Membrane Fusion Induced by the Catalytic Activity of a Phospholipase C/Sphingomyelinase from *Listeria monocytogenes*†

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ABSTRACT: *Listeria monocytogenes* is a bacterium responsible for localized and generalized infections in humans and animals. It has the ability to spread from the cytoplasm of an infected cell to neighboring cells without becoming exposed to the extracellular space. The bacterium secretes a phospholipase C (PLC_{LM}) that is active on glycerophospholipids, e.g., phosphatidylcholine, and on sphingomyelin; thus, PLC_{LM} should be described more appropriately as a phospholipase C/sphingomyelinase. We have obtained PLC_{LM} free from a frequent contaminant, listeriolysin O, using an improved purification procedure. PLC_{LM} has been assayed on large unilamellar liposomes of defined lipid composition. The enzyme is activated by K^+ and Mg^{2+}, and readily degrades phospholipids in bilayer form, in the absence of detergents. Enzyme activity is accompanied by important changes in the structure of the phospholipid vesicles, namely, vesicle aggregation, intervesicular mixing of lipids, and mixing of aqueous contents, with very low leakage of vesicular contents. The data are interpreted as indicative of PLC_{LM}-induced vesicle fusion. This is confirmed by the demonstration of intervesicular mixing of inner monolayer lipids, using a novel procedure. The observation of PLC_{LM}-induced membrane fusion suggests a mechanism for the cell-to-cell propagation of the bacterium, which requires disruption of a double-membrane vacuole.

Listeria are Gram-positive bacteria, closely related to *Bacillus*. *Listeria monocytogenes*, which is predominantly food-borne, causes serious infections in humans and other vertebrates. *L. monocytogenes* can invade various types of cells that are not normally phagocytic, e.g., epithelial cells, fibroblasts, or hepatocytes, and after internalization, the bacteria multiply and spread to adjacent cells (see ref *1* for a review on *Listeria* pathogenesis).

Cell-to-cell spread occurs by actin-based motility and phagocytosis of a bacterium within an induced pseudopod by a neighboring cell, resulting in the formation of a phagocytic vesicle with a double membrane (*2*, *3*). It has been proposed that escape from the double-membrane vacuole, thus intercellular spread, is potentiated by the product of the *L. monocytogenes* gene *plcB*, a 29 kDa protein with phospholipase C and sphingomyelinase activities (*4*−*6*). The product of gene *plcB* (PLC_{LM})[1] is secreted as an inactive proenzyme and activated by proteolysis (*7*, *8*). When assayed on detergent−phospholipid mixed micelles, PLC_{LM} is found to be very sensitive to the ionic composition of the medium, being stimulated by 0.5 M NaCl and 0.05 mM $ZnSO_4$. The enzyme is active on a variety of glycerophospholipids and, to a smaller extent, on sphingomyelin (*4*, *9*). Replacement of certain amino acid residues near the PLC_{LM} active site by the corresponding ones in the *Bacillus cereus* ortholog phospholipase C (PCL_{BC}) yields mutants with close to wild-type activities and spreading efficiencies. However, replacing *L. monocytogenes* PLC_{LM} with the *B. cereus* ortholog leads to a significantly lower spreading capacity (*10*).

From a different perspective, Alonso and co-workers have established the ability of PLC_{BC} to aggregate and fuse large unilamellar liposomes as a result of its catalytic activity (*11*, *12*). PLC_{BC} has no sphingomyelinase activity. Moreover, the above authors have shown that *B. cereus* sphingomyelinase (SMase), structurally related to both PLC_{LM} and PLC_{BC}, which is inactive on glycerophospholipids, produces extensive leakage of aqueous vesicular contents, but little or no fusion (*13*, *14*). Studies involving model membranes, particularly liposomes, have shed light on many important aspects of cell membrane structure and function. In this case, we intend to apply our previous studies with liposomes and the *B. cereus* enzymes to test a hypothesis related to the pathology of *Listeria* infection. Specifically we suggest that

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[1] Abbreviations: ANTS, 1-aminonaphthalene-1,3,6-trisulfonic acid; Ch, cholesterol; DPX, *p*-xylenebis(pyridinium bromide); LUVs, large unilamellar vesicles; NBD $(C_{16})_2$, 4-dihexadecylamino-7-nitrobenz-2-oxa-1,3-diazole; NPPC, *p*-nitrophenylphosphorylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLC_{LM}, phospholipase C/sphingomyelinase from *L. monocytogenes*; PLC_{BC}, phospholipase C from *B. cereus*; PS, phosphatidylserine; R18, octadecylrhodamine; SM, sphingomyelin; SMase, sphingomyelinase from *B. cereus*.

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the effects of PLC_{LM}, whose properties are intermediate between those of PLC_{BC} and SMase, on the aggregation, fusion, and permeability of phospholipid liposomes could provide information on the molecular mechanism of *Listeria* intercellular spreading.

In the present paper, we have prepared PLC_{LM} free from its frequent contaminant listeriolysin, and optimized its activity on lipids in the form of large unilamellar vesicles (LUVs), in the absence of detergent. Our results demonstrate that the catalytic activity of PLC_{LM} induces aggregation and fusion of vesicles under conditions that are compatible with the proposed pathogenic role of this enzyme.

MATERIALS AND METHODS

Egg PC and egg PE were purchased from Lipid Products (South Nutfield, U.K.). Brain PS (sodium salt) and egg SM were from Avanti Polar Lipids (Alabaster, AL). 1-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylenebis(pyridinium bromide) (DPX), octadecylrhodamine B chloride (R_{18}). and 4-dihexadecylamino-7-nitrobenz-2-oxa-1,3-diazole (NBD dihexadecylamine) were supplied by Molecular Probes, Inc. (Eugene, OR). Cholesterol (Ch) and *o*-phenanthroline were from Sigma.

