Minireview

Biophysical mechanisms of phospholipase A2 activation and their use in liposome-based drug delivery

Kent Jørgensen[a], Jesper Davidsen[a], Ole G. Mouritsen[b,*]

[a] *MEMPHYS – Center for Biomembrane Physics, LiPlasome Pharma A/S, Technical University of Denmark, Building 207, DK-2800 Lyngby, Denmark*

[b] *MEMPHYS – Center for Biomembrane Physics, Physics Department, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*

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Abstract Secretory phospholipase A2 (PLA2) is a ubiquitous water-soluble enzyme found in venom, pancreatic, and cancerous fluid. It is also known to play a role in membrane remodeling processes as well as in cellular signaling cascades. PLA2 is interfacially active and functions mainly on organized types of substrate, e.g. micelles and lipid bilayers. Hence the activity of the enzyme is modulated by the lateral organization and the physical properties of the substrate, in particular the structure in the nanometer range. The evidence for nano-scale structure and lipid domains in bilayers is briefly reviewed. Results obtained from a variety of experimental and theoretical studies of PLA2 activity on lipid-bilayer substrates are then presented which provide insight into the biophysical mechanisms of PLA2 activation on lipid bilayers and liposomes of different composition. The insight into these mechanisms has been used to propose a novel principle for liposomal drug targeting, release, and absorption triggered by secretory PLA2.

Key words: Phospholipase A2; Lipid domain; Stealth liposome; Pro-drug; Pro-enhancer; Anti-cancer drug

1. Introduction

Secretory phospholipase A2 (PLA2) refers a large class of interfacially active enzymes that catalyze the hydrolysis of glycerophospholipids in the *sn*-2 position, leading to the formation of lysolipids and fatty acids [1,2]. Since there is a substantial structural similarity between different secretory PLA2s it is surmised that they have a common catalytic mechanism. PLA2 is only weakly active at monomeric substrate but very active on organized types of substrate, e.g. micelles or bilayers, and it is therefore not surprising that the enzyme's activity and mode of action to a certain extent are controlled by the physical properties of the substrate [3–5].

Results obtained from a variety of experimental and theoretical studies of PLA2 activity on lipid-bilayer substrates have provided insight into the dependence of the enzyme activity on bilayer composition, lateral structure, and thermodynamic conditions. The enzyme activity is often measured via its peculiar lag-burst kinetics in terms of the lag time. In particular, it has been studied how the lag time and the degree of hydrolysis can be varied by changing the physical properties of the substrate [4,5].

There is accumulating evidence that small-scale structure and lipid domains persist in lipid bilayers in the nanometer range [6–8]. Furthermore, it has been found that PLA2 activity is sensitive to this small-scale structure, which can be varied systematically by varying acyl-chain length of the phospholipids, temperature, and lipid composition [3,8–12]. It has been discovered that PLA2 activity is enhanced at polymer-covered Stealth liposomes [13]. Detailed and systematic studies have assessed the effects of polymer coverage and polymer length, liposome surface charge, and lipid composition [14,15]. Furthermore, significant enhancer effects of the hydrolysis products on drug release have been observed [16].

In the present Minireview we report on recent progress in the field with focus on work that has been carried out in the authors' own laboratory. First, we describe the present theoretical and experimental evidence for nano-scale structure and lipid domains in bilayers. Second, results obtained from a variety of experimental and theoretical studies of PLA2 activity on lipid-bilayer substrates are presented which provide insight into the biophysical mechanisms of PLA2 activation on lipid bilayers and liposomes of different composition. The various results have been obtained from a wide range of methods and techniques, including fluorescence spectroscopy, real-time HPLC analysis, atomic force microscopy, and molecular modeling and computer-simulation calculations. Finally, we describe how the insight into the biophysical mechanisms of PLA2 recently has been used to propose a novel principle for liposomal drug targeting, release, and absorption by secretory PLA2 [17]. This principle takes advantage of an elevated level of secretory PLA2 in the diseased tissue [18].

