Phospholipase C Hydrolysis of Phospholipids in Bilayers of Mixed Lipid Compositions†

M. Begoña Ruiz-Argüello, Félix M. Goñi, and Alicia Alonso*

Grupo Biomembranas (Unidad Asociada al C.S.I.C.), Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain

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ABSTRACT: Phosphatidylcholine phospholipase C (EC 3.1.4.3) from *Bacillus cereus* has been assayed with substrates in the form of large unilamellar vesicles. Phosphatidylcholine, phosphatidylethanolamine (also a substrate for the enzyme), sphingomyelin, and cholesterol have been mixed in various proportions, in binary, ternary, and quaternary mixtures. A lag period, followed by a burst of enzyme activity, has been found in all cases. The activity burst was always accompanied by an increase in turbidity of the vesicle suspension. Varying lipid compositions while keeping constant all the other parameters leads to a range of lag times extending over 2 orders of magnitude (from 0.13 to 38.0 min), and a similar variability is found in maximal enzyme rates (from 0.40 to 55.9 min^{-1}). Meanwhile, the proportion of substrate that is hydrolyzed during the lag period remains relatively constant at 0.10% moles of total lipid, in agreement with the idea that enzyme activation is linked to vesicle aggregation through diacylglycerol-rich patches. Phosphatidylethanolamine and cholesterol enhance the enzyme activity in a dose-dependent way: they reduce the lag times and increase the maximal rates. The opposite is true of sphingomyelin. These lipids exert each its own peculiar effect, positive or negative, either alone or in combination, so that the susceptibility of a given mixture to the enzyme activity can be to some extent predicted from its composition. Phospholipase C activity is not directly influenced by the formation of nonlamellar structures. However, the presence of lipids with a tendency to form nonlamellar phases, such as phosphatidylethanolamine or cholesterol, stimulates the enzyme even under conditions at which purely lamellar phases exist. Conversely sphingomyelin, a well-known stabilizer of the lamellar phase, inhibits the enzyme. Thus phospholipase C appears to be regulated by the overall geometry and composition of the bilayer.

Phospholipases C hydrolyze phospholipids yielding diacylglycerol. Apart from their intrinsic interest as enzymes in lipid metabolism, they have become particularly conspicuous after the identification of their reaction product diacylglycerol as a metabolic regulatory signal (*1*). Among the various known isozymes, phospholipase C from *Bacillus cereus* (PLC),[1] variously known as PC-specific PLC, or PC-preferring PLC, has been extensively studied because of its ready availability and apparent similarity to mammalian analogues (*2*, *3*). PLC (EC 3.1.4.3) is a monomeric, 28.5 kDa, Zn-containing enzyme that has been recently cloned and expressed at high levels (*4*).

From the point of view of its mechanism of action, PLC shares with other lipases the intriguing property of changing its activity as a result of the presence of lipids that are not specifically bound to the enzyme. They may not even be substrates for the enzyme, yet they influence greatly the enzyme performance. As a result, a number of phenomena such as lag periods, activations, etc., are observed. In the particular case of PLC, several of these kinetic peculiarities have been detected with substrates in the form of micelles or vesicles (*5*−*9*). This behavior of PLC and related enzymes has been variously attributed to the lipases being sensitive to the lipid phase (bilayer or nonbilayer) (*9*, *10*), or to a pretransitional packing stress (*11*), or to frustrated lamellar phases (*12*), or to lipid packing defects, in turn related to the structural microheterogeneity of the bilayer (*13*, *14*).

In a recent study from this laboratory, the origin of the lag period that is observed when phospholipase C attacks phosphatidylcholine in bilayers has been examined (*15*). The enzyme appears to act at a very low rate until a certain proportion of the end-product diacylglycerol is formed in the bilayer, at which point a burst of enzyme activity occurs, presumably related to structural defect formation. As a further step in elucidating the mechanism of PLC activity, we have now performed a detailed analysis of PLC function when the substrate is organized in bilayers consisting of mixtures of two, three, or four components, some of them enzyme substrates and some not, some being bilayer-stabilizing lipids along with other nonbilayer promoters. In all cases the substrate was in the form of large unilamellar vesicles obtained by extrusion, in the absence of surfactants, organic solvents, or other agents that could modify enzyme behavior. The experimental results support the idea that the

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* Corresponding author. Fax: +34-4-4648500.

[1] Abbreviations: Ch, cholesterol; DAG, diacylglycerol; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLC, bacterial phosphatidylcholine-preferring phospholipase C.

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enzyme activity is modulated by the physical properties of the bilayer as a whole, although the various lipid components contribute in a predictable way, each with their distinct, individual properties to the overall result.

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.3) from *Bacillus cereus* was supplied by Boehringer-Mannheim. Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), and 1,2-diacylglycerol (DAG) obtained by phospholipase C hydrolysis of egg PC were grade I from Lipid Products (South Nutfield, U.K.). Egg sphingomyelin (SM) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol was from Sigma (St. Louis, MO).

Phospholipid dispersions were prepared by rehydrating lipid films dried from organic solvents under high vacuum. Large unilamellar vesicles (LUVs) were prepared by the extrusion method of Mayer et al. (*16*) using Nuclepore filters of 0.1 μm pore diameter, at room temperature. Vesicle size was estimated by quasi-elastic light scattering (QELS) using a Malvern Zeta-Sizer instrument. In all cases vesicles had an average diameter of $\approx$100−115 nm.

All the measurements were carried out in 100 mM NaCl, 10 mM $CaCl_2$, 10 mM Hepes, pH 7.0. All experiments were performed at 37 °C and with continuous stirring. Lipid concentration was 0.3 mM, and enzyme was used at 1.6 units/mL.

