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Rapid report

Synthesis and membrane behavior of a new class of unnatural phospholipid analogs useful as phospholipase A2 degradable liposomal drug carriers

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Abstract

A new and unnatural type of lipid analogs with the phosphocholine and phosphoglycerol head groups linked to the C-2 position of the glycerol moiety have been synthesized and the thermodynamic lipid membrane behavior has been investigated using differential scanning calorimetry. From the heat capacity measurements, it was observed that the pre-transition was abolished most likely due to the central position of the head groups providing better packing properties in the low temperature ordered gel phase. Activity measurements of secretory phospholipase A2 (PLA2) on unilamellar liposomal membranes revealed that the unnatural phospholipids are excellent substrates for PLA2 catalyzed hydrolysis. This was manifested as a minimum in the PLA2 lag time in the main phase transition temperature regime and a high degree of lipid hydrolysis over a broad temperature range. The obtained results provide new information about the interplay between the molecular structure of phospholipids and the lipid membrane packing constrains that govern the pre-transition. In addition, the PLA2 activity measurements are useful for obtaining deeper insight into the molecular details of the catalytic site of PLA2. The combined results also suggest new approaches to rationally design liposomal drug carries that can undergo a triggered activation in diseased tissue by overexpressed PLA2.

Keywords: Liposomes; Lipid synthesis; Ripple phase formation; Phospholipase A2; Enzyme lipid interaction; Liposomal drug carrier; Lipid membrane heterogeneity; Calorimetry; Fluorescence

Fully hydrated lipid membranes composed of saturated phospholipids can undergo several thermotropic phase transitions, which are considered to be of relevance for the functional behavior of biological membranes [1–3]. In addition, the structural biomaterial properties of phospholipid membranes undergoing phase transitions are of importance for the development of new lipid-based drug delivery systems with improved therapeutic effects [3–7]. The most extensively studied lipid membrane phase transition is the gel-to-fluid chain melting transition, which takes the membrane from a low temperature gel phase with the acyl chains in a predominantly ordered configuration to a fluid phase with the acyl chains in a highly disordered conformation [1,5]. The ripple phase, ($P_{\beta'}$), which is characterized by corrugations in the lipid membrane plane with a periodicity ranging from 10 to 30 nm, exists in a temperature range between the pre-transition and the main transition [8,9]. The ripple phase is one of the more

Abbreviations: DPPC, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoglycerol; 1-*O*-DPPC′, (*S*)-1-*O*-hexadecyl-3-hexadecanoyl-glycero-2-phosphocholine; 1-*O*-DPPG′, (*S*)-1-*O*-hexadecyl-3-hexadecanoyl-glycero-2-phosphoglycerol; 1-*O*-DPPC, 1-*O*-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine; 1-*O*-DPPG, 1-*O*-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphoglycerol; PLA2, phospholipase A2; DSPE-PEG_{2000}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol)$_{2000}$

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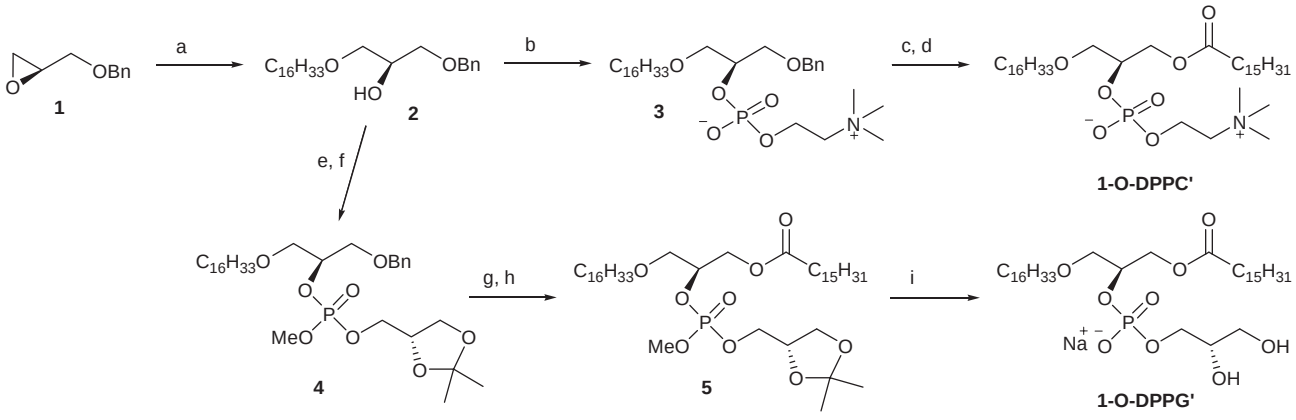
intriguing ordered lamellar phases and its structural behavior has been extensively studied by a large number of experimental techniques including freeze fracture electron microscopy, differential scanning calorimetry, and atomic force microscopy [10–15]. The ripples have an asymmetric saw-tooth profile with the acyl chains tilted with respect to the lipid membrane normal [10]. The molecular origin of ripple formation has traditionally been associated with the lipid head group region and for this reason phospholipids are normally divided into ripple forming lipids and non-ripple-forming lipids. One of the most extensively studied ripple-forming lipid families is the phosphatidylcholine lipids [9,10,14]. Several attempts have been made to explain the reason for ripple phase formation in a lipid membrane. Examples of some of the molecular mechanisms that have been proposed to play an important role for the formation of a corrugated lipid membrane structure include an electrostatic coupling between the water molecules and the polar lipid head groups [16] and a coupling between membrane curvature and molecular tilt [17]. Other models assume that ripples form in order to relieve packing frustrations that arise whenever the relationship between the cross-sectional area of the bulky head group and the cross-sectional area of the long apolar tail exceeds a certain threshold value [18,19].

