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Synergistic permeability enhancing effect of lysophospholipids and fatty acids on lipid membranes

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Abstract

The permeability-enhancing effects of the two surfactants, 1-palmitoyl-2-lyso-*sn*-gycero-3-phosphocholine (lysoPPC) and palmitic acid (PA), on lipid membranes that at physiological temperatures are in the gel, fluid, and liquid-ordered phases were determined using the concentration-dependent self-quenching properties of the hydrophilic marker, calcein. Adding lysoPPC to lipid membranes in the gel-phase induced a time-dependent calcein release curve that can be described by the sum of two exponentials, whereas PA induces a considerably more complex release curve. However, when lysoPPC and PA were added simultaneously in equimolar concentrations, a dramatic synergistic permeability-enhancing effect was observed. In contrast, when both lysoPPC and PA are added to liposomal membranes that are in the fluid or liquid-ordered phases, no effect on the transmembrane permeation of calcein was observed.

Keywords: Drug-delivery; Permeability; Membrane; Surfactant; Enhancer; Lysophospholipid; Fatty acid; Liposome

1. Introduction

The lipid bilayer part of biological membranes is composed of a large variety of different types of phospholipids, sphingolipids, and cholesterol [1–3]. In addition, small concentrations of water-soluble single chain surfactants, e.g. lysophospholipids and fatty acids (FA) are found in the lipid membrane. The transmembrane barrier properties of the lipid membrane are of key interest in drug research in relation to penetration and permeation of drugs across cell membranes [4]. For example, the permeation and transport of new macromolecular drugs as well as small molecular drugs across cell membranes are of crucial importance for the ability of the drugs to reach their intracellular target sites [4]. The major determinants of the transmembrane permeability are the microscopic and macroscopic phase behavior of the lipid membrane, which is affected by the membrane composition and external thermodynamic conditions such as temperature, degree of hydration, and ionic strength [5,6]. It is, however, possible to enhance the transmembrane permeability of the lipid membranes by adding surfactants in the vicinity of the membrane and thereby increase the transport of drugs across the membrane. FA and in particular lysophosphatidylcholines (lysoPC), which can be produced by enzymatic hydrolysis of phosphatidylcholine, are examples of surfactants that can act to lower the passive transmembrane barrier properties of biological membranes [7]. Several studies have shown that the incorporation of small concentrations of lysoPCs or FAs into red blood cells or well-defined vesicle membranes enhances the transmembrane permeability [8–15]. However, only a limited number of studies have in a systematic way dealt with the combined effects of the simultaneous incorporation of lysoPC and FA on the physical properties of lipid membranes [9].

Exogenously added lysoPCs are preferentially localized in the outside monolayer of cellular or vesicular membranes, and movement of lysoPCs across the lipid membrane (flip-flop) is extremely slow as compared to the flip-flop of FAs [16–18]. Incorporation of lysoPCs or FAs into the lipid membrane can lead to the formation of small-scale phase separated structures, and it has been suggested that the observed increase in the transmembrane permeability is intimately related to the formation of a heterogeneous local

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membrane structure composed of coexisting small-scale lipid structures with leaky boundaries [4]. The disruptive and permeability-enhancing properties of lysoPCs and FAs depend to some extent on the length and degree of saturation of the attached single hydrocarbon chain [7]. This can be understood in terms of the nonbilayer forming molecular shape of, e.g. lysoPCs, which effectively can be described by the inherent imbalance between volume and cross-sectional area of the polar head-group region and the nonpolar hydrocarbon chain region. The presence of other lipid components that have a molecular shape opposite to that of lysoPC, for example FA and cholesterol, can counteract the lipid membrane destabilizing effects of lysoPCs [19–21].