Enzyme activity was assayed by measuring phosphorus contents in the aqueous phase of an extraction mixture (chloroform/methanol, 2:1) after addition of aliquots from the reaction mixture at different times. Phosphorus contents of the samples were determined by the method of Böttcher et al. (*17*). The specificity of the enzyme was checked by thin-layer chromatography. For this purpose, aliquots of the aqueous phase were separated on TLC Silica Gel 60 plates, using the solvent chloroform:methanol:water (60:30:5). After charring with an H_2SO_4 reagent, the spot intensities were quantified with a CS-930 dual wavelength TLC scanner from Shimadzu Corp.

Vesicle aggregation was monitored continuously as turbidity (absorbance at 500 nm) in a Cary 3 Bio Varian UV−vis spectrophotometer, or as an increase in light scattering at 90° in a LS-50 B Perkin-Elmer spectrofluorimeter with both monochromators set at 520 nm. Lag times for the aggregation events were computed from the individual time-course plots as the time at which the maximum slope line intersects with the "0% effect" baseline.

^{31}P NMR spectra were recorded in a KM360 Varian spectrometer operating at 300 MHz for protons. Spectral parameters were 45° pulses (10 μs), 3 s pulse interval, 16 kHz sweep width, and full proton decoupling. Two thousand free induction decays were routinely accumulated from each sample; the spectra were plotted with a line broadening of 80 Hz. Samples ($\sim$200 mM in lipid) were equilibrated at 37 °C for 10 min before data acquisition.

RESULTS

Preliminary Questions. A number of experiments were performed with the aim of answering the following questions: (a) What is the range of PLC substrates, among the three phospholipids used in this study, namely egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), and egg sphingomyelin (SM), under our experimental conditions? (b) Does the parallelism between phosphohydrolase activity and increase in suspension turbidity, which was observed when pure PC was the substrate (*15*), still hold for the different lipid compositions used in this study? The answers are contained in Figure 1. Under our conditions, and starting with a PC:PE:SM equimolar mixture, the enzyme hydrolyzes PC and PE at similar rates, while SM is unaffected (Figure 1A). A similar behavior had been found for these lipids in detergent mixed micelles (*5*), and is now seen in bilayers. With respect to the parallel change in phospholipid hydrolysis and suspension turbidity, the same was found with all lipid mixtures under study. Three examples are shown in Figure 1B. The increase in turbidity is due to vesicle aggregation (*18*) that appears to accompany the burst in activity (*15*).

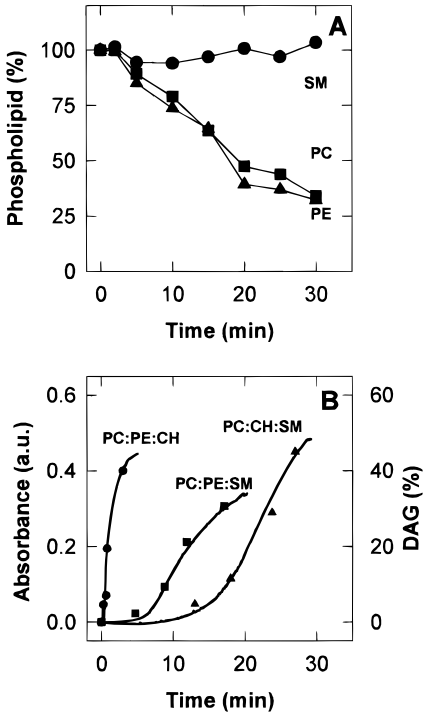

FIGURE 1: Phospholipase C hydrolysis of ternary mixtures of PC, PE, SM, and/or Ch in the form of large unilamellar vesicles. (A) Time-course of the cleavage of individual phospholipids by phospholipase C. The starting mixture was PC:PE:SM (1:1:1, mole ratio). (B) Examples of time-courses of three ternary lipid mixtures (2:1:1, mole ratio in all cases) followed by changes in turbidity (continuous line) or through chemical analysis (data points). Note the good correlation of both procedures.

Binary Mixtures. The behavior of PLC in binary mixtures of PC with PE, SM, or cholesterol (Ch) at different molar ratios is summarized in Figures 2−4. In each case, the length of the lag period, the amount of diacylglycerol (DAG) that had been formed during the lag period, and the maximum rate of enzyme activity have been measured as a function of the proportion of non-PC component. PE produces a clear decrease in the lag time (Figure 2A), but the amount of DAG required to put an end to the lag phase is virtually unchanged (Figure 2B). Thus, PE is increasing the rate of enzyme activity already during the lag phase. After the burst in activity, PE also increases enzyme rates, at least when its mole fraction in the bilayer is above $\sim$12%.

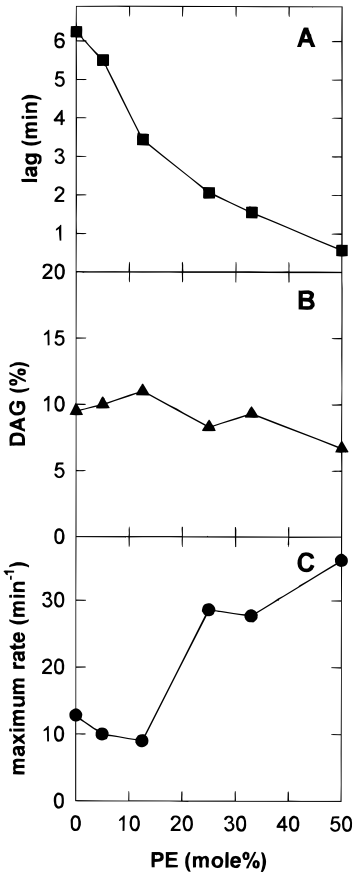

FIGURE 2: Phospholipase C hydrolysis of PC:PE mixtures in the form of large unilamellar vesicles. The results are plotted as a function of PE mole percent concentration in the bilayers. (A) Lag times. (B) Proportions of DAG in the bilayers (as mole percent of total lipids) at the end of the latency period. (C) Maximum enzyme rates. In (A) and (C), the experimental points are derived from turbidity measurements.

