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An enzyme caught in action: direct imaging of hydrolytic function and domain formation of phospholipase A_2 in phosphatidylcholine monolayers

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Phospholipase A_2, a ubiquitous lipolytic enzyme that actively catalyzes hydrolysis of phospholipids, has been studied as a model for enzyme-substrate reactions, as a membrane structural probe, and as a model for lipid-protein interactions. Its mechanism of action remains largely controversial. We report here for the first time direct microscopic observation of the lipolytic action of fluorescently marked phospholipase A_2 (*Naja naja naja*) against phosphatidylcholine monolayers in the lipid phase transition region. Under these conditions, phospholipase A_2 is shown to target and hydrolyze solid-phase lipid domains of L-α-dipalmitoylphosphatidylcholine. In addition, after a critical extent of monolayer hydrolysis, the enzyme itself aggregates into regular, visible proteinaceous domains within the lipid monolayer. Solid-phase lipid hydrolysis indicates a preferential hydrolytic environment for phospholipase A_2 while enzyme domain formation points to a possible allosteric inhibition mechanism by hydrolysis products.

Monolayer; Enzymatic hydrolysis; Fluorescence microscopy; Phospholipid; Phospholipase A_2; Domain

1. INTRODUCTION

Phospholipase A_2 (PLA_2, EC 3.1.1.4) constitutes a family of widely studied, ubiquitous, small, water-soluble lipolytic enzymes [1]. PLA_2 is responsible for catalyzing hydrolysis of the 2-acyl ester bond of 3-*sn*-glycerophospholipids to yield the acyl fatty acid and corresponding lysolipid. The remarkable activation of this enzyme in response to organized interfacial phospholipid substrates as opposed to dispersed lipid monomers [2,3] has made it an ideal investigative tool for probing membrane structure [4,5] as well as model enzymatic behavior. However, despite several known high-resolution crystal structures of several of these enzymes [6], an array of other structural data, and countless kinetic studies of enzymatic hydrolysis [1], the mechanism of action of PLA_2 remains speculative. To elucidate, in physicochemical terms, the many biochemical processes mediated by this enzyme as well as its catalytic action at membrane surfaces, an understanding of its interaction with simplified interfaces in phospholipid model membranes must first be achieved. The drastic enhancement of PLA_2 hydrolytic activity in the lipid phase transition region [7,8] as well as a higher hydrolytic activity against gel-phase lipids as opposed to liquid-crystalline phases [9,10] are important examples of how critical the physical state of the lipid substrate is to enzymatic action [11,12].

Because the influence of the physical state of the lipid monolayer on PLA_2 hydrolytic behavior has, until now, only been indirectly inferred, in the present study, the monolayer physical state and its relation to subsequent PLA_2 hydrolysis of the model membrane was directly observed by

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PLA_2, phospholipase A_2

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fluorescence microscopy of monolayers. Fluorescence microscopy has already proven to be a sensitive optical tool to observe directly and to analyze monolayer physical behavior at gas/water interfaces [13–17], particularly in distinguishing formation of various solid-phase lipid domains from fluid lipid phases in the monolayer phase transition region [16,17]. By extending this strategy of monolayer physical state visualization with the addition of PLA_2 into the subphase, we can record the interaction between enzyme and monolayer substrate optically under various conditions. Real-time observations demonstrate the elegance of the method to show that PLA_2 hydrolysis is directed regularly against solid analogue lipid domains in the monolayer phase transition region. Corresponding enzyme aggregation and visible, regular enzyme domain formation implicate hydrolytic end products in altering the physical nature of the enzyme and monolayer to inhibit enzyme action.

2. MATERIALS AND METHODS

2.1. *Materials*

Phospholipase A_2 (*N. naja naja*), L-α-dipalmitoylphosphatidylcholine and D-α-dipalmitoylphosphatidylcholine were purchased from Sigma. Dienoylphosphatidylcholine 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. [18]. Phospholipids were of greater than 99% purity and showed single spots by thin-layer chromatography (TLC) analysis (chloroform/methanol/water, 65:25:4 as eluant solvent). Fluorescein isothiocyanate was purchased from Aldrich and used as supplied. A fluorescent lipid probe containing sulforhodamine in its head group was synthesized using purified dioctadecylamine and sulforhodamine isothiocyanate, and shown to be pure by TLC. This lipid was shown to partition preferentially into the fluid phase of monolayers.

2.2. *Labeling of phospholipase A_2*

PLA_2 was dissolved from the supplier's bottle in buffer and labeled [19], providing fluorescein labels on 2 of the estimated 6 lysine residues [20] on each PLA_2 molecule. The resulting enzyme was indistinguishable from the unlabeled form in its ability to hydrolyze DMPC monolayers. However, labeling was shown previously by a more sensitive method to reduce hydrolytic activity slightly by binding to nonessential lysine amine residues on *N. naja naja* PLA_2 [21]. Labeled PLA_2 was separated from unreacted label and buffer salts on Pharmacia PD-10 chromatography columns (Sephadex G-25M) equilibrated with pure water, and lyophilized under protection from light and heat. Product yields were always over 90% and were stored in amber glass vials at -22°C. Enzyme solutions for interaction with monolayer experiments were made by dissolving 0.36 mg labeled PLA_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 use. For each experiment, 0.5 ml thawed PLA_2 buffer solution was removed from a vial with a glass syringe for injection under the monolayer.

