J. Membrane Biol. 114, 195–203 (1990)

The Journal of
Membrane Biology

Surface Dielectric Constant, Surface Hydrophobicity and Membrane Fusion

S. Ohki and K. Arnold*
Department of Biophysical Sciences, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214

Summary. Membrane fusion induced by ions and its associated membrane property, surface dielectric constant, were studied with the use of acidic and neutral phospholipid vesicles. The fusion of vesicles was monitored by utilizing two fluorescence fusion assays: fluorescence content mixing method and fluorescence labelled membrane component dilution method. For the surface dielectric constant measurements, a fluorescence method was used which detected the environmental effect on the membrane surface upon the addition of various fusogenic cations. Also, the effects of poly-(ethylene glycol) on both fusion and surface dielectric properties were examined. It was found that the extent of fusion correlated well with the degree of lowering in the dielectric constant of the surface membrane, which corresponds to the increase in hydrophobicity of the membrane surface. This agrees with the previously obtained experimental results that the increase in interfacial tension of the membrane, which also corresponds to the increase in surface hydrophobicity, correlates with the extent of membrane fusion.

Key Words lipid vesicle fusion · surface dielectric constant · hydrophobicity · cations · poly-(ethylene glycol)

Introduction

Membrane fusion is an important molecular event which is involved in many cellular processes [24, 26]. In order to gain insight into the molecular mechanisms of membrane fusion occurring in such biological systems, membrane fusion studies using model lipid membranes have been extensively made for the last decade [25, 27]. A number of methods to induce fusion of the model lipid membranes have been developed and several different theories accounting for mechanisms of membrane fusion have been put forward [19].

Among others, Ohki [16, 20] extensively studied ion-induced lipid membrane fusion and found that the extent of membrane fusion correlated with the degree of the increase in interfacial tension of the membrane. Also, he proposed a theory that in order for two membranes to fuse, it was necessary for the membrane surfaces to attain a certain degree of hydrophobicity [17, 18]; the energy of dehydration of the membrane should become small enough to be overcome by other attractive interaction energies. The increase in interfacial tension of the membrane, corresponds to the increase in surface hydrophobicity and also to the decrease in dehydration energy. In this paper, the surface dielectric constant, another physico-chemical quantity of the membrane, was studied for various lipid vesicle systems with respect to certain fusogenic and non-fusogenic cation concentrations and poly-(ethylene glycol) in terms of membrane fusion.

Materials and Methods

Chemicals

Phospholipids (bovine brain phosphatidylserine (PS), egg-phosphatidylcholine (PC), phosphatidylethanolamine (PE) derived from egg-phosphatidylcholine) were obtained from Avanti Polar Lipids (Birmingham, AL). Each phospholipid showed a single spot on a thin layer chromatographic plate. Fluorophore-labeled phospholipids (dansylphosphatidylethanolamine (DPE), 1-4-nitrobenzo-2-oxa-1,3-diazole-PE (NBD-PE) and lissamine rhodamine B sulfonyl-PE (Rh-PE) were also obtained from Avanti Polar Lipids and used as they were. Organic solvents (methanol, ethanol, L-propanol, chloroform, L-octanol, *n*-butylamine and *n*-decane) were all of reagent grade and were obtained from Baker Chemical. $TbCl_3$, $6H_2O$ and $LaCl_3$-$7H_2O$ were of the highest purity and were purchased from Aldrich Chemical. One other organic solvent (octylamine) was also purchased from Aldrich. Dipicolinic acid (DPA) and polyamines (spermine, spermidine) were obtained from Sigma Chemical. HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, ultrol grade, Calbiochem) was used as a buffer to all solutions. Poly-ethylene glycols (MW 1000 and 6000, Fluka Chemical, Switzerland) were used without further purification. All other chemicals used were of reagent grade and obtained from Baker Chemical. Some solutions contained a small amount of EDTA to remove possible divalent and

* *Present address:* Institute of Biophysics, Karl-Marx University, Leipzig 7010, DDR.

polyvalent cation contaminants in the experimental solutions. The water used was distilled three times, including an alkaline permanganate process.

Small Unilamellar Vesicle Preparation

Small unilamellar lipid vesicles were prepared by hydrating with 0.1 M NaCl/5 mM HEPES/pH 7.4, and then vortexed for 10 min and sonicated for 1 hr in a bath-type sonicator. The lipid concentration of such stock vesicle suspensions was normally 5 mM lipid. The details are described in an earlier paper [4]. The average sizes of prepared vesicles were determined to be about 300 Å in diameter by use of a photon-correlation spectrometer (Coulter-N4). The preparation of fluorophore incorporated vesicles are described in each section.

Measurements of Dielectric Constant of Membrane Surface by Use of Fluorescence Probe (DPE)

Dansylphosphatidylethanolamine (DPE) was either suspended in organic solvents (15 μg/ml), or incorporated in small unilamellar phosphatidylserine vesicle membranes at the molar ratio of phospholipid/DPE ~ 200–300, and suspended in 0.1 M NaCl buffer solution (total lipid concentration of 5 mM) by the co-sonication method. The latter was kept as the vesicle stock suspension. An aliquot of the vesicle stock was suspended in 0.1 M NaCl buffer (0.05 mM lipid) for the experiment. For trivalent cation (La^{3+}, Tb^{3+}) one-tenth of the above mentioned lipid concentration was used. The fluorescent signal of DPE was detected by a spectrofluorimeter (Perkin-Elmer, LS5) equipped with a temperature-controlling device. Excitation and emission spectra of DPE were obtained in various organic solvents, where the maximum of the excitation spectra was not altered by the various organic environments, whereas those of emission spectra were shifted according to different dielectric media. The intensity of fluorescence emission of DPE incorporated in the phosphatidylserine vesicle suspended in 0.1 M NaCl having various amounts of other ions and/or polyethylene glycols (PEG), was obtained in the range of 400–600 nm by exciting at 340 nm. From the shifts of emission spectra maxima obtained in the various organic solvents having different dielectric constants, the dielectric constants of the DPE environment in the lipid membrane were calculated using a Stokes shift equation which relates the wavelength at the maximum value of the emission spectrum and its dielectric properties [12, 15].

