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Hydrolytic action of phospholipase A_2 in monolayers in the phase transition region: direct observation of enzyme domain formation using fluorescence microscopy

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Phospholipase A_2, a ubiquitous lipolytic enzyme highly active in the hydrolysis of organized phospholipid substrates, has been characterized optically in its action against a variety of phospholipid monolayers using fluorescence microscopy. By labeling the enzyme with a fluorescent marker and introducing it into the subphase of a Langmuir film balance, the hydrolysis of lipid monolayers in their liquid-solid phase transition region could be directly observed with the assistance of an epifluorescence microscope. Visual observation of hydrolysis of different phospholipid monolayers in the phase transition region in real-time could differentiate various mechanisms of hydrolytic action against lipid solid phase domains. DPPC solid phase domains were specifically targeted by phospholipase A_2 and were observed to be hydrolyzed in a manner consistent with localized packing density differences. DPPE lipid domain hydrolysis showed no such preferential phospholipase A_2 response but did demonstrate a preference for solid/lipid interfaces. DMPC solid lipid domains were also hydrolyzed to create large circular areas in the monolayer cleared of solid phase lipid domains. In all cases, after critical extents of monolayer hydrolysis in the phase transition region, highly stabile, organized domains of enzyme of regular sizes and morphologies were consistently seen to form in the monolayers. Enzyme domain formation was entirely dependent upon hydrolytic activity in the monolayer phase transition region and was not witnessed otherwise.

Introduction

Phospholipase A_2 is a small, water soluble enzyme that catalyzes hydrolysis of an acyl ester bond in glycerophospholipid substrates to produce a free fatty acid and a corresponding lysophospholipid. Phospholipase A_2 from a wide variety of sources, both intra- and extracellularly, has been isolated and characterized as the focus for research in a number of different fields. Its ubiquitous nature, increasing significance in a wide spectrum of biochemical processes, and basic utility as a probe of biomembrane structure has produced an overwhelming amount of information about its enzymatic action (for reviews see Refs. 1–4).

An important aspect of phospholipase A_2 interaction with its substrate is that enzymatic hydrolysis is both regio- and stereoselective. Naturally occurring phosphoglycerides are hydrolyzed exclusively in the 2-acyl position by phospholipase A_2. In addition, except in cases of extremely short acyl chain substrates [1,5], catalysis occurs only with L-isomeric forms (such as naturally occurring lipids that compose biomembranes). Of particular significance is that phospholipase A_2's hydrolytic action, although active against dispersed monomeric phospholipid substrates, manifests itself fully (activation) only against organized lipid interfaces such as monolayers, micelles and bilayers. It is probably for this reason that the exact catalytic mechanism for such a well-documented and exhaustively studied enzyme remains speculative. Despite amino-acid sequence, structural and crystallographic data for several phospholipase A_2 forms [6,7], issues regarding the physical and structural nature of the enzyme and enzyme-substrate complex during activation and catalysis are un-

Abbreviations: Tris, tris(hydroxymethylamino)methane; DPPC, L-α-dipalmitoylphosphatidylcholine; DPPE, L-α-dipalmitoylphosphatidylethanolamine; DMPC, L-α-dimyristoylphosphatidylcholine; DMPE, L-α-dimyristoylphosphatidylethanolamine; lysoPPC, L-α-lysopalmitoylphosphatidylcholine.

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confirmed. In fact, structural studies are a compelling priority [4] and critical to elucidating phospholipase A_2's mechanism of action at interfaces [8]. To date, no highly diffracting crystals of phospholipase A_2 containing either substrate or substrate analogue bound to the enzyme's catalytic site have been reported. Around questions over the basic functional mechanism of the enzyme against its substrate necessarily revolve a number of issues about substrate character: specifically, the phospholipid's water-insoluble nature and the nature of the resulting lipid/water interface.

In contrast to water-soluble enzymes acting against water-soluble substrates, clarification of phospholipase A_2 action against lipids requires not only a knowledge of the substrate but also, because of the organized nature of the substrate, a knowledge of how phospholipase A_2 molecules interact with and physically recognize its interfacially-organized substrate. Although several models of physical association and subsequent catalytic action between phospholipase A_2 and lipid interfaces have been forwarded, based mostly on kinetic evidence (all theories reviewed in Ref. 4), no one theory has yet proven convincingly better than any other. In addition to uncertainty surrounding interfacial activation, mechanisms of enzyme inhibition are also poorly characterized. Because of phospholipase A_2's role as a mediator of inflammatory response and thrombosis as well as membrane turnover (reviewed in Ref. 2), understanding physiological control and regulation of phospholipase A_2 action is essential.

The present study has concentrated on gathering new information on the physical nature of phospholipase A_2 interaction with various phospholipid monolayer interfaces using newly developed optical techniques. Direct optical visualization of phospholipase A_2 hydrolysis of phospholipid domains in monolayers has recently been reported by this group [9], using a combination of epifluorescence microscopy and Langmuir film balance techniques. By virtue of a dual fluorescent labeling method and selective optical filtering, new information about action of phospholipase A_2 against a phospholipid substrate in its liquid-solid phase transition region in monolayers was presented. Selective hydrolysis of solid phase lipid domains was accompanied simultaneously by formation of visible phospholipase A_2 enzyme domains in the monolayer [9].

Optical investigations of enzyme action have now been extended to other phospholipid substrates and show herein that the exact nature of hydrolytic effects depends on the lipid substrate. Results indicate that different lipids showing different packing characteristics and domain morphology in the phase transition regions respond differently to enzyme hydrolysis. Lipid solid domains seem to be more susceptible to hydrolysis at defects and interfacial regions where packing is irregular. Particularly, lipid domains formed by rapid monolayer compression show an entirely different hydrolytic response than slowly-compressed equilibrium shapes, despite effects of time-dependent annealing. Moreover, regardless of the phospholipid, if hydrolysis occurs in the phase transition region, the enzyme systematically aggregates into proteinaceous domains of regular size and morphology in the monolayer. Enzyme domains are possibly mediated by and related to interactions with critical concentrations of hydrolytic end products that influence both the enzyme and monolayer physical states. Kinetic and spectroscopic evidence has indicated that snake venom phospholipase A_2 such as *Naja naja naja* forms dimers and larger aggregates upon hydrolytic activation [8,10,11]. Further evidence that large, visible, regularly ordered, stable enzyme aggregates presented herein could result from allosteric inhibition of phospholipase A_2 by hydrolytic end products are discussed.

Materials

Phospholipase A_2 (*Naja naja*), L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dipalmitoylphosphatidylcholine (L-α-DPPC), D-α-dipalmitoylphosphatidylcholine (D-α-DPPC), L-α-dimyristoylphosphatidylethanolamine (DMPE), L-α-dipalmitoylphosphatidylethanolamine (DPPE) and L-α-lysopalmitoylphosphatidylcholine (lysoPPC) were purchased from Sigma. All phospholipids were of greater than 99% purity and showed single spots by TLC analysis (chloroform/methanol/water (65 : 25 : 4, v/v) as eluting solvent). Palmitic acid was purchased from Fluka (puriss grade) and used as supplied after checking purity in monolayers over pure water at 20 °C. A polymerizable phospholipid analogue, dienoylphosphatidylcholine (dienoyllecithin) containing C-18 alkyl chains, each with butadiene groups conjugated to the glycerol backbone ester linkage in the acyl 1 and 2 positions, was synthesized by extending the methods of Patel et al. [12]. Fluorescein isothiocyanate was purchased from Aldrich and used as supplied. Water for buffers was distilled on glass and purified through a Millipore filtration apparatus (18 Mohm resistivity). Isotonic Tris buffer (10 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, chloroform washed, pH 8.9) was prepared by dissolution of salts in 10-times concentration in acid cleaned glass flasks and diluted with pure water to proper concentration for monolayer subphases for each experiment.