2.3. *Fluorescence microscopy of monolayers*

Water for buffer subphases was distilled three times and purified through a Millipore filtration apparatus (18 MΩ resistivity). Tris buffer (10 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 8.9, chloroform washed) was prepared by dissolution of the various salts in 10-times concentration in acid-cleaned glass flasks. Buffer for each experiment was made by diluting the concentrated, washed stock 1:9 with pure water. Monolayers were spread over buffer (30°C) from lipid solutions in chloroform containing 0.25–1.0 mol% of the fluorescent sulforhodamine lipid probe, a probe concentration dilute enough so that no changes in isotherm behavior of the main lipid component could be detected. Before spreading, the buffer surface was cleaned by suction. After spreading, the monolayer was immediately compressed by a computer-controlled barrier at a rate of 2.5 Å^2/molecule per min. At a surface pressure approx. 3–5 dyn/cm below the start of the phase transition of each lipid under the respective conditions, the barrier was stopped and the enzyme solution (0.5 ml) was injected into the mask from a syringe immersed from behind the barrier (fig.1a). After injection the barrier was restarted and the monolayer compressed until domains of dark solid-phase lipids in a bright, fluid lipid matrix filled the majority of the microscope field. Another strategy giving similar results involved injection of enzyme under a DPPC monolayer in the lipid phase transition region after solid lipid domains had formed. At this point, the field was observed alternately through two interchangeable fluorescent filters, corresponding respectively to the monolayer (sulforhodamine) marker and the PLA_2 (fluorescein) marker. To eliminate surface flow in the plane of the monolayer, a circular mask (fig.1b) was constructed from Teflon (diameter 20 mm) and inserted into the subphase below the microscope objective. The size and design were chosen to eliminate meniscal effects on the focal plane of the microscope and to make local observations independent of trough geometry. In addition, 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 have been shown to influence strongly film morphology [22], could virtually be eliminated. Flow, over the time scale of observation of the enzyme, is also minimized so that specific areas could be monitored over hour-long experimental time scales. 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. Specific details regarding the epifluorescent microscope and associated Langmuir film balance equipment have been recently published elsewhere [16].

3. RESULTS AND DISCUSSION

The sulforhodamine-containing L-α-DPPC monolayer in the phase transition region was displayed by the imaging system as dark solid-

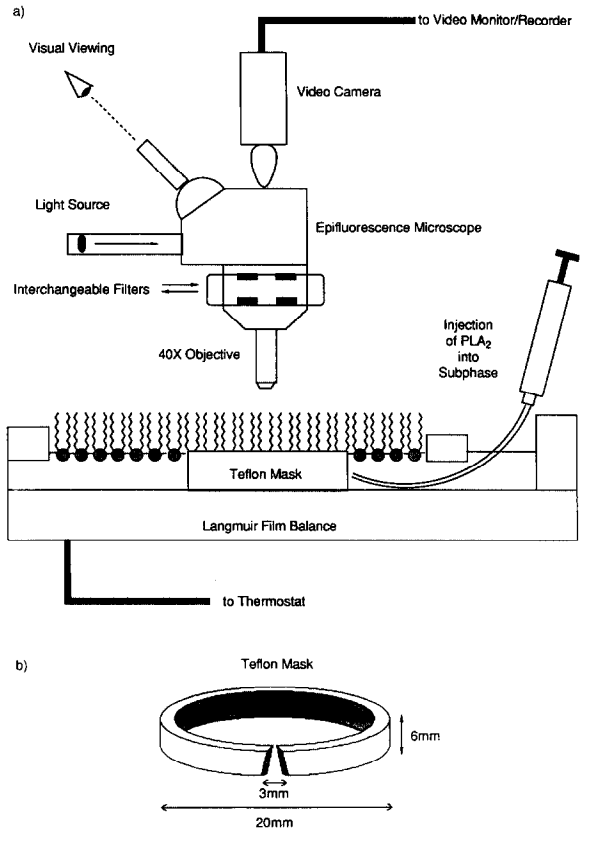
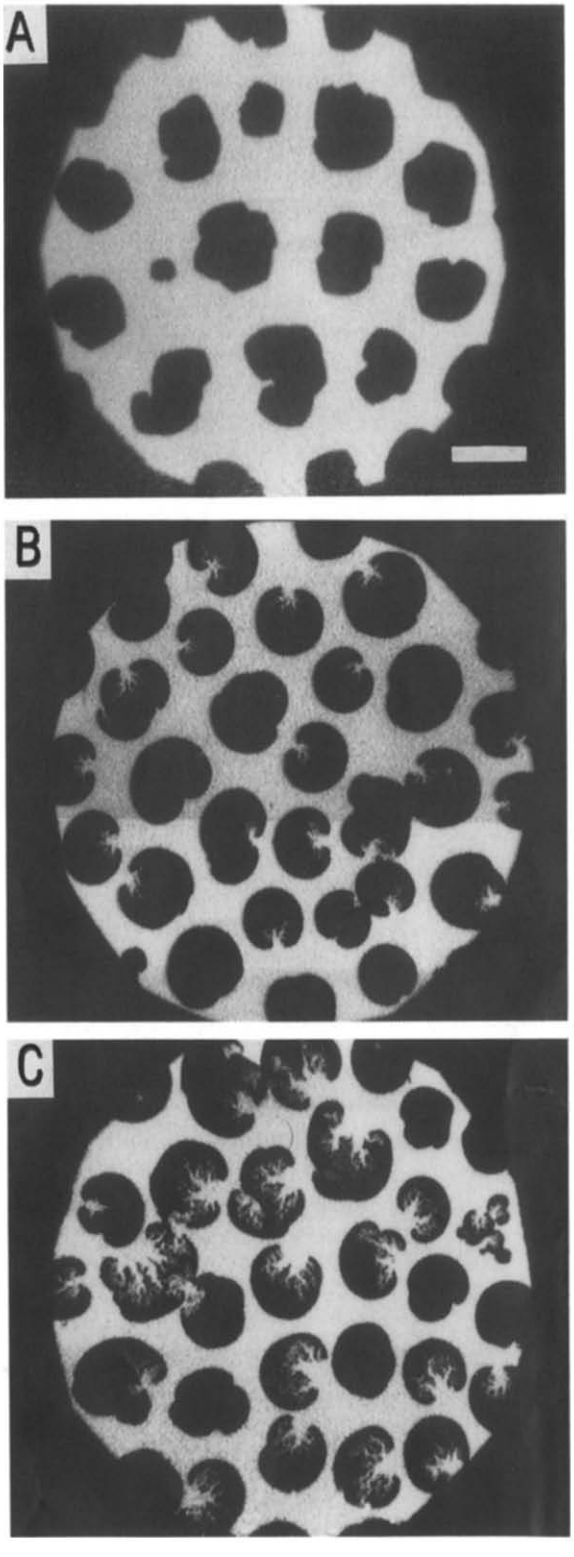