In this paper, we have synthesized a new class of unnatural phospholipid analogs where the phosphocholine (PC) and phosphoglycerol (PG) head groups are linked to the C-2 position and with an alkyl chain ether-linked to the C-1 position of the glycerol backbone of the phospholipids. Using differential scanning calorimetry, we have investigated the thermodynamic phase behavior and shown that the pre-transition that usually characterizes the thermodynamic lipid membrane phase behavior of natural PC and PG phospholipids is abolished for the unnatural phospholipid analogs. From the PLA2 activity measurements we have furthermore learned that these unnatural phospholipids behave as excellent substrates for PLA2 catalyzed hydrolysis. These findings are in agreement with earlier thermodynamic studies and PLA2 activity measurements carried out on 1,3-diacyl-*sn*-2-phosphocholines [20–25]. Our PLA2 activity measurements on the novel 1-*O*-DPPC′ and 1-*O*-DPPG′ liposomes furthermore revealed a minimum in the lag time in the gel-to-fluid transition region and a high degree of hydrolysis over a broad temperature range. Incorporation of small amounts of negatively charged PG phospholipids or DSPE-PEG_{2000} polymer lipids resulted in a reduction of the PLA2 lag time in accordance with earlier results on polymer grafted DPPC lipid membranes containing negatively charged polymer lipids [26]. In addition, human secretory PLA2 showed a high activity towards liposomes formed by the 1-*O*-DPPG′ lipids.

Our combined approach involving chemical synthesis of new phospholipid analogs followed by thermodynamic bulk investigations of the lipid membrane phase behavior suggests new strategies to learn about molecular details of phospholipids that are of importance for ripple formation in a lipid membrane. Furthermore, the results provide information about the non-selective catalytic behavior of PLA2 that can be used, e.g. in combination with future molecular dynamic simulations, to map out conformational details of the catalytic active site of PLA2. In addition, the unnatural lipids offer new ways to rationally design soft lipid-based composite biomaterials such as liposomal drug carrier systems that can be degraded specifically by overexpressed PLA2 in diseased tissue [26–30].

The unnatural PC and PG phospholipids (1-*O*-DPPC′, 1-*O*-DPPG′) with the phosphocholine and phosphoglycerol head groups linked to the C-2 position of the glycerol moiety were synthesized utilizing (*S*)-*O*-benzyl glycidol as a versatile starting material [27,28] as shown in Scheme 1. Opening of the epoxide 1 under basic conditions, using



Scheme 1. Synthesis of the novel phospholipid analogs 1-*O*-DPPC′ and 1-*O*-DPPG′ with the phosphate in the C-2 position from (*S*)-*O*-benzyl glycidol. (a) $C_{16}H_{33}OH$, NaH, DMF/THF, rt (71%); (b) i. $POCl_3$, Et_3N, DCM, rt, ii. pyridine, choline tosylate, rt (65%); (c) H_2, Pd/C, MeOH, rt (99%); (d) $C_{15}H_{31}COCl$, DMAP, Et_3N, DCM, rt (83%); (e) (i-Pr) $_2$NP(OMe)Cl, TMP, DCM, rt; (f) i. (*R*)-isopropylidene glycerol, 5-phenyl-1H-tetrazole, DCM, 0 °C, ii. *t*-BuOOH, 0 °C (67% over 2 steps); (g) H_2, Pd/C, MeOH, rt (99%); (h) palmitic acid, DMAP, DCC, DCM, rt (92%); (i) i. CH_3CN, isopropanol, Me_3N, DCM, rt, ii. HCl, MeOH, DCM, H_2O, rt, iii. $NaHCO_3$, DCM, rt (70%).

