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Quenching and dequenching of octadecyl Rhodamine B chloride fluorescence in Ca^{2+}-induced fusion of phosphatidylserine vesicles: effects of poly(ethylene glycol)

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Abstract

Ca^{2+}-induced fusion of SUV and LUV composed of ox brain phosphatidylserine (PS) was studied as a function of temperature and concentration of Ca^{2+} using octadecyl Rhodamine B chloride (R-18). Ca^{2+} was added to a 1:1 mixture of labelled (8 mol%) and unlabelled vesicles (assay conditions) or to samples containing only labelled liposomes (control conditions). Both, in SUV and LUV the dependence of differences in fluorescence between assay and control samples on temperature can be divided into three regions. At temperatures lower than 20°C the differences in fluorescence increase only slightly in SUV or remain unchanged in LUV after the addition of Ca^{2+}. At 28°C and higher temperatures the differences of fluorescence intensities increase much more drastically, whereby SUV exhibit higher fusion rates than LUV. Between 20°C and 28°C exists an intermediate region for both SUV and LUV. Here the fluorescence changes continuously from one behaviour to the other independent of the concentration of Ca^{2+}. A drastic quenching of R-18 fluorescence occurs in LUV composed of PS below 10°C, where the lipids are in the gel state. In SUV the fluorescence is only weakly changed in this temperature region. It is assumed that a demixing between dye and phospholipid molecules occurs below phase transition. During fusion the phase transition of PS is shifted from 8–10°C to about 24–28°C as revealed by polarization measurements using diphenylhexatriene. Because the differences in R-18 fluorescence between assay and control samples depend strongly on temperature we assume that the shift in phase transition temperature of PS occurs immediately after the addition of Ca^{2+} to SUV or LUV. Poly(ethylene glycol) 6000 accelerates fusion in both SUV and LUV under all conditions where a fusion takes place. Further, the threshold concentration of Ca^{2+} to induce fusion is diminished from about 1 mmol/l without polymers to about 0.5 mmol/l in the presence of 10% (w/v) PEG 6000. The intermediate region of changes in fluorescence properties of R-18 in the Ca^{2+}-induced fusion of PS is not changed by PEG.

Key words: Membrane fusion; Phospholipid vesicle; Octadecyl Rhodamine B chloride; Fluorescence quenching; Fluorescence dequenching; Phase transition; Poly(ethylene glycol)

1. Introduction

Ca^{2+}-induced fusion of phosphatidylserine (PS) vesicles is often used to model cellular fusion processes such as the fusion of intracellular vesicles with the plasma membrane during exocytosis [1,2]. Fusion of PS vesicles starts only after formation of 'trans' complexes between Ca^{2+} and PS molecules of different bilayers [3]. Although, Ca^{2+}-PS 'trans' complexes are stable at micromolar concentrations of Ca^{2+} [4,5], they are only generated after the electrostatic repulsion of PS bilayers and the hydration of phospholipid headgroups are overcome using millimolar concentrations of Ca^{2+} [6]. At lower concentrations only 'cis' complexes between PS molecules neighboured in a lipid monolayer and Ca^{2+} are formed and no fusion takes place [7].

Parallel to fusion induced by Ca^{2+} alterations in thermal properties of PS molecules are known to occur. LUV and MLV composed of ox brain PS exhibit a phase transition at 8–10°C [7–9]. This transition point

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Abbreviations: SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; R-18, octadecyl Rhodamine B chloride; DPH, diphenylhexatriene; PS, phosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; PEG, poly(ethylene glycol).

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is shifted moderately to higher temperatures in LUV and MLV due to the formation of Ca^{2+}-PS 'cis' complexes. At the same time the phase transition region is broadened and the DSC peak is flattened [8,9]. On the other hand, nearly anhydrous Ca^{2+}-PS 'trans' complexes prepared from fused material show a phase transition at temperatures higher than 100°C [4,8]. But, the mechanisms how the phase transition characteristics in the Ca^{2+}-PS system are changed immediately after initiation and during the first minutes of fusion remains unknown up to now.

Previously we have used the fluorescent dye octadecyl Rhodamine B chloride (R-18) to detect phase transitions in DMPC and DPPC vesicles [10]. The fluorescence of R-18 increases drastically by heating the samples in the region of phase transition of these lipids as revealed by comparative experiments with DSC. The high quenching of fluorescence in the gel state of lipids was explained by a demixing of R-18 and lipid molecules and an accumulation of dye molecules in so-called gel defect structures [10].

These results have prompt us to examine alterations in phase transitions during Ca^{2+}-induced fusion of PS vesicles using R-18. Usually R-18 is used to study the kinetics in cell-virus fusion at 37°C [11–13]. This assay is based on the dynamical quenching properties of the dye incorporated in membranes. The fluorescence quenching is linearly dependent on the concentration of dye in membranes up to 9 mol% [14]. Membrane systems labelled with R-18 and unlabelled are mixed. Their fluorescence increases after initiating fusion due to dilution of R-18 in the lipid matrix.

