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Phospholipase A_2 promotes raft budding and fission from giant liposomes

Galya Staneva [a], Miglena I. Angelova [b,*], Kamen Koumanov [a]

[a] *Institute of Biophysics, Bulgarian Academy of Sciences, Acad. G. Bonchev St., Bl.21, 1113 Sofia, Bulgaria*
[b] *Laboratoire de Physicochimie Biomoléculaire et Cellulaire (LPBC), Université Pierre et Marie Curie, CNRS UMR 7033, Case 138, 4 Place Jussieu, 75252 Paris Cedex 05, France*

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Abstract

Cellular processes involving membrane vesiculation are related to cellular transport and membrane components trafficking. Endocytosis, formation of caveolae and caveosomes, as well as Golgi membranes traffic have been linked to the existence and dynamics of particular types of lipid/protein membrane domains, enriched in sphingolipids and cholesterol, called rafts [Nature 387 (1997) 569; Trends Cell Biol. 12 (2002) 296; Biochemistry 27 (1988) 6197]. In addition, the participation of phospholipases in the vesiculation of Golgi and other membranes has been already established [Traffic 1 (2000) 504] essentially in their role in the production of second messenger molecules. In this work we illustrate with raft-containing giant lipid vesicles a mechanism for raft-vesicle expulsion from the membrane due to the activity of a single enzyme-phospholipase A_2 (PLA_2). This leads to the hypothesis that the PLA_2, apart from its role in second messenger generation, might play a direct and general role in the vesiculation processes underlying the intermembrane transport of rafts through purely physicochemical mechanisms. These mechanisms would be: enzyme adsorption leading to membrane curvature generation (budding), and enzyme activity modulation of the line tension at the raft boundaries, which induces vesicle fission.

Keywords: GUV; Rafts; PLA_2; Budding; Vesiculation; Fission

1. Introduction

The existence of raft-type domains in biological membranes and their role in numerous cellular processes have become recently a subject of intense interest. Formation of rafts is based on the specific lipid–lipid interactions between sphingolipids (e.g., SM) and Chol, that promote the formation of a new lipid layer phase—the liquid-ordered (L_o)-phase (Ipsen et al., 1987; Schroeder et al., 1994; Bittman et al., 1994). The L_o-phase coexists laterally in the plane of the membrane with the liquid-disordered (L_d) lipid phase, as two fluid phases, characterised by inter-phase boundary lines. Raft-type domains isolated from trans-Golgi network seem to play an important role in sorting processes (Simons and van Meer, 1988;

Abbreviations: GUV, giant unilamellar vesicle; PLA_2, phospholipase A_2; PC, egg yolk L-α-phosphtidylcholine; SM, egg yolk sphingomyelin; Chol, cholesterol; PC*, fluorescent lipid analogue C12-NBD-PC

* Corresponding author. Present address: INSERM U538, CHU St. Antoine, 27 rue Chaligny, 75012 Paris, France.
Fax: +33-1400-11390.
E-mail address: angelova@ccr.jussieu.fr (M.I. Angelova).

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Simons and Ikonen, 1997). In the same instance, the Golgi complex is probably the place in the cell where the process of transport vesicles formation is most intense. Another recent finding, the possibility for endocytosis to occur by a clathrin-independent mechanism, raised the question of alternative pathways involving lipid rafts (van der Goot and Gruenberg, 2002). On the other hand, the role of certain enzymes as biochemical signal-generating molecules in several budding and vesiculation processes, including those in Golgi membranes, has already been established. For example, Simon et al. (1996) showed the protein kinase C implication in Golgi membranes vesiculation as an activator of phospholipase D (PLD). The authors suggested that the phosphatidic acid, produced as a result of PLD activity, triggers budding and vesiculation from the Golgi complex. De Figueredo et al. (2000) demonstrated the participation of PLA_2 in the process of tubule-mediated Golgi-to-endoplasmic reticulum retrograde trafficking. This retro-transport insures the transport of cholesterol but not that of sphingolipids.

