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# Spontaneous domain formation of phospholipase $A_2$ at interfaces: fluorescence microscopy of the interaction of phospholipase $A_2$ with mixed monolayers of lecithin, lysolecithin and fatty acid

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Fluorescence microscopy has recently been proven to be an ideal tool to investigate the specific interaction of phospholipase $A_2$ with oriented substrate monolayers. Using a dual labeling technique, it could be shown that phospholipase $A_2$ can specifically attack and hydrolyze solid analogous L-α-DPPC domains. After a critical extent of monolayer hydrolysis the enzyme itself starts to aggregate forming regular shaped protein domains (Grainger et al. (1990) Biochim. Biophys. Acta 1023, 365–379). In order to confirm that the existence of hydrolysis products in the monolayer is necessary for the observed aggregation of phospholipase $A_2$, mixed monolayers of D- and L-α-DPPC, L-α-lysoPPC and palmitic acid in different ratios were examined. The phase behavior and the interaction of these films with phospholipase $A_2$ were directly visualized with an epifluorescence microscope. Above a certain critical concentration of lysolecithin and palmitic acid in the monolayer, compression of these mixed films leads to phase separation and formation of mixed domains of unknown composition. Their high negative charge density is evidenced by preferential binding of a cationic dye to these phase-separated areas. Introduction of fluorescence-labeled phospholipase $A_2$ underneath these mixed domains results in rapid binding of the protein to the domains without visible hydrolytic activity, regardless of whether the L-form or the D-form of the DPPC were used. In binary mixtures, only those with DPPC/palmitic acid show formation of phase-separated areas which can be specifically targeted by phospholipase $A_2$ leading to a rapid formation (within 2 min) of protein domains. Experiments with pyrenedecanoic acid containing monolayers give the first direct evidence that acid is located above the enzyme domains. These results show that a locally high negative charge density of the phase-separated domains is one of the prerequisites for the binding of phospholipase $A_2$. In addition, however, small amounts of D- or L-α-DPPC headgroups within the domains of the monolayer seem to be necessary for recognition followed by fast binding of the protein to the domains. This is confirmed by experiments with mixed monolayers of diacetylene carboxylic acid and D-α-DPPC. The acid – immiscible with lecithin – forms well defined pure acid domains in the monolayer. While the cationic dye can be docked rapidly to these phase separated areas, no preferential enzyme binding and thus no protein domain formation below these acid domains can be induced.

## Introduction: phospholipase $A_2$ domains at lipid monolayers

Phospholipase $A_2$ (EC 3.1.1.4) belongs to an important class of hydrolytically active enzymes which all catalyze cleavage of ester linkages of membrane-forming phospholipids to release biological active substances [1]. Phospholipase $A_2$ itself catalyzes hydrolysis of the ester linkage in C-2-position of a glycerolipid releasing the corresponding lysolipid and a fatty acid. Its importance in many biological processes like rebuilding and modification of membranes and release of arachidonic acid has produced an overwhelming amount of information about its enzymatic action (for reviews, see Refs. 2–8).

Several models of physical association and subsequent catalytic action between phospholipase $A_2$ and lipid interfaces have been forwarded [2–9]. Up to now most results are based on kinetic evidence [10–15], site specific mutagenesis [16,17], crystallographic data [18–

Abbreviations: L-α-DMPC, L-α-dimyristoylphosphatidylcholine; L-α-DPPC, L-α-dipalmitoylphosphatidylcholine; D-α-DPPC, D-α-dipalmitoylphosphatidylcholine; lysoPPC, L-α-lysopalmitoylphosphatidylcholine; SR-DPPE, *N*-(Texas red sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; $C_6$-NBD PC, 3-palmitoyl-2-[6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl]-L-α-phosphatidylcholine; Tris, tris(hydroxymethylamino)methane.

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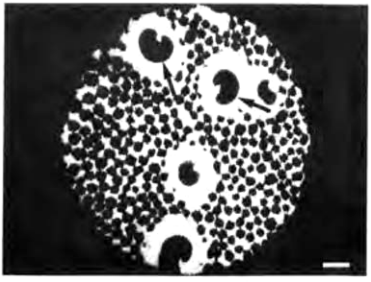


Fig. 1. Phospholipase $A_2$ domains (arrow) within the matrix of solid analogous DMPC domains and liquid analogous DMPC doped with SR-DPPE after 40 min of hydrolysis [27]; Scale bar is 20 $\mu$m.

20] and research about potent phospholipase $A_2$ inhibitors [21–26]. Recently fluorescence microscopy has proven to be very well suited to study the interaction of proteins with functionalized monolayers [27–32]. Using this method it could be shown that phospholipase $A_2$ can specifically recognize and hydrolyze solid analogous lipid domains of e.g. L-α-DPPC or L-α-DMPC. After a critical extent of monolayer hydrolysis the enzyme itself aggregates into regular shaped domains as shown in Fig. 1 [27,28]. It has to be pointed out that the phospholipase $A_2$ aggregated into these domains, formed after the active phase of the enzyme, is not active any more and does not interact with adjacent L-α-DPPC domains [28,31]. There is no relation to the aggregates (dimers or oligomers) discussed by Jain et al. in their careful mechanistic studies showing that only the monomer is active [10f].