In the present paper Ca^{2+}-induced fusion of SUV and LUV composed of PS is studied as a function of temperature and Ca^{2+} concentration using R-18 incorporated in lipid bilayers. Furthermore, effects of poly(ethylene glycol) (PEG) are investigated under these conditions. PEG is often used for lipid vesicle fusion [15]. It lowers the dielectric constant of the membrane surface [16,17], causes a strong aggregation of vesicles [18], and diminishes the threshold concentration of Ca^{2+} to induce fusion in PS vesicles [19].

Changes in R-18 fluorescence after initiation of Ca^{2+}-induced fusion of PS depend strongly on temperature. At temperatures lower than 28°C the results are influenced by a phase separation between dye and phospholipid molecules. The same temperature dependence is observed in the presence of PEG.

2. Materials and methods

2.1. Chemicals

Ox brain phosphatidylserine was obtained from Sigma, Germany, and used without further purification. R-18 was a product of Molecular Probes, U.S. DPH was purchased from Serva, Germany. PEG 6000 was obtained from Fluka, Switzerland. All other chemicals were from Laborchemie Apolda, Germany and used in p.A. quality.

2.2. Liposome preparations

SUV. Lipid films of PS with or without fluorescence compounds were suspended in 0.14 mol/l NaCl, 0.01 mol/l sodium barbital (pH 7.4). This suspension was sonified for 6 min using a Branson sonifier. SUV was diluted to a concentration of 50 nmol PS/ml.

LUV. Large unilamellar vesicles were prepared as reverse phase evaporation liposomes by a modified procedure [20] of the original method [21] and extruded through a polycarbonate filter with a pore diameter of 200 nm using a Lipex Biomembranes (Vancouver, Canada) high-pressure extrusion device. Liposomes suspended in 0.14 mol/l NaCl, 0.01 mol/l sodium barbital (pH 7.4) were diluted to a final concentration of 50 nmol PS/ml.

2.3. Monitoring of fusion processes

Changes in fluorescence intensities of vesicle suspensions labelled with R-18 were followed fluorimetrically using a Perkin-Elmer LS 50 fluorescence spectrophotometer. The samples were measured at 590 nm with an excitation wavelength of 560 nm. Both slits were set at 5 nm. Fusion was monitored using the Time Drive regime of the Fluorescence Data Manager software (Perkin Elmer).

Usually liposomes labelled with R-18 and unlabelled were mixed in a ratio of 1:1. For control purposes only labelled liposomes were used. After the equilibration of samples (volume 3 ml) to a constant temperature the fluorescence was recorded for 15 s and then 100 μl $CaCl_2$ (final concentrations are indicated in the figures) was added. Samples were stirred magnetically during all procedure. In the presence of PEG samples were mixed thoroughly for 5 s after the addition of Ca^{2+}.

2.4. Phase transitions

Phase transitions in PS liposome preparations were detected using the polarization relief of diphenylhexatriene (DPH). SUV composed of PS/DPH (200:1) were used in these experiments. The wavelengths for excitation and emission were set at 360 nm and 430 nm, respectively. Slits widths were 5 nm. The degree of polarization was calculated under consideration of the G-factor according to [22,23]. Polarization values were measured before and 30–60 s after the addition of

Ca^{2+} to samples at different temperatures. All measurements were repeated four times.

3. Results

3.1. Fusion of SUV

First fusion experiments of SUV composed of PS were carried out in the absence of PEG. Vesicles labelled with 8 mol% R-18 were mixed with unlabelled SUV in a ratio of 1:1 and then Ca^{2+} was injected. For control purposes instead unlabelled vesicles the same amount of buffer solution was added. Examples of fluorescence profiles are given as a function of temperature for assay (Figs. 1a–c) and control (Figs. 1d–f) conditions using 1.61, 2.58, and 6.45 mmol/l Ca^{2+} (final concentrations).

The addition of 2.58 mmol/l Ca^{2+} causes a fast increase in fluorescence which remains nearly unchanged during the next two minutes under assay conditions at 36°C. It is assumed that R-18 is diluted due to lateral diffusion in lipid monolayers after fusion with a subsequent dequenching of fluorescence [14]. However, another behaviour is found at lower temperatures using this Ca^{2+} concentration. The fluorescence decreases all the time at 9°C, whereas an intermediate behaviour is observed at 24°C. Fluorescence values in all control samples decrease also after the addition of Ca^{2+}, whereby the most pronounced effect is found at 9°C. It is impossible to explain these changes by a dequenching of fluorescence due to dilution of R-18 during fusion.

