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Activity of phospholipase A_2 on a fluorescent substrate incorporated into non-hydrolyzable phospholipid liposomes

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Abstract

The activity of phospholipase A_2 (PLA_2) on phospholipid liposomes depends on the physicochemical properties of the aggregated substrate, which are subject to continuous modification by the products released during hydrolysis. We propose here an experimental design that, by means of the incorporation of a fluorescent substrate at very low molar ratio ($\leq 1:500$) into a nonhydrolizable liposomal matrix of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC), allows the study of hydrolysis by porcine pancreatic phospholipase A_2, in virtual absence of physical perturbations of the lamellar phase, by the released products. We have been able to measure immediate hydrolysis of the fluorescent substrate 1,2-di-[ω(1'-pyreno)-decanoyl]-*sn*-glycero-3-phosphocholine when the sonicated liposomal matrix is in the gel phase. In the liquid crystalline state, in contrast, hydrolysis is very poor even after 80 min of adding the enzyme. Both in the gel and liquid-crystalline phases, incorporation of unlabeled PLA_2 products activates the hydrolysis rate to comparable levels. It appears that the conformation adopted by the substrate immersed in the gel or liquid crystalline matrix is especially important in determining its susceptibility to hydrolysis in the absence of products.

Key words: Phospholipase A_2; Fluorescent substrate; Liposome physical state

1. Introduction

The hydrolytic activity of phospholipase A_2 (PLA_2) toward the ester bond at the *sn*-2 position of *sn*-3 phosphoglycerides increases markedly when the phospholipid substrate is aggregated [1]. Although it is possible that an interaction with the lipid–water interface results in a conformational change in the enzyme leading to increased catalytic efficiency, there is strong evidence that upon aggregation, phospholipids adopt a conformation showing enhanced susceptibility to PLA_2 action [2–4].

The phenomenon of interfacial activation of PLA_2 presents several peculiarities which depend on the properties of the phospholipid aggregate. For instance, an important feature of the hydrolysis of phosphatidylcholine liposomes catalyzed by porcine pancreatic PLA_2 is that, under a number of conditions, the apparent activation of the enzyme is preceded by a lag period of low hydrolytic activity and variable duration [5–10]. Triggering of the high-rate phase of hydrolysis that follows the lag phase has been attributed to several factors such as dimerization of the enzyme [11] or promotion of enzyme binding by a critical fraction of products generated through low enzymatic activity during the latency [6,12]. In our laboratory we have identified the negative surface charge density generated by the released fatty acid and the resulting interfacial calcium ion concentration as important modulators in the activation of hydrolysis [8–10].

Hydrolysis of sonicated dipalmitoylphosphatidylcholine liposomes in the gel phase proceeds immediately after addition of porcine pancreatic PLA_2 [5,7,8,11]. In contrast, when the substrate is in the liquid crystalline state, long latencies are found [5,7,8]. Addition of products reverses the latency. Sequestration of products by defatted bovine serum albumin

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Abbreviations: DiPyDPC, 1,2-di-[ω-(1'-pyreno)decanoyl]-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; T_m, gel–liquid-crystal phase transition temperature of liposomes; PLA_{22}, phospholipase A_2.

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induces a latency in gel phase vesicles of dipalmitoylphosphatidylcholine [8]. Thus, if participation of products is also important in the hydrolysis of the gel phase vesicles, the question remains as to why these liposomes are immediately attacked by PLA_2.

Usually the aggregated phospholipid substrate determines its own lipid environment. During hydrolysis, the generated products will modify this situation. Since the activity of PLA_2 is extremely sensitive to the physicochemical properties of the organized substrate [1,8], the changes induced by the products released during hydrolysis in the properties of the remaining substrate can be expected to continuously modify the enzymatic action. To study the activity of PLA_2 at constant macroscopic physicochemical properties of the substrate environment, we propose an experimental design based on the use of the fluorescent substrate 1,2-di-[ω-(1'-pyreno)-decanoyl]-*sn*-glycero-3-phosphocholine (DiPyDPC) incorporated into liposomes of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC), the non-hydrolyzable diether analogue of dipalmitoylphosphatidylcholine. By fixing the molar ratio of substrate to lipid matrix at a value equal to or lower than 1:500, the influence of the products on the physical state of the liposomes should be negligible.

The ratio between the excimer (I_e) and monomer (I_m) fluorescence shown by the substrate DiPyDPC, allows to monitor the physical phase of the liposomal matrix into which it is inserted [13]. In addition, cleavage of this type of molecule by PLA_2 increases the monomer fluorescence and therefore allows the follow-up of the hydrolysis time-course, as demonstrated by Hendrickson and Rauk for dipyrendibutanoylphosphatidylcholine micelles [14].

Using this approach, we have studied if, in virtual absence of any perturbation by the released products, hydrolysis proceeds differently when the substrate matrix is in the gel or liquid-crystalline state. We have also investigated in this system the effect of externally added unlabeled products on PLA_2 action. The results could contribute to a better understanding of the hydrolysis process in the gel and liquid-crystalline phases of sonicated liposomes composed of the authentic, pure unlabeled substrate. The experimental design described here could also be used in the future for studies on hydrolysis of large unilamellar vesicles.

2. Materials and methods

The fluorophore-labeled lipids 1,2-di-[ω-(1'-pyreno)-decanoyl]-*sn*-glycero-3-phosphocholine (DiPyDPC) and 10-(1'-pyreno)-decanoic acid, were purchased from Molecular Probes. The ether-linked phospholipid 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) was from Serdary. Porcine pancreatic phospholipase A_2, lysopalmitoylphosphatidylcholine and palmitic acid, were from Sigma. All other reagents were analytical grade. Triple distilled water was used throughout. The enzyme, which was obtained in a 3.2 M ammonium sulfate solution, was extensively dialyzed just before use, against 10 mM NaCl. It shows an activity of about 600 units according to the pH-stat egg yolk assay performed with a Radiometer titration system [8–10]. The pyrene-labeled lipids were checked by TLC and the spots visualized with UV illumination through their intrinsic fluorescence. Comparison with standards of dipalmitoylphosphatidylcholine and palmitic acid, revealed with ANS [15], shows similar R_f values.

