Critical Micelle Concentrations and Stirring Are Rate-Limiting in the Loss of Lipid Mass during Membrane Degradation by Phospholipase A_2

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ABSTRACT In phospholipid membranes attacked by phospholipase A_2 (PLA_2), accumulation of degradation products influences the binding affinity as well as the catalytic activity of PLA_2. Such accumulation in its turn depends on the rate of membrane degradation and the efflux of degradation products from the membrane, the latter being influenced by the stirring conditions in the system. This complicated process was investigated with a new ellipsometric technique for in situ measurement of membrane mass in a well-defined flow system. Planar phospholipid bilayers were formed on rotating silicon discs in buffer solution. After the addition of 0.05–100 ng/ml of PLA_2 (from *Naja mocambique mocambique*) to the buffer, mass desorption could be measured with a precision of 3–5 ng/cm^2, that is, about 1% of the surface mass of a single bilayer. Using radiolabeled phospholipids and thin-layer chromatography, it was verified that only the degradation products desorb from the membrane, which was confirmed by the desorption of mixtures of phospholipids, lysophospholipids, and fatty acids. The rotating disc allows the exact calculation of the mass transfer constant for transport-limited exchange of lipid between fluid and disc surface, as a function of rotation rate. By using the mass transfer constant, the critical micelle concentrations, and the mole fractions of products, desorption kinetics could be fully described. The amount of degraded phospholipid could be continuously monitored as the sum of the product mass still present in the membrane, as inferred from the desorption rate, and the mass already lost from the surface. It is concluded that ellipsometry is a suitable tool for studying the effects of PLA_2 on membranes.

INTRODUCTION

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of phospholipids at the sn-2 position, yielding fatty acids and lysophospholipids. Natural substrates of PLA_2 are usually present in the form of phospholipid membranes, to which PLA_2 can adsorb to become enzymatically active. Whether this process concerns the degradation of cell membranes by intracellular PLA_2 activity, as observed in ischemic muscle injury (Van Bilsen et al., 1989; Van der Vusse et al., 1992), or by external attack of cells by added or circulating PLA_2 (Gul and Smith, 1974); Speijer et al., unpublished results), efflux of the degradation products from the membrane will be hampered by the accumulation of lysophospholipids and fatty acids in the fluid phase in contact with the membrane. Such accumulation will also depend on the transport conditions in the fluid phase, that is, on diffusion and convection (stirring). The concentration of degradation products in the membrane in its turn influences the binding affinity of PLA_2 (Apitz-Castro et al., 1982; Jain et al., 1982) and, by product inhibition, the catalytic activity of PLA_2 (Smith et al., 1972; Kupferberg et al., 1981). As a result, the rate of phospholipid hydrolysis by PLA_2 will generally become a complicated function of intrinsic properties, such as binding affinity and catalytic efficiency, as well as of the transport conditions in the system.

Ellipsometry is an optical technique allowing the sensitive detection of organic substances at liquid-solid interfaces (Azzam and Bashara, 1977). Phospholipid bilayers on reflecting slides can be prepared by the Langmuir-Blodgett technique of dipping the slide into a monolayer-covered film balance (Cuypers et al., 1983; Kop et al., 1984), or by exposure of the slide to a suspension of phospholipid vesicles (Brian and McConnell, 1984; Giesen et al., 1991). The phospholipid mass per unit surface area of such bilayers can be continuously measured in buffer solution, under well-defined flow conditions.

In the present study we investigated the use of ellipsometry for unraveling the kinetics of membrane degradation by snake venom PLA_2. Phospholipid membranes were formed on rotating silicon discs, which form so-called uniformly accessible surfaces, allowing the exact calculation of the rate of mass exchange between the disc surface and the buffer solution (Willems et al., 1993). After the addition of PLA_2 to the buffer, phospholipid mass on the disc was measured in situ, and mass desorption curves could be fully described with a model incorporating the rotation rate of the disc, the diffusion constant, and the critical micelle concentration (CMC) of the lipid products. This model also explains the stability of bilayers consisting of intact phospholipids.

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MATERIALS AND METHODS

Materials

Dioleoyl-phosphatidylcholine (DOPC) and 1-oleoyl-lysophosphatidylcholine (LPC) were obtained from Avanti Polar Lipids (Alabaster, AL).

1-Stearyl-2-arachidonoyl-phosphatidylcholine (SAPC), oleic acid, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Di[1-^{14}C]oleoyl-PC (4.2 GBq/mmol) was from Dupont NEN (Wilmington, DE). 1-Stearoyl-2-[1-^{14}C]arachidonoyl-PC (2.15 GBq/mmol) was from Amersham Laboratories (Amersham, UK). Silicon wafers were obtained from Aurel GmbH (Landsberg, Germany). Thin-layer chromatography (TLC) plates (20 × 20 cm, 0.5 mm silica gel 60) were from Merck (Darmstadt, Germany). All chemicals used were of the highest grade available.

Phospholipase

PLA_2 from the venom of *Naja mocambique mocambique* (isoenzyme with pI = 8.8) was obtained from Sigma. It was solubilized and dialyzed against 5 mM HEPES, 10 mM NaCl, pH 7.4. The concentration was determined from the absorbance at 280 nm, using $E_{1\%} = 23\ cm^{-1}$ (Jain et al., 1991).

Phospholipid vesicles and planar bilayers

Phospholipid concentrations were determined by phosphate analysis (Böttcher et al., 1961). Phospholipid vesicles and planar bilayers on silicon discs were prepared as described before (Giesen et al., 1991). Briefly, small unilamellar vesicles were prepared by sonication of lipid dispersions (1 mM) in HEPES buffer (50 mM, pH 7.4, 100 mM NaCl). Adsorption of these vesicles (usually 20 μM phospholipid) to rotating hydrophilic silicon discs results in the formation of continuous planar bilayers (Giesen et al., 1995).

Ellipsometry

The surface mass Γ of lipid adsorbed on the silicon surface was determined by ellipsometry at 20°C (Cuypers et al., 1983). The bilayer-coated disc was mounted in a cuvette filled with 5 ml of buffer, PLA_2 was added to the cuvette, and changes in Γ were measured directly on the rotating disc (Willems et al., 1993). Under these circumstances, the mass transfer between membrane and solution can be described by $d\Gamma/dt = \Delta(C_{bulk} - C_0)$, where Δ is the mass transfer coefficient, C_{bulk} is the concentration of lipid in bulk solution, and C_0 is the concentration of lipid in the buffer at the membrane surface. The rotating disc has the special feature of uniform accessibility, that is, the value of Δ is the same over the entire disc surface. This implies that total adsorbed or desorbed quantities can simply be calculated by multiplication of changes in Γ by the total surface area of the disc (2 cm^2). The mass transfer coefficient is a function of the diffusion constant D of the exchanged substance, the kinematic viscosity ν of the fluid, and the angular velocity of rotation ω, that is, $\Delta = 0.62\ D^{[2/3]}\nu^{-1/6}\omega^{[1/2]}$ (Willems et al., 1993). With the values of $\nu = 0.01\ cm^2/s$ and $\omega = 78$ rad/s used in the experiments, this equation is reduced to $\Delta = 11.8\ D^{[2/3]}$ cm/s.