PLC_{LM} was purified as described previously (*4*), from the culture supernatants of *L. monocytogenes* DP-L1553. It contains an in-frame deletion in plcA and was derived from a wild-type hypersecreting strain SLCC 5764 (*9*, *15*). To separate a small impurity of listeriolysin O (LLO) contained in these preparations, a further step of purification was performed, using a 25 mL Superdex HR75 column (10 × 300 mm) (Pharmacia) with an Amersham Biosciences FPLC system. Gel filtration was performed in 10 mM HEPES, 145 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, pH 7.0, buffer. The peak fractions corresponding to PLC_{LM} were pooled (fractions 22−29, 2.5 mL), and concentrated by Centricon YM-10 systems (Amicon) to a final volume of 500 μL. PLC_{LM} activity was assayed after all purification steps with the NPPC assay (*16*).

LUVs of diameters 100−150 nm were prepared by the extrusion method (*11*) using Nuclepore filters of 0.1 μm pore diameter at room temperature, in 10 mM HEPES, 145 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, pH 7.0. Quantitative analysis of the LUV preparations, as described by Ruiz-Argüello et al. (*13*) , showed that their composition did not differ significantly from that of the initial lipid mixture. All experiments were performed at 39 °C. Lipid concentration was 0.3 mM, and PLC was used at 20 μg/mL.

PLC_{LM} activity was assayed by determining phosphorus contents in the aqueous phase of an extraction mixture (chloroform/methanol/HCl, 66:33:1), which was obtained after addition of aliquots from the reaction mixture to an appropriate volume of the solvent mixture at different times. To calculate the percentage of individual lipids hydrolyzed by PLC_{LM}, aliquots were extracted at different times with chloroform/methanol (2:1). Each organic phase was concentrated and separated on thin-layer chromatography silica gel 60 plates, using successively in the same direction and for the whole plate the solvents chloroform/methanol/acetic acid (65:25:8, v/v/v) and petroleum ether/ethyl ether/acetic acid (60:40:1, v/v/v). Plates were then stained with iodine vapors, and after sublimation, the phosphorus contents of the spots were measured.

To test PLC_{LM} sensitivity to ionic compositions, enzyme activity was assayed using a K^+-rich buffer (that mimics the intracellular ionic conditions), consisting of 10 mM HEPES, 145 mM KCl, 10 mM NaCl, pH 7.0, supplemented with 1 or 2 mM $MgCl_2$. Alternatively, when the hydrolytic activity of PLC_{LM} was assayed under conditions that mimick the extracellular ionic conditions, Na^+-rich buffer, consisting of 10 mM HEPES, 145 mM NaCl, 5 mM KCl, pH 7.0, supplemented with 1 or 2 mM $MgCl_2$, was used.

Liposome aggregation was estimated as an increase in absorbance at 500 nm, measured in a Cary Bio 3 spectrophotometer.

Fusion was assayed as mixing of aqueous contents, using the ANTS/DPX fluorescent probe system described by Ellens et al. (*17*). Vesicle efflux was also measured with the ANTS/DPX system. Calibration of these procedures has been described in detail in previous papers (*11*, *18*). Lipid mixing was assayed by the octadecylrhodamine B method (*19*). The 0% fluorescence level (or 0% mixing) was determined from a 1:4 mixture of 8 mol % R18-containing liposomes and R18-free liposomes. The fluorescence of the same amount of liposomes with the diluted probe uniformly distributed, i.e., 1.6 mol % R18-containing liposomes, was taken as the 100% fluorescence level, or 100% lipid mixing. Alternatively, lipid mixing was measured by the resonance energy transfer method (*20*), except that dihexadecyl-NBD and octadecylrhodamine B were used instead of NBD-PE and octadecylrhodamine B, to avoid PLC hydrolysis of the probes. Vesicles containing in their bilayer composition 0.6% dihexadecyl-NBD and 0.6% octadecylrhodamine B were mixed with probe-free liposomes at a 1:4 ratio. NBD emission was followed at 530 nm (excitation wavelength at 465 nm) with a cutoff filter at 515 nm. Zero percent mixing was established as the equilibrium fluorescence emission in the absence of enzyme. One hundred percent mixing was set after addition of 1 mM Triton X-100. All fluorescence measurements were performed in an Aminco Bowman Series 2 luminescence spectrometer.

RESULTS

PLC_{LM} Purification. Previously published procedures for PLC_{LM} purification yielded enzyme preparations that contained a small impurity of LLO, another *Listeria* virulence factor, that inserts into membranes and forms pores. Since LLO could perturb our measurements with liposomes loaded with aqueous solutions of fluorescent probes, a further purification step was performed, as detailed in the Materials and Methods, using a Superdex 75 gel filtration column. The result of a typical purification step on Superdex 75 is shown in Figure 1. LLO elutes in fractions 16−18, clearly separated from the maximum of PLC_{LM} elution at fraction 22. PLC_{LM} elutes together with a certain proportion of its inactive precursor, which contains a 26-residue N-terminal peptide that is removed during activation of the enzyme (*4*, *21*). Preliminary experiments showed that the phosphohydrolase activity of PLC_{LM} was not modified by LLO; however, all the experiments in this paper were performed with the purest PLC_{LM} preparation, e.g., the pooled fractions 22−29 in the chromatographic run corresponding to Figure 1.