Liposomes were prepared as previously described [8–10,15–17], by sonication and further annealing above the T_m of the phospholipid, to avoid structural defects in the resulting bilayers [18]. Briefly, a chloroform/methanol (9:1, v/v) solution of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine previously mixed with the appropriate amount of fluorophore-labeled lipid, was dried with nitrogen. Thereafter, to help remove the last traces of retained chloroform, benzene was added and evaporated with nitrogen [15–18]. After addition of the aqueous solution containing the required concentrations of buffer and salts, as indicated in each experiment, the mixture was ultrasonically irradiated in a bath-type sonicator (Cole Parmer Model 8851) for 15 min. During sonication the sample was kept at 51°C, i.e., above the phase transition temperature, by means of a Lauda K-4/RD open circulating bath. The vesicles were annealed at this temperature for 30 min and then kept at room temperature until used (about 1 h). To prepare vesicles containing different proportions of unlabeled products, appropriate aliquots of palmitic acid and lysopalmitoylphosphatidylcholine dissolved in chloroform/methanol (9:1, v/v) were thoroughly mixed with DHPC and DiPyDPC in the same solvent, before drying with nitrogen. The rest of the procedure was as described above. For the experiments using cholate micelles, the fluorescent substrate dissolved in ethanol was dried and subsequently, the aqueous detergent solution was added. The mixture was sonicated for 15 min at 37°C in the above-mentioned bath.

The fluorescence measurements were performed in an Aminco Bowman spectrofluorometer equipped with a thermostated sample chamber, to which a magnetic stirrer was adapted. The sample was continuously stirred. Temperature was measured directly in the cuvette using a microprobe attached to a Digital Thermometer Bat 8 from Bailey Instruments. Fluorescence spectra as well as time-courses of fluorescence changes at fixed wavelengths, were registered using a x-y/x-t 7004B Hewlett Packard recorder.

The hydrolysis of DiPyDPC incorporated into the appropriate matrix was started by quick addition of 10 μl of PLA_2 solution to give a final concentration of

2.75 μg/ml. At time zero, PLA_2 was added and the progress of the enzymatic reaction was monitored by recording the increase of the relative fluorescence intensity at 393 nm with excitation at 343 nm [14]. The relative fluorescence intensity increments were calculated with respect to a baseline, registered during 10 min prior to enzyme addition. Calibration of the fluorescence increments was performed for some experiments using mixtures of DiPyDPC and pyrenedecanoic acid according to a theoretical treatment described in Section 3.

The phase transition temperature (T_m) of DHPC liposomes was determined by temperature scans of the ratio of fluorescence intensities of DiPyDPC at 470 nm (I_e) and 393 nm (I_m) with excitation at 343 nm. The scattered light at the same wavelengths, measured in a control experiment with DHPC liposomes in the absence of fluorophore labeled lipid, was subtracted from the fluorescence intensities. No further corrections due to instrumental response at different wavelengths were considered. The T_m was taken at the midpoint of the abrupt change of I_e/I_m which appears in the temperature scans, as described by Sunamoto et al. [13]. Alternatively, the T_m was evaluated from temperature scans of the 90° light scattering at 400 nm of DHPC samples in the absence of DiPyDPC [8,9,16–17]. In both cases, the lipid dispersions were cooled from 60°C to 24°C at an average sweep rate of approx. −0.7°C to −0.8°C/min by circulation of water through the cell compartment. Temperature was sensed with a microprobe (see above) inserted directly into the cuvette without perturbing the lightpath.

Results shown are representative of at least three separate experiments.

3. Results

The fluorescence of DiPyDPC cosonicated with DPPC shows monomer and excimer components [13]. Fig. 1 shows that, when this dipyrene-labeled phospholipid is incorporated into inert DHPC liposomes upon excitation at 343 nm, it also presents monomer (I_m) and excimer (I_e) fluorescence at 393 and 470 nm, respectively. In this experiment the molar ratio of DiPyDPC to DHPC is 1:500. If this proportion of dipyrene-labeled lecithin to unlabeled phospholipid is increased or decreased by 50%, the I_e to I_m relation does not vary. This is in agreement with previous results of Zachariasse et al. [19] indicating that at such a low proportion of a dipyrene-labeled molecule to unlabeled matrix, excimer formation is a monomolecular process, independent of concentration. Thus, fluorescence at 470 nm can be attributed to intramolecular excimer formation, i.e., to the excimer formed by two pyrenes linked to the same molecule. The relative

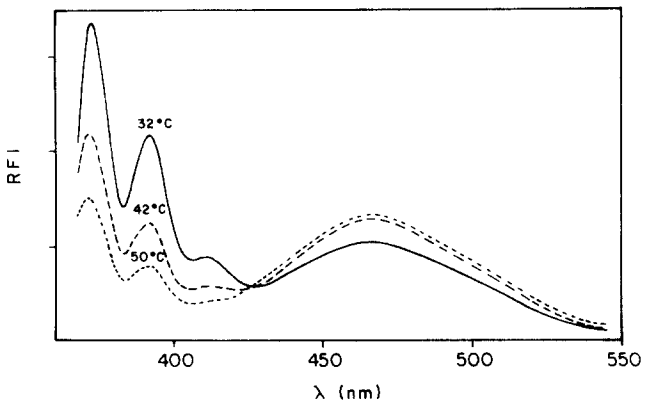

Fig. 1. Emission spectra (uncorrected) of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes, at different temperatures, as indicated. Excitation wavelength: 343 nm. Liposomes were prepared in 1 mM NaCl, 5 mM Tris-HCl (pH 8.0).

intensities of I_e and I_m vary with temperature and, especially, with the physical state of liposomes (Fig. 1). As described elsewhere [13], by measuring the I_e/I_m quotient as a function of temperature, a phase transition can be detected at the midpoint of the abrupt change of that ratio. In this way the T_m of DHPC liposomes can be estimated as equal to 43°C (Fig. 2), similar to values reported in the literature [20]. It is worth mentioning that this sensitivity of the I_e/I_m ratio of DiPyDPC to the gel–liquid-crystalline phase transition temperature of bilayers is also observed with other phospholipids such as dipalmitoylphosphatidylcholine or dimyristoylphoshatidylcholine [13,22], giving T_m values comparable to those determined by other methods such as differential scanning calorimetry or light scattering.

