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Biochimica et Biophysica Acta 1609 (2003) 95–101

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Secreted phospholipase A_2 as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue

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Received 15 July 2002; received in revised form 23 October 2002; accepted 5 November 2002

Abstract

Polymer-coated liposomes can act as versatile drug-delivery systems due to long vascular circulation time and passive targeting by leaky blood vessels in diseased tissue. We present an experimental model system illustrating a new principle for improved and programmable drug-delivery, which takes advantage of an elevated activity of secretory phospholipase A_2 (PLA_2) at the diseased target tissue. The secretory PLA_2 hydrolyses a lipid-based proenhancer in the carrier liposome, producing lyso-phospholipids and free fatty acids, which are shown in a synergistic way to lead to enhanced liposome destabilization and drug release at the same time as the permeability of the target membrane is enhanced. Moreover, the proposed system can be made thermosensitive and offers a rational way for developing smart liposome-based drug delivery systems. This can be achieved by incorporating specific lipid-based proenhancers or prodestabilisers into the liposome carrier, which automatically becomes activated by PLA_2 only at the diseased target sites, such as inflamed or cancerous tissue.
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Keywords: Drug delivery; Liposome; Phospholipase A_2; Permeability; Membrane; Surfactant; Enhancer; Lyso-phospholipid; Fatty acid

1. Introduction

Drug-delivery systems based on liposomal carriers in the 100-nm range [1,2] are one of the modern microcarrier therapeutic systems that holds a promise for coming close to realising Paul Erlich's early vision of a 'magic bullet' for treatment of diseases [3]. Liposomes made of biocompatible, nontoxic phospholipids provide a system for efficient formulation and encapsulation of toxic drugs that can effectively evade the immune system [4]. The drug assumes the altered pharmacokinetics of the liposomal carrier and can in principle be targeted to the diseased tissue by using a combination of physicochemical and pathophysiological factors at the sites of the liposome carrier and the target membrane, respectively. Liposomes incorporated with lipopolymers, such as polyethylene glycol (PEG)-lipids, known as 'Stealth' liposomes [1], display an improved stability in the vascular system, possibly due to steric protection caused by the polymer coating. The prolonged circulation time of these liposomes, combined with increased vascular porosity of diseased tissue [5], has formed the basis for positive clinical results for specific systems, including anticancer drugs like doxorubicin [6] as well as antibacterial [7] and anti-inflammatory [8] drugs.

Liposomes are self-assembled lipid systems and their stability is therefore, to a large extent, controlled by non-specific physical interactions. Insight into the molecular control of the physical properties of liposomes is therefore important for manipulating and tailoring the liposomal

Abbreviations: PEG, polyethylene-glycol; PLA_2, phospholipase A_2; 1,2-di-*O*-SPC, 1,2-*O*-octadecyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-hexadecanoyl-*sn*-glycero-3-phosphocholine; DCPC, 1,2-capryl-*sn*-glycero-3-phosphocholine; DPPE-PEG_{2000}, 1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; DPPE-PEG_{350}, 1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-350]; lysoPPC, 1-hexadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; PA, palmitic acid; 1-*O*-DPPC, 1-*O*-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine; 1-*O*-DPPE-PEG_{350}, 1-*O*-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-350]; bis-py-DPC, 1,2-bis-(1-pyrene-decanoyl)-*sn*-glycero-3-phosphocholine; MLV, multilamellar vesicles; LUV, large unilamellar liposomes

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PII: S0005-2736(02)00659-4

properties in relation to specific drug-delivery purposes. As an example, the thermally induced gel–fluid lipid transition has been exploited [9] and optimized [10] to design systems for enhanced release of drugs due to hyperthermia. Recently, it has been suggested that specific peptide-linked lipids incorporated into the carrier liposome can be turned into fusogenic agents by the action of proteases in the tumour thereby promoting the intracellular drug transport [11]. It would be desirable if an intelligent and versatile drug-delivery system could be designed which has built in a dual virtual trigger mechanism of simultaneous (i) enhanced drug release selectively at the target tissue and (ii) enhanced transport of the drug into the diseased cells.