2. Lipid bilayers display small-scale structure and lipid domains

Whereas the distinct trans-bilayer structure and lateral pressure profile of lipid bilayers are well-established features of membranes, the lateral organization, in particular on scales

*Corresponding author. Fax: (45)- 6615 8760.
E-mail address: ogm@memphys.sdu.dk (O.G. Mouritsen).

Abbreviations: PC, phosphatidylcholine; DMPC, dimyristoyl PC; DPPC, dipalmitoyl PC; DSPC, distearoyl PC; PE, phosphatidylethanolamine; PEG, poly(ethylene-glycol); PLA2, phospholipase A2

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ranging from 10 to 1000 nm, is much less well-characterized [19]. However, in recent years an increasing amount of indirect and direct evidence from studies of model membranes as well as biological membranes has suggested that membranes are often laterally structured in terms of domains and so-called rafts [20]. The length and timescales of these domains remain controversial, and domain diameters from a few nanometers to many micrometers have been reported. The general observation is that the techniques that have the higher spatial resolution also tend to find domains of a smaller size. Lipid domains and rafts are stabilized by a number of different interactions, including membrane–cytoskeleton, lipid–protein, and lipid–lipid interactions, and the organization can be both of an equilibrium as well as a non-equilibrium nature [19].

We shall focus here on lipid-domain formation caused by the fundamental interactions between lipid molecules and the derived cooperative behavior that is of particular importance for PLA2 activity in the present context. In the top panel of Fig. 1 is shown a gallery of the lateral organization of simple one- and two-component lipid bilayers as obtained from computer-simulation calculations and atomic force microscopy. A pronounced degree of lateral heterogeneity in terms of lipid domains is found. The domains are caused by the lateral density and compositional fluctuations that are associated with the underlying phase transitions and phase equilibria in the lipid bilayers. The lipid domains are either solid lipid patches in fluid bilayers or fluid lipid patches in solid bilayers. In the case of lipid mixtures, the domains may reflect incomplete phase separation. The size, morphology, and topology of the domain pattern depend on the lipid composition and the thermodynamic conditions. The domains can be enhanced or suppressed by adding further lipid components or solutes [21].

Similar lipid-domain patterns have been found in other lipid-bilayer systems using a variety of experimental techniques, including fluorescence spectroscopy [22], fluorescence microscopy [23], single-particle tracking techniques [24], scattering techniques [25], and atomic force microscopy [26,27]. In particular, direct-imaging techniques like atomic force microscopy applied to solid-supported bilayers and fluorescence microscopy applied to solid-supported lipid bilayers or giant unilamellar vesicles have revealed lipid domains in the range from tens of nanometers to micrometers, depending on the lipid membrane in question.

3. PLA2 is sensitive to the lateral structure of lipid bilayers

Being presented to a substrate that is laterally heterogeneous like those shown in the top panel of Fig. 1, PLA2 has the option of binding to and hydrolyzing lipid bilayers in very different states: the domains, the background phase, and the domain interfaces. It is important to note that the enzyme only senses structure and structural variations over length scales that are smaller or comparable to the scale over which the enzyme diffuses within the time-frame of an

