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Calcium-Induced Membrane Microdomains Trigger Plant Phospholipase D Activity

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*Plant α-type phospholipase D proteins are calcium-dependent, lipolytic enzymes. The morphology of the aggregates of their phospholipid substrate fundamentally defines the interaction between the enzyme and the surface. Here we demonstrate that the Ca^{2+}-induced generation of membrane microdomains dramatically activates α-type phospholipase D from white cabbage. 500-fold stimulation was observed upon incorporation of 10 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA) into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles in the presence of Ca^{2+} ions. Enhanced association of PLDα2 with phospholipid surfaces containing anionic components was indicated by lag phase analysis and film balance measurements. Differential scanning calorimetry showed that the POPA-specific activation correlates with the phase behavior of the POPC/POPA vesicles in the presence of Ca^{2+} ions. We conclude from the results that the Ca^{2+}-induced formation of POPA microdomains is the crucial parameter that facilitates the binding of PLD to the phospholipid surface and suggest that this effect serves as a cellular switch for controlling PLD activity.*

Introduction

Phospholipase D proteins (PLDs) are a group of lipolytic enzymes that catalyze the hydrolysis of the terminal phosphodiester bond of glycerophospholipids to release phosphatidic acid (PA) and the alcohol of the head group. They are ubiquitously found within plants, animals, fungi and bacteria, and accomplish a variety of functions such as membrane remodeling and trafficking, stress response and cell signaling.[1] In the presence of appropriate alcohols, they also catalyze the transesterification at the terminal phosphate ester bond. This transphosphatidylation potential is exploited in biotechnology for the synthesis of phospholipids with special head groups.[2] Most PLDs belong to the so-called PLD superfamily,[3] which is characterized by four conserved regions in the primary structure.[4] As derived from the only crystal structure of PLD, the prokaryotic PLD from *Streptomyces* sp. strain PMF, two of the conserved regions (called HKD motifs after the amino acids found within them) form the active site.[5] In addition, most mammalian PLDs contain Phox (PX) or Pleckstrin (PH) domains, while most plant PLDs are provided with an N-terminal C2 domain.

Despite the increasing number of PLDs identified in different organisms on the genetic level, and the numerous studies on their physiological functions,[1a] the manifold mechanisms of the regulation of their activities in response to the composition and structure of membrane lipids have hitherto been poorly understood. Most PLDs need Ca^{2+} ions for activity; the role of these Ca^{2+} ions is not yet clarified. As generally typical of lipid converting enzymes, PLDs need interfaces for full activity. In comparison with phospholipase A_2 (PLA_2) proteins, in which the interfacial activation has been intensively studied by many authors,[6] information on the enzyme-membrane interaction with PLDs is poor. Kinetic measurements on artificial substrate aggregates, such as mixed micelles or liposomes, showed the typical increase of activity above the critical micelle concentration (CMC).[7] From very early studies on PLD from cabbage, it was concluded that binding of the enzyme to phospholipids and their subsequent hydrolysis requires both an appropriate charge and a gel-to-liquid crystalline phase transition in the substrate.[8] In aqueous-organic two-phase systems, the initial rates of PLD could be correlated with the interfacial pressure of the substrate molecules.[9] For plant PLDs, anionic surfactants such as SDS,[10] PA,[11] and other anionic compounds[12] have a strong activating effect. Detailed studies on the effects of PA and Ca^{2+} ions on membrane binding have been performed for an enzyme with low PLD activity from *Streptomyces chromofuscus*.[13] This enzyme, however, is not a member of the PLD family but homologous to bacterial alkaline phosphatases and should, therefore, not be used as PLD model.[14]

In the present paper we demonstrate the significance of calcium-induced microdomain formation in the regulation of PLD activity. The isoenzyme PLDα2 from cabbage, which is the most traditional plant PLD and will be abbreviated as PLD in the following text, was recombinantly produced and extensively purified as described recently.[15] The activity of the enzyme toward small unilamellar vesicles (SUVs) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and the anionic phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) or 1-

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palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) in the presence of Ca^{2+} ions could be correlated with the characteristics of the membrane structure and interfacial binding as deduced from experiments using differential scanning calorimetry (DSC) and the monomolecular film technique.

