Effect of Cholesterol, Diacylglycerol and Phosphatidylethanolamine on PEG 6000 Induced Lipid Mixing and Surface Dielectric Constant of Phosphatidylcholine Vesicle

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Abstract. Using the NBD/Rh fluorescence assay the PEG 6000 induced phospholipid mixing of phosphatidylcholine liposomes containing cholesterol, diacylglycerol or phosphatidylethanolamine was measured. All 3 components shifted the PEG 6000 concentration necessary to induce 50% of maximal phospholipid mixing to lower concentrations. After the addition of PEG cholesterol containing PC liposomes exhibit different values of the surface dielectric constant as measured by the stokes shift of the fluorophore dansyl-PE compared to pure PC, whereas in DAG- and PE-containing PC liposomes no differences were observed. It is concluded that the incorporation of cholesterol leads to a different surface dielectric constant after PEG addition. The changed surface dielectric properties are a prerequest for the onset of fusion, as shown by Ohki and Arnold (1990). The incorporation of DAG and PE into PC membranes leads to structural instabilities as proposed by Siegel et al. (1989). This additional structurally unstable region created by molecules like PE or DAG may shift the onset of fusion to lower PEG concentration.

Key words: Membrane fusion — Diacylglycerol — Cholesterol — Phosphatidylethanolamine — Fluorescence

Introduction

A large number of studies concerning bilayer vesicle fusion processes induced by poly(ethylene glycol) were published (Boni et al. 1981, 1984, MacDonald 1985,

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Parente and Lentz 1986). It is now generally accepted that the first step of fusion, the aggregation of bilayers, is modulated by several factors, such as the initial surface charge of the liposomes, the molecular weight of PEG, the ionic strength and the pH of the solution (Boni et al. 1984). On the other hand, a detailed description of the change of the physicochemical properties such as surface dielectric constant and surface hydrophobicity induced by PEG, was given recently (Ohki and Arnold 1990). The degree of changes of these properties is related to the close adhesion of two interacting membranes. The authors concluded that a correlation between the change of surface dielectric constant and the onset of fusion occurs. The systems used were pure PS or PC liposomes. Now it is known from studies of Ca^{2+}- and Mg^{2+}-induced fusion processes that such membrane components as cholesterol (Shavnin et al. 1988), diacylglycerol (Ellens et al. 1989) and phosphatidylethanolamine (Siegel et al. 1989) have a strong influence on the fusion process under certain conditions. Nieva et al. (1989) measured a phospholipase C induced fusion of PC/PE/Chol LUV. The phospholipase C treatment leads to increased amounts of diacylglycerol in the membrane, the authors claimed this as the fusion inducing event.

It is the aim of this study to investigate the influences of components which were claimed as fusogenic (Ahkong et al. 1973) on the surface dielectric constant and the PEG induced fusion in neutral lipid membranes.

Abbreviations: phosphatidylcholine, PC; phosphatidylethanolamine, PE; dansylphosphatidylethanolamine, DPE; diacylglycerol, DAG; 1-4-nitrobenzo-2-oxa--1,3-diazole-PE, NBD-PE; lissamine rhodamine B sulfonyl-PE, Rh-PE; poly-ethylene glycol, PEG.

Materials and Methods

Egg phosphatidylcholine and egg phosphatidylethanolamine (Avanti Polar Lipids, USA) were chromatographically pure as shown by TLC. PL molecular weights were obtained by suppliers information. Cholesterol (Fisher, USA) was recrystallized from ethanol. 1,2-Dimyristoyl-rac-glycerol (Sigma) was used without further purification.

Fluorophore-labelled phospholipids dansylphosphatidyl- ethanolamine (DPE), 1-4-nitrobenzo-2-oxa-1,3-diazole-PE (NBD-PE) and lissamine rhodamine B sulfonyl-PE (Rh-PE) were obtained from Avanti Polar Lipids. HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid], Ultrol grade, Calbiochem) was used as buffer to all solutions. Poly-ethylene glycol (MW 6000) from Fluka Chemical Co., Switzerland, was used without further purification. All other chemicals used were of reagent grade and obtained from Baker Chemical Company. The water used was distilled three times, including an alkaline permanganate process.

Small unilamellar vesicles were prepared by hydrating lipids in 3 mmol/l HEPES/ 0.1 mol/l NaCl/ 0.05 mmol/l EDTA/ pH 7.5, vortexing for 10 min and then sonicating for 40 min. using a bath type sonifier.

The formation of vesicles and their size was checked using a Coulter N4S submicrometer particle size analyzer at 25 °C.