A fluorometric continuous assay for the detection of PLA_2 activity on dipyrenedibutanoylphosphatidylcholine micelles was proposed by Hendrickson and Rauk [14], who observed that hydrolysis produces dramatic changes in the spectra of this substrate due to disappearance of the excimer fluorescence and to appearance of monomer emission. Using this approach, we

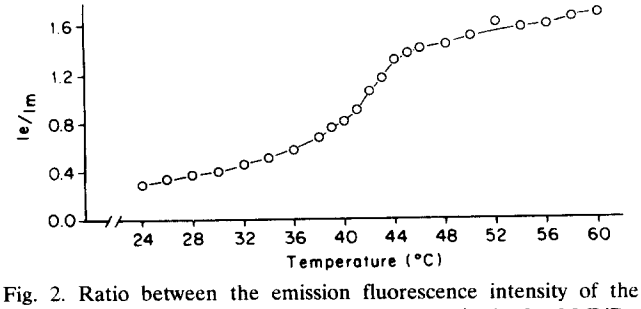

Fig. 2. Ratio between the emission fluorescence intensity of the excimer (I_e: 470 nm) and monomer (I_m: 393 nm) of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes, as a function of temperature. Conditions similar to those of Fig. 1.

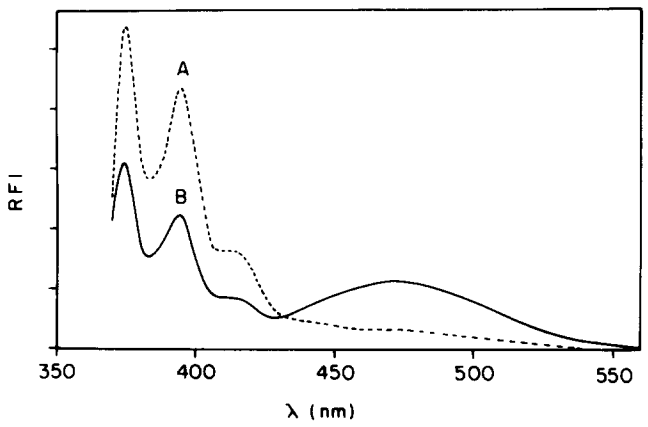

Fig. 3. Emission spectra (uncorrected) of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes, before (B: ———) and after (A: — — —) hydrolysis by PLA_2 (2.7 μg/ml) during 60 min at 34°C. Excitation wavelength: 343 nm. Liposomes were prepared in 1 mM NaCl, 5 mM CaCl, 5 mM Tris-HCl (pH 8.0). Addition of PLA_2 in the absence of free calcium ions (EDTA 7 mM) does not modify spectrum B.

have found that after hydrolysis catalyzed by PLA_2 during 60 min, the emission spectrum of the sample containing DiPyDPC incorporated into DHPC vesicles presents a fluorescence increment at 393 nm and a decrement at 470 nm (Fig. 3). No spectral change upon addition of the enzyme is found in the absence of free calcium ions, i.e., if Ca^{2+}, which is a cofactor of the enzyme, is complexed by EDTA. As reported by Hendrickson and Rauk [14], the progress of the enzymatic reaction can be best monitored by recording the increase in monomer fluorescence. Thus, in the present case, we chose the 393 nm increment to follow hydrolysis.

We have shown that the ratio between the excimer and monomer fluorescence of DiPyDPC is extremely dependent on the physical state of the matrix into which the fluorophore is inserted (Figs. 1 and 2) and that it also changes when the pyrene-labeled phospholipid is hydrolyzed (Fig. 3). Thus, if the hydrolysis by PLA_2 is to be followed by the fluorescence increment at 393 nm, accuracy of the measurement should depend on the constancy of the physical state of liposomes. If the physical state is not constant, the fluorescence at 393 nm would be affected not only by the cleavage of the substrate molecule but also by the variation of its environment. Thus, from a strict point of view this precludes the fluorometric detection of PLA_2 action on DiPyDPC when this molecule is incorporated into liposomes of unlabeled authentic substrate. In such a system, it would not be easy to discern the changes in monomer fluorescence due to cleavage of the dipyrene-labeled substrate from those originated by the modifications of the macroscopical physical state of a matrix that can also be hydrolyzed. In the experiments reported here, we fix the physical ambient of the fluorescent substrate using a liposomal matrix of a non-hydrolyzable lipid, the properties of which must remain constant, despite of the hydrolysis of the very low amount of fluorescent substrate used with respect to the inert lipid ($\leq 1:500$). This allows us to study hydrolysis in virtual absence of product-induced perturbations.

Fig. 4 shows the hydrolysis of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes at 34°C (gel phase) or 45°C (liquid crystalline state) as measured by the time-course of the relative fluorescence intensity increment at 393 nm. The assay mixture con-