Results

Interfacial activation of PLD

Figure 1 exemplifies the activity of PLD toward the short chain substrate 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (diC_6PC) in a concentration range that covers monomeric and micellar structures. The CMC of diC_6PC is 9.6 ± 0.2 mM.[12] In the presence of 10 mM $CaCl_2$, PLD activity as function of the diC_6PC concentration increases up to saturation at 20 mM substrate. Below the CMC, the initial rates of hydrolysis adopt a sigmoidal shape. When the CMC is exceeded, the curve merges into a second, sigmoidal phase, demonstrating the interfacial activation. In the absence of Ca^{2+}, there was only marginal activity (≤ 0.03 μmol min^{-1} mg^{-1}).

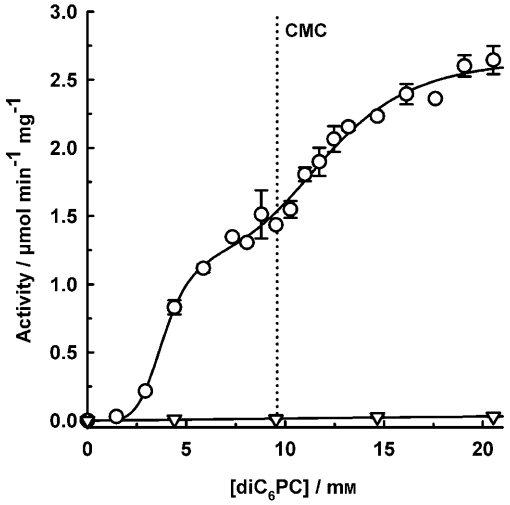

Figure 1. PLD activity as a function of diC_6PC concentration in the absence (▽) and presence (○) of 10 mM $CaCl_2$. The activity of PLD was determined by the continuous fluorescence assay monitoring choline release as described in the Experimental Section. The reactions were performed in 10 mM Pipes, pH 7.0. CMC refers to the critical micelle concentration.

Activation of PLD by anionic phospholipids

As anionic phospholipids are known to be involved in mediating the membrane binding of membrane enzymes,[16] we examined PLD activity towards SUVs from POPC/POPG, POPC/DOPA and POPC/POPA as a function of the mol percentage of the anionic components (Figure 2A). The reaction mixture included 10 mM $CaCl_2$. The most remarkable effect was observed in the POPC/POPA system. The activity toward pure POPC was 0.6 μmol min^{-1} mg^{-1}. Up to about 2 mol% POPA, PLD activity was only slightly enhanced. However, an increase in POPA concentration resulted in a cooperative transition yielding a 500-fold stimulation at 10 mol% POPA (309.8 μmol min^{-1} mg^{-1}). Compared to this dramatic activation, DOPA and POPG caused only small-scale effects. At the maximum, DOPA stimulated PLD activity about 20-fold (11.6 μmol min^{-1} mg^{-1}) and POPG about twofold (1.2 μmol min^{-1} mg^{-1}).