THF/DMF (1:1) as a solvent system that minimizes dimerization gave **2** in 71% yield after purification by column chromatography. The phosphorylation was performed using phosphorous oxychloride in CH_2Cl_2 [27], which gave **3** in 65% yield. Debenzylation under H_2 atmosphere with Pd/C as catalyst followed by a simple acylation using palmitoyl chloride gave the target 1-*O*-DPPC′ lipid. The synthesis of 1-*O*-DPPG′ was carried out from **2** using $(i\text{-}Pr)_2NPClOMe$ [31] as the phosphorylation reagent. The phosphorylation using TMP as base in the lipid coupling followed by (*R*)-isopropylidene glycerol with 5-phenyl-1H-tetrazole as a weak proton donor gave the protected phospholipid **4** in 67% yield after oxidation. Debenzylation followed by acylation using DCC gave **5** in 92% yield. Deprotection of lipid **5** was carried out with Me_3N to remove the methyl protection group, followed by stirring in CH_2Cl_2/MeOH/0.5M HCl (65:25:4) resulting in removal of the isopropylidene group. Finally, the proton on the phosphate was exchanged with sodium using $NaHCO_3$, which gave the desired 1-*O*-DPPG′ in 70% yield after purification by column chromatography.

By replacing the ester linkage in the C-1 position with an ether bond, these unnatural phospholipids can furthermore be used as novel prodrug anticancer lipids to make liposomal microcarriers that can be degraded to active anticancer lysoetherlipids by PLA2 [27]. The lipid prodrug analogs (1-*O*-DPPC and 1-*O*-DPPG) were synthesized as described earlier [27,28]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocho-