Vesicle Fusion Assay

The fusion of the PS vesicles was followed by using two different fluorescence fusion assay methods: one of them was to use the fluorescence energy transfer method, using NBD-PE and Rh-PE; the other was the vesicle internal content mixing method using Tb^{3+} and DPA^{3-}. For the latter assay [16, 31], small, unilamellar lipid vesicles were prepared by sonication, as described above, in the presence of either 10 mM $TbCl_3$/100 mM sodium citrate/5 mM HEPES (pH 7.4) or 100 mM DPA/5 mM HEPES (pH 7.4). Nonencapsulated materials were separated from the vesicles by gel filtration on a Sephadex G-75 column. The elution buffer was 100 mM NaCl/5 mM HEPES (pH 7.4). For the assay of fusion, approximately equimolar amounts (0.1 μmol lipid each) of the Tb-encapsulated vesicles and DPA-encapsulated vesicles were mixed at final concentration of about 0.05 mM of lipids in 2 ml of 100 mM NaCl buffer solution with and without various amounts of PEG. Small aliquots of the stock fusogenic ion solutions were added (usually in steps of 10 μl about 0.2-mM or 2-μM increments) and mixed well. After each addition, fluorescent measurements were made on the solution using a Perkin-Elmer LS-5 spectrofluorimeter equipped with filters in the excitation (transmission wavelength 200–340 nm) and emission (transmission wavelength 420–700 nm) arms. The excitation was set at 273 nm. Fluorescence was recorded in the range of 450–580 nm. The total time which elapsed between the two consecutive fusogenic ion additions was about 1 min. The fluorescent intensity at 545 nm in the emission spectra was used to determine the extent of vesicle fusion. The fluorescent intensity due to vesicle fusion was obtained by subtracting the background scattering contribution from the total intensity.

For the former assay [28], the vesicles were composed of the mixture of phosphatidylserine and 1% (mol/mol) of both NBD-PE and Rh-PE. They were prepared in 0.1 M NaCl buffer solution by co-sonication (5 mM lipids). One part (0.05 μmol lipid) of the fluorophore-incorporated vesicles and two parts (0.1 μmol lipid) of the unlabeled vesicles were suspended in 2 ml of 0.1 M NaCl buffer with or without various amounts of PEG and then the fusogenic cations were added to the vesicle suspension solution in small increments successively as described above. The fluorescence measurements of these suspensions were done by exciting at 460 nm and recording the fluorescence from 500 to 620 nm. Fusion was evaluated from the intensity of NBD at 525 nm. The degree of fusion, F, was expressed by

$$F = \frac{I_{525} - I^0_{525}}{I^0_{525}} \tag{1}$$

where I_{525} was the fluorescence from the experimental solution containing fusogens, and I^0_{525} without fusogens. All experiments were done at 24 ± 1°C.

Experimental Results

In order to establish a baseline to estimate the dielectric constant of the polar region of the lipid bilayers, first a relationship between known dielectric constants of various organic solvents, and the wavelength at the maximum in the fluorescent emission spectrum of DPE in each organic solvent should be obtained. For this purpose, the emission spectra (400–600 nm) of DPE in various organic solvents (methanol, ethanol, 1-propanol, 1-octanol, *n*-butylamine, octylamine and *n*-decane) were obtained by exciting the fluorophore at 340 nm. According to the theory [12, 15], the wavelength at the spectrum maximum of the fluorophore is related to the dielectric constant and the refractive index of the medium in the following formula:

$$\frac{1}{\lambda_m} = K\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right) \tag{2}$$

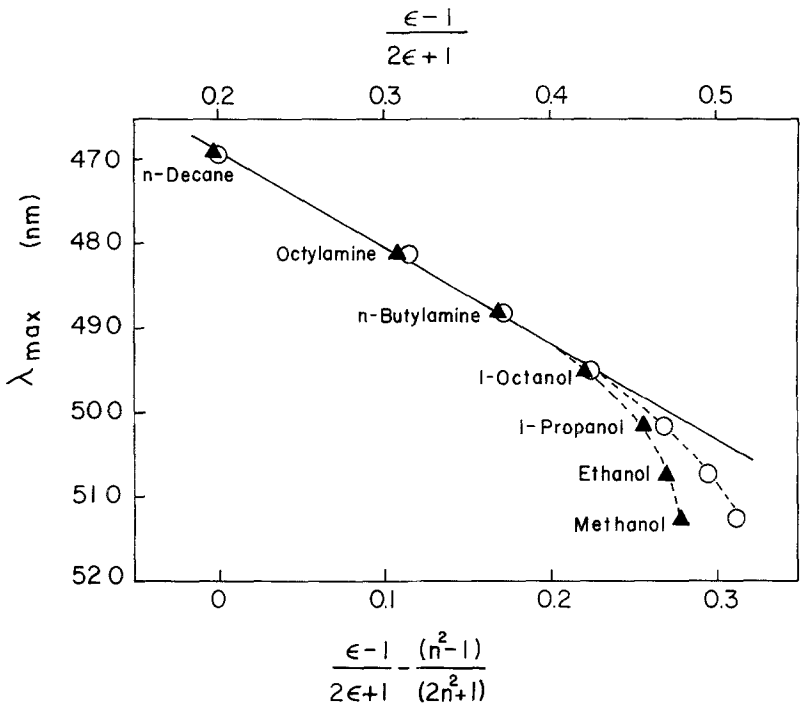

Fig. 1. A relation of dielectric constant and λ_{max} of DPE fluorescence determined in various organic solvents: methanol (ε = 32.6, n = 1.329), ethanol (24.3, 1.361), 1-propanol (20.1, 1.385), 1-octanol (10.3, 1.431), n-butylamine (5.4, 1.403), octylamine (3.4, 1.429), and n-decane (1.991, 1.410). The values of dielectric constants and refractive index are taken from [30]. ▲ refers to the case for λ_{max} *versus* $(\varepsilon - 1)/(2\varepsilon + 1)$ and ○ refers to the case for λ_{max} *versus* $(\varepsilon - 1)/(2\varepsilon + 1) - (n^2 - 1)/(2n^2 + 1)$