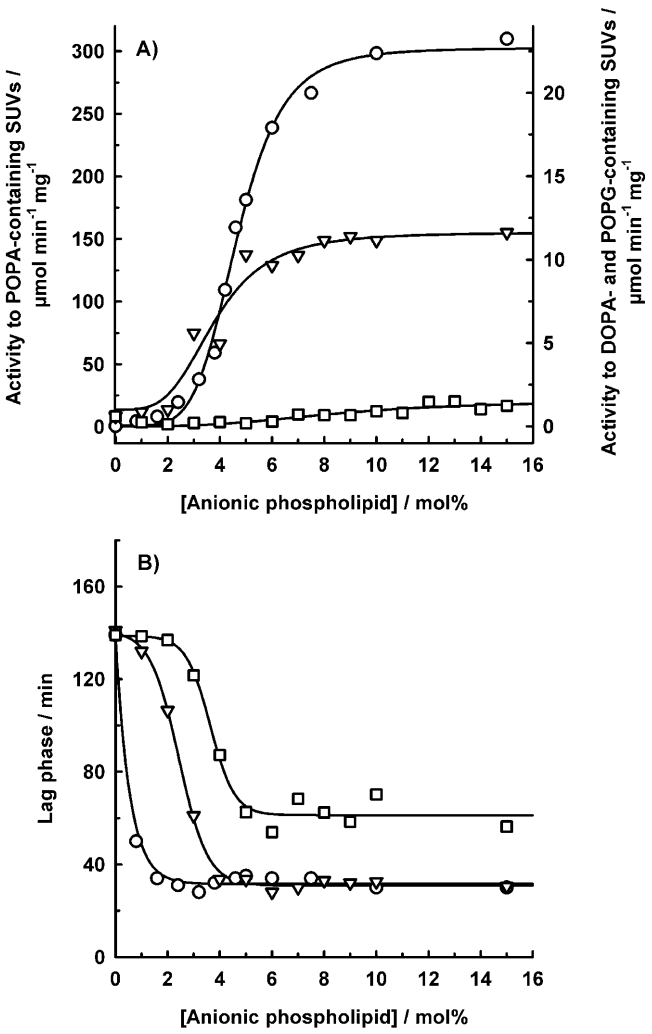

Figure 2. A) PLD activity and B) lag phases as a function of anionic phospholipid component. PLD was incubated with POPC SUVs containing increasing proportions of POPA (○), DOPA (▽) and POPG (□). The phospholipid concentration was 1 mM and the PLD concentration was 17 pM PLD. All measurements were performed in 10 mM Pipes, pH 7.0, 10 mM $CaCl_2$ at 25 °C.

The presence of anionic lipids in the substrate SUVs was also reflected in the lag phase that occurred at the beginning of the reaction (Figure 2B). POPA and DOPA decreased the lag phase nearly fivefold, whereas POPG decreased the lag phase about 2.5-fold. Interestingly, the mol percentages at which the lag phases reached their minimum values increased for the three anionic lipids from POPA to POPG (POPA < DOPA < POPG); these values increased in the same order as the activity data decreased. The thresholds were 2 mol% for POPA, 4 mol% for DOPA, and about 6 mol% for POPG. This finding

strongly indicates an enhanced attraction of PLD to the interface in the presence of anionic lipids.

To examine the interdependence of the activation effects by Ca^{2+} ions and anionic phospholipids, the activities of PLD toward POPC and POPC/POPA (85:15) SUVs were compared at different concentrations of $CaCl_2$ (Figure 3). The coincidence of the relative activities as function of the Ca^{2+} ion concentrations in the absence and presence of POPA indicates that the two activation effects do not interact.

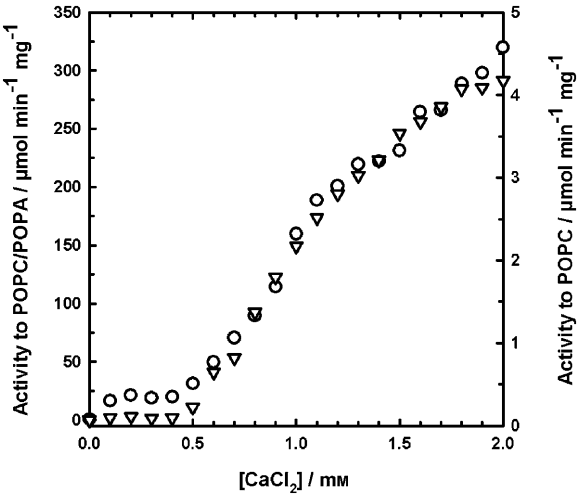

Figure 3. PLD activity toward POPC and POPC/POPA (85:15) SUVs as a function of [$CaCl_2$]. PLD was incubated with POPC (▽) and POPC/POPA (○) SUVs at different $CaCl_2$ concentrations. Phospholipid concentration was 1 mM and PLD concentration was 17 pM. All measurements were performed in 10 mM Pipes, pH 7.0 at 25 °C.

Binding of PLD to non-hydrolyzable monolayers containing POPA

To analyze the influence of POPA on the association of PLD with the phospholipid surface, the monolayer film balance technique was employed. As phospholipase lipid binding experiments that measure the increase in the monolayer surface pressure are hampered by the counteracting pressure changes arising from phospholipid hydrolysis, we probed 1,3-diacylglycerophosphocholines, such as 1,3-dimyristoyl-glycero-2-phosphatidylcholine (1,3-diC_{14}-PC), as substrate analogues. These compounds, which carry the fatty acids in the *sn*1- and *sn*3-positions, have similar physicochemical properties as the natural 1,2-diacyl-*sn*-glycero-3-phosphocholines, but they are not cleaved by PLD and have been shown to inhibit this enzyme.[17] Injection of PLD into the subphase of a 1,3-diC_{14}-PC monolayer led to an increase in surface pressure that was proportional to the PLD concentration in the subphase (data not shown). Thus, this method proved to be suitable for the determination of PLD binding to phospholipid surfaces at different fractions of POPA.