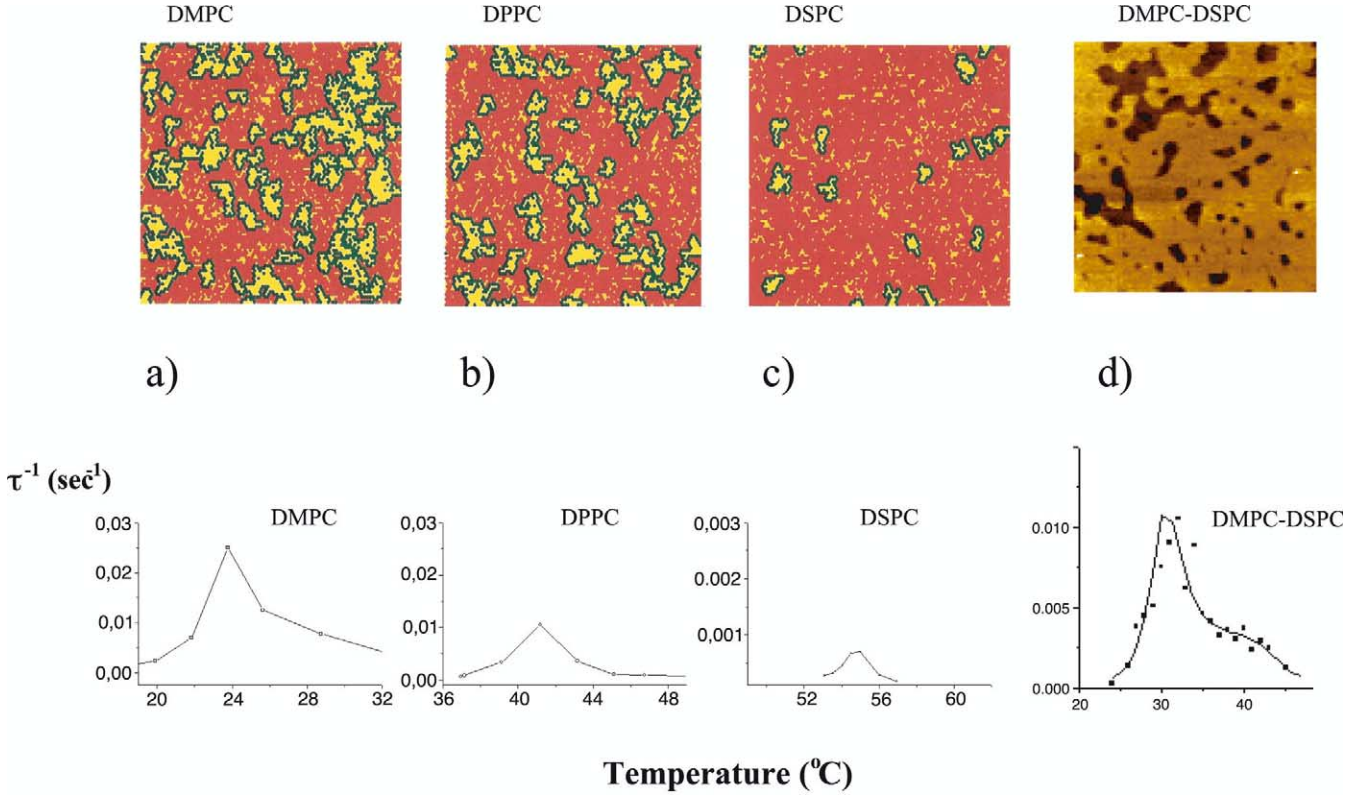

Fig. 1. Small-scale structure and domain formation in lipid bilayers (top panel) and the corresponding temperature dependence of PLA2 activity (bottom panel) measured by the inverse lag time, τ^{-1}. a: DMPC; b: DPPC; c: DSPC; d: 1:1 DMPC–DSPC (courtesy of Pernille Høyrup). The domain structures in cases a, b, and c are obtained from computer-simulation calculations (frames are 100 nm×100 nm), and in case d by atomic force microscopy of a solid-supported bilayer (frame is 1 μm×1 μm; courtesy of Thomas Kaasgaard). The lag-time data are obtained for snake venom PLA2 (*Agkistridon piscivorus piscivorus*) from a combination of fluorescence spectroscopy, light-scattering, real-time HPLC analysis, and pH-stat titration.

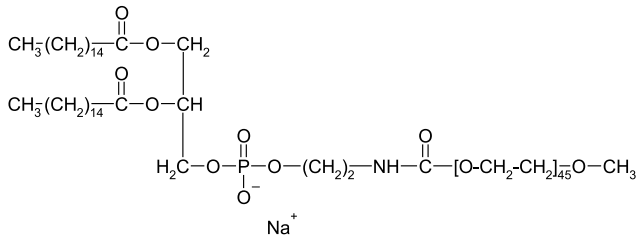

Fig. 2. Chemical structure of the lipopolymer DPPE–PEG2000.

actual hydrolytic event. Hence the enzyme does not care about the large-scale thermodynamic behavior of the substrate, but only the small-scale structure.