The phospholipase A_2 is an enzyme catalyzing the *sn*-2 ester bond hydrolysis of glycerophopholipids. It is well established that the PLA_2 activity increases significantly when acting on lipid bilayers which contain membrane defects, e.g., due to the co-existence of L_d and H_{II}, or L_d and L_o phases (Koumanov et al., 2002). SM has an inhibitory effect on PLA_2 due to the binding of the enzyme to SM. This inhibitory effect is abolished by adding to the substrate of equimolar quantities of Chol (Koumanov et al., 1998) because of the high affinity of Chol for the SM (Bittman et al., 1994) and the displacement of the latter from its association with PLA_2. The lateral phase separation in lipid bilayers corresponding to raft formation has for consequences the occurrence of lipid packing defects and molecular density fluctuations (Mouritsen, 1991; Jorgensen et al., 2002). Therefore, it might be anticipated that the kinetics of PLA_2 adsorption and its enzymatic activity would be different on the raft and non-raft regions of the membrane, as well as in the vicinity of the inter-phase boundary lines, due to their different molecular composition and packing.

In order to test this idea we have used GUVs as a model system. GUVs represent a potential model system for mimicking cell membranes (Menger and Angelova, 1998). Enzymatic reactions on individual GUVs can be directly visualized and studied by optical microscopy (Wick et al., 1996; Holopainen et al., 2000, 2002). The dynamics of membrane domains, vesicle shape and morphology transformations can be clearly followed as well. For example, we studied by direct optical microscopy observation the DNA induced endocytic vesicles formation and the dynamics of membrane morphology transformations upon local microinjection of DNAs to individual giant unilamellar cationic vesicles (Angelova and Tsoneva, 1999). Giant unilamellar vesicles can be prepared by the liposome electroformation method (Angelova and Dimitrov, 1986), which can quickly supply a large number of 50–100 μm diameter vesicles having predetermined lipid compositions (Angelova, 2000). This approach has allowed us and others (Dietrich et al., 2001), to visualise rafts by fluorescence microscopy.

Here we have studied the dynamics of the direct interactions of PLA_2 with raft-GUVs under conditions of local delivery of the enzyme to the vesicle membrane by phase contrast and fluorescence optical microscopy, micromanipulation and microinjection.

2. Materials and methods

2.1. Reagents

PC, SM, Chol, and the PLA_2 from bee venom were purchased from Sigma. The fluorescent lipid analogue C12-NBD-PC (PC*) was from Avanti Polar Lipids. The PLA_2, microinjected to GUVs, was at 0.83 mg/ml (activity ∼ 1 U/μl) in 0.5 mM HEPES buffer, pH 7.4, and 10 mM $CaCl_2$ (if not otherwise stated).

2.2. Giant unilamellar vesicles (GUVs)

GUVs were prepared by electroformation (Angelova and Dimitrov, 1986; Angelova, 2000), see Fig. 1A, and were made of PC/SM/Chol 45:45:10 mol/mol (unless otherwise indicated). The lipid composition used for phase separation visualisation in fluorescence was PC/PC*/SM/Chol 40:5:45:10 mol/mol. Briefly, the specific electroformation protocol was the following: the lipid mixture was prepared in diethyl ether/methanol/chloroform 72:10:18 v/v, at 0.5 mg/ml total lipid. A droplet of lipid solution (1 μl) was deposited (avoiding sliding) on each of the two parallel

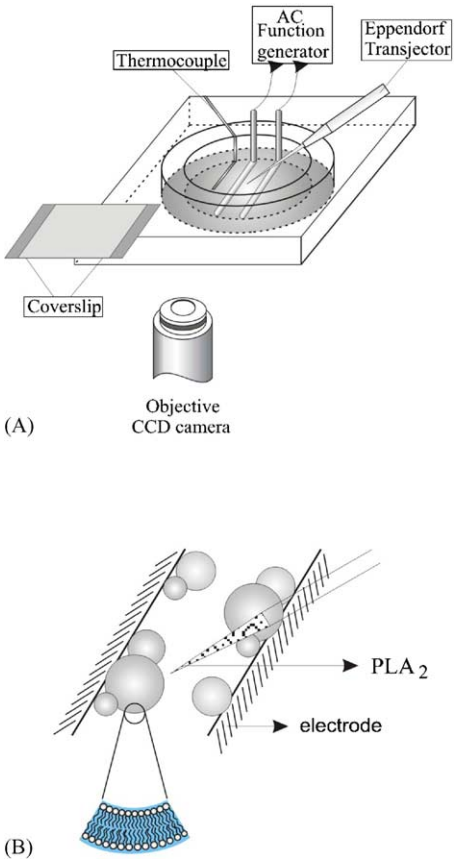

Fig. 1. Schematic presentation of (A) the thermostated chamber for electroformation and micromanipulation of GUVs, and (B) the microinjection of PLA_2 to a GUV.

