A Two-Photon View of an Enzyme at Work: *Crotalus atrox* Venom PLA_2 Interaction with Single-Lipid and Mixed-Lipid Giant Unilamellar Vesicles

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ABSTRACT We describe the interaction of *Crotalus atrox*-secreted phospholipase A_2 ($sPLA_2$) with giant unilamellar vesicles (GUVs) composed of single and binary phospholipid mixtures visualized through two-photon excitation fluorescent microscopy. The GUV lipid compositions that we examined included 1-palmitoyl-2-oleoyl-phosphatidylcholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (above their gel-liquid crystal transition temperatures) and two well characterized lipid mixtures, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE):DMPC (7:3) and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC)/1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (DAPC) (1:1) equilibrated at their phase-coexistence temperature regime. The membrane fluorescence probes, 6-lauroyl-2-(dimethylamino) napthalene, 6-propionyl-2-(dimethylamino) naphthalene, and rhodamine-phosphatidylethanolamine, were used to assess the state of the membrane and specifically mark the phospholipid domains.

Independent of their lipid composition, all GUVs were reduced in size as $sPLA_2$-dependent lipid hydrolysis proceeded. The binding of $sPLA_2$ was monitored using a fluorescein-$sPLA_2$ conjugate. The $sPLA_2$ was observed to associate with the entire surface of the liquid phase in the single phospholipid GUVs. In the mixed-lipid GUV's, at temperatures promoting domain coexistence, a preferential binding of the enzyme to the liquid regions was also found. The lipid phase of the GUV protein binding region was verified by the introduction of 6-propionyl-2-(dimethylamino) naphthalene, which partitions quickly into the lipid fluid phase. Preferential hydrolysis of the liquid domains supported the conclusions based on the binding studies. sPLA2 hydrolyzes the liquid domains in the binary lipid mixtures DLPC:DAPC and DMPC:DMPE, indicating that the solid-phase packing of DAPC and DMPE interferes with $sPLA_2$ binding, irrespective of the phospholipid headgroup. These studies emphasize the importance of lateral packing of the lipids in *C. atrox* $sPLA_2$ enzymatic hydrolysis of a membrane surface.

INTRODUCTION

Secreted phospholipases A_2 ($sPLA_2$) are a group of soluble enzymes that catalyze the hydrolysis of the 2-acyl group in phospholipids, producing fatty acid and lysophospholipid as reaction products. As a class, $sPLA_2$ enzymes have a high degree of structural similarity and are believed to have a common catalytic mechanism (Heinrikson et al., 1977; Welches et al., 1993). Because of their stability and the simplicity of the purification protocols, $sPLA_2$s have been used as models to understand the more complicated cellular $sPLA_2$ groups.

The lateral organization of membrane lipids has been known to be an important parameter in the enzymatic hydrolysis of membrane phospholipids by $sPLA_2$. It was recognized early that the phospholipase enzymes preferred an organized lipid substrate near the lipid's phase transition and were particularly active against micellar lipids. To explain the activity of $sPLA_2$ against lipid bilayers, many researchers postulated the existence of transient bilayer "surface defects." These defects were imagined as membrane gaps that served to increase the affinity of the enzyme for the interface and facilitate enzyme access to the *sn*-2 position fatty acid through the disruption of the tightly organized lipid lattice. (Bell et al., 1996; Burack et al., 1997a, 1993; Grainger et al., 1989, 1990; Grandbois et al., 1998; Kensil and Dennis 1979; Op den Kamp et al., 1975). The exact molecular details of how the lipid packing influences $sPLA_2$ activity are still being investigated today.

Physical evidence of membrane defects and their correlation with $sPLA_2$ function is rare, but a number of studies have addressed this issue. One excellent example is the detailed work on mixed cholesterol-phospholipid in which a strong correlation between the formation of lipid superlattices and the precipitous decrease in $sPLA_2$ activity was clearly shown (Liu and Chong, 1999). Also, in phospholipid monolayer studies, physical spaces between phospholipid domains were identified as the starting points for $sPLA_2$ hydrolysis (Grainger et al., 1989). Along similar lines, products accumulated during the enzymatic lag period have been shown to lead to a characteristic burst in $sPLA_2$ hydrolysis activity, an effect which has been attributed to phase separation between substrate and products (Burack et al., 1997a, 1993; Dahmen-Levison et al., 1998; Grainger et al., 1989; Maloney and Grainger, 1993). Transient membrane irregularities created by lipid phase coexistence have been of interest to the phospholipase research community since the discovery that vesicles equilibrated at temperatures within their phase-coexistence region show the highest susceptibility to $sPLA_2$ attack. These types of defects are of likely

Submitted August 28, 2001, and accepted for publication January 7, 2002.

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0006-3495/02/04/2232/12 $2.00

biological significance, as natural membranes contain a variety of lipids, and the potential for forming packing arrangements containing mismatched borders is high.

Studies on the interaction of sPLA$_2$ and organized lipid interfaces have been conducted using a variety of systems, including small and large unilamellar vesicles, (SUVs and LUVs), multilamellar vesicles (MLVs), as well as monolayers at the air-water interface. Because of their particular characteristics (size and lamellarity) these model membrane systems are not necessarily accurate descriptions of cell membranes. Giant unilamellar vesicles (GUVs) with a mean diameter of 30 μm have a minimum curvature and mimic cell membranes in this respect. GUVs are ideal for studying lipid/lipid and lipid/protein interactions using microscopy techniques (Holopainen et al., 2000; Longo et al., 1998; Mengar and Keiper, 1998; Wick et al., 1996).

In this work, we explore two questions about the interaction of sPLA$_2$ with organized lipid interfaces: 1) does the interaction of the enzyme with organized lipids such as GUVs produce morphological change in the substrate vesicles, and 2) when the enzyme interacts with vesicles showing lipid domain separations and "defects," does the enzyme preferentially bind to a particular area, namely a particular domain or to the border between different domains?

In our experimental approach, we use two-photon microscopy, *Crotalus atrox* sPLA$_2$ and two classes of GUVs; namely, single lipid GUVs above their transition temperatures and mixed-lipid GUVs showing domain separation. In both mixed and homogeneous GUVs, we directly visualize the interaction of the vesicles with the enzyme by two-photon microscopy. To measure the binding of the enzyme to the vesicles, we used fluorescein-labeled sPLA$_2$ in the presence of Ba^{2+} (to inhibit activity but retain enzyme association with the interface). Activity measurements were done by addition of active enzyme (sPLA$_2$ plus Ca^{2+}) to the GUVs. Fluorescent dyes were used to visualize the GUVs and to study the packing changes in the membrane (6-lauroyl-2-(dimethylamino) napthalene (LAURDAN)), and to visualize and identify liquid crystal and gel domains (Lissamine Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (*N*-Rh-DPPE) and 6-propionyl-2-(dimethylamino) naphthalene (PRODAN)).

Our results show, for *C. atrox* sPLA$_2$, *Naja naja naja*, and *Agkistrodon piscivorus piscivorus*, that the interaction of the enzyme with GUV induces dramatic morphological changes in the vesicles. The vesicles shrink until the liquid phase disappears and, in the case of GUVs presenting liquid/gel-phase separation (for *C. atrox* sPLA$_2$), only the solid domains remain at the end of the shrinkage processes. Binding experiments show homogeneous binding of the *C. atrox* sPLA$_2$ enzyme to the liquid crystal phospholipid domains with no measurable preference for the borders between domains. The hydrolysis kinetic results are consistent with this conclusion.