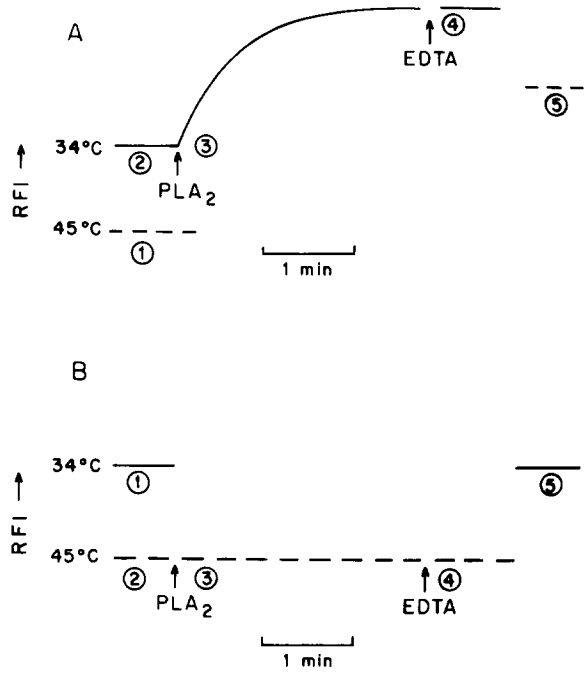

Fig. 4. Hydrolysis by PLA_2 (2.7 μg/ml) of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes at 34°C (gel phase) and 45°C (liquid crystalline state), as detected by the relative fluorescence intensity (RFI) increment at 393 nm. Liposomes were prepared in 5 mM $CaCl_2$, 1 mM NaCl, 60 mM Tris-HCl (pH 8.0). Tracings represent recordings of RFI vs. time at 34°C: (———) or 45°C: (— — —). (A) Fluorescence baselines (in the absence of enzyme) at 45°C (1) and 34°C (2); hydrolysis at 34°C after addition of PLA_2 (3); termination of the reaction by addition of 7 mM EDTA and recording of the final fluorescence level at 34°C (4) and 45°C (5). The final fluorescence increment due to hydrolysis at 34°C can be measured at 34°C and 45°C as the difference between the fluorescence in (4) and the basal fluorescence in (2) and between the fluorescence in (5) and the baseline in (1), respectively. The ratio between the increments measured at 34°C and 45°C is 0.97. (B) Fluorescence baselines (in the absence of enzyme) at 34°C (1) and 45°C (2); hydrolysis at 45°C, after addition of PLA_2 (3); inactivation of the enzyme by addition of 7 mM EDTA and recording of the final fluorescence level at 45°C (4) and 34°C (5). The final fluorescence increment due to hydrolysis at 45°C, can be measured at 45°C and 34°C as the difference between the fluorescence in (4) and the basal fluorescence in (2) and between the fluorescence in (5) and the baseline in (1), respectively. The final increments measured at both temperatures are close to zero.

tains the cofactor Ca^{2+}. At 34°C (Fig. 4A) there is immediate hydrolysis as judged by the increase of the fluorescence upon addition of PLA_2, whereas at 45°C (Fig. 4B), the fluorescence change is almost negligible. The distinct profiles of the 393 nm fluorescence tracings obtained after addition of PLA_2 at 34°C and 45°C seem to be due to different susceptibility to hydrolysis of the pyrene-substituted substrate when inserted into gel and liquid-crystalline liposomes. Another possibility is that hydrolysis proceeds similarly in the two cases but is not reflected in a fluorescence increment at 45°C in the liquid crystalline state of the matrix. This point is clarified by the control experiments self-contained in Fig. 4. The increment of fluorescence for the hydrolysis of DiPyDPC incorporated into gel phase liposomes of DHPC at 34°C (Fig. 4A) is registered, and after a certain time, the reaction is stopped with EDTA. When the sample temperature is shifted to 45°C, the fluorescence increment with respect to a baseline previously recorded at this temperature is nearly similar to that obtained at 34°C. The ratio between the final increments measured at 34 and 45°C is 0.97. In the same way (Fig. 4B), when the fluorescent substrate is submitted to hydrolysis in liquid crystalline DHPC liposomes at 45°C, the insignificant increment of fluorescence obtained in that state remains unchanged if, after stopping the reaction with EDTA, temperature is shifted to 34°C and comparison is done with a fluorescence baseline, previously recorded at 34°C. Thus, the fluorescence increment obtained by hydrolysis at 34°C does not vary if the measurement of the hydrolysis products generated at this temperature is performed at 45°C (Fig. 4A). Similarly, the virtual absence of fluorescence increment that seems to indicate null or negligible hydrolysis at 45°C (Fig. 4B) persists if, after inhibiting any enzymatic activity by addition of EDTA, the sample fluorescence variation is measured at 34°C. These controls refute the possibility of an artifact due to poor fluorescence increment but good hydrolytic activity in the liquid crystalline state at 45°C.

Although it is known that PLA_2 is extremely resistant to high temperatures [21,23], another control was performed in order to verify that the negligible enzymatic activity obtained at 45°C for the substrate inserted into liquid crystalline liposomes is not due to inactivation of the enzyme at this temperature. Fig. 5 shows that, when the fluorescent substrate is incorporated into cholate micelles which do not suffer a phase change, increase of the temperature to 45°C results in higher hydrolytic activity as judged from the fluorescence increment.

In order to quantify the hydrolysis by PLA_2 detected through the fluorometric procedure, a calibration method is needed. Addition of aliquots of pyrene-fatty acid to the substrate mixture in the absence of enzyme, and corroboration of the linearity of the

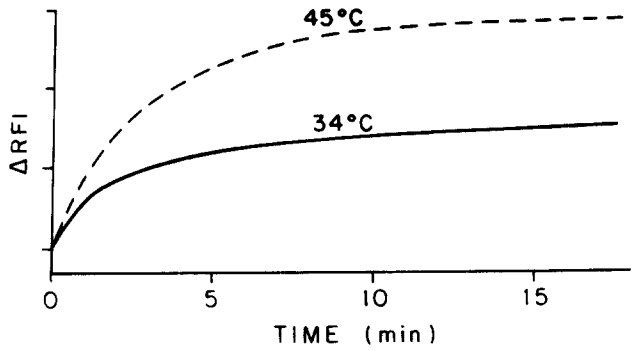

Fig. 5. Hydrolysis by PLA_2 (0.27 μg/ml) of 1.2 μM DiPyDPC incorporated into 6 mM cholate micelles, at 34°C (———) and 45°C (— — —), as followed by the time-course of the relative fluorescence intensity increment at 393 nm. Micelles were prepared in 5 mM $CaCl_2$, 1 mM NaCl, 5 mM Tris-HCl (pH 8.0).

monomer fluorescence increment in the concentration range employed, are the usual procedures [24–26]. Due to close similarity of the fluorescence spectra of both pyrene-labeled products, fatty acid and lysolecithin [14], this calibration protocol is good enough as long as the unhydrolyzed fluorescent substrate does not show any monomer emission. This is the case for aqueous, pure micellar dispersion of dipyrenedibutanoylphosphatidylcholine which gives only excimer emission [14]. However, the fluorescence of DiPyDPC inserted in a lamellar phase exhibits both monomer and excimer components in different ratios, depending on the physical state of the matrix [13,22], as shown in Figs. 1 and 2. Thus, a formalization of the calibration procedure is needed to take into account the contributions to the 393 nm fluorescence change of the released pyrene-labeled products as well as the stoichiometric reduction of dipyrenelecithin concentration through the hydrolytic process, as described in the following.