PLC_{LM} Phosphohydrolase Activities. Prior to the membrane fusion assays, it was important to optimize the

Table 1: Influence of Bilayer Lipid Composition on the Phosphohydrolase Activity of PLC_{LM}[a]

lipid composition (mole ratio)	hydrolyzed phospholipid (pmol/min)	lipid composition (mole ratio)	hydrolyzed phospholipid (pmol/min)	lipid composition (mole ratio)	hydrolyzed phospholipid (pmol/min)
PC	392 ± 46	SM/Ch (2:1)	223 ± 51	PC/PE/PS (2:5:3)	11870 ± 445
PC/Ch (2:1)	406 ± 60	SM/PE/Ch (2:1:1)	217 ± 11	PC/PE/PS/Ch (2:5:3:2.5)	12120 ± 509
PC/PE/Ch (2:1:1)	582 ± 46	PC/SM/PE/Ch (1:1:1:1)	782 ± 29		

[a] LUVs of different compositions (total lipid concentration 0.3 mM) were incubated with PLC_{LM} and aliquots removed at fixed time intervals. Average values ± SD ($n = 3$).

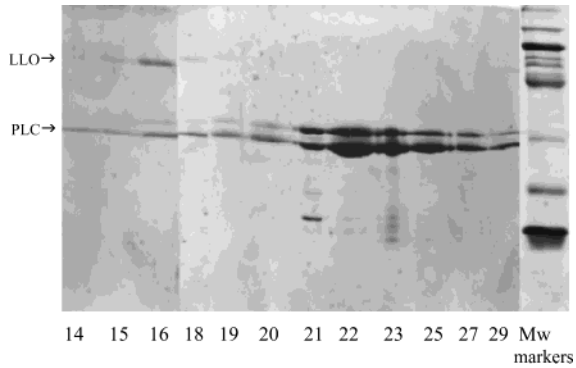

FIGURE 1: SDS−PAGE gel of a representative PLC_{LM} purification step in the Superdex 75 column. Only the fractions containing PLC_{LM} and LLO are shown.

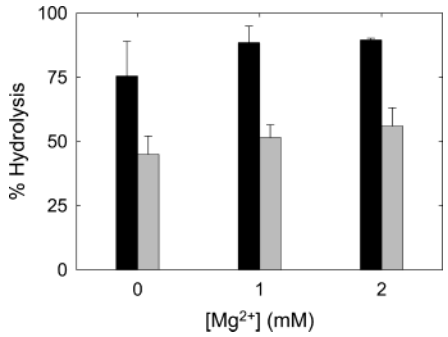

FIGURE 2: Effect of buffer cations on PLC_{LM} activity. K^+-rich buffer: 10 mM HEPES, 145 mM KCl, 10 mM NaCl, pH 7.0. Na^+-rich buffer: 10 mM HEPES, 145 mM NaCl, 5 mM KCl, pH 7.0. The substrate was an equimolar mixture of PC/SM/PE/Ch, in the form of LUVs. Average values ± SD ($n = 3$).

hydrolytic activities of PLC_{LM} with substrates in the form of LUVs. Because of the enzyme sensitivity to ionic compositions, a number of experiments were performed in which the enzyme was assayed either in a K^+-rich buffer, which mimics the intracellular ionic conditions (see the Materials and Methods for details), or in a Na^+-rich medium, more similar to the extracellular conditions. In addition the effect of Mg^{2+} ions, which can activate certain sphingomyelinases (*22*), was also tested. The results are summarized in Figure 2. The K^+-rich buffer allowed higher hydrolytic activities than the Na^+-rich medium. The activating effect of Mg^{2+} was smaller. In all further experiments the assay medium corresponded to the K^+-rich buffer supplemented with 2 mM Mg^{2+}.

In a different series of experiments, the influence of bilayer lipid composition on the PLC_{LM} phosphohydrolase activity was tested. The results are summarized in Table 1. Of the lipid mixtures examined in this table, the highest activities were found, by far, with the PE,PS-rich mixtures. These compositions are separately discussed below. Apart from those, comparison of the PC/Ch and SM/Ch mixtures suggests that the enzyme is more active on PC than on SM, although both lipids are substrates. This confirms previous observations (*9*), and is in agreement with the structural similarities of PLC_{LM} with both PLC_{BC} and SMase from *B. cereus*, which are only active on glycerophospholipids and on SM, respectively. Higher hydrolytic activities were observed on the PC/PE/Ch (2:1:1) mixture (Table 1), as found for PLC_{BC} (*11*). However, at variance with the *Bacillus* enzyme, which has a very low activity on the equimolar PC/SM/PE/Ch mixture (*23*), PLC_{LM} was highly active on this quaternary mixture. Considering that the latter mixture contains four of the most abundant lipids in mammalian cell membranes, and that PLC_{LM} has both a phospholipase C and a sphingomyelinase activity, aggregation and fusion assays were carried out on LUVs composed of PC/SM/PE/Ch (1:1:1:1 mol ratio).

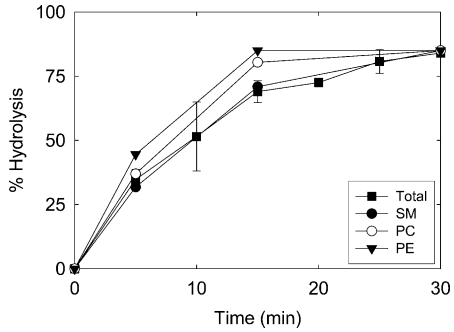

FIGURE 3: Time course of hydrolysis of total and individual lipids by PLC_{LM}. Substrate: LUVs of PC/SM/PE/Ch (1:1:1:1). Total lipids (■): average values ± SD ($n = 3$). Individual lipids: average values of two closely similar experiments. Key: PC (○), SM (●), PE (▼).