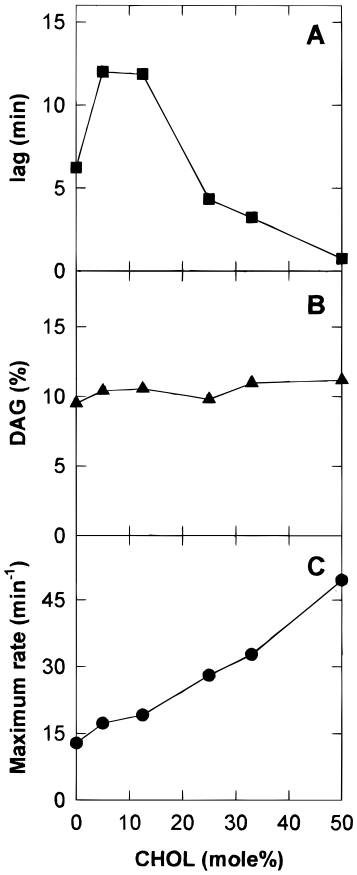

FIGURE 3: Phospholipase C hydrolysis of PC:Ch mixtures in the form of large unilamellar vesicles. The results are plotted as a function of Ch mole percent concentration in the bilayers. (A) Lag times. (B) Proportions of DAG in the bilayers (as mole percent of total lipids) at the end of the latency period. (C) Maximum enzyme rates. In (A) and (C), the experimental points are derived from turbidity measurements.

Cholesterol, obviously not a PLC substrate, has a dual effect on the lag time, increasing it at mole ratios up to ≈12%, then decreasing it at higher proportions (Figure 3A). Again the proportion of DAG required for the activity burst remains constant at ≈10% (Figure 3B), while the enzyme rate after the lag period increases monotonically with the proportion of cholesterol in the bilayers (Figure 3C).

Sphingomyelin, a lipid that tends to stabilize the lamellar phase (*19*) has a seemingly opposite effect compared to PE or Ch, it increases the lag time of the PLC reaction (Figure 4A) and decreases the post-burst rate (Figure 4C). Again, the amount of DAG at the end of the lag period remains largely unchanged by the presence of SM in the bilayer (Figure 4B).

Ternary and Quaternary Mixtures. The results of PLC activity on a number of three-component mixtures are summarized in Table 1. Four groups of mixtures have been used: (a) those based on PC:PE:Ch, combining two lipids PE and Ch that, in the binary mixtures, had shown to increase PLC activity, (b) PC:PE:SM, and (c) PC:Ch:SM contain one of the enzyme-enhancing lipids, PE or Ch, and the enzyme-inhibiting SM, and (d) SM:PE:Ch, similar to (a) except that PC has been substituted by SM. Four different lipid molar ratios have been explored for each ternary mixture, as detailed in Table 1.

Enzyme activities are particularly high when PC:PE:Ch mixtures are used. Lag times are very short and comparable to those in the equimolar PC:PE or PC:Ch mixtures. Note that these are lag times of the order of a few seconds, that are usually seen only as a slight sigmoidicity in the activity vs time curves. Remarkably, the amount of DAG required for the activity burst is also clearly decreased to values about 5%. The enzyme rates after the lag period are also very high. All four mixtures based on PC:PE:Ch behave similarly, with the 2:1:1 composition allowing a somewhat higher activity and a shorter lag time. The latter mixture is the one that was used by Nieva et al. (*22*) in their studies of PLC-induced liposomal fusion.

As soon as SM takes the place of either PE or Ch in the ternary system (PC:PE:SM or PC:Ch:SM mixtures) the situation changes drastically, lag times become longer and maximum rates lower, each by 1 order of magnitude (Table 1) (see also Figure 1B). The required amount of DAG for the activity burst also changes to the more usual values around 10%. In those two groups of samples, SM exerts a clearly inhibitory effect on the enzyme, that is particularly visible in the 1:1:2 compositions, i.e., when SM is present at 50 mol % in the bilayers. In these samples the lag time increases enormously, and the post-burst enzyme rate decreases, to the point that, under these conditions, the word "burst" can only be used by analogy (Figure 5).

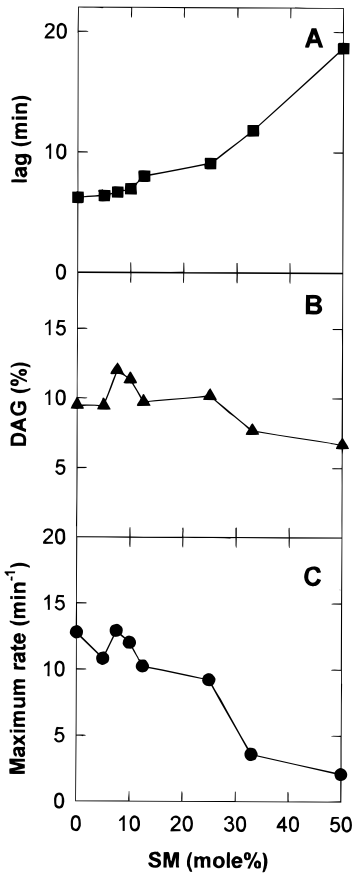

FIGURE 4: Phospholipase C hydrolysis of PC:SM mixtures in the form of large unilamellar vesicles. The results are plotted as a function of SM mole percent concentration in the bilayers. (A) Lag times. (B) Proportions of DAG in the bilayers (as mole percent of total lipids) at the end of the latency period. (C) Maximum enzyme rates. In (A) and (C), the experimental points are derived from turbidity measurements.