Chromatographic analysis of lipids

Radiolabeled lipids adsorbed on the discs, and those released into the buffer due to PLA_2 activity were analyzed by TLC. To this end, the disc was removed from the cuvette, avoiding exposure to air, and lipids remaining on the disc were collected in 4 ml methanol containing unlabeled lipids (25 μg PC, 25 μg LPC, and 25 μg oleic acid per ml). The cuvette buffer was collected and the cuvette walls were washed with 4 ml methanol containing unlabeled lipids. Lipids were extracted according to a modified Folch extraction procedure (Van der Vusse et al., 1980). Fatty acids, PC, and LPC were separated by TLC, using chloroform/methanol/acetic acid/water (95/50/5.5/5.5). The plate was sprayed with rhodamine-6G (0.05% in methanol) and 2,7-dichlorofluorescein (0.04% in 80% methanol). Lipids were visualized under UV light (366 nm) and removed from the plate for liquid scintillation counting (Beckman LS 3801).

Quantitative description of desorption curves

If during desorption of lipid from the membrane C_{bulk} is kept at zero, for instance by constant flushing, the expression for mass transfer is reduced to $d\Gamma/dt = -\Delta C_0$. Assuming that during desorption of a pure lipid component a local equilibrium is maintained at the membrane (Corsel et al., 1986), the value of C_0 will be equal to its CMC, that is, the threshold concentration at which lipid monomers form aggregates. According to theory (Tanford, 1973), C_0 will be lower for a mixture of components, and in that case will be equal to $C_0 = CMC \cdot X$, where X is the mole fraction of the component. If only lysophospholipids (lpc) and fatty acids (fa) desorb from a membrane that also contains intact phospholipid (pl), we have $d\Gamma/dt = -\Delta_{lpc}CMC_{lpc}X_{lpc} - \Delta_{fa}CMC_{fa}X_{fa}$. For products with identical chains we have (Tanford, 1973) $CMC_{lpc} \approx CMC_{fa}$ and $D_{lpc} \approx D_{fa}$, and this expression is reduced to $d\Gamma/dt = -\Delta CMC\ (X_{lpc} + X_{fa}) = -\Delta CMC\ (1 - X_{pl})$. To relate this expression to measurable variables, mole fractions are replaced by weight fractions (W), and because each substrate molecule splits into two product molecules, the ratio of mole fractions $(X_{lpc} + X_{fa})/X_{pl}$ will be twice the ratio of weight fractions $(W_{lpc} + W_{fa})/W_{pl}$, or $(1 - X_{pl})/X_{pl} = 2(1 - W_{pl})/W_{pl}$. This relation can be rewritten as $X_{pl} = W_{pl}/(2 - W_{pl})$. As intact phospholipid molecules do not desorb, we have for all times $\Gamma W_{pl} = (\Gamma W_{pl})_{t=0} = \Gamma_{0pl}$. Substitution of the latter two relations, and the value of Δ, into the expression for $d\Gamma/dt$ yields $d\Gamma/dt = -11.8\ D^{[2/3]}\ CMC\ (2\Gamma - 2\Gamma_{0pl})/(2\Gamma - \Gamma_{0pl})$. Using standard computer software (function solver of Microsoft Excel 5.0; Frontline Systems), the simple numerical approximation $\Gamma(t_i) = \Gamma(t_{i-1}) - 11.8\ D^{[2/3]}\ CMC\ (t_i - t_{i-1})(2\Gamma(t_{i-1}) - 2\Gamma_{0pl})/(2\Gamma(t_{i-1}) - \Gamma_{0pl})$ was fitted to the desorption curves.

RESULTS

Formation of planar lipid bilayers and subsequent degradation by PLA_2

A typical adsorption curve is shown in Fig. 1. Addition of DOPC vesicles (16 μg/ml phospholipid) to the cuvette resulted in rapid adsorption of vesicles, with an initial adsorption rate of 5.6 ng/cm^2/s, and formation of a stable membrane with an adsorbed lipid mass of 0.40–0.43 μg/cm^2. Removal of nonadsorbed vesicles by flushing of the cuvette did not cause mass desorption, showing that the bilayer on the silicon disc was stable.

In the further experiments shown in Fig. 1, supported bilayers on silicon discs were prepared in a separate vessel (Giesen et al., 1995) and then transferred to the ellipsometer cuvette, avoiding exposure of the membrane to air. This procedure prevents adsorption of lipid on the inner cuvette surface. After recording the baseline level of phospholipid mass, membrane degradation was started by the addition of PLA_2. To prevent depletion of PLA_2 from the bulk phase, the cuvette was constantly flushed (5 ml/min) with buffer containing the appropriate PLA_2 concentration. This procedure had the additional advantage that it facilitated desorption of lipids from the membrane by prevention of accumulation of desorbed products in the bulk phase. For increasing PLA_2 concentrations (Fig. 1), the increase in membrane degradation rate became progressively smaller, until a limiting value of about 1.5 ng/cm^2/s was reached for the steepest part of the desorption curve. This aspect is explained below.

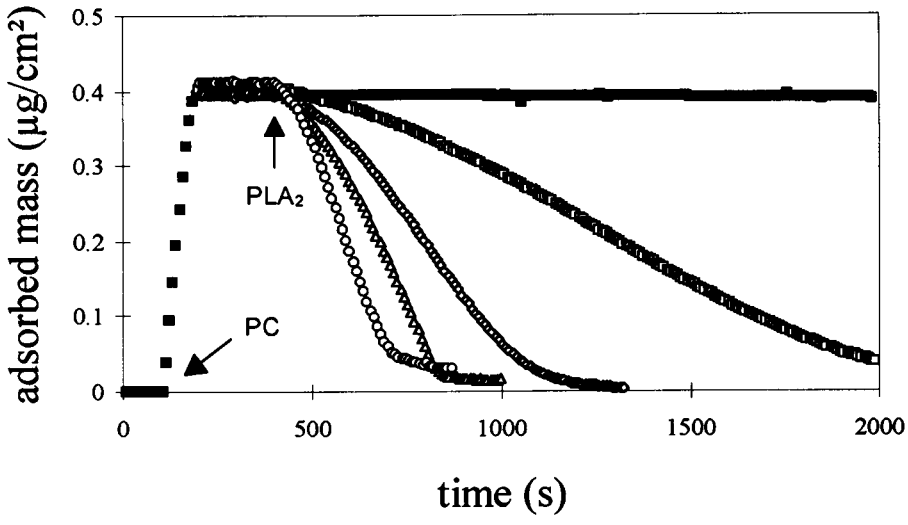

FIGURE 1 Formation of planar PC membranes and subsequent degradation by PLA_2. Planar membranes were formed by adsorption of DOPC vesicles (16 μg/ml) to silicon discs in HEPES buffer (5 ml) containing 1 mM $CaCl_2$. Nonbound vesicles were removed by flushing the cuvette (■) or by transferring the disc to the ellipsometer cuvette after vesicle adsorption in a separate vessel. At 400 s, PLA_2 was added in final concentrations of 0 (■), 0.1 (□), 1 (◇), 10 (△), and 100 (○) ng/ml, and the cuvette was subsequently flushed with the same concentrations of PLA_2 at 5 ml/min.