Membranes fuse with a lower rate at 36°C using only 1.61 mmol/l Ca^{2+}. There is a continuous increase of fluorescence during all the time. However, after a fast increase of fluorescence it starts to decrease using concentrations of Ca^{2+} higher than 3 mmol/l at 36°C. Examples are given using 6.45 mmol/l. Similar to 2.58 mmol/l Ca^{2+}, the behaviour of fluorescence at lower or higher Ca^{2+} concentrations is drastically changed carring out experiments at 24°C or 9°C.

Fusion events in PS vesicles are complex phenomena including aggregation, multiple membrane fusion steps and final destructions to anhydrous Ca^{2+}-PS complexes [24,25]. In order to get more information about fusion the differences between assay and control curves were calculated (Figs. 1g–i). We assume that aggregation-induced changes in fluorescence, fusion between labelled vesicles, and fusion between unlabelled vesicles can be eliminated by this procedure. Differences should include only information about changes in fluorescence due to fusion between labelled and unlabelled vesicle membranes.

The fluorescence differences obtained for 1.61 mmol/l Ca^{2+} increase continuously all the time at all temperatures investigated. They increase faster using 2.58 mmol/l Ca^{2+} and reach a maximum value 10–20 s after the addition of Ca^{2+}. Then they remain unchanged or decrease slightly. However, difference

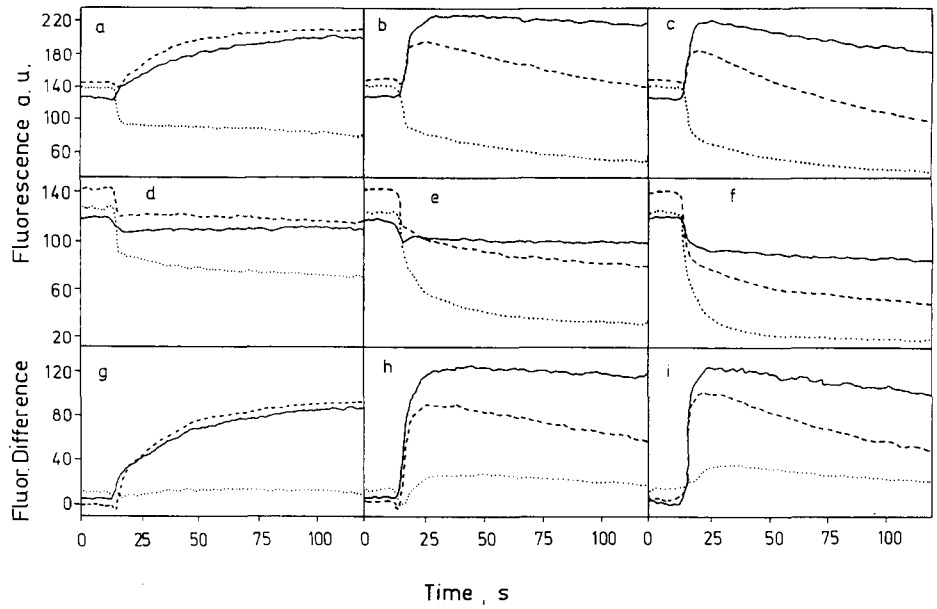

Fig. 1. Changes in fluorescence of SUV composed of PS upon addition of Ca^{2+} as a function of Ca^{2+} concentration and temperature. 100 μl Ca^{2+} was added to a 1:1 mixture of labelled (8 mol%) and unlabelled liposomes (panels a–c) or only labelled vesicles (d–f) 15 s after the start of registration. The final concentrations of Ca^{2+} were 1.61, 2.58, and 6.45 mmol/l, respectively (from left to right). PS was used in a final concentration of 25 nmol/ml in each fraction. Experiments were done at 36°C (unbroken curves), 24°C (dashed curves) or 9°C (dotted curves). In panels g–i the difference curves between fluorescence values obtained under assay and control conditions are given. All measurements were repeated four times. Data were taken from a representative experiment.