platinum wires (diameter 0.8 mm, distance between axes 3 mm) of the working chamber and dried under vacuum for 30 min. An alternating current (ac) electrical field, 10 Hz, 100 mV pp, was applied to the electrodes at room temperature (about 21 °C). A thermocouple positioned at a distance of about 0.5 mm from the place of observation was used to monitor the temperature. 1.2 ml of HEPES buffer solution (0.5 mM, pH 7.4, conductivity $\sigma = 59\,\mu$S/cm) was added (avoiding agitation) into the working chamber. The temperature and the applied voltage were gradually increased (during 10 min) up to 30 °C, and 200 mV pp, respectively, and then only voltage during 2 h up to 400 mV pp. After 1 h, at least 10 GUVs of diameters 60–90 μm were present in each preparation.

2.3. Video microscopy

A Zeiss Axiovert 135 microscope, equipped with Narishige MMN-1 plus MMO-22 micromanipulator, and Hamamatsu B/W chilled CCD camera (C5985-10) connected to an image recording and processing system were used. The morphology transformations and dynamics of the heterogeneous GUV membrane were followed in phase contrast and in fluorescence by Zeiss filter set 16 (Ex/Em = 485/>520 nm).

2.4. Microinjection of PLA_2

The microinjection was carried out with an Eppendorf Transjector 5246. The microcapillary for performing *local* microinjection to a GUV had a 0.5–0.8 μm inner diameter. Injected volumes were of the order of a few pl (1 p1 = 1×10^{-12} l). The injection was performed from a distance of about 15 μm from the GUV, see Fig. 1B. The observations presented below are based on at least five experiments of the same kind.

3. Results

Fig. 2A and B illustrate the very phenomenon of a raft-vesicle expulsion after local delivery of PLA_2 onto a raft-containing GUV composed of PC/PC*/SM/Chol 40:5:45:10 mol/mol at 30 °C. This lipid composition and the temperature correspond to the conditions where the L_o-phase occurs. The co-existence of two phases is visualized by fluorescence (see Fig. 2A(a) and B(a)) since the chain-labeled lipid analogue PC* is excluded from the condensed and L_o phase and partitions predominantly into the L_d phase (Ruano et al., 1998; Worthman et al., 2000 and our preliminary experiments). The occurrence of a dark round shaped domains, floating laterally within the plane of the bright lipid membrane, suggests the presence of the liquid ordered (L_o) phase, co-existing laterally with the liquid disordered (L_d) phase of the vesicle membrane. The number, the dimensions and the dynamics of the dark domains (representing the L_o-phase in a GUV membrane) were strongly dependent on the temperature and lipid composition. Usually, fewer and larger domains were observed at lower temperatures (down to 10 °C), and a larger