Chromatographic analysis of lipids desorbing from the disc

The stability of the bilayer of intact phospholipids during flushing with buffer was shown by experiments, as illustrated in Fig. 1. Loss of membrane mass after addition of PLA_2 therefore probably reflected only desorption of degradation products from the membrane. However, partial degradation of phospholipids could also cause destabilization of the membrane, resulting in co-desorption of intact DOPC with LPC and oleic acid. To study this aspect, a planar DOPC membrane containing radiolabeled DOPC (5000 dpm) was prepared. Shortly after the addition of PLA_2 (5 ng/ml), that is, after about 3.5% of lipid mass had desorbed, the cuvette was flushed to remove nonadsorbed PLA_2 from the bulk phase and to prevent degradation of possibly desorbed intact DOPC. After partial further degradation of the membrane, PC hydrolysis was stopped by the addition of EDTA. Lipids recovered in the buffer phase and those remaining on the disc were extracted, and DOPC was separated from LPC and oleic acid by thin-layer chromatography. Less than 1.5% of total recovered counts were found in the buffer DOPC fraction. In a similar experiment, a SAPC membrane was prepared containing radiolabeled SAPC (5000 dpm) and degraded with PLA_2 (200 ng/ml). Again, undegraded PC appearing in the buffer was negligible. Therefore, it is concluded that PLA_2 action did not promote desorption of undegraded PC from the membrane.

Desorption from DOPC/LPC/oleic acid membranes

For straightforward interpretation of the desorption curves in Fig. 1 in terms of phospholipid degradation, intact phospholipids should not only remain adsorbed, but the degradation products should also readily desorb from the membrane. This aspect was studied for bilayers prepared from mixtures of DOPC, LPC, and oleic acid. Vesicles were prepared from an equimolar mixture of LPC and oleic acid. Exposure of a silicon disc to this vesicle suspension resulted in the rapid formation of a membrane (Fig. 2). Because LPC and oleic acid have considerable aqueous solubility (see below), we used a high lipid concentration (40 μg/ml) to ensure that the bulk of the lipid was added in the form of aggregates. Furthermore, a higher calcium concentration (3 mM) was used to prevent slow adsorption in the final phase of membrane formation. This higher calcium concentration probably compensates for the net charge of oleic acid in the vesicles. The resulting adsorption, however, was too fast for accurate determination of the initial adsorption rate. An equilibrium level of about 0.41 μg/cm^2 was observed, similar to the membrane mass obtained from pure DOPC. Removal of nonbound vesicles by flushing of the cuvette now caused rapid loss of adsorbed mass, at a rate of 1.86 ng/cm^2/s, and virtually no lipid remained adsorbed to the disc (Fig. 2, *open squares*). Note also the remarkable linearity of the desorption curve, explained below. Some delay in the start of desorption after flushing is apparent in Fig. 2 and was caused by the time required for sufficient dilution of the high lipid concentrations in the buffer.

Next, the behavior of ternary mixtures of DOPC, LPC, and oleic acid was studied. Vesicles were prepared from mixtures of DOPC and equimolar amounts of LPC and oleic acid, together forming 12.5%, 25%, 50%, and 75% of total lipid mass. All preparations readily adsorbed to the discs, resulting in about 0.41 μg/cm^2 adsorbed mass (Fig. 2). Upon flushing of the cuvette with buffer, partial desorption of lipids occurred. The desorbed lipid mass of 11.2%, 21.6%, 50.5%, and 75.3% closely correlated with the weight fraction of LPC + oleic acid originally present in the vesicle preparations. This indicated that a) DOPC, LPC, and oleic acid were incorporated quantitatively into the membrane and b) LPC and oleic acid quantitatively desorbed from the slide after flushing with buffer, while DOPC remained adsorbed.

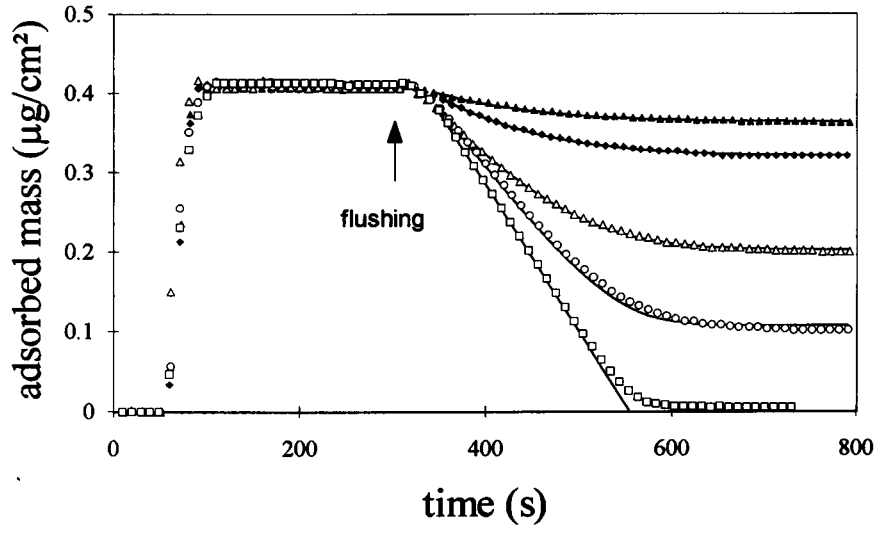

FIGURE 2 Desorption of LPC and oleic acid from ternary mixtures with PC. Planar membranes were formed in HEPES buffer containing 3 mM $CaCl_2$ by adsorption to silicon discs of vesicles (40 μg/ml total liquid) of mixtures of DOPC and equimolar LPC/oleic acid. The weight ratios of PC/LPC + oleic acid were 87.5/12.5 (▲), 75/25 (◆), 50/50 (△), 25/75 (○), and 0/100 (□). At 300 s, nonbound vesicles were removed by flushing the cuvette at 50 ml/min with HEPES buffer containing 1 mM $CaCl_2$. The solid lines are fitted curves according to the model described in Materials and Methods.