Figure 4 shows the rates of the surface pressure increases of 1,3-diC_{14}-PC monolayers monitored as a function of the proportion of POPA incorporated into the monolayer. With increasing amounts of POPA, the binding of PLD to the monolayer was accelerated up to 30-fold. Hence, POPA notably facilitates the binding of PLD to the phospholipid surface.

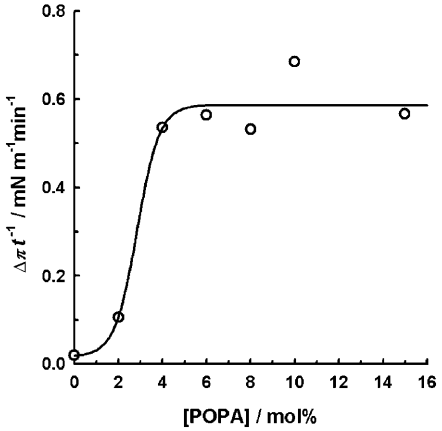

Figure 4. Binding of PLD to non-hydrolyzable phospholipid monolayers. Rate of surface pressure increase ($\Delta\pi t^{-1}$) within 1,3-diC_{14}-PC/POPA monolayers as a function of POPA concentration upon injection of 23 nM PLD. The measurements were performed at an initial surface pressure of 25 mN m^{-1} min^{-1} and at a temperature of 25 °C. The subphase contained 10 mM Pipes, pH 7.0 and 10 mM $CaCl_2$.

Lateral phase separation within POPC/anionic phospholipid SUVs induced by Ca^{2+} ions

DSC was applied to determine the thermotrophic phase behavior of pure POPC SUVs in comparison to POPC/POPA, POPC/DOPA and POPC/POPG (1:1) SUVs between 7 and 70 °C. The thermal transitions were measured in the absence and presence of 10 mM $CaCl_2$ at pH 7.0. Without Ca^{2+} ions, no transition peaks were observed in this temperature range (Figure 5), indicating that under these conditions the lipid components are completely miscible and the gel-to-fluid tran-

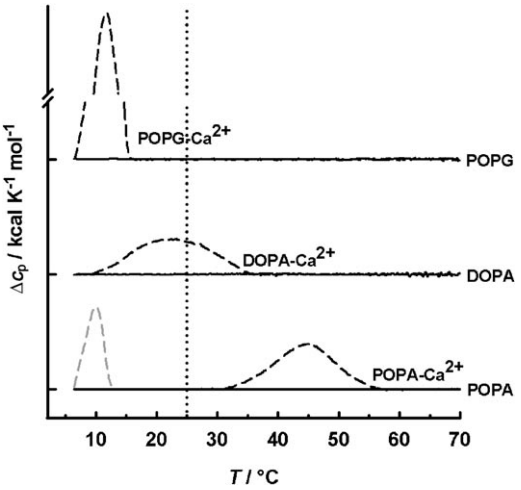

Figure 5. Influence of $CaCl_2$ on the thermotrophic phase behavior of POPC/POPA, POPC/DOPA and POPC/POPG (1:1) SUVs. DSC measurements were performed in the absence (full lines) and in the presence of 10 mM $CaCl_2$ (broken lines). The dotted vertical line marks the temperature where activity measurements (Figure 2) were performed.

sition temperatures lie below 7 °C, which was the lower temperature limit of the DSC instrument. The addition of 10 mM $CaCl_2$ led to the appearance of transition peaks in all vesicle preparations. As these transitions emerged only in the presence of Ca^{2+} ions and differ for the different anionic components, they can clearly be assigned to the gel-to-fluid transitions of the anionic phospholipid complexes with Ca^{2+}. Thus, it can be concluded that below the transition temperatures of the anionic phospholipid/Ca^{2+} complexes, the SUVs are characterized by nonideal mixing of the phospholipid constituents with the tendency to form microdomains composed of anionic phospholipid/Ca^{2+} complexes. Interestingly, the temperatures of the transition maxima (T_m) dropped in the order POPA > DOPA > POPG with T_m values of 44.4, 23.2 and 11.7 °C. Accordingly, at 25 °C—the temperature of the activity assay—the membrane of the POPC/POPG SUVs should be in the fluid phase, whereas the membrane of the POPC/DOPA SUVs should be in the transition region of the DOPA/Ca^{2+} complexes; only the membrane of the POPC/POPA SUVs should be characterized by the coexistence of gel-like POPA/Ca^{2+} complexes and a fluid phase. The second Ca^{2+} induced peak in the POPC/POPA samples probably arises from the melting of solitary POPA/Ca^{2+} complexes surrounded by POPC.