MATERIALS AND METHODS

Chemicals

Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification: phospholipids used were: 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC); 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (DAPC); 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE); 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE); 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC); PRODAN; LAURDAN; and *N*-Rh-DPPE. These were purchased from Molecular Probes (Eugene, OR). EDTA and Tris (hydroxymethyl) aminomethane hydrochloride (Trizma HCl) were obtained from Sigma Biochemicals (St. Louis, MO).

PLA$_2$ and fluorescent conjugates

The dimeric sPLA$_2$ from *C. atrox* venom (Miami Serpentarium, Punta Gorda, FL) was purified in our laboratory by using published procedures (Hachimori et al., 1971). The monomeric sPLA$_2$ from *N. n. naja* venom was a gift from Dr. Edward Dennis (Dept. Chemistry, University of California, La Jolla, CA). The monomeric sPLA$_2$ from *A. p. piscivorus* venom was a gift from Dr. John Bell (Brigham Young University, Provo, UT). sPLA$_2$ from *N. n. naja* venom was obtained from Sigma, without further purification. The concentration of *C. atrox*, *A. p. piscivorus*, and *N. n. naja* sPLA$_2$ was determine by their extinction coefficients of ϵ_{280} = 25,000 M^{-1} cm^{-1} (Brunie et al., 1985), ϵ_{280} = 30,800 M^{-1} cm^{-1} (Bell et al., 1996) and ϵ_{280} = 29,300 M^{-1} cm^{-1}(Darke et al., 1980), respectively.

The fluorescein conjugates of the *C. atrox* sPLA$_2$ were prepared by labeling the enzyme with fluorescein succinimidyl ester (Molecular Probes) as previously described (Sanchez et al., 2001). Conjugates were routinely labeled with an average of 1 fluorescein (ϵ_{499} = 70,000 M^{-1} cm^{-1} (Jablonski et al., 1983) per sPLA$_2$ monomer. The sPLA$_2$ specific activity was measured using a pH-stat, and mixed micelle assay at pH = 8.0 (Dennis, 1973). No significant differences in enzyme activity between the fluorescein-labeled and unlabeled sPLA$_2$ species were found.

Vesicle preparation

Phospholipid stock solutions were prepared in chloroform at concentrations of 0.2 mg/ml. In our experiments we formed GUVs with POPC, DMPC, and DPPC and the binary mixtures DMPE/DMPC (7:3 molar ratio) and DLPC/DAPC (1:1 molar ratio).

The electroformation method, developed by Angelova and Dimitrov (Angelova and Dimitrov, 1986; Angelova et al., 1992; Dimitrov and Angelova, 1987) was used to prepare the vesicles. GUVs were formed in a temperature-controlled chamber that allows a working temperature range from 9°C to 80°C (Bagatolli and Gratton, 1999, 2000a). GUVs were prepared using the following steps: ~2 μl of the lipid stock solution was spread on each of the two sample chamber platinum wires under a stream of dry N_2. The chamber was then lyophilized for ~1 h to remove any remaining trace of organic solvent. The chamber and the buffer (Tris 0.5 mM, pH = 8) were separately equilibrated to temperatures above the lipid mixture phase transition(s) (~10°C over the corresponding transition temperature) and then 4 ml of buffer was added to cover the wires. Immediately after buffer addition, the platinum wires were connected to a function generator (Hewlett-Packard, Santa Clara, CA) and a low-frequency alternating field (sinusoidal wave function with a frequency of 10 Hz and an amplitude of 2V) was applied for 90 min. The AC field was turned off after GUVs were formed and the temperature was adjusted to the desired temperature. In the binary lipid mixtures the temperature was decreased to the gel/fluid phase-coexistence temperature regime (44°C for DMPE/DMPC and 46.6°C for DLPC/DAPC). The temperature was measured

inside the chamber at the platinum wires, using a digital thermometer (model 400B, Omega, Stamford, CT) with a precision of 0.1°C. The experiments were carried out in the same chamber after the vesicle formation with an inverted fluorescence microscope (Axiovert 35, Zeiss, Thornwood, NY). The vesicles remained attached to the platinum wires, which allowed us to perform the particular experiments on single GUVs without vesicle drifting. A charge-coupled device video camera (CCD-Iris, Sony, Tokyo, Japan) was used to follow GUV formation and to select the target vesicle. For experiments involving LAURDAN and PRODAN, fluorophore (in dimethyl sulfoxide (DMSO)) was added to the sample chamber after the vesicles were formed. The final bilayer phospholipid:fluorophore ratio was kept greater than 100:1. The vesicles were homogeneously labeled within 15 min of fluorophore addition. The final concentration of DMSO in the sample was $<0.1\%$. In the case of *N*-Rh-DPPE, the fluorescent phospholipid was premixed in chloroform with the primary lipid(s) and spread onto the sample chamber wires before GUVs were formed. The concentration of *N*-Rh-DPPE in the samples was 0.5 mol%.The mean diameter of the GUVs was $\sim 30\ \mu$m, as previously reported (Bagatolli and Gratton 2000a, 2000b).

Two-photon intensity images

Images were collected on a scanning two-photon fluorescence microscope designed in our laboratory (So et al., 1995, 1996). A LD-Achroplan 20X long working distance air objective with a N.A. of 0.4 (Zeiss, Holmdale, NJ) was used. A mode-locked titanium-sapphire laser (Mira 900, Coherent, Palo Alto, CA) pumped by a frequency-doubled Nd:vanadate laser (Verdi, Coherent) set to 780 nm, was used as the two-photon excitation light source. A galvanometer-driven *x-y* scanner was positioned in the excitation path (Cambridge Technology, Watertown, MA) to achieve beam scanning in both the *x* and *y* directions. The samples received from 5 to 9 mW of 780 nm excitation light, and a frame rate of 9 s/frame was used to acquire the 256×256 pixel images. A quarter wave-plate (CVI Laser Corporation, Albuquerque, NM) was aligned and placed before the light entered the microscope to minimize the polarization effects of the excitation light. The fluorescence emission was observed through a broad band-pass filter from 350 nm to 600 nm (BG39 filter, Chroma Technology, Brattleboro, VT). A miniature photomultiplier (R5600-P, Hamamatsu, Bridgewater, NJ) was used for light detection in the photon counting mode. A commercialized version (ISS card) of a card designed in our lab (Eid et al., 2000) was used to acquired the counts. A two-channel detection system was attached for generalized polarization function (GP) image collection (see below for details).

The $sPLA_2$, either native or labeled with fluorescein, was prepared in the buffer used to prepare the vesicles (0.5 mM Tris pH = 8). Ca^{2+} or Ba^{2+} was added to the buffer as needed for the specific protocol. The desired amount of PLA_2 was then deposited with a microinjector needle or with a pipette into the chamber containing the target GUV. The addition of buffer and salts in the absence of the $sPLA_2$ was used as the control. For the binding experiments, the GUV images were collected following the addition of the fluorescein-labeled $sPLA_2$ (in presence of Ba^{2+}). Imaging the fluorescein-labeled $sPLA_2$ bound on the GUV surface required integration of $\sim 10-80$ frames (9 s/frame) because of the low fluorescence intensity from the bound fluorescein-$sPLA_2$. The lipid phase-state of the enzyme-bound region was determined through the addition of PRODAN, which shows a preferential partition into the lipid fluid phase (Bagatolli and Gratton, 2000b; Krasnowska et al., 1998). For the protein activity experiments, successive images of LAURDAN and *N*-Rh-DPPE labeled GUV were taken immediately after the addition of the active $sPLA_2$ (in presence of Ca^{2+}). Single lipid vesicles, LAURDAN-doped and *N*-Rh-DPPE-labeled, were sufficiently bright to image with one frame.