Determination of the CMC of an equimolar LPC/oleic acid mixture

The rapid formation of membranes at high lipid concentrations (Fig. 2) showed that the lipid aggregates (vesicles) had high affinity for the hydrophilic silicon surface. In contrast, complete desorption of the LPC/oleic acid mixture (Fig. 2) proved that monomers have little affinity for the surface. A plot of initial adsorption rates against lipid concentrations could thus provide a means for determination of the CMC by showing a corresponding discontinuity. Fig. 3 shows the adsorption curves of freshly prepared equimolar LPC/oleic acid mixtures in a concentration ranging from 0.1 to 6 μg/ml. Clearly, adsorption started at about 1 μg/ml total lipid. A more accurate estimation was obtained by plotting the initial

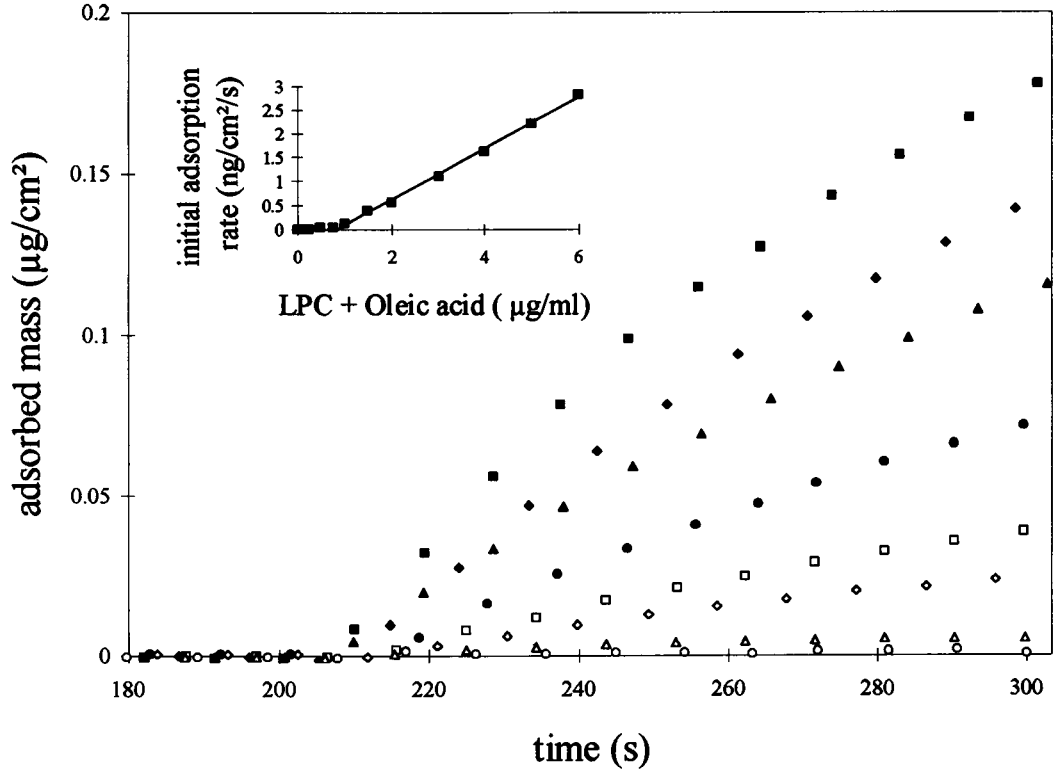

FIGURE 3 Determination of the CMC of equimolar LPC/oleic acid. Vesicles of a pure, equimolar mixture of LPC and oleic acid were prepared. Adsorption of these vesicles to silicon discs was determined in HEPES buffer containing 1 mM $CaCl_2$ at lipid concentrations of 0.1, 0.25, 0.5, 0.75 (○), 1 (△), 1.5 (◇), 2 (□), 3 (●), 4 (▲), 5 (◆), and 6 (■) μg/ml. (*Inset*) Estimated initial adsorption rates were plotted as a function of the lipid concentration and were analyzed by linear regression.

adsorption rates as a function of the lipid concentration (Fig. 3, *inset*). A clearly biphasic curve was obtained. Using a linear fit on the data from 1 to 6 μg/ml, the value of the CMC was estimated from the intercept with the abscissa as 0.86 μg/ml, or 1.1 μM. Alternatively, one could determine the CMC by estimating the bulk concentration of products required to maintain a stable membrane. Indeed, it was found that LPC/oleic acid membranes readily desorbed during flushing with a bulk concentration of 0.25 μg/ml, but remained stable at a bulk concentration of 1.5 μg/ml.

Build-up of degradation products in the membrane

Fig. 2 shows that the initial desorption rate was related to the amount of products in the membrane. It was assumed that a similar relationship holds for desorption rates at any time during desorption because of the action of PLA_2 (see also Discussion). This assumption was verified by termination of PLA_2 action with EDTA and measurement of the amount of products desorbing from the membrane beyond this point, as shown in Fig. 4. For a relatively low PLA_2 concentration of 0.1 ng/ml (*upper curve*), the ongoing desorption proved limited. However, for a high PLA_2 concentration of 100 ng/ml (*lower curve*), almost all PC appeared to be hydrolyzed at the moment of PLA_2 inhibition, although only 33% of lipid mass had actually desorbed at that time. From the ongoing desorption in these curves, the weight fraction of products at the moment of EDTA addition was estimated to be 0.07, 0.37, and 0.91, respectively. The actual desorption rates, measured just before the time of EDTA addition, were 0.20, 0.93, and 1.8 ng/cm^2/s. As shown in Fig. 5, these data agreed with the data on desorption of membranes prepared from ternary mixtures, as presented in Fig. 2. It was therefore concluded that the amount of hydrolyzed phospholipid in the membrane can be estimated at any time from the desorption rate. The best-fit straight line in Fig. 5 had a slope of 1.89 ng/cm^2/s. Using the relation 11.8 $D^{[2/3]}$ CMC = 1.89 (see Materials and Methods) and CMC = 860 ng/cm^3, a value of $D = 2.5 \times 10^{-6}$ cm^2/s was obtained.

Fig. 4 also shows the effect of recalcification. Although nonbound PLA_2 was removed by continuous flushing with EDTA-buffer, PC hydrolysis continued at almost identical rates upon recalcification (*dashed arrows*). Apparently PLA_2 has considerable affinity for the membrane, even in the absence of calcium ions.