To establish whether some phospholipid class is preferentially cleaved by PLC_{LM}, aliquots of the reaction mixture were removed at fixed time intervals after enzyme addition, and the corresponding organic extracts subjected to thin-layer chromatography for the separation of the different phospholipid classes. Lipid phosphorus was assayed in the scraped phospholipid spots for quantification, with the result that the relative hydrolysis rates of PC, PE, and SM, when PLC_{LM} is active on LUVs composed of the quaternary mixture mentioned above, are similar (Figure 3). These results support the inclusion of PLC_{LM} as a phospholipase C/sphingomyelinase in the classification of sphingomyelinases by Goñi and Alonso (*22*).

PLC_{LM}-Induced Vesicle Aggregation and Fusion. When LUVs composed of equimolar amounts of PC, SM, PE, and Ch are incubated with PLC_{LM}, phospholipid hydrolysis proceeds gradually (Figure 4). Hydrolysis is accompanied by an increase in suspension turbidity, from <0.05 to ~0.37 absorbance unit in 30 min, an indication of vesicle aggregation.

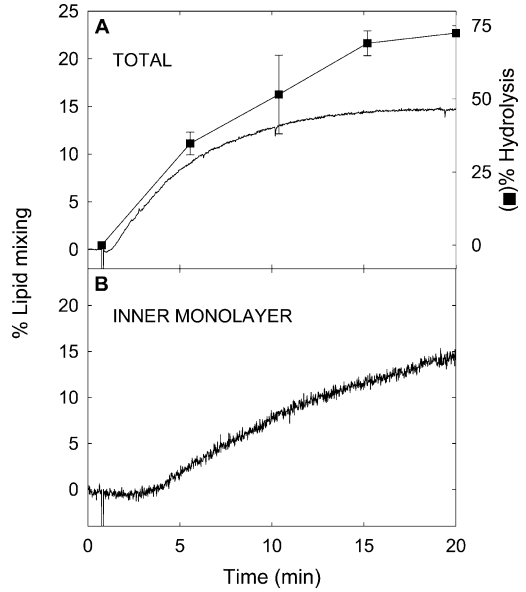

FIGURE 4: PLC_{LM}-induced phospholipid hydrolysis and intervesicular lipid mixing. Substrate: LUVs of PC/SM/PE/Ch (1:1:1:1). (A) Hydrolysis (■): average values ± SD ($n = 3$). Continuous curve: total lipid mixing. (B) Inner monolayer lipid mixing. See the text for details. Total lipid concentration: 0.3 mM. Enzyme concentration: 20 μg/mL.

Octadecylrhodamine (R18) is a fluorescence probe that partitions into lipid bilayers and, above a certain concentration, displays the property of fluorescence self-quenching; i.e., fluorescence emission intensity decreases with increasing concentrations of R18 in the bilayer. R18 can be used to detect intervesicular lipid mixing because, in a mixture of R18-free and R18-labeled (and self-quenched) vesicles, lipid mixing will lead to R18 dilution and a subsequent increase in fluorescence emission intensity. As shown in Figure 4A, the R18 dilution method reveals that PLC_{LM} hydrolysis is accompanied by intervesicular lipid mixing. This phenomenon requires previous intimate vesicle−vesicle contact. Vesicle aggregation does not necessarily lead to lipid mixing, but lipid mixing occurs only after aggregation. Figure 4A shows that intervesicular lipid mixing starts almost immediately after PLC_{LM} addition. Like all amphiphiles, R18 has a certain tendency to partition into the water phase, even if the highest proportion of the molecule remains membrane-bound. To ensure that spontaneous intervesicular diffusion of R18 through the aqueous phase is not responsible for the observed increase in R18 fluorescence, the measurement was repeated in the absence of PLC_{LM}. The results in Figure 4A correspond actually to the difference between the enzyme-induced and the (small) spontaneous diffusion. Thus, the increase in fluorescence in Figure 4A is exclusively due to intervesicular lipid mixing induced by PLC_{LM}.

Intervesicular lipid mixing reaches an equilibrium, under our conditions, at approximately 15% mixing (Figure 4A), and this may mean either that about 15% of the vesicles in the system have exchanged lipids from both the inner and outer monolayers or that about 30% of the vesicles have exchanged lipids from their outer monolayers. (In these estimations the approximation is made that in LUVs the outer and inner monolayers contain the same number of phospholipids.) When only the outer monolayers mix, the phenomenon is described as "hemifusion" (*24*) or "close apposition" (*25*). Mixing of the inner monolayers is instead an indication of vesicle fusion. Inner monolayer mixing in our system was detected by fluorescence resonance energy transfer (FRET) between the fluorescence energy donor dihexadecyl-NBD [NBD $(C_{16})_2$] and the acceptor R18. For this purpose LUVs were made, either probe-free or containing NBD $(C_{16})_2$ and R_{18} (see the Materials and Methods). The preparation technique leads to an approximately even distribution of the probes between the inner and outer monolayers in the labeled vesicles. Probe concentration is such that, when NBD fluorescence is excited, most of the energy is transferred to R18, so that the fluorescence emission intensity of NBD $(C_{16})_2$ is low. LUVs were then treated with 10 mM sodium dithionite, virtually impermeant through the liposomal membranes. Dithionite reduces the probes in the outer monolayers of the labeled vesicles, and their fluorescence thereby becomes abolished. LUVs were then passed through a Sephadex G-75 column to remove the excess dithionite in the medium. The resulting LUVs contained NBD $(C_{16})_2$ and R18 only in their inner monolayers. When they were incubated with probe-free LUVs and PLC_{LM}, the NBD fluorescence emission increased in parallel with the total lipid mixing (Figure 4B), indicating probe dilution due to intervesicular mixing of inner monolayer lipids, and consequently vesicle−vesicle fusion. The extent of inner monolayer mixing after 20 min was about 15% (100% being the signal for complete mixing of *inner* monolayer lipids), in good agreement with the 15% mixing of total lipids observed in Figure 4A. A lag time of ca. 3 min is seen for inner monolayer mixing (Figure 4B), suggesting a period of hemifusion before complete fusion occurs.

