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Ca^{2+}-induced fusion of large unilamellar phosphatidylserine / cholesterol vesicles

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The effect of cholesterol on the Ca^{2+}-induced aggregation and fusion of large unilamellar phosphatidylserine (PS) vesicles has been investigated. Mixing of aqueous vesicle contents was followed continuously with the Tb / dipicolinate assay, while the dissociation of pre-encapsulated Tb / dipicolinate complex was taken as a measure of the release of vesicle contents. Vesicles consisting of pure PS or PS / cholesterol mixtures at molar ratios of 4:1, 2:1 and 1:1 were employed at three different lipid concentrations, each at four different Ca^{2+} concentrations. The results could be well simulated in terms of a mass-action kinetic model, providing separately the rate constants of vesicle aggregation, c_{11}, and of the fusion reaction itself, f_{11}. In the analyses the possibility of deaggregation of aggregated vesicles was considered explicitly. Values of both c_{11} and f_{11} increase steeply with the Ca^{2+} concentration increasing from 2 to 5 mM. With increasing cholesterol content of the vesicles the value of c_{11} decreases, while the rate of the actual fusion reaction, f_{11}, increases. Remarkably, the effect of cholesterol on both aggregation and fusion is quite moderate. The presence of cholesterol in the vesicle bilayer does not affect the leakage of vesicle contents during fusion.

Introduction

Cholesterol is a major constituent of many biological membranes. Numerous studies have been carried out on sterol-containing model membranes in order to delineate the possible functional role of cholesterol in membranes. These have demonstrated that cholesterol has profound effects on the fluidity and barrier properties of lipid bilayers [1]. Cholesterol has also been shown to affect the polymorphic phase behaviour of membrane lipids [2]. However, relatively little is known about a possible role of cholesterol in membrane fusion.

Perhaps one of the best characterized cases that point to a specific function of cholesterol in membrane fusion is that of fusion of certain viruses with target membranes. For example, it has been demonstrated that fusion of Semliki Forest virus at mildly acidic pH requires the presence of cholesterol in the target membrane [3,4], while recent studies on Sendai virus indicate that for virus-induced fusion and lysis of liposomes the presence of cholesterol in the liposomal bilayer is essential [5–7]. It has also been demonstrated that

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicles; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; PS, phosphatidylserine; SUV, small unilamellar vesicles.

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enrichment of erythrocytes with cholesterol enhances their susceptibility to fusion induced by Sendai virus [8]. Another example of biological membrane fusion suggesting a specific involvement of cholesterol is the fusion of myoblasts which has been shown to be inhibited when the cholesterol content of the cells is lowered [9].

A great deal of information regarding the molecular mechanisms of interaction and fusion between lipid bilayer membranes has been obtained from investigation of model systems [10–12]. Utilizing PS vesicles, Fraley et al. [13] have shown that cholesterol greatly reduces the leakage of encapsulated contents during fusion of the vesicles induced by calcium phosphate. Recently, it was found that cholesterol reduces the overall rate of Ca^{2+}-induced fusion in small unilamellar vesicle systems, consisting of various PS/cholesterol mixtures [14]. Kinetic analyses demonstrated that the observed reduction in the overall rate of fusion was due to a decrease of the rate of vesicle aggregation, while the rate of the actual fusion reaction was somewhat enhanced in the presence of cholesterol [14].

In the above study [14] small vesicles with a strongly curved bilayer and a high intrinsic fusion capacity were used. Thus, large NaCl concentrations [15,16] had to be employed in order to slow down the rate of fusion. The present study provides a more complete answer to the question of the effect of cholesterol on the aggregation and fusion of PS vesicles in the presence of Ca^{2+}. Large unilamellar vesicles with an unstrained bilayer and a lower intrinsic fusion capacity were employed. Thus, the effect of bilayer curvature was eliminated and the use of high NaCl concentrations could be avoided. In addition, vesicle deaggregation processes, which could affect the rate constants, were considered explicitly.

Materials and Methods

Materials. Bovine brain phosphatidylserine (PS, grade I) was obtained from Lipid Products (Nutfield, UK) and cholesterol from Sigma Chemical Co. (St. Louis, MO). *N*-NBD-PE was from Avanti Polar Lipids, Inc. (Birmingham, AL). The lipids were stored as a chloroform solution under argon, at −20 °C. $TbCl_3 \cdot 6H_2O$ was obtained from Aldrich (Brussels), dipicolinic acid from Sigma and [^{14}C]sucrose from Amersham International (Amersham, U.K.). All other chemicals were of the highest purity available. Water was distilled twice.