In the present study, we have investigated the permeability enhancing effect of 1-palmitoyl-2-lyso-*sn*-gycero-3-phosphocholine (lysoPPC) and palmitic acid (PA) on membranes that at physiological temperatures are in the solid-ordered (gel), liquid-disordered (fluid), and liquid-ordered phases, using the self-quenching properties of a hydrophilic fluorescent calcein marker. The di-ether-1,2-SPC phospholipids (1,2-*O*-distearoyl-*sn*-glycero-3-phosphocholine) composing the gel (solid-ordered) phase are characterized by conformationally ordered acyl chains and a low lateral mobility of the lipids in the membrane. The fluid-phase membranes were made of SOPC phospholipids (1-steroyl-2-oleyl-*sn*-glycero-3-phosphocholine) having an unsaturated hydrocarbon chain in the *sn*-2 position. The SOPC lipids in the fluid-phase membranes have a high degree of lateral mobility and the hydrocarbon chains are much more flexible and conformationally disordered than the chains in the gel-phase di-ether-1,2-SPC membranes. Finally, the membranes in the liquid-ordered phase were made of a mixture of SOPC and cholesterol. The SOPC lipids in these membranes have a high degree of lateral mobility but the hydrocarbon chains are conformationally ordered by the presence of cholesterol [5].

2. Materials and methods

2.1. Materials

The lipids, 1,2-*O*-distearoyl-*sn*-glycero-3-phosphocholine (di-ether-1,2-SPC), 1-steroyl-2-oleyl-*sn*-glycero-3-phosphocholine (SOPC), cholesterol, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysoPPC), and PA were purchased from Avanti Polar Lipids (Alabaster, AL). 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). All chemicals were used without further purification.

2.2. Preparation of liposomes containing entrapped calcein

Multilamellar (MLV) liposomes were made by dispersing a weight 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 were 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 di-ether-1,2-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 unilamellar liposomes (LUV) with a narrow size distribution [22]. 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 a final lipid concentration of 20 μM. This dilution ensured 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 in concentrations up to 1 μM. The high calcein concentration (20 mM) of the encapsulated calcein led to self-quenching of its fluorescence, resulting in low background fluorescence intensity of 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. Calcein release measurements

The permeability enhancing effects of lysoPPC and PA as well as equimolar mixtures of lysoPPC and PA were determined by measuring the amount of entrapped calcein that was released from the liposomes as a function of time, *t*. LysoPPC was added to the liposome suspension directly from a 1 mM aqueous stock solution containing 20% methanol, and PA was added from a 5 mM 100% methanol stock solution. The 1 mM lysoPPC and 1 mM PA equimolar mixture was added from a 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. The fluorescence intensity of calcein in the samples was measured at excitation wavelength of 492 nm and emission wavelength of 520 nm using a SLM DMX-1100 spectrofluorometer. The 90° static light-scattering, which reflects morphological changes of the lipid system [23,24], was measured simultaneously with the fluorescence intensity.

Each liposome sample was equilibrated at 39 °C for 10 min before the background fluorescence intensity (I_B) was measured. The surfactants were added to the liposomes under vigorously stirring to obtain final global concentra-

tions of 2.5, 5.0, or 10.0 μM corresponding to 12.5, 25.0, and 50.0 mol% of the di-ether-1,2-SPC concentration. It is expected that lysoPPC and PA, which are added in concentrations above their cmc, are incorporated rapidly into the bilayer [7]. The enhanced permeability of the lipid membranes was monitored by the time-dependent fluorescence intensity ($I_{F(t)}$) increase, which results from diluting of the encapsulated self-quenching calcein into the surrounding buffer. Total fluorescence (I_{tot}), corresponding to release of all the entrapped calcein from the liposomes, was measured after complete solubilization of the liposomal membranes by adding Triton X-100 to the samples. The time-dependent release of calcein from the di-ether-1,2-SPC liposomes was calculated as: $\%R(t)=100\ (I_{F(t)}-I_B)/(I_{tot}-I_B)$.

The experiments on the di-ether-1,2-SPC gel-phase liposomes were repeated at least three times and the standard deviations are shown on the release curves. Experiments on the di-ether-1,2-SPC liquid-ordered state liposomes with 40 mol% cholesterol and on the fluid-phase SOPC liposomes were repeated twice.