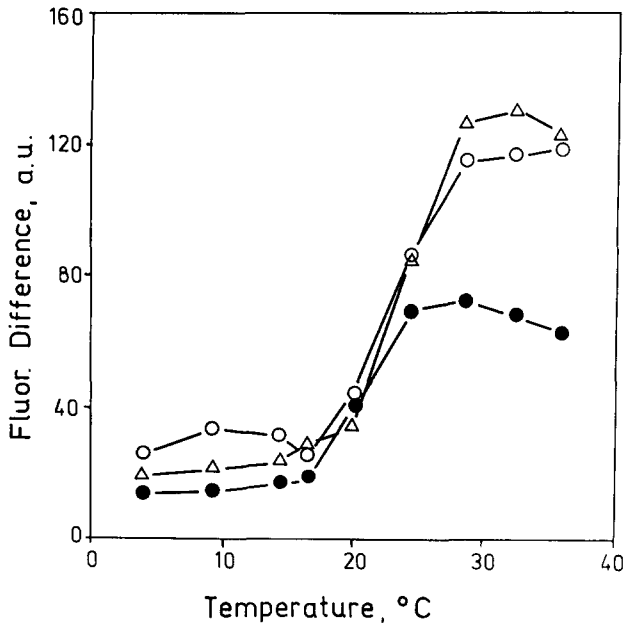

Fig. 2. Differences between fluorescence values obtained under assay and control conditions 30 s after the addition of Ca^{2+} to SUV as a function of temperature. Final concentrations of Ca^{2+} were 1.61 (●), 2.58 (△), and 6.45 mmol/l (○), respectively. PS was used in a final concentration of 25 nmol/ml in each fraction. Measurements were repeated four times. Data were taken from a representative experiment.

curves exhibit a maximum always 10 s after the start of fusion using 6.45 mmol/l Ca^{2+}. The values decrease then more markedly. Highest difference values are found at 36°C.

Moreover, examining the temperature dependence in more detail three regions of temperature according to the behaviour of difference curves can be found. In Fig. 2 fluorescence values of difference curves determined 30 s after the addition of Ca^{2+} are given as a function of temperature. Below 17°C nearly the same changes are observed for all curves. Similarly, identical curves are also obtained between 28°C and 36°C. It exists a third intermediate region of temperature where a continuous increase of changes in fluorescence occurs. This region is localized between nearly 17°C and 24°C at 1.61 mmol/l Ca^{2+}. It is shifted slightly (from about 19°C to 28°C) at 2.58 mmol/l and 6.45 mmol/l Ca^{2+}.

3.2. Fusion of LUV

LUV composed of PS were also examined for Ca^{2+}-induced changes in fluorescence as a function of temperature and Ca^{2+} concentration. Examples are given at 36°C using Ca^{2+} in final concentrations of 1.61, 2.58, and 6.45 mmol/l (Fig. 3). A fast decrease of fluorescence by about 12% (assay conditions) or 17% (control conditions) is observed immediately after the addition of Ca^{2+} in all curves. Then the fluorescence increases continuously with time under assay conditions at Ca^{2+} concentrations lower than 3 mmol/l. At 6.45 mmol/l Ca^{2+} a maximum of fluorescence is found 20 s after the initiation of fusion. In all control samples the fluorescence values continue to decrease with time, but pronounced changes in fluorescence are only observed at 6.45 mmol/l Ca^{2+}.

Difference curves from these data (Fig. 3c) show clearly an increase of fluorescence in LUV after the initiation of fusion as a function of Ca^{2+} concentration at 36°C. The more Ca^{2+} is added to LUV, the faster and more pronounced are the changes in fluorescence. However, Ca^{2+}-induced changes in SUV are much more expressed under the same conditions.