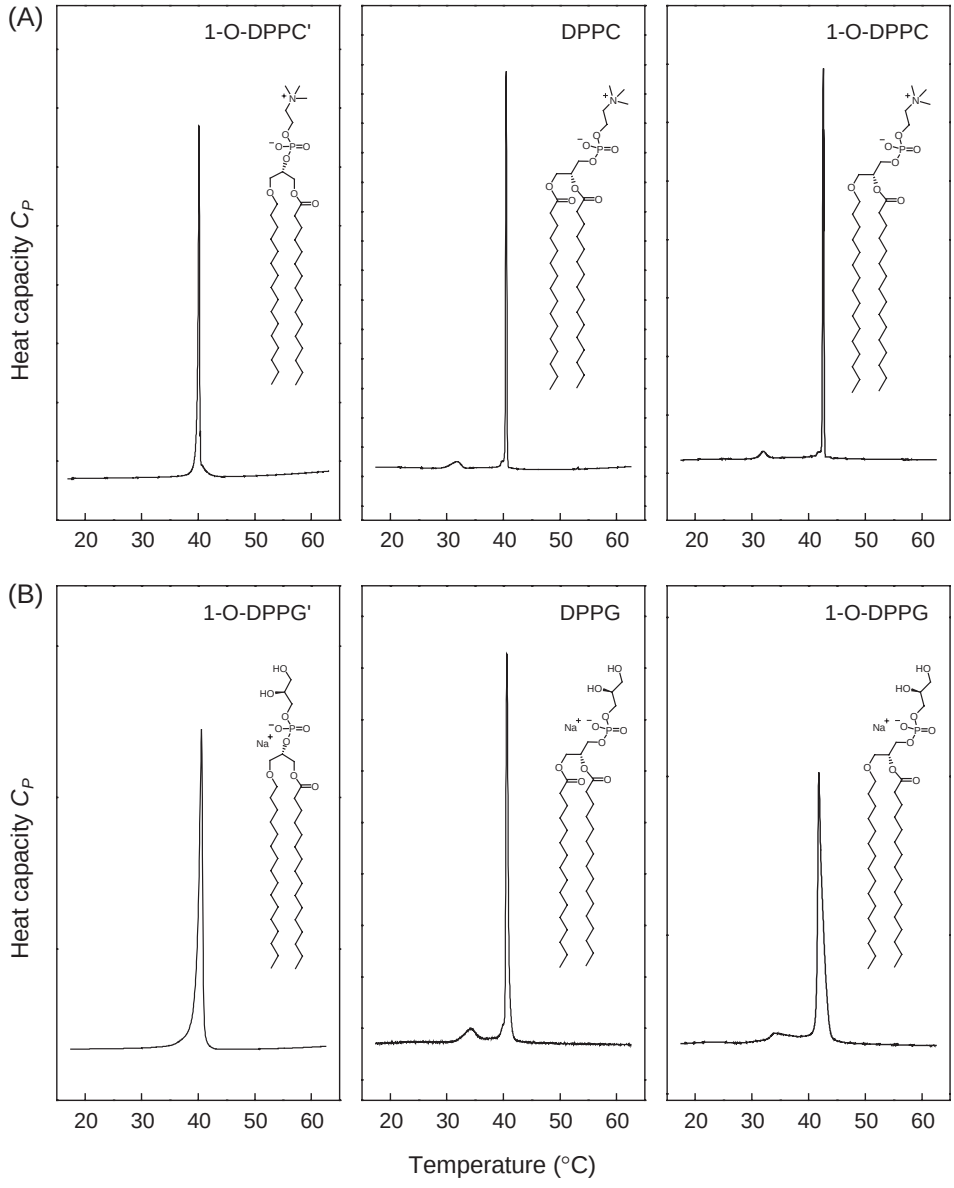


Fig. 1. Heat capacity, C_P, obtained using differential scanning calorimetry at a scan rate of 20 °C/h for 5 mM multilamellar liposomes: (A) 1-*O*-DPPC′ (left), DPPC (middle), and 1-*O*-DPPC (right). (B) 1-*O*-DPPG′ (left), DPPG (middle) and 1-*O*-DPPG (right).

line (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) were purchased from Avanti Polar Lipids, AL, USA. Multi- and unilamellar liposomes were hydrated in a HEPES buffer solution (10 mM HEPES, 110 mM KCl, 1 mM NaN_3, 30 μM $CaCl_2$, 10 μM NaEDTA, pH 7.5) and prepared as described in previous work [26,27]. Differential scanning calorimetry was carried out in the upscan mode at a scan rate of 20 °C/h using multilamellar liposomes [26,27]. The PLA2 lag time measurements on 1-*O*-DPPC′ unilamellar liposomes incorporated with small amounts of 1-*O*-DPPG′ or DSPE-PEG$_{2000}$ lipids were performed in the gel-to-fluid main phase transition range and the degree of lipid hydrolysis was analyzed using HPLC techniques [26–28].

The heat capacity (C_P) curves obtained using differential scanning calorimetry are shown in Fig. 1 for one-component 1-*O*-DPPC′ and 1-*O*-DPPG′ multilamellar liposomes. The C_P curves show a main peak at 40.1 and 40.6 °C reflecting the chain disordering gel-to-fluid main phase transition of the 1-*O*-DPPC′ and 1-*O*-DPPG′ lipid membranes, respectively. As clearly demonstrated by the C_P curves, the pre-transition that usually characterizes the lipid membrane phase behavior of natural DPPC and DPPG lipids (Fig. 1A and B) is abolished for the unnatural 1-*O*-DPPC′ and 1-*O*-DPPG′ lipids. This leads to the formation of low temperature ordered and non-corrugated gel phase (L_β) that prevails until the unset of the main transition giving rise to a concomitant increase in the integrated melting enthalpy of the main transition (Table 1). Since the 1-*O*-DPPC′ and 1-*O*-DPPG′ lipids undergo a transition from an ordered configuration in the low temperature non-corrugated gel phase to a disordered configuration in the high temperature fluid phase without passing through the ripple phase, the integrated melting enthalpy is expected to be higher than for the natural DPPC and DPPG ripple phase forming lipids, which undergo a transition to the fluid phase from a ripple phase with a higher energy than the non-corrugated gel phase. In order to exclude the possibility that the abolished ripple transition is due to having an ether bond in the C-1 position, we have in Fig. 1 also shown C_P curves obtained on 1-*O*-DPPC and 1-*O*-DPPG multilamellar liposomes. Clearly, these C_P curves show the same characteristic phase behavior as the natural DPPC and DPPG lipids with a pre-transition a few degrees below the main phase transition peak underlining that it is the position of the phosphate head group in the C-3 position on the natural phospholipids that is crucial for ripple formation. Our results therefore suggest that ripples form in order to relieve packing frustrations that arise due to the flanked position of the bulky hydrophilic head group relative to the long apolar acyl chains on the natural DPPC and DPPG lipids, whereas an imbalance between the cross-sectional area of the head and tail regions [18,19] and an electrostatic interaction between water molecules and the polar lipid head groups [16] seem to be less crucial for ripple formation in line with results reported earlier on 1,3-diacyl-phosphocholine lipids [20,21].