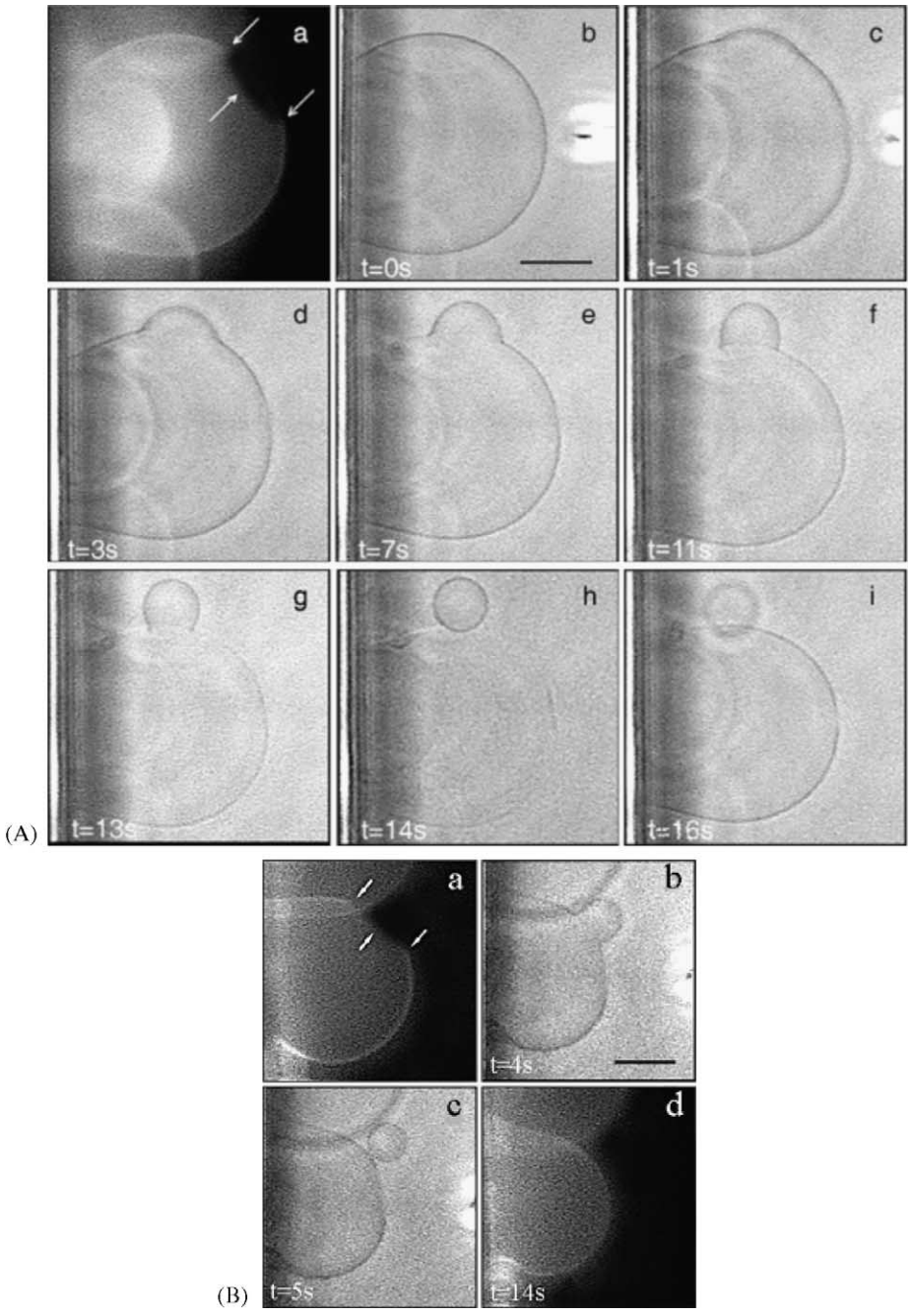

Fig. 2. Raft-vesicle budding and fission promoted by phospholipase A_2 from a GUV made of PC/PC*/SM/Chol 40:5:45:10 mol/mol at 30 °C. (A(a) and B(a)) Initial raft-GUV: the raft is visualized in fluorescence as a round-shaped dark domain (arrows pointed) within the bright lipid non-raft membrane since the C12-NBD-PC is excluded from the raft-phase; (A(b)) PLA_2 local delivery to the GUV by the micropipette (phase contrast image); (A(c)) emergence of a bud at the raft location; (A(d)–(g) and B(b)) progressive growth of the bud to a spherical vesicle; (A(h), (i) and B(c), (d)) final closure and separation of the raft-vesicle and parent GUV. (B(d)) The detached vesicle was not visible in fluorescence, and on the other hand, no dark domain left in the mother vesicle. Bar = 20 μm.

number of smaller ones (a few μm in diameter) at higher temperatures, e.g., at 37 °C. It was still possible to resolve in fluorescence minute discrete dark domains up to 39 °C (not shown). Stable L_o-phases, and their visualization at physiological temperatures, 37–39 °C, are made possible by the particular lipid composition of GUVs we used for this study, PC/SM/Chol 45:45:10 mol/mol. Recently, Kahya et al. (2003) showed the L_o domain formation at room temperature in GUV composed of DOPC/SM/Chol in the same ratio as in our experiment. In previous works rafts were not observed above 30 °C in GUVs made of DOPC/BrainSM/Chol 1:1:1 mol/mol (see Dietrich et al., 2001). It might be due to the smaller molar part of SM they used. And, in addition, the SM used contained some unsaturated chains. (BrainSM contains about 9% unsaturated chains, egg SM contains all saturated chains, see, e.g., the Avanti Polar Lipids web site). Some of the vesicles in our samples were tension free, and thermal fluctuations were visible in the phase contrast regime. Qualitatively, one observes that the membrane is more rigid within the raft-domain. One second after the PLA_2 delivery to the GUVs membrane (Fig. 2A(b)), budding is initiated at the raft location (Fig. 2A(c)). The bud grows continuously, becoming a spherical vesicle, (Fig. 2A(d–g) and B(b)), which ultimately closes and disconnects from the parent GUV (Fig. 2A(h) and B(c)). The process takes about 15 s under the conditions described in Fig. 2A and 5 s as in Fig. 2B. The detached vesicle is not fluorescent, and on the other hand, no dark domain is left in the parent vesicle (Fig. 2B(d)). The latter regains the quasi-spherical shape, but with a smaller diameter (Fig. 2A(i) and B(c)). That suggests that the expulsed vesicle is entirely made of the raft domain of the initial raft-GUV membrane. This is well illustrated also in the images in the moment of raft budding (Fig. 4B(a)) and the lack of fluorescent signal in the bud region (Fig. 4B(b)).