Phospholipase A_2 was dissolved from the supplier's bottle (without further purification) with buffer and labeled with fluorescein isothiocyanate by the method of Nargessi and Smith [13]. Labeled phospholipase A_2 was separated from unreacted label and buffer salts on Pharmacia PD-10 columns (Sephadex G-25M) equilibrated in pure water, and lyophilized under protection from light and heat. Labeling on a statistical basis

provided fluorescein molecules on 2 of an estimated 6 lysine residues [14]. Product yields were always over 90% and were stored in amber glass vials at −22° C. Enzyme solution for monolayer studies were made by dissolving 0.36 mg labeled phospholipase A_2 in 26 ml buffer to make a 0.014 mg/ml solution. Aliquots of 2.1 ml were frozen in polypropylene vials at −22° C until thawed ambiently before use. Labeling of the same enzyme by a similar method was shown to lower the degree of hydrolytic activity depending on the degree of labeling [11]. Experiments on the kinetics of hydrolysis of DMPC monolayers could not distinguish any activity differences before or after labeling, however (method described elsewhere [15]). A fluorescent lipid probe containing Texas red (sulforhodamine) in its headgroup was synthesized using pure dioctadecylamine and sulforhodamine isothiocyanate. This lipid was shown by monolayer measurements to partition preferentially into the fluid phase of lipid monolayers [16].

Methods

A complete description of the epifluorescent microscope and associated Langmuir film balance equipment have been recently published elsewhere [17]. Briefly, a thermostated, motor-driven Langmuir film balance is placed below the objective head of an epifluorescence microscope. Excitation of fluorescent monolayer probe (sulforhodamine) and fluorescein-labeled enzyme is achieved using a high-pressure mercury lamp (100 watt). Discrimination of excitation and emission from each fluorescent probe is regulated by conventional dichroic mirrors and interchangeable cut-off filters. Observation of the emitted fluorescence signal is possible either visually or via a low-level light video camera. Analysis and image recording is achieved via an interfaced processing system. Film balance measurements and manipulations are controlled through an interfaced personal computer using a home-designed, menu-written software program.

Surface flow in the plane of the monolayer was eliminated by constructing a circular mask from Teflon (diameter 20 mm) and inserting it into the subphase below the microscope objective. Size and design were chosen to eliminate meniscal effects on the monolayer focal plane and make local observations independent of trough geometry. The mask contained an inverted V-shaped slot that faced toward the movable barrier of the trough to maintain exchange of localized subphase and monolayer components with the external trough at all times. With this arrangement, flow problems, which can strongly influence film morphology [18], could virtually be eliminated. Flow, over the time scale of observation of the enzyme, is also minimized so that specific areas are monitored undisturbed for hours. Video recording of the monolayer within the mask with each filter through an SIT TV camera was initiated at various time points of film hydrolysis. Photographs shown are taken directly from the video screen.

A dual fluorescent labeling strategy was utilized to distinguish monolayer lipid domain morphology from phospholipase A_2 events occurring in the aqueous subphase under the monolayer [9]. Phospholipid solutions in chloroform (0.6 μM) were doped with 0.5 mol% fluorescent sulforhodamine lipid probe, a probe concentration so dilute that no detectable changes in isotherm behavior of the primary phospholipid component were evidenced. Lipid monolayers were spread over a buffer subphase at temperatures necessary to reach the monolayer liquid-solid phase transition at 22 mN/m for each respective lipid. In this way, the surface pressure for each phospholipid liquid-solid phase transition was held constant. Before monolayer spreading, the surface of the buffer subphase was cleaned by suction. After spreading, solvent was evaporated and the monolayer was immediately compressed at a rate of 2.5 $Å^2$/mol per min for DMPC, DMPE, and DPPE monolayers and both 2.5 and 10 $Å^2$/mol per min for DPPC monolayers. At a surface pressure approx. 6 mN/m below the liquid-solid phase transition (16 mN/m), the barrier was stopped and the enzyme solution in buffer (up to 0.5 ml, 7 μg phospholipase A_2) was injected into the mask subphase from a syringe immersed from behind the barrier. After enzyme injection was completed, monolayer compression was continued into the phase transition region until dark domains of solid phase lipid could be distinguished in a bright field of fluid lipid. Another strategy resulting in a virtually identical effect involved injection of the enzyme into the subphase under the phospholipid monolayers already in the phase transition region, that is, forming lipid solid domains before enzyme addition. This method was found to be unsatisfactory in that surface flow induced by subphase stirring from injection of enzyme visibly perturbed solid lipid domain morphology and aggregated lipid domains. However, subsequent hydrolysis resulted in the formation of enzyme domains having a shape indistinguishable from those obtained with the other method.

After addition of enzyme, the monolayer and subphase were directly observed with the epifluorescence microscope mounted over the Langmuir film balance. The field was observed alternately through two interchangeable cut-off filters, corresponding to signals from the sulforhodamine labeled monolayer (Zeiss filter 487714) and fluorescein-labeled phospholipase A_2 (Zeiss filter 487709), respectively.

Results and Discussion

Monolayer imaging under epifluorescence microscopy

Formation of solid lipid domains during the main phase transition in lipid monolayers has been described,

visualized and analyzed before using a number of different fluorescent microscopy systems [17,19–22]. Suffice it here only to mention that enantiometrically-pure, chiral phospholipids form circular or non-circular equilibrium solid phase domains within the lipid liquid-solid phase transition region in monolayers. Such chiral structures of solid phase lipid phases can be readily distinguished in monolayers using the epifluorescent technique [21,23–26].

Using the epifluorescent microscope system, the fluid lipid phase appears bright through the sulforhodamine filter because of the high concentration of the fluorescent lipid sulforhodamine marker present in the fluid lipid monolayer matrix. Contrast between dark solid domains and bright fluid lipid matrix is due to fluorescent marker exclusion from solid lipid domains. The fluid lipid phase remains greatly marker-enriched, providing high contrast optical imaging of lipid physical states.

Imaging of hydrolysis of DPPC monolayers by phospholipase A_2

DPPC monolayers containing the sulforhodamine lipid probe were compressed over a buffer subphase at 30 °C to its main liquid-solid phase transition region and held at 22 mN/m surface pressure. As recently reported [9], the pressure-area isotherms and DPPC domain morphology in phase transitions were similar, if not identical, in form and time-dependent annealing behavior to those already reported [27,28]. The sulforhodamine-containing L-α-DPPC monolayer in the phase transition region is imaged as dark solid phase L-α-DPPC domains in a bright matrix of fluid L-α-DPPC and sulforhodamine probe (see Fig. 1B). Inversion of the cut-off filter to view fluorescein emission provides no signal (figure not shown, completely dark field). Immediately upon injection of fluorescein-marked phospholipase A_2 into the subphase, however, a diffuse, homogeneous fluorescent signal is seen under the lipid