Mixtures consisting of SM:PE:Ch resemble more any of the other SM-containing compositions than the apparently more similar PC:PE:Ch (Table 1). Particularly striking is the comparison between PC:PE:Ch (2:1:1) and SM:PE:Ch (2:1:1), the former allowing optimal enzyme activity, while the latter requires a lag time 2 orders of magnitude longer and allows a maximal rate 2 orders of magnitude lower than the PC-containing mixture. The structural similarity between PC and SM is somewhat misleading in this case. Not only is SM not a PLC substrate, it is obviously an enzyme inhibitor. SM is also very different from PC in its phase behavior when mixed with PE and Ch (*18*, *19*, *41*).

Taking a further step into the complexity of biological membranes, a series of studies were performed with quaternary lipid mixtures containing PC, SM, PE, and Ch, always in the form of large unilamellar vesicles. Some of these results are summarized in Table 2. The four-component mixtures are designed as modifications of the PC:PE:Ch (2:1:1) composition. The effect of adding SM is clearly seen in Table 2, lag times increase considerably, and the maximal rates decrease. Also the amount of DAG required for the burst of activity returns to the usual values of ≈10% as soon as SM is present in the bilayers.

The inhibitory effect of SM was studied in more detail by preparing a series of mixtures in which SM was gradually increased from 0% (PC:SM:PE:Ch, 2:0:1:1) to 25% (PC:SM:PE:Ch, 1:1:1:1), while the PC proportion was correspondingly decreased. The results in Figure 6 show that the effect of SM is not linear, 5% of this lipid causes already a major decrease in catalytic rate (Figure 6C). Interestingly, in this case, the main increase in lag time occurs with SM concentrations above 12.5% (Figure 6A). The proportion of DAG at the burst also increases with SM concentrations, although less steeply (Figure 6B).

Table 1: Kinetic Data of Phospholipase C Activity on Bilayers Consisting of Ternary Lipid Mixtures[a]

lipidcomposition (mole ratio)	lag[b] (min)	DAG[c] (%)	maximal rate[d] (min^{-1})
pure PC	6.2 ± 0.41	9.5 ± 0.28	12.8 ± 0.47
PC:PE:Ch			
2:1:1	0.13 ± 0.047	5.9 ± 0.06	55.9 ± 5.05
1:1:1	0.18 ± 0.002	5.2 ± 0.04	39.2 ± 1.76
1:2:1	0.21 ± 0.011	5.7 ± 0.07	20.6 ± 3.40
1:1:2	0.18 ± 0.016	5.2 ± 0.06	32.4 ± 19.18
PC:PE:SM			
2:1:1	8.8 ± 0.18	9.3 ± 0.09	2.9 ± 0.15
1:1:1	7.0 ± 0.05	11.1 ± 0.06	2.7 ± 0.23
1:2:1	4.5 ± 0.25	10.1 ± 0.10	3.3 ± 0.40
1:1:2	36.5 ± 1.29	10.0 ± 0.12	1.2 ± 0.07
PC:Ch:SM			
2:1:1	13.1 ± 0.54	8.2 ± 0.12	2.9 ± 0.22
1:1:1	9.2 ± 0.22	10.4 ± 0.31	2.4 ± 0.14
1:2:1	18.2 ± 0.51	13.2 ± 0.62	2.6 ± 0.02
1:1:2	38.0 ± 1.54	9.4 ± 0.47	1.8 ± 0.27
SM:PE:Ch			
2:1:1	16.6 ± 0.66	11.1 ± 1.71	0.4 ± 0.07
1:1:1	5.8 ± 0.62	13.3 ± 1.82	1.1 ± 0.02
1:2:1	3.9 ± 0.44	12.9 ± 2.14	1.9 ± 0.16
1:1:2	1.6 ± 0.13	13.1 ± 2.05	0.8 ± 0.04

[a] Data are average values ± S.E.D. ($n = 4-6$). [b] Measured from turbidity plots; see Materials and Methods for details. [c] DAG generated at the end of the lag period; expressed as percentage with respect to total lipid. [d] Measured from turbidity plots, at their maximum slope; expressed as (absorbance units times 100)min^{-1}.

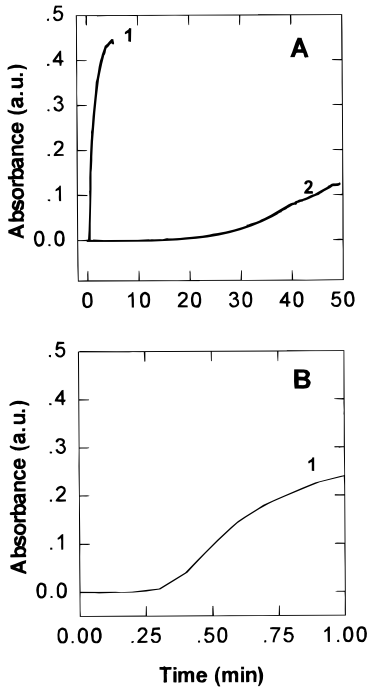

FIGURE 5: Turbidity vs time plots illustrating quantitative differences in enzyme behaviour depending on the bilayer lipid compositions. Note the different time scale in A and B. (1) PC:PE:Ch, 2:1:1; (2) PC:PE:SM, 1:1:2.