The PLA_2 activity-induced expulsion of raft vesicles also occurs at 20, and at 37 °C but the size of the expulsed vesicles as well as the kinetics are drastically different. At 20 °C, expulsion of a large raft vesicle (about 15 μm in diameter) could take a few minutes, while at 37 °C expulsion of several small raft vesicles (few microns in diameter) could take place within few seconds after PLA_2 injection.

Raft vesicle fission, but not raft budding, is critically dependent on the PLA_2 enzymatic activity. So, addition of inactive or poorly active enzyme still initiates raft budding from the initial raft-GUV but the process does not develop to the completion of the final fission and raft vesicle expulsion. As the PLA_2 is a Ca^{2+} dependent enzyme, the addition of EDTA to the enzyme solution is a common way to inhibit the enzyme activity (Fig. 3). Lowering the temperature decreases the enzyme activity as well (Fig. 4A). In both cases initial budding at the raft sites appear temporally after local PLA_2 addition, but then reverses upon enzyme diffusion. The raft-domains remain in the original vesicle.

GUV *continuous* fission and vesicle expulsion as a result of the PLA_2 activity occurs from the parent GUV only for particular lipid compositions (e.g., PC/SM/Chol 45:45:10 mol/mol). With pure PC GUVs a large variety of discontinuous vesicle shape and topology transformations were observed: opening and closing of micrometric holes in the vesicle membrane, vesicle diameter fluctuations and shrinking, vesicle fragmentation, expulsion of small liposomes, etc. (Wick et al., 1996; Staneva, Angelova, Koumanov—article in preparation, 2003). The presence of SM significantly increases membrane resistance to PLA_2. For PC/SM 50:50 mol/mol, most of the GUVs (about 80%) remained intact after the PLA_2 injection, and the other 20% slightly shrank (not shown). The observed resistance was presumably due to the inhibitory effect of SM on PLA_2. It is likely that in the case of PC/SM/Chol 45:45:10 GUVs, the predominant Chol–SM interaction partially relieves the inhibitory effect of the latter, (Koumanov et al., 1998), while still ensuring considerable membrane protection towards PLA_2. Such balance permits vesicle fission and raft expulsion to take place in a continuous manner, i.e., without hole opening or membrane fragmentations. Thereby, the significant mixing of internal GUV volume with the external aqueous space was prevented during fission, which mimics properly the vesiculation processes of biological membranes.

As the enzyme-generated lyso-lipid seams to be the key to the fission mechanism, we injected simply lyso-phosphtidylcholine (LysoPC) solution at different concentrations onto the raft sites. At exogenous LysoPC low concentrations we observe rise of thermal fluctuations of both raft and non-raft regions of the membrane which was not the case when PLA_2 was