In contrast, lipid domain containing monolayers of L-α-DPPC (non-hydrolyzable substrate) do not induce any enzyme domain formation even after 1–2 h. This indicates that formation of these protein domains is dependent on the hydrolysis reaction. However, the protein aggregation may be a secondary rather than primary consequence of the hydrolysis process. Protein aggregation may be induced purely as a result of interaction of phospholipase $A_2$ with the cleavage products of the hydrolysis. In order to investigate whether the existence of the hydrolysis products is crucial for the observed aggregation of phospholipase $A_2$ and to find out if the lecithin itself plays an additional role in this process, the effect of lysolipid and fatty acid content in the monolayer has to be investigated separately from the hydrolysis process. This can be achieved by using mixed monolayers of L- or D-α-lecithin, lysolecithin and palmitic acid in various ratios. Such mixtures simulate the membrane composition after different extents of enzymatic hydrolysis. Assuming that, besides lecithin itself, only its cleavage products and not additional reactions during the hydrolysis process (e.g. acylation [33]) induce enzyme domain formation, one can expect spontaneous development of protein domains induced just by a monolayer of the suitable composition.

On the basis that the fatty acid, which is known to highly influence phospholipase $A_2$ activity [8], plays an important role in protein domain formation, three additional sets of experiments were performed. Pyrenedecanoic acid in a mixture with L-α-DPPC was used to investigate what happens with the acid after the hydrolysis process. The pyrene label allows to directly observe inhomogeneously distributed acid within the monolayer after phospholipase $A_2$ hydrolysis. Further information about possible clustering of the fatty acid and thus about the charge distribution of the lipid layer above the protein domains was obtained by studying the interaction of a cationic dye with the monolayer. Areas of high negative charge density become apparent due to binding of the dye. In addition the influence of negative charges on protein domain formation was investigated with the help of a diacetylene carboxylic acid which is immiscible with lecithin.

## Materials

Phospholipase $A_2$ (*Naja naja*), L-α-DPPC, D-α-DPPC and 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. The diacetylene carboxylic acid was prepared as described elsewhere [34]. Fluorescence labeled lipids (SR-DPPE, pyrenedecanoic acid and $C_6$-NBD-PC) as well as the cationic dye (1,1',3,3,3',3'-hexamethylindocarbocyanine iodide) and sulforhodamine isothiocyanate were purchased from Molecular Probes and used as supplied after checking purity by TLC analysis. 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 $CaCl_2$, pH 8.9) was prepared by dissolution of salts in 10-time concentration and diluted with pure water to the proper concentration for monolayer subphases for each experiment.

Phospholipase $A_2$ was dissolved from the supplier's bottle (without further purification) in buffer and labeled with fluorescein isothiocyanate or sulforhodamine isothiocyanate by the method of Nargessi and Smith [35]. Labeled phospholipase $A_2$ was separated from unreacted fluorescence probe and buffersalts on Pharmacia PD 10 columns (Sephadex G-25M) equili-

brated in pure water, and lyophilized under protection from light and heat. Labeling on a statistical basis provided about two marker molecules on an estimated six lysine residues. Labeling of the same enzyme by a similar method was shown to lower the degree of hydrolytic activity depending on the degree of labeling [36]. Experiments on the kinetics of hydrolysis of L-α-DMPC monolayers show only slight differences in activity before and after labeling (method described elsewhere [37]). Enzyme solutions for monolayer studies were made by dissolving the labeled enzyme in buffer to give 0.01 mg/ml solutions. Aliquots of 1 ml were frozen at −22°C until thawed ambiently before use.

Phospholipid monolayers were spread from chloroform solutions (0.1–0.5 mg/ml). For the mixed monolayers, appropriate volumes of equimolar stock solutions of the pure lipids were premixed and spread. All lipid solutions were doped with 0.5 mol% fluorescence lipid (SR-DPPE or $C_6$-NBD-PC), a probe concentration so dilute that no detectable changes in isotherm behavior of the primary phospholipid component were observed.

Solutions of the cationic dye were made by dissolving the dye in buffer to give $1.3 \cdot 10^{-4}$ μg/ml solutions ($2.75 \cdot 10^{-7}$ mol/l).

## Methods

### *Fluorescence filmbalance*

A complete description of the epifluorescence microscope and associated Langmuir film balance which is placed below it have been published elsewhere [38]. Excitation of fluorescence monolayer probe and labeled enzyme is achieved using a high-pressure mercury lamp. Discrimination of excitation and emission from the different fluorescence probes 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 video camera. Film balance measurements are controlled through an interfaced personal computer. Video recording of the monolayer through the video camera was initiated at various time points of film observation. Photographs shown are directly taken from the video screen.