Measurements of the dielectric constant

DPE was solved together with the phospholipids, cholesterol or diacylglycerol in chloroform at a molar ratio of phospholipid/DPE ~ 200–300. The samples were evaporated, then suspended in the buffer solution. After vortexing (10 min) the lipid suspension was sonicated for 40 min. An aliquot of this vesicle stock solution was suspended in the appropriate buffer solution. The fluorescence signal of DPE was detected by a spectrofluorimeter (Perkin-Elmer, LS-5). The excitation wavelength was 340 nm, the emission was measured in the range of 400–600 nm. From the shifts of the emission spectra maxima the dielectric constants of the DPE environment in the lipid membrane were calculated using the Stokes shift equation which relates the wavelength at the maximum value of the emission spectrum and its dielectric properties (Kimura and Ikegami 1985, Ohki and Arnold 1990).

Phospholipid mixing assay

The fusion of the vesicles was followed by utilization the fluorescence energy transfer method, using NBD-PE and Rh-PE (Struck et al. 1981). The vesicles were composed of PC/Cholesterol, PC/DAG or PC/PE and 1% (mol/mol PC) of both NBD-PE and Rh-PE. They were prepared by sonication in the above mentioned buffer solution. One part (0.05 μmol lipid) of the fluorophore-incorporated vesicles and two parts (0.1 μmol lipid) of the unlabelled vesicles were suspended in at least 0.25 ml of the appropriate buffer solution without PEG 6000. PEG 6000 (40 wt%) containing buffer solution was then added to achieve the PEG concentration required. After intense shaking, the fluorescence measurements were carried out by exciting at 470 nm and recording the fluorescence from 500 to 620 nm. The extent of fusion F was evaluated from the intensity of NBD at 525 nm:

$$F = \frac{I_{525} - I_{525}^0}{I_{525}}$$

where I_{525} was the fluorescence amplitude from the solution containing PEG 6000, and I_{525}^0 without PEG. The experiments were done at room temperature. The 100% value of fusion was defined as the value obtained by solubilization of the vesicles in 0.2% Triton X–100. This value was corrected by the factor 1.5 because of the quenching effect of Triton X–100 (Keren-Zur et al. 1989; Beigel et al. 1988).

The threshold concentration of PEG was defined as at the PEG concentration for 50% of the maximal fluorescence value.

Results

Cholesterol-containing PC liposomes

It is known that the incorporation of cholesterol into egg PC liposomes is possible up to 50 mol% cholesterol (Barton 1976, Ladbrooke et al. 1968). In this range we prepared egg PC liposomes containing cholesterol. In Fig. 1 the surface dielectric constants of the cholesterol containing egg PC liposomes in dependence on the PEG 6000 concentration is given. The surface dielectric constants were calculated

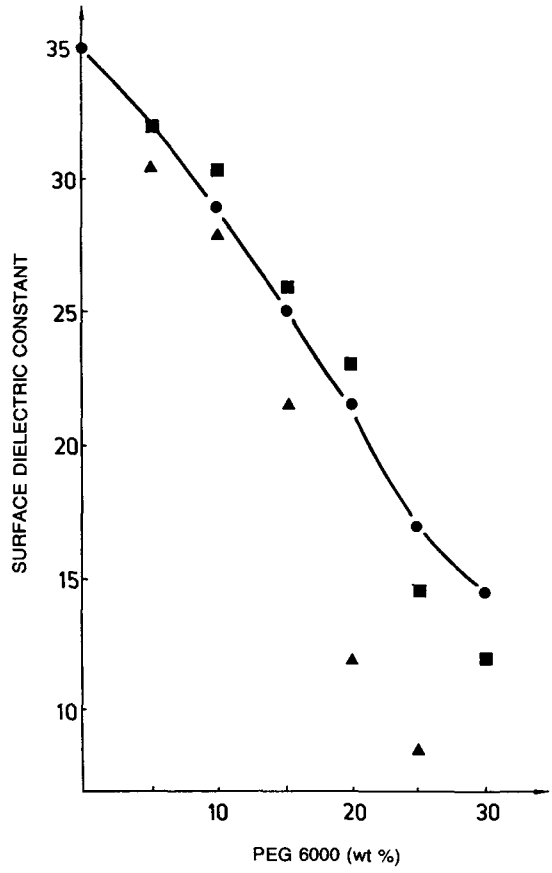

Figure 1. Surface dielectric constant of egg-PC/ Chol SUV as measured by the Stockes shift of DPE in dependence on the PEG 6000 concentration in the solution. ● 0 mol% Chol, ■ 17 mol% Chol, ▲ 42 mol% Chol.