where λ_m is the wavelength of the emission spectra maximum, K is a constant, and ε and n are the dielectric constant and refractive index of the medium, respectively. The experimental relationship between the wavelength λ_m and the dielectric properties of the media was obtained. The results are shown in Fig. 1. The inverse of the wavelength was roughly proportional to the quantity $(\varepsilon - 1/2\varepsilon + 1)$ in the range of dielectric constant below 20, which was similar to those obtained earlier [10].

Then, the emission spectrum of DPE incorporated in the phosphatidylserine vesicle was measured as a function of various salt concentrations. As the fusogenic ion concentration was increased, the emission spectra as well as its maximum position were shifted towards the higher frequency and the fluorescence intensity also increased. These changes (the blue shift of the emission spectrum maximum and an increase in fluorescent emission intensity) indicate that the local environment around the fluorophore was altered to a lower dielectric medium. Since the fluorophore (dansyl group) of DPE is to detect the dielectric medium of the glycerol backbone region of lipid bilayers [29], the observed changes in fluorescent signal indicate the surface polar region of the bilayer to be more hydrophobic upon interaction with fusogenic ions. A typical emission spectra of DPE in the PS vesicle is shown in Fig. 2. From the relationship between λ_m and known dielectric media and the measured values of the emission spectra maxima of DPE in the small, unilamellar phosphatidylserine vesicle, we can deduce the dielectric constant of the lipid polar region where the DPE molecule is possibly situated. Such surface dielectric constants of the phosphatidylserine vesicle are shown as a function of various fusogenic cation concentrations in Fig. 3. The surface dielectric constant of the phosphatidylserine membrane in 0.1 M NaCl was about ε = 30. As the fusogenic ion concentration in the vesicle suspension solution increased, the dielectric constant of the DPE environment decreased. There is a good correlation between the extent of vesicle fusion and the degree of decrease in dielectric constant of the DPE environment (Figs. 3 and 4). The dielectric constant of the membrane surface detected by the DPE probe at the concentration corresponding to the fusion threshold concentration for the small, unilamellar phosphatidylserine vesicle was about the same value (~12) for all fusogenic ions examined (*see also* Table 1). An interesting observation was that in the La^{3+}, Tb^{3+}, Mg^{2+} and Ca^{2+} cases, the shift of spectrum maximum was greatly enhanced toward the higher frequency side and also the fluorescence intensity increased greatly as these fusogenic ions exceeded their fusion threshold concentrations (*see* Fig. 3). The surface dielectric constants were reduced to as low as 4. On the other hand, in the Mg^{2+} case, the shift did not change further and rather saturated at concentra-

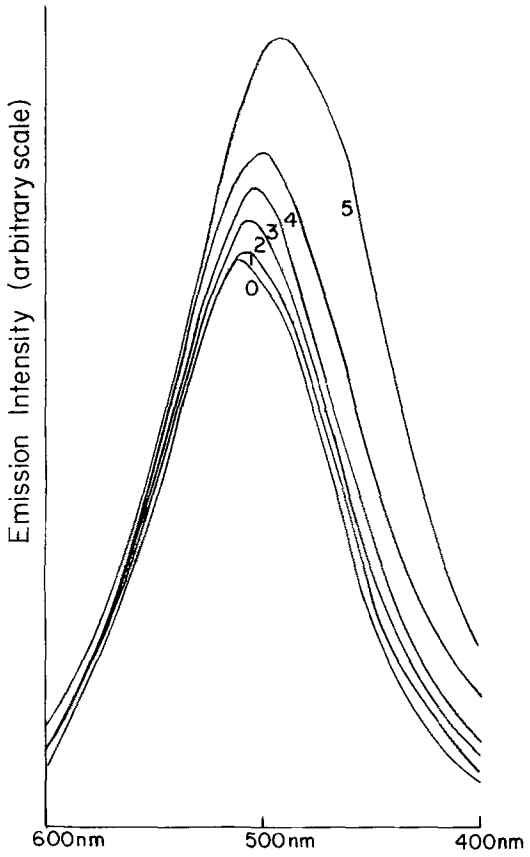

Fig. 2. Emission spectra of DPE in PS vesicles suspended in 0.1 M NaCl buffer containing various Ca^{2+} concentrations (*0:* 0 mM, *1:* 0.5 mM, *2:* 0.7 mM, *3:* 0.9 mM, *4:* 1.1 mM and *5:* 1.4 mM). Excitation: 340 nm

tions above its fusion threshold concentration (8 mM Mg^{2+} in 0.1 M NaCl), and also the fluorescence intensity did not increase as much as those for the other fusogenic ions mentioned above.

For nonfusogenic ions (Na^+, K^+ and polyamines), no shift in the spectra maxima was observed up to the concentrations of 1 M for Na^+ and 1.5 M for K^+ and no appreciable shift was observed up to the concentration of 300 mM spermine (*experimental data not given*). At these concentrations, however, a great deal of aggregation of phosphatidylserine vesicles was observed [20]. It should be pointed out that in these concentration ranges examined, the magnitudes of the surface potentials of the phosphatidylserine membranes were reduced from −80 to −30 mV, according to the previous work [20]. As for monovalent cations, the intensity of fluorescence did not change over the entire spectrum at various monovalent cation concentrations. For spermine, a slight increase in the fluorescent intensity was observed as the spermine concentration was increased. Contrary to other monovalent cations, as the concentration of hydrogen ion was increased (pH was decreased), the spectrum maximum of DPE was shifted to the same direction (blue shift) as the other fusogenic cations. However, the change in the spectrum shift was rather gradual with respect to H^+ concentration, while the fusogenic divalent and trivalent cations induced a large shift within the small range of their respective concentrations. The fluorescent intensity was reduced as the pH was lowered.