At a certain time t, let X_L be the ratio between the molar concentration of unhydrolyzed DiPyDPC (C_t) and the initial molar concentration of this substrate (C_i); let X_S be the ratio between the molar concentration of pyrene-lysoderivative ($C_i - C_t$) and the maximal molar concentration of pyrene-lysoderivative that can be generated by total hydrolysis of the *sn*-2 ester of DiPyDPC; let X_A be the ratio between the molar concentration of pyrenedecanoic acid ($C_i - C_t$) and the maximal molar concentration of the acid that can be reached by total hydrolysis of the *sn*-2 ester of DiPyDPC. Then, the 393 nm fluorescence change (ΔF) due to generation of products (pyrene-fatty acid and pyrenelysophosphatidylcholine) and to the concomitant disappearance of dipyrenelecithin by the hydrolytic action of PLA_2 on DiPyDPC, is given by the following equation:

$$\Delta F = [F_L X_L + F_S(1 - X_L) + F_A(1 - X_L)] - F_L \quad (1)$$

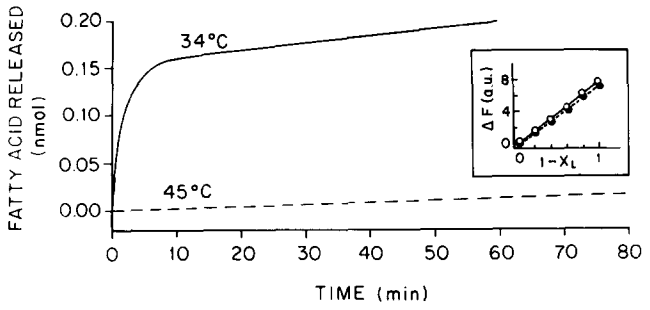

Fig. 6. Calibrated time-courses of hydrolysis by PLA_2 (2.7 μg/ml) of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes in the gel phase at 34°C (———) and in the liquid crystalline state at 45°C (— — —). Hydrolysis was followed by the time-course of the relative fluorescence intensity change at 393 nm. Liposomes were prepared in 5 mM $CaCl_2$, 1 mM NaCl, 60 mM Tris-HCl (pH 8.0). Inset: Calibration curves (see Eq. (2)) obtained from mixtures of DiPyDPC and pyrenedecanoic acid incorporated into 0.6 mM DHPC liposomes at 34°C (o———o) and 45°C (•— — —•). The equivalence between a certain fluorescence increment (in arbitrary units, a.u.) due to hydrolysis and the amount of released products was obtained from interpolation in these curves.

where F_L is the fluorescence intensity of DiPyDPC for $X_L = 1$; F_S, the fluorescence intensity of the pyrene-lysoderivative when $X_S = 1$, and F_A, the fluorescence intensity of pyrenedecanoic acid when $X_A = 1$. By assuming that $F_S = F_A$, Eq. (1) is reduced to:

$$\Delta F = [F_L X_L + 2F_A(1 - X_L)] - F_L \quad (2)$$

Calibration curves were constructed by plotting the fluorescence of samples containing variable amounts of DiPyDPC and pyrenedecanoic acid, inserted into the inert DHPC liposomal matrix, as a function of $(1 - X_L)$, according to Eq. (2). Separate calibrations were done at 34°C and 45°C. The experimental calibration points obey the linear relationship predicted by this equation (see inset to Fig. 6). A linear dependence is also found when the calibration curves are constructed using an inert matrix of DHPC containing different molar fractions of externally added unlabeled products, such as palmitic acid and lysopalmitoylphospatidylcholine. These additional calibrations (not shown) are required to quantify experiments on the influence of products on the initial rate of hydrolysis, which will be shown in Fig. 7.

Fig. 6 shows experiments similar to those of Fig. 4 but followed for longer periods and calibrated according to Eq. (2). It can be observed that DiPyDPC is immediately attacked by PLA_2 when inserted into gel phase DHPC liposomes but very slowly hydrolyzed in the liquid crystalline state of the matrix. The profiles of the calibrated reaction progress curves are similar to those of Fig. 4, indicating that the conclusions drawn by direct comparison of the 393 nm fluorescence increments obtained at 34 and 45°C, as a means of judging the PLA_2 hydrolytic activity, are essentially correct. It should be noted that the extremely low activity of PLA_2 on DiPyDPC in liquid crystalline liposomes was undetectable in the experiment of Fig. 4 due to the short period of time (less than 3 min) during which the reaction was followed. Another important conclusion that can be drawn from the experiments in Fig. 6 is that even after a long incubation with the enzyme (80 min), there is no activation of the slow hydrolysis observed in liquid crystalline state at 45°C. This could be due to the lack of accummulation of significant concentrations of products, since our experimental system contains only traces of the substrate.

In order to investigate the effect of significant concentrations of representative products of PLA_2 hydrolysis in our assay with the fluorescent substrate, we performed the experiments of Fig. 7. This figure shows the initial rate of hydrolysis by PLA_2 of DiPyDPC incorporated in a 1:1000 molecular proportion, into DHPC liposomes containing variable mole fractions of the unlabelled products, palmitic acid plus lysopalmitoylphosphatidylcholine (1:1). It is apparent that in both the gel and the liquid crystalline state, there is an activating effect which results in similar rates of hydrolysis above 8 mol% added products. However, it has to be emphasized that, as shown in Figs. 6 and 7, even in the absence of products there is significant and immediate activity on DiPyDPC incorporated into gel phase vesicles but extremely low hydrolytic activity in the liquid crystalline state.