Fig.1. Experimental design. Phospholipid monolayers containing a small amount of fluorescent sulforhodamine lipid probe were compressed over a buffer subphase to the phase transition region. Fluorescein-marked PLA was then introduced into the subphase under the lipid monolayer (a) directly into a mask, (b) placed in the subphase.

phase L-α-DPPC domains in a field of bright mixture of fluid L-α-DPPC and sulforhodamine lipid (see fig.2A). Pressure-area isotherms and DPPC domain morphology in phase transitions were similar, if not identical, in form and time-dependent annealing behavior to those reported by Albrecht et al. [23] and McConnell et al. [24], respectively. No signal (i.e. completely dark field) was seen upon inverting the optical filter to view fluorescein emission. Immediately upon injection of fluorescein-marked enzyme into the subphase, however, a diffuse, homogeneous fluorescent signal could be seen through the fluorescein filter (fig.3A). No changes in the sulforhodamine-labeled L-α-DPPC layer could be detected at this point. After 10 min, this enzyme fluorescein signal changes little (fig.3B) while observation of the L-α-DPPC layer through the sulforhodamine filter (fig.2B) shows the first evidence of L-α-DPPC solid domain hydrolysis, displayed as small, frayed indentations in the domain sides, frequently located at L-α-DPPC domain morphological discontinuities. This evidence indicates that active enzyme attaches, often at only one point, to the edge of each L-α-DPPC domain throughout the layer, even though no change in the PLA_2-fluorescein signal is yet seen. That hydrolysis starts exclusively here also indicates that this point on the L-α-DPPC domain has a unique lipid environment. In addition, that the enzyme molecules bind to the edges of the domains indicates a preferable binding environment between liquid and solid regions. This visual evidence, shown here for the first time, is strongly supported by numerous accounts from other groups of enhancement of PLA_2 lipolytic and binding activity in lipid substrate phase transition regions in vesicles [7,10,25], and accompanying enzyme aggregation in its activated state [21,26,27]. These reports of activity enhancement in vesicle phase transition regions induced by temperature cycling are more specifically and directly observed here, using more exact isothermal, pressure-induced phase transition states with defined lipid domains instead. Furthermore, the hypothesis that membrane defects caused by the phase transition pro-

Fig.2. Real-time epifluorescent observation of L-α-DPPC monolayer solid domain hydrolysis by PLA_2 (sulforhodamine filter), constant surface pressure = 22 mN/m, buffer subphase; temperature 30°C. (A) Time = 0 (immediately after PLA_2-fluorescein injection in subphase, (B) 10, (C) 15, (D) 20, (E) 25, (F) 30, (G) 35, (H) 40, (I) 45, (J) 50, (K) 60, (L) 75 min. Scale: white bar in A = 20 μm.

Fig.3. Formation of PLA_2 domains in L-α-DPPC monolayer during phospholipid hydrolysis as observed through epifluorescence microscope (fluorescein filter, corresponding photographs with same scale as time points in fig.2), constant surface pressure = 22 mN/m, buffer subphase, temperature, 30°C. (A) Time = 0 (immediately after PLA_2-FITC injection in subphase), (B) 10, (C) 15, (D) 20, (E) 25, (F) 30, (G) 35, (H) 40, (I) 45, (J) 50, (K) 60, (L) 75 min.