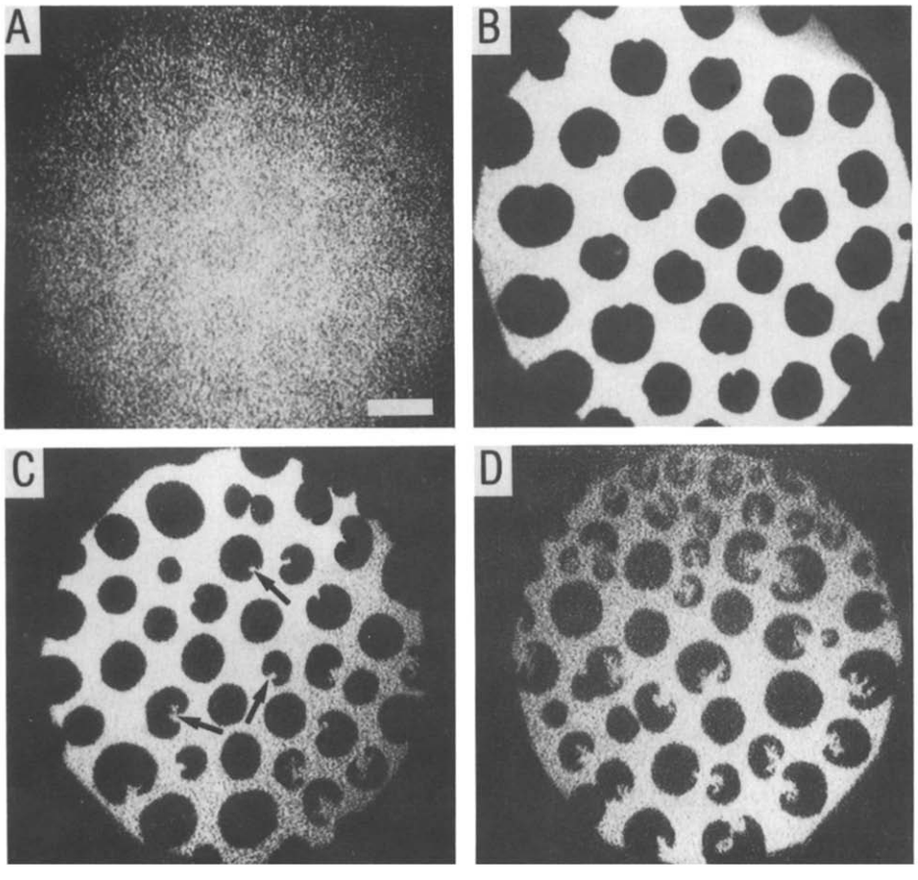


Fig. 1. Epifluorescence imaging of L-α-DPPC monolayer phase transition region after slow compression and subsequent lipid hydrolysis by injection of phospholipase A_2 into the aqueous subphase under the monolayer. All photos are recorded through conventional dichroic cut-off filters as described in Methods. (A) At time zero (immediately after enzyme injection). Imaged through fluorescein filter to observe homogeneous subphase signal of fluorescein-labeled phospholipase A_2. (B) At time zero, but imaged through sulforhodamine filter in L-α-DPPC phase transition region. Dark regions are lipid solid domains in a matrix of bright, fluorescing fluid phase lipid as detailed in text. (C) At 20 min after enzyme injection. View as in (B) through sulforhodamine filter. Arrows indicate attack of phospholipase A_2 at lipid domain discontinuities. (D) At 40 min. View as described in (B) imaged through sulforhodamine filter. Scale bar in (A) is 20 μm. Temperature 30 °C, surface pressure 22 mN/m.

monolayer through the fluorescein filter (Fig. 1A). As seen in Fig. 1A, this fluorescein subphase signal is weaker than that for the corresponding monolayer image seen through the sulforhodamine filter (Fig. 1B). Through the monolayer sulforhodamine filter, no changes in the monolayer state are detected immediately after enzyme addition (figure not shown).

After 20 min, observation of the L-α-DPPC layer through the sulforhodamine filter demonstrates specific evidence of L-α-DPPC solid domain hydrolysis: small, frayed openings in the domain sides (Fig. 1C, see arrows), frequently located at L-α-DPPC domain indentations. As noted before [9], this evidence indicates that active enzyme attaches to the edge of each L-α-DPPC domain throughout the layer though observation of the phospholipase A_2 fluorescent signal at this point shows no apparent changes (figure not shown). Further hydrolysis (Fig. 1D) of lipid solid domains at later times details the specific nature of enzyme action against these types of L-α-DPPC solid domains. That hydrolysis starts consistently at one point on each domain indicates also that certain points on L-α-DPPC domains have unique physical or energetic arrangements of lipid molecules at these locations, an interesting point which will be discussed further in a later section. Furthermore, it is clearly visible that the enzyme starting at one point of each domain hydrolyses its way through the interior in a seemingly regular manner. This can be explained by the fact that once started the enzyme creates a locally high concentration of hydrolysis products (lysoPPC and fatty acid) which autocatalytically activate the protein. In addition, the observation that enzyme molecules are seen to bind to the edges of the domains indicates a preferable binding environment at the liquid/solid interface [9]. Kinetic evidence detailing the enhancement of phospholipase A_2 lipolytic and binding activity in lipid substrate phase transition regions in vesicles [29–32], and accompanying enzyme dimer- and oligomerization in its activated state [8,10,11,33–35] supports optical evidence presented herein. However, activity enhancement in vesicle phase transition regions induced by temperature cycling has usually been kinetically determined. The techniques in this study are unique in that more physically precise, isothermal monolayer

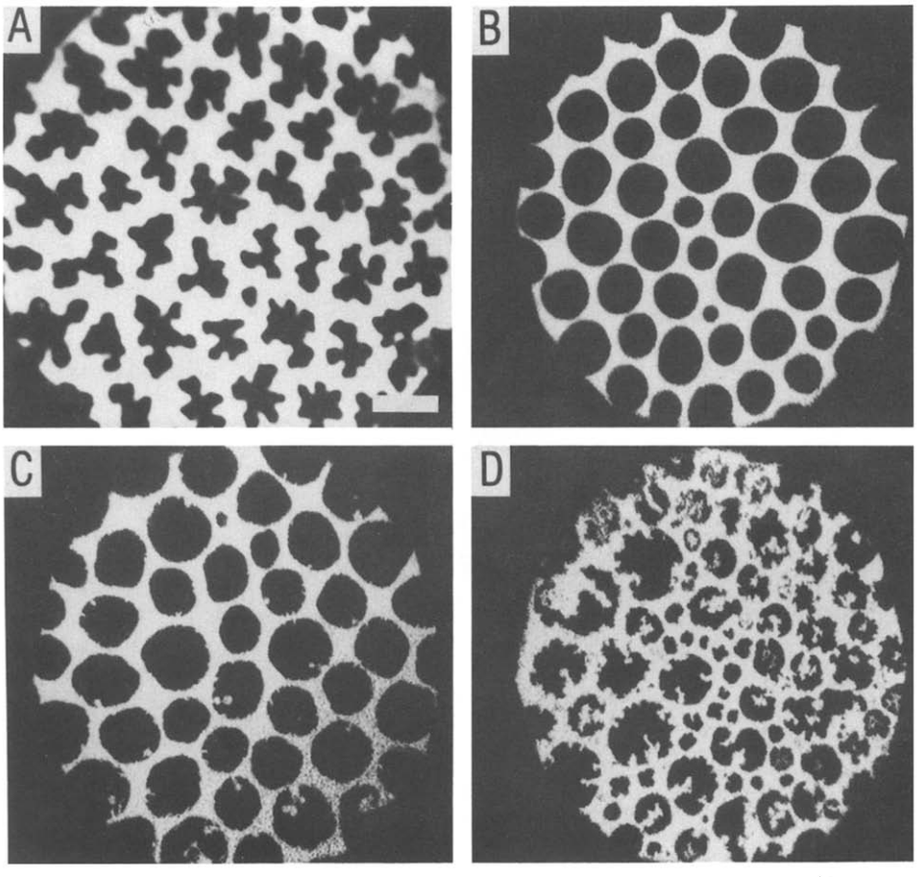


Fig. 2. Real time observation and imaging of L-α-DPPC monolayer hydrolysis in monolayers compressed at 10 Å^2/mol per min and annealed as described in text. All photographs are imaged through the sulforhodamine filter; other conditions remain as described in Fig. 1. (A) Dendritic solid lipid domains immediately after compression. (B) Round lipid solid domains in L-α-DPPC monolayer after 1 h annealing at 30 °C. (C) Hydrolysis seen at multiple points around lipid solid domains after 20 min incubation with enzyme in subphase. (D) After 40 min hydrolysis by enzyme. Scale bar in (A) is 20 μm.

compression is used to produce visible, stable and spatially defined monolayer transition states of solid lipid domains. Moreover, kinetic data showing membrane defects from gel-liquid crystal transition disordering in the phase transition process [11,31,34,36,37] increase phospholipase A_2 binding affinity and hydrolytic activity support this new optical evidence of preferential phospholipase A_2 binding in the solid-liquid L-α-DPPC interfacial regions.

The entire hydrolytic process in the monolayer, simultaneous destruction of L-α-DPPC solid domains together with the unique growth of phospholipase A_2 domains, has been recently reported [9]. Further evidence for a rather different type of solid domain DPPC hydrolysis by phospholipase A_2 on annealed dendritic lipid domains is shown in Fig. 2. Using faster compression rates (10 $Å^2$/mol per min), DPPC domains having a dendritic morphology (Fig. 2A) could be formed, which upon annealing, transform to large, dark round lipid domains (Fig. 2B). During enzyme hydrolysis, secondary sites of phospholipase A_2 attack are often observed in the case of annealed dendritic domains; that is, some lipid domains sustain hydrolysis from more than one site on their domain edges (Figs. 2C, D), compared to the previous case where slowly compressed DPPC solid domains were hydrolyzed only at single lipid domain morphological discontinuities (Figs. 1C, D) [9].