This is supported by the data shown in the bottom panel of Fig. 1, which shows the inverse lag time as a function of temperature for the same four lipid substrates as in the top panel. For the one-component bilayers there is a dramatic increase in activity at the phase transition temperature [3], and for the binary mixtures there is enhanced activity at the phase boundaries and in the phase-coexistence region [12,28]. These observations suggest that the activity of PLA2 is modulated by the small-scale lateral structure of the bilayer substrate. The relationship between PLA2 activity and lateral bilayer heterogeneity can be made semi-quantitative via comparison with results from computer-simulation calculations [3,9]. This comparison suggests that PLA2 may be particularly active at domain interfaces that are sites of structural defects and hence good points of attack for the enzyme. Similar conclusions have been drawn from early work on lipid monolayers [29].

This general picture of PLA2 activation is supported by detailed analyses of atomic force microscopy images of solid-supported bilayers in the presence of PLA2 in the various stages before and after the lag phase [8,10,11]. These analyses strongly suggest that PLA2 attacks at areas with structural defects, such as at domain boundaries, at regions with accumulated hydrolysis products, and at the edges of pre-existing holes in the bilayer. This is also in line with findings suggesting that PLA2 activity is enhanced on lipid vesicles of decreasing size, i.e. increasing curvature [30]. In the case of bilayers made of lipids with different chain length, it has been found that, although the enzyme seems to bind uniformly across solid and fluid domains enriched in the long- and short-chain lipid species, respectively, the enzyme has a preference for the short-lipid species which is first hydrolyzed [8,12,31]. It is possible that the preference for the short-chain lipids, which are in the fluid phase, is due to the lower surface area density of the lipid head groups in this phase. This would be in line with observations of enhanced PLA2 activity on giant unilamellar liposomes which are subject to an osmotic stress that lowers the surface density [32].

4. PLA2 activity is enhanced at Stealth liposomes

Bare unilamellar liposomes are poor candidates for drug delivery systems because the liposomes are quickly caught and degraded by the immune system. It has been known for some time that by covering the liposomes by an appropriate polymer coat the liposomes become invisible to the macrophages and therefore enjoy long circulation times in the blood stream. These so-called Stealth liposomes [33] contain a certain fraction of lipopolymers often of the type poly(ethyleneglycol) (PEG) which is covalently attached to a phosphatidylethanolamine lipid head group as illustrated in Fig. 2.

It would intuitively be expected that the polymer coat should inhibit the access of enzymes like PLA2 to the liposome surface and hence lower the enzyme activity. In contrast, it was found that the presence of the lipopolymers decreases the lag time of PLA2 as shown in Fig. 3a [13]. Subsequent systematic studies of the dependence of the lag time on lipopolymer content and polymer length suggested that the enhancement of PLA2 activity is an electrostatic effect caused by the attraction between the positively charged PLA2 and the negative charges of the phosphate head groups of the lipopolymers [14] (cf. Fig. 2). This was confirmed by the finding of a suppression of activity when a methyl group is coupled to the phosphate head group, rendering it uncharged. In this case the screening of the enzyme from the liposome surface by the polymer coat becomes effective [15].

Stealth liposomes maintain the lipid phase transition of lipid bilayers and are therefore subject to the same fluctuations and domain-formation phenomena as described above (see Fig. 1). This implies that the degradation of Stealth liposomes can be made thermosensitive. This effect is illustrated in