In the present study, we propose a new principle for liposomal targeting to diseased regions that assumes elevated activity of secreted phospholipase A_2 (PLA_2) at the diseased sites as is the case in inflamed [12–16] and cancerous tissue where the level of secretory PLA_2 are often enhanced manyfold over normal levels [17–20]. Upon exposure to PLA_2, the phospholipids of the PEG-liposomes have been shown to suffer enhanced hydrolysis compared to conventional bare liposomes [21,22]. This leads to destabilisation of the PEG-liposome and enhanced release of the encapsulated drug. The hydrolysis products, lyso-phospholipids and free fatty acids, act in turn as absorption enhancers for drug permeation across the target membrane.

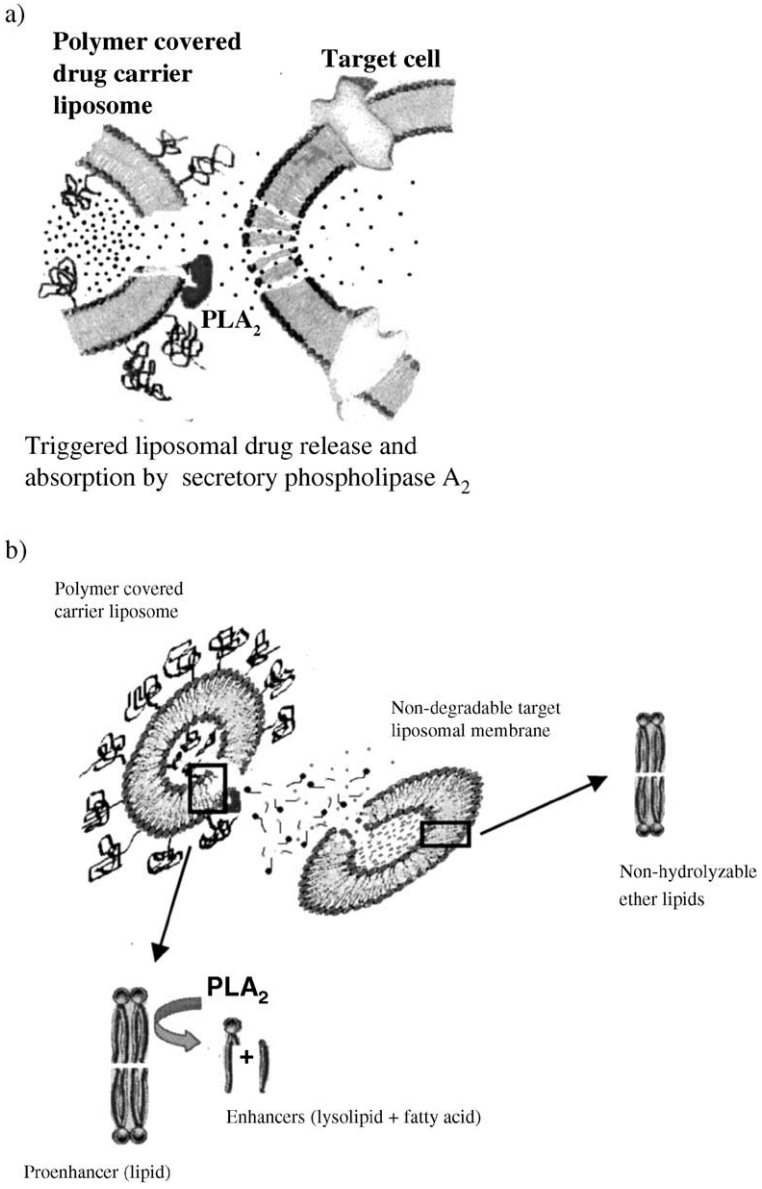

Fig. 1. (a) Schematic illustration of the new liposomal drug-targeting principle involving accumulation of liposomal drug carriers in porous diseased tissue and subsequent drug release and transport across the target membrane via endogenous PLA_2 activity. (b) Schematic illustration of a molecular-based biophysical model system where the phospholipids of the carrier liposome, via the PLA_2-catalysed hydrolysis, act as prodestabilisers at the site of the carrier and as proenhancers at the site of the target.

The phospholipids of the carrier liposome thereby behave as prodestabilisers at the site of the carrier and as proenhancers at the site of the target membrane. The new principle for localized liposomal drug release and absorption that is based on elevated levels of PLA_2 in the diseased tissue is illustrated schematically in Fig. 1a.