Moreover, a few solid phase lipid domains are seen to undergo 'fraying' and 'scalloping' of entire edges as hydrolysis ensues (Figs. 2C, D), indicating that the edges of some domains are susceptible to hydrolysis at more than one location. This type of reaction has not been witnessed before. Similarly to the first reports [9], however, this difference is explained on the basis of lipid domain physical structure and localized lipid packing differences. By compressing the DPPC monolayer at higher rates than before, dendritic solid phase lipid domains having arm-like structures extending in multiple points from the primary domain bodies are formed as shown in Fig. 2A. Upon annealing, however, these arms gradually disappear, resorbing into the main solid phase lipid domains to form rounded structures (Fig. 2B). It is postulated that the local solid lipid packing

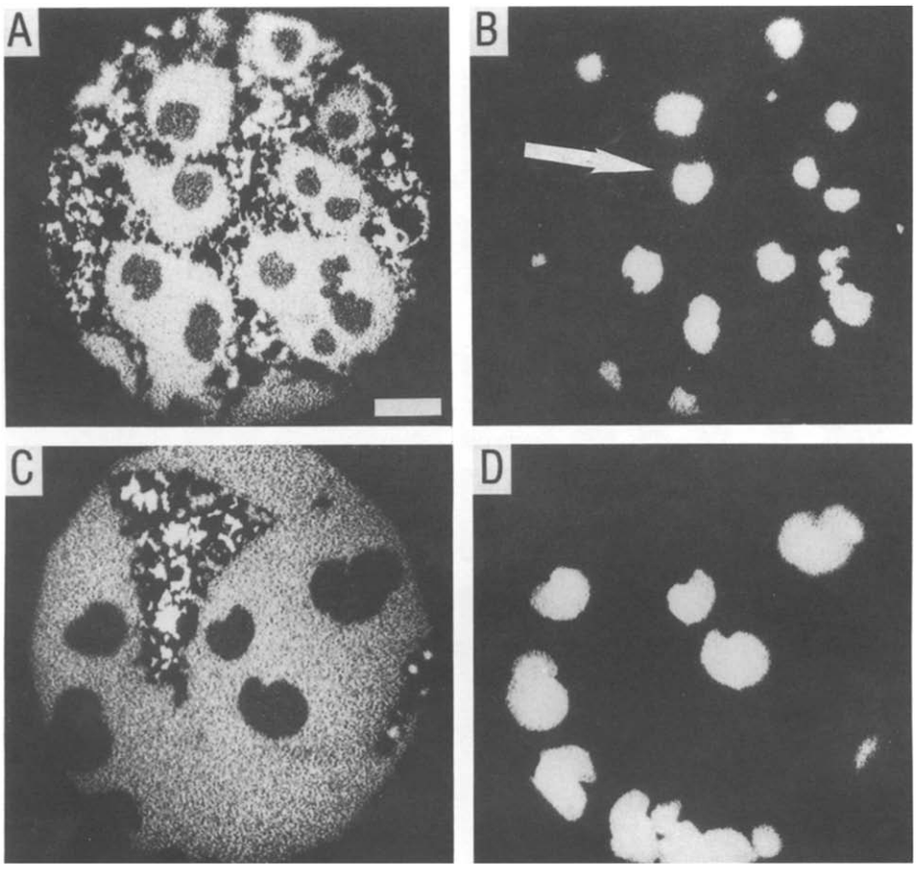


Fig. 3. Formation of enzyme domains of fluorescein-labeled phospholipase A_2 in a L-α-DPPC monolayer after extended hydrolysis times. Photos correspond to the same view seen through two different filters as detailed in the text. In (A) and (C) (after approx. 60 and 90 min hydrolysis time, respectively), the monolayer is imaged through the sulforhodamine filter (monolayer) where the bright signal is the fluid phase of the lipid monolayer and the dark areas are solid phase lipid and enzyme domains. In (B) and (D), the corresponding views at the respective time points are seen through the fluorescein filter, where the bright areas are specifically the enzyme domains (example: see arrow) from (A) and (C). Scale bar in (A) is 20 μm.

structure that promotes these arms to extend from certain points on domain edges upon compression are retained on domain edges after annealing, even though the arm extensions have disappeared. Due to their unique and perhaps irregular packing state, these localized lipid packing 'defects' promote enzyme attachment and hydrolysis at these points around each lipid domain edge. This would explain secondary sites of hydrolysis seen in rapidly compressed domains (high packing defect density) compared to the previous case [9] where slow compression showed no dendritic morphology and only one primary site of enzyme attachment and hydrolysis was observed (Figs. 1C, D). It is interesting to note that almost all lipid domains demonstrate a first and primary site of enzyme hydrolysis in the major morphologic cleft or discontinuity typical of these chiral lipid forms, while secondary sites of hydrolysis come at other locations on lipid domain edges (Fig. 2C).

As monolayer hydrolysis continues over the course of 60 min, enzyme domains appear and grow within the monolayer. Numerous kidney- or crescent-shaped fluorescent areas seen through the fluorescein filter (Figs. 3A–D) increase in size over time. This curious kidney or crescent shape that typifies enzyme domain morphology (see arrow, Fig. 3) is retained from their first discernible appearance through the time course of their development within the monolayer. At long times (2–4 hours), the solid phase lipid areas are completely hydrolyzed away to leave a monolayer of hydrolytic products (lysoPPC and fatty acid) and fluid analogue DPPC, swimming with hundreds of enzyme domains (figure not shown). Low amounts of added enzyme delay hydrolysis reaction progress without influencing the ultimate result, that is, the identical enzyme domain formation.

Enzyme domain formation may be a direct result of the physical state of the lipid substrate, a physical arrangement based on the direct influence of hydrolytic by-products in the layer [9]. In fact, very recent evidence [38] indicates lateral segregation and phase separation of fatty acid in phospholipase A_2-hydrolyzed DMPC vesicles induced by critical concentrations of fatty acid products in the bilayers. In monolayers, by hydrolyzing its way from one point through the center of L-α-DPPC domains, the enzyme creates an increased, perturbed solid-liquid L-α-DPPC interfacial area. This effect, in combination with a resulting local concentration and segregation of lipid *sn*-2 ester hydrolytic end-products (production of the corresponding lysoPPC and free fatty acid) promotes further enzyme-substrate binding, interfacial activation, and aggregation sites for more enzyme in a relatively localized area. This environment could serve than as a site for a self-potentiated phospholipase A_2 aggregation mechanism. The fatty acid and lysoPPC are not readily pushed out of the monolayer and, therefore, remain in the layer after substantial hydrolysis has taken place. Complexes of fatty acids with Ca^{2+} ions present in the subphase (soap formation) may concentrate or hinder free diffusion of end products, resulting in localized concentrations of these molecules in the layer [10] and perhaps phase separation. Because phospholipase A_2 domains form well before most of the monolayer is hydrolyzed, only critical, localized concentration increases in these end-products together perhaps with the unhydrolyzed L-α-DPPC in a ternary mixture may be required to induce localized enzyme domain formation in phase separated regions of the lipid monolayer. Enzyme domain formation would be prompted then by physical changes (lipid segregation and local charge density changes) in the lipid monolayer by the introduction of hydrolytic end products, resulting in altered enzyme activity and subsequent aggregation.

This phenomenon suggests a type of allosteric enzyme inhibition. In fact, *N. naja naja* phospholipase A_2 is inhibited by increasing levels of free fatty acids [10,11]. Furthermore, recent evidence indicating autocatalyzed self-acylation of the snake venom phospholipase A_2. *A. p. piscivorus* by fatty acid hydrolysis products with subsequent enzyme self-association [39] could explain larger domain formation through increased enzyme hydrophobization. Direct visual observation of actual enzyme domains in regular morphologies strongly supports hydrolysis-induced changes in enzyme-substrate binding affinity [10,40] and enzyme structural changes [8,11,34,41] to promote enzyme–enzyme interaction. This mechanism of aggregation might indeed be a contributing mechanism of allosteric enzyme inhibition [10] in the monolayer as well as in native cell membranes, and is further addressed below. The time-dependent nature of the enzyme domain appearance suggests a mechanism associated with the time- and hydrolysis-dependent inhibition of phospholipase A_2 by end-products together with a substrate-dependent (L-α-DPPC) activation of the enzyme. When interfacial recognition is altered by changing both the chemical and physical nature of the lipid interface through the introduction of fatty acid and lysoPPC, the enzyme may be prompted to aggregate. That this may be due to fatty acid-associated changes in enzyme structure and action is indicated, as shown in Fig. 4.