PLD activity in relation to thermal phase transition

To relate the gel-to-fluid phase transitions of POPC and POPC/POPA SUVs to PLD activity, PLD activity toward POPC and POPC/POPA (85:15) SUVs was measured as a function of temperature. In both cases curves with a maximum in the activity were obtained (Figure 6). However, in the POPC/POPA system the decrease in activity occurred at a temperature 5 °C lower than in the POPC system and coincided with the thermally induced phase transition of the POPA/POPC SUVs (Figure 6). Interestingly, the experimental data in the temperature range of phase transition, particularly at 32.5–40 °C, show large standard deviations, which might be the result of the highly dynamic state of the substrate in this temperature region.

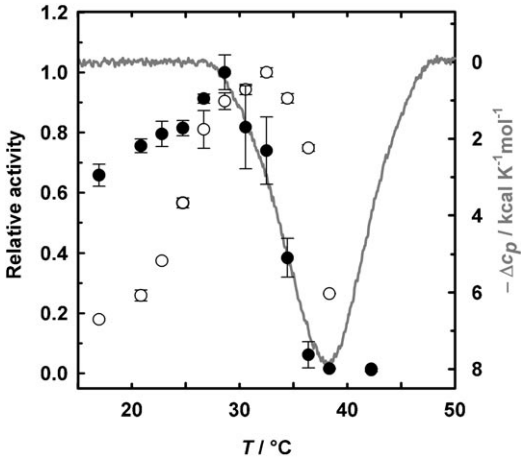

Figure 6. Relative PLD activity towards POPC (○) and POPC/POPA (85:15) SUVs (●) as a function of temperature. All measurements were performed in 10 mM Pipes, pH 7.0, 10 mM $CaCl_2$ at 25 °C. To indicate the gel/fluid transition of the POPC/POPA system, the corresponding DSC curve (Figure 5) was overlaid in grey.

The effect of phase separation within neutral membranes on PLD activity

To analyze whether the activation of PLD generally arises from the presence of phospholipid domains, we tested the effect of uncharged vesicles containing a typical "raft"-like ternary lipid mixture[18] on PLD activity. A low melting temperature lipid (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC), a high melting temperature lipid (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, DPPC) and cholesterol (Chol) were used to prepare SUVs, which represent membranes in ordered, disorderd or coexistent states, respectively. No significant activation could be observed with SUVs in which ordered and disordered domains coexist (DOPC/DPPC/Chol, 40:40:20) as compared to the SUVs in disordered (DOPC/DPPC, 85:15) or ordered (DOPC/DPPC/Chol, 3:62:35) states (Figure 7).

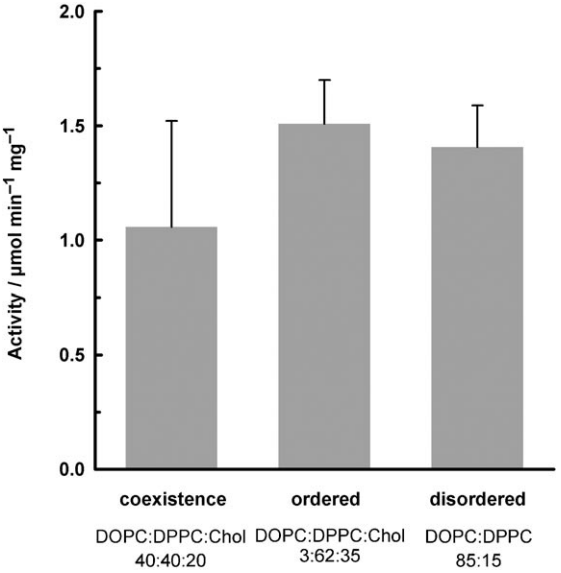

Figure 7. PLD activity towards uncharged SUVs. PLD was incubated with DOPC/DPPC/Chol (40:40:20), DOPC/DPPC/Chol (3:62:35) and DOPC/DPPC (85:15) SUVs. Phospholipid concentration was 1 mM and PLD concentration was 17 pM PLD. All measurements were performed in 10 mM Pipes, pH 7.0, 10 mM $CaCl_2$ at 25 °C.