### *Monolayer investigations*

A dual labeling technique was used for all experiments to distinguish monolayer lipid domain morphology from phospholipase $A_2$ action or dye behavior occurring in the aqueous subphase [27,28]. The experimental procedure is almost the same for all investigations. It only differs in the labels used for the monolayer and the enzyme:

(1) Interaction of phospholipase $A_2$ with mixed monolayers of lecithin, lysolecithin and palmitic acid was studied using either SR-DPPE as the label in the monolayer and fluorescein as the label on the enzyme (as shown in Figs. 1–3) or vice versa.

(2) Binding of the cationic dye to mixed monolayers was examined using a $C_6$-NBD-PC label in the monolayer.

(3) Sulforhodamine-labeled enzyme was used for studying hydrolysis of L-α-DPPC monolayers mixed with pyrenedecanoic acid. In this case, an additional fluorescence lipid in the monolayer is not necessary because the pyrene labeled acid itself can directly be visualized.

(4) Interaction of phospholipase $A_2$ and the cationic dye with mixed monolayers of D-α-DPPC/diacetylene carboxylic acid was performed using the $C_6$-NBD-PC label in the monolayer and sulforhodamine-labeled enzyme.

Phospholipid solutions were spread on a buffer subphase at 30°C. After spreading, the monolayer was immediately compressed at a rate of 2.5 Å²/mol per min. At a surface pressure of 17–20 mN/m the barrier was stopped and either the enzyme solution (0.2 ml = 2 μg phospholipase $A_2$) or the dye solution (0.5 ml = 0.07 μg dye) were injected into the subphase under the monolayer. From this time on the pressure was kept constant. Especially mixed films with a high content of lysoPPC and palmitic acid are unstable on standing and the pressure decreases within 10 min. This may be caused by the relatively high water solubility of the lysoPPC.

During compression and after addition of enzyme or dye, the monolayer and subphase were directly observed with the fluorescence microscope. Observation was performed using alternately two interchangeable cut-off filters, corresponding to signals from the sulforhodamine label (Zeiss filter 487714) and the fluorescein label (Zeiss filter 487709), respectively. Hydrolysis of the pyrene acid containing films was visualized using a pyrene filter (Zeiss filter 487702) and the sulforhodamine filter.

## Results and Discussion

Formation of solid analogous lipid domains during the main phase transition in lipid monolayers has been described using a number of different fluorescence microscopy systems [38–43]. Although many investigations of pure systems have been forwarded [44–47] only little attention has been given to mixed monolayers which therefore are not well characterized by this method [48].

### *Investigations of ternary mixtures of DPPC, L-α-lysoPPC and palmitic acid*

Different mixtures of DPPC (L-form and D-form), L-α-lysoPPC and palmitic acid were used to study their

TABLE I

*Domain formation in ternary mixed monolayers of DPPC, lysoPPC and palmitic acid*

The ratio of lysoPPC/palmitic acid is always 1:1 [a]; compression speed 2.5 Å²/mol per min.

| DPPC [b] (mol%) | Hydrolysis products lysoPPC/palmitic acid (mol%) | Monolayer behavior at 18 mN/m [c] |
|---|---|---|
| 80 | 20 | homogeneous |
| 70 | 30 | homogeneous |
| 60 | 40 | homogeneous |
| 50 | 50 | domain formation; size ≈ 5 μm |
| 40 | 60 | domain formation; size ≈ 10 μm |
| 30 | 70 | domain formation; size ≈ 20 μm |
| 20 | 80 | domain formation; size ≈ 30 μm |
| 15 | 85 | domain formation; size ≈ 40 μm |
| 10 | 90 | domain formation; size ≈ 60 μm |

[a] This corresponds to the ratio achieved in the hydrolysis of DPPC.
[b] Both the D- and the L-form were used
[c] If the surface pressure is further increased an additional 2D-crystallization of DPPC is induced.

interaction with phospholipase $A_2$ simulating the composition of the monolayer after different extents of hydrolysis (Table I). The ratio of L-α-lysoPPC/palmitic acid is always 1:1 as it is formed during the hydrolysis process.

After spreading of the ternary mixtures (given in Table I) doped with 0.5 mol% DPPE-SR on buffer subphase the liquid analogous monolayers appear homogeneously bright under the fluorescence microscope (sulforhodamine filter, Fig. 2A). Monolayer images of films with 20%, 30% and 40% of hydrolysis products do not change during compression up to 20 mN/m (compression speed: 2.5 Å²/mol per min). However, compression of mixed monolayers containing more than 40% of the 1:1 mixture of lysoPPC/palmitic acid lead to phase separation and formation of grey domains of unknown composition. Similar results indicating phase separation have been obtained with liposomes of DPPC/lysoPPC/palmitic acid [49]. The mixed domains appear grey because, unlike the black domains of pure DPPC, the fluorescence dye is not completely excluded (Fig. 2B). Further compression leads to an increase in number and size of these regular shaped mixed domains. The compression speed plays an important role for monolayer morphology. Higher compression speeds (20–10 Å²/mol per min) induce an additional 2D-crystallization of DPPC at lower pressures (10–16 mN/m). These black lipid domains are not stable on standing and slowly disappear forming the same mixed grey domains, directly visible using the slow compression speed.