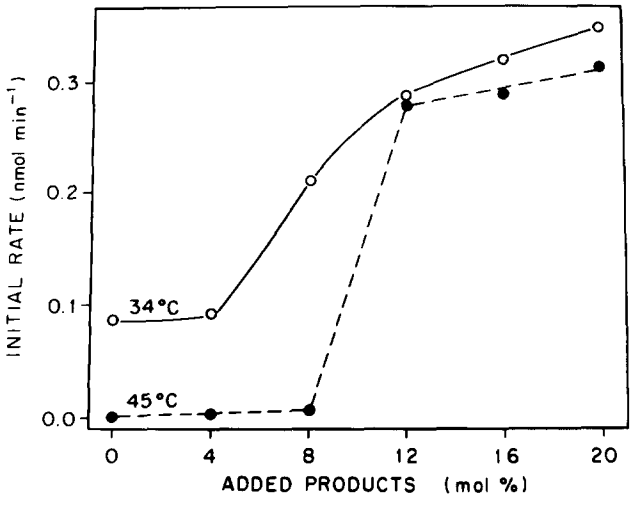

Fig. 7. Initial rates of hydrolysis by PLA_2 (2.7 μg/ml) of 0.6 μM DiPyDPC inserted into 0.6 mM DHPC liposomes containing variable molar percentages of the unlabelled products, palmitic acid plus lysopalmitoylphosphatidylcholine (1:1). Liposomes were in the gel (o———o) or liquid-crystalline phase (•— — —•) at 34°C or 45°C, respectively. The fluorescence increments were interpolated in calibration curves obtained from mixtures of DiPyDPC and pyrenedecanoic acid (see Eq. (2)), incorporated into 0.6 mM DHPC liposomes containing the corresponding molar percentages of palmitic acid and lysopalmitoylphosphatidylcholine.

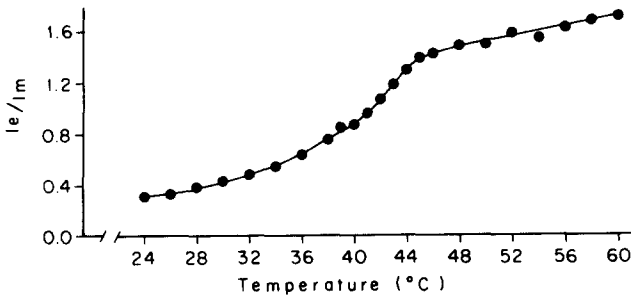

Fig. 8. Ratio between the emission fluorescence intensity of the excimer (I_e: 470 nm) and the monomer (I_m: 393 nm) of 1.2 μM DiPyDPC incorporated into 0.6 mM DHPC liposomes containing 20 mol% unlabeled products (palmitic acid plus lysopalmitoylphosphatidylcholine, 1:1). Other conditions are as in Figs. 1 and 2.

A control experiment was necessary to determine the effect of the incorporation of unlabeled products in DHPC liposomes, on the temperature dependence of the I_e/I_m ratio of DiPyDPC. Although there are reports in the literature on the influence of fatty acids and lysolecithin on the thermotropic behavior of dialkyl- and diacylphospatidylcholine lamellae [27,28], none of the available data corresponds precisely to the experimental conditions of the present work, regarding salts employed, buffer composition and pH value of the lipid dispersion. Aside from providing information concerning the phase transition temperature, the I_e/I_m values obtained in the presence of products may help in the interpretation of hydrolysis results in terms of substrate conformation, which is presented in Section 4. The control experiment is depicted in Fig. 8. Comparison with Fig. 2, shows that the temperature dependence of I_e/I_m is not significantly affected by incorporation of products. Although the change in the I_e/I_m ratio is not as abrupt as the observed in Fig. 2 for the phase transition in the absence of products, it is clear that 34°C and 45°C are still temperatures corresponding to the gel and liquid-crystalline states, respectively. Possibly, the broader phase transition observed could be related to the phase separation phenomenon detected by differential scanning calorimetry by Jain and De Haas [28] for DPPC/palmitic acid/lysolecithin ternary lamellar phases. Perhaps this phase separation cannot be resolved with the DiPyDPC probe, but results in a wider transition than that of pure DHPC. It is also apparent that the I_e/I_m values are very similar in the absence or presence of products both in the gel and liquid-crystalline phases.

4. Discussion

The experimental model presented in this article has been designed for the study of the hydrolysis by PLA_2 of a fluorescent substrate inserted in lamellar phases, in virtual absence of product-induced perturbations. Attempts to minimize the influence of the hydrolysis products on the liposome structure, have been made in an NMR study of Bhamidipaty et al. [29]. However, they used between 10 and 25 mol% of substrate, with respect to the inert lipid matrix, a proportion far from negligible. In contrast, in the current work, due to the sensitivity of the fluorometric detection of hydrolysis, the concentration of the substrate inserted into the inert matrix can be reduced to trace levels. Thus, it can be considered that the substrate is present at 'infinite' interfacial dilution, so that the generated products will be also 'infinitely' dilute. In this way, products will not affect the liposome physical properties since a significant macroscopic concentration will never build up. The substrate is incorporated into a bilayer that determines its phase state (Fig. 2), consisting of an inert phospholipid, the diether linked DHPC, which bears a close structural relationship to the hydrolyzable diester analogue, DPPC [20,29].

In this system, there is immediate activity of PLA_2 when the liposomal matrix is in the gel phase but poor hydrolysis (Figs. 4, 6), when it is in the liquid crystalline state. The conformation of the substrate might not be the same when inserted into a lipid matrix in the gel or liquid crystalline phase, and this could lead to different susceptibilities to hydrolysis. Since the unhydrolyzed dipyrene-labeled phospholipid is by itself an indicator of the phase state of its matrix through the I_e/I_m ratio, it could be interesting to analyze whether the I_e/I_m values give some information about the possible relation between substrate conformation and susceptibility to hydrolysis.

Sunamoto et al. [13] were the first to report that when DiPyDPC is cosonicated with dipalmitoylphosphatidylcholine, there is a thermotropically reversible increase of I_e/I_m in passing from the gel to the liquid-crystalline state. They suggested that a temperature-dependent equilibrium associated with a conformational change of the liposomal bilayer can be followed through this ratio. Sensitivity of DiPyDPC I_e/I_m ratio to the phase transition of other phospholipids has also been reported [22].

At the concentrations used in the current investigation, DiPyDPC excimer can be expected to be only of the intramolecular type. Formation of intramolecular excimers is related to the motional freedom of the two pyrene moieties. This motion should be influenced by the configuration of the acyl chains to which the pyrenes are linked, and by the fluidity properties of the environment [19,22,30].