Lipid domain area calculations were carried out using procedures written within Igor (Wave Metrics, Lake Oswego, OR). Pixels were defined as "gel" or "liquid" by their GP values and summed to determine the corresponding areas (see below for GP description).

LAURDAN GP measurements

LAURDAN was used as a membrane probe because of its ability to report the extent of water penetration into the bilayer surface. Water penetration has been strongly correlated with lipid packing and membrane fluidity (Parasassi et al., 1990, 1998; Parasassi and Gratton, 1995). The emission spectrum of LAURDAN in a single phospholipid bilayer is centered at 490 nm when the membrane is in the gel phase and at 440 nm when in the liquid crystalline phase. The GP gives a mathematically convenient and quantitative way to measure the emission shift. The function is given by:

$$GP = \frac{I_B - I_R}{I_B + I_R}$$

where I_B and I_R are the blue (440 nm) and red (490 nm) emission intensities, respectively. A full discussion on the use and mathematical significance of GP can be founded in the literature (Bagatolli and Gratton, 1999; Parasassi et al., 1991). In our two-photon, dual-channel instrument the excitation wavelength used for the two-photon images was 780 nm. The fluorescence was split into red and blue channels by using a Chroma Technology 470DCXR-BS dichroic beam splitter in the emission path. Interference filters, either an Ealing 490 or an Ealing 440, were placed in the appropriate emission paths to further isolate the red and blue parts of the emission spectrum. Separate detectors were used for each channel to simultaneously collect the 490 nm and 440 nm emission. Corrections for the wavelength dependence of the emission detection system was accomplished through the comparison of known solutions (LAURDAN in DMSO or LAURDAN in DMPC vesicles at 20°C (Bagatolli and Gratton, 1999)), taken on an ISS Inc (Champaign, Urbana, IL) model PC1 steady-state fluorometer. The separate red and blue images were simultaneously collected and then recombined to form the GP image of the sample.

RESULTS

Interaction of *C. atrox* $sPLA_2$ with single lipid GUVs

GUVs were visualized with the membrane fluorophore LAURDAN during experiments on $sPLA_2$ hydrolysis. LAURDAN integrates into the membrane and emits with sufficient intensity to follow the changes produced on the GUVs after the addition of the $sPLA_2$. LAURDAN also allows us to quantify the fluidity changes in the membrane by measuring GP (Parasassi et al., 1991). Fig. 1 *a* shows the time sequence of fluorescence intensity images obtained before and after the addition of dimeric *C. atrox* $sPLA_2$ to DPPC GUVs at 53°C in the liquid phase. Calcium cofactor, essential for enzymatic activity, was added together with the enzyme. After the addition of the protein, the vesicle shrinks until there is virtually nothing of the vesicle remaining. Time-dependent changes in the estimated surface area of the DPPC GUV as hydrolysis proceeds are plotted in Fig. 1 *b*. Shrinking responses were also observed with DMPC (Fig. 2) and POPC (data not shown) vesicles.

GUV membrane-phase state was evaluated during the hydrolysis process through the LAURDAN GP images. A series of GP images that follow the time course for $sPLA_2$ hydrolysis of a DMPC vesicle cluster is shown in Fig. 2. The GUV cluster was equilibrated at 26.1°C, several degrees above the main transition phase for this phos-

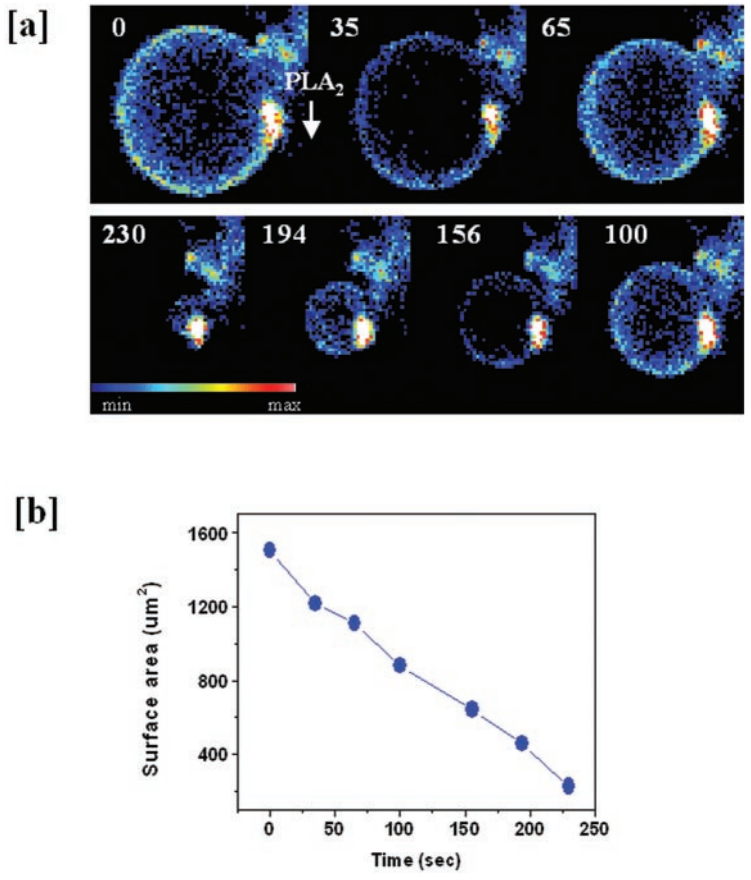

FIGURE 1 PLA$_2$-dependent shrinking of a DPPC GUV at 53°C is shown. Fluorescence intensity images of LAURDAN-containing DPPC GUV were collected before and after the addition of *C. atrox* sPLA$_2$. The point of enzyme addition (10 μl of 8.5$\times$ 10^{-6} M sPLA$_2$ in 100 μM Ca^{2+}) is noted by an arrow. The images are shown using false color representation according to the figure scales, and the numbers indicate the time in seconds. The vesicle radii, in pixels, were determined directly from the vesicle cross-sections. The surface areas were then calculated assuming a spherical shape, and a plot of the surface area as a function of time is shown in (*b*).

pholipid. The GP images (Fig. 2) show an increase in the observed average membrane GP from 0.37 to 0.47 (Fig. 3, *a* and *b*) as the bilayer phospholipids are hydrolyzed. The GP histograms for these images have a clear GP center and distribution, which can be plotted as a function of time (Fig. 3 *b*). The width of the distribution remains, within experimental error, constant throughout the hydrolysis process.

The binding of the sPLA$_2$ to the single lipid GUVs was observed using a fluorescein-labeled *C. atrox* sPLA$_2$ conjugate. Because sPLA$_2$ activity on the GUV's interface would alter the membrane character, which would influence the sPLA$_2$ interfacial binding, Ba^{2+}, a mimic of the Ca^{2+} cofactor, known to inhibit enzymatic activity but permit interfacial association (Yu et al., 1998, 1993), was used in the binding studies. The association of the fluorescein-sPLA$_2$ conjugate to single lipid vesicles in the liquid phase is shown in Fig. 4. The cross-section and surface images of POPC and DPPC vesicle were obtained by changing the position of the *z*-focus in the microscope. Under conditions in which the phospholipids were in the liquid crystalline state, the signal from the *C. atrox* fluorescein-labeled sPLA$_2$ was found to evenly cover the POPC and DPPC vesicle surfaces.