When initially injected under the phospholipid monolayer in the phase transition region, the enzyme binds to the interfacial regions between the fluid and the solid lipid phases (Figs. 4A, B). Hydrolysis of lipids in this region ensues, evidenced optically as the preferential attack on solid lipid phases from their external edges (Fig. 4C). Fatty acids or lysolipids (or both) from the hydrolysis remain to some extent in the monolayer and induce a change in the physical arrangement as well as the electrostatic environment at the interface. This eventually could result in phase separation in a hydro-

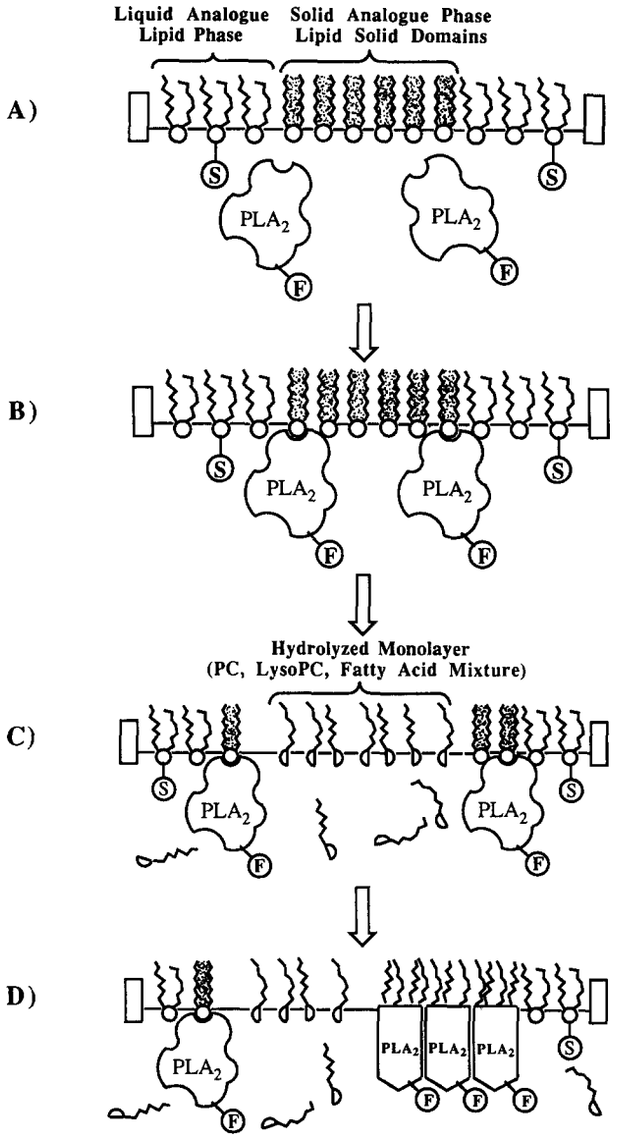


Fig. 4. Proposed mechanism for recognition, binding, hydrolysis and subsequent phospholipase A_2 domain formation prompted by critical concentrations of hydrolytic end-products mixed with substrate lipids in phospholipid monolayers. (A) Injection of phospholipase A_2 into aqueous subphase under lipid monolayer. (B) Recognition and binding of phospholipase A_2 to lipid interface. (C) Hydrolysis of monolayer by phospholipase A_2 with subsequent build-up of hydrolytic products (fatty acid and lysolipid) within the layer. (D) Organization of bound phospholipase A_2 into protein domains at the lipid/water interface prompted by critical concentrations and phase separation of hydrolytic endproducts in the monolayer.

lyzed monolayer. Such physical changes are, in turn, sensed by the enzyme which is prompted to aggregate in two dimensions at the interface (Fig. 4D). In this regard, it is believed that enzyme interfacial recognition of certain physical monolayer quality, activation and a certain degree of hydrolysis that changes the substrate physical character and charge density are, therefore, necessary to prompt formation of these microscopically observable enzyme domains.

Local changes in monolayer phase transition caused by increases in lysoPPC and fatty acid content in the layer produce effects that could strongly influence monolayer morphology and enzyme interaction as shown in vesicle systems [33,41,42]. Quantitative compositional effects of these products on the L-α-DPPC phase transition are not yet available but the evidence to date strongly suggests that L-α-DPPC domains remain solid until actively hydrolyzed into fragments. Slow decompressions of the L-α-DPPC layer, simulating gradual refluidization of L-α-DPPC solid domains, demonstrate an entirely different morphological behavior [28]. A further point concerns concentration-dependent aggregation of phospholipase A_2 [43]. That the enzyme domain phenomenon was observed extensively and homogeneously throughout the layer, on nearly all L-α-DPPC domains refutes the possibility that enzyme domain formation was an artifact of poorly dispersed, locally concentrated, and hence, aggregated enzyme. Amounts of phospholipase A_2 added (7 μg in 0.75 ml mask volume) could not even approach the aggregation concentration limit already documented (> 50 μg/ml) [43]. In addition, the fact that all domains sustain independently directed hydrolytic vectors, all starting from a point between the liquid and solid phase L-α-DPPC directed through the center of each solid domain confirms the reality of the phenomenon. Further examples with other lipid substrates detailed below emphasize this point.

Imaging hydrolysis of DPPE monolayers by phospholipase A_2

To investigate the effects of lipid polar head group and lipid solid domain morphology on enzyme domain formation, imaging of DPPE hydrolysis by phospholipase A_2 in monolayers was also investigated. DPPE is known to be actively hydrolyzed by *N. naja naja* phospholipase A_2 in micelles [10]. Morphologies of DPPE domains formed at 37°C after compression of lipid monolayers are shown in Fig. 5A (surface pressure, 22 mN/m). These solid lipid domains are distinctly different from those of DPPC, showing typical dendritic appearance which anneals to rounded forms only very slowly with time (many hours compared to minutes for DPPC). Thus, any relationship between lipid substrate solid domain morphology and subsequent enzyme hydrolysis could be compared on the basis of headgroup influences and distinguished from the phosphatidylcholine cases mentioned above.

After 15 min of enzyme hydrolysis, the lipid domains, as seen through the sulforhodamine filter, show irregular, rough edges and spikes (Fig. 5B), indicating transformation of the lipid domain edges through the effects of enzyme action. At this time, small patches of enzyme domains have appeared in the layer (arrow, Fig.