Table 1
Differential scanning calorimetry data showing the melting enthalpy, ΔH, half width, $T_{1/2}$, and peak position, T_m, of the pre- and main phase transitions

	Pre-transition			Main transition		
	ΔH (kcal/mol)	$T_{1/2}$ (°C)	T_m (°C)	ΔH_m (kcal/mol)	$T_{1/2}$ (°C)	T_m (°C)
1-*O*-DPPC′	–	–	–	12.8	0.25	40.1
DPPC	1.2	1.9	31.9	8.7	0.21	40.5
1-*O*-DPPC	0.84	1.23	32.0	8.5	0.24	42.6
1-*O*-DPPG′	–	–	–	11.3	0.71	40.6
DPPG	1.31	2.2	34.3	9.8	0.41	40.6
1-*O*-DPPG	0.58	2.7	34.1	9.1	0.86	41.8

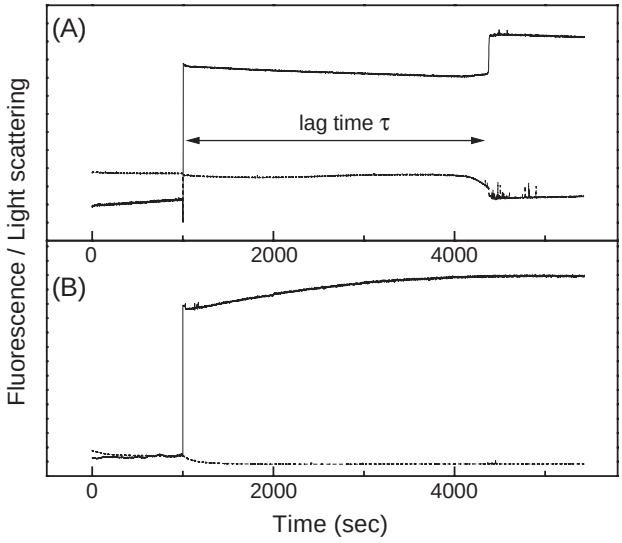


Fig. 2. Characteristic reaction time profiles at 40 °C for PLA2 hydrolysis of unilamellar. (A) 0.15 mM 1-*O*-DPPC′ liposomes incubated with 150 nM PLA2 (*A. piscivorus piscivorus*). (B) 0.15 mM 1-*O*-DPPG' liposomes incubated with human secretory PLA2 from 20 μL freshly prepared human tear fluid [42]. The PLA2 hydrolysis reaction is monitored by intrinsic fluorescence (solid line) from the enzyme and 90° static light scattering (dashed lines) from the lipid suspension. After adding PLA2 to the equilibrated liposome suspension a characteristic lag time, τ, follows before a sudden increase in the catalytic activity takes place (A). This lag time is observed for the neutrally charged 1-*O*-DPPC′ liposomes but not for the negatively charged 1-*O*-DPPG′ liposomes (B) where the enzyme becomes active immediately after addition.

Typical PLA2 (*A. piscivorus piscivorus*) hydrolysis time course profiles obtained at 40 °C are shown in Fig. 2 for 1-*O*-DPPC′ and 1-*O*-DPPG′ unilamellar liposomes. The lag time, τ, shown in Fig. 2A is derived on basis of the time elapsed after addition of PLA2 to the lipid suspension until the unset of rapid lipid hydrolysis. The rapid increase in PLA2 activity is reflected by a sharp rise in the intrinsic PLA2 tryptophan fluorescence intensity [32,33] followed by a concomitant decrease in the 90° static light scattering caused by a change in the morphology of the lipid system when a large amount of the phospholipids are converted to