Vesicle−vesicle fusion can also be assayed through intervesicular mixing of aqueous contents. For this purpose two LUV preparations were separately made, one containing the water-soluble fluorescence probe ANTS, and the other containing the fluorescence quencher DPX. DPX has a great affinity for ANTS, largely abolishing the fluorescence of the latter. When the two vesicle preparations were mixed and incubated with PLC_{LM}, fusion was observed as a decrease in ANTS fluorescence (Figure 5A). Note that in the figure a decrease in ANTS fluorescence is indicated as an *increase* in fusion. About 22% fusion was achieved in 20 min. This value is somewhat higher than expected, considering the 15% inner monolayer lipid mixing observed after 20 min (Figure 4B). Mixing of aqueous contents is frequently lower, but should never be higher than mixing of lipids. We shall discuss this issue further in the next section on PS,PE-rich vesicles. A lag time of ~4 min was observed for the mixing of aqueous contents (Figure 5A). The time elapsed between the onset of inner monolayer mixing (Figure 4B) and the onset of contents mixing (Figure 5A) is an indication of the period at which the vesicles are separated by a diaphragm consisting of a single bilayer involving the inner monolayer lipids, immediately before the fusion pore is opened (*24*, *26*).

False positive results of vesicle−vesicle fusion detected through mixing of aqueous contents can be obtained when the putative fusogenic agent is causing, either in addition to or instead of fusion, extensive leakage of liposomal contents. In the present case, the observation of both inner monolayer mixing and contents mixing is a clear indication of true fusion. However, PLC_{LM}-induced leakage was measured as a further control. For this purpose vesicles containing ANTS complexed with DPX were treated with the enzyme. Leakage

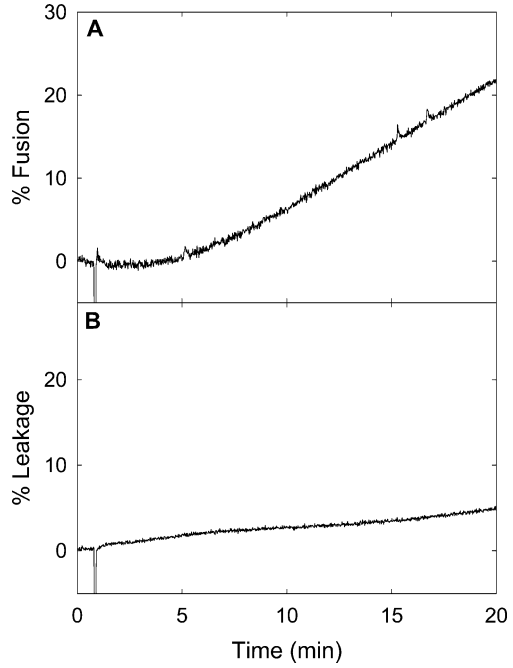

FIGURE 5: PLC_{LM}-induced vesicle−vesicle fusion. LUVs composed of PC/SM/PE/Ch (1:1:1:1). (A) Fusion, observed as intervesicular mixing of aqueous contents. (B) Virtual absence of enzyme-induced vesicle leakage. Lipid and enzyme concentrations as in Figure 4.

would mean dilution and dissociation of the complex and, thus, an increase in ANTS fluorescence. In fact, only a residual leakage was found as a consequence of PLC_{LM} activity (Figure 5B), confirming that the enzyme induces true fusion of pure lipid vesicles.

PS,PE-Rich Vesicles. When the bacterium is contained within the double membrane vacuole in the in vivo situation, the monolayer closest to the bacterium is the cytoplasmic monolayer of the cell plasma membrane, which is expected to be rich in PS and PE. Consequently, a series of experiments were performed on LUVs composed of (a) PC/PE/PS (2:5:3, mole ratio) and (b) PC/PE/PS/Ch (2:5:3:2.5, mole ratio). The results were very similar in both cases, and only those corresponding to the former composition are shown. The main difference between the PS,PE-rich vesicles and the PC,SM-rich ones described up to now is the much higher enzyme rates, about 1 order of magnitude, with the LUVs representing the cytoplasmic monolayer (Table 1). Both PS and PE were known to be good substrates for PLC_{LM} when present in the form of detergent mixed micelles (*9*). Otherwise the effects of PLC_{LM} are very similar, and the enzyme appears to modify the vesicles to a similar extent, although at a faster rate. Absorbance at 500 nm increases from <0.05 to ~ 0.6 au in the 300 s after enzyme addition, indicating vesicle aggregation. Total lipid mixing (Figure 6A) starts with a lag time of about 20 s and reaches equilibrium at 26%. Mixing of inner monolayer lipids (Figure 6B) has a somewhat longer lag time (ca. 30 s) and proceeds until 20% of the lipids in the LUV inner monolayers have intermixed. Mixing of vesicular aqueous contents (Figure 7A) provides an unexpected result, since it reaches a value of ca. 50% 200 s after enzyme addition, when only 20% mixing of inner monolayer lipids has occurred (see Figure 6B). A similar, though less pronounced, disparity between lipid and contents mixing was already observed for the PC,SM-rich vesicles (Figures 4B and 5A). Also in both cases contents mixing