Table 2: Kinetic Data of Phospholipase C Activity on Bilayers Consisting of Quaternary Lipid Mixtures[a]

PC:SM:PE:Ch (mole ratio)	lag[b] (min)	DAG[c] (%)	maximal rate[d] (min^{-1})
2:0:1:1	0.058 ± 0.0016 (6)	5.9 ± 0.06 (6)	355 ± 42.6 (6)
1:1:1:1	13.0 ± 0.65 (6)	14.2 ± 0.28 (4)	0.24 ± 0.006 (6)
2:0.5:0.5:1	1.2 ± 0.018 (4)	14.5 ± 0.3 (3)	2.0 ± 0.36 (4)
2:0.5:1:0.5	1.8 ± 0.061 (4)	13.5 ± 0.2 (3)	2.0 ± 0.06 (4)

[a] Data are average values ± S.E.D; number of experiments in parentheses. [b] Measured from light scattering plots; see Materials and Methods for details. [c] DAG generated at the end of the lag period; expressed as percentage with respect to total lipid. [d] Measured from light scattering plots, at their maximum slope; expressed as units·min^{-1}.

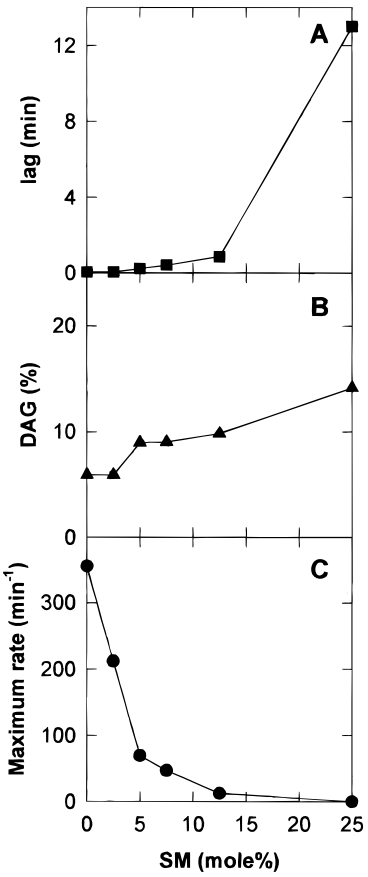

FIGURE 6: Phospholipase C hydrolysis of PC:SM:PE:Ch in the form of large unilamellar vesicles. The compositions range from 2:0:1:1 mole ratio (0% SM) to 1:1:1:1 mole ratio (25% SM), SM gradually substituting PC. (A) Lag times. (B) Proportions of DAG in the bilayers (as mole percent of total lipids) at the end of the latency period. (C) Maximum enzyme rates. In (A) and (C), the experimental points are derived from light scattering measurements.

Phase Studies. Since phospholipase activities have often been related to the presence of certain lipid phases or phase transitions (see Introduction), the phase behavior of several PC:PE, PC:Ch and PC:SM mixtures, with or without DAG, was studied by ^{31}P NMR. Representative results are shown in Figure 7. It should be noted that ^{31}P NMR studies are carried out under conditions very different from those of the enzyme assays summarized in Figures 1−4. Essentially, lipid concentration is much higher in the NMR experiments, and the lipid is present in the form of multilamellar dispersions. Also, when DAG is added to the NMR samples, it is mixed in organic solvent, then codispersed in water with the other lipids, thus homogeneously mixed, while DAG generated in situ by PLC is believed to be asymmetrically distributed and localized in patches (*20*, *21*). With these considerations in mind, examination of Figure 7 may help in interpreting the enzyme activity results.

Aqueous dispersions of equimolar mixtures of PC:PE, PC:Ch or PC:SM give rise to ^{31}P NMR spectra that are compatible with phospholipids in a lamellar organization. Addition of DAG has different effects, depending on the mixture. In PC:PE systems, 10% DAG is enough to induce the complete transition from a lamellar to an isotropic (probably cubic) (*21*) phase, while the same proportion of DAG in a PC:Ch mixture gives rise to a largely lamellar system, although with a nonlamellar component. Systems containing PC:SM are purely lamellar under all conditions tested, even with 20% DAG. Thus, each of the three binary systems under study displays its own peculiar behavior in the presence of DAG, PC:PE giving rise most easily to a nonlamellar phase, and PC:SM, at the opposite end, remaining lamellar under all the studied conditions.