Interaction of *C. atrox* sPLA$_2$ with mixed lipid GUVs

The effect of enzyme activity toward mixed-lipid GUVs was examined using *N*-Rh-DPPE, which has been shown to partition into the liquid domains in these binary lipid GUVs (Bagatolli and Gratton, 2000b). Images were collected after the addition of sPLA$_2$ to single GUVs composed of DMPE/DMPC (Fig. 5 *a*) and DLPC/DAPC (Fig. 5 *b*) at 44°C and 46°C, respectively. For both lipid mixtures, and before the addition of the enzyme, the GUVs displayed leaf-shaped gel domains surrounded by a fluid phase. This phase separation is stable if the temperature is kept constant, in agreement with previous observation (Bagatolli and Gratton, 2000b). After the addition of the sPLA$_2$ (*arrow* in Fig. 5, *a* and *b*), the vesicles were observed to decrease in size to a small mass of lipid. For both binary lipid mixtures, as the vesicle size decreased, the solid domains became deformed and the fluorescent (liquid) domains disappeared. Fig. 6, *a* and *b*, shows the time-dependent changes of the area corresponding to the total, the gel and the fluid regions of the fluorescent images illustrated in Fig. 5. A faster decrease of the liquid crystalline phase with respect to the gel phase is observed in both mixtures. The time needed to eliminate the

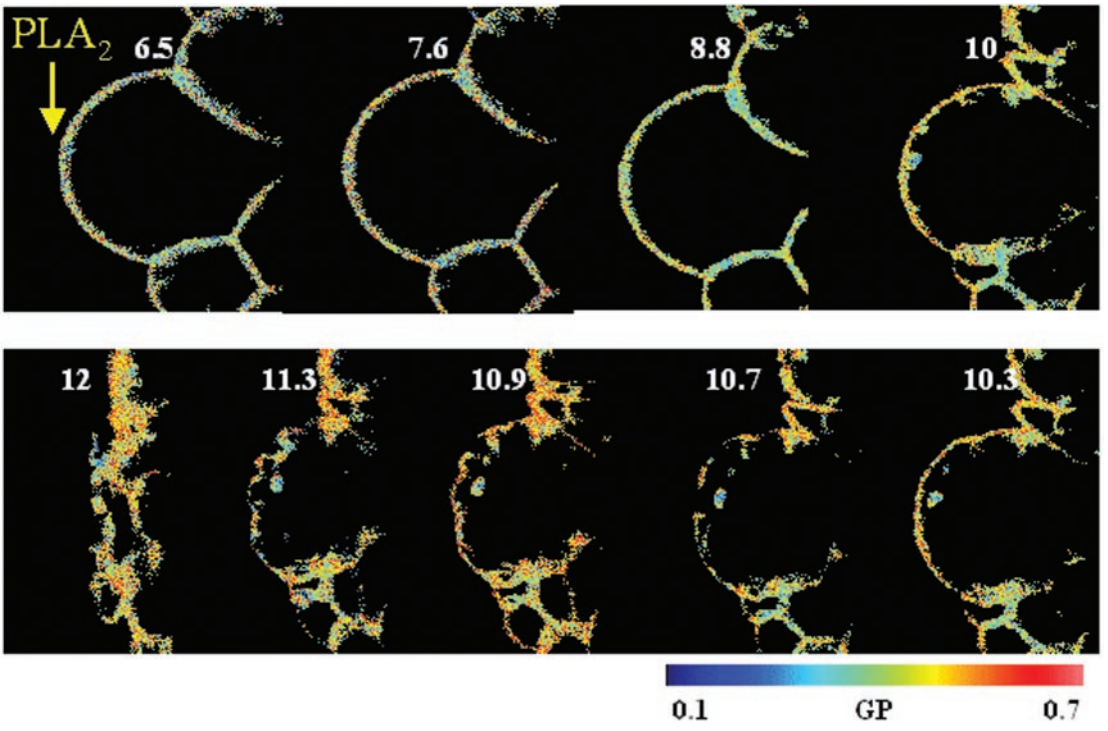

FIGURE 2 Time-dependent changes in the LAURDAN GP of DMPC GUVs after the addition of *C. atrox* $sPLA_2$. Numbers indicates the time in minutes, and the arrow indicates enzyme addition (of 7×10^{-7} M $sPLA_2$ in 100 μM Ca^{2+}). The temperature was kept constant at 26.1°C during the experiment. The GP images are obtained by processing the simultaneously collected red and blue images as described in the Materials and Methods section.

liquid crystalline part of the vesicle was ~300 s for both experiments.

Direct observation of $sPLA_2$ binding to the GUVs was monitored using a fluorescein-labeled $sPLA_2$ (with Ba^{+2}). Labeled enzyme was added to unlabeled GUVs composed of DMPE/DMPC (7:3 molar ratio) and DLPC/DAPC (1:1 molar ratio) mixtures at temperatures corresponding to the gel/fluid phase coexistence. After addition, the enzyme was found to decorate a defined region of the binary GUVs (Fig. 7, *a* and *b*, left panel). To determine the phase-state of the protein-labeled membrane region, PRODAN was added to the vesicle immediately after $sPLA_2$ binding was observed. In a bilayer membrane, PRODAN partitions into the lipid liquid crystalline phase 35-fold greater than the lipid gel phase (Bagatolli and Gratton, 2000b; Krasnowska et al., 1998). PRODAN was found to label the same GUV region that contained the fluorescein-$sPLA_2$, indicating that the $sPLA_2$ was binding to the liquid crystalline lipid domains (Fig. 7, *a* and *b*, right panel).

DISCUSSION

C. atrox $sPLA_2$ activity and binding on GUVs

Single lipid GUVs

The primary effect of *C. atrox* $sPLA_2$ on DPPC GUVs (at 53°C in the liquid phase) or DMPC GUVs (at 26.1°C in the liquid phase) was to gradually diminish the size of the target vesicle (Figs. 1 and 2). The binding experiment (Fig. 4) would suggest that the activity derives from $sPLA_2$, which is evenly bound across the vesicle (Fig. 1 *b*). The monomeric $sPLA_2$ isolated from *A. p. piscivorus* (data not shown) also produced shrinkage of the GUVs in this manner. In contrast, an article by Wick et al. (1996) had reported that the $sPLA_2$ from *N. n. naja* venom caused vesicle shrinking when the enzyme was injected inside a GUV, but that the vesicles would rupture if the enzyme was added to the outside of the vesicle, as was done here. We could successfully reproduced the vesicle rupturing using *N. n. naja* $sPLA_2$ from Sigma Biochemicals, also the source of the authors' $sPLA_2$. We tested a second source of *N. n. naja* $sPLA_2$ (Dr. E. Dennis, UC at San Diego) and found vesicle shrinking, not rupture. This discrepancy might be explained by the presence of contaminating lytic factors that had been found to co-purify with *N. n. naja* venom $sPLA_2$ (Vogel et al., 1981). These factors work at trace levels with $sPLA_2$ to lyse red blood cells and may be responsible for the observed GUV rupturing effect. The small internal volume might explain why Wick et al. did not observe the vesicle collapse when injecting the $sPLA_2$ into the vesicle. As hydrolysis begins, the fatty acid produced would quickly lower the GUV's internal pH, which would then inhibit the activity of the $sPLA_2$ and lead presumably to reduced activity. In contrast, $sPLA_2$ hydrolysis on the exterior surface would