After injection of fluorescein-labeled phospholipase $A_2$ below the domain containing monolayer (at 18 mN/m), spontaneous binding of the enzyme onto these mixed grey domains can be visualized by switching the filter to view the fluorescein fluorescence. Domains which are grey in the sulforhodamine filter appear bright in the fluorescein filter already after 1–2 min (Fig. 2C). This rapid binding of the enzyme to the monolayer is even more noteworthy when it is compared with the slow formation of protein domains during hydrolysis of L-α-DPPC or L-α-DMPC monolayers [27,28,31] by phospholipase $A_2$ (about 40 min, see Fig. 1). The mixed domains therefore seem to represent a prefered environment for enzyme binding. Such a process is demonstrated in Fig. 2 for a mixture of 10% L-α-DPPC with 90% lysoPPC/palmitic acid (1:1) simulating nearly complete hydrolysis of a lecithin monolayer. The shape of the mixed domains and therefore also of the protein domains is nearly the same as that observed for hydrolysis of L-α-DPPC after 70 min [27] (see also Fig. 1).

Waiting for some time after phospholipase $A_2$ injection (30 min) leads to slow hydrolysis of the lecithin in the liquid analogous phase due to the excess of free enzyme in the subphase. This causes a slow increase of protein domain size like during the hydrolysis of a lecithin monolayer [27,31].

It is important to note that the same phase separation of mixed domains within the monolayer and also the fast binding of phospholipase $A_2$ to this domains can be obtained with the non-hydrolyzable D-α-DPPC instead of the L-α-DPPC in the mixtures with the only difference that the protein domains do not increase in size with time. The D-form of the lecithin can be recognized but not hydrolyzed by phospholipase $A_2$ [21]. Thus it appears that recognition of the headgroup of the lecithin, but not its cleavage, is necessary for rapid aggregation of the protein under the mixed monolayer. On the other hand the lecithin headgroup may not be needed at all to form the protein domains. Thus the question is still open which components really induce the rapid enzyme domain formation without enzyme activity.

*Investigations of binary mixtures*

In order to obtain information about which of the hydrolysis products in the ternary mixtures are necessary to induce phase separation followed by rapid enzyme aggregation at the monolayer, binary mixtures doped with 0.5 mol% SR-DPPE were investigated (Table II).

All mixtures containing *D-α-DPPC and palmitic acid* show nearly the same behavior as the ternary ones. Directly after spreading on buffer subphase the monolayer looks homogeneously bright in the sulforhodamine filter. After compression small grey domains sometimes bright at the borders appear if the amount of palmitic acid is higher than 30% (Fig. 3A). This phenomenon of phase separation in DPPC/palmitic

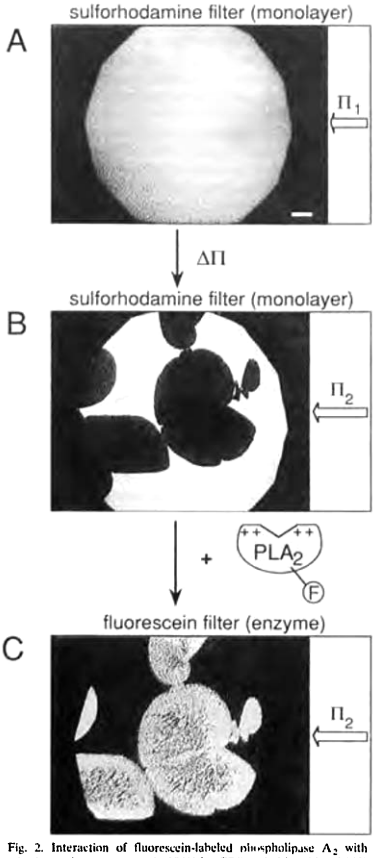



Fig. 2. Interaction of fluorescein-labeled phospholipase $A_2$ with mixed monolayers composed of 90% lysoPPC/palmitic acid and 10% D-α-DPPC doped with 0.5 mol% SR-DPPE: (A) homogeneous fluorescence of the liquid analogous mixed monolayer ($\pi_1$ = 1 mN/m); (B) compression leads to phase separation of grey domains of unknown composition ($\pi_2$ = 18 mN/m); (C) fluorescein-labeled phospholipase $A_2$ injected underneath this monolayer binds very quickly to these mixed domains which thus appear bright in the fluorescein filter. Subphase: Tris buffer (pH 8.9); Temperature = 30°C; Scale bar in (A) is 20 μm.