PLA_2-dependent membrane degradation in the presence of bovine serum albumin

To facilitate desorption of the products from the membrane we introduced BSA into the buffer. It was determined that the BSA preparation did not contain PLA_2 activity, by verifying the stability of DOPC bilayers in BSA solutions. BSA binds both fatty acids and lysophospholipids, and it has been shown to dramatically increase diffusional fluxes of lipids across water layers (Weisiger et al., 1989). If, as further discussed below, desorption rates are limited because the concentrations of lipid monomers at the surface cannot exceed critical micelle concentrations, the addition of BSA may shift lipid desorption from transport-limited to intrinsic rates. Fig. 6 shows that BSA (0.5 mg/ml) indeed enhanced the product desorption rates compared to rates in absence of BSA. Already with 1 ng/ml PLA_2 the desorption rate (2.5 ng/cm^2/s) exceeded the transport limit of a pure LPC/oleic acid mixture in the absence of BSA. With 10 ng/ml PLA_2 lipid desorption was even faster (5 ng/cm^2/s). Lipid desorption from a pure LPC/oleic acid membrane in BSA was too fast for accurate measurement (>10 ng/cm^2/s;

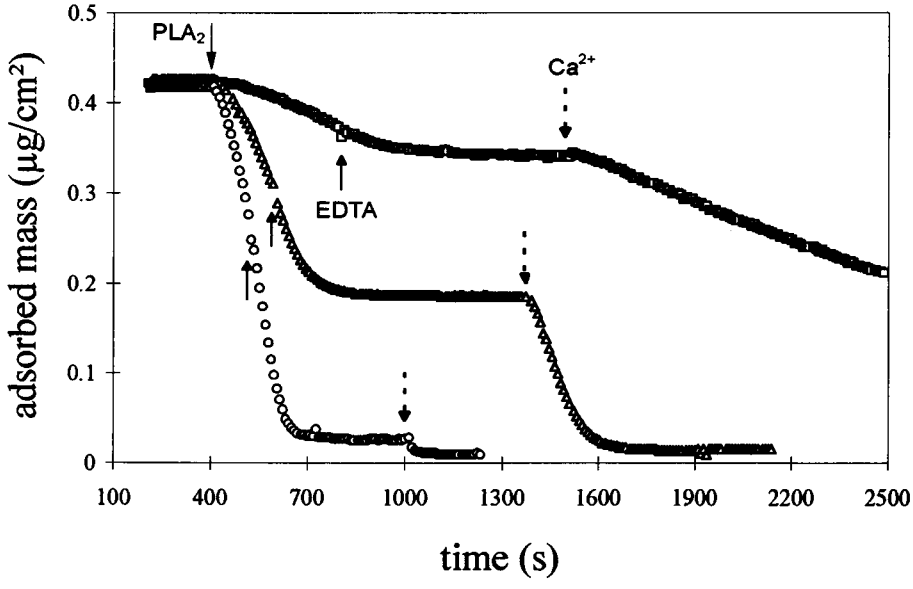

FIGURE 4 Effect of EDTA on PLA_2-dependent membrane degradation. Planar DOPC membranes were prepared as described for Fig. 1. At 400 s, PLA_2 was added with a final concentration of 0.1 (□), 10 (△), or 100 (○) ng/ml, and the cuvette was subsequently flushed with the same PLA_2 concentration at 5 ml/min. At the time indicated by the solid arrows, PLA_2 activity was inhibited by the addition of EDTA (2 mM), and flushing was continued with HEPES buffer containing 1 mM EDTA. PLA_2 activity was restored by the addition of $CaCl_2$ (2 mM, *dashed arrows*) and subsequent flushing with HEPES buffer containing 1 mM $CaCl_2$.

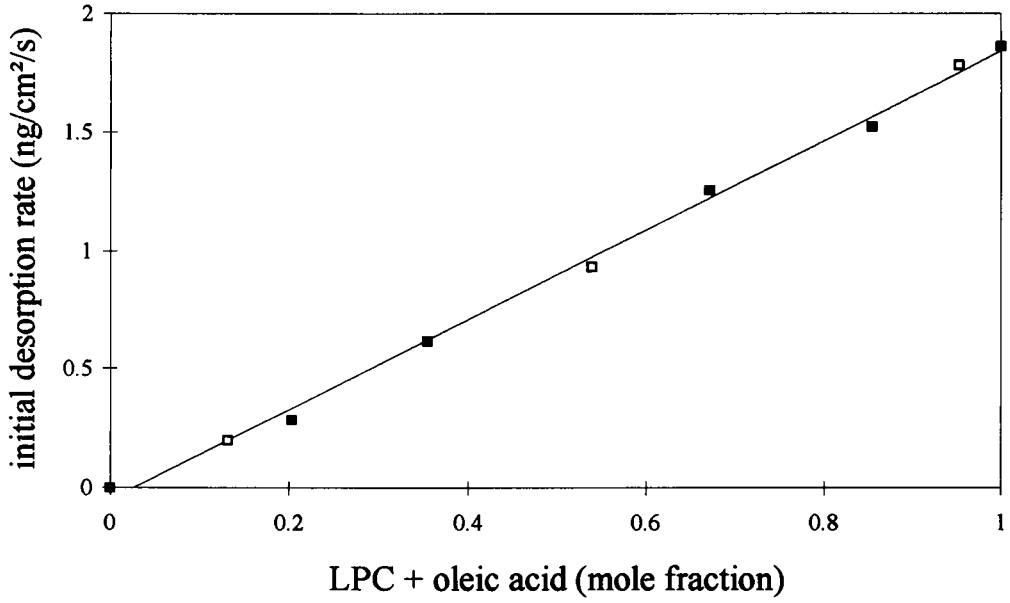

FIGURE 5 Desorption rates as a function of the mole fraction of products. The estimated initial desorption rates from the curves in Fig. 2 are plotted as a function of the mole fraction of LPC + oleic acid in the membrane (■). Also shown are the observed desorption rates from the curves in Fig. 4 just before EDTA addition (□).

not shown). At the end of the degradation curves some adsorption of BSA to the exposed silicon surface was apparent. Direct comparison of desorption curves in the absence and presence of BSA revealed that not only product desorption rates were increased by BSA, but also the activity of the enzyme itself appeared to be enhanced. For instance, degradation with 1 ng/ml PLA_2 in BSA was completed after about 200 s, whereas total hydrolysis of phospholipids in the absence of BSA did not exceed 36% during this period, even when the amount of nondesorbed products was taken into account (Fig. 6, *inset*).