The possible contribution of the conformation of the dipyrene-labeled molecule to the excimer formation rate, and its relation with hydrolysis by PLA_2, have been considered by Thuren et al. in studies of monolayers and micellar systems [24,31]. These authors have

proposed that for a dipyrenelecithin with saturated acyl chains of equal length, a change from the kinked to the extended conformation would result in a marked increase of the I_e/I_m quotient. In the kinked conformation, the glycerol backbone and the *sn*-1 chain are almost parallel to the bilayer normal. The *sn*-2 chain begins perpendicularly to the glycerol moiety but bends off after the second carbon, so that it becomes parallel to the *sn*-1 acyl chain, but showing a vertical displacement equivalent to 3 methylene units. Thus, the *sn*-1 chain penetrates deeper into the bilayer than the *sn*-2 chain. It is worth mentioning that this is the conformation proposed for phosphatidylcholine crystals from X-ray diffraction studies [32] and the one that best fits into the hydrophobic catalytic pocket of PLA_2 [3,33]. As a consequence of the vertical displacement of chains, pyrenes attached to the acyl terminal carbons will be out of step, resulting in poor efficiency of intramolecular excimer formation and in low I_e/I_m ratios. In contrast, in the so-called extended conformation, glycerol lies parallel to the interface, with both the *sn*-1 and *sn*-2 chains extended and aligned, such that pyrenes are not spatially displaced, formation of intramolecular excimers is favored and higher I_e/I_m values are detected. Following this line of interpretation, it is possible that the abrupt increase in I_e/I_m obtained in the gel to liquid-crystalline phase transition of the DHPC matrix reflects in part changes in the preferred conformation of DiPyDPC, from kinked to extended. In that case, the hydrolysis of DiPyDPC inserted in the gel phase matrix and the poor hydrolysis in liquid crystalline state, in the absence of products, could be readily explained in view of the proposed higher susceptibility to PLA_2 hydrolysis of the substrate in the kinked conformation as compared to the extended state [24,31]. This interpretation disregards local lateral diffusion effects on the excimer formation rates. However, short-range lateral diffusion coefficients as determined by analysis of intermolecular excimer formation by monopyrene-didecanoylphosphatidylcholine in dimyristoylphosphatidylcholine liposomes, according to the 'milling crowd' model, show only a smooth increase with temperature and no discontinuities in the gel to liquid crystalline transition of the matrix [34]. In fitting the model, the optimal probability factor PE for the intermolecular excimer formation is constant and equal to 0.25 throughout the gel and liquid-crystalline states. In contrast, when the experimental parameters obtained with the monopyrenyl probe were used to analyze data of intramolecular excimer formation by DiPyDPC, incorporated in liposomes of the same phospholipid, the estimated probability factor PE decreased from 0.26 to 0.15, in going from the liquid-crystalline to the gel phase [22]. This change in the probability of intramolecular excimer formation seems compatible with the interpretation discussed above of preferred extended and kinked conformations for the substrate in liquid-crystalline and gel phase matrices, respectively.

Recently, it has been reported that in liquid crystalline state, the vertical displacement of saturated phosphatidylcholine acyl-chains is equivalent to only 0.84 methylene units, such that the glycerol backbone is only moderately tilted with respect to the bilayer [35]. This is similar to rotamer B_γ, one of the four minimum-energy possible conformations of glycerophospholipids proposed by Hauser et al. [36]. Such orientation of the glycerol moiety nearly parallel to the interface is quite different from the perpendicular orientation observed previously for phosphatidylcholine crystals [32] and considered in all X-ray diffraction models of the interaction of phospholipids with PLA_2 as an absolute requirement for the substrate to fit into the catalytic site of enzyme [3,33]. This would make the substrate in the liquid crystalline state less susceptible to hydrolysis by the enzyme.

In view of the above discussed evidence, our results in the absence of products seem to indicate that the physical state of the lipid matrix may determine a preferred conformation of the dipyrene-labeled substrate, which is reflected both in the I_e/I_m ratio and in the susceptibility to hydrolysis by PLA_2. The substrate in the gel phase characterized by the low I_e/I_m ratio, seems to adopt the preferred kinked conformation that satisfies the steric requirements of PLA_2 catalytic site. Thus, it is more susceptible to hydrolysis than in the extended conformation of the liquid crystalline state which shows the high I_e/I_m quotient. A preferential binding of PLA_2 to lipid bilayers in the gel phase has been reported by Bell and Biltonen [37]. They have found that the intrinsic fluorescence of the *Agkistrodon piscivorus piscivorus* PLA_2 is increased by addition of DPPC or DHPC liposomes, the increment induced by gel phase vesicles being larger than that obtained with the liquid crystalline vesicles. This preferential interaction has been related to the phenomenon of immediate activation that takes place in the presence of sonicated gel phase liposomes [37]. Although it is very tempting to give a similar interpretation to our results with the porcine PLA_2, there is no evidence indicating better interaction of the pancreatic enzyme with gel phase liposomes than with liquid crystalline vesicles. In fact, Jain et al. [38] did not find any interaction of porcine pancreatic PLA_2 with ditetradecylgycerophosphocholine vesicles in the absence of products, as judged by the lack of fluorescence increment obtained, regardless of the phase state of liposomes [38]. A different behavior of the porcine PLA_2 with respect to venom enzymes has also been observed in studies of the change in the intrinsic fluorescence during hydrolysis [39]. It has been found that, in contrast to the behavior of the venom enzymes, the pan-

creatic PLA_2 does not show a change in fluorescence upon activation, possibly because of the position of its single tryptophan residue in the amino acid sequence [39]. From an analysis of the fluorescence of four different enzymes, Bell et al. suggest that while the increase of the intrinsic PLA_2 fluorescence in the presence of liposomes indicates interaction of the protein with the bilayer, the fraction of adsorbed enzyme responsible for the fluorescence increment does not necessarily correspond to the 'activated enzyme' [39]. Thus, it appears that there is no correlation between fluorescence increment and activation.