In order to substantiate and support the novel idea of localized liposomal drug release and absorption by means of elevated levels of PLA_2, we have developed a simple and operative experimental biophysical model system that sustains such a dual mechanism to be triggered at the pathological target sites. The experimental model system consists of a polymer-coated liposome carrier and a model target membrane. Molecular details of the experimental model system are illustrated schematically in Fig. 1b. The carrier is a 100-nm unilamellar liposome made of 1,2-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) lipids incorporated with 1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DPPE-PEG_{2000}). The target membrane is another liposome made of 1,2-*O*-octadecyl-*sn*-glycero-3-phosphocholine (1,2-di-*O*-SPC), which is a phospholipid where the acyl linkages of the stearoyl chains are ether bonds. In contrast to DPPC, 1,2-di-*O*-SPC is inert towards PLA_2-catalysed hydrolysis thereby mimicking the stability of a target cell membrane towards degradation by its own enzymes. This experimental assay, which permits simultaneous as well as separate investigation of the effect of destabilisers at the carrier liposomes and the effect of enhancers at the target membrane, involves entrapment of a water-soluble fluorescent calcein model drug, in a self-quenching concentration, in the interior of the nonhydrolysable target liposome, rather than in the carrier liposome. The enhanced level of PLA_2 at the target membrane can then be simulated by adding PLA_2 to initiate the hydrolytic reaction in a suspension of the carrier and target liposomes. The permeation of calcein across the 1,2-di-*O*-SPC target membrane is subsequently monitored by the increase in fluorescence. In order to investigate the effect of the presence of the PEG-lipids in the carrier liposome, a similar experiment was performed with conventional bare DPPC liposomes. Furthermore, in order to compare and discriminate the permeability-enhancing effect of lyso-phospholipids from that of free fatty acids, experiments without enzymes were carried out where 1-hexadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysoPPC) and palmitic acid (PA) were added simultaneously or separately to the target liposomes.

2. Materials and methods

2.1. Materials

The lipids, 1,2-di-*O*-SPC, DPPC, 1,2-capryl-*sn*-glycero-3-phosphocholine (DCPC) and 1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol) 2000] (DPPE-PEG_{2000}), 1,2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine *N*-[methoxy(polyethylene glycol)-350] (DPPE-PEG_{350}), lysoPPC and PA were purchased from Avanti Polar Lipids (Alabaster, AL). 1-*O*-hexadecyl-2-hexadecanoyl-sn-glycero-3-phosphocholine (1-*O*-DPPC) and 1-*O*-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-350] (1-*O*-DPPE-PEG_{350}) were synthesized in our laboratory. PLA_2 hydrolysis of the 1-*O*-DPPC and 1-*O*-DPPE-PEG_{350} monoether lipids leads to the release of etherlysolipids, which are nondegradable by, e.g. lysophospholipases rendering the generated lysolipid permeability enhancers more stable in biological fluids. Calcein (2,4-bis-(*N*,*N*′-di(carboxymethyl)aminomethyl)-fluorescin) was purchased from ICN Biochemicals (Costa Mesa, CA) and Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden). 1,2-bis-(1-pyrene-decanoyl)-*sn*-glycero-3-phosphocholine (bis-py-DPC) was from Molecular Probes (Eugene, OR). All chemicals were used without further purification. Purified snake venom PLA_2 (*Agkistrodon piscivorus piscivorus*) [23] was a generous gift from Dr. R.L. Biltonen. This PLA_2 enzyme belongs to the class of low molecular weight, 14 kDa secretory enzymes that display structural similarity to human secretory PLA_2 [24–26].

2.2. Preparation of target liposomes containing entrapped calcein

Multilamellar (MLV) target liposomes were made by dispersing a weighed amount of lipid 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) containing the water-soluble marker, calcein, in a self-quenching concentration (20 mM). Calcein was dissolved in water and pH were adjusted with NaOH to pH = 7.5 prior to adding the calcein solution to the 10 mM HEPES buffer. The lipid suspension was kept at a temperature 10 °C above the main phase transition temperature of 1,2-di-*O*-SPC (T_m = 55.5 °C) for 1 h in order to ensure complete hydration. During this period, the lipid suspension was vortexed every 15 min. The multilamellar liposomes were extruded 10 times through two stacked 100 nm pore size polycarbonate filters forming large unilamellar liposomes (LUV) with a narrow size distribution [27]. Untrapped calcein was removed from the liposome suspension by gel filtration through a column packed with Sephadex G-50 using a HEPES buffer solution (10 mM HEPES, 150 mM KCl, 1 mM NaN_3, 30 μM $CaCl_2$, 10 μM NaEDTA, pH 7.5) as eluent. The lipid concentration was determined by HPLC, and the samples were diluted with eluent to the final lipid concentration ensuring a calcein concentration of less than 1 μM after release of all the calcein from the liposomes. Our standard curve for calcein showed that the fluorescence intensity of calcein displays a linear dependency on concentrations up to 1 μM. The high concentration (20 mM) of the encapsulated calcein led to self-quenching of

its fluorescence, resulting in low background fluorescence intensity from the liposomes. Control experiments showed that the calcein fluorescence was unaffected by the presence of PA and lysoPPC at concentrations used in the experiments.