Although BSA had a stimulatory effect on PLA_2 activity, it may also reduce the affinity of PLA_2s for the membrane, because this affinity is markedly enhanced by the presence

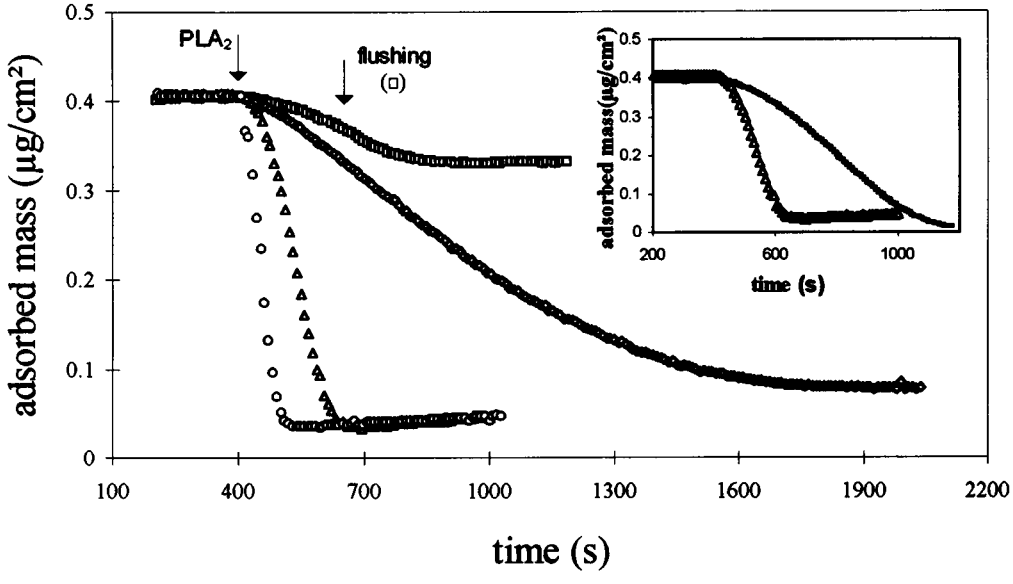

FIGURE 6 PLA_2-dependent membrane degradation in the presence of BSA. Planar DOPC membranes were prepared as described for Fig. 1. Membrane degradation was measured in HEPES buffer containing 1 mM $CaCl_2$ and 0.5 mg/ml BSA after the addition (at 400 s) of 0.05 (□), 0.1 (◇), 1 (△), or 10 (○) ng/ml PLA_2. In the upper curve, nonbound PLA_2 was removed by flushing the cuvette (*arrow*) at 50 ml/min for 75 s; flushing was subsequently continued at 5 ml/min. For direct comparison of the effect of BSA, the inset shows a replot of the curves with 1 ng/ml PLA_2 in BSA (△), and in the absence of BSA (−) as taken from Fig. 1.

of LPC and oleic acid in the membrane (Apitz-Castro et al., 1982; Jain et al., 1982). Indeed, flushing of the cuvette with BSA buffer, thereby removing nonbound PLA_2, resulted in a rapid decrease in product desorption rates (Fig. 6, *upper curve*). Apparently, PLA_2 dissociated from the membrane, an effect that was not observed when a similar experiment was performed in the absence of BSA (result not shown, but see also Fig. 4).

DISCUSSION

Transport-limited formation of membranes

The initial adsorption rate of 5.6 ng/cm^2/s, measured for membrane formation in Fig. 1, is transport limited (Giesen et al., 1995), that is, the intrinsic adsorption rate of the vesicles to the silicon surface is much higher than the maximum transport rate of vesicles from buffer toward the disc surface. Under these circumstances, the concentration C_0 of vesicles at the surface will approach zero and the adsorption rate equals the maximum transport rate of $d\Gamma/dt = 11.8\ D^{[2/3]}\ C_{bulk}$ (see Materials and Methods). Inserting the observed initial adsorption rate and C_{bulk}, a value of 1.6×10^{-7} cm^2/s is found for the diffusion constant D of the vesicles, similar to the values of $(1.8–2.2) \times 10^{-7}$ cm^2/s reported in the literature (Huang and Thompson, 1974; Watts et al., 1978; Wei et al., 1982). The slope of the regression data in Fig. 3 yields a mass transfer coefficient for adsorbing LPC/oleic acid vesicles of 0.54×10^{-3} cm/s. This corresponds to a diffusion constant of $D = 3.1 \times 10^{-7}$ cm^2/s. Apparently, LPC/oleic acid vesicles are smaller than DOPC vesicles, which could be related to their ionic charge. As discussed by Giesen et al. (1995), membranes formed by adsorption of small unilamellar phospholipid vesicles on hydrophilic silicon slides, as used in the present study, probably consist of continuous bilayers because the average mass of 0.41 μg/cm^2 exactly equals the mass of a bilayer deposited by the classical Blodgett stacking technique, and complete mixing of lipids occurs. Assuming such a bilayer structure, the membrane mass of 0.41 μg/cm^2 corresponds to an average surface area of 0.64 nm^2 per molecule.

Quantitative description of desorption

It has been demonstrated that aqueous mixtures of lysophospholipids and fatty acids form bilayer structures, whereas the individual components form micelles (Jain et al., 1980). Together with the finding in the present study of identical mass (0.41 μg/cm^2) for bilayers formed by pure DOPC and membranes formed by ternary mixtures, this indicates that the latter mixtures also formed bilayers. In view of the different CMC values, it might seem surprising that the three different components are incorporated quantitatively into the membrane. However, the lipid concentrations used for producing these membranes were so high (40 μg/ml) that, even for the lysophospholipids and fatty acids, less than a few percent was present as monomers. The adsorbing aggregates therefore had about the same composition as the overall mixture. It should be mentioned that preparations of erythrocytes (Gul and Smith, 1974) and vesicles (Jain and Berg, 1989) will often have high lipid monomer concentrations, preventing efflux of degradation products from the membrane. This has caused the impression that these products have an inherent tendency to remain in the membrane, sometimes visualized by LPC/fatty acid complexes behaving like intact PC molecules. The present study proves this impression false by showing that, in efficiently stirred systems, even long-chain degradation products will readily desorb from the membrane if the bulk concentration is kept sufficiently low, for instance, by constant flushing.

The remarkable linearity of the desorption curve of a pure LPC/oleic acid mixture in Fig. 2 suggested a constant value of C_0 during desorption. This could be explained by assuming local equilibrium at the membrane, with a build-up of C_0 during desorption limited to the value of the CMC. During flushing of the cuvette ($C_{bulk} = 0$), the desorption rate reduces to $d\Gamma/dt = -11.8\ D^{2/3}\ C_0$. From a desorption rate of 1.86 ng/cm^2/s and a CMC of 0.86 μg/ml (see Results), a diffusion constant $D_{20} = 2.5 \times 10^{-6}$ cm^2/s is obtained, in agreement with the value calculated from Fig. 5 (see Results) and with reported values for oleate of $D_{25} = (2.8–3.3) \times 10^{-6}$ cm^2/s (Patil et al., 1973; Stewart et al., 1991). This also explains the stability of the original DOPC membranes. The CMC of long-chain phospholipids is on the order of 0.1–1 nM (Tanford, 1973), and this would correspond to desorption rates for DOPC monomers of less than 1 pg/cm^2/s, which are only observable after several hours.