Discussion

Ca^{2+} ions are essential for PLD activity independent of the association state of the substrate

The essential role of Ca^{2+} ions for the activity of most PLDs has been known for many years.[19] In cabbage PLD, which is the best characterized plant PLD and is also used in this study, two binding sites with affinity differences of two orders of magnitude have recently been reported.[15b] In the present paper we demonstrate that the need for Ca^{2+} ions is inde-

pendent of the substrate and its state of aggregation. With the short-chain substrate diC_6PC, no activity is found above or below the CMC if Ca^{2+} ions are absent, whereas in the presence of 10 mM $CaCl_2$, two sigmoidal curve sections (below and above the CMC) are observed in the plot of activity versus substrate concentration (Figure 1). These curves are similar to those described before for cabbage PLD and short-chain substrates.[7] Previous curves, however, did not show sigmoidicity in the two concentration ranges. In a detailed study of PLD activity in the monomer range of diC_6PC, sigmoidicity with a Hill factor of 1.8 was reported; this was hypothesized to be due to an additional substrate binding site.[12]

PA facilitates the association of PLD with the phospholipid surface

In addition to the Ca^{2+}-mediated activation of PLD, anionic phospholipids were confirmed to stimulate enzyme activity. The 500-fold increase of PLD activity from 0.6 to 309.8 $\mu mol\,min^{-1}mg^{-1}$ induced by POPA (Figure 2A) is the highest activation of this enzyme described thus far. Jung et al.[11] found a maximum activation factor of about 20 for cabbage PLD with PA. This lower activation might be caused by the fatty acid composition of the PA, which is not specified in this paper. As demonstrated in Figure 2A, the stimulation of PLD activity is strongly dependent on the fatty acid structure and decreases to the factor of 20 if DOPA (with two unsaturated oleoyl fatty acids) is incorporated into the vesicles instead of POPA (with one saturated palmitoyl and one unsaturated oleoyl fatty acid). POPG showed an even smaller activation effect (twofold maximum activation; Figure 2A). From the decrease of the lag phases due to the incorporation of POPA, DOPA or POPG into the zwitterionic neutral POPC SUVs, (Figure 2B) it can be concluded that the association of PLD to the interfaces is facilitated by these anionic components. The increase of the binding affinity of PLD to membranes containing small fractions of POPA was also confirmed in the experiments on the monolayers of 1,3-dimyristoylglycero-2-phosphocholine which are substrate-analogous compounds but nonhydrolyzable by PLD (Figure 4). Interestingly, there are no synergisms between the activation by Ca^{2+} ions and the activation by POPA (Figure 3). POPA cannot act as activating component in the absence of Ca^{2+} ions.

In the presence of Ca^{2+} ions anionic phospholipids induce lateral heterogeneity within POPC vesicles

DSC analysis of the SUVs composed of POPC and POPA, DOPA or POPG revealed striking differences, which can be correlated with the activation data of PLD as discussed below. In the absence of Ca^{2+} ions, all the anionic phospholipids were readily miscible with POPC without showing any indication of phase transitions (Figure 5). Obviously, the lipids were in the fluid state in the considered temperature range (7-70 °C). The T_m for the gel-to-fluid transition was below 7 °C, presumably near the T_m of POPC (−3 °C).[20] In contrast, lateral phase separation is reflected in the DSC diagrams of the SUVs with anionic phospholipids in the presence of 10 mM $CaCl_2$. According to physicochemical studies on the domain formation by phosphatidic acid in the presence of Ca^{2+} ions,[21] the endothermal peaks in Figure 5 can be assigned to the gel-to-fluid transition of complexes of Ca^{2+} ions with POPA, DOPA or POPG. Obviously, Ca^{2+} ions induced partitioning of the anionic phospholipids into microdomains with T_m values which are higher than 7 °C and could, therefore, be observed in the DSC thermograms. While the fluid POPC molecules remained nearly unchanged, the anionic phospholipids are obviously tightly packed as also indicated by monolayer experiments with L-α-dimyristoylphosphatidic acid.[22] Only with POPC/POPA was a shift of the gel-to-fluid transition of the POPC-rich phase to higher temperatures indicated by the appearance of an additional peak at T_m = 10 °C (Figure 5). Remarkably, the T_m values of the microdomains strongly differed, dependent on the kind of anionic phospholipid. Interestingly, the exchange of one saturated fatty acid (in POPA) by an unsaturated one (in DOPA) provoked a decrease in T_m of about 21 °C, whereas, even more strikingly, the head group exchange from POPA to POPG caused a decrease in T_m of about 32 °C. The strong influence of the acyl chain composition of PA on T_m is in good accordance with the findings of Silvius,[23] who used carbazole-labeled fluorescent PCs to monitor lateral phase separations of PC/anionic phospholipid vesicles.

