO ₄ (0.10)	1120
	TX-100	KCl (0.15)	1030
PI ^b	-	-	1.3
	-	KCl (0.10)	54
	-	KCl (0.20)	330
	-	KCl (0.30)	511
	POPC	-	9.0
	POPC	(NH ₄) ₂ SO ₄ (0.10)	127
diC ₄ PI ^c	-	-	0.7 ^d
	-	KCl (0.075)	1.2 ^d
	TX-100	-	1.8 ^d
	TX-100	KCl (0.075)	11.2
	diC ₆ PC	-	1.2
	diC ₆ PC	KCl (0.075)	10

^a Assays towards 8 mM PI presented in TX-100 (32 mM) mixed micelles used 0.2 $\mu\text{g/ml}$ PI-PLC in 50 mM HEPES, pH 7.0. All specific activities are the average of at least two samples; errors in these values are less than 15%.

^b Assays towards PI (8 mM) in SUVs in the absence or presence of an equimolar amount of POPC used 1.6 $\mu\text{g/ml}$ *L. monocytogenes* PI-PLC; cIP was the sole product.

^c Monomeric diC₄PI (2 mM) in 50 mM HEPES, pH 7.0, treated with 1.6 $\mu\text{g/ml}$ PI-PLC. Amphiphiles such as TX-100 (32 mM) or diC₆PC (5 mM, monomeric in the absence of enzyme) added had little effect on PI-PLC specific activity in the absence of added salt.

^d Both cIP and I-1-P were generated, so the specific activity represents the sum of both cIP and I-1-P production.

Effect of citraconic anhydride modification of Lys residues on the kinetic activity of recombinant *L. monocytogenes* PI-PLC for PI cleavage.

Table 3

PI-PLC (μg/ml)	X _{det}	KCl (M)	Specific Activity ^a		(μmol min ⁻¹ mg ⁻¹)
			Control	CA-1 ^b	CA-2 ^b
0.2	0.80	0.0	303	165	109
1.6	0.80	0.0	60.6	82.5	32.7
0.2	0.92	0.0	1150		534
0.2	0.80	0.15	1030	990	403

^aThe phosphotransferase activity was measured towards 8 mM PI solubilized in 32 mM or 96 mM TX-100; specific activities of duplicate assays were within 20%.

^bTwo different batches of citraconylated PI-PLC were prepared. About 5–7 lysine residues of the 29 total were modified according to 2D gels.