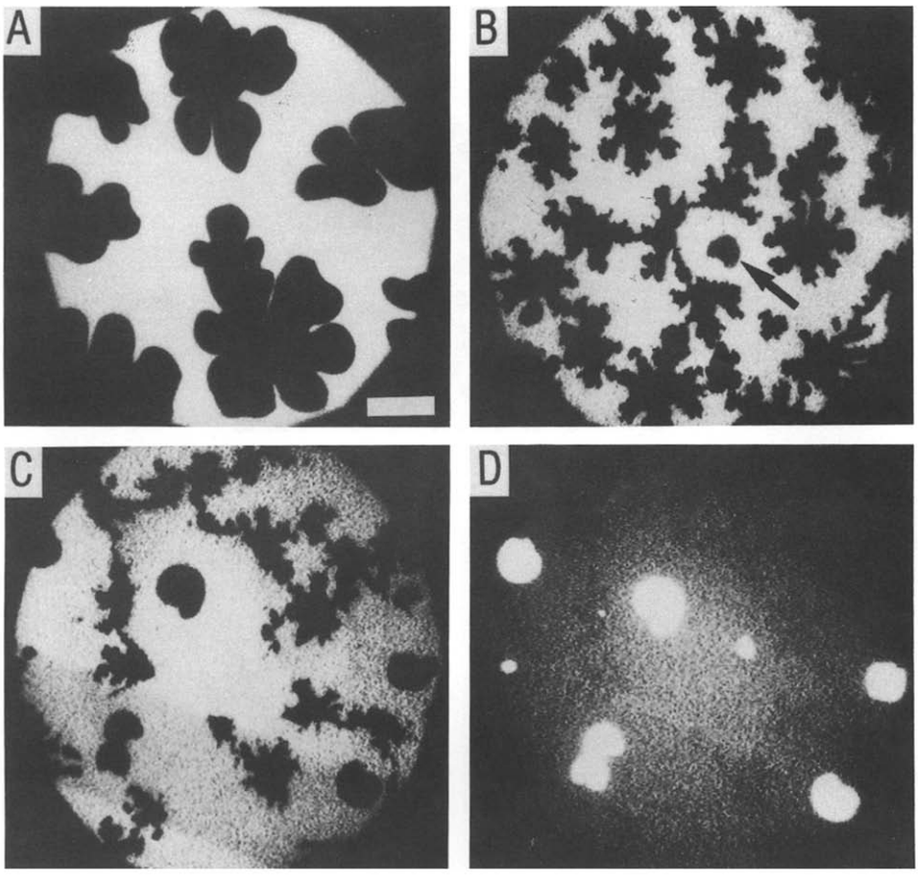
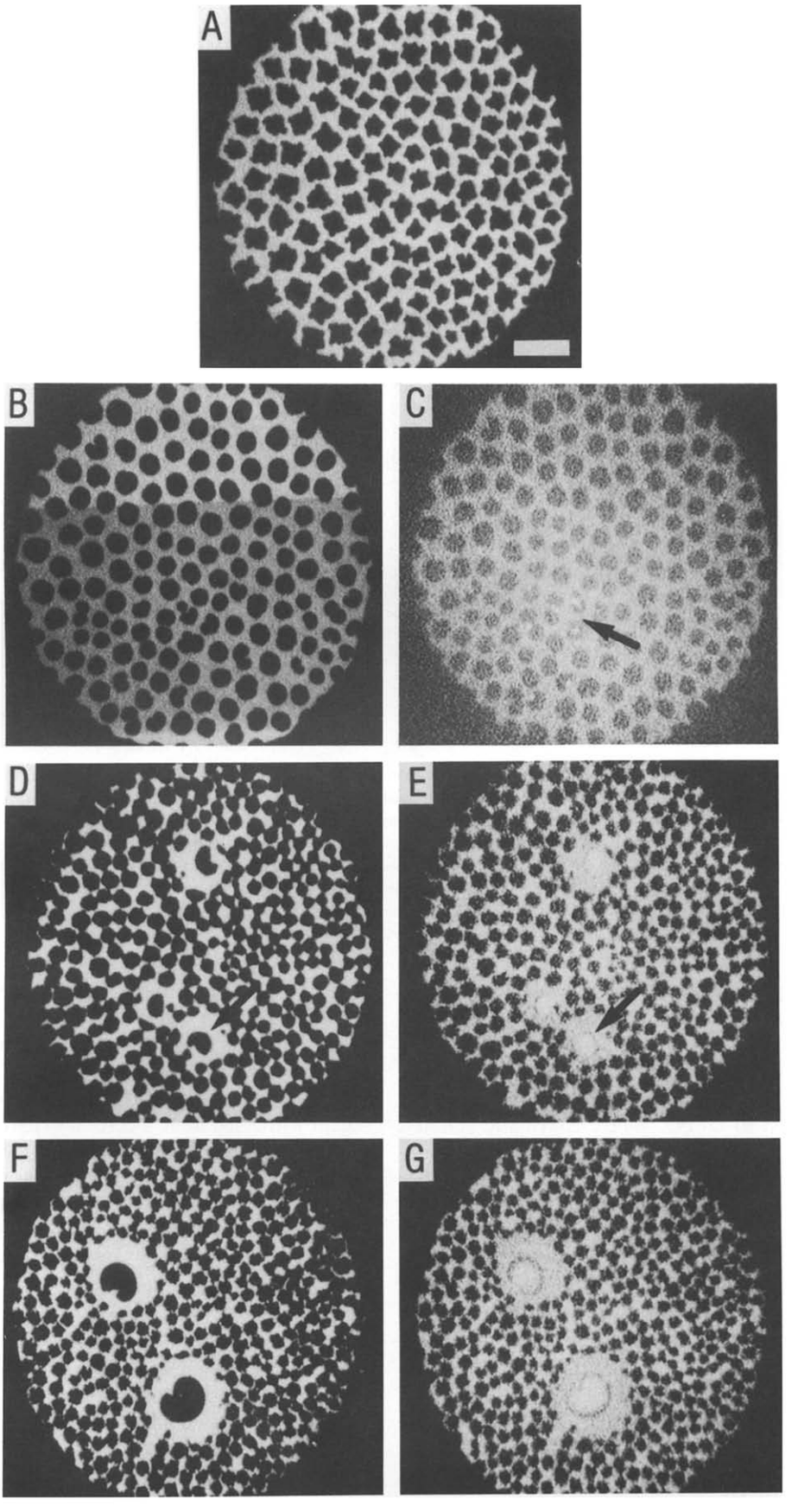


Fig. 5. Epifluorescence imaging of L-α-DPPE monolayer phase transition region and subsequent hydrolysis by injection of phospholipase A_2 into the aqueous subphase under the monolayer. Identical experimental conditions as in Fig. 1 except that the temperature is 37°C. (A) L-α-DPPE monolayer in the phase transition region before enzyme addition showing typical dendritic type of domains for this solid phase. (B) through (D) show progress of enzyme hydrolysis: (B) At 30 min hydrolysis time, imaged through the sulforhodamine filter. Note frayed, spiked lipid solid domain edges and formation of small enzyme domains (arrow). (C) At 45 min hydrolysis time. Lipid solid domains are nearly completely destroyed and many enzyme domains can be seen in the layer. (D) Identical view as in (C) except seen through the fluorescein filter. Bright areas are corresponding enzyme domains. Scale bar in (A) is 20 μm.

5B). Changes in lipid domains are now readily apparent and hydrolytic damage is quite extensive. Whether hydrolysis occurs directly on or within the solid domains is difficult to prove. However, it is clear that the mechanism of enzymatic hydrolysis is qualitatively different in this case in contrast to DPPC. Where enzymatic action was witnessed to start at the DPPC lipid liquid/solid interface and progress to penetrate each solid lipid domain specifically in a directional manner, hydrolysis of DPPE domains appears to have no optically detectable phase preference (liquid or solid lipid) and no specific, directional hydrolytic path. One piece of evidence hints that enzymatic hydrolysis may, on the contrary, be directed uniformly against solid DPPE domains. Simple decompression of DPPE monolayers in the phase transition region demonstrates, similar to the DPPC case, a totally different refluidization behavior of solid domains, that is, no textured edges, edge discontinuities or spikes are seen. That hydrolysis only gives these edge effects suggests that the enzyme prefers the solid liquid phase interface, but by a less-optically evident mechanism. Again, this is entirely in agreement with enhanced enzyme activity in the phase transition region detailed above.

Moreover, not only is enzyme domain formation again witnessed after a certain extent of enzyme hydrolysis, but enzyme domain morphology is identical to that seen for hydrolysis of DPPC, despite the great difference in solid lipid domain morphologies and hydrolysis patterns. The enzyme is seen to form regular, crescent-shaped aggregates within the monolayer (Fig. 5C and the corresponding Fig. 5D) which, by comparison of the same microscope field through both cut-off filters (compare the different micrographs of Fig. 5) are easily discerned as enzyme domains (bright areas, Fig. 5D) coexisting with lipid domains (dark domains, Fig. 5D). One difference in the DPPE case seems to be that the enzyme domains readily aggregate to a greater extent than seen in relation to DPPC.

Previous work has kinetically differentiated the hy-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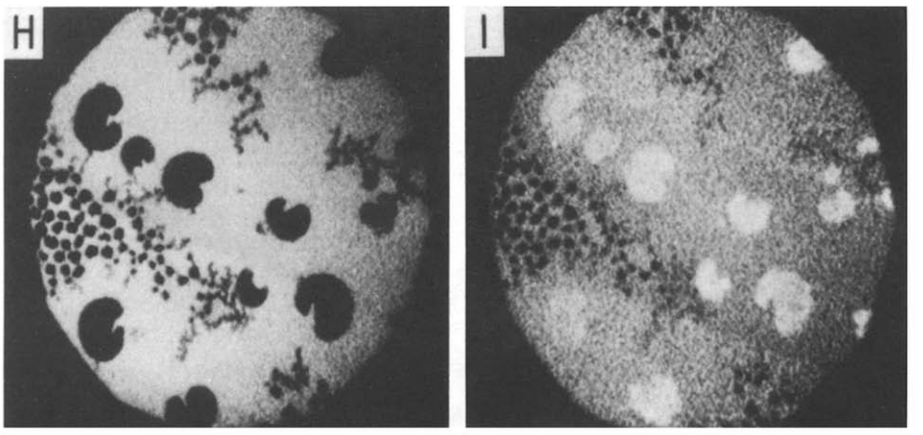