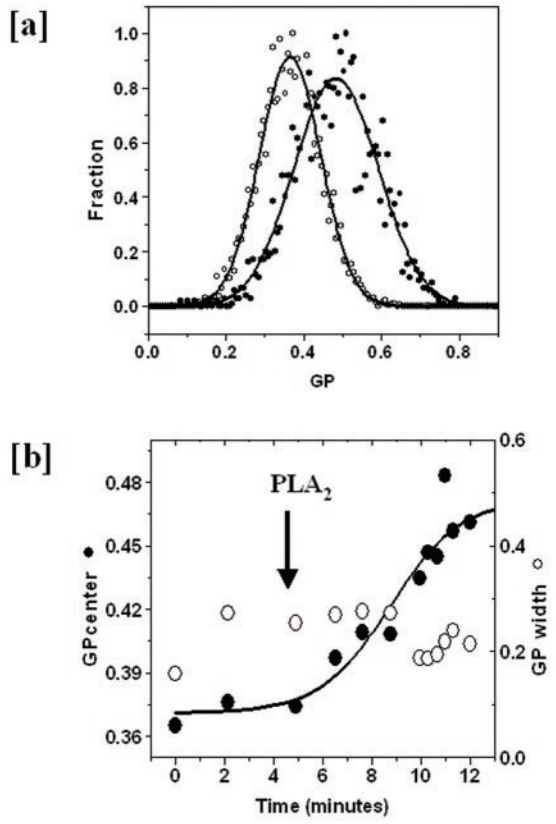

FIGURE 3 The membrane GP histograms shown were calculated from the images in Fig. 2. In (*a*) the GP histograms sPLA$_2$ before sPLA$_2$ addition (○) (first image in Fig. 2) and during the hydrolysis but before vesicle collapse (●) (second to last image in Fig. 2). The Gaussian center and width of the GP histograms are shown as a function of time in (*b*).

dilute the pH change over a larger external volume. It has been our observation that the shrinking effect is the common expression of the sPLA$_2$ enzymatic activity for these three sPLA$_2$ and is independent of the lipids used to form the GUVs.

A second observation on the hydrolysis of membrane phospholipids by sPLA$_2$, which may be relevant here, is the activity lag phase (Bell and Biltonen, 1989; Bell et al., 1996; Henshaw et al., 1998). The lag phase is a phenomenon observed for a variety of secreted PLA$_2$s, which manifests as a delay period marked by low lipid hydrolysis rates and then followed by a sudden burst of activity. In the GUV membrane model system, the time for the shrinkage to start and the total time for the vesicle disappearance varies from GUV to GUV (data not shown). If this is attributable to the lag phase, the variability is not surprising if we consider that previous studies have been performed in bulk solution and correspond to an average time. In this report, we are measuring the response of individual vesicles and our results may not be directly comparable with an average response. However, the advantage in looking at single vesicles is akin to single molecule approaches, in that we can look at a single vesicle and piece together what constitutes the "average" response. In this manner we can potentially get a more complete picture of the basic molecular behavior of these processes. With this in mind, one can attribute the fact that not all vesicles behave similarly to be an inherent variability in the vesicles themselves. We have looked at vesicle shape and LAURDAN GP values, but, at the present time, we can not distinguish between vesicles with short lag phases (8, 17, 37 s) and vesicles that have long delay times (2–5 min). In some cases, a few vesicles appeared to be impervious to sPLA$_2$ attack, suggesting that the GUVs, although made in what one would consider identical conditions, can still show a range of behaviors.

In the hydrolysis process, it is of interest to examine the change in membrane composition and/or organization during sPLA$_2$-dependent hydrolysis. An article by Bell et al. (Henshaw et al., 1998) had examined this process in bulk solution with LUVs using the membrane probes LAURDAN and PRODAN. The authors concluded that LAURDAN was more sensitive to later stages of hydrolysis when more profound changes in vesicle structure occurred. Our goal here was to use LAURDAN GP and intensity measurements to examine the time-dependent and spatial distribution of the sPLA$_2$-induced membrane effects on a single vesicle. As hydrolysis proceeded, the average membrane GP increased from 0.37 to 0.47, indicating an overall decrease in membrane polarity. The image GP values are organized into GP histograms in Fig. 3 *a*. These data generally agree with the bulk solution studies carried out by Bell and coworkers (Bell et al., 1996; Henshaw et al., 1998; Wilson et al., 1999), who reported LAURDAN GP changes during hydrolysis profiles for DPPC vesicles. In our case, however, we can visualize individual vesicles and determine what specific changes may be occurring at the vesicle surface. The detailed look of the GP images in Fig. 2 shows the progressive formation of small solid domains (orange dot accumulations) telling us that the average GP values in Fig. 3 *b* are the consequence of small product-rich domains formed as the hydrolysis proceeds. Presumably, the fatty acid and lysolipid that remain in the bilayer form the basis for these small, high-GP regions. A high GP is indicative of low water penetration. An increase in lipid order, domain formation, or interdigitation of the hydrolysis products in the surrounding phospholipids would be consistent with these results. This observation agrees with work on monolayer studies by Grainger et al. (1989, 1990). In their studies the authors used surface pressure to create mixed domain monolayers and explored the interaction, binding, and hydrolysis, with *N. n. naja* sPLA$_2$. They concluded that the enzyme hydolyzes the lipid to form fatty acid domains that sPLA$_2$s then preferentially bind. Our hydrolysis-induced, higher-GP domains (Fig. 2) are consistent with this hypothesis, but we have not as yet been able to identify preferential binding of the *C. atrox* sPLA$_2$ to these regions. It should be noted that the *C. atrox* sPLA$_2$ is one of the few dimeric

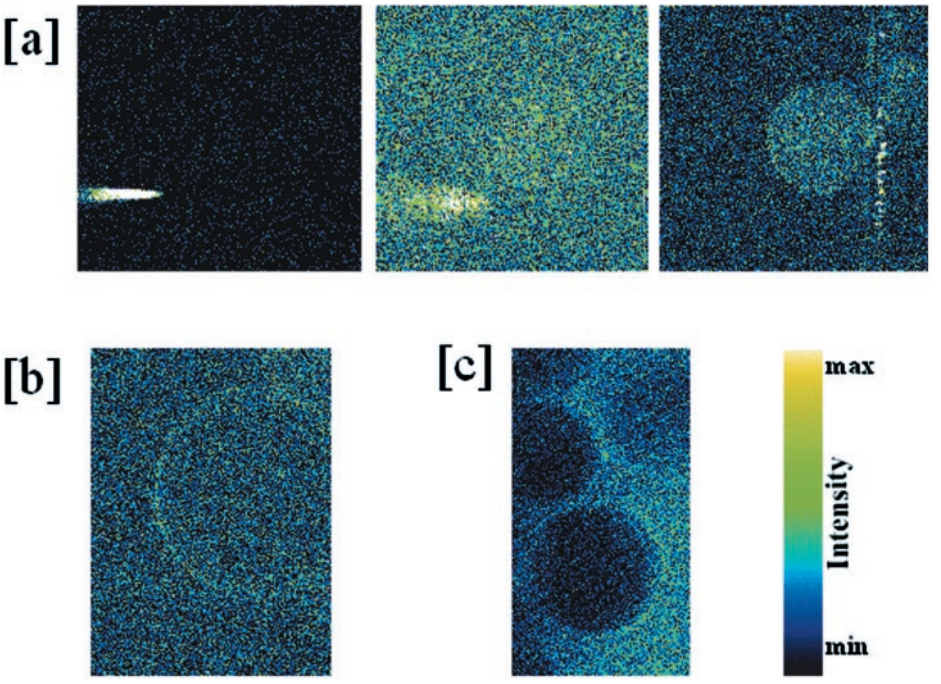

FIGURE 4 Two-photon excitation images of single lipid GUVs after addition of *C. atrox* fluorescein-sPLA$_2$. (*a*) Sequence of events when a microinjector needle was used to spread the labeled protein on the surface of a POPC GUV; the needle is located close to the target vesicle (just the needle containing the fluorescent protein can be seen) (*left panel*), the labeled enzyme is spread on the GUV surface which can already be visualized as a diffuse bright region in the center panel, and the needle is removed and the homogeneous labeled GUV is observed after some of the excess labeled protein has diffused away (*right panel*). (*b*) The cross-section view of POPC vesicles after the addition of 63×10^3 fmol (added by microinjector) of *C. atrox* sPLA$_2$ 8×10^{-6} M in buffer containing 150 mM Ba^{2+} on the GUV surface. Temperature was maintained at 51°C. (*c*) DPPC vesicles after the addition (added by pipette) of 20 μl of sPLA$_2$ 3.2×10^{-6}M in 100 μM Ba^{2+}.

sPLA$_2$s and may have unique membrane-association properties because of its dimeric structure.