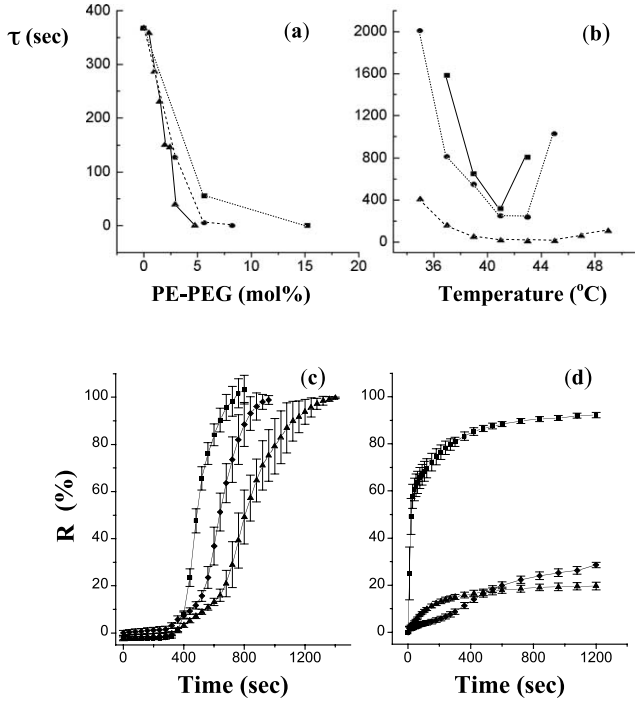

Fig. 3. a: Dependence of the lag time, τ, of PLA2 activation on lipopolymer coverage of DPPC liposomes incorporated with increasing amounts of DPPE–PEG750 (squares), PE–DPPEG2000 (circles), and DPPE–PEG5000 (triangles). The numbers denote the molar weight of the polymers, corresponding to 17, 45, and 114 monomers of ethylene oxide units. b: Dependence of the lag time, τ, of PLA2 activation in the neighborhood of the phase transition of DPPC liposomes incorporated with increasing amounts of DPPE–PEG2000 lipopolymers (0, 2.5, and 5 mol% from top to bottom). c: Drug-release characteristics of DPPC liposomes incorporated with increasing amounts of DPPE–PEG2000 lipopolymers (0, 2.5, and 5 mol% from right to left). d: The effect on calcein release from liposomes in the presence of the hydrolysis products, lysolipids (triangles), free fatty acids (diamonds), and equimolar amounts of lysolipids and fatty acids (squares).

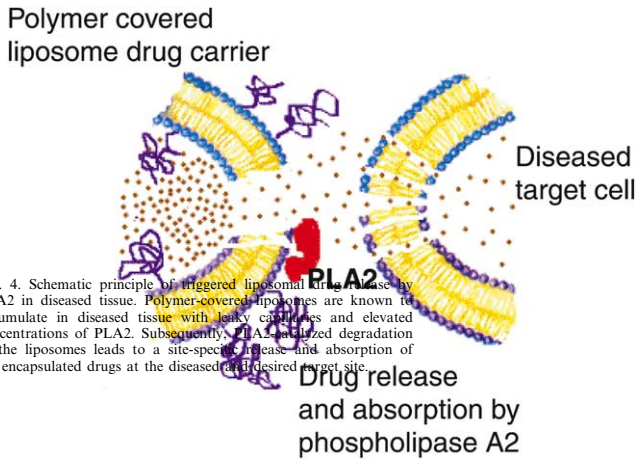

Fig. 4. Schematic principle of triggered liposomal drug release by PLA2 in diseased tissue. Polymer-covered liposomes are known to accumulate in diseased tissue with leaky capillaries and elevated concentrations of PLA2. Subsequently, PLA2-catalyzed degradation of the liposomes leads to a site-specific release and absorption of the encapsulated drugs at the diseased and desired target site.

Fig. 3b, which shows the action of PLA2 on Stealth liposomes in the temperature region of the main phase transition.

The action of PLA2 on liposomes can be used as a mechanism to decrease their permeability barrier, open them up, and possibly release molecular compounds (e.g. drugs) that have been encapsulated in their aqueous lumen or in their bilayer wall. This effect is illustrated in Fig. 3c, in the case of calcein, which is a self-quenching fluorescent molecule whose release from the liposomes can be monitored by fluorescence intensity measurements. The figure shows that the release of the encapsulated calcein model drug becomes faster for increasing polymer coverage.