TABLE II

*Domain formation in binary mixed monolayers of DPPC/lysoPPC, lysoPPC/palmitic acid and DPPC/palmitic acid*

The compression speed is 2.5 Å²/mol per min.

| D-α-DPPC (mol%) | LysoPPC (mol%) | Palmitic acid (mol%) | Monolayer behavior at 18 mN/m |
|---|---|---|---|
| 0 | 50 | 50 | homogeneous |
| 70 | 30 | 0 | homogeneous [a] |
| 50 | 50 | 0 | homogeneous [a] |
| 25 | 75 | 0 | homogeneous [a] |
| 80 | 0 | 20 | only DPPC domains |
| 70 | 0 | 30 | domain formation [b]; size ≈ 3 μm |
| 60 | 0 | 40 | domain formation; size ≈ 10 μm |
| 50 | 0 | 50 | domain formation; size ≈ 25 μm |
| 40 | 0 | 60 | immiscible with labeled lipid |
| 25 | 0 | 75 | immiscible with labeled lipid |
| 17 | 0 | 83 | immiscible with labeled lipid |

[a] If the surface pressure is further increased crystallization of DPPC is induced.

[b] Very small domains only visible in the fluorescein filter after injection of fluorescein-labeled phospholipase $A_2$.

acid mixtures is already known from liposomes [50–52]. During further compression up to 18 mN/m these mixed domains grow in size and number (Fig. 3).

In contrast to ternary mixtures where only grey domains appear in the monolayer during compression up to 18 mN/m, use of binary mixtures of D-α-DPPC/fatty acid leads to an additional crystallization of DPPC (Fig. 3B). Thus at 18 mN/m one finds a coexistence between lecithin domains (black in the sulforhodamine filter) and mixed domains (grey in the sulforhodamine filter). In the following experiments only the behavior of the mixed grey domains will be discussed. Size of these mixed domains depends strongly on the amount of fatty acid used in the mixture. In the case of 50% palmitic acid in the monolayer, the phase separated areas are of regular shape and clearly visible (diameter about 25 μm). Decrease of the amount of acid results in decreasing size of the domains and at a concentration of 30% palmitic acid no phase separation of mixed grey domains can be detected. Increasing amounts of fatty acid (> 50%) cause immiscibility of the lipids with the applied fluorescence dye and therefore these mixtures could not be examined with this method.

If fluorescein-labeled phospholipase $A_2$ is injected under a mixed monolayer of DPPC/palmitic acid which contains phase separated mixed domains (Fig. 3B,C; arrow) as well as black DPPC domains the enzyme rapidly binds to the grey domains within 2 min as already shown for the ternary mixtures in Fig. 2. These mixed domains can now be easily distinguished from the black lipid domains of pure lecithin by switching the filter to view the fluorescein fluorescence (Fig. 3C).

Only the mixed domains appear bright due to enzyme binding and no protein can be found below the black lipid domains. In addition, the fluorescein-labeled phospholipase $A_2$ allows detection of phase separation also in the mixture with 30% palmitic acid. The mixed domains are very small and therefore only visible in the fluorescein filter as small pinpoints of light after enzyme binding. The whole process of phase separation