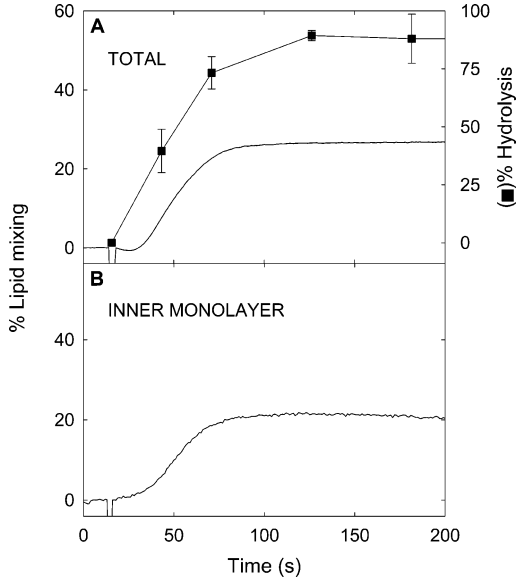

FIGURE 6: PLC_{LM}-induced phospholipid hydrolysis and intervesicular lipid mixing. Substrate: LUVs of PC/PE/PS (2:5:3). (A) Hydrolysis (■): average values ± SD ($n = 3$). Continuous curve: total lipid mixing. (B) Inner monolayer lipid mixing. See the text for details. Lipid and enzyme concentrations as in Figure 4.

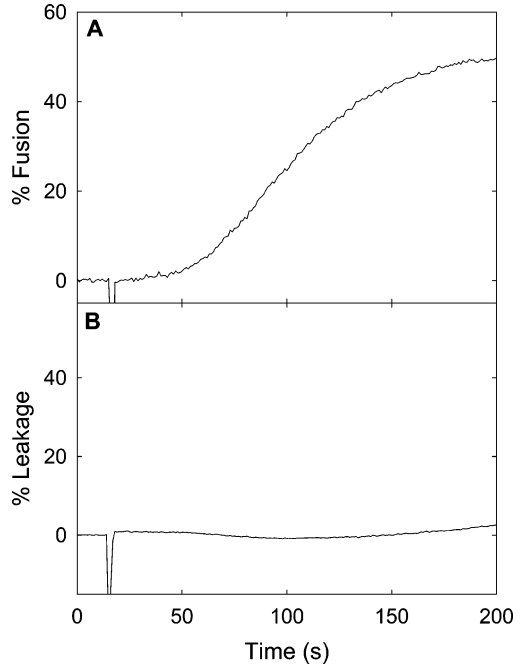

FIGURE 7: PLC_{LM}-induced vesicle−vesicle fusion.. LUVs composed of PC/PE/PS (2:5:3). (A) Fusion, observed as intervesicular mixing of aqueous contents. (B) Virtual absence of enzyme-induced vesicle leakage. Lipid and enzyme concentrations as in Figure 4.

reached plateau values at a later stage than the other observed phenomena. We cannot explain at present this behavior of the intervesicular contents mixing, which must be attributed to a hitherto undetected phenomenon. A shrinking of the fused vesicles could explain the observation, since it would lead to an effective increase in ANTS/DPX concentration, but no methodology is available for the reliable measurement of volume changes in highly polydispersed, aggregated vesicles. Alternatively, volume mixing might reflect the true fusion, whereas a part of the surface probe did not fully dilute from areas in which it was more concentrated. Thus, the results in Figure 7A should be taken mainly as a qualitative

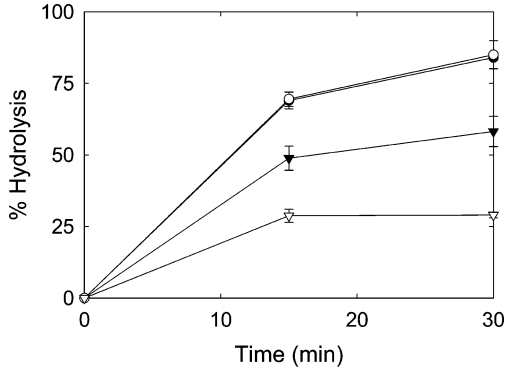

FIGURE 8: Effects of Zn^{2+} and *o*-phenanthroline on PLC_{LM} activity. Time course of hydrolysis of PC/SM/PE/Ch LUVs. Experimental data as in Figure 3. Average values ± SD ($n = 3$). Key: (●) control, (○) +0.1 mM Zn^{2+}, (▼) +2 mM *o*-phenanthroline, (▽) enzyme preincubated with 2 mM *o*-phenanthroline for 10 min.

indication that vesicle fusion, as measured by the intervesicular mixing of aqueous contents, does take place. Note that, as in the PC,SM-rich vesicles (Figure 5A), the lag time of contents mixing is somewhat longer than that of inner monolayer lipid mixing (~40 vs 30 s). An important observation is that, as in the PC,SM-rich vesicles, fusion induced by PLC_{LM} in PS,PE-rich LUVs is leakage-free (Figure 7B).

Zn^{2+} and PLC_{LM} Activity. PLC_{LM} is activated by 0.1 mM Zn^{2+} when tested with phospholipid−detergent mixed micelles as substrate (*9*). However, under our experimental conditions, when the substrate is in the form of bilayers, 0.1 mM Zn^{2+} does not lead to enzyme activation (Figure 8, circles). The *Bacillus* homologous enzyme PLC_{BC} does contain Zn^{2+} in its structure, but no additional Zn^{2+} in the assay medium is required (*11*). PLC_{BC} however is inhibited by the zinc chelator *o*-phenanthroline (*27*). The effect of *o*-phenanthroline on PLC_{LM} is also seen in Figure 8. At variance with PLC_{BC}, which is completely inhibited by 2 mM *o*-phenanthroline (*27*), PLC_{LM} retains a substantial fraction of its activity even after preincubation of the enzyme with the chelator (Figure 8, triangles). This is consistent with either a very tightly bound Zn^{2+} ion in the enzyme structure or a Zn^{2+}-binding region secluded from the aqueous medium.