Fig. 6. Dual epifluorescence imaging of L-α-DMPC monolayer phase transition region and subsequent hydrolysis by injection of phospholipase A_2 into the aqueous subphase under the monolayer. (A) L-α-DMPC monolayer seen through the sulforhodamine filter after compression into phase transition region as described in text. (B) Same monolayer as in (A) after 30 min annealing time and after enzyme addition. (C) Monolayer as in (B) seen through the fluorescein filter after enzyme addition. Note small bright points (arrow) indicating enzyme attachment to lipid monolayer. (D) Same monolayer at 25 min hydrolysis time seen through the sulforhodamine filter. Hydrolyzed areas are clearly seen along with accompanying enzyme domain formation. (E) Same image as in (D) but seen through the fluorescein filter. Bright areas (arrow) are enzyme domains. (F) Same monolayer at 40 min hydrolysis time imaged through the rhodamine filter. Monolayer hydrolysis is substantial and enzyme domains have grown in size but maintain original morphology. (G) Same image as in (F) but seen through the fluorescein filter. Bright areas are enzyme domains. (H) Same monolayer after 55 min hydrolysis time imaged through rhodamine filter. The black solid phase DMPC domains are nearly completely destroyed and numerous domains of enzyme (larger kidney-shaped domains in the bright fluid monolayer phase) have assembled at the interface. (I) Corresponding image to (H) seen through the fluorescein filter. Enzyme domains are readily identified as fluorescing, bright areas. Scale bar in (A) is 20 μm. Temperature 10 °C, surface pressure 22 mN/m.

drolysis of DPPC versus DPPE by phospholipase A_2 *N. naja naja* in micelles [10]. In this system, *N. naja naja* prefers, as a substrate, ethanolamine lipids to choline lipids by a factor of 36. Moreover, hydrolysis of ethanolamine substrates is enhanced 20–30-times in the presence of choline substrates in micelles. However, the effects of hydrolytic end product concentrations (free fatty acid and lysolipid) serve to decrease or eliminate the lag period of phospholipase A_2 hydrolytic action against ethanolamine lipids, while fatty acid strongly inhibits phospholipase A_2 hydrolysis of choline substrates, where no lag period in micelles is observed [10]. Based on this evidence, it would have been predicted from DPPC monolayer results that phospholipase A_2 domains were strictly a result of fatty acid content in the monolayer, resulting in aggregation. However, the fact that fatty acid end products activate DPPE hydrolysis [10], in combination with the appearance of the identical enzyme domains during monolayer hydrolysis with both DPPC and DPPE substrates, suggests a more complicated phenomenon. The observed enzyme domains seem to constitute organized, inactive surface-bound protein. That fatty acid activates the phospholipase A_2 against DPPE and inhibits hydrolysis of DPPC, yet promotes enzyme domain formation in both cases is difficult to clarify. The only factor in common in the hydrolysis of both these substrates is the production of the same fatty acid, which induces enzyme domains in both systems. Perhaps it is only that fatty acid effects on phospholipase A_2 during DPPE hydrolysis run a different (longer?) time course than with DPPC, with fatty acid-induced aggregation at much higher fatty acid levels in the monolayer, but with the same ultimate fate of the enzyme (domain formation). Differences between enzyme hydrolysis of micellar substrates [10] compared to monolayer substrates could also account for this discrepancy [44].

Imaging hydrolysis of DMPC monolayers by phospholipase A_2

In contrast to DPPC and DPPE monolayers, DMPC has a shorter acyl chain length (14-C chains versus 16-C for DPPC and DPPE) and its lipid monolayers show a phase transition at lower temperatures. An experimental temperature of 10 °C was chosen so that the liquid-solid lipid phase transition would occur at approx. 22 mN/m, similar to the other lipid systems. At this temperature on this subphase with the given barrier speed, the phase transition of DMPC produces small, star-like solid domains (Fig. 6A) which anneal quickly to round domains showing hexagonal long range ordering (Fig. 6B). Before enzyme injection, the mciroscope field, as seen through the sulforhodamine filter, is completely dark. A few minutes after enzyme addition, bright pinpoints of fluorescein signal, indicating enzyme, appear on various DMPC lipid domains in the monolayer (Fig. 6C, arrow). demonstrating again the enzyme attachment to the monolayer, specifically onto the edges of the monolayer solid DMPC domains. At longer exposure times, these pinpoints grow in both size and number to rapidly give

many visible enzyme domains of regular morphology scattered throughout the layer and seen through both filters (Figs. 6D–G). This is accompanied by changes in lipid domains from round to irregular, pointed morphologies (Figs. 6D–G). The enzyme domains (arrow, Fig. 6E) assemble simultaneously as the DMPC solid domains are destroyed. The enzyme domains appear with the same morphology as seen in both DPPC and DPPE in response to hydrolytic action. In addition, enzyme domains seem to form in areas free of solid phase lipid. Hydrolysis action first produces a 'hole' in the monolayer that, although filled with fluid phase DMPC, hydrolytic end-products, and sulforhodamine probe, contains no solid phase lipid. Phospholipase A_2 domains seem to center themselves in the middle of these areas, away from solid phase contact. Whether this phenomenon has particular significance is difficult to say at this point. Domain location could result from concentrations of fatty acids in these hydrolyzed areas, resulting in localized changes in monolayer charge density and physical packing that have been shown to influence enzyme action and induce lipid phase separation [38].

As mentioned above, evidence for fatty acid induced allosteric inhibition of *N. naja naja* [10] indicates close association and even binding of fatty acid by the enzyme. Newer evidence of self-catalyzed acylation of enzyme lysine residues with free fatty acid resulting from hydrolysis, although from another snake venom phospholipase A_2 [39], strongly suggests that fatty acid plays an integral role in enzyme activation and control. That perhaps each substrate has a different critical concentration of hydrolytic end products for enzyme activation [45,46], inhibition, and resulting domain formation reported here could explain differences in lipid solid domain hydrolysis behaviors and effects of hydrolytic products on monolayer lipid packing structure. The fate of hydrolyzed fatty acid appears to be a critical point but must not directly effect the enzyme alone. The influence of hydrolytically-produced fatty acid in lipid bilayers on lipid phase separation with subsequent activation of phospholipase A_2 has recently demonstrated a new mechanism of interaction between the enzyme and its action on changing the hydrolytic susceptibility of its substrate [38]. Fatty acid seems to be a third party to the interaction of enzyme and lipid substrate that may act on both enzyme and lipid interfaces to produce two distinct effects, namely, direct association with enzyme molecules at the interface and influence of the packing and segregation of lipid substrate.

Hydrolysis of monolayers of DMPE by phospholipase A_2

In contrast to the other lipid systems reported here, the hydrolysis of DMPE monolayers at 22 mN/m and 30 °C occurred so quickly that maintaining constant surface pressure was not possible. This meant, in addition, that solid lipid domains created in the liquid-solid phase transition region disappeared quickly as well, leaving no liquid-solid lipid interface. Thus, observation of hydrolysis in the phase transition region could not be imaged for this lipid. Although small amounts of aggregated enzyme could be found after solid lipid domains had disappeared, no similarities to previous systems were observed.

Control experiments

Control experiments using solid lipid domains of nonhydrolyzable phosphatidylcholines (D-α-DPPC and dienoylphosphatidylcholine) in monolayers show absolutely no detectable hydrolysis or enzyme domain formation similar to that of L-α-DPPC, even after hours of enzyme exposure. D-α-DPPC, the stereoisomer of the L-form, blocks phospholipase A_2 hydrolytic activity [47], but shows identical surface monolayer properties and domain characteristics as the L-form [28]. Lack of any detectable fluorescence of the lipid/water interface clearly indicates that non-specific adsorption does not play any role in the described phenomenon. Moreover, monolayers of DMPC at 37 °C show only fluid phase behavior. These monolayers were actively hydrolyzed by phospholipase A_2 but showed only small, barely-distinguishable enzyme aggregates of no particular regular morphology or apparent relation to those witnessed in phase transition regions (see Fig. 6). Monolayers of 1 : 1 molar mixtures of palmitic acid and lysoPPC showed no ability to induce enzyme domain formation under identical monolayer conditions, indicating that, in addition, presence of non-hydrolyzed DPPC is necessary. Furthermore, monolayer experiments on DPPC spread over buffer in a noncompressed gas-analogue phase also underwent hydrolysis but showed no detectable enzyme domain formation in the monolayer after 30 min. Furthermore, when more DPPC was spread on top of this already-spread lipid film, the monolayer, by virtue of excess lipid at the interface, was forced into the liquid-solid phase transition region, forming solid phase lipid domains. As soon as this occurred, enzyme domains were observed within 5 min. This unequivocally demonstrates the requirement for a liquid-solid phase transition lipid interface before or even after hydrolysis to precipitate the subsequent events leading to enzyme domain formation.