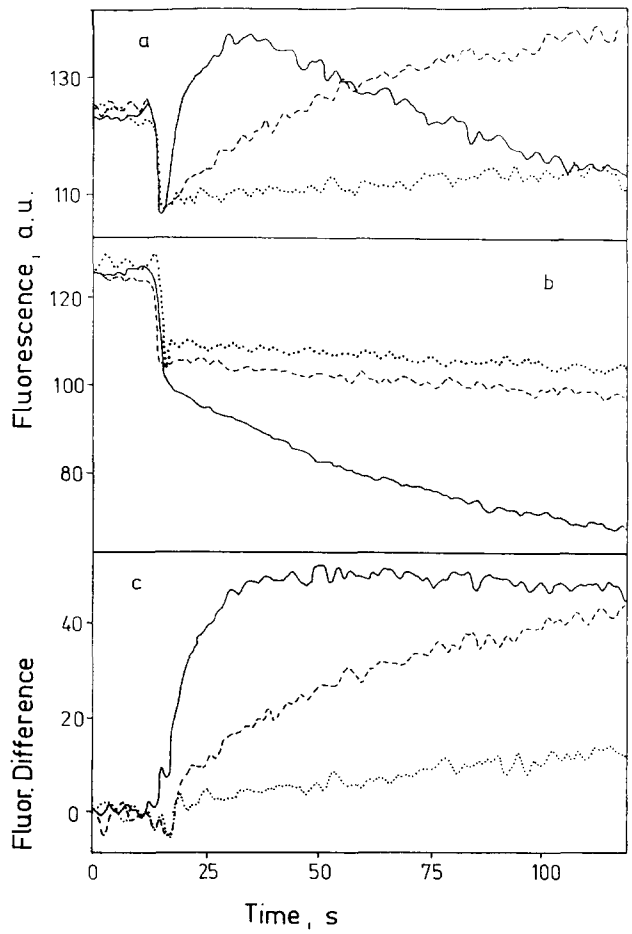

Fig. 3. Changes in fluorescence of LUV composed of PS upon addition of Ca^{2+} as a function of Ca^{2+} concentration at 36°C. 100 μl Ca^{2+} was added to a 1:1 mixture of labelled (8 mol% R-18) and unlabelled LUV (panel a) or only labelled vesicles (b) 15 s after the start of registration. The final concentrations of Ca^{2+} were 1.61 (dotted curves), 2.58 (dashed curves), and 6, 45 mmol/l (unbroken curves), respectively. PS was used in a final concentration of 25 nmol/ml in each fraction. The difference curves between fluorescence values obtained under assay and control conditions are given in panel c. All measurements were repeated three times. Data were taken from a representative experiment.

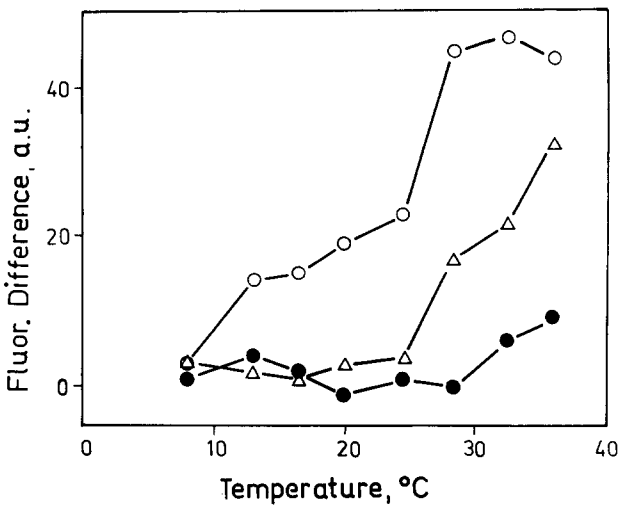

Fig. 4. Differences between fluorescence values obtained under assay and control conditions 30 s after the addition of Ca^{2+} to LUV as a function of temperature. Final concentrations of Ca^{2+} were 1.61 (•), 2.58 (△), and 6.45 mmol/l (○), respectively. PS was used in a final concentration of 25 nmmol/ml in each fraction. Measurements were repeated three times. Data were taken from a representative experiment.

No significant differences are found between assay and control samples using 1.61 or 2.58 mmol/l Ca^{2+} at temperatures below 24°C, whereas only moderate values are observed at 6.45 mmol/l Ca^{2+} (Fig. 4). An intermediate region of behaviour of difference curves exists between 20°C and 28°C for the later Ca^{2+} concentration. The differences of fluorescence values increase clearly in this region. At lower final concentrations of Ca^{2+} there is a continuous increase of difference values with temperature starting at about 24°C to 28°C.

Results concerning fusion rates agree with literature data. Fusion rates of LUV are much smaller than those of SUV [7,26,27] and only an aggregation but no fusion occurs in LUV at lower temperatures [7]. As in experiments with SUV an enhanced fluorescence quenching is observed in LUV especially at high Ca^{2+} concentrations and at low temperatures. This quenching effects can not be explained by dilution of R-18 in the lipid matrix due to fusion.

3.3. Phase transitions

The enhancement of fluorescence quenching observed in a part of our experiments can be only explained by a lateral separation between dye and phospholipid molecules in the plane of bilayers. We described such a quenching of fluorescence for DMPC/R-18 and DPPC/R-18 for temperatures where lipids are in the gel state [10].

Fig. 5 demonstrates the dependence of fluorescence intensities of SUV and LUV composed of PS and 8 mol% R-18 on temperature. Fluorescence decrases slowly with increasing temperature in both vesicle fractions at temperatures higher than 12°C. We attribute this decrease to a dynamic quenching due to higher mobility of dye molecules. Intensities are markedly quenched in LUV below 12°C, whereas only moderate changes are expressed in SUV. Vesicles composed of ox brain PS exhibit a phase transition at approximately 8–10°C [8,9]. Our results indicate that a lateral phase separation between dye and phospholipid molecules occurs below the phase transition. The small changes observed in SUV are not surprising because the phase transition is only weakly expressed in SUV [7]. In this