Desorption curves for ternary mixtures in Fig. 2 were fitted to the equation $d\Gamma/dt = -11.8\ D^{[2/3]}\ CMC\ (2\Gamma - 2\Gamma_{0pl})/(2\Gamma - \Gamma_{0pl})$ (derived in Materials and Methods), using the obtained values of $D = 2.5 \times 10^{-6}$ cm^2/s and CMC = 0.86 μg/ml, and adjustment of the parameter Γ_{0pl}. As shown by the fitted (continuous) curves in Fig. 2, good agreement between theory and experiments was obtained, not only for the initial desorption rates but for the entire desorption curves. Apparently desorption rates remain proportional to the mole fractions of desorbing products, as also shown in Fig. 5. It also follows from Fig. 5 that desorption from ternary mixtures and desorption caused by the action of PLA_2 both conform to the same model. This model assumes that higher PLA_2 concentrations cause higher mole fractions X of degradation products in the membrane, in its turn causing higher $C_0 = CMC \cdot X$ values for the concentration in the buffer at the surface (see Materials and Methods) and higher desorption rates. For very low PLA_2 activities the rate of product efflux from the membrane could become too low to reach these C_0 values. In that case the desorption rate would reflect the true intrinsic rate of membrane hydolysis. However, this situation apparently does not occur for the PLA_2 concentrations used in the present study.

The laser beam of the ellipsometer had a cross section surface area of about 1 mm^2, and ellipsometry thus measured mean parameters for such areas. It could not discriminate between continuous films or membranes consisting of

micro-patches. Using adsorption of annexin V, a phospholipid-binding protein, to partially degraded membranes, we found a reduction of annexin V adsorption roughly proportional to the loss of membrane mass (unpublished results). We therefore propose that PLA_2 causes formation of bare patches devoid of lipids. The number and size of these patches may increase up to a stage where the membrane only consists of a few remaining lipid islands, further decreasing in size. Thus, the degradation of the membrane is viewed as a reversal of its formation (Giesen et al., 1995).

Adsorption of PLA_2 to the membrane could not be observed in the present study

During PLA_2-dependent membrane degradation, the rates of mass desorption initially increased (Fig. 1). This is probably caused by increasing amounts of PLA_2 bound to the membrane, because removal of PLA_2 from the bulk phase resulted in almost linear kinetics (Fig. 4). The latter experiment also suggested that the affinity of PLA_2 for the membrane was high. Therefore, adsorption of PLA_2 to the membrane could be transport limited. With the low PLA_2 concentrations used in most of our experiments, however, adsorption of PLA_2 mass was negligible, even for transport-limited PLA_2 adsorption. For instance, with 10 ng/ml PLA_2, a concentration in the medium of the range used in Fig. 1, and an assumed value of 10^{-6} cm^2/s for the diffusion constant of PLA_2, the maximum rate of PLA_2 adsorption would be 0.012 $ng/cm^2/s$, only about 1% of the observed lipid desorption rate of 1.2 $ng/cm^2/s$.

With high PLA_2 concentrations, PLA_2 adsorption rates would become considerable and could even exceed the initial desorption rates of the products. Indeed, using a PLA_2 concentration of 1 $\mu g/ml$ (not shown), initial adsorption was observed, and the desorption rate appeared delayed compared to the curve with 100 ng/ml. It should be realized, however, that these PLA_2 activities are extremely high. Even the desorption rate observed with 0.1 ng/ml PLA_2 (0.3 $ng/cm^2/s$ in the steepest part of the curve) considerably exceeds physiological PLA_2 activities. For instance, maximum phospholipid degradation rates in anoxic myocardial tissue are in the range of 1%/min (Van der Vusse et al., 1989).

Comparison of ellipsometry with existing techniques

Several methods for the assay of PLA_2 activity have been developed, of which the so-called pH-stat method, the monolayer trough, and chromatographic separation of products from substrates are frequently used. In the pH-stat method, PLA_2 activity is measured by the titration of released protons from the generated fatty acids at a constant pH (Nieuwenhuizen et al., 1974). Because the method only permits the use of weakly buffered systems, the pH at the charged phospholipid/water interface is not well defined, and titration of the generated fatty acids may not be fully achieved at lower pH. In spite of this limitation, and a limited sensitivity, the method is often used because of its ease and because it allows continuous measurement, and has been called the "workhorse" of lipolytic research (Reynolds et al., 1991).

PLA_2 activity can also be measured from degradation of phospholipid monolayers spread at the air/water interface in a monolayer trough (Ransac et al., 1991). Activity is usually measured from the change in phospholipid surface area at a constant surface pressure, and is continuously monitored. Only very low amounts of lipid are needed, and the method has high sensitivity. Because the surface pressure used in this technique is in principle arbitrary, molecular spacing in the monolayer may deviate from the spacing in natural membranes, and this may influence PLA_2 activity (Verger and De Haas, 1976). Furthermore, the lability of the monolayer at the air/water interface prevents efficient stirring and only permits the use of nonphysiological short-chain phospholipids with degradation products that readily dissolve in the aqueous phase (Zografi et al., 1971). In contrast, bilayers stacked on hydrophilic silicon discs can be exposed to high shear stress without destabilization of the membrane. Thus, even long-chain lysophospholipids and fatty acids may readily desorb from the membrane.

Another sensitive method for the measurement of PLA_2 activity employs chromatographic separation (TLC, HPLC) of degradation products from intact phospholipids (Blank and Snyder, 1991), and subsequent quantitation, for instance, by phosphate assay. The sensitivity of the method can be further increased with radiolabeled substrates. However, this method is quite laborious. In this respect it should be mentioned that the ellipsometric technique presented in this study also does not allow more than five or six experiments to be performed per day.

Ellipsometry could be particularly useful for investigating the influence of membrane-binding proteins, like the annexin family, on PLA_2 activity. Adsorption of these proteins, for instance annexin V, proceeds rapidly (Andree et al., 1990) and can be measured during membrane degradation. This may be especially interesting for PLA_2s requiring anionic phospholipids like phosphatidylserine in the membrane, mimicking the redistribution of lipids upon cell activation (Zwaal et al., 1992), which may increase both the binding affinity of annexins and the activity of PLA_2s on the plasma membrane of, for instance, blood platelets.

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REFERENCES

Andree, H. A. M., C. P. M. Reutelingsperger, R. Hauptmann, H. C. Hemker, W. Th. Hermens, and G. M. Willems. 1990. Binding of vascular anticoagulant-α (VAC-α) to planar phospholipid bilayers. *J. Biol. Chem.* 265:4923–4928.

Apitz-Castro, R., M. K. Jain, and G. M. De Haas. 1982. Origin of the latency phase during the action of phospholipase A_2 on unmodified phosphatidylcholine vesicles. *Biochim. Biophys. Acta.* 688:349–356.

Azzam, R. M. A., and N. M. Bashara. 1977. Ellipsometry and Polarized Light. North Holland Publishing, Amsterdam.

Blank, M. L., and F. Snyder. 1991. Chromatographic analysis of phospholipase reaction products. *Methods Enzymol.* 197:158–165.

Böttcher, C. J. F., C. M. Van Gent, and C. Pries. 1961. A rapid and sensitive sub-micro phosphorus determination. *Anal. Chim. Acta* 24: 203–207.

Brian, A. A., and H. M. McConnell. 1984. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. USA.* 81:6159–6163.