PLD activity is regulated by membrane morphology

The striking activation of PLD (Figure 2A) and its promoted binding to phospholipid vesicles (Figure 2B and Figure 4) in the presence of anionic phospholipids and Ca^{2+} ions can be clearly correlated with the thermotropic behavior of the corresponding SUVs (Figure 5). The stimulation of PLD activity at 25 °C is largest (500-fold) with POPC/POPA SUVs, which are characterized by the coexistence of gel-like POPA/Ca^{2+} microdomains and the fluid POPC phase at this temperature. In contrast, the POPC/DOPA microdomains (20-fold activation) are in the process of melting at 25 °C and no POPC/POPG microdomains (two-fold activation) are present any more at 25 °C. The suggested relationship between Ca^{2+}-induced lateral membrane heterogeneity and PLD activation is further supported by the strong activity decrease of PLD toward POPC/POPA SUVs at temperatures > 32.5 °C (Figure 6). At this temperature, the Ca^{2+}-POPA complexes start to melt. The declining branch of the activity-temperature curve exactly corresponds to the endothermic DSC peak (Figure 6). Thermal unfolding, which normally causes the declining branch in the activity-temperature profiles, can be ruled out as reason for the steep inactivation above 32.5 °C in the POPC/POPA system because PLD is stable at this temperature.[24] The temperature optimum for pure POPC SUVs is 37 °C.

The stimulation of enzyme activity by local enrichment of anionic lipids has also been observed and extensively analyzed for secretory PLA_2s.[25] In these studies, the phase separations were attributed to immiscible lipids differing in charge[25b] or fatty acid composition.[25a] However, the nonideal mixing in our experiments has been proved to emerge from calcium-induced

microdomain formation, which is the result of the concurrent presence of anionic phospholipids and Ca^{2+} ions. The importance of Ca^{2+} ions in the microdomain-triggered activation was accentuated by the result that in the ternary "raft"-model system (DOPC/DPPC/Chol), PLD activity was not increased (Figure 7). Therefore, lateral phase separation per se is not able to stimulate PLD activity.

In cells PA microdomains may act through three modes:[26] 1) altering the membrane structure; 2) acting as messengers by specific interactions with proteins; 3) tethering a protein to a membrane and/or modulating the catalytic activity. Potentially, all of these mechanisms are feasible in the case of plant PLDs. On the basis of the present results, however, it seems most probable that the amplification of the local Ca^{2+} concentration within the microdomains satisfies the unusually high Ca^{2+} requirement of PLD. In the absence of PA, PLD from cabbage needs 40–100 mM $CaCl_2$ for optimum activity.[15] Anchoring the negatively charged amino acid residues of PLD to the phospholipid surface may be facilitated by Ca^{2+}-enriched membrane regions. In any case, Ca^{2+} ions are assumed to serve as a biological switch that enables the cell to transiently regulate PLD activity. A Ca^{2+}-triggered PLD regulation in close cooperation with PA appears reasonable considering that unrestrained PLD activity would cause a collapse of the cellular membrane network. Particularly for plants, elaborated modifications of the membrane lipid composition are essential in the adaptation to specific environments and physiological circumstances.[27] However, an interplay between Ca^{2+} ions and PA has been also reported for many other enzymes such as the extracellular phosphoesterase with PLD-like activity from *Streptomyces chromofuscus*.[13] Therefore, an ingenious interplay between Ca^{2+} ions and PA seems to be a general mechanism of membrane-associated enzymes.