3. Results and discussion

Typical measurements of the time-dependent calcein permeation through di-ether-1,2-SPC lipid membranes in the gel-phase, monitored by the fluorescence intensity increase of calcein released from the interior of the liposomes into the aqueous bulk are shown in Fig. 1 (solid lines). Also shown is the 90° static light scattering, which reflects morphological changes in the lipid system (dashed lines). The solid line in Fig. 1A shows that lysoPPC added to the calcein containing di-ether-1,2-SPC liposomes at time zero results in a fast initial increase in the permeation of calcein across the liposomal membranes. This is clearly seen as an abrupt increase in the fluorescence intensity, $I_{F(t)}$, compared to the background level, I_B. The initial fast increase is followed by a second stage where the transmembrane permeability decreases and the release curve reaches a level of saturation. The total fluorescence intensity, I_{tot}, which corresponds to the release of all the entrapped calcein, was determined by complete solubilization of the liposomes by Triton X-100. The time course of the increase in the transmembrane permeability induced by PA is shown in Fig. 1B. The initial increase in the transmembrane permeability induced by PA is slower than that induced by lysoPPC. However, the transmembrane permeability slowly increases and after about 600 s, the total amount of calcein released by PA is actually higher than the total amount released by lysoPPC.

In order to investigate the combined effect of lysoPPC and PA, the two surfactants were added simultaneously from an equimolar mixture to the calcein containing gel-phase di-ether-1,2-SPC liposomes. Fig. 1C shows that the simultaneous addition of lysoPPC and PA induces a fast and large increase in the transmembrane permeability, resulting in the release of almost all the encapsulated calcein. The data in Fig. 1C strongly indicates that PA and lysoPPC act in a synergistic fashion to increase the permeability of the gel-phase di-ether-1,2-SPC membranes. A similar fast increase in the transmembrane permeability was observed when the liposomes were added to a buffer solution containing the same global concentration of the two surfactants. Therefore, it can be excluded that the unexpected fast increase is a response to high local concentrations of surfactants in the immediate surroundings of a minor population of the di-ether-1,2-SPC liposomes.

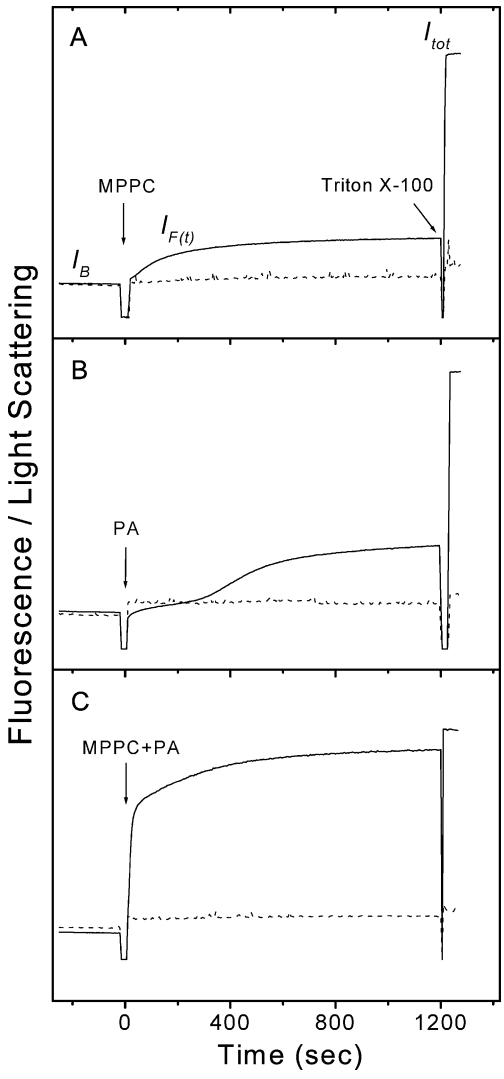

Fig. 1. Calcein released from 20 μM di-ether-1,2-SPC liposomes in HEPES buffer at 39 °C, induced by adding (A) 10 μM lysoPPC, (B) 10 μM PA, and (C) 10 μM mixture of PA and lysoPPC (1:1). I_B is the background fluorescence intensity from self-quenched calcein loaded in the di-ether-1,2-SPC liposomes, $I_{F(t)}$ is the fluorescence intensity versus time after adding the surfactant, and I_{tot} is the total fluorescence intensity after adding triton X-100. The dashed lines in (A) through (C) represent the 90° static light scattering.