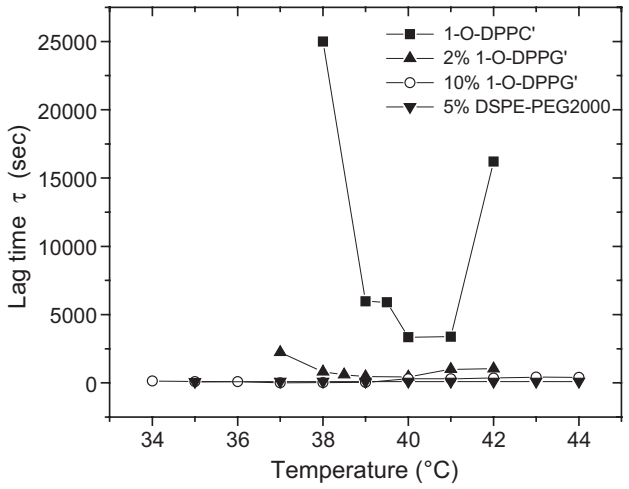


Fig. 3. PLA2 (*A. piscivorus piscivorus*) lag time, τ, as a function of temperature for the hydrolysis of 1-*O*-DPPC′ unilamellar liposomes incorporated with 0 mol% (■), 2 mol% (▲), and 10 mol% (○) 1-*O*-DPPG′ lipids, and 5 mol% (▼) DSPE-PEG_{2000} polymer lipids. The concentration of the liposomes was 0.15 mM and the concentration of PLA2 was 150 nM.

non-bilayer forming lysolipids and free fatty acids [32,33]. It has earlier been argued that this lag time reflects the time required to slowly accumulate a threshold amount of lysolipid and fatty acid hydrolysis products in the lipid membrane needed to cause a sudden activation of PLA2 [32]. Both the fluorescence and light scattering data clearly indicate that the unnatural phospholipids are excellent substrates for PLA2 catalyzed hydrolysis. Apparently, these synthetic lipids are easily degradable by PLA2. This is especially seen for the negatively charged 1-*O*-DPPG′ liposomes (Fig. 2B) where immediate hydrolysis is observed when PLA2 derived from human tear fluid was added to the suspension. A high activity of human secretory PLA2 is expected towards these negatively charged liposomes in accordance with earlier results showing that a threshold amount of negatively charged lipids is needed to activate the human PLA2 [28]. Fig. 3 shows the lag time, τ, as a function of temperature in the gel-to-fluid transition range of unilamellar 1-*O*-DPPC′ liposomes. The appearance of a miminum in the lag time at the gel-to-fluid phase transition is most likely caused by the formation of a heterogeneous membrane structure composed of fluctuating gel and fluid domains as earlier described for phosphocholine lipid membranes [26,27,33,34]. The results in Fig. 3 furthermore show that the PLA2 activity increases with increasing amounts of negatively charged 1-*O*-DPPG′ lipids incorporated into the liposomes.

Since one of our aims was to develop new liposomal carriers that can be used for PLA2 triggered drug delivery [27,28] we have also tested the influence of DSPE-PEG_{2000} polymer lipids on PLA2 degradation. For drug delivery purposes it is necessary to incorporate a small amount of DSPE-PEG_{2000} lipids in order to obtain a sufficient blood circulation time [3]. When 5 mol% DPPE-PEG_{2000} lipopolymers are incorporated into the 1-*O*-DPPC′ liposomes a very short lag time is observed as shown in Fig. 3 in accordance with similar studies using natural DPPC liposomes incorporated with DSPE-PEG_{2000} lipids [26]. Fig. 4A shows the degree of PLA2 lipid hydrolysis 1000 s after the unset of the burst for the unnatural 1-*O*-DPPC′ phospholipids containing 0, 2 and 10 mol% 1-*O*-DPPG′ lipids, and 5 mol% DPPE-PEG_{2000} polymer lipids. A remarkably high degree of lipid hydrolysis is observed over a broad temperature range clearly demonstrating that PLA2 is able to hydrolyze the unnatural 1-*O*-DPPC′ and 1-*O*-DPPG′ phospholipids. For the negatively charged 1-*O*-DPPG′ lipids, which are superior substrates for the human secretory PLA2, the degree of lipid hydrolysis is furthermore determined as a function of time after addition of PLA2 to the lipid suspension (Fig. 4B). Although PLA2 is