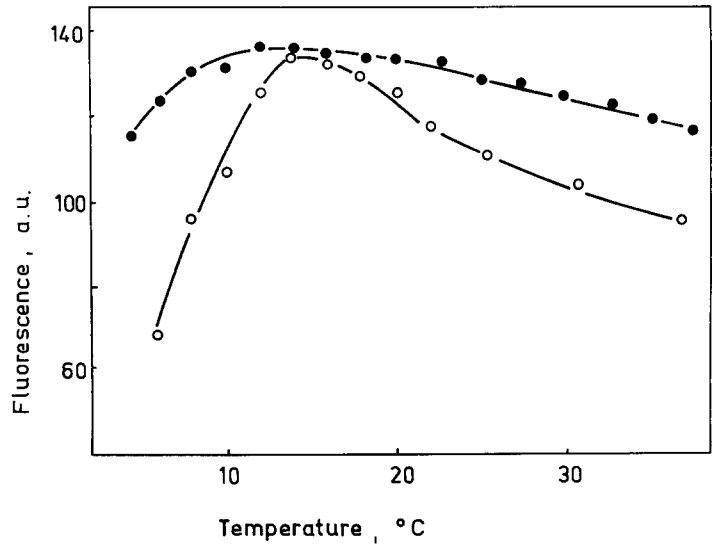

Fig. 5. Fluorescence intensities of SUV (•) and LUV (○) composed of PS and labelled with 8 mol% R-18 as a function of temperature. The PS concentration was 50 nmol/ml. Each value represents the mean of four measurements (S.D. < 4%).

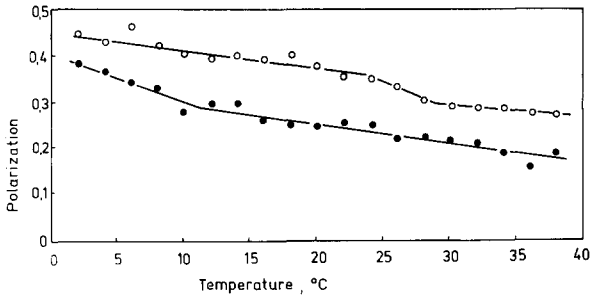

Fig. 6. The degree of polarization of SUV composed of PS and containing DPH in a mole ratio of 200:1 (PS/DPH) as a function of temperature before (●) and 30–60 s after the addition of Ca^{2+} (○). The PS concentration was 50 nmol/ml. $CaCl_2$ was added in a final concentration of 2.58 mmol/l. Each value represents the mean of four measurements (S.D. < 8%).

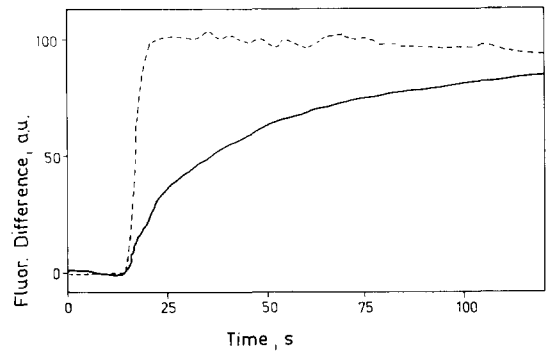

Fig. 7. Difference curves between fluorescence values obtained under assay and control conditions upon addition of Ca^{2+} to SUV in the presence (dashed curve) and absence (unbroken curve) of 10% (w/v) PEG. Ca^{2+} (1.61 mmol/l final concentration) was added 15 s after the start of registration. The concentration of PS was 25 nmol/ml in each fraction. Data are given for a representative measurement of four experiments.

fraction the transition is broadened and shifted to lower temperatures due to the high curvature of vesicle membrane [28,29].

The binding of Ca^{2+} to PS bilayers shifts the phase transition region at nonfuseogenic concentrations of Ca^{2+} by nearly 10 K to higher temperatures [8,9]. In order to determine the shift of phase transition in PS membranes after the initiation of fusion Ca^{2+} (2.58 mmol/l final concentration) was added to SUV composed of PS (50 nmol/ml) and labelled with DPH (PS/DPH, 200:1). The polarization of DPH flourescence was measured immediately before and 30–60 s after the initiation of fusion. Our SUV experience a very weakly expressed transition at 10°C (Fig. 6). The addition of Ca^{2+} shifts the transition region to 24–28°C. This transition becomes markedly expressed possibly due to the lower curvature of membranes in vesicles fused. The experiments given in Fig. 2 reveal that the character of fluorescence profiles in SUV was changed in the same temperature region.

Therefore, our finding of an enhancement of fluorescence quenching of R-18 incorporated in SUV or LUV composed of PS after the addition of Ca^{2+} can be explained by a demixing of dye and phospholipid molecules in the plane of membrane at temperatures below the phase transition. The shift of transition temperature to higher values caused by Ca^{2+} supports this conclusion. Under our experimental conditions the phase transition temperature of Ca^{2+}-PS complexes is in the range from 24°C to 28°C during the first seconds after the initiation of fusion.

3.4. Effects of PEG

The influence of PEG on Ca^{2+}-induced fusion of PS monitored by R-18 was investigated. Experiments were done as described above but unlike that the buffer used to dilute liposome samples contains PEG 6000 (10% (w/v) final concentration). Owing to the high viscocity of the PEG solution, samples have to mix violently after the addition of Ca^{2+}.