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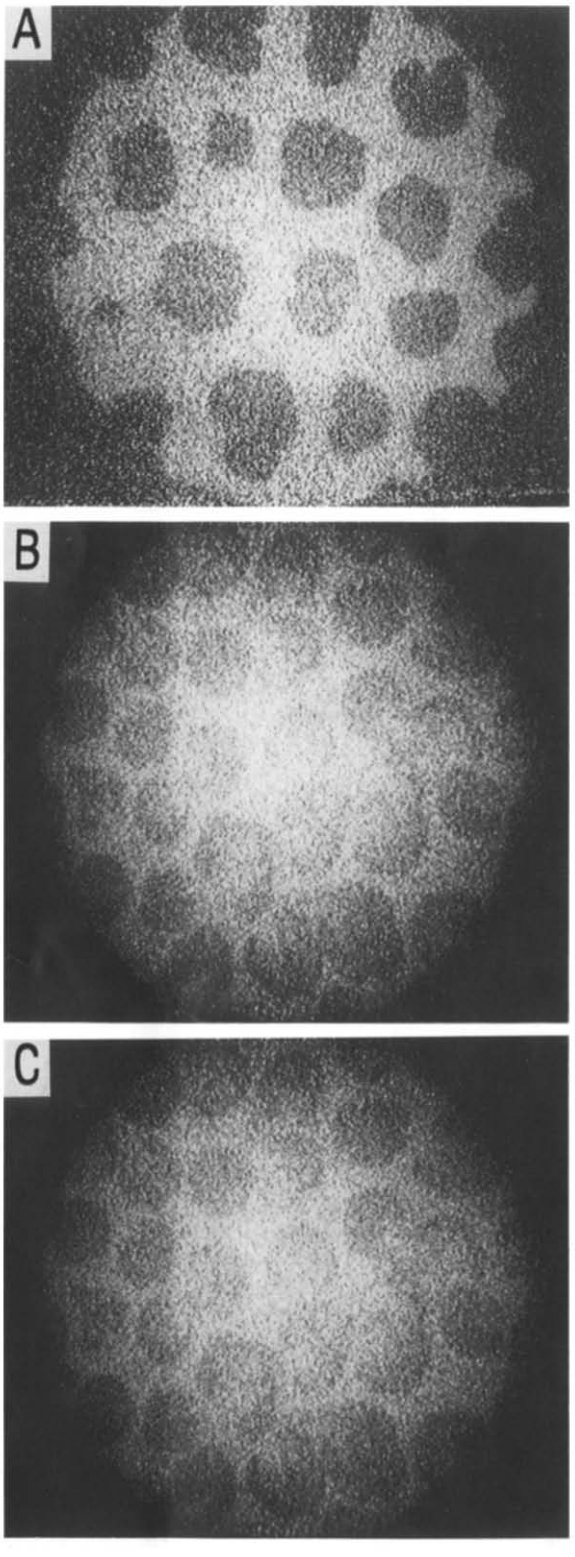

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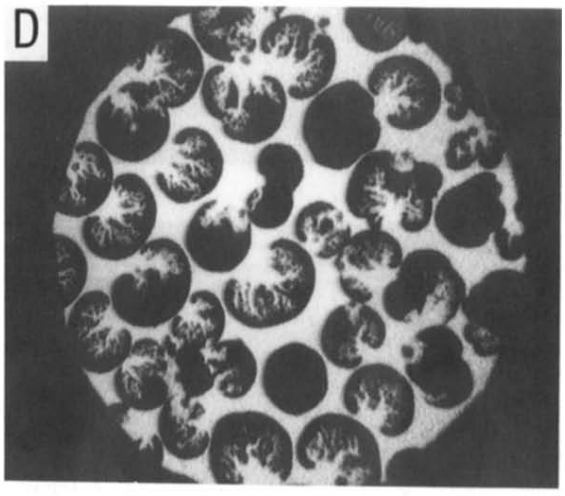