The synergistic effect induced by PA and lysoPPC was studied systematically by adding increasing concentrations of lysoPPC, PA, and equimolar mixtures of the two surfac-

tants to gel-phase di-ether-1,2-SPC liposomes, measuring the transmembrane permeability. Fig. 2A shows the time-dependent calcein release, $R(t)$, induced by adding 2.5 μM lysoPPC, PA, and an 2.5 μM equimolar mixture to 20 μM di-ether-1,2-SPC liposomes. LysoPPC and PA added separately induce a slow increase in the transmembrane permeability, and after 20 min, only 4% to 5% of the encapsulated calcein was released from the di-ether-1,2-SPC liposomes. However, when PA and lysoPPC were added simultaneously, a fast instantaneous increase in the transmembrane permeability was observed, leading to release of around 24% of the encapsulated calcein. Fig. 2B,C shows the time-dependent release of calcein from the liposomes after increasing the concentrations of added surfactants to 5 and 10 μM, corresponding to 25 and 50 mol%, respectively, of the di-ether-1,2-SPC lipid concentration. The simultaneous addition of increasing amounts of the equimolar mixture of PA and lysoPPC induces the release of around 47% and 92% of the encapsulated calcein in 20 min, respectively. In contrast, the addition of the same concentrations of lysoPPC result in the release of only around 10% and 20%, whereas PA induces the release of approximately 18% and 27% of the encapsulated calcein after 20 min. It is interesting to observe that significantly different time-dependent release curves are induced by lysoPPC as compared to those of PA, suggesting that different mechanisms are involved in the permeability increasing effect mediated by PA and lysoPPC.

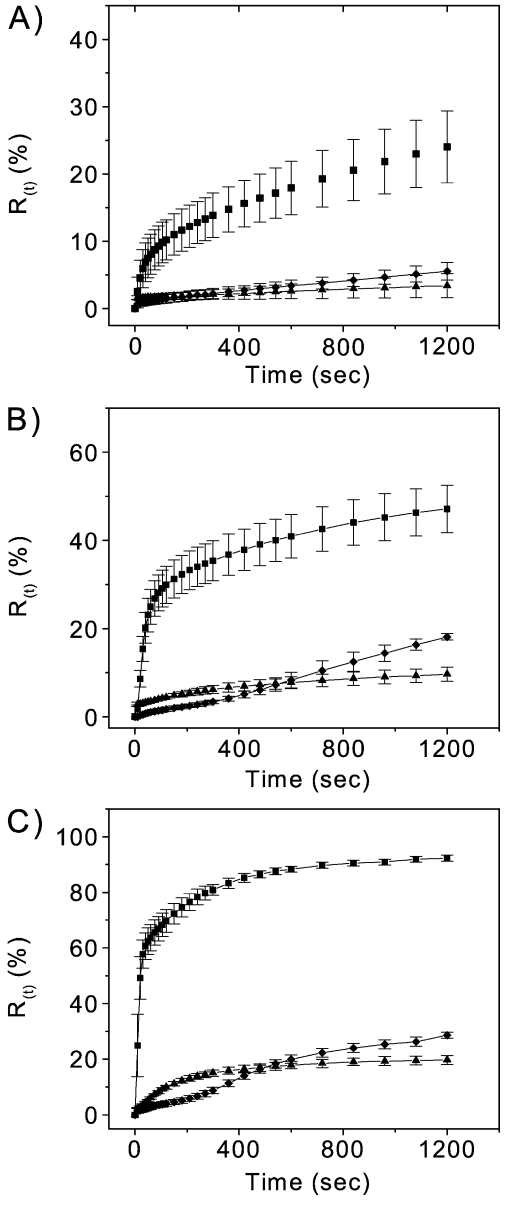

Fig. 2. Percent calcein release from di-ether-1,2-SPC liposomes versus time after adding PA (diamonds), lysoPPC (triangles), and an equimolar mixture of PA and lysoPPC (squares) in 2.5 μM (A), 5.0 μM (B), and 10 μM (C) concentrations, corresponding to 12.5, 25 and 50 mol% of the total di-ether-1,2-SPC lipid concentration. Each measurement was repeated at least three times and the error bars represent the S.D. of the measurements.