PEG accelerates all fusion events in SUV and LUV induced by Ca^{2+}. Examples are given using 1.61 mmol/l Ca^{2+} (final concentration) at 36°C in Fig. 7. We chose this concentration for presentation because under these conditions the fluorescence increases only slightly, whereas in the presence of PEG a strong increase in fluorescence can be observed.

A fusion occurs also in the presence of PEG using nonfusogenic concentrations of Ca^{2+}. Fig. 8 demon-

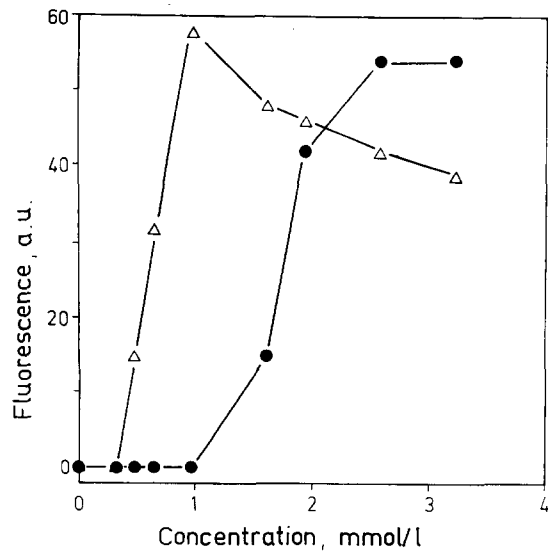

Fig. 8. Changes in fluorescence of SUV measured 30 s after the addition of Ca^{2+} as a function of Ca^{2+} concentration. The samples contain labelled (8 mol% R-18) and unlabelled vesicles in a ratio of 1:1 and 10% (w/v) PEG 6000 (△) or no PEG (●). The concentration of PS was 50 nmol/ml. All experiments are performed at 36°C and repeated three times. Data are given for a representative experiment.

strates the extent of changes in fluorescence in SUV as a function of Ca^{2+} at 36°C. Whereas no fusion is observed at concentrations lower than 1.0 mmol/l, 10% (w/v) PEG 6000 diminishs the threshold concentration of Ca^{2+} up to about 0.4 mmol/l. This value corresponds to data of others published for 25°C [16,17]. Moreover, the same threshold concentration is found carrying out experiments mentioned above at 24°C or 9°C (data not shown).

Finally, difference curves between assay and control samples for Ca^{2+}-induced fusion of SUV and LUV were examined in the presence of 10% (w/v) PEG 6000 as a function of temperature and Ca^{2+} concentration. We used 0.80, 1.61, and 2.58 mmol/l Ca^{2+}. As in experiments without polymers independent of the Ca^{2+} concentration again three regions for the temperature with different behaviour of difference curves for SUV could be found. A strong increase in fluorescence values occurs in the intermediate region of temperature between about 20°C and 28°C. Similar results are obtained for LUV. These results correspond to data given in Figs. 2 and 4 for PEG-free systems. PEG does not influence the shift of the phase transition region in PS vesicles induced by fuseogenic concentrations of Ca^{2+}.

4. Discussion

R-18 is a fluorescence molecule mainly used in probe dilution assays to study fusion between viruses and biological membranes [11–14]. A prerequisite of its use is a homogeneous mixing of dye and lipid molecules during all stages of fusion. However, in liposome samples a demixing between R-18 and PC molecules was recently observed at temperatures below the phase transition [10]. The present results support also this view for R-18 and PS.

Phospholipids are arranged in gel state in the form of regularly packed lipid clusters with all-*trans* chain configuration. Neighboured clusters differ in their orientation. Therefore, grain boundaries and vacancies are formed in the lipid matrix [30,31]. These structural elements are absent in the liquid-crystalline state. We assume that below phase transition temperatures phospholipid and R-18 molecules are demixed and the R-18 probes accumulate in such gel defect structures.

Although the mechanism of fluorescence quenching in R-18 is not well understood up to now, it was found that the self-quenching occurs mainly due to an energy transfer from exited monomers to nonfluorescent dimers of the dye [32]. The extent of self-quenching is a linear function on dye concentration if the lipids are in the liquid-crystalline state. However, fluorescence quenching obeys an exponential dependence in PC vesicles with a high content of cholesterol or below the phase transition [32]. This means that the concentration of nonfluorescent dimers of R-18 increases under these conditions. Because the fluorescence of R-18 incorporated in PC or PS vesicles is highly quenched in the gel state of lipids it can be assumed that the dye molecules exists in gel defect structures mainly as dimers.