Our observation that the enzyme binds to liquid crystalline phase phospholipid is counter to the conclusions of the monolayer work mentioned above and also of fluorescence studies by Bell et al. (1996). Both of these groups have found binding of sPLA$_2$ to the gel lipid state. We have not observed this with the *C. atrox* sPLA$_2$. Although the monolayer interface may differ in many respects to a true unsupported bilayer, the LUV work reported by Bell would be expected to be similar to the GUV system. Information on the association of sPLA$_2$ with vesicles has come from observed changes in the sPLA$_2$ intrinsic fluorescence and the appearance of energy transfer from a membrane fluorophore probe to a bound sPLA$_2$ tryptophan. Although these data do suggest that the solid lipid phase may promote sPLA$_2$ binding, a report by Gadd and Biltonen (2000) has suggested that the sPLA$_2$ may bind the interface in at least two separate conformations. The authors further demonstrated that sPLA$_2$ can bind to the interface without appreciable change in fluorescence and that earlier studies may not have accurately measured sPLA$_2$ and vesicle interactions. In addition, it has been reported that given the "right sequence of events", DPPC LUVs in the liquid crystalline phase can be instantaneously hydrolyzed at 48°C (Lichtenberg and Biltonen, 1986). We clearly see sPLA$_2$ binding and activity at temperatures in which the phospholipid resides in a liquid crystalline state.

Mixed lipid GUVs

The binding of sPLA$_2$ to lipid interfaces is dependent on many properties of the membrane surface. It has been a generally accepted hypothesis that the secreted PLA$_2$s are particularly active in the presence of transient "membrane defects," and borders between coexisting lipid phases have been postulated to be a source of these defects (Burack et al, 1997a, 1997b; Maloney and Grainger, 1993). To address this issue, we examined two different phospholipid binary mixtures: DMPC/DMPE and DLPC/DAPC. These lipid systems exhibit a broad temperature range in which large fluid and gel phospholipid domains can be observed (Bagatolli and Gratton, 2000a). These conditions are ideal for examining sPLA$_2$ interactions with the different lipid domains in single, free-standing bilayers. One of the recurrent questions in this respect is that concerning the preferential interactions of this enzyme with different lipid domains or with the lipid domain borders. Our experimental approach can help to answer this question by direct visualization of the enzyme on unsupported lipid bilayers (GUVs).

The two-photon excitation intensity images obtained using *N*-Rh-DPPE and LAURDAN-labeled mixed lipid GUVs (Fig. 5) show the shrinking of the GUV, as observed in the single lipid GUVs, and a preferential hydrolysis of the fluid lipid domains. Visualizing the domains through the presence (liquid phase) and absence (gel phase) of rhodamine-DPPE after addition of active sPLA$_2$ (Fig. 6) strongly suggested that the fluid-phase domains are hydrolyzed faster than the solid-phase domains. This observation was made with both the DMPE: DMPC and the DLPC:DAPC lipid binary mixtures. Consistent with these results, a preferential binding of *C. atrox* sPLA$_2$ to the fluid phase domains was also observed (Fig. 7). The most straightforward interpretation of these data would be to conclude that the dimeric *C. atrox* sPLA$_2$ has a greater affinity for the less packed liquid

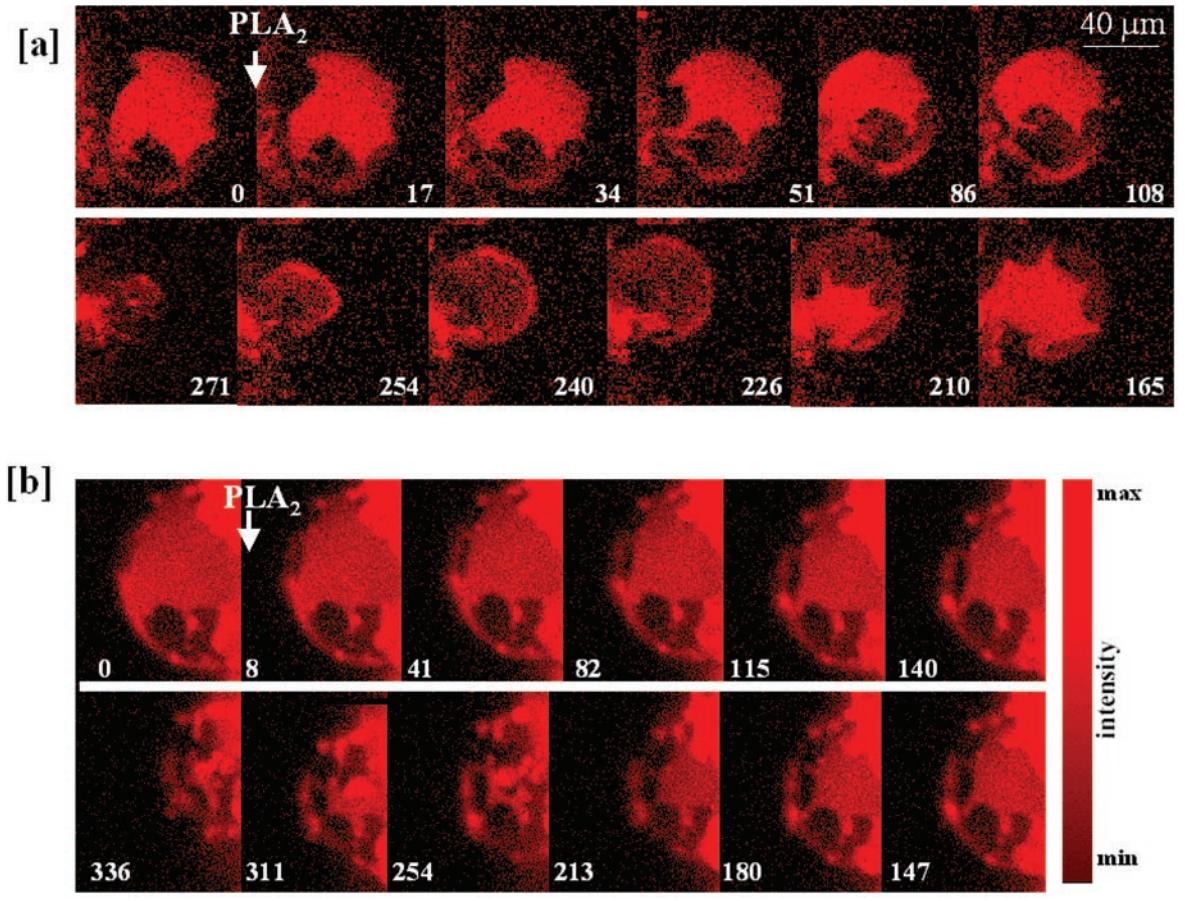

FIGURE 5 Series of two-photon excitation images of DMPE:DMPC (*a*) and DLPC:DAPC (*b*) GUVs before and after the addition of a *C. atrox* sPLA$_2$. The red and the dark areas in the vesicles correspond to the fluid and gel phases, respectively (false color representation is according to the scale on the bottom of the figure). The fluorescent images were taken at the vesicle surface. (*a*) The addition of 15 μl of sPLA$_2$ 2 mg/ml in 150 mM Ca^{2+} at 44°C. (*b*) The addition of 20 μl of sPLA$_2$ 2 mg/ml in 150 mM Ca^{2+} at 46°C. *N*-Rh-DPPE final concentration was 0.5%. Numbers indicate the time in seconds.