2.3. Preparation of carrier liposomes

Weighed amounts of lipids were dissolved in chloroform. The solvent was removed by a gentle stream of N_2. The lipid films were then dried overnight under low pressure to remove trace amounts of solvent. Multilamellar vesicles were made by dispersing the dried lipids in a buffer solution containing: 150 mM KCl, 10 mM HEPES, 1 mM NaN_3, 30 μM $CaCl_2$ and 10 μM EDTA, pH = 7.5. The multilamellar vesicles were extruded 10 times through two stacked 100 nm pore size polycarbonate filters as described above.

2.4. Calcein release measurements

The permeability-enhancing effect of PLA_2-catalysed hydrolysis products were measured by incubating 100 nm unilamellar carrier liposomes composed of DPPC incorporated with DPPE-PEG_{2000} or DCPC, together with calcein-containing target liposomes in a HEPES buffer with pH = 7.5 at 37 °C. The snake venom PLA_2 (*A. piscivorus piscivorus*) was added at time $t = 0$ in order to initiate the hydrolytic reaction. As calcein is released from the 1,2-di-*O*-SPC target liposomes, a linear increase in the fluorescence at 520 nm after excitation at 492 nm is observed when calcein is diluted into the surrounding buffer. Fluorescence was measured using a SLM DMX-1100 spectrofluorometer. The time-dependent release of calcein from the 1,2-di-*O*-SPC target liposomes was calculated as: $\%R(t) = 100\ (I_{F(t)} - I_B)/(I_{tot} - I_B)$, where $I_{F(t)}$ is the measured fluorescence at time t after addition of the enzyme, I_B is the background fluorescence, and I_{tot} is total fluorescence, measured after addition of Triton X-100 and complete release of calcein.

In order to discriminate the permeability-enhancing effect of lysoPPC from that of PA, experiments were carried out where lysoPPC and PA were added separately and as an equimolar mixture to the 1,2-di-*O*-SPC target liposomes. The permeability enhancers were added to the target liposomes under vigorous stirring to obtain final global concentrations of 10.0 μM corresponding to 50.0 mol% of the 1,2-di-*O*-SPC concentration. LysoPPC was added directly from a 1 mM aqueous stock solution containing 20% methanol, and PA was added from a 5 mM 100% methanol stock solution. The equimolar mixture was added from a 1 mM 20% methanol stock solution. The final concentration of methanol in the experimental samples did not exceed 1% (v/v). Control experiments showed that methanol by itself did not cause any significant calcein release.

2.5. PLA_2 from rat peritoneal inflammatory exudate

Cell-free peritoneal fluid from rat with casein-induced acute inflammation was prepared by injecting 5 ml 1% sodium caseinate into the peritoneal cavity of a SPRD male rat weighing 250–260 g. The rat was sacrificed after 24 h and the inflammatory fluid was collected from the peritoneum and centrifuged at $1500 \times g$ for 20 min in order to obtain a cell-free peritoneal fluid [28].

2.6. PLA_2 bis-py-DPC assay

Unilamellar liposomes with a narrow size distribution were prepared from 89 mol% DPPC, 10 mol% DPPE-PEG_{350} and 1 mol% bis-py-DPC. Bis-py-DPC is a PLA_2 substrate with two adjacent pyrene fluorophores that form dimers (excimers) emitting at 470 nm upon excitation at 342 nm. PLA_2 catalysed lipid hydrolysis separates the two fluorophores resulting in an increased monomer emission at 380 nm [29].