according to Ohki and Arnold (1990). At 0 wt% PEG 6000, there were only slight differences in the surface dielectric constant for pure egg PC liposomes and in 42 mol% cholesterol containing egg PC liposomes. PC liposomes without cholesterol show a surface dielectric constant of about 35, with 17 or 42 mol% cholesterol a surface dielectric constant of about 34. For clarification these points are not shown in Fig. 1. The higher cholesterol content in the liposomes did not lead to lower surface dielectric constants of the membrane. By increasing the PEG 6000 concentration in the surrounding medium an increase in the difference of the surface dielectric constant of cholesterol containing and cholesterol free membranes was observed. Cholesterol containing membranes exhibited a further decrease in sur-

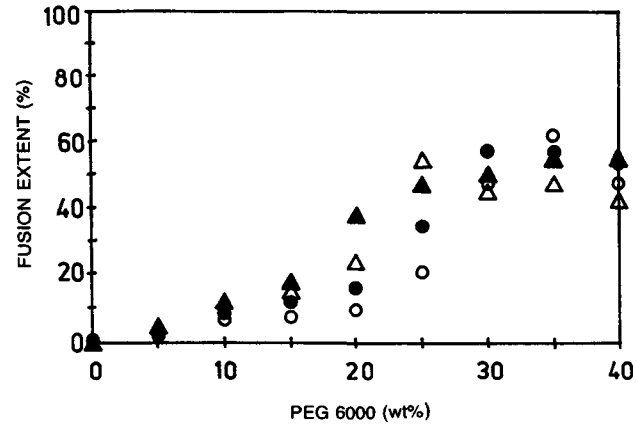

Figure 2. Fusion of egg-PC/ Chol SUV induced by PEG 6000 as measured by the NBD/Rh fluorescence assay. o 0 mol% Chol, • 16.9 mol% Chol, Δ 28.9 mol% Chol, ▲ 37.9 mol% Chol.

face dielectric constant when exposed to high (30 wt%) PEG 6000 concentrations; pure PC liposomes exhibited a surface dielectric constant of about 15 in the presence of 30 wt% PEG 6000, whereas samples of PC liposomes containing 17 mol% cholesterol showed a dielectric constant of about 12 at this PEG concentration and for PC liposomes containing 42 mol% cholesterol the dielectric constant value was below 10.

Fig. 2 shows the results of the application of the NBD/Rh phospholipid mixing assay. Various amounts of PEG 6000 were added to egg PC liposomes containing 0–48 mol% cholesterol. Pure egg PC liposomes started with fusion at about 20 wt% PEG 6000 (threshold concentration 25 wt%). Cholesterol-containing liposomes (42 mol%) started at lower PEG 6000 concentrations with fusion (threshold 17.5 wt%).

Diacylglycerol (DAG) containing liposomes

An appreciable difference of the surface dielectric constant of DAG containing egg PC vesicles in comparison to PC liposomes was not observed (data not shown). In the presence of PEG 6000 there were also no great differences in the surface dielectric constant of egg PC liposomes with different contents of DAG, although in all cases PEG 6000 lowered the surface dielectric constant of the PC/ DAG SUV investigated.

The results of the NBD/ Rh assay are given in Fig. 3. In the investigated range of DAG content in the egg PC liposomes, differences in the fusion characteristics were observed. The higher the DAG concentration in the membrane, the lower was the threshold PEG 6000 concentration.

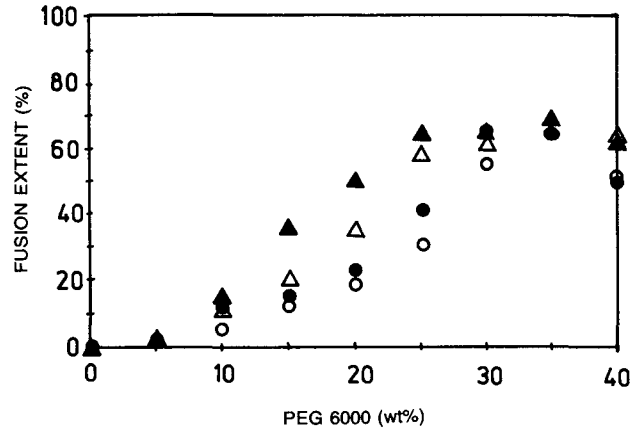

Figure 3. Fusion of egg-PC/ DAG SUV induced by PEG 6000 as measured by the NBD/Rh fluorescence assay. o 0 mol% DAG, • 7 mol% DAG, Δ 13.3 mol% DAG, ▲ 18.7 mol% DAG.