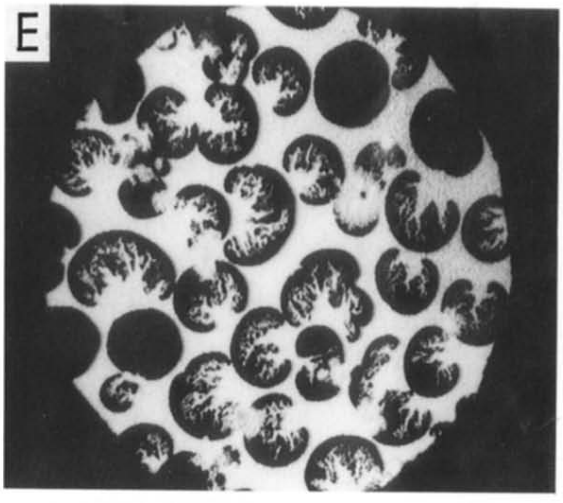

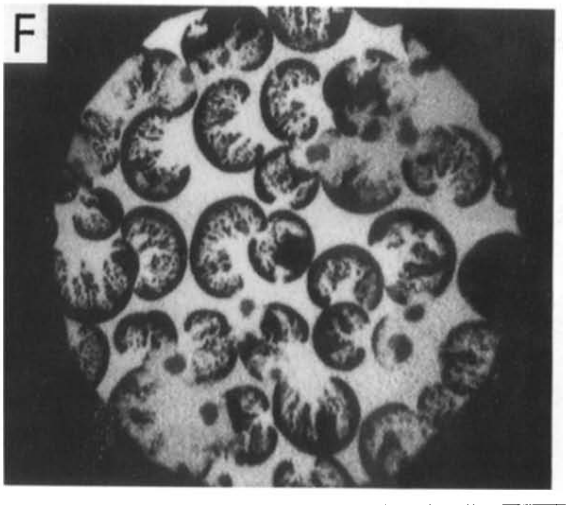

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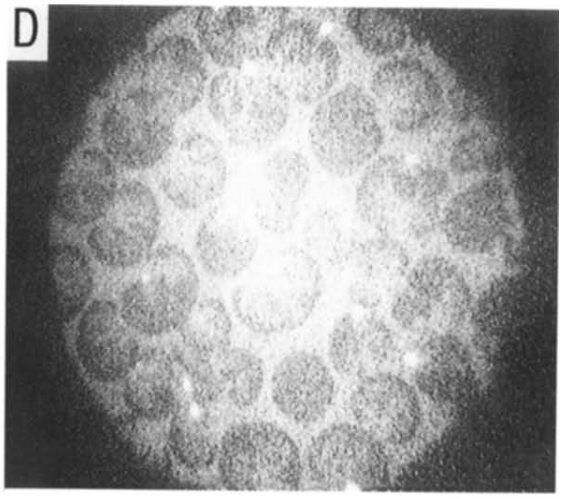

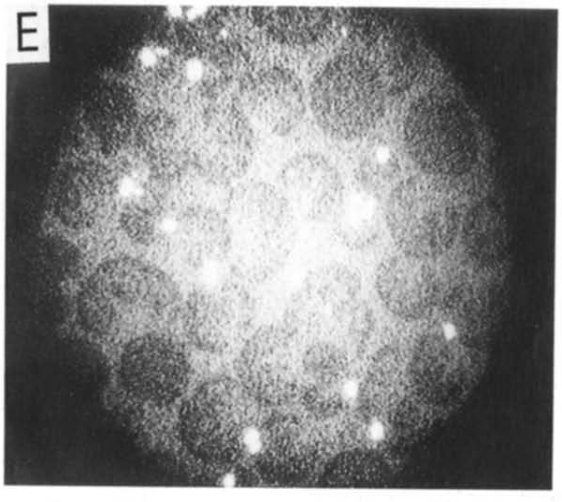

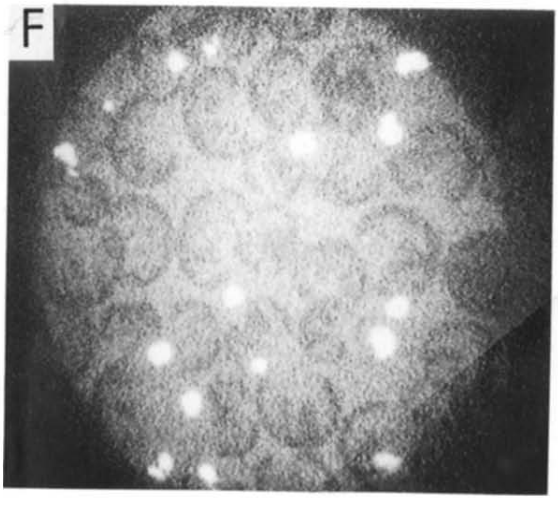

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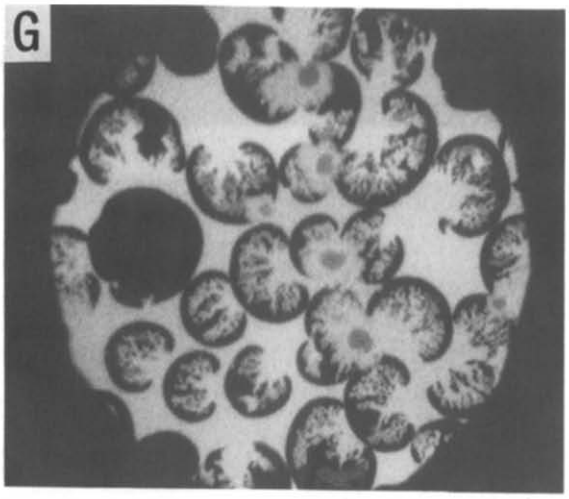