3. Results and discussion

3.1. Triggered liposomal release and absorption by PLA_2

The results in Fig. 2a show the release of calcein as a function of time after adding PLA_2 to the system. The reaction time course of the particular PLA_2 used has a characteristic lag-burst behavior with a so-called lag time, which can be used conveniently as a measure of the enzymatic activity [30]. A dramatic decrease in the lag time and a concomitant enhancement of the rate of release are observed when the carrier liposomes contain the lipopolymers, DPPE-PEG_{2000}, in accordance with previous findings of enhanced PLA_2 degradation of polymer-coated liposomes [21,22]. The calcein release results suggest that the products of the PLA_2-catalysed hydrolysis of the DPPC lipids of the carrier, lysoPPC and PA, which are produced in a 1:1 ratio, are incorporated into the target membrane [31], leading to a large increase in membrane permeability. These products, which have very low water solubility, are known, due to their noncylindrical molecular shapes, to induce a curvature stress field in the membrane [24,32] or small-scale lateral phase separation [23], which induce membrane defects and increased permeability. This is substantiated by the data in Fig. 3 which show that the addition of lyso-phospholipid or fatty acid separately to the present target system, in the absence of PLA_2, leads to an increased rate of calcein release across the target membrane. However, the crucial effect is that if lysoPPC and PA are added simultaneously in a 1:1 mixture, a dramatic enhancement in the rate of release is observed as shown in Fig. 3 [33]. This strongly suggests that the two enhancers act in a synergistic fashion, thereby highlighting the unique possibility in exploiting PLA_2-catalysed hydrolysis for combined destabilisation of the

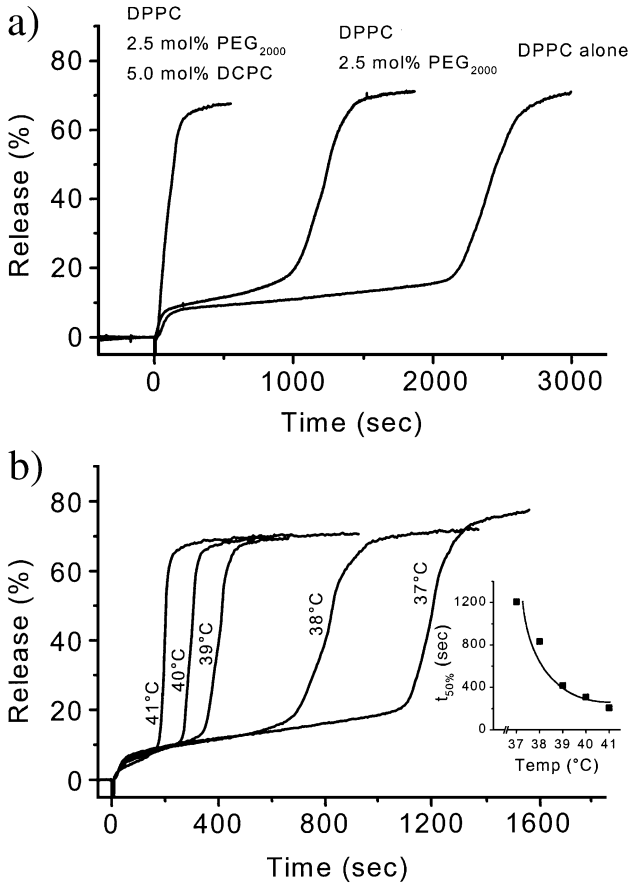

Fig. 2. (a) PLA_2-controlled release of the fluorescent model drug calcein across the 1,2-di-*O*-SPC target membrane as a function of time for different compositions of the carrier liposome. The temperature is 37 °C. In comparison with bare DPPC carriers, the rate of release of the model drug is dramatically enhanced for the polymer-coated carriers, DPPC + 2.5 mol% DPPE-PEG_{2000}. A further augmentation of the rate of release is obtained if the carrier also contains a short-chain phospholipid, DCPC, which acts as a local activator for the enzyme. (b) PLA_2-controlled release of the fluorescent model drug calcein across the target membrane as a function of time for different temperatures. As the temperature is raised, the rate of release is enhanced due to increased activity of the enzyme induced by structural changes in the lipid bilayer substrate of the carrier liposome, which consists of DPPC + 2.5 mol% DPPE-PEG_{2000}. In the present assay, a maximum release of about 70% is achieved in all cases. The insert shows the time of 50% calcein release, $t_{50\%}$, as a function of temperature.

carrier liposome and enhancement of drug transport across the target membrane. The synergistic effect is further augmented by the fact that PLA_2 is activated by its own hydrolysis products [23,32], revealing the degradable phospholipids of the carrier liposome as a kind of proactivators.