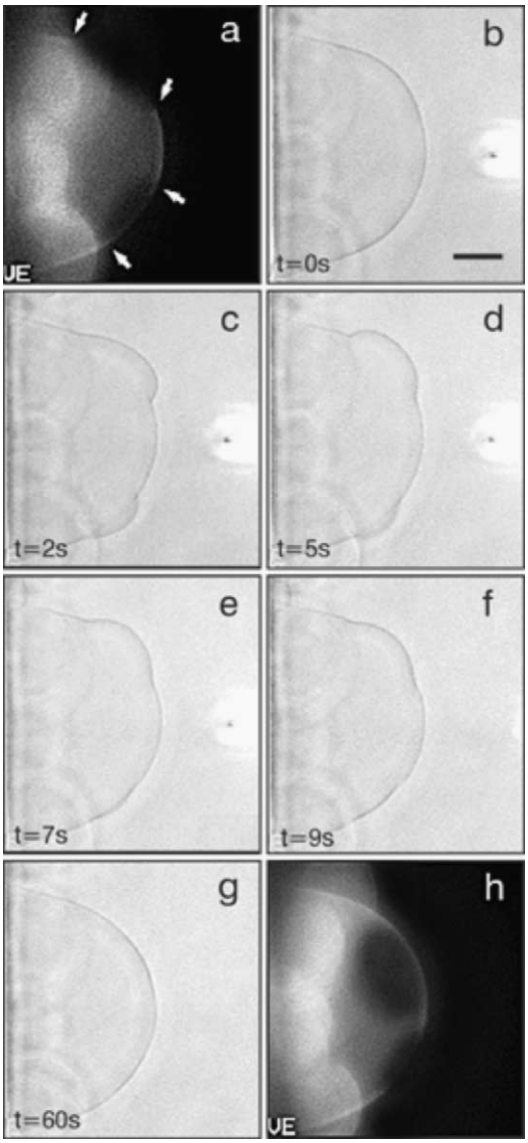

Fig. 3. Raft budding without vesicle fission promoted by non-active PLA_2 (inhibited by EDTA, 10 mM). (a) Initial raft-GUV in fluorescence (two rafts are pointed by the arrows), and (b) in phase contrast; (c, d) two buds emerging at raft locations after the local delivery of non-active PLA_2; (e–h) vesiculation process reverses the initial buds retract, and the raft remains in the parent vesicle. Bar = 20 μm.

injected. That suggests distribution of the exogenous LysoPC into both L_d and L_o phases. At high enough exogenous LysoPC concentrations we observed raft budding and fission, or, only reversible budding depending on the size of the particular raft: budding and fission in the case of small rafts, and reversible budding (but not fission) in the case of large ones (article in preparation).

4. Discussion

4.1. A possible mechanism for raft vesicle expulsion due to PLA_2 activity

A possible mechanism we propose for the PLA_2-induced vesiculation of rafts relates first, to several distinct physico-chemical properties of a multicomponent lipid membrane which contains domains (the rafts), and second, to the enzyme and its products—LysoPC and free fatty acids. Indeed, budding appears to be an intrinsic property of multicomponent membranes (Lipowsky, 2002). In a two-phase membrane, the difference in composition of the two phases is usually associated with a difference in spontaneous curvature, as well as with a free energy term, which is proportional to the length of the inter-phase boundary (the density of this free energy term being the line tension at inter-phase boundary). In general, both the spontaneous curvature and the line tension provide a driving force for budding of one domain from the lipid bilayer matrix, since this process allows for difference in spontaneous curvature and leads to a decrease of the length of the inter-phase boundary. Whether budding actually occurs is related to the interplay with the opposing effect of bending free energy, and, in the general case, on any mechanical lateral tension applied on the membrane (Lipowsky, 2002). Our experiments indicate that the presence of a liquid ordered domain (raft) per se is not sufficient for budding to appear even from a tension-free (fluctuating) GUV. In contrast, the adsorption of PLA_2 triggers budding at the raft sites. The destabilising effect of the PLA_2 might be due to modification of the membrane local spontaneous curvature in both (L_o and L_d) phases. Externally added PLA_2 adsorbs onto the vesicle membrane in an asymmetrical way since the enzyme cannot cross the bilayer. Due to the asymmetrical constraint generated on the coupled lipid bilayer the partial penetration of PLA_2 molecules into the external leaflet is likely to induce a spontaneous curvature (i.e., a budding towards the exterior).