The effect of the *o*-phenanthroline inhibitor was also tested on the intervesicular lipid mixing activity induced by PLC_{LM}. The results in Figure 9 show that preincubating the enzyme with 2 mM *o*-phenanthroline for 10 min, which considerably decreased the enzyme phosphohydrolytic activity (Figure 8), also diminishes the initial rate and extent of lipid mixing (Figure 9). This confirms that it is the catalytic activity of PLC_{LM}, and not a stoichiometric factor, that induces membrane fusion. In a different experiment, corresponding to curve PHEN (2) in Figure 9, the enzyme was added at time zero in the absence of inhibitor and then 2 mM phenanthroline was added at time 3 min, with the result that lipid mixing stopped immediately. One possible explanation for this behavior would be that substrate binding changes the enzyme conformation in such a way that Zn^{2+} now becomes more accessible to the water-soluble inhibitor.

DISCUSSION

The above results are interesting from the point of view of bacterial phospholipases C/sphingomyelinases and their effects on membrane architecture. They can also be analyzed from the point of view of the pathogenic mechanisms in *L. monocytogenes* infection.

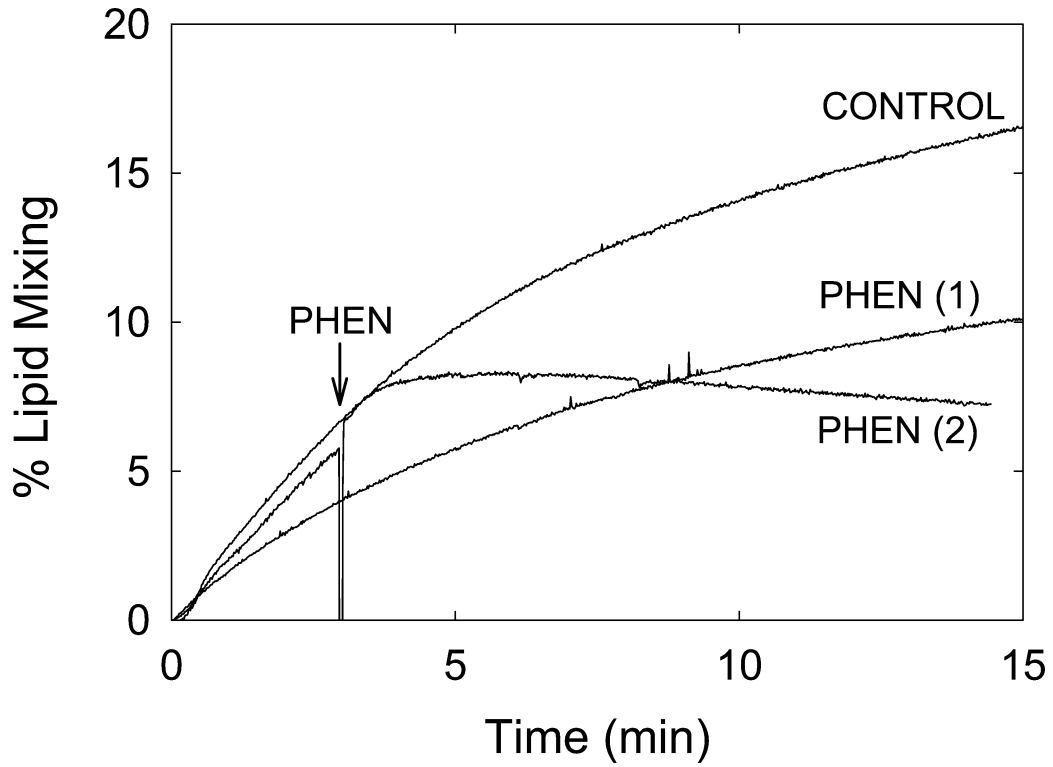

FIGURE 9: Effect of *o*-phenanthroline on intervesicular lipid mixing induced by PLC_{LM}. Experimental details as in Figures 4 and 5A. Key: control, experiment in the absence of inhibitor; PHEN (1), experiment in which the enzyme has been incubated with 2 mM *o*-phenanthroline for 10 min; PHEN (2), experiment started in the absence of chelator, which is added as indicated by the arrow 3 min after the onset of the experiment.

PLC_{LM} and Phospholipases C/Sphingomyelinases. The behavior of PLC_{LM} on substrates in the form of liposomes is best understood in the light of previous studies on its *B. cereus* orthologs PLC_{BC} and SMase (see refs *12* and *22* for reviews). As suggested by Zückert et al. (*10*), PLC_{LM} from *L. monocytogenes* has properties intermediate between those of the other two enzymes. In fact, this phospholipase C degrades both glycerophospholipids and sphingomyelin (Table 1, Figure 3). Ruiz-Argüello et al. (*23*) showed that PLC_{BC} is very active on PC/PE/Ch (2:1:1) mixtures, and SMase readily degrades SM/PE/Ch (2:1:1) bilayers, but neither of the enzymes acting separately is active on both PC and SM. The fact that PLC_{LM} rapidly hydrolyzes all three phospholipids in the quaternary mixture supports the 2-fold nature of the enzyme, as a phospholipase C and as a sphingomyelinase.