The increase in transmembrane permeability induced by lysoPPC can be described by a sum of two exponentials with fast and slow components. The fast component is likely to be dominated by the transfer of lysoPPC from the aqueous bulk into the outer monolayer of the lipid membranes, and by lateral diffusion and rearrangement of lysoPPC in the outer monolayer of the lipid membranes [25]. This might lead to the formation of nonequilibrium small-scale phase separated lipid structures similar to those that earlier have been reported in lipid membrane mixtures [6]. At the same time, transient nanometer-size defects could form whereby the entrapped calcein can leak through the membrane [6,25–27]. The second slow component is thought to be dominated by the slow rate of lysoPPC transfer across the lipid bilayer from the outer to the inner monolayer, thereby reducing the packing constrains and leading to resealing of the membranes [16–18]. Fig. 2C shows that addition of the same concentrations of PA to the di-ether-1,2-SPC liposomes induces a considerably more complex release curve. The difference in the permeability-enhancing effects probably reflects differences in the kinetics of the incorporation of the FAs and lysoPCs in the membranes [7]. However, when PA is added simultaneously with lysoPPC, an unexpected synergistic effect is observed as described above. The general shape of the release curves for the equimolar mixture is similar to that induced by lysoPPC, although the instantaneous increase is much more dramatic. The synergistic effect of PA and lysoPPC on the transmembrane permeability is further demonstrated in Fig. 3, which in a quantitative way shows the percentage of calcein release 20 min after adding increasing concentrations of the surfactants.

It is clear from the data in Figs. 1 and 2 that the barrier properties of the gel-phase di-ether-1,2-SPC lipid membranes are reestablished quite fast after addition of the surfactants. Fig. 4 shows how consecutive additions of surfactants to the same population of di-ether-1,2-SPC liposomes induce a stepwise increase in the transmembrane permeability followed by a resealing of the membranes. However, the second addition of the 2.5 μM equimolar

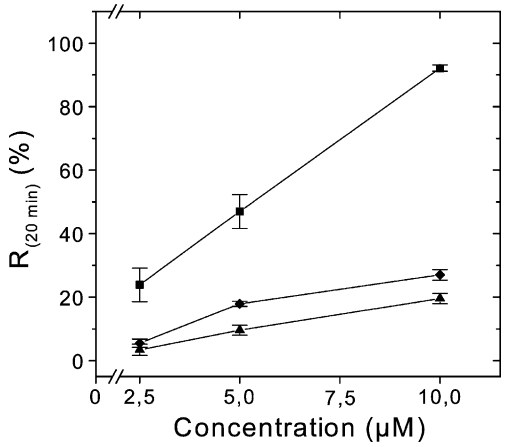

Fig. 3. Percent calcein release after 20 min as a function of increasing concentrations of lysoPPC (triangles), PA (diamonds), and an equimolar mixture of lysoPPC and PA (squares), to 20 μM di-ether-1,2-SPC liposomes at 39 °C.

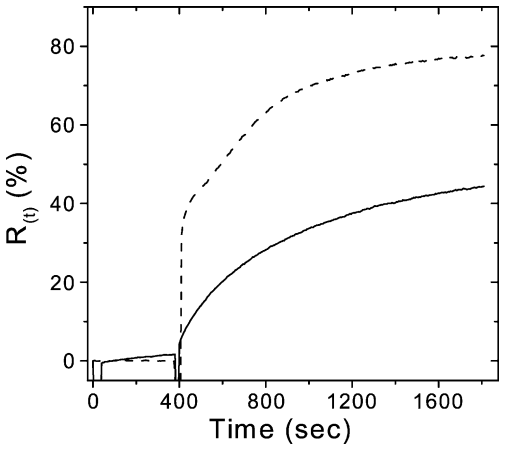

Fig. 5. Addition of 10 μM lysoPPC to 20 μM di-ether-1,2-SPC liposomes preincubated with 2.5 μM PA (dashed line) and addition of 10 μM PA to 20 μM di-ether-1,2-SPC liposomes preincubated with 2.5 μM lysoPPC (solid line).