The zwitterionic phosphatidylcholine (PC) phospholipids that are substrates for PLA2 are good bilayer formers. In contrast, the hydrolysis products, free fatty acids and lysolipids, are molecules that have effective shapes like a cone and an inverted cone, respectively. Hence they have propensity for forming non-lamellar phases [34] and therefore tend to destabilize lipid bilayers and lower their permeability barrier. This effect is illustrated in Fig. 3d, which shows that the calcein release from liposomes is enhanced upon addition of either of the two products. Moreover, a dramatic synergistic effect is observed when the two products are added simultaneously [16]. This suggests that the permeability enhancing effects of lysolipids and free fatty acids are optimal when the two species are introduced at the same time. Hence, PLA2 action is an extremely convenient way of producing permeability enhancers at the site where they are needed.

We shall discuss below how one can take advantage of these physico-chemical effects on PLA2 activity when constructing smart liposome-based drug-delivery systems.

5. Triggered liposomal drug release by PLA2 in diseased tissue

One of the most important questions when administering highly potent but also very toxic drugs is how to achieve a targeted and localized drug delivery at the diseased target tissue [35,36]. In particular, the clinical use of anti-cancer drugs, which have shown promising in vitro efficacy, is often prohibited due to insufficient tumor delivery and severe systemic side effects. Polymer-covered Stealth liposomes can be used as promising particulate microcarriers of encapsulated drugs to diseased sites in the body [37]. The protective effect induced by the lipopolymers against degradation by, e.g. macrophages, in the vascular system is generally understood in terms of a steric barrier induced by the flexible polymer chains attached to the surface of the liposomes [37,38]. The long-circulating PEG–liposomes will accumulate in pathological tissue due to leaky capillaries [39,40]. Although PEG–liposomes solve some of the important carrier problems in drug delivery, it is still a major task to control and trigger the degradation of the drug carrier liposomes to occur predominantly at the target site. In recent years several promising liposomal drug-delivery strategies have appeared for site-specific drug release in pathological tissues. Promising examples include temperature sensitive liposomes and peptidase targeting of liposomes [35,36].

The insight into the biophysical mechanisms of PLA2 activation at lipid bilayer interfaces has recently led to the design of a novel principle for liposomal drug targeting, release and absorption by secretory PLA2, as illustrated in Fig. 4 [16,17]. The phospholipase hydrolyzes a lipid-based pro-drug or pro-enhancer liposome, leading to products that in a synergistic fashion promote liposome destabilization and drug release at the same time that the permeability of the target membrane is enhanced. The new liposomal drug-delivery system offers a rational way of developing smart lipid-based drug-delivery systems by incorporating into the liposome carrier-specific lipid-based pro-enhancers, pro-destabilizers or pro-drugs, e.g. anti-cancer ether lipids [41], that automatically become activated by elevated concentrations of PLA2 at the diseased target sites, such as inflamed or cancerous tissue [42–44].

6. Conclusions and perspectives

The combined theoretical and experimental studies reported in this Minireview provide information about the many-particle behavior of the lipid bilayer that might be of relevance for a deeper understanding of the relationship that exists between the structural and dynamical properties of lipid bilayers and the activity of the small lipolytic PLA2 enzyme. In particular, a detailed insight into the influence of lipid composition on structural and functional biomaterial properties of composite liposome systems is of relevance for a rational development of liposomes as targeted drug-carrier systems. The biophysical studies presented above might advantageously be used to design and optimize the site-specific in vivo degradation of drug-loaded liposomes at the desired pathological site, which is characterized by leaky capillaries [37,39] and elevated levels of PLA2 [42–44]. The biophysical results suggest that long-circulating and polymer-grafted liposomes, which are known to accumulate in diseased tissue with leaky capillaries [39,40], can also be designed to become susceptible to PLA2-catalyzed degradation leading to a remote and site-specific release of the encapsulated drugs specifically at the diseased target site [16,17].

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