Table 1. Fusion threshold concentrations of various fusogenic ions for small, unilamellar PS vesicles and surface dielectric constants at their corresponding threshold concentrations in 0.1 M NaCl buffer solution (Figs. 3 and 4)

Fusogenic ions	Threshold concentrations	Surface dielectric constant
Tb^{3+}	3 ~ 5 μM	11 ~ 14
La^{3+}	4 ~ 6 μM	11 ~ 14
Mn^{2+}	0.65 mM	12.5
Ba^{2+}	0.8 mM	12
Ca^{2+}	1.0 mM	12
Sr^{2+}	1.6 mM	11
Mg^{2+}	8 mM	15
H^+	pH 3.0 ~ 2.7	~12

Surface dielectric constant experiments, similar to those described above, were done for the phosphatidylserine vesicle suspended in 0.1 M NaCl buffer containing various amounts of poly-(ethylene glycol) (PEG-6000) as a function of Ca^{2+} concentration (Fig. 5). In the presence of the same amounts of Ca^{2+} in the vesicle suspension, the higher the PEG concentration was, the lower the surface dielectric constant became. The experimental data using PEG-1000 (*experimental data not given*) were similar to those mentioned above with PEG-6000. PEG in the vesicle suspension alone also affected the surface dielectric constant of the lipid vesicles. As the PEG concentration was increased, the surface dielectric constants of lipid vesicles made of either PC or PS were reduced. At the same concentration of PEG, the surface dielectric constant of the PC membrane was slightly greater than that of the PS membrane. The dielectric constant of a small, unilamellar PC vesicle in 0.1 M NaCl buffer was approximately 35 while that for the PS vesicle was 30. The results are shown in Fig. 6.

Figure 7 shows the results of fusion experiments measured by the NBD-Rh energy transfer fusion assay on phosphatidylserine vesicles induced by Ca^{2+} in the presence of various PEG concentrations in the vesicle suspension. As the PEG concentration was increased, the concentration of Ca^{2+} required to induce the same extent of PS vesicle fusion was decreased. This tendency parallels the

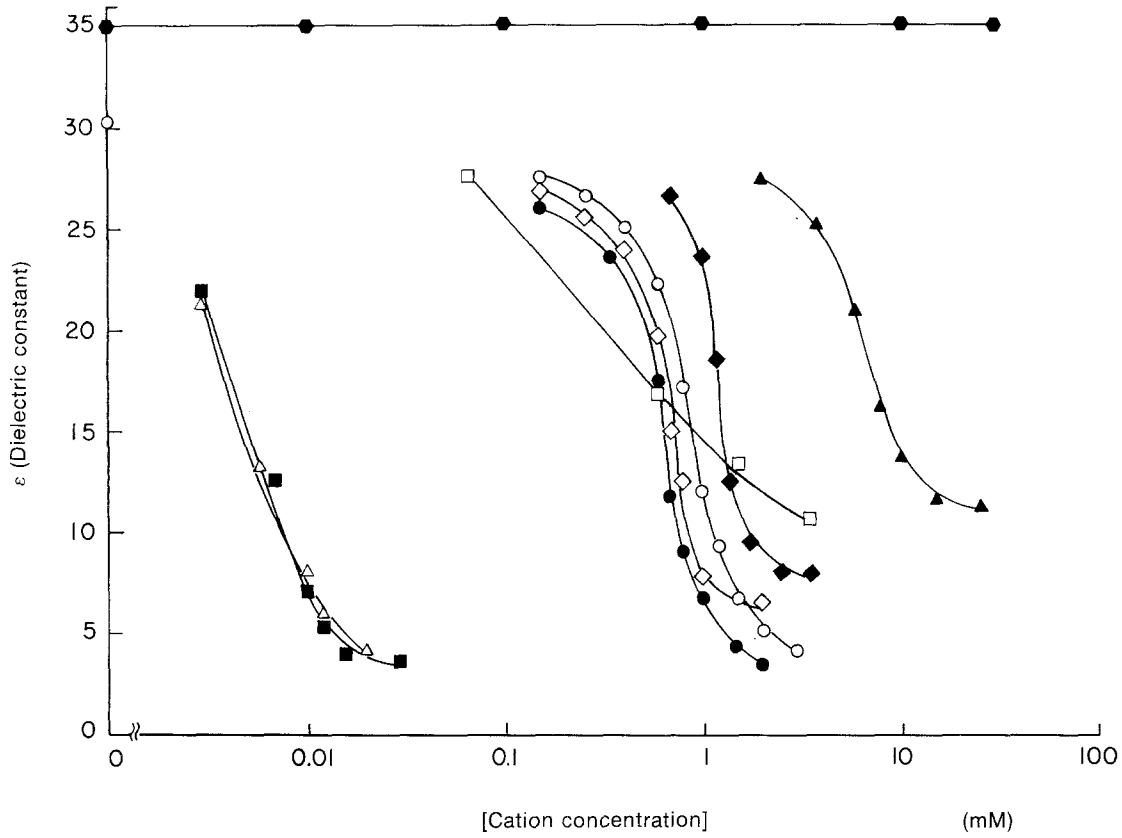

Fig. 3. Surface dielectric constant of the environment for DPE in PS vesicle suspended in 0.1 M NaCl buffer containing various fusogenic cation concentrations (△: La^{3+}, ■: Tb^{3+}, ●: Mn^{2+}, ◇: Ba^{2+}, ○: Ca^{2+}, ◆: Sr^{2+}, ▲: Mg^{2+}, □: H^{+}). A control experiment using PC vesicle with respect to Ca^{2+} is given with a symbol ⬣