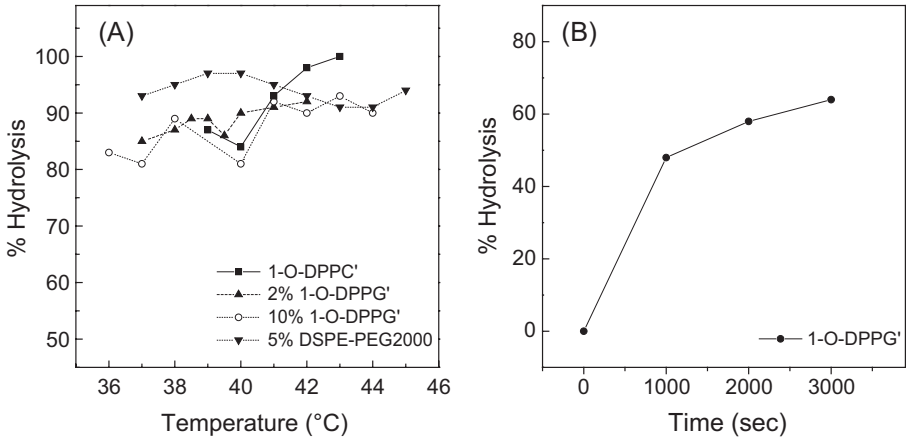


Fig. 4. (A) PLA2 (*A. piscivorus piscivorus*) hydrolysis of 1-*O*-DPPC′ liposomes containing 0 mol% (■), 2 mol% (▲), and 10 mol% (○) 1-*O*-DPPG′ lipids, and 5 mol% (▼) DSPE-PEG_{2000} polymer lipids determined by HPLC [1]. The percent lipid hydrolysis is determined 1000 s after the unset of the burst. The concentration of the liposomes was 0.15 mM and the concentration of PLA2 was 150 nM. (B) Hydrolysis of 1-*O*-DPPG′ unilamellar liposomes determined by HPLC [1]. The PLA2 catalyzed lipid hydrolysis is determined as a function of time after addition of 20 μL freshly prepared human tear fluid [42] to 0.15 mM 1-*O*-DPPG′ liposomes.

classified as an enzyme that is able to specifically catalyze the hydrolysis of the ester linkage in the C-2 position of natural phospholipids, our results demonstrate that the catalytic activity is sustained when the phosphate head group is linked to the C-2 position and the hydrolysable acyl chain is linked to the C-3 position in accordance with earlier work carried out on 1,3-diacyl-phosphocholine lipids [22–24]. For the 1,3-diacyl-phosphocholine lipids it has earlier been suggested that both acyl chains assume a bent conformation in the lipid membrane similar to the C-2 acyl chain in natural 1,2-diacyl-phosphocholine lipid substrates, and that it is this bent that is important for PLA2 hydrolysis [20]. Chupin et al. [35] have exploited PLA2 in the synthesis of platelet-activating factor analogs with the phosphate headgroup attached to the C-2 position.

The ability of PLA2 to hydrolyze the unnatural phospholipids can advantageously in future studies in combination with, e.g. molecular dynamic simulations be used to learn more about molecular details of the active site of PLA2 [36,37]. The non-selective behavior of PLA2 towards the natural/unnatural phospholipids furthermore raises an interesting question related to why natural lipids are designed as ripple-forming lipids. Although our results are obtained on model lipid membranes, it is tempting to speculate that the ability of phospholipids to form ripple structures might be of importance for the functional and structural behavior of the lipid membrane part of cell membranes with locally ordered and differentiated lipid regions [38]. Interestingly, it has recently been demonstrated that PLA2 displays an increased activity towards the undulated ripple phase compared to the rather flat and non-corrugated gel phase [39].

In a broader perspective, the results presented above may have implications for a deeper understanding of the regulation of enzymatic activity via a modification of the structural phase behavior and physical properties of the lipid membrane part of biological membranes. Furthermore, a fundamental understanding of the influence of phospholipid composition on the functional biomaterial properties of lipid membranes is of relevance for a rational modification and optimization of liposomal drug carrier systems that can undergo a triggered activation [40–42] at diseased sites with high levels of PLA2 [29,30]. The unnatural phospholipids, which we have designed as prodrug lipids of anticancer lysolipids [27,28] can in addition be used as suitable building blocks to create novel liposomal prodrug and drug delivery systems that can undergo a PLA2 mediated degradation and activation [29,30].

Acknowledgements

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