mixture of PA and lysoPPC leads to a much larger increase in the transmembrane permeability as compared to the initial addition of the 2.5 μM equimolar mixture of lysoPPC and PA. This indicates that the incorporation of PA and lysoPPC from the first addition has changed the physical properties of the membranes, thereby facilitating the further incorporation of lysoPCs and FAs. The third addition induces an increase in the transmembrane permeability that leads to the release of nearly all the calcein remaining in the interior of the liposomes.

The effect of adding PA and lysoPPC on the transmembrane permeability of the di-ether-1,2-SPC lipid membranes was further investigated in experiments where di-ether-1,2-SPC liposomes were preincubated with 2.5 μM PA prior to adding 10 μM lysoPPC. The dashed line in Fig. 5 shows that 10 μM lysoPPC induces a fast increase in the transmembrane permeability of the di-ether-1,2-SPC lipid membranes. Conversely, adding 10 μM PA to di-ether-1,2-SPC liposomes preincubated with 2.5 μM lysoPPC induces a much smaller increase in the transmembrane permeability, as shown by the solid line in Fig. 5. This indicates that the permeability-enhancing effect of lysoPPC is increased by the presence of small concentrations of FAs. This effect can possibly be ascribed to the ability of FA to perturb the dense head-group region of the gel-phase membranes, thereby making space for fast local incorporation of the more bulky lysoPCs [28–30]. The results reported so far demonstrate that PA and lysoPPC can act to enhance the transmembrane permeability of gel-phase membranes. Interestingly, the results show that lysoPPC and PA can act in a synergistic fashion to induce an unexpected fast increase in the transmembrane permeability of gel-phase membranes.

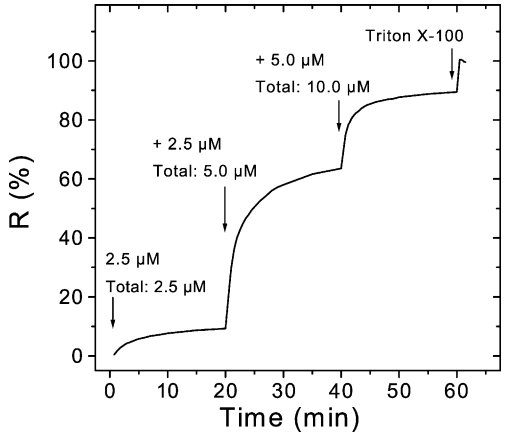

Fig. 4. Percent calcein release versus time. Equimolar mixtures of PA and lysoPPC were added stepwise to a population of 20 μM di-ether-1,2-SPC liposomes at 39 °C.

We have furthermore investigated the permeability-enhancing properties of lysoPPC and PA on membranes in both the fluid- and liquid-ordered phases. The physical properties of these fluid-phase membranes are considerably different than the physical properties of gel-phase membranes [31]. Fig. 6A shows the fluorescence of calcein encapsulated in fluid-phase SOPC liposomes versus time (solid line). Also, the 90° static light scattering from the liposomes versus time are shown (dashed line). Equimolar mixtures of lysoPPC and PA (20 μM) were added as indicated by the arrows. The added PA and lysoPPC have no effect on the transmembrane permeability of the fluid-phase SOPC membranes. This is in contrast to the dramatic permeability-enhancing effect observed on di-ether-1,2-SPC membranes in the gel phase. However, a pronounced increase in the static light scattering from the fluid-phase liposomes is observed, indicating a change in the overall morphology of the liposomes. This is possibly due to aggregation or fusion of the liposomes induced by PA and lysoPPC [23]. Experiments with di-ether-1,2-SPC liposomes incorporated with 40 mol% cholesterol, which is in the liquid-ordered phase [32], yield similar results, although there were no changes in the light scattering (Fig. 6B). The relationship between the permeability-enhancing effect of in