When acting on PC/PE/Ch, PLC_{BC} activity efficiently induces vesicle aggregation and fusion in the absence of leakage (*11*). Correspondingly SMase, acting on SM/PE/Ch vesicles, causes extensive aggregation and leakage, but no fusion (*13*). When the substrate consisted of the quaternary mixture PC/PE/SM/Ch at equimolar ratios, neither PLC_{BC} nor SMase was able to induce aggregation, fusion, or leakage, but a mixture of the two enzymes, acting together, did cause extensive vesicle fusion, with a delayed leakage (*23*). Again from this point of view, the behavior of PLC_{LM} resembles that of the mixture PLC_{BC} + SMase rather than that of either of the components. When vesicles composed of PE, PS, and PC are used as substrate (Table 1, Figures 6 and 7), only the "phospholipase C aspect" of the *Listeria* enzyme is observed. The high enzyme activity with these bilayers, containing 50 mol % PE, resembles what was observed with the *B. cereus* phospholipase C at high PE concentrations (*11*).

Many mammalian sphingomyelinases are structurally related to SMase, PLC_{BC}, and PLC_{LM}, in fact the bacteria may have derived the corresponding gene(s) from an infected host (*28*), but no mammalian sphingomyelinase is known with a significant phospholipase C activity (*22*). This is understandable since the end products of both enzyme activities, ceramide and diacylglycerol, have such diverse physiological roles (*29*, *30*). In bacteria however these enzymes have a purely extracellular degrading role; thus, evolution may have favored a wider substrate range. At least six bacterial species secrete this kind of phospholipase C/sphingomyelinase (*1*, *22*).

PLC_{LM} and Listeria Pathogenesis. PLC_{LM} was suspected to be a virulence factor of *L. monocytogenes* since its discovery in 1962 (*31*). More recently, it has been shown to participate in efficient escape of *L. monocytogenes* from the double-membrane vacuole resulting from cell-to-cell spread. LLO plays an essential role, and PI−PLC also aids in this process (*5*, *6*, *32*). When membrane-bound protrusions containing *L. monocytogenes* are taken up by adjacent cells, the double-membrane vacuole formed consists of an inner membrane from the donor cell and an outer membrane from the recipient cell membrane. These two membranes are topologically of opposite asymmetry. When the pH in the vacuole is decreased, the pro form of PLC_{LM} is released from the bacterial cell wall and is cleaved by the metalloprotease Mpl to provide active enzyme (*33*). In MDCK epithelial cells the double-membrane vacuole lyses within 3−5 min of its formation (*34*).

On the basis of the results presented here, we propose that vacuolar lysis may be preceded by fusion of the inner and outer membranes of the vacuole induced by PLC. Several experiments have shown that PLC_{LM} can induce fusion of bilayers rich in PC and SM, or rich in PS and PE. This underlines the wide fusogenic properties of the enzyme, and its capacity to attack, in principle, both inner and outer leaflets of the plasma membrane. However, our studies with model membranes cannot reproduce the asymmetric lipid distribution found in the cell membranes.

Fusing membranes involves opening of a pore, thus communicating two previously separated compartments. The pore, initially a few nanometers wide, may expand its diameter to allow the passage of large molecules (*24*). The facts that PLC_{LM} is more active in K^+-rich buffers that resemble the intracellular medium (Figure 1) and that it readily hydrolyzes different lipid mixtures, representing the most abundant lipids in mammalian cell membranes (Table 1), also point in the direction of PLC_{LM}-induced fusion as a pathogenic mechanism of *L. monocytogenes*. When *plcB* was replaced by the gene for PLC_{BC}, which does not have sphingomyelinase activity, cell-to-cell spread was reduced, but not to the level observed with a *plcB* deletion strain (*10*). Thus, sphingomyelinase activity of PLC_{LM} plays a significant, but not essential, role in cell-to-cell spread.

Quantitative considerations are important in understanding the relevance of studies on model membranes for the cellular systems. In general model membrane studies, such as the one described above, depict an ideal laboratory situation in which the process under consideration is greatly amplified. In our model system (Figures 4 and 6) the enzyme produces ∼15−20% fusion (according to measurements of inner monolayer lipid mixing); i.e., 15−20% of the vesicles are involved, on average, in a successful fusion event in the time interval considered in our experiments. The reason fusion does not proceed until 100% of the vesicles fuse is 2-fold: first, the enzyme end product diacylglycerol inhibits phospholipase C (*35*) and, second, above a certain concentration of diacylglycerol in the lipid mixture, lipid structures are formed that prevent fusion from going on (*36*). In any case, if the fusion rates and extents observed in our experiments were translated to the in vivo situation, the high number of fusion events would rapidly convert all the infected tissues into syncytia, which is certainly not the case. Not only is the difference between the in vivo and in vitro situations quantitative, it is also one of topology. In the test tube vesicles and enzyme are mixed, and all the lipids in the bilayer (or, initially, in the outer monolayer) are equally accessible to the phospholipase. In the cell, only a very small and localized part of the membrane is degraded by PLC_{LM}. At variance with PLC_{BC}, which can induce over 50% vesicle fusion after hydrolyzing less than 10% of the phospholipid (*11*), PLC_{LM} requires extensive lipid degradation before significant fusion occurs (Figures 4 and 6). This requirement produces relatively low damage to the cell precisely because the perturbation is localized to a specific vacuole. The comparatively low fusogenic efficiency of PLC_{LM} per amount of degraded phospholipid, as compared to PLC_{BC}, may possibly be a safety mechanism to prolong the life of the host cell, since extensive and indiscriminate membrane fusion could probably endanger host cell survival. Consequently, our studies of PLC_{LM}-induced fusion of model membranes

(liposomes) are in agreement, also in a quantitative way, with the idea that, in the in vivo situation, PLC_{LM} is instrumental in spreading *L. monocytogenes* infection from cell to cell by inducing fusion of the double-membrane vacuoles that contain the bacterium in infected cells.

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