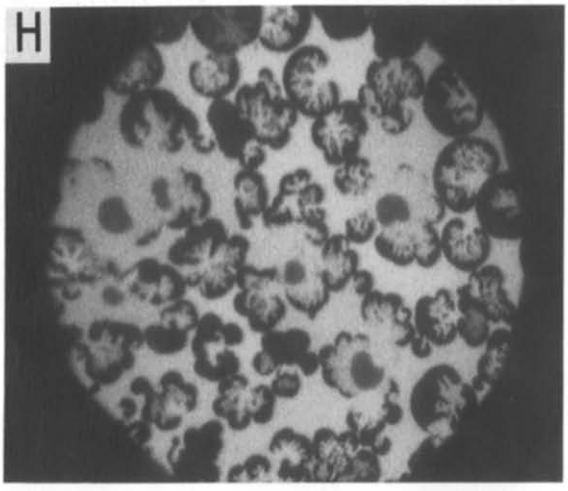

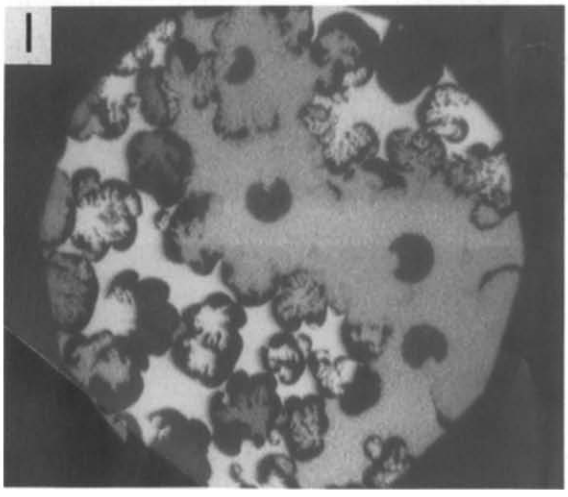

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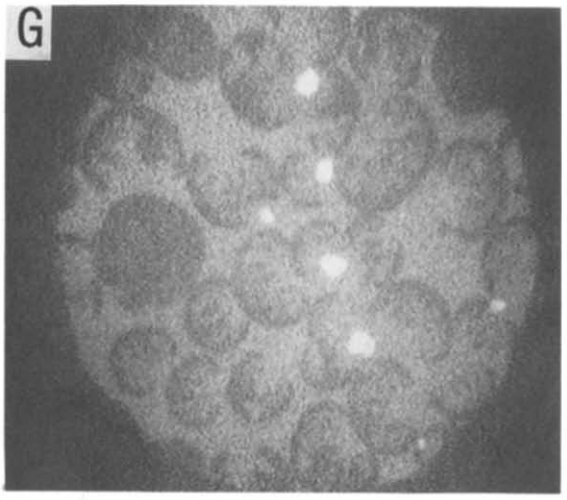

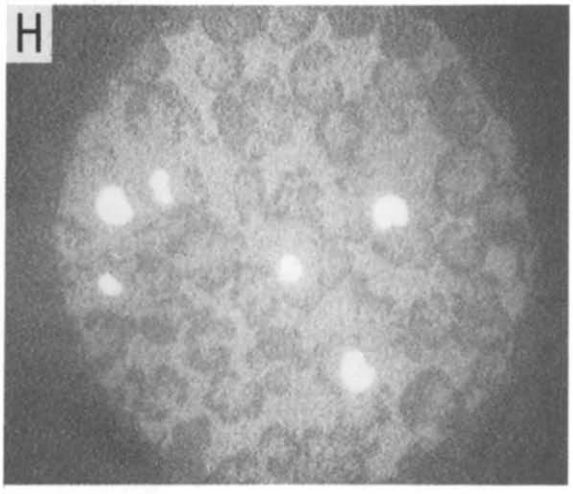

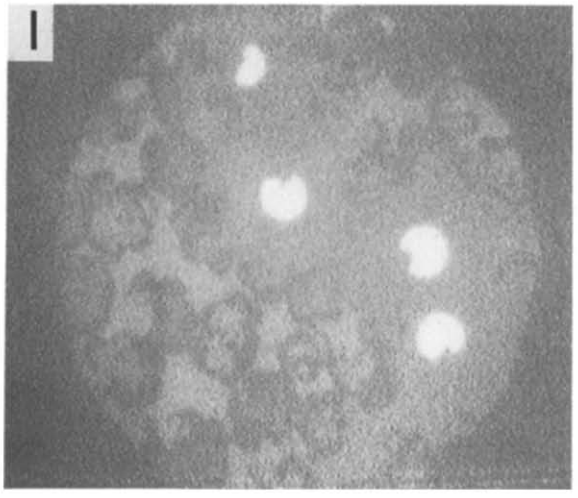

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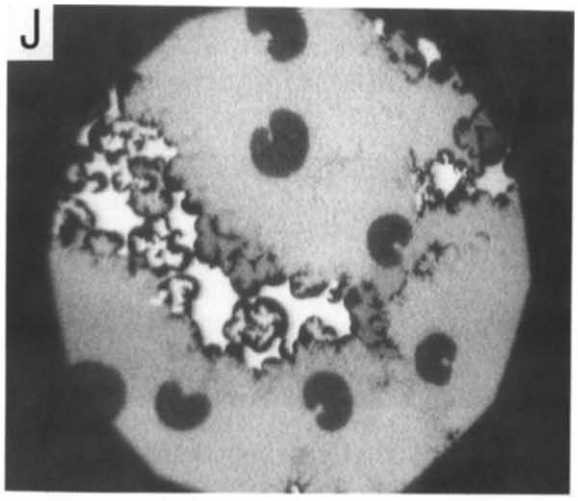