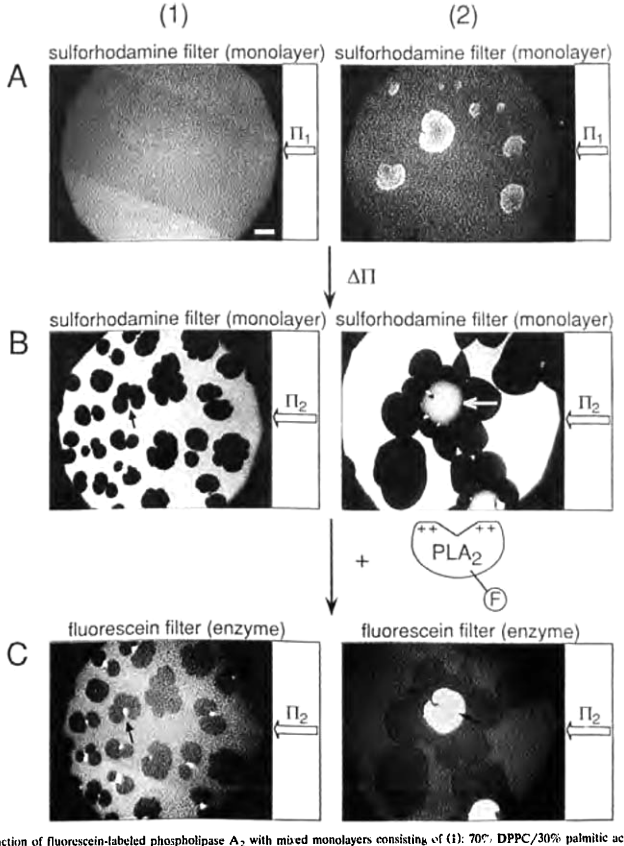


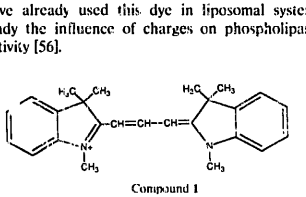

Fig. 3. Interaction of fluorescein-labeled phospholipase $A_2$ with mixed monolayers consisting of (1): 70% DPPC/30% palmitic acid; (2): 50% DPPC/50% palmitic acid doped with 0.5 mol% SR-DPPE: (A) phase separated mixed domains of unknown composition ($\pi_1$ = 12 mN/m); (B) compression ($\pi_2$ = 18 mN/m) leads to an additional crystallization of solid analogous DPPC around the mixed grey domains (arrow); (C) fluorescein-labeled phospholipase $A_2$ injected under this monolayer binds very quickly (within 2 min) to these mixed grey domains which thus appear bright in the fluorescein filter (arrow); Subphase: Tris buffer (pH = 8.9); Temperature = 30°C; Scale bar in (A) is 20 μm.

and enzyme interaction is shown in Fig. 3 for monolayers which contain 30% palmitic acid (1) and 50% palmitic acid (2).

In mixed systems of *lysoPPC / palmitic acid* and *DPPC / lysoPPC* no phase separation of mixed domains can be detected. These results are in good agreement with results obtained with liposomes [49,53] showing complete miscibility of the two components in both cases. The mixed monolayers appear homogeneously bright in the sulforhodamine filter and nothing changes during compression up to 25 mN/m. Injection of fluorescein-labeled phospholipase $A_2$ at 18 mN/m results in homogeneous fluorescein fluorescence with no domain formation occurring even after a 60 min wait. This is a further hint that both palmitic acid, as well as uncleaved lecithin, are necessary to induce enzyme aggregation. However, it can not be excluded that the lysoPPC in the mixture leads to an increase of phospholipase $A_2$ binding to the whole monolayer as discussed for liposomes [54].

*Interaction of mixed monolayers with a cationic dye*

For further characterization of the mixed domains which induce preferential binding of phospholipase $A_2$, interaction of these mixed domains with 1,1',3,3,3',3'-hexamethylindocarbocyanine-iodide (**1**) was examined. This dye has a positive charge and binds very strongly to negatively charged interfaces [55]. Jain et al. have already used this dye in liposomal systems to study the influence of charges on phospholipase $A_2$ activity [56].




**1**

Compound 1

The fluorescence spectra of the cationic dye has a broad emission peak at 560 nm detectable with the sulforhodamine filter [57]. Therefore a $C_6$-NBD-PC label visible with the fluorescein filter was used to study the interaction of the cationic dye with the mixed monolayers.

After spreading of the appropriate mixture on buffer subphase the monolayer looks homogeneously bright in the microscope (fluorescein filter). Compression leads to phase separation of domains which are slightly brighter than the matrix because of a higher content of labeled lipid ($C_6$-NBD-PC) within the domains. Injection of 0.5 ml of the dye solution under the monolayer leads to direct binding of the dye to the phase separated areas indicating that negative charge (fatty acid) is concentrated in these areas. The cationic dye **1** and phospholipase $A_2$ show rapid binding to the same phase separated areas suggesting so far that preferential binding of phospholipase $A_2$ is the result of charge–charge interactions. It is pertinent in this respect that the active site of phospholipase $A_2$ is surrounded by cationic amino acids [8] which are important for enzyme binding to interfaces [8,58–62].

These findings are in agreement with results from Jain et al. showing that hydrolysis of DMPC vesicles with phospholipase $A_2$ leads to preferential binding of the cationic dye after a certain extent of hydrolysis [56]. Their results are interpreted to imply that the hydrolysis products (especially the fatty acid) phase separate above a certain concentration and that this leads to binding of the cationic dye as well as to binding of a higher amount of enzyme. All these results, indicating phase separation of acid containing mixtures, are obtained by indirect evidences and domain formation of the enzyme is not mentioned. Fluorescence microscopy gives the first direct evidence of phase separation of negative charged domains. In addition one can directly visualize that these domains can specifically bind the cationic dye as well as the labeled phospholipase $A_2$. At this point of the discussion it is still unclear if the high negative charge density is the only reason for the fast docking of the enzyme to the phase separated mixed domains or if the lipid headgroup has an additional influence.

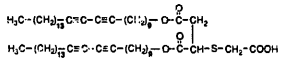
*Hydrolysis of a mixed monolayer of L-α-DPPC / pyrenedecanoic acid*

Visualization of the hydrolysis of a mixed film of L-α-DPPC with pyrenedecanoic acid (ratio 5:1) by phospholipase $A_2$ offers the possibility to directly see what happens with the acid during hydrolysis reaction. Images of different times during hydrolysis of this mixed monolayer are given in Fig. 4.