Vesicle preparation. Large unilamellar vesicles (LUV), composed of PS or PS/cholesterol mixtures, were prepared by reverse-phase evaporation and sized to 0.1 μm by successive extrusion through 0.2 μm and 0.1 μm Unipore polycarbonate filters as described before [17]. The internal aqueous contents of the vesicles were either (i) 5 mM $TbCl_3$/50 mM sodium citrate (Tb-vesicles), (ii) 50 mM dipicolinate (sodium salt)/20 mM NaCl (dipicolinate-vesicles), or (iii) 2.5 mM TbCl/25 mM sodium citrate/25 mM dipicolinate (sodium salt)/10 mM NaCl (Tb/dipicolinate-vesicles). In addition, all aqueous media contained 5 mM Hepes, adjusted to a final pH of 7.4. Vesicles were separated from non-encapsulated material by gel filtration on Sephadex G-50, using 100 mM NaCl/5 mM Hepes/1.0 mM EDTA (pH 7.4) as elution buffer. Lipid phosphorus was determined according to the method of Böttcher et al. [18].

Fusion measurements. The fusion assay was carried out as described before [17,19,20]. Briefly, the method invovles the encapsulation of Tb as the Tb (citrate)$_3^{6-}$ complex in one population of vesicles, and the anion of dipicolinic acid in another. Vesicle fusion results in mixing of the vesicle contents and fast formation of the fluorescent Tb(DPA)$_3^{3-}$ complex (designated Tb/dipicolinate complex). Fusion is followed continuously by monitoring the Tb/dipicolinate fluorescence intensity. EDTA (0.1 mM) and Ca^{2+} (> 1 mM), included in the external medium, effectively prevent the formation of the Tb/dipicolinate complex outside the vesicles.

Fluorescence measurements were carried out in a Perkin-Elmer MPF 44 fluorimeter. The experiments were carried out in a final volume of 2.0 ml 100 mM NaCl/5 mM Hepes/0.1 mM EDTA (pH 7.4) which was magnetically stirred and maintained at 25 °C. Routinely, the medium in the cuvette contained the desired $CaCl_2$ concentration. Fusion was initiated by injecting an aliquot (100 μl) of a concentrated 1 : 1 mixture of Tb- and dipicolinate-vesicles, to obtain final lipid phosphorus concentrations of 5, 20 or 100 μM. Excitation of the Tb/dipicolinate complex was at 276 nm; fluorescence emission was measured at 545

nm, with a cutoff filter (< 525 nm) between the sample and the emission monochromator [17]. Excitation and emission slits were set at 10 nm. The fluorescence scale was calibrated such that the 100% value corresponded to the maximal fluorescence intensity that could be obtained with the vesicle concentration used [17]. Therefore, the fluorescence intensity at any time during the fusion reaction represents the percentage of the total amount of Tb that is associated with dipicolinate. All experiments were performed in duplicate. Correction for Tb/dipicolinate complex dissociation due to its release to the external medium or due to entry of Ca^{2+} and/or EDTA into the vesicles was made by following the decrease in the complex fluorescence intensity upon injection of the appropriate concentration of vesicles containing the pre-encapsulated Tb/dipicolinate complex (Tb/dipicolinate-vesicles) into the Ca^{2+}-containing buffer, under the same conditions that were employed in the fusion measurements [16].

Lipid phase separation measurements. The extent of lipid phase separation in PS/cholesterol vesicles was determined according to the method of Hoekstra [21]. The fluorescent probe *N*-NBD-PE was incorporated in the lipid bilayer of the vesicles and the degree of its self-quenching determined by measuring fluorescence before and after addition of Triton X-100 (1%, v/v) with appropriate correction of the latter value for the effect of Triton on the fluorescence quantum yield of *N*-NBD-PE [21]. Excitation was at 465 nm and emission was measured at 530 nm. The average surface density of *N*-NBD-PE in the different vesicle preparations was kept at 1 molecule per 1460 Å^2, which corresponds to 4.8 mol% in pure PS vesicles and 3.6 mol% in PS/cholesterol (molar ratio, 1 : 1), given an area per molecule of 70 Å^2 for PS and 35 Å^2 for cholesterol [22].

Determination of the encapsulated volume and the molar concentration of the vesicles. Encapsulation of Tb, dipicolinate and Tb/dipicolinate in the vesicles was determined as described before [17]. For comparison, 1 mM [^{14}C]sucrose (0.5 Ci/mol) was added to the media in which the vesicles were made and its encapsulation in the vesicles was determined by radioactivity measurements. Encapsulated volumes calculated on the basis of the entrapped [^{14}C]sucrose were essentially the same as the corresponding internal volumes based on the entrapment of either Tb, dipicolinate or Tb/dipicolinate.

The molar concentration of the vesicles was determined on the basis of the encapsulated volume of the vesicles. The inner vesicle radius was calculated from a set of two equations

$$\frac{4\pi N}{3} R^3 = v$$

$$4\pi N [R^2 + (R + d)^2]/A = N_l$$

in which R is the inner vesicle radius, v the encapsulated volume per unit volume, N the number of vesicles per unit volume, d the bilayer thickness (40 Å), A the average area per PS molecule and N_l the number of PS molecules per unit volume. The average area per PS molecule in a PS/cholesterol vesicle was calculated by taking 70 Å^2 for a PS molecule and half of that value (35 Å^2) for cholesterol. Thus, for example at PS/cholesterol molar ratios of 4 : 1 and 2 : 1 the values for A used were 78.8 Å^2 and 93.3 Å^2, respectively.