The observation of isotropic signals in Figure 7A,B, but not in 7C, i.e., in the presence of the activating lipids PE or Ch (Figures 2, 3), but not with the inhibitor lipid SM (Figure 4), rules out the involvement of nonlamellar phases in the lag−burst transition, since lag and burst occur as well in the presence of SM. However, nonlamellar structures could be related to the post-burst enzyme activity, causing an increase in enzyme rates. This possibility was explored by recording ^{31}P NMR spectra of sixteen lipid mixtures, as detailed in Table 3. Of these, eight had been studied in enzyme assays (Figures 2−4 and 6, Tables 1 and 2); they are listed at the left side of the Table, in order of increasing post-burst enzyme rates (the latter are also included in the Table). Another eight mixtures contain DAG, they are derived from the eight previous ones, substituting PC (and, when required, PE) for DAG to mimic the compositions that, according to the data in Figures 2-4 and 6, and Tables 1 and 2, exist at the beginning of the burst period of enzyme activity, i.e., when the enzyme rates given in Table 3 are observed. In the absence of DAG, all eight mixtures gave ^{31}P NMR spectra compatible with a fully lamellar organization. With the mixtures containing DAG, nonlamellar components were detected only in ^{31}P NMR spectra of two PC:PE:Ch:DAG mixtures (Table 3). Of two mixtures, PC:Ch:DAG (76:12:12) and PC:PE:Ch:DAG (23:46:25:6), that gave rise to very similar enzyme rates, respectively 18.5 and 20.6 min^{-1}, only the latter produced spectra with nonlamellar components. More significantly, PC:PE:Ch:DAG (46:23:25:6), that was able to induce the highest detected activity (55.9 min^{-1}), gave off fully lamellar ^{31}P NMR signals. The conclusion of these experiments is that the presence or absence of nonlamellar phases, and the rates of phospholipase C activity, while being both very sensitive to lipid composition, are unrelated phenomena.

DISCUSSION

The phospholipase C assays described above, that were carried out on a number of lipid mixtures of varying complexity, allow some generalizations to be made on the behavior of this enzyme.

(a) Different Lipids Modify the Enzyme Activity in Specific Ways. On the basis of the behavior of PLC on pure egg PC

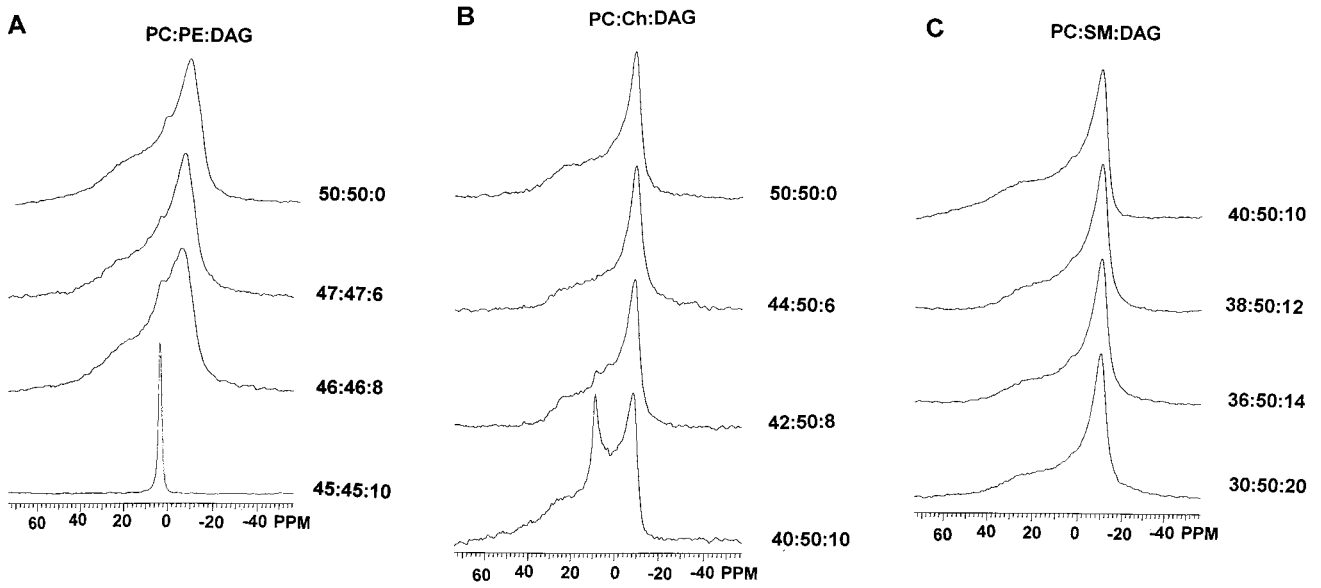

FIGURE 7: ^{31}P NMR spectra of aqueous lipid dispersions. (A) PC:PE:DAG mixtures. (B) PC:Ch:DAG mixtures. (C) PC:SM:DAG mixtures. The composition of each lipid mixture is indicated for each spectrum. Line broadening: 80 Hz.

Table 3: Presence of Nonlamellar Components in ^{31}P NMR Spectra of Aqueous Lipid Dispersions Representing Bilayer Compositions at the Start of the Phospholipase C Activity Burst[a]

mixtures without DAG[b] (mole percentages)		mixtures with DAG[c] (mole percentages)		post-burst rates[d] (min^{-1})	nonlamellar component[e]
PC:SM:PE:Ch	50:12:5:12:5:25	PC:SM:PE:Ch:DAG	38:12.5:9.5:25:15	0.3	NO
PC:Ch:SM	33.3:33.3:33.3	PC:Ch:SM:DAG	23.3:33.3:33.3:10	2.4	NO
PC:PE	88:12	PC:PE:DAG	77.4:10.6:12	8.0	NO
PC	100	PC:DAG	90:10	12.8	NO
PC:Ch	88:12	PC:Ch:DAG	76:12:12	18.5	NO
PC:PE:Ch	25:50:25	PC:PE:Ch:DAG	23:46:25:6	20.6	YES
PC:PE:Ch	33.3:33.3:33.3	PC:PE:Ch:DAG	29.3:31.3:33.3:6	39.2	YES
PC:PE:Ch	50:25:25	PC:PE:Ch:DAG	46:23:25:6	55.9	NO