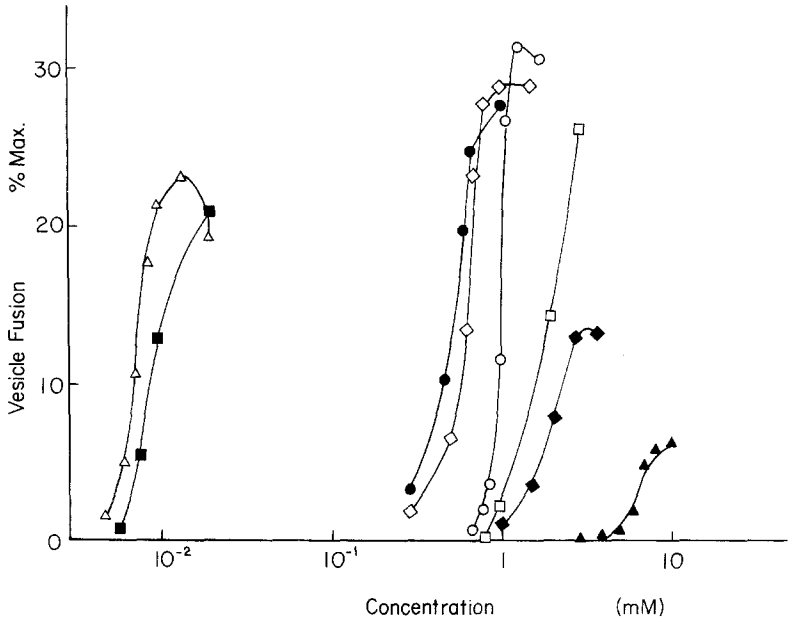

Fig. 4. Extent of fusion of PS vesicles suspended in 0.1 M NaCl buffer with respect to various fusogenic cation concentrations determined by the internal content mixing fluorescent fusion assay (△: La^{3+}, ■: Tb^{3+}, ●: Mn^{2+}, ◇: Ba^{2+}, ○: Ca^{2+}, ◆: Sr^{2+}, ▲: Mg^{2+}, □: H^{+})

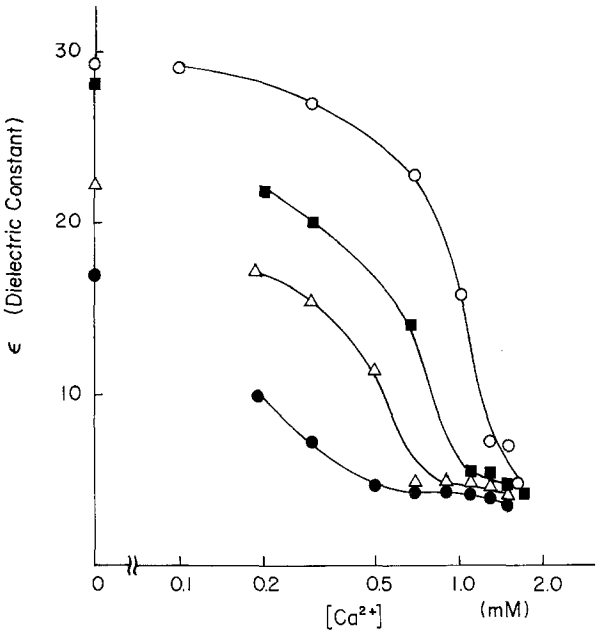

Fig. 5. Surface dielectric constant of the environment for DPE in PS vesicle suspended in 0.1 M NaCl buffer containing various concentrations of PEG (6000) with respect to the variation of Ca^{2+} concentration. (○: no PEG, ■: 5 wt% PEG, △: 10 wt% PEG, ●: 15 wt% PEG)

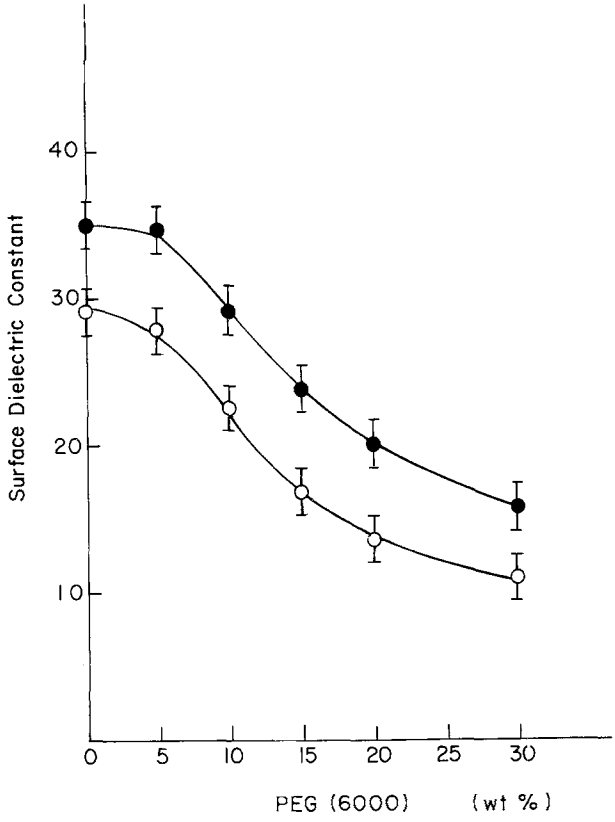

Fig. 6. Surface dielectric constants of the environment for DPE in PC and PS vesicles suspended in 0.1 M NaCl buffer as a function of PEG (6000) concentrations (●: PC vesicles and ○: PS vesicles)

results obtained for the surface dielectric constant experiments. From Figs. 5 and 7, the dielectric constant of the membrane surface at the Ca^{2+} concentration corresponding to the fusion threshold for the small, unilamellar PS vesicles is about 12, which is approximately the same as those (~12) obtained in the absence of PEG in the vesicle suspension. The results of the fusion experiments on the PS vesicles using the Tb-DPA assay were similar to those obtained by the fluorescence energy transfer (NBD-Rh) fusion assay, as mentioned above. The threshold concentrations of Ca^{2+} to induce PS vesicle fusion in the presence of various amounts of PEG are summarized in Table 2.