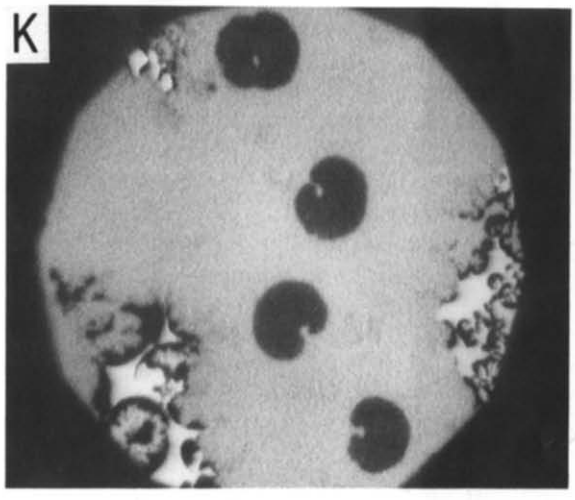

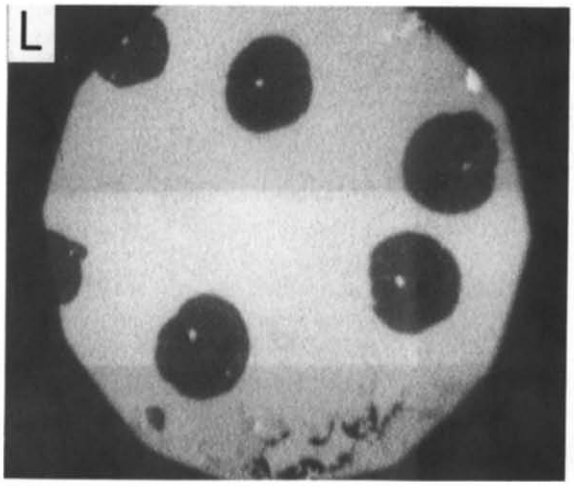

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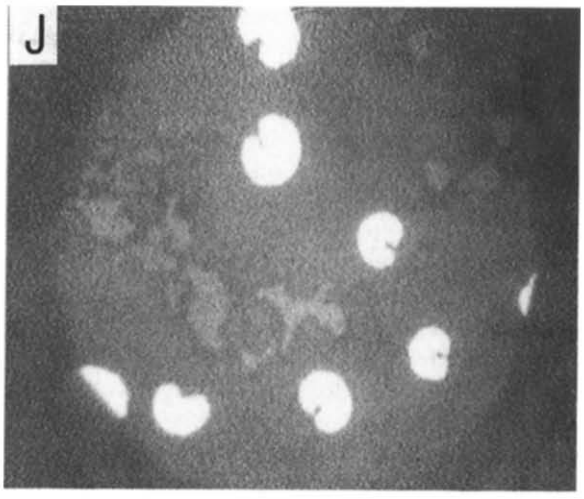

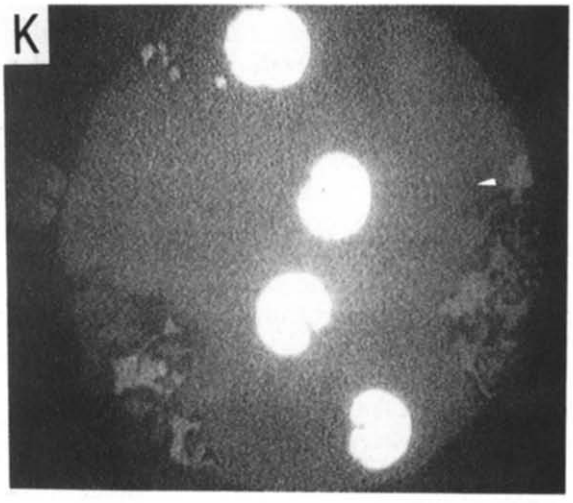

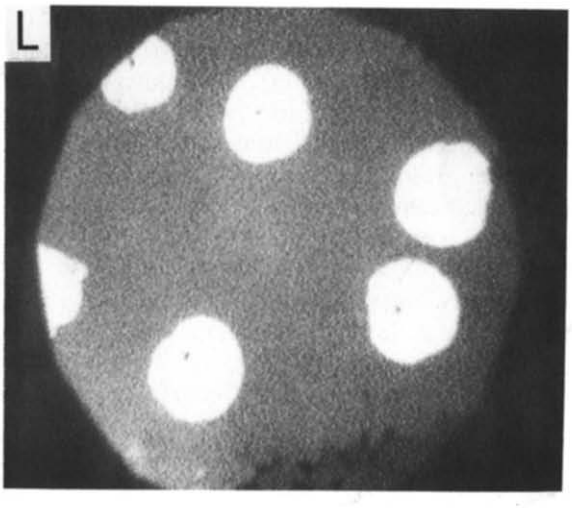

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cess [28–30] account for the increased binding affinity and hydrolytic activity of PLA_2 support our observations of preferential PLA_2 binding at the solid-liquid L-α-DPPC interfaces.