[a] Mixtures in order of increasing enzyme rates. [b] These mixtures have all been tested for phospholipase activity in the experiments shown in Figures 2−4 and 6, and Tables 1 and 2. [c] The composition of these mixtures has been calculated (from data in Figures 2−4 and 6, and Tables 1 and 2) to mimic the situation of the mixtures in the left-hand column at the start of the activity burst. PC and PE are assumed to be equivalent substrates for phospholipase C (see Figure 1A). [d] Maximal rates, measured by the turbidity method, for liposomes consisting of the lipids at the left-hand column. [e] Presence (YES) or absence (NO) of nonlamellar components in ^{31}P NMR spectra of multilamellar vesicles of the corresponding DAG- containing mixture.

bilayers, as described in ref *15*, it can be stated that addition of some lipids to the bilayer, e.g., PE or Ch, activates the enzyme in the sense that (with few exceptions) lag times are decreased and post-burst enzyme rates are increased (Figures 2, 3), while other lipids are inhibitory, e.g., SM (Figure 4). Note that the two activating lipids bear very little structural resemblance, in fact one of them is also a PLC substrate, while the other one is not. It should also be noted that both activatory molecules belong to the class of lipids that favor nonlamellar phase structures (*23*−*25*), while SM is a stabilizer of the lamellar phase (*19*, *26*, *27*).

(b) The Effects of the Various Lipids Are Additive. The stimulatory or inhibitory properties of the lipids with respect to PLC activity are additive, at least in a semiquantitative way. PE and Ch, either alone or in combination, increase the enzyme activity whenever they are incorporated into a bilayer. Compare, e.g., the maximal rates of the 1:1 PC:SM mixture in Figure 4 with those of the PC:PE:SM or PC:Ch:SM mixtures in Table 1, or with the quaternary mixtures in Table 2. Conversely, SM causes the enzyme activity to decrease when present in a bilayer, as seen in Figures 4 and 6. Compare also the 1:1 PC:PE (Figure 2) or 1:1 PC:Ch (Figure 3) mixtures with the PC:PE:SM or PC:Ch:SM composition in Table 1, or the PC:PE:Ch with the SM:PE:Ch mixtures in the same Table. Enhancers may be neutralized by inhibitors when mixed together; PC:PE:SM (1:1:1) or PC:Ch:SM (1:1:1) behave in a rather similar way to pure PC, although the maximal rates are somewhat lower in the mixtures (Table 1). The constancy of the positive or negative effects of these lipids on the PLC activity endows our observations with an important predictive power that may help in guiding future experiments with this enzyme. These results may also be important in interpreting in vivo studies of the enzyme; e.g., cell membranes with external monolayers rich in SM should be relatively resistant to this enzyme.

(c) Lag Times and Maximal Rates Are Inversely Related. This is a rather straightforward conclusion from the whole of the enzyme assays described above. Experimental conditions that tend to shorten the lag times also allow high post-burst enzyme rates. The only clear exception to this rule is the increased lag times that occur together with increased rates at PC:Ch ratios ≈90:10, that may be attributed to the complexity of the PC−cholesterol phase diagram under these conditions (*42*). The observed inverse correlation, together

with the fact that the proportion of DAG required for the burst of activity is fairly constant (see below), means that whatever agent that increases the post-burst rate is also favoring the (much slower) enzyme activity during the lag period. This in turn would be consistent with the idea, that was outlined in our previous paper (*15*), that PLC catalyzes the hydrolytic reaction in a similar way both before and after the burst, but at very different rates. In fact, in some experiments with SM (e.g., SM:PE:Ch 2:1:1, Table 1) the maximal rate is so low that there are hardly any clearly defined lag time and burst.

(d) The Proportion of Diacylglycerol That Is Required for the Burst of Enzyme Activity Is Remarkably Constant. In a previous study (*15*) it was found that, for bilayers consisting of pure PC in the form of large unilamellar vesicles, PLC activation occurred once a fixed molar fraction of DAG (≈10%) had been produced by the enzyme. We have now extended this observation to bilayers with a large number of lipid compositions. In the 42 lipid mixtures tested, the average proportion of DAG at the burst was 9.5 ± 0.44 mol % total lipid. In 34 mixtures out of those 42, the proportion of DAG was in the 7−13% range. A significant exception to this rule is the behavior of the PC:PE:Ch compositions, in which case the critical proportion of DAG is around 5−6%. These mixtures are particularly prone to nonlamellar phase formation (*21*) and, although 6% DAG is not enough to induce the phase transition in all of them, at least at 37 °C (Table 3), the proximity to the phase boundary may be related to the end of the lag period. However, this is a hypothesis that requires more careful experimental testing. The fraction of DAG that, with the noted exceptions, remains constant irrespective of lipid composition is calculated with respect to the total amount of lipid, not with respect to hydrolyzable lipid (no constant value is found when the latter calculation is performed). This is in agreement with our hypothesis that the role of the DAG generated in the lag phase is to induce some kind of phase separation, with the formation of DAG-rich domains, that in turn facilitate vesicle aggregation, and enzyme activation (*15*).

(e) Vesicle Aggregation Marks the End of the Lag Phase. As stated in the Introduction, this work was partly conceived as an extension of our previous study on the origin of the lag periods in phospholipase C (*15*). There it was concluded that enzyme activation occurs concomitantly with vesicle aggregation, that is usually detected as an increase in liposome suspension turbidity. The previous report was based on observations with pure PC liposomes. The present results confirm and extend this finding as a general feature of phospholipase C, irrespective of lipid composition in the bilayers. Vesicle aggregation has been detected as an increase in turbidity at the end of the lag phase in all the examined samples (see, e.g. Figure 1B). In light of our previous study (*15*), in which enzyme and substrate concentration, vesicle size, temperature, and other parameters were independently varied, it can be stated that the burst period is not merely the result of the enzyme being provided with more substrate at the time of vesicle aggregation. Instead, the diacylglycerol that is produced at low rate during the lag period gives rise to in-plane phase separation, then the resulting structural defects (eventually stabilized by vesicle−vesicle contacts) lead to enzyme activation.