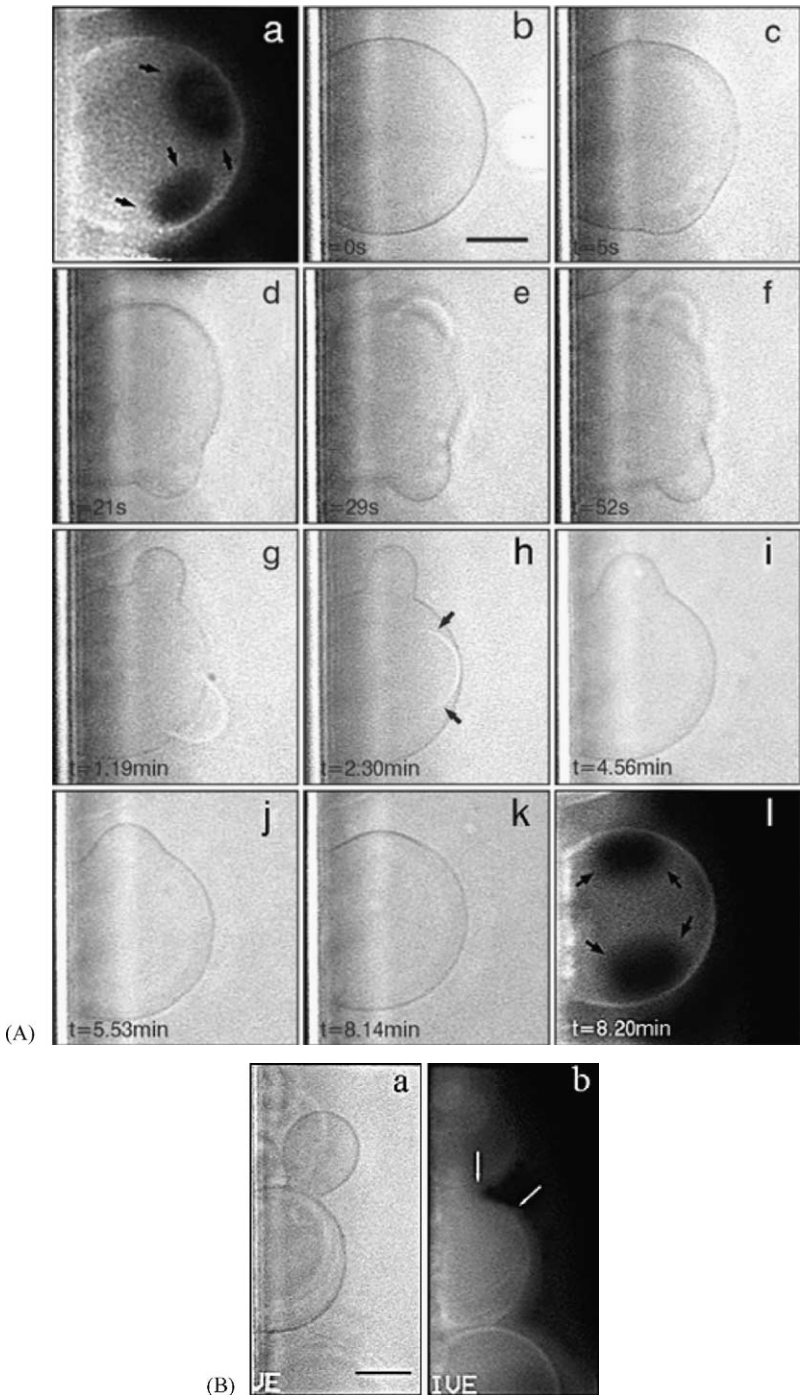

Fig. 4. Raft budding without vesicle fission promoted by PLA_2, being poorly active at low temperature (14 °C). A: (a) Initial raft-GUV in fluorescence (two rafts are pointed by the arrows), and (b) in phase contrast; (c–g) temporal budding after local delivery of poorly active PLA_2; (h–l) progressive retraction of the initial buds, the rafts remain in the parent vesicle. B: (a) GUV at the moment of raft budding (phase contrast image) and (b) the same raft budding GUV in fluorescence (bud location is pointed by arrows). Bar = 20 μm.

Difference in the protein adsorption between L_o and L_d phases, as well as surface tension modifications at lipid bilayer/water interface, might also contribute to the effect. In our previous publication (Koumanov et al., 1998) we showed the affinity of PLA_2 to SM-containing liposomes. As L_o-phase is enriched in SM, logically injected PLA_2 binds preferentially to this phase.