Directly after spreading the film looks homogeneously bright in the pyrene filter (label in the monolayer). Compression leads to formation of L-α-DPPC domains which may contain small amounts of pyrene decanoic acid (Fig. 4A). After injection of sulforhodamine-labeled phospholipase $A_2$ at 20 mN/m the enzyme starts to interact with the lipid domains [28]. They decrease in size with time (Fig. 4B) and after 30 min. large enzyme domains can be visualized (Fig. 4C,D). These protein domains are now not only bright in the sulforhodamine filter (label of the enzyme) but also in the pyrene filter (pyrenedecanoic acid: label in the monolayer). This clearly indicates that the acid itself phase separates out of the mixed film and is concentrated above the protein domains as proposed above. Whether the monolayer above the protein domains consists only of the acid, or of a mixture of acid and DPPC, can still not be clearly distinguished with these measurements alone.

*Interaction of phospholipase $A_2$ and the cationic dye 1 with pure carboxylic acid domains within a liquid analogous D-α-DPPC matrix*

To further study the influence of the lecithin headgroup on the rapid binding of phospholipase $A_2$ to the mixed grey domains, experiments were performed with long chain carboxylic acid immiscible with DPPC. For these investigations a diacetylene carboxylic acid **2** was used.



**2**

Compound **2**

This acid has a high tendency to crystallize and the pressure/area diagram shows only a solid analogous phase [34]. Directly after spreading of a mixture of D-α-DPPC/diacetylene carboxylic acid (1:1) doped with 0.5 mol% $C_6$-NBD-PC, large, well defined, characteristically shaped solid analogous acid domains [63] are visible ($\Pi$ = 1–2 mN/m). At this low pressure the DPPC is far away from any tendency to crystallize. Due to its immiscibility with the diacetylene carboxylic acid one can assume that the black domains visible in the film consist of pure acid. Further compression leads to an increase in size of these domains and at a pressure of 18 mN/m the barrier is stopped to prevent cocrystallization of DPPC. Up to this pressure the monolayer thus consists only of solid analogous acid domains in a liquid analogous matrix of DPPC mixed with $C_6$-NBD-PC (Fig. 5A). Injection of the cationic dye **1** leads to rapid binding (1 min) of the dye to the acid domains showing the high negative charge density within the domains as expected for pure acid (Fig. 5 (1) B). After addition of sulforhodamine-labeled phospholipase $A_2$ no binding of the enzyme to the acid domains can be detected (Fig. 5 (2) B). This is in contrast to the mixed systems with DPPC/lysoPPC/palmitic acid and DPPC/palmitic acid, where dye and phospholipase $A_2$ show the same preferential and fast binding to the

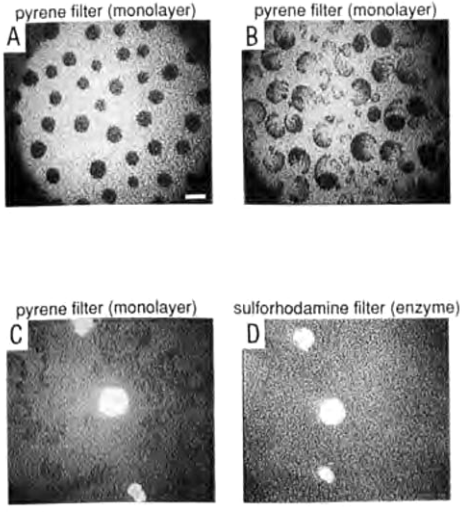



Fig. 4. Hydrolysis of a mixed monolayer of DPPC with pyrenedecanoic acid (5:1) by sulforhodamine-labeled phospholipase $A_2$; the monolayer is seen through the pyrene filter and the enzyme is seen through the sulforhodamine filter: (A) dark solid analogous lipid domains in a bright liquid analogous matrix; (B) phospholipase $A_2$ has partially hydrolyzed the solid analogous lipid domains; (C), (D) protein domains in coexistence with residues of solid analogous lipid in a liquid analogous matrix, enzyme domains appear bright in the pyrene filter (enrichment of pyrenedecanoic acid above the protein domains) as well as in the sulforhodamine filter (fluorescence of the labeled enzyme); (A): $t$ = 0 min; (B): $t$ = 10 min; (C), (D): $t$ = 30 min; $\pi$ = 20 mN/m; Subphase: Tris buffer (pH 8.9); Temperature = 30°C; Scale bar in (A) is 20 μm.

mixed domains. The different interaction of the cationic dye **1** and the sulforhodamine-labeled enzyme with the diacetylene carboxylic acid (**2**) domains within the fluid D-α-DPPC matrix are compared in Fig. 5.