The above generalizations can be the basis for future exploration of the in vivo and in vitro activities of PLC and other lipases. Two other aspects deserve separate discussion, namely the putative relationship between lipid phase behavior and PLC activity, and a comparison with studies using other phospholipases.

Enzyme Activity and Nonbilayer Lipids. Lipids with an inherent tendency to destabilize the lamellar phase (e.g., PE, Ch, or DAG itself) have often been found to modify the activity of PLC and other lipases, in agreement with the observations in this paper (*9*−*12*, *28*−*32*). Such effects of nonbilayer lipids have been attributed to (a) the actual formation of nonbilayer phases in the system (*9*), or (b) a pretransitional packing stress, and related packing defects (*11*), or else (c) to a "frustration" of lipid bilayers resulting from the bending stress imposed by the presence of lipids with negative spontaneous curvature (*12*).

In our case, the observed activation by PE and/or Ch cannot be attributed to lamellar-to-nonlamellar phase transitions, as seen particularly from the studies on binary mixtures (Figures 2−4 and 7). The burst of enzyme activity requires in all these cases the formation of ≈7−10% DAG while, as seen in Figure 7, the three systems have a totally different phase behavior, PC:PE becoming isotropic with ≈10% DAG while PC:SM mixtures remain purely lamellar even with 20% DAG, and PC:Ch represent an intermediate situation. The increase in post-burst enzyme rates in the presence of PE or Ch (Figures 2, 3 and Table 1) cannot be interpreted either in relation to nonlamellar phases since catalytic rates vary independently from the lamellar-to-nonlamellar transition (Table 3).

De Boeck and Zidovetzki (*31*) have argued, for phospholipase A_2, that the enzyme is sensitive to bilayer packing stress, rather than to actual nonbilayer structures. Sen et al. (*11*) also proposed for phospholipase A_2 that it is not the actual transition, but the bilayer packing stress and structural defects at the onset of the bilayer-to-nonbilayer transition that increases the phospholipid susceptibility to the enzyme. While the idea of activation by packing stress may be valid for PLC this does not seem to be the case with the "onset of the transition" suggestion, essentially for the same reasons given in the previous paragraph. Basáñez et al. (*15*) also described experimental data for PLC and pure PC bilayers that were not compatible with an activation at the onset of the transition.

More recently, Kinnunen (*12*) has suggested a hypothesis, altogether not removed from some of the proposals mentioned above (*11*, *31*), according to which a number of enzymes that interact with membranes as peripheral proteins would be activated by a certain propensity of lipid bilayers to adopt the inverted hexagonal disposition, while remaining in the lamellar phase. Such propensity would be given by the presence of nonbilayer lipids in the membrane, that would induce a frustrated lamellar state. The results in the present paper can certainly be interpreted in the light of this hypothesis. Thus, PLC would join in a large group of enzymes, reviewed in ref *12*, whose activities are enhanced by the presence of nonbilayer lipids in essentially lamellar systems. This hypothesis appears to be physiologically relevant, since it allows the possibility of enzyme regulation in cell membranes without loss of the bilayer structure and of its barrier properties.

The studies of Rao and Sundaram (*9*) are surprising since, opposite to ours and other observations, they describe that PLC is *less* active in PC:PE mixtures than in pure PC. However, their observations should be taken with caution, since enzyme activity measurements consist in their paper of a single point, taken 10 min after enzyme addition. Considering the very long lag times that are often seen with PLC, single point observations can be very risky. Also surprising is the lack of changes in light scattering reported by these authors in their enzyme-treated samples. An increase in light scattering accompanies PLC activity in each one of our experiments, in this and previous papers (refs *15*, *18* and references therein).

A Comparison with Other Phospholipases. Our results reveal a number of similarities between PLC and other phosphohydrolases. A lag time is observed in phospholipase A_2 under a variety of conditions, until a certain fraction of the reaction products, free fatty acids and lysolecithin, accumulates in the bilayer (*33*−*35*, *43*), just like PLC. Phospholipase A_2 activation by long-chain DAG has already been mentioned above (*31*, *32*) as well as the role of bilayer microheterogeneities in promoting the burst of activity of this enzyme (*14*).

Perhaps more relevant are the properties observed in parallel in different phospholipases C, particularly in the bacterial PC-preferring PLC and the various phosphatidylinositol (PI)-specific phospholipases. Zhou et al. (*36*, *37*) have recently demonstrated that PI-specific phospholipase C from *Bacillus* exhibits interfacial activation, very much like PLC, and that PC and PE provide optimal interfaces for this purpose. Mammalian phospholipases C have been shown to be activated by DAG and inhibited by PC in sonicated phospholipid dispersions (*28*). Phospholipase Cδ from various mammalian sources is inhibited by sphingomyelin in detergent micelles and liposomes (*38*, *39*) although at SM/PI ratios much higher than most SM/PC ratios used in this study. While these data may appear as rather scattered and difficult to rationalize in the absence of a more detailed structure−function knowledge of phospholipases, they certainly support the hypothesis, very important in membrane biology, that the collective physical properties of the lipid bilayer can modulate the activities of membrane-associated proteins (*14*, *40*).

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