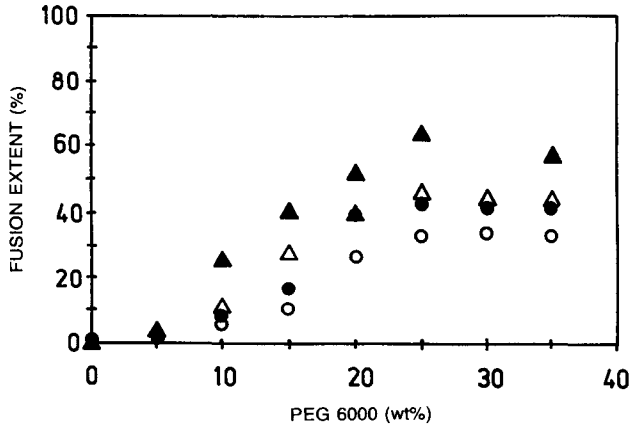

Figure 4. Fusion of egg-PC/ egg-PE SUV induced by PEG 6000 as measured by the NBD/Rh fluorescence assay. o 0 mol% PE, • 30 mol% PE, Δ 39 mol% PE, ▲ 46 mol% PE.

Phosphatidylethanolamine containing liposomes

Between the samples containing different PE contents no differences in the surface dielectric constant were detected. Additionally we checked the influence of different PE concentrations in PC SUV at different pH, no changes in surface dielectric

constant could be detected (data not shown). PEG 6000 caused a reduction of the surface dielectric constant of the PC/PE SUV. The changes in dielectric constant for PC/PE liposomes of different molar ratios after addition of PEG 6000 were about the same.

The fusion characteristics of the PC/PE SUV are shown in Fig. 4. Compared to pure PC vesicles which fuse at about 15–20 wt% PEG 6000 (threshold), the threshold PEG 6000 concentration for PC/PE SUV (46 mol% PE) was about 12.5 wt% PEG 6000. The extent of fusion of the PE containing liposomes was increased compared to pure PC SUV.

Discussion

The influence of PEG on phospholipid membranes seems to be mediated on various indirect ways such as alteration of the dielectric constant of the aqueous phase (Arnold et al. 1985) and changes in the hydration of the phospholipid headgroups (Arnold et al. 1988). The results of a possible PEG binding are contradictory (Arnold et al. 1988, Boni et al. 1981, Boss 1983).

Cholesterol itself does not change drastically the surface dielectric constant of the egg PC membranes. But in the presence of PEG the incorporation of cholesterol results in a stronger change of the dielectric constant. This may be due to the property of cholesterol to release hydrated water easier from the surface when PEG is present. An analog effect was observed by adding PEG to egg PC liposomes in the presence of Ca^{2+} ions (Hoekstra 1982). These more hydrophobic properties of the membrane surface should influence the fusion properties. Indeed, the extent of fusion increased significantly in the presence of cholesterol after addition of PEG. At PEG concentrations, where fusion starts, the dielectric constant at the membrane surface is about 15, indicating that the surface is still hydrophilic. This requires an additional mechanism for bringing together the opposite membrane surfaces.

The effects of cholesterol on the cation induced fusion of PS phospholipid membranes were investigated by Shavnin et al. (1988). They found a decrease of the rate of fusion induced by Ca^{2+} and Mg^{2+} at 25 °C by increasing the cholesterol content in the membranes, whereas at 40 °C the Mg^{2+} induced fusion of PS vesicles is increased by cholesterol. Braun et al. (1985) showed that PS/Cholesterol SUV exhibit a higher fusion rate constant than pure PS vesicles induced by Ca^{2+} at 24 °C. The solution contained 660 mmol/l NaCl to reduce the fusion rate constant in order to allow an accurate determination. Bental et al. (1987) determined the effect of cholesterol on PS LUV fusion induced by Ca^{2+} at 100 mmol/l NaCl. They observed an increased fusion rate constant with increasing cholesterol content, but the overall rate of fusion decreases.

Our observation that the surface dielectric constant of the PC/cholesterol vesicles was not appreciably affected by the amounts of cholesterol incorporated into

the PC membrane in the absence of PEG suggests that cholesterol *per se* may not alter the surface dielectric constant but the cholesterol molecule may more easily release the adsorbed water molecules from its surface compared to the phosphatidylcholine molecule when an external driving force to remove adsorbed water is applied.