Corsel, J. W., G. M. Willems, J. M. M. Kop, P. A. Cuypers, and W. Th. Hermens. 1986. The role of intrinsic binding rate and transport rate in the adsorption of prothrombin, albumin and fibrinogen to phospholipid bilayers. *J. Colloid Interface Sci.* 111:544–554.

Cuypers, P. A., J. W. Corsel, M. P. Janssen, J. M. M. Kop, W. Th. Hermens, and H. C. Hemker. 1983. The adsorption of prothrombin to phosphatidylserine multilayers quantitated by ellipsometry. *J. Biol. Chem.* 258:2426–2431.

Giesen, P. L. A., H. C. Hemker, and W. Th. Hermens. 1995. Production of thrombin as a probe for mixing of phospholipids in membranes on solid supports. *Biochim. Biophys. Acta.* 1237:43–48.

Giesen, P. L. A., G. M. Willems, and W. Th. Hermens. 1991. Production of thrombin by the prothrombinase complex is regulated by membrane-mediated transport of prothrombin. *J. Biol. Chem.* 266:1379–1382.

Gul, S., and A. D. Smith. 1974. Haemolysis of intact human erythrocytes by purified cobra venom phospholipase A_2 in the presence of albumin and Ca^{2+}. *Biochim. Biophys. Acta.* 367:271–281.

Huang, C., and T. E. Thompson. 1974. Preparation of homogeneous, single-walled phosphatidylcholine vesicles. *Methods Enzymol.* 32: 485–489.

Jain, M. K., and O. G. Berg. 1989. The kinetics of interfacial activation: hopping versus scooting. *Biochim. Biophys. Acta.* 1002:127–156.

Jain, M. K., M. R. Egmond, H. M. Verheij, R. Apitz-Castro, R. Dijkman, and G. H. De Haas. 1982. Interaction of phospholipase A_2 and phospholipid bilayers. *Biochim. Biophys. Acta.* 688:341–348.

Jain, M. K., G. R. Ranadive, B.-Z. Yu, and H. M. Verheij. 1991. Interfacial catalysis by phospholipase A_2: monomeric enzyme is fully catalytically active at the bilayer interface. *Biochemistry.* 30:7330–7340.

Jain, M. K., C. J. A. Van Echteld, F. Ramirez, J. De Gier, G. H. De Haas, and L. L. M. Van Deenen. 1980. Association of lysophosphatidylcholine with fatty acids in aqueous phase to form bilayers. *Nature.* 284: 486–487.

Kop, J. M. M., P. A. Cuypers, Th. Lindhout, H. C. Hemker, and W. Th. Hermens. 1984. The adsorption of prothrombin to phospholipid monolayers quantitated by ellipsometry. *J. Biol. Chem.* 259:13993–13998.

Kupferberg, J. P., S. Yokoyama, and F. J. Kézdy. 1981. The kinetics of the phospholipase A_2-catalyzed hydrolysis of egg phosphatidylcholine in unilamellar vesicles. Product inhibition and its relief by serum albumin. *J. Biol. Chem.* 256:6274–6281.

Nieuwenhuizen, W., H. Kunze, and G. H. De Haas. 1974. Phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4) from porcine pancreas. *Methods Enzymol.* 32B:147–154.

Patil, G. S., R. H. Matthews, and D. G. Cornwell. 1973. Kinetics of the processes of desorption from fatty acid monolayers. *J. Lipid Res.* 14: 26–31.

Ransac, S., H. Moreau, R. Rivière, and R. Verger. 1991. Monolayer techniques for studying phospholipase kinetics. *Methods Enzymol.* 197: 49–65.

Reynolds, L. J., W. N. Washburn, R. A. Deems, and E. A. Dennis. 1991. Assay strategies and methods for phospholipases. *Methods Enzymol.* 197:3–23.

Smith, A. D., S. Gul, and R. H. S. Thompson. 1972. The effect of fatty acids and of albumin on the action of a purified phospholipase A_2 from cobra venom on synthetic lecithins. *Biochim. Biophys. Acta.* 289: 147–15.

Stewart, J. M., W. R. Driedzic, and M. Berkelaar. 1991. Fatty-acid-binding protein facilitates the diffusion of oleate in a model cytosol system. *Biochem. J.* 275:569–573.

Tanford, C. 1973. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. Wiley and Sons, New York.

Van Bilsen, M., G. J. Van der Vusse, P. H. Willemsen, W. A. Coumans, T. H. M. Roemen, and R. S. Reneman. 1989. Lipid alterations in isolated, working rat hearts during ischemia and reperfusion: Its relation to myocardial damage. *Circ. Res.* 64:304–314.

Van der Vusse, G. J., M. J. M. De Groot, P. H. M. Willemsen, M. Van Bilsen, A. H. G. J. Schrijvers, and R. S. Reneman. 1989. Degradation of phospholipids and triacylglycerol, and accumulation of fatty acids in anoxic myocardial tissue, disrupted by freeze-thawing. *Mol. Cell. Biochem.* 88:83–90.

Van der Vusse, G. J., J. F. Glatz, H. C. Stam, and R. S. Reneman. 1992. Fatty acid homeostasis in the normoxic and ischemic heart. *Physiol. Rev.* 72:881–940.

Van der Vusse, G. J., T. H. M. Roemen, and R. S. Reneman. 1980. Assessment of fatty acids in dog left ventricular myocardium. *Biochim. Biophys. Acta.* 617:347–352.

Verger, R., and G. H. De Haas. 1976. Interfacial enzyme kinetics of lipolysis. *Annu. Rev. Biophys. Bioeng.* 5:77–117.

Watts, A., D. Marsh, and P. F. Knowles. 1978. Characterization of dimyristoylphosphatidylcholine vesicles and their dimensional changes through the phase transition: molecular control of membrane morphology. *Biochemistry.* 17:1792–1801.

Wei, G. J., V. A. Bloomfield, R. M. Resnick, and G. L. Nelsestuen. 1982. Kinetic and mechanistic analysis of prothrombin-membrane binding by stopped-flow light scattering. *Biochemistry.* 21:1949–1959.

Weisiger, R. A., S. M. Pond, and L. Bass. 1989. Albumin enhances unidirectional fluxes of fatty acid across a lipid-water interface: theory and experiments. *Am. J. Physiol.* 257:G904-G916.

Willems, G. M., P. L. A. Giesen, and W. Th. Hermens. 1993. Adsorption and conversion of prothrombin on a rotating disc. *Blood.* 82:497–504.

Zografi, G., R. Verger, and G. H. De Haas. 1971. Kinetic analysis of the hydrolysis of lecithin monolayers by phospholipase A. *Chem. Phys. Lipids.* 7:185–206.

Zwaal, R. F. A., P. Comfurius, and E. M. Bevers. 1992. Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis. *Biochim. Biophys. Acta.* 1180:1–8.