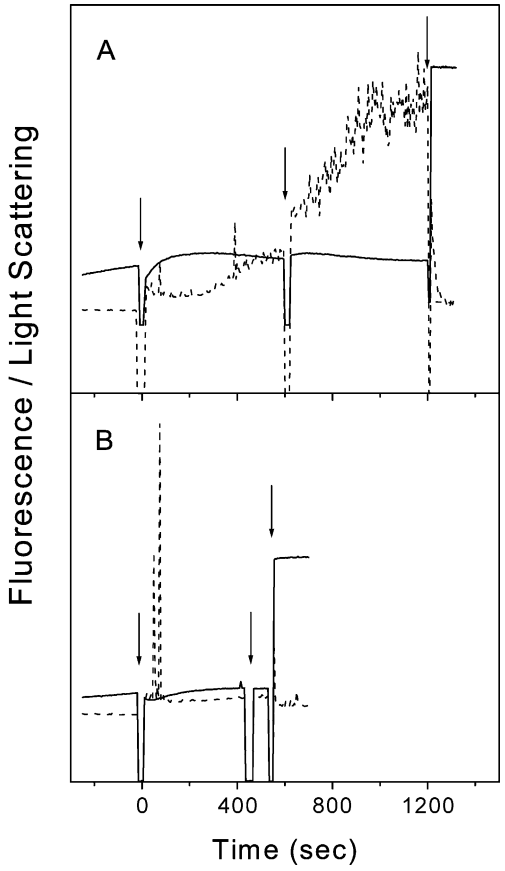

Fig. 6. Consecutive additions of 20 μM equimolar mixture of PA and lysoPPC indicated by the arrows to (A) 20 μM SOPC liposomes and to (B) 20 μM SOPC/cholesterol (60:40) liposomes incubated at 39 °C.

particular lysoPCs and the composition and physical state of lipid membranes has previously been investigated using red blood cells [15,33,34]. These experiments showed that the permeability-enhancing effect of lysoPCs on red blood cells was also drastically increased when the red blood cells were cooled down from a fluid state at physiological temperatures to a gel-like state close to 0–5 °C [15]. The difference in the permeability-enhancing effect of the surfactants on membranes in the gel- and fluid-phases can possibly be explained in terms the lateral diffusion of the lipids, which for gel-phase membranes is at least two orders of magnitude smaller than in the fluid-phase [27]. This probably means that the membranes in the fluid- and liquid-ordered phases reseal on a much faster time scale than the membranes in the gel phase.

4. Conclusion

The permeability-enhancing effect of lysoPPC and PA on membranes in the gel, fluid- and liquid-ordered phases were studied using the self-quenching properties of the hydrophilic fluorescent marker, calcein. The experimental results demonstrate that lysoPPC and PA act in a synergistic fashion to enhance the transmembrane permeability of gel-phase membranes. Furthermore, the data revealed that the time-dependent release curves induced by PA were significantly different from the release curves induced by lysoPPC, suggesting that the mechanisms behind the increased transmembrane permeability are different for the two surfactants. In contrast to the dramatic permeability-enhancing effect of lysoPPC and PA on gel-phase membranes, no changes in the transmembrane permeability of fluid- and liquid-ordered phase membranes were observed. However, a significant increase in the 90° static light scattering from liposomes in the fluid state was observed when lysoPPC and FA were added, possibly due to aggregation or fusion of the liposomes. Apparently, the results of the present study indicate that lysoPCs and free FAs cannot be expected to increase the permeability of the lipid bilayer part of biological membranes, which often is assumed to be in the fluid- or liquid-ordered phase at physiological temperatures [4]. If, however, small domains in the biological membrane are composed of lipids that are in the gel-phase, lysoPCs and free FAs are likely to decrease the barrier properties of those local membrane regions, resulting in an increase in the drug permeation across the target membrane. Furthermore, the synergistic permeability-enhancing effect of lysoPC and FA can advantageously be used to trigger a rapid drug release from drug transporting vehicles composed of gel-phase lipid membranes.

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