phospholipid domains and by its presence then hydrolyzes these regions directly. The more closely packed gel domains of either the DMPE or DAPC seemed to resist significant binding and were not hydrolyzed, or hydrolyzed as fast, as the liquid regions. Thus, our findings suggest that for interfaces composed of zwitterionic polar head groups phospholipids (PC and PE at pH 8) the phase state of the lipid domains, and not the nature of phospholipid headgroup, is the critical factor for *C. atrox* sPLA$_2$ association (Figs. 5–7).

A more subtle point suggested by our data is that the activity of the enzyme is uniform over the area of the liquid crystalline phase and not restricted to the border regions between the domains. To support this point we note that for vesicles composed of only one lipid, Fig. 1, the surface area, which is proportional in this case to the amount of lipid, decreases linearly with time. This observation indicates that the hydrolysis reaction, under the given conditions, is zeroth order. For vesicles composed of two phospholipids and showing phase coexistence (Fig. 6), the kinetics of the reaction is similar, i.e., the surface area of the fluid phase is approximately linear with time and it occurs during a comparable time scale. In addition, the shape and size of the solid domains do not change appreciably during the hydrolysis until after much of the liquid domain has been removed. The most straightforward interpretation of these data is that hydrolysis occurs homogeneously across the liquid domains, rather than solely at the domain borders. Therefore, we conclude that in this system, the hydrolysis proceeds through a primarily area-dependent process and not an exclusively perimeter-dependent process. Our binding studies support this interpretation, showing that the enzyme homogeneously binds the liquid domain. We would like to note that these results should not necessarily be extrapolated to all sPLA$_2$-bilayer systems, as the particular phospholipid mixtures and the influence of the hydrolysis products on the domains could influence the kinetics. In our case, much of the product leaves the membrane because of partitioning and is not present in sufficient quantities to significantly alter the hydrolysis rates (although our GP data shows at least some level of product retention).

Our observations, homogenous binding and hydrolysis of the liquid domains, conflict with earlier results reported in

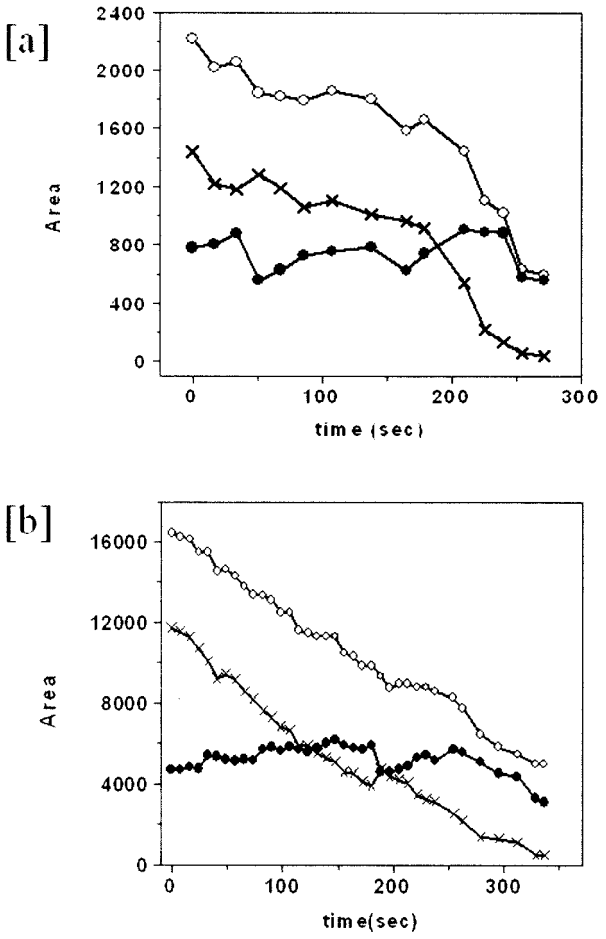

FIGURE 6 Time-dependent changes of the area corresponding to the total (○), the gel (●) and the fluid (black Xs) regions of the *N*-Rh-DPPE-labeled GUVs fluorescent images from Fig. 5. (*a*) DMPE/DMPC 7:3 mol at 44°C. (*b*) DLPC/DAPC 1:1 mol at 46°C.

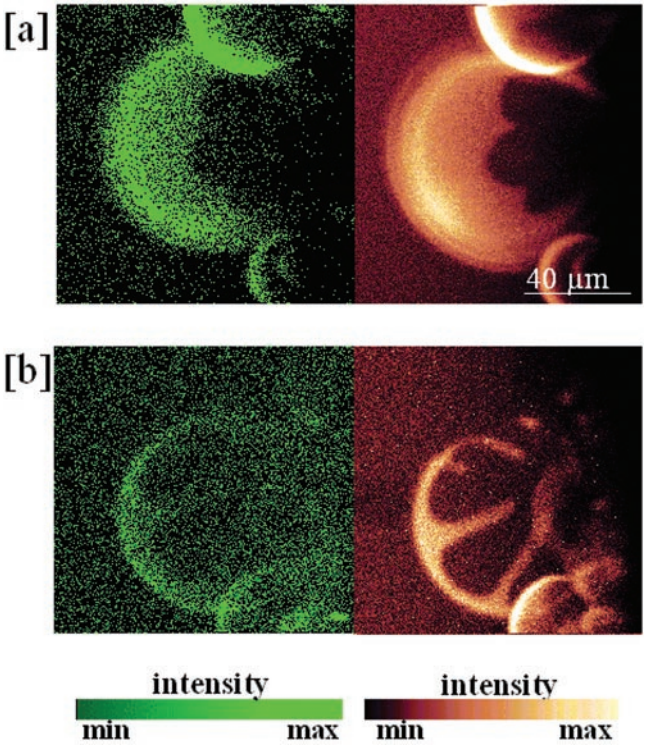

FIGURE 7 Two-photon excitation images of (*a*) DMPC/DMPE 7:3 mol GUV at 46.6°C and (*b*) DLPC/DAPC 1:1 mol GUV at 48.1°C after the addition of 10 μl of *C. atrox* sPLA$_2$ fluorescein-labeled (12×10^{-6} M in buffer containing 150 mM Ba^{2+}) (*left panel*) and after PRODAN addition (*right panel*). The fluorescent images were taken at the vesicle surface with the false color representation denoted in the figure scale.

the literature based on data collected using a monolayer membrane model system. The general view from those studies has been that the solid phase DPPC is hydrolyzed by sPLA$_2$ and that the sPLA$_2$ preferentially binds to this phase (Grainger et al., 1989, 1990; Reichert et al., 1992). In these studies DPPC, DMPC, and DMPE monolayers were formed and kept at conditions in which a single phospholipid demonstrates both liquid and solid phases. Phospholipid monolayers studies have provided an interesting and well controlled environment to study sPLA$_2$. However, it is far from clear whether the domains formed in a monolayer, through lateral pressure, and at temperatures below the main lipid phase transitions (10°C for DMPC, 30°C for DPPC), are similar to those formed in unsupported bilayers as studied here (GUVs). The observation that bilayer phospholipids show strong interleaflet communication suggests significant differences in phospholipid packing for the bilayer and monolayer systems (Bagatolli and Gratton, 2000b). Even subtle differences in the lipid packing could strongly influence the interaction of sPLA$_2$ with the lipids. The lack of a clear relationship between the two model systems makes an accurate comparison of the existing reports problematic.