By constantly inverting filters back and forth over time, we were able to follow the simultaneous destruction of L-α-DPPC solid domains (fig.2B–I) together with the unique growth of PLA_2 domains (fig.3D–L) in identical regions. Each identity could not be mistaken because, as seen by comparing figs 2 and 3, specific signals from each filter are inverted as the filters are exchanged, indicating positive identification of each domain with either L-α-DPPC/sulforhodamine or PLA_2-fluorescein, respectively, within the monolayer. PLA_2 domains, which appear bright through the fluorescein filter, are absolutely dark in the sulforhodamine filter, while the L-α-DPPC domains remain dark in both filters. Control experiments using solid lipid domains of nonhydrolyzable phosphatidylcholines – D-α-DPPC and dienoylphosphatidylcholine [4] – 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 PLA_2 hydrolytic activity [31], but shows surface monolayer properties and domain characteristics identical to those of the L-form [15]. Moreover, enzyme domain formation was not limited exclusively to L-α-DPPC monolayers. PLA_2 domains could also be observed in L-α-dimyristoylphosphatidylcholine as well as L-α-phosphatidylethanolamine monolayers in the phase transition region (unpublished), indicating the validity and applicability of the phenomenon. In addition, the necessity for a solid-liquid interface is indicated by hydrolysis experiments in the monolayer gas-liquid expanded analogue phase. Under such conditions, no enzyme domains are observed, even after 2 h hydrolysis. However, when sufficient additional amounts of lipid are subsequently spread on this monolayer, prompting formation of solid-phase lipid domains, hydrolysis is accelerated and enzyme domains appear within a few minutes. We believe that interfacial recognition, activation and a certain degree of hydrolysis are therefore necessary for formation of these microscopically observable enzyme domains.

Over the course of 60 min, the number and size of enzyme domains, as demonstrated by more numerous crescent- or kidney-shaped fluorescent areas seen through the fluorescein filter (fig.3D–L) drastically increase. These enzyme domains retain this crescent morphology through the time course of their development. Most interestingly, this is coupled with corresponding hydrolysis of the L-α-DPPC solid domains which degrade from fully intact, round forms to sequentially and continuously hydrolyzed solid-phase, albeit drastically degraded, remnants (fig.2B–I). That the lipolytic enzyme starts at one point on the L-α-DPPC domain exterior and hydrolyzes its way through the interior in a seemingly regular and directional manner is intriguing and linked possibly to a unique physical arrangement of solid-phase L-α-DPPC molecules. One possible mechanism of subsequent enzyme domain formation is that, by hydrolyzing its way literally through the cross-section of the L-α-DPPC domains, the enzyme creates an increased, perturbed interfacial solid L-α-DPPC border area in contact with the fluid phase lipid. This effect, in combination with a local concentration of lipid *sn*-2 ester hydrolytic end-products, namely production of the corresponding lysophosphatidylcholine and free fatty acid, promotes further potential binding and aggregation sites for more enzyme in a relatively localized area, thereby serving as a site for a self-potentiated PLA_2 aggregation mechanism. Critical, localized concentration increases in these end-products together perhaps with the unhydrolyzed L-α-DPPC in a ternary mixture are speculated to induce localized enzyme domain formation and, might we speculate, two-dimensional crystallization in specific regions of the lipid monolayer. This would suggest an allosteric type of enzyme inhibition. In fact, *N. naja naja* PLA_2 is inhibited by increasing levels of free fatty acids [32]. Furthermore, recent evidence indicating autocatalyzed self-acylation of snake venom PLA_2 with subsequent enzyme self-association [33] could explain larger domain formation through increased enzyme hydrophobization. Our direct observation of actual enzyme domains in regular morphologies strongly supports changes in enzyme-substrate binding affinity [32] and enzyme structural changes [21] induced perhaps by critical changes in the lipid monolayer physical state due to the presence of hydrolytic end products. This mechanism of aggregation might

indeed be a contributing mechanism of allosteric enzyme inhibition [32] in the monolayer as well as in native cell membranes, and remains an interesting question that we are pursuing. The time-dependent nature of the enzyme domain appearance suggests a mechanism associated with the time- and hydrolysis-dependent inhibition of PLA_2 by end-products together with a substrate-dependent (physical state) activation of the enzyme.

In addition to possible monolayer structural changes, another consideration regards the local changes in monolayer phase transition caused by increases in lysolipid and fatty acid content in the layer [10]. Although we cannot yet address specifically any quantitative compositional effects of these products on the L-α-DPPC phase transition, our evidence strongly suggests that L-α-DPPC domains remain solid until actively hydrolyzed. Slow decompressions of the L-α-DPPC layer, simulating gradual refluidization of L-α-DPPC solid domains, show an entirely different morphological behavior [24]. That the enzyme domain phenomenon was witnessed throughout the layer, on nearly all L-α-DPPC domains refutes the possibility that domain formation was an artifact of poorly dispersed, locally concentrated enzyme. 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.

We have witnessed, for the first time, the unique self-association and domain formation of interfacially activated PLA_2 directly in phospholipid monolayers. We propose, in conjunction with Hazlett and Dennis [21], that interfacial recognition, enzyme aggregation, and hydrolytic activation are closely interrelated and interdependent requirements for PLA_2 behavior. In this regard, activation at the membrane interface leads to further enzyme domain growth for small oligomer aggregates into observable domains, presumably by some type of allosteric inhibition mechanism. We further conclude that delays in the formation of these domains results from time-dependent hydrolytic end-product build ups that stimulate enzyme conformational changes with resulting enzyme domain formation in localized areas.

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