Several models on PLA_2 mechanism of action emphasize the role of the phospholipid conformation at the lipid-water interface, in its susceptibility to hydrolysis [3,4]. However, the very well known activating effect of products has to be included in any explanation of hydrolysis catalyzed by this enzyme [6,12]. Although in the absence of products, there is quite a different behavior of the hydrolysis of DiPyDPC when inserted in gel or liquid-crystalline sonicated liposomes, we have also found that incorporation of lysolecithin and fatty acid activates the hydrolysis of DiPyDPC in both phases to comparable rate levels (Fig. 7). However, the values of the I_e/I_m ratio in the gel or liquid-crystalline vesicles are not significantly modified by incorporation of unlabelled products (Figs. 2, 8). Thus, the activating effect of products cannot be explained in terms of any conformational change induced in the substrate, as detected by the I_e/I_m ratio. It appears that the conformation of the substrate is especially important in determining different susceptibilities to hydrolysis in the absence of products.

It is known that products – especially the negatively charged fatty acids – give rise to a dramatic increase in the binding constant of the enzyme to the lipid–water interface of sonicated zwitterionic phospholipids [40]. It is possible that through this increase in affinity that would favor a change in PLA_2 mechanism of action from the hopping to the scooting mode [40], the difference in susceptibility to hydrolysis of the substrate in gel and liquid crystalline states could be overcome. In this regard, it is worth mentioning that Jain and Berg [40] have reported that progress of hydrolysis in the scooting mode of aggregated negatively charged substrates does not show any latency period, irrespective of the phase state of the lamellae.

As mentioned in the Introduction section, previous work from this and other laboratories [2,7,8] showed that PLA_2 acting on gel state, pure substrate sonicated liposomes made of DPPC presents immediate activity. In contrast, in the liquid crystalline phase, the time-course of hydrolysis is characterized by a long latency period after which triggering of the high activity phase occurs [2,7,8]. Among other explanations, latency periods in the hydrolysis of phosphatidylcholine by PLA_2 have been interpreted in terms of an induction time during which a critical mole fraction of the reaction products is accumulated [6]. This critical concentration of products induces phase segregation in the lamellar substrate and favors binding of the enzyme to the interface [12,41]. The critical concentration of products required to trigger the high-rate phase of hydrolysis has been estimated to be in the range from 5 to 10 mol% [6,10,12]. Obviously, in our study with DHPC liposomes doped with traces of the substrate DiPyDPC, such 'critical' concentrations will never be reached and therefore, the slow hydrolysis observed in liquid crystalline liposomes will continue indefinitely. This is precisely what we observe in Fig. 6 in which the time-course of hydrolysis was studied for 80 min but no activation was detected.

On the basis of our present findings, a more complete interpretation of the time-course of PLA_2 action on sonicated liposomes made of pure DPPC substrate [8], in which the macroscopic composition does change during hydrolysis, can be attempted. The immediate activity observed in sonicated, gel phase liposomes of DPPC could be explained in terms of an early step of hydrolysis due to the favorable substrate conformation. This early step does not require activation by products, but generates a fraction of them which should be enough to ensure sustained product-activation of the hydrolytic process, so that no lag phase is observed. In contrast, in liquid-crystalline DPPC liposomes, since the conformation of the substrate does not favor its own hydrolysis, the initial rate of product formation is extremely low. Therefore, a relatively long period of time – the lag period – is needed for the accumulation of the critical fraction of products leading to the apparent activation of hydrolysis.

The procedure presented here for the study of the hydrolysis of lamellar phases by PLA_2 in virtual absence of product-induced perturbations allows to follow the enzymatic action at constant interfacial macroscopic properties of the liposome matrix. This approach can be used to dissect the participation in the hydrolysis time-courses of different parameters such as phase state, surface charge density or interfacial calcium concentration which, as shown previously [8–10], are correlated with the interfacial activation of this enzyme.

Although the present study has been conducted on sonicated liposomes, it could also be applied in the future to investigate hydrolysis of large unilamellar vesicles. It is known that the temperature dependence of the hydrolysis of DPPC large vesicles by PLA_2 is different from that of the smaller liposomes [42,43]. While in DPPC sonicated liposomes the lag phase is seen only above T_m [8], with large vesicles a latency prior to rapid hydrolysis is observed at all temperatures [42]. The duration of the latency is shortest near the

T_m. It is also striking that hydrolysis of DPPC large vesicles, below or above the T_m, is not activated by the addition of external products [42]. Only in the phase transition temperature range can the latency length be reduced by addition of products.

In the absence of experiments with large vesicles similar to those performed in the present article with sonicated liposomes, only speculative explanations of the dissimilarities observed between the hydrolysis of both types of structures can be attempted. It has been reported by Lichtenberg et al. [42] that incubation of PLA_2 with large DPPC liposomes in the gel phase followed by a temperature upshift to the T_m range results in immediate and large hydrolysis. However, if incubation is done with vesicles in the liquid crystalline state and subsequently the temperature is downshifted to the T_m range, there is no increase in activity. It is necessary to add products to the liquid-crystalline liposomes in order to see rapid hydrolysis after the temperature is changed to the T_m value. Thus, it appears that during the incubation in the absence of added products, the hydrolysis, albeit small, is larger in the gel phase vesicles than in the liquid-crystalline ones suggesting that also in large unilamellar liposomes the substrate conformation may determine the initial susceptibility to hydrolysis.

As observed in Fig. 7, the initial activity of PLA_2 on DiPyDPC in gel phase sonicated vesicles can be amplified up to four times by external addition of products. It seems that in large vesicles this amplification does not occur in the gel state but is still operative near the phase transition temperature. In support of this are the findings that addition of products does not affect the lag period in the gel or liquid-crystalline states but reduces the latency length in the T_m range. This differential effect could be due to a much lower tendency of the DPPC/lysolecithin/fatty acid ternary system to segregate products at temperatures corresponding to gel or liquid-crystalline states, than in the phase transition range. Phase separation of products, especially of the fatty acids, is considered an essential requirement for the enzyme binding to phosphatidylcholine sonicated liposomes [12]. Detailed studies of the effect of fatty acids and lysolecithin on large phosphatidylcholine vesicles are needed to determine whether lateral segregation of products can account for some of the peculiarities shown by the temperature dependence of the hydrolysis of these vesicles by PLA_2.

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