To support our observations, many reports on PLA$_2$ activity show that sPLA$_2$s will hydrolyze phospholipid vesicles in the liquid crystalline phase temperature regime and, therefore, we must assume that this enzyme also binds to the liquid phase. Along these lines, an interesting work by Liu and Chong (1999) not only demonstrated sPLA$_2$ hydrolysis of liquid-crystalline phase phospholipid-cholesterol vesicles but also clearly showed that the regular superlattice packing of the membrane phospholipids interferes with the enzyme's activity. In their work, the authors varied the cholesterol concentration of their vesicles and found that at certain critical cholesterol mole fractions activity of sPLA$_2$ precipitately decreased. They interpreted their results in terms of a regular packing of cholesterol into hexagonal or centered rectangular superlattices to which sPLA$_2$ could not effectively bind.

Reports on the influence of mixed lipid vesicles, SUVs, or LUVs, containing domain coexistence on sPLA$_2$ hydrolysis and binding have not been common. One report on the activity of sPLA$_2$ on mixed chain length lipids found that the short chain lipids were hydrolyzed preferentially to the

long chain lipids in SUVs (Gabriel and Roberts, 1987). This result is consistent with our DLPC:DAPC mixed GUV results. Unfortunately, SUVs have a much higher curvature than large vesicles which directly influences the hydrolysis kinetics of the sPLA$_2$s and thus the result may be particular to the SUVs and not an accurate prediction of events on GUVs. At present, we can only report our observations and suggest that differences in the nature of the bilayers and of the enzymes must be the source of the discrepancies between this work and others.

Morphology changes in GUVs

Our primary observation of sPLA$_2$-dependent GUV morphology change has been the shrinking response to sPLA$_2$. Gradual loss of vesicle size because of sPLA$_2$ is surprising. Historically, the general view in PLA$_2$ research is that membranes, normally SUVs, LUVs, or erythrocytes, remain intact and unaffected as sPLA$_2$ hydrolysis of the outer leaflet phospholipids proceeds (Gul and Smith, 1974; Jain et al., 1991, 1980). In fact, studies on the hydrolysis kinetics of sPLA$_2$ toward vesicle phospholipids have often rested on the assumption that the vesicles will remain intact (Jain et al., 1991). Our results here would bring into question some of these long-standing beliefs.

How general are our observations? Can our observation on GUVs be extrapolated to standard bulk experiments on LUVs? Reduction in GUV dimensions indicates a loss of lipid mass and a dramatic change in vesicle volume. The GUVs in the absence of *C. atrox* sPLA$_2$ are stable for many hours and thus the mass loss clearly must be attributable to the formation of fatty acid and lysophospholipid from enzymatic digestion by sPLA$_2$. Lipid loss is likely to occur through the escape of hydrolysis products, alone or in mixtures with undigested phospholipids, in the form of monomers, micelles, or smaller vesicles. The lysophospholipids have a sensible solubility in the aqueous phase and have been shown to desorb from the membrane surface (Kupferberg et al., 1981; Speijer et al., 1996). Let us consider the DPPC GUVs and LUVs, as DPPC is a common lipid used in vesicle studies. Palmitic acid, which has a lower solubility in the aqueous phase than the 1-palmitate-lysophospholipid, would be expected to remain in the GUV at higher concentrations, but it too must eventually be removed for the vesicle to continue the shrinking process. It is our belief that the vesicle initially shrinks through loss of monomer and micellar lysophospholipid. The loss of lysolipid results in a decrease in the GUV internal volume stressing the GUV bilayer, which serves to further extrude mixtures of fatty acid, lysophospholipid, and phospholipid in the form of micelles or smaller vesicles below the resolution of our microscope. Indeed, the partition coefficient measured for palmitate and that estimated for 1-palmitate-lysophospholipid (Anel et al., 1993; Bent and Bell, 1995; Brown et al., 1993) would suggest that these species will significantly desorb from the membrane under the low concentrations of phospholipid used in our studies, ~0.3 uM. In fact, ~99% of the lysolipid and 70% of the fatty acid would be expected to leave the membrane under equilibrium conditions. In contrast, the phospholipid concentrations generally used for bulk vesicle studies are almost three orders of magnitude and the vesicles should retain most of the hydrolyzed product. In retaining the hydrolysis products, the membranes could very well stay intact, as indicated in a number of studies (Kupferberg et al., 1981). However, care should be exercised, as the partition coefficients are temperature dependent and may be affected by the ionic strength and pH of the buffer used. In the low lipid concentration case, a buildup of products will still occur but will be limited by the rate of hydrolysis, the product desorption rates, and the partition coefficients.

The hypothesis that there may be profound effects on vesicle structure because of PLA$_2$ is not new and has been discussed by Biltonen et al. (Burack et al., 1997a, 1995). Through the use of electron microscopy, ^{13}PNMR, and light-scattering techniques, Biltonen collected data that clearly indicated fundamental changes in bulk LUVs attributable to *A. p. piscivorus* sPLA$_2$ addition. Their data are consistent with the observed shrinking we report here and suggest that LUVs and GUVs have similar properties with respect to sPLA$_2$ hydrolysis and vesicle morphology change.

Our results demonstrate that the addition of *C. atrox* sPLA$_2$ to GUVs of single-lipid (DPPC and DMPC) and mixed-lipid at their domain's coexisting temperature (DPPC/DPPE, DLPC/DAPC) shows gross morphological changes, both distortions in the vesicle membrane (data not shown) and shrinking of the vesicle size. These effects will likely disturb the local packing structure of the lipids, potentially affecting the binding and hydrolysis of the surface phospholipids by sPLA$_2$ and thus influence the kinetic profiles collected.

In conclusion, we would like to make two general remarks. First, *C. atrox* sPLA$_2$ is a dimeric enzyme unlike most of the sPLA$_2$s under active investigation and may have its own unique membrane association and interactions. Based on the common opinion that the secreted PLA$_2$s share a common mechanism of hydrolysis, we may speculate that the interaction observed for C. *atrox* sPLA$_2$ and the GUVs may reveal part of that mechanism which is common. However, particular differences depending on the enzyme's origin may change this scenario. We conclude from this study that the *C. atrox* sPLA$_2$ binds to the liquid region of zwitterionic vesicles and hydrolyzes the liquid crystalline phase lipids in a homogeneous fashion. Second, independent of the sPLA$_2$ issues, we show here that two-photon microscopy, as a technique, together with the GUVs as an ideal model for biological membranes, represent a powerful methodology to study the interaction of proteins with organized, unsupported bilayer membranes.

This work is supported by a grant from the National Institute of Health (RR03155 to S.A.S., E.G., and T.L.H.) and Fundacion Antorchas (L.A.B). L.A.B. is a member of the CONICET (Argentina) Investigator Career.

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