Our experiments give information about the dynamics of phase transitions during fusion. At nonfusogenic concentrations of Ca^{2+} the phase transition temperature of PS is shifted in Ca^{2+}-PS 'cis' complexes to approximately 18°C. Under these conditions nearly 70% of negative charges of PS molecules are neutralized [4,33]. However, at fusogenic concentrations of Ca^{2+} changes in fluorescence properties of R-18 were found in the range from about 20°C to 28°C in SUV and LUV, whereby the strongest changes occur between 24°C and 28°C. We assume that PS molecules exhibit a phase transition at these temperatures in vesicles membranes fused by Ca^{2+}. Correspondingly, a phase transition was observed in polarization measurements with DPH in the range from 24°C to 28°C immediately after the start of fusion and during the first minute. We assume that fusogenic concentrations of Ca^{2+} cause a nearly complete neutralization of electrical charge of PS molecules. Interestingly, at acid pH values PS exhibits also a phase transition at 20–26°C in its electrically neutralized form [34].

In SUV the demixing between fluorophor and phospholipid molecules takes place immediately after the addition of Ca^{2+} at lower temperatures. Then fluorescence declines further with a much lower time constant indicating that phase separation proceeds. On the other hand, the initiation of fusion is correlated to the formation of Ca^{2+}-PS 'trans' complexes [4,5], which results in a collapse of vesicle membranes. Phospholipids are arranged by the end in closely packed cochleate-like structures [24,25]. We assume that the decline of fluorescence indicates these processes. However, the phase transition temperature of Ca^{2+}-PS complexes in cochleate structures is higher than 100°C [4,8]. Because in our experiments the phase transition occurs always in the range of 20–28°C after the initiation of fusion it is suspected that R-18 and presumably also DPH are localized in lipid areas which do not represent cochleate-like structures.

Interestingly, such a decrease in fluorescence starting approximately 5–10 s (SUV) or 20–30 s (LUV) after the addition of Ca^{2+} is also observed at 30°C and higher temperatures using Ca^{2+} concentrations higher than 4 mmol/l. This would mean that the rearrangement of vesicles fused and aggregated to cochleate structures is enhanced under these conditions. In this regard LUV and SUV give similar results. However, there are great differences in the behaviour of LUV and SUV concerning the rate of probe dilution and

ability to initiate fusion. Results obtained in our study are comparable to literature data [7,26,27].

It is known that chain melting properties are regulated by the surface of lipid bilayers [35]. The shift of phase transition temperature due to changes in polar region, e.g. induced by binding of divalent cations, can be represented as a sum of different contributions including headgroup hydration, lipid-lipid bonds and others [36]. In addition to the neutralization of negative charges by binding of Ca^{2+} to PS molecules a dehydration of these molecules occurs resulting in a new phase transition [34].

Our results show also that the phase transition temperature in fused PS membranes is independent of the concentration of Ca^{2+} and the presence of PEG. At a certain concentration of Ca^{2+} all PS molecules should be electrically neutralized and involved in Ca^{2+}-PS 'trans' complexes. Higher Ca^{2+} concentrations in the incubation system are unable to cause a further binding of Ca^{2+} to PS molecules. Only at relatively low concentrations of Ca^{2+} (1.60 mmol/l in experiments with SUV, Fig. 1) the phase tranisition temperature of fused PS vesicles is somewhat lower (from about 17°C to 24°C). Maybe PS molecules are not fully neutralized in this case.

Higher concentrations of Ca^{2+} and the presence of PEG accelerate fusion. Furthermore, PEG lowers the threshold concentration of Ca^{2+} to initiate fusion at all temperatures in SUV and LUV, where a fusion has taken place. This is in accordance to data of others published for 25°C [16,17]. The independence of phase transition in fused PS membranes on the presence of PEG supports the view that the fusion itself depends only on Ca^{2+} and PS. Although vesicles are strongly aggregated by PEG, the polymer is excluded from liposome surfaces [37]. However, concentrations of PEG higher than 22% (w/v) initiate fusion in the absence of Ca^{2+} due to high mechanical stress [38]. The lowering of the threshold concentration of Ca^{2+} necessary to fuse vesicles in the presence of PEG can be explained by an additional attractive force between vesicles exerted by PEG. Therefore, lower amounts of Ca^{2+} are sufficient to induce fusion [17].

Experiments with octadecyl Rhodamine B chloride can also serve as a model for lipid impurities or mixed lipid systems. There are several reports indicating that in liposomes composed of two or more different kinds of phospholipids a lateral phase separation between these lipids occurs as the result of fusion [30,39]. For example Ca^{2+} induces phase separations in PS/PC [30,40] and PS/PE [41] mixtures. It is assumed that phase separation is mainly caused by formation of anhydrous Ca^{2+}-PS complexes [39]. Finally, an enhanced quenching of R-18 fluorescence was observed in membranes of lipids extracted from Sendai virus [42]. Maybe, here the lipids are arranged in the membrane in different phases and not homogeneously mixed.

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