On the other hand, the fission process can be explained by different types of arguments. Several studies on the PLA_2 suggest that the enzyme activity is inherently dependent on, and modulated by, the physical state of substrate. Any event increasing lipid packing disorder, e.g., membrane components segregation and domain formation, undergoing phase transitions (Jorgensen et al., 2002), favors PLA_2 enzymatic activity. Therefore, this activity on the membrane is likely to be higher at the L_o/L_d domain interface where LysoPC, which is a product of PLA_2 activity, destabilizes the lipid bilayer structure. Indeed, formation of hydrophilic pores, progressive thinning or rupture of bilayer, decrease of membrane bending and stretching rigidity, as well as water permeability increase, have been observed (see, e.g., van Echteld et al., 1981; Needham and Zhelev, 1995). Recently, Grandbois et al. (1998) visualised by atomic force microscopy the membrane heterogeneity (formation of channels (annealing)), which appeared in dipalmitoyl-PC (DPPC) supported bilayers as a result of hydrolysis by PLA_2. They concluded that there is a segregation into intact bilayer, and thinner LysoPC-enriched bilayer. The difference in their thickness was 1.5 nm. On the other hand, using the same technique, Rinia et al. (2001) studied supported lipid bilayers composed by dioleoyl-PC/SM/Chol (45:45:10 mol/mol). They found that the average area covered by domains in L_o phase was about $28 \pm 5\%$ of the total lipid bilayer area. The bilayer thickness of L_o phase was (0.9 ± 0.1) nm larger then that L_d phase. In our case, PLA_2 acts on L_d lipid bilayer containing a L_o phase domain. Therefore, we may expect that the difference in the thickness between the L_o phase, and the LysoPC-enriched L_d phase would be at least larger then 1.5 nm, due to accumulation of the two effects.

Bearing in mind such properties, we suggest the following mechanism for raft vesicle expulsion due to PLA_2 activity: PLA_2 adsorption triggers the initial membrane budding at the raft-sites. The direction of the bending (the budding direction) is determined by the asymmetrical adsorption and partial penetration of protein into the membrane outer monolayer.

The enzyme activity is most efficient around the boundary between the raft and the rest of the membrane. The accumulation of enzymatic hydrolysis products at this line perturbs lipid packing, leaving space for water molecules to penetrate deeper into the lipid bilayer. The bilayer thickness difference at the raft boundary is increasing in parallel.

This process leads to an increase of the free energy, and the corresponding line tension, at the raft boundary, which is relaxed by forcing further membrane bending, raft-vesicle formation and fission. As suggested above, this process might occur without intensive water flux and mixing between inner and outer aqueous compartments and there by might represent a proper model for membrane budding and vesicle formation.

4.2. A model for membrane budding and vesicle formation

Membrane budding is an ubiquitous phenomenon related to biological cell functions and represents the initial stage of transport vesicle formation for intermembrane trafficking of lipids and proteins (Muniz et al., 1997). The Golgi complex appears to be a central sorting device for the endocytic/exocitic pathways. The biosynthesis of certain important lipid components of rafts, e.g., the sphingolipids, is taking place in the Golgi complex. The SM–Chol-based raft-domains generated in the Golgi membrane, as well as the proteins inherently coupled to them, might be sorted and trafficked to the plasma membrane by means of membrane fission and transport vesicle formation (Pagano, 1990). For example, sphingolipid transporting vesicles were isolated from permeabilized cells (Kobayashi et al., 1992). On the other hand, it seems that the lipids and proteins constituting the Golgi complex microdomains are efficiently segregated from the COPI-coated vesicles (Brügger et al., 2000). Rafts appear to be also involved in endocytosis processes and retrograde transport of plasma membrane components to intracellular compartment. Of particular importance is the recent observation that the latter processes might also occur by clathrin-independent

mechanisms, which might involve lipid rafts per se (van der Goot and Gruenberg, 2002).

The exact nature of the cellular mechanisms involved in the vesicular trafficking of lipid rafts is still a matter of debate (van der Goot and Gruenberg, 2002; Ikonen, 2001). In this work we have presented model experiments and described a possible mechanism for raft vesicle expulsion due to the activity of a single enzyme-PLA_2. This leads us to the proposal that, apart from other possible factors (i.e., aminophospholipids translocase creating a transient asymmetry in lipid concentration, Farge et al., 1999), the presence of active PLA_2 in the cell is an important factor for triggering, developing and finalisation the process of rafts-transporting vesicles formation from organelle and plasma membranes. For example, PLA_2 might play an important role in the direct transport of rafts from Golgi to plasma membrane by means of trafficking vesicles. The efficiency of this possible direct transport would be of crucial importance for ensuring rafts functions, in the plasma membrane, as platforms concentrating a large variety of biologically active proteins.

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