This direct comparison of dye and enzyme binding shows that not only the high negative charge density of the acid domains can be the reason for the rapid phospholipase $A_2$ domain formation. This points to the fact that besides ionic interactions the specific interaction of the lecithin headgroup with the enzyme seems to be necessary for the fast docking process at preformed acid and lecithin containing domains. This assumption is schematically shown in Fig. 6, illustrating two facts:

(1) Interaction of labeled phospholipase $A_2$ with pure acid domains leads to an equilibrium between bound and free enzyme due to ionic interaction but no enzyme domain formation can be detected.

(2) Injection of the enzyme below monolayers containing acid domains with incorporated lecithin headgroups leads to fast binding of the protein to these domains.

Thus rapid protein domain formation seems to be induced by both, charge-charge interaction with the

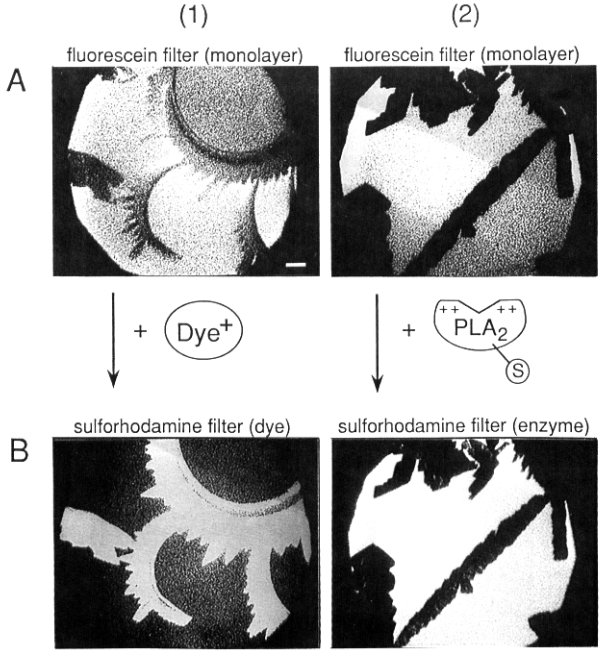



Fig. 5. Interaction of the cationic dye (**1**) and sulforhodamine-labeled phospholipase $A_2$ (**2**) with a mixed monolayer of diacetylene carboxylic acid **2** and D-α-DPPC (1:1) doped with 0.5 mol% $C_6$-NBD-PC: (A) black solid analogous acid domains in the liquid analogous matrix of D-α-DPPC ($\pi$ = 18 mN/m), (B) interaction of the dye (**1**)/the enzyme (**2**) with the mixed monolayer: (1) after injection of the dye **1** the acid domains immediately appear bright in the sulforhodamine filter due to direct binding of the dye to the acid domains; (2) after injection of the enzyme no preferential binding of phospholipase $A_2$ to the acid domains can be detected; Subphase: Tris buffer (pH 8.9); Temperature = 30°C; Scale bar in (A) is 20 $\mu$m.

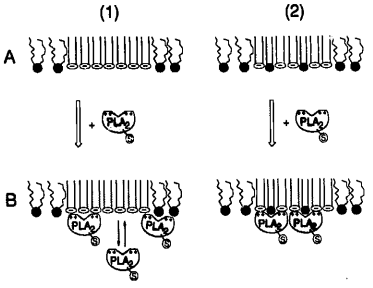



Fig. 6. Schematic representation of the proposed influence of recognizable headgroups on the induction of phospholipase $A_2$ domain formation; (1) A: monolayer with pure acid domains; B: injection of labeled enzyme leads to an equilibrium between bound and free enzyme due to charge–charge interaction – no domain formation of the protein is detectable; (2) A: monolayer with acid domains which contain a small amount of recognizable headgroups; B: injection of labeled phospholipase $A_2$ leads to direct binding and thus domain formation of the enzyme due to specific interaction with lecithin headgroups and charge–charge interaction with the anionic lipids present in the monolayer.

anionic monolayer as well as specific interaction with the headgroup. In the generally accepted scheme for interfacial catalysis of phospholipase $A_2$ at interfaces [2,4,8], three different equilibria can be distinguished: at first the equilibrium between free enzyme and bound enzyme, secondly the equilibrium between bound enzyme and the bound enzyme-substrate complex and at last the equilibrium between the bound enzyme-substrate complex and bound enzyme and product.

$$E \rightleftharpoons E^* + S \rightleftharpoons E^*S \rightleftharpoons E^* + P$$

Considering the first two equilibria, one possible explanation for enzyme domain formation can be proposed: incorporation of recognizable headgroups within the acid domains leads to a shift of the E to E* equilibrium to the $E^* + S$ to $E^*S$ equilibrium because now substrate molecules are present in the interface and formation of the $E^*S$ complex is possible. Without substrate molecules only the first equilibrium exists. In addition it would be interesting to find out if the carboxylic acid has a specific influence on enzyme domain formation or if it can be replaced by other anionic additives like sulfonic acid or phosphonic acid.

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