Experimental Section

Materials: Phospholipids were purchased from Avanti Polar Lipids, (Alabaster, AL, USA). 1,3-Diacylglycero-2-phosphocholine was synthesized by Haftendorn et al.[17] All other chemicals were the purest ones commercially available.

Preparation of PLD: PLD, which is one of two α-type isoenzymes from white cabbage and has been designated as PLD2 or PLDα2 in previous publications, was expressed in *E. coli* as described by Schäffner et al.[15a] and purified according to Stumpe et al.[15b] Before use, PLD was stored in Pipes buffer (10 mM, pH 7.0) at $-80\,^{\circ}C$.

Determination of protein concentration: The concentration of PLD was determined spectrophotometrically (Ultrospec 3000, Pharmacia Biotech, Uppsala, Sweden), using the molar extinction coefficient ($\varepsilon_{280} = 123\,720\ M^{-1}cm^{-1}$) calculated according to Gill and von Hippel.[28]

SUV preparation: The lipids, dissolved in chloroform, were dried by rotatory evaporation to yield a thin film on the inner surface of a round bottom flask. The lipid film was then suspended in Pipes buffer (10 mM, pH 7.0) in an ultrasound bath at a temperature about 15–30 K higher than the transition temperature of the lipids. The cloudy multilamellar vesicle suspensions were sonicated for 100 s with 3 s pulses (using a Vibra-Cell 72442, Bioblock-Scientic Instruments, Freiburg, Germany), yielding a clear solution of SUVs.

Determination of PLD activity and lag time: PLD activity was quantified by an enzyme coupled assay using 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent, Molecular Probes Inc., Eugene, OR, USA). In this assay, choline released from PC is oxidized by choline oxidase. In the presence of horseradish peroxidase, the resulting H_2O_2 reacts with the Amplex Red reagent in a 1:1 stoichiometry to generate the highly fluorescent resorufin. The intensity of resorufin fluorescence was monitored with a microplate reader (Polarstar Galaxy, BMG Labtechnologies, Offenburg, Germany) at 25 °C or—in the case of the temperature-dependent measurements—with a Jasco Spectrofluorometer FP-6500 (Jasco International, Tokyo, Japan), using an excitation wavelength of 542 nm and an emission wavelength of 590 nm. The reaction mixture contained Pipes buffer (10 mM, pH 7.0), $CaCl_2$ (10 mM), phospholipids (1 mM), Amplex Red reagent (50 μM), horseradish peroxidase (1 U ml^{-1}), choline oxidase from *Alcanigenes* sp. (0.1 U ml^{-1}) and PLD (17 pM). The activity was calculated from the initial velocity of the fluorescence increase (after the lag phase). The lag time was defined as the time interval from starting the measurement up to the time when 5% of the maximum fluorescence intensity was reached.

Differential scanning calorimetry: DSC measurements were performed with a differential scanning calorimeter (MC-2, MicroCal Inc., Northampton, MA, USA) at a heating rate of 1 °C min^{-1} and a time resolution of 3 s. SUVs were prepared in Pipes buffer (10 mM, pH 7.0) as described above. For the measurements in the presence of calcium ions, $CaCl_2$ (10 mM) was added to the sample tubes followed by rapid vortexing. In all experiments the lipid concentration in the calorimetric cell was 2.5 mM, and Pipes buffer (10 mM, pH 7.0) was used as reference. Four heating scans were performed for each sample to prove reproducibility. All presented curves show the third heating scan.

Monolayer measurements: Surface pressure measurements were performed using a homebuilt film balance with a constant area and a Wilhelmy plate surface pressure measuring device (Riegler & Kirstein, Mainz, Germany). Lipid mixtures were dissolved in chloroform/methanol (4:1 v/v) yielding a concentration of 0.1 mM. The subphase contained Pipes buffer (10 mM, pH 7.0) and $CaCl_2$ (10 mM). Lipids were spread with a microsyringe at the air-buffer interface. Surface films were equilibrated until surface pressure remained constant at about 25 $mN\,m^{-1}$. PLD was then injected into the subphase and the increase in surface pressure was followed until a plateau was reached. During the measurement the subphase was slowly stirred by a magnetic stirrer. All experiments were performed at 25 °C.

Data analysis: All measurements were conducted at least threefold in independent experiments. Data were analyzed with MS Excel or Sigma Plot 8.0.

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