For PC vesicles, the emission spectrum maximum did not shift nor did the intensity change by the addition of Ca^{2+} up to 200 mM in 0.1 M NaCl buffer solution (shown in Fig. 3). This means that the surface dielectric constant remains unchanged in the presence of various concentrations of Ca^{2+}. In these Ca^{2+} concentration ranges, the fusion of PC vesicle was not observed. In addition, the effect of PE on the surface dielectric constant of PC vesicle was examined. No significant shift of the emission spectrum was observed for vesicles composed of various mixtures of PC and PE (the molar ratio of PC/PE was varied from 1 : 0 to 2 : 8).

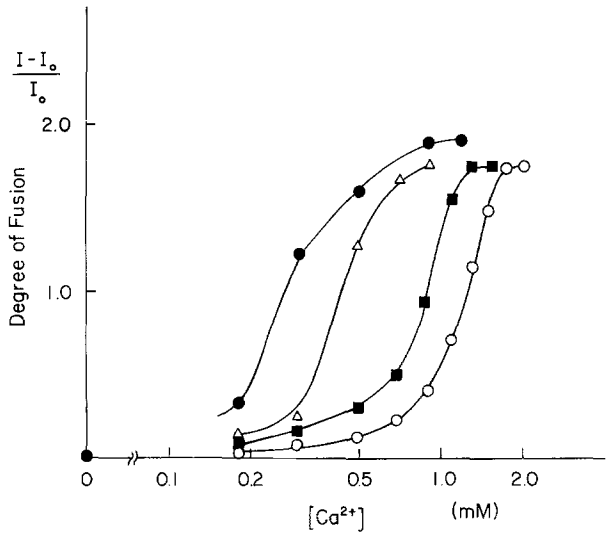

Fig. 7. The extent of fusion of PS vesicles suspended in 0.1 M NaCl and various PEG concentrations/pH 7.4, where fusion was induced by Ca^{2+} concentration in the vesicle suspension solution. Fusion was monitored by use of the fluorescence energy transfer (NBD-Rh) assay. (○: no PEG, ■: 5 wt% PEG (6000), △: 10 wt% PEG, ●: 15 wt% PEG). The degree of fusion refers to Eq. (1)

Table 2. Surface dielectric constants of PS vesicles in the presence of either Ca^{2+} or PEG, or both Ca^{2+} and PEG in 0.1 M NaCl buffer solutions[a]

Variation of both Ca^{2+} and PEG conc.			Variation of PEG conc. only		Variation of Ca^{2+} conc. only (Figs. 3 and 4)	
PEG	Ca^{2+}_{th}	Surface dielectric constant	PEG	Surface dielectric constant	Ca^{2+}	Surface dielectric constant
0	1.1 (mM) (1.2)	12*	0	30	1.0 (mM)	12**
5%	0.8 (0.8)	10	5%	26	0.8	18
10%	0.45 (0.5)	12	10%	22	0.5	24
15%	0.22 (0.25)	8.5	15%	17	0.25	26

[a] Ca^{2+}_{th} refers to the threshold concentration of Ca^{2+} to induce PS vesicle fusion. The vesicle fusion was monitored by either the fluorescence energy transfer assay (Figs. 5 and 7) or the internal content mixing assay (Tb-DPA assay). The values obtained by the latter assay are given in the parentheses. *Note:* The slight discrepancies in the threshold values (* and **) obtained from Figs. 5 and 7 and Figs. 3 and 4, respectively, may be due to different preparations of vesicle samples and different vesicle concentrations used in the experimental solution.

Discussion

It was found that the increase in fusogenic ion concentration in the aqueous medium of the phosphatidylserine vesicle caused the decrease in surface dielectric constant of the surface membrane of the lipid vesicle. The lowering of the surface dielectric constant indicates the increase in hydrophobicity of the membrane surface. This decrease in surface dielectric constant of the membrane has a good correlation with the extent of membrane fusion (*see* Figs. 3–5 and 7, and Tables 1 and 2). At the fusion threshold concentration of each fusogenic cation, the dielectric constant of the DPE fluorophore environment was reduced to about the same value (~12) for all fusogenic metal ions. It has been shown earlier [16, 20], that the increase in interfacial tension of the membrane also has a good correlation with the extent of membrane fusion. The increase in the interfacial tension is considered to be due to the strong bindings of these fusogenic cations to the negatively charged polar groups of phosphatidylserine molecules [21]. It is noted that in the cases of strong fusogenic cations, such as Ca^{2+}, Mn^{2+}, La^{3+} and Tb^{3+}, at or above the threshold concentrations of these fusogenic cations, the fluorescence intensity of DPE increased significantly and the maximum position of the emission spectrum shifted to lower wavelength (for example, *see* Fig. 2). Consequently, the surface dielectric constant reduced to lower values, as low as 4. These results suggest that when these strong fusogenic ions interact with acidic lipid membranes, they may form nearly anhydrous complexes of cation and lipid polar groups [5]. They may form a Ca^{2+}-interbridged lipid complex [6, 7]. On the other hand, although Mg^{2+} can induce fusion of the PS vesicles, the surface dielectric constant was not reduced lower than 11 by Mg^{2+}. Mg^{2+} may lower the surface dielectric constant just enough to produce membrane fusion but may not remove membrane-bound waters thoroughly enough to produce such anhydrous Mg-PS complexes. The earlier experiments also support this point [23].