Ellens et al. (1989) found an increase of membrane fusion events in PC/PE/ /DAG membranes induced by Mg^{2+}. The concentration of DAG was about 1 mol%. This enhancement in fusion was reasoned in preffered forming of nonbilayer phases of PE by DAG. These nonbilayer phases should act as point defects in the fusion process. However, we were unable to find under our experimental conditions an enhancement of fusion by 1 mol% DAG. There were no differences of DAG containing PC SUV compared to pure PC SUV in respect to the surface dielectric constant with and without PEG addition. From these measurements it can be concluded that the onset of fusion at lower PEG concentrations is not a result of the decrease in dielectric constant. It seems more evidence is given for the above mentioned destabilization of the membrane, probably caused by formation of nonbilayer structures within the membrane. This is supported by the observation that certain diacylglycerols promote the Ca^{2+} induced fusion of PS/PC (molar ratio 1 : 1) (Gomez-Fernandez et al. 1989). ^{31}P-NMR investigations brought evidence that the promotion of the Ca^{2+} induced fusion by these diacylglycerols is connected with the occurence of isotropic structures within the membrane. DAG induces a solid phase/fluid phase immiscibility (Ortiz et al. 1988) and alters the bilayer to hexagonal phase transition temperature of PE (Epand 1985). For the PEG induced fusion of PC/DAG liposomes it seems that point defects occur and have also an influence on the PEG induced fusion process. Lentz et al. (1992) measured the leakage, lipid mixing and vesicle content mixing of DPPC LUV containing up to 5 mol% of different diacylglycerols. Interestingly, they found no content mixing, but lipid mixing after addition of more than 30 wt% PEG 8000.

Burgess et al. (1991, 1992) have observed that PEG does not induce fusion of LUV composed of a single synthetic PC (DPPC) but that is does cause close contact and rapid lipid transfer between these vesicles (Wu and Lentz 1991). PE-containing LUV (DLPE/DOPC) did fuse and the concentration of PEG required to induce fusion decreased as the percentage of PE increased (Burgess et al. 1992). PE is known as bilayer destabilizing component because of its molecular structure like cone shape (Israelachvili et al. 1980). PE decreases the hydration repulsion of PL bilayers (Rand et al. 1988). From this point of view the lower PEG concentrations necessary for induction of lipid mixing of PL vesicles with increased PE content are reasonable.

DPPC LUV can be induced to fuse when they contain small amounts of certain amphipathic compounds such as lyso-PC, platelet activating factor and palmitic acid (Lentz et al. 1992). The common property of these additives is the positive

intrinsic curvature. These fusogenic amphipaths disrupt the normal lamellar lipid packing within their immediate environment, especially the interfacial region of the membrane. This imperfect packing could expose hydrophobic regions of the membrane to water and thereby raise the surface energy of the bilayer (Lentz et al. 1992). In contrast to the usual assumption that fusion is promoted by additives favouring nonbilayer structures, the ability of these amphipaths to enhance fusion is better correlated with the tendency to change lipid packing.

In our measurements no changes in the surface dielectric constant of DAG and PE containing PC SUV were determined whereas the PEG induced fusion was strongly influenced by the presence of PE.

In comparison of the effect of three investigated membrane components, cholesterol, DAG and PE it seems reasonable to offer different mechanisms for the description of the PEG faciliated fusion of SUV composed of PC and these components. Cholesterol changes the surface dielectric constant compared to pure PC SUV after PEG addition. The earlier onset of fusion can be connected with this observation by applying the theory of Ohki and Arnold (1990) concerning surface hydrophobicity and membrane fusion. On the other hand, other membrane components such as DAG and PE would create structurally unstable regions in the membranes, e.g. non-bilayer structures, which would facilitate membrane fusion. However, it cannot be expected that close correlation of fusion extent with the decrease of surface dielectric constant is a property of all fusion systems. Especially in systems where the fusion event is strongly localized in small areas, the delocalized fluorescence probe is unable to detect these changes of surface properties. Moreover, when molecules contributing a high surface energy or defects to a membrane, are mixed with the lipid composing vesicle membranes, the fusion can start at an overall lower surface hydrophobicity if these molecules are located at the fusion site (Ohki 1991; Lentz et al. 1992). The application of the method is restricted to the occurrence of large areas of contacting membranes. Very recently a fluorescence method was developed for the detection of the polarity decrease at the adhesive junction between two model membranes (Brewer 1992).

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