Phospholipase A_2 domain stability

Experiments varying the lateral surface pressure in the monolayer after enzyme domain formation demonstrate the stability of these domains under extreme surface pressure conditions. After monolayer hydrolysis occurs to the extent that lipid solid domains had entirely disappeared L-α-DPPC and L-α-DMPC monolayers were recompressed into their respective phase

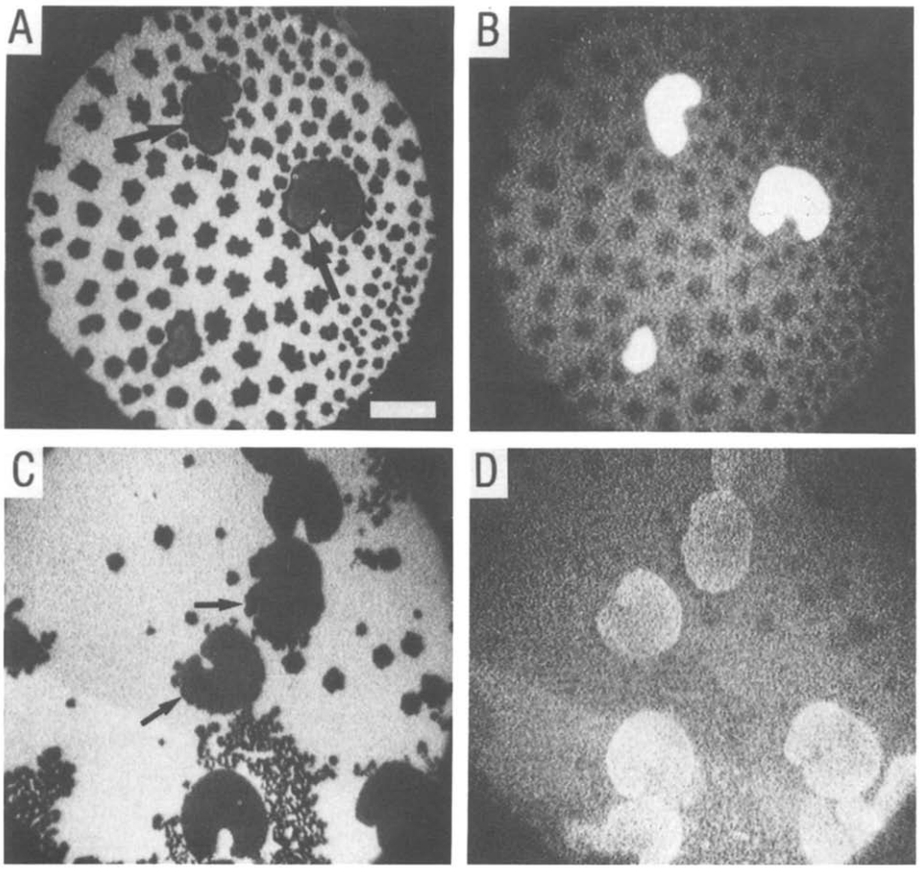

Fig. 7. Dual epifluorescent imaging of phospholipid recrystallization onto the edges of phospholipase A_2 domains in partially-hydrolyzed phosphatidylcholine monolayers by further compression of the lipid monolayer. (A) L-α-DMPC monolayer after 90 min hydrolysis time seen through the sulforhodamine filter. Temperature 10 °C, lateral surface pressure of 35 mN/m, Dark crescent areas are enzyme domains outlined with a black border of DMPC solid phase (see arrows). (B) Same image as in (A) but seen through the fluorescein filter to visualize enzyme domains. (C) L-α-DPPC monolayer after 120 min hydrolysis, temperature 30 °C, lateral surface pressure of 50 mN/m. Arrows indicate lipid recrystallization of DPPC on enzyme domains. (D) Same image as in (C) but seen through the fluorescein filter to specifically observe enzyme domains. Scale bar in (A) is 20 μm.

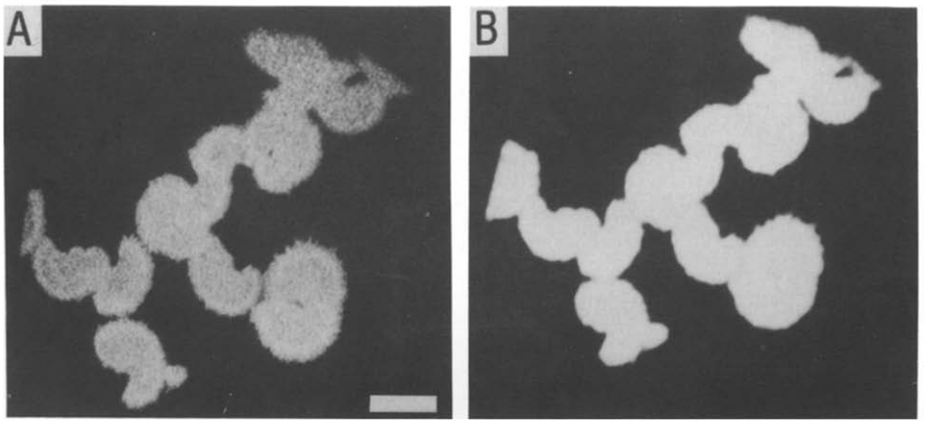

Fig. 8. Epifluorescent images of fluorescein-labeled phospholipase A_2 domains in a L-α-DPPC monolayer gas-analogue state at 0 mN/m surface pressure after slow expansion from the phase transition region after 90 min. Hydrolysis time. Temperature 30 °C. (A) Image seen through the sulforhodamine filter. Grey clusters are rhodamine lipid-rich enzyme domains in the dark background gas-analogue state. (B) Enzyme domains seen directly through fluorescein filter. Scale bar in (A) is 20 μm.

transition regions. It should be noted that, although dependent on the extent of hydrolysis, these phase transitions always occur at substantially higher lateral surface pressures than the respective phase transitions of the nonhydrolyzed monolayers due to the presence of hydrolytic end-products. The recompression produces new solid lipid domain nuclei crystallizing both out of the fluid lipid matrix as well as directly onto the edges of the enzyme domains seen (see arrows, Fig. 7). This experiment shows that there still exists plenty of unhydrolyzed fluid DPPC and DMPC in the monolayer after lipid solid domains are hydrolyzed and disappear from the monolayer. Enzyme domain shape and integrity are not affected by increasing lateral surface pressure up to 50 mN/m, indicating a remarkable protein domain stability. In addition crystallization of solid lipid domains on the edges of protein domains points to the interesting fact that the protein domains do not seem to be hydrolytically active any more. This is another hint that on forming protein domains after a certain degree of hydrolysis phospholipase A_2 undergoes a conformational change into the inactive form.

A further indication of phospholipase A_2 domain stability is the observation that enzyme aggregates are stable to very low surface pressures (0 mN/m) and do not readily dissociate. Fig. 8 shows phospholipase A_2 domains at 0 mN/m surface pressure surrounded by the hydrolyzed DPPC monolayer in the gas-analogue state after expansion of the hydrolyzed monolayer from the phase transition region. The domains retain their morphology even after expansion. It appears, therefore, that the enzyme domains are stable and structurally coherent, able to exist independent of a supporting monolayer, lending further evidence for a regular mechanism for their assembly and organization at the monolayer interface.

Formation of enzyme domains to date has required the presence of a solid-liquid phase boundary. Experiments in the monolayer gas-analogue and liquid-analogue phases below the main phase transition region demonstrate hydrolysis but no enzyme domain formation. The first two properties make the system ideal for further structural investigations on the enzyme domains themselves. Short or long range structural orders, even two-dimensional crystallinity, might be expected for such a small enzyme (mol. wt. 14000) stabilized by seven disulfide bonds.

That the domain formation requires a lipid coexistence region indicates an energetic or structural substrate preference or specificity for enzyme action. Moreover, evidence from DPPC that shows clearly that this substrate specificity, i.e., the directional nature of solid domain hydrolysis following specific attachment of enzyme to the solid domain edge discontinuity. Specific enzyme attack of the equilibrium solid lipid domain consistently at this one point (i.e., the indentation present in each chiral lipid form (see Fig. 1)) indicates a preferential energetic or structural substrate environment here. This is supported by theoretical analyses [23,48–53] that describe the growth of these lipid chiral equilibrium structures in terms of energy equilibrium between repulsive long-range molecular forces, line tension, and an anisotropic crystallization force. The morphological discontinuity (indentation) resulting from the so-called 'equilibrium non-circular domain shape' is also an energy discontinuity, possibly inducing preferential enzyme action here. In this regard, possible use of the enzyme then as a probe of lipid domain structure and energies, particularly in the phase transition region where kinetic optimization of enzyme action is witnessed and where such effects can be visibly imaged by fluorescence microscopy is proposed.

Acknowledgements

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