The surface dielectric constant of the PS membrane was not altered appreciably by the change in concentrations of Na^+, K^+ or spermine^{4+} up to 1 M, 1.5 M and 300 mM, respectively. However, in their corresponding ion concentration ranges, the surface potentials of the PS membrane were reduced greatly (i.e., from −80 mV at 0.1 M NaCl to −30 mV) and a great deal of vesicle aggregation occurred [20]. This assures that the observed dielectric constant changes were not due to the surface potential changes of the membranes.

The presence of PEG also induced similar changes in surface dielectric constant by lowering its value as fusogenic cations did. In the presence of 30 wt% of PEG, where fusion of either PC or PS vesicles was observed [13, 22], the surface dielectric constant decreased to about 12 for the PS membrane which is approximately the same value as

those for cation-induced fusion. However, for the PC membrane it decreased to only 16, indicating that the membrane surfaces are still more hydrophilic than the PS membrane surface when fusion occurs. This suggests that additional mechanisms are necessary for membrane fusion in the PEG system where PEG destabilizes the region of contacting membranes besides the Ca^{2+} effect on the hydrophobicity of the surface. The withdrawal of free water from the vesicles by PEG could cause them to flatten. Thus, two lipid vesicles might fuse due to their stress on the highly curved boundaries as proposed by MacDonald [13], and Parente and Lentz [22]. The detergent-like properties of PEG are another possibility of the bilayer destabilization [2].

The presence of PEG lowered the fusion threshold concentration of Ca^{2+} for the PS membranes. Hoekstra [9] has measured membrane fusion for the membrane system described above, by use of the membrane probe mixing method (NBD-Rh energy transfer assay). He found that the PS vesicle fusion was induced at 0.5 mM Ca^{2+} in the presence of 10 wt% PEG, and in the case of the presence of 20 wt% PEG, the extent of fusion at 0.5 mM Ca^{2+} was the same as that for 5 mM Ca^{2+} alone. These results compare well with our experimental results. Similar experiments were done with PEG of molecular weight 1000 instead of 6000. Although the results are not shown, the results obtained were similar to those mentioned above (PEG 6000). The reduction of surface dielectric constant in the presence of PEG in the vesicle suspension was not due to the direct interaction of PEG with the membrane surfaces, but due to the reduction of the interlayer water between the two membranes by PEG in the solution. Arnold et al. [1, 3] have shown that PEG 6000 does not interact with the membrane in the concentration range employed here.

As we have proposed earlier [18], the membrane surface hydrophobicity is an essential factor for two membranes to come to close adhesion (molecular contact of membrane) by reducing dehydration energy of the membrane surface. The forces exerted on the two interacting membranes may consist of various types [8, 11]: van der Waals, electrostatic and hydration forces, etc. Among these, the hydration force [11, 14], which may arise from the water associated with the membrane surface through hydrogen bonding, is a major force and usually inhibits the two interacting membranes from coming into close contact. However, the energy of the adhesion of water onto (or dehydration of water from) the membrane surface depends on the nature of the membrane surface [17, 18]. According to our recent work, if the membrane surfaces remain strongly hydrophilic, the hydration energy is much greater (order of magnitude) than the other attractive and repulsive interaction energies between the two membranes [17, 18]. Many biological membrane interfaces, including lipid membrane surfaces, are usually strongly hydrophilic in nature and their water association energies are much greater than the other attractive interaction energies. Therefore, many biological interfaces are not likely to come into close molecular contact by squeezing out the intermembraneous water associated with the membrane surfaces by any intermembraneous attractive forces. However, such repulsive interaction forces due to hydration water on the membrane surface (so called "hydration pressure") can be reduced by altering membrane surfaces to a more hydrophobic nature, so that the intermembrane attractive forces overcome the repulsive hydration pressure and the two membranes can come into close contact. Binding of divalent and polyvalent cations to the acidic phospholipid membranes will make membrane surfaces strongly hydrophobic [18]. The increase in surface tension and decrease in surface dielectric constants make membrane surfaces hydrophobic in a similar manner.

The earlier study measuring the time-resolved fluorescence of DPE in lipid membranes [10] showed that the diffusion constant of the DPE local environment was not much affected by the presence of divalent cations in the vesicle suspension solution (e.g., 1.8 mM Ca^{2+} in the PS vesicle suspension). These results support that the estimate of the local dielectric constant of the DPE environment in the lipid membrane obtained from the Stokes shift of DPE fluorescence is reasonable. Although it is not clear which region of dielectric environment in the lipid membrane is measured by the DPE probe method, the earlier work suggested that the signal of the DPE probe reflected the dielectric media around the glycerol backbone in lipid membranes [29]. We may say that in order to induce the close adhesion of the membranes (or molecular contact of the membranes), it is necessary for the dielectric constant at the membrane surface region, where the probe is located, to become sufficiently lower. The critical value of the surface dielectric constant, which is related to the membrane close adhesion condition may vary with different membrane systems, as well as types of fusogenic agents. A mere membrane close adhesion is not necessarily sufficient for the two membranes to fuse. In the case of divalent and trivalent cation-induced membrane fusion of acidic lipid membranes, these cations bind the lipid polar groups and tie them together, making the membrane surface molecules physically rigid or constrained. In such cases, the membrane molecules at the boundary between the close contact and

noncontact regions of the two membranes may experience greater physical stress exerted by bending pressure or molecular expansion. This may lead to increases in interfacial tension of these membrane regions, or to enhanced intermembraneous molecular exchanges mediated by the fusogenic ions. Thus, the molecules in these regions which are affected by the mechanical stress or chemical forces would become a possible site of membrane fusion in the case of polyvalent metal cation-induced membrane fusion. Similar mechanisms for membrane fusion induced by PEG were given earlier. Although the molecular processes involved in the two membrane fusion systems (polyvalent cations *vs.* PEG) are quite different, the results of the withdrawal of hydrated water from the intermembraneous space and the reduction of the surface dielectric constant are in accordance for both cases.