It should be pointed out that the effect in the present drug-delivery model system of using lipids as proenhancers and prodestabilisers via PLA_2 activity is dynamic and refers to an intrinsic time scale. This time scale is the effective retention time of the carrier liposomes near the target membrane. The more rapidly the enzyme is activated, the faster is the drug release and the larger the drug absorption during the time which the carrier spends near the target. Furthermore, the faster the enzyme works, the more readily it becomes available for hydrolysis of other drug-carrying liposomes that approach the diseased target site.

Once it has been established that PLA_2 activity can be used to control drug release, several rational ways open up for intelligent improvements of the proposed drug-delivery system via use of well-known mechanisms of altering PLA_2 activity by manipulating the physical properties of the lipid bilayer [30,34]. Hence the strategy is to modify certain physical properties of the carrier liposomes without significantly changing their vascular circulation time. We shall illustrate this general principle by demonstrating the effects of both a physicochemical factor, the lipid composition of the carrier, and an environmental (thermodynamic) factor, the local temperature at the target site.

3.2. Effect of lipid composition on liposomal release and absorption by PLA_2

Short-chain phospholipids, such as DCPC are known to activate PLA_2 [35]. The effect on calcein permeation across the target membranes induced by incorporation of a small amount of DCPC into the carrier PEG-liposomes is also shown in Fig. 2a. The release is very fast due to an almost instantaneous activation of the enzyme. We have furthermore found that PLA_2 becomes deactivated (data not shown) when a large amount of cholesterol (>20 mol%) is incorporated into PEG-liposomes. These significant findings are of particular interest since the blood circulation time of PEG-liposomes has been reported to be almost the same without cholesterol as with large amounts of cholesterol [36].

3.3. Effect of temperature on liposomal release and absorption by PLA_2

Temperature is known to have a dramatic and highly nonlinear effect on PLA_2 activation in the region of the gel–fluid phase transition of saturated phospholipid bilayers

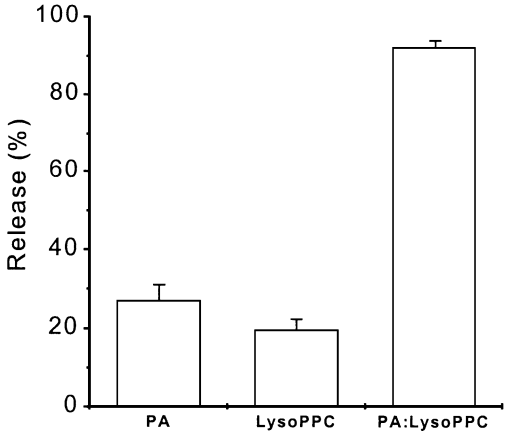

Fig. 3. Total release of calcein across the target membrane 20 min after the addition of 10 μM PA and lysoPPC, separately, and as an equimolar mixture. The concentration of the 1,2-di-*O*-SPC target membranes was 20 μM in a HEPES buffer with pH = 7.5 at a temperature of 39 °C.

[30,37]. This effect is not caused by changes in the enzyme but by dramatic lateral structural changes in the lipid bilayer [38]. It is possible to take advantage of this effect in the present drug-delivery system as suggested by the data in Fig. 2b. As the temperature approaches the main phase transition temperature at 41 °C, the rate of calcein release is progressively enhanced as quantified by the time of 50% calcein release, $t_{50\%}$, shown in the insert to Fig. 2b. It has previously been suggested that hypertermia could be exploited to enhance drug release, and that local heating at predefined tumour areas could be used to locally destabilise drug-carrying liposomes, by exploiting the enhanced leakiness of liposomes at their phase transition [9].

3.4. Liposomal release and absorption as a function of PLA_2 concentration

The release of calcein entrapped in carrier liposomes composed of 1-*O*-DPPC incorporated with 10 mol% 1-*O*-DPPE-PEG_{350} was measured as a function of enzyme concentration. The results in Fig. 4 demonstrate that rapid release, within minutes, can be obtained even at 1 nM PLA_2 (~ 14 ng/ml). Interestingly, the PLA_2 concentrations found in effusions from patients with various cancer types were in the range from 9 to 188 ng/ml [17]. It should be noted that PLA_2 is able to hydrolyse the 1-*O*-DPPC and 1-*O*-DPPE-PEG_{350} monoether lipids leading to the release of lysolipids that are stable against hydrolysis by, e.g. lysophospholipases. The released etherlysolipids are expected to display an increased in vivo stability thereby having the potential to induce a more pronounced permeability enhancing effect in the diseased tissue.