This work was supported by a grant from the U.S. National Institutes of Health (GM24840). Parts of this work were presented at both the 5th International Symposium of Cell Surface, Kuhlungborn, GDR, October, 1988, and the 33rd American Biophysical Society Meeting, Cincinnati, Ohio, February, 1989.

References

1. Arnold, K., Herrmann, A., Gawrisch, K., Pratsch, L. 1988. Water-mediated effects of PEG on membrane properties and fusion." *In:* Molecular Mechanisms of Membrane Fusion. S. Ohki, D. Doyle, T.D. Flanagan, S.W. Hui, and E. Mayhew, editors. pp 255–272. Plenum, New York
2. Arnold, K., Herrmann, A., Pratsch, L., Gawrisch, K. 1985. The dielectric properties of aqueous solution of poly-(ethylene glycol) and their influence on membrane structure. *Biochim. Biophys. Acta* **815:**515–518
3. Arnold, K., Zschornig, O., Herold, W., Barthel, D. 1987. Effect of PEG on the electrophoretic mobility of liposomes. *Mol. Cryst. Liq. Cryst.* **152:**357–362
4. Duzgunes, N., Ohki, S. 1977. Calcium-induced interaction of phospholipid vesicles and bilayer lipid membranes. *Biochim. Biophys. Acta* **467:**301–308
5. Ekerdt, R., Papahadjopoulos, D. 1980. Intermembrane contact affects calcium binding to phospholipid vesicles. *Proc. Natl. Acad. Sci. USA* **79:**2273–2277
6. Feigenson, G.W. 1986. On the nature of calcium ion binding between phosphatidylserine lamellae. *Biochemistry* **25:**5819–5825
7. Hauser, H., Finer, G.E., Darke, A. 1977. Crystalline anhydrous Ca-phosphatidylserine bilayers. *Biochem. Biophys. Res. Commun.* **76:**267–274
8. Hiemenz, P.C. 1977. Principles of Colloid and Surface Chemistry. Marcel Dekker, New York
9. Hoekstra, D. 1982. Role of lipid phase separation and membrane hydration in phospholipid vesicle fusion. *Biochemistry* **21:**2833–3840
10. Kimura, Y., Ikegami, A. 1985. Local dielectric properties around polar region of lipid bilayers membranes. *J. Membrane Biol.* **85:**225–231
11. LeNeveu, D.M., Rand, P., Parsegian, A. 1976. Measurement of forces between lecithin bilayers. *Nature (London)* **259:**601–603
12. Lippert, E. von. 1957. Spektroskopische Bestimmung des Dipolmoments Aromatischer Verbindungen im ersten Angeregten Singulettzustand. *Z. Electrochem.* **61:**962–975
13. MacDonald, R.I. 1985. Membrane Fusion Due to Dehydration by Polyethylene Glycol, Dextran or Sucrose. *Biochemistry* **24:**4058–4066
14. Marcelja, S., Radic, N. 1976. Repulsion of interfaces due to bound water. *Chem. Phys. Lett.* **42:**129–130
15. Mataga, N., Kaifu, Y., Koizumi, M. 1955. The solvent effect on fluorescence spectrum. Changes of solute-solvent interaction during the life time of excited solute molecule. *Bull. Chem. Soc. Jpn.* **28:**690–691
16. Ohki, S. 1982. A mechanism of divalent ion-induced phosphatidylserine membrane fusion. *Biochim. Biophys. Acta* **689:**1–11
17. Ohki, S. 1985. Membrane fusion: Theory and experiments. *Studia Biophys.* **111:**95–104
18. Ohki, S. 1988. Surface tension, hydration energy and membrane fusion. *In:* Molecular Mechanism of Membrane Fusion. S. Ohki, D. Doyle, T.D. Flanagan, S.W. Hui, and E. Mayhew, editors. pp. 123–138. Plenum, New York
19. Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W., Mayhew, E. (editors) 1988. Molecular Mechanisms of Membrane Fusion, Plenum, New York
20. Ohki, S., Duax, J. 1986. Effects of cations and polyamines on the aggregation and fusion of phosphatidylserine membranes. *Biochim. Biophys. Acta* **861:**177–186
21. Ohki, S., Ohshima, H. 1984. Divalent cation-induced surface tension increase in acidic phospholipid membranes: Ion binding and membrane fusion. *Biochim. Biophys. Acta* **776:**177–182
22. Parente, T.A., Lentz, B.R. 1986. Rate and extent of poly-(ethylene glycol)-induced large vesicle fusion monitored by bilayer and internal content mixing. *Biochemistry* **25:**6678–6688
23. Portis, A., Newton, C., Pangborn, W., Papahadjopoulos, D. 1979. Studies on the mechanism of membrane fusion: Evidence for an intermembrane Ca^{2+}-phospholipid complex synergism with Mg^{2+} and inhibition by spectrin. *Biochemistry* **18:**780–790
24. Poste, G., Allison, A.C. 1973. Membrane Fusion. *Biochim. Biophys. Acta* **300:**421–465
25. Poste, G., Nicolson, G.L. (editors) 1978. Membrane Fusion. Elsevier, Amsterdam
26. Rasmussen, H. 1970. Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* **170:**404–412
27. Sowers, A.E. (editor) 1987. Membrane Fusion. Plenum, New York
28. Struck, D.K., Hoekstra, D., Pagano, R.E. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20:**4093–4099
29. Waggoner, A.S., Stryer, L. 1970. Fluorescent probes of biological membranes. *Proc. Natl. Acad. Sci. USA* **67:**579–589
30. Weast, R.C. (editor). 1968. Handbook of Chemistry and Physics. The Chemical Rubber Co., Cleveland (OH)
31. Wilshut, J., Papahadjopoulos, D. 1979. Ca^{2+}-induced fusion of phospholipid vesicles monitored by mixing of aqueous contents. *Nature (London)* **281:**690–692

Received 9 June 1989; revised 27 September 1989