3.5. Activity of PLA_2 from inflammatory exudate

Finally, it was investigated whether DPPC drug carrier liposomes incorporated with 10 mol% DPPE-PEG_{350} were

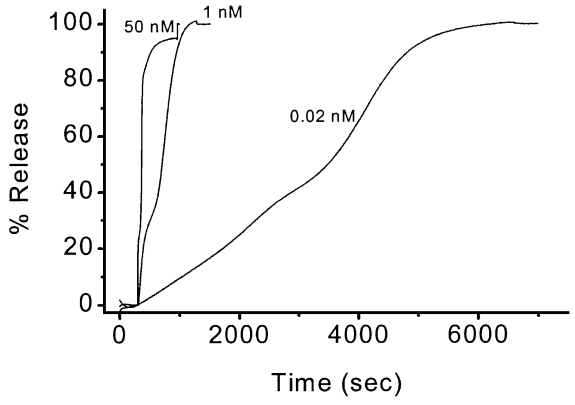

Fig. 4. PLA_2-controlled release of calcein from liposomes composed of 25 μM 90 mol% 1-*O*-DPPC and 10 mol% 1-*O*-DPPE-PEG_{350} suspended in a 10 mM HEPES-buffer (pH = 7.5), as a function of time. Decreasing concentrations of PLA_2 (*A. piscivorus piscivorus*) was added at time 300 s. The temperature was 35.5 °C.

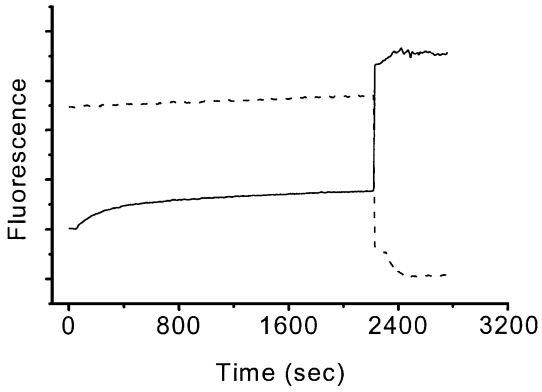

Fig. 5. Characteristic reaction time profile at 38.5 °C for rat phospholipase catalyzed hydrolysis of DPPC liposomes incorporated with 10 mol% DPPE-PEG_{350}. The catalytic reaction was initiated by adding cell-free peritoneal fluid at $t = 60$ s to 2.5 ml of the thermostated liposome suspension. The hydrolysis reaction is monitored by monomer fluorescence (solid line) and eximer fluorescence (dashed line) from bis-py-DPC.

degraded by mammal secretory PLA_2 obtained from rat peritoneum fluid. In Fig. 5, a sudden increase in monomer fluorescence and a simultaneously decrease in excimer fluorescence is observed approx. 2200 s after adding rat peritoneal fluid to the equilibrated suspension, indicating that bis-py-DPC is hydrolysed by PLA_2 originating from the peritoneal inflammatory fluid [28,29,39]. Interestingly, it has recently been shown that group V PLA_2 and group X PLA_2 are upregulated and secreted by macrophages in inflammatory tissue [12,16], and that these two enzymes display a high hydrolyzing activity against zwitterionic phosphatidylcholine lipids as it is reflected by the results shown in Fig. 5.

4. Conclusion

Polymer-grafted liposomes are known to accumulate in diseased tissue with leaky capillaries due to a long vascular circulation time. The results reported in the present paper suggest that polymer-grafted liposomal drug carriers can also be constructed to become susceptible to PLA_2 catalysed degradation in the diseased tissue, which is characterised by increased levels of PLA_2. Through this, it is possible to achieve a remote and site-specific release and absorption of the encapsulated drugs specifically at the diseased target site. This new principle for targeted liposomal drug delivery to diseased tissue implies that the PLA_2 controlled release will be enhanced where both temperature and PLA_2 concentration are elevated, e.g. in inflamed tissue, independent of the size of the diseased region and without requiring a preceding localisation of the diseased tissue. This is in contrast with the case where the thermic effect can only be achieved by a local temperature increase using external heating sources at a predetermined tumour site of some minimal size [10].

Acknowledgements

This work was supported by the Hasselblad Foundation and by the Medical Research Council via the Centre for Drug Design and Transport. MEMPHYS Center for Biomembrane Physics is supported by the Danish National Research Foundation.

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