The size of the vesicles was not significantly affected by the presence of cholesterol. For example, the average encapsulated volume of vesicles consisting of pure PS was 3.0 litre per mol of phospholipid. This value, according to the above equations, corresponds to an inner vesicle radius of 460 Å and a vesicle concentration at 100 μM PS of $1.2 \cdot 10^{-9}$ M. The encapsulated volume of PS/cholesterol (molar ratio, 1 : 1) vesicles was 4.5 litre per mol of phospholipid. In this case the inner vesicle radius is 465 Å, corresponding to a vesicle concentration at 100 μM PS of $1.8 \cdot 10^{-9}$ M.

Theoretical analysis. The overall fusion reaction is modeled to consist of two main steps: (i) the aggregation of the vesicles, and (ii) the fusion reaction itself, resulting in coalescence of the inner vesicle volumes. The initial stage of the fusion process, in which a fused doublet, denoted by F_2, is formed through fusion of an aggregated dimer V_2, is given by

$$V_1 + V_1 \underset{d_{11}}{\overset{c_{11}}{\rightleftarrows}} V_2 \xrightarrow{f_{11}} F_2$$

The rate of formation of V_2 from two single

vesicles V_1, is of second order in the vesicle concentration and is described by the rate constant c_{11} ($M^{-1} \cdot s^{-1}$). The dimers V_2 can either fuse or deaggregate. These reactions are of first order with rate constants f_{11} (s^{-1}) and d_{11} (s^{-1}), respectively. The kinetic equations, describing the disappearance of V_1 and the formation of V_2 and F_2, can be solved by numerical integration. This provides a simulation of the time-course of fluorescence intensity determined experimentally. Thus, the kinetic parameters of the aggregation and fusion process can be determined by fitting the experimental data to the theoretical calculation.

Measurements were performed at vesicle concentrations varying from 5 to 100 μM phospholipid. The dilute vesicle concentration should satisfy the relation $c_{11}V_0 \ll f_{11}$, in which V_0 is the molar concentration of vesicles. Such a condition enables the determination of the rate constant c_{11}, since in this case the overall fusion process is rate limited by the aggregation. The rate of fusion in concentrated vesicle suspensions is more sensitive to the value of f_{11}, and can be used to determine f_{11}, provided that $c_{11}V_0 > f_{11}/100$.

The first stage in the analysis consists of obtaining the time-course of the quantity $I = F + 0.5\,D$, in which F is the % fluorescence of the Tb/dipicolinate complex in the fusion assay and D the dissociation of the pre-encapsulated complex due to its leakage and entry of EDTA and Ca^{2+} into the vesicles [16]. The factor 0.5 in the expression for I is due to the fact that only 50% of the fused doublets are productive in terms of fluorescence development. Subsequently, the experimental results at different vesicle concentrations are simulated in terms of the model, the essential aspects of which are outlined above, thus providing the values for the parameters c_{11}, d_{11} and f_{11}.

Details of the model and the procedure employed in the simulation of the experimental results for I values up to 20% are given in [10,15,16,23,24]. An extension of the procedure to higher I values is described in Ref. 25 and has been employed in the analysis of the kinetics of bilayer lipid mixing and internal contents mixing during fusion of cardiolipin- or PS-containing vesicles [26,27]. It should be noted that the current procedure of numerical calculation of the time-course of fluorescence intensity does not just consider doublet formation, but takes into account aggregation-fusion products consisting of up to 16 original vesicle monomers. To limit the number of adjustable parameters to a minimum, we employed for the higher-order aggregation, deaggregation and fusion reactions the same rate constants (c_{11}, d_{11} and f_{11}), respectively as for the initial dimerization. By doing so, we do not intend to suggest that the higher-order rate constants are identical to those of the initial dimerization reaction. However, up to I values of approx. 25%, as considered here, fluorescence is contribted almost exclusively by doublets.

Results

Measurements of the kinetics of vesicle fusion (Tb/dipicolinate complex formation) and the dissociation of pre-encapsulated Tb/dipicolinate complex included four vesicle compositions: pure PS and PS/cholesterol mixtures at molar ratios of 4:1, 2:1 and 1:1. Three vesicle concentrations were employed, corresponding to PS concentrations in pure and mixed vesicles of 5, 20 and 100 μM. For brevity we present only a sample of the results. The data points in Fig. 1 show the observed enhancement of the overall rate of fusion with increasing vesicle concentrations and the effect of

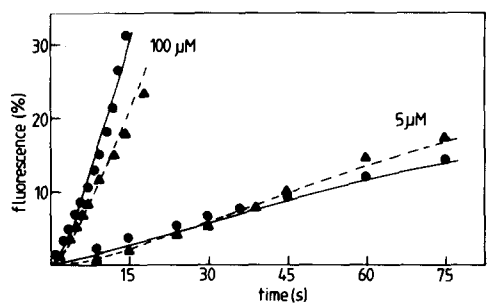

Fig. 1. Effect of cholesterol on the fusion of PS LUV at different vesicle concentrations in the presence of 5 mM Ca^{2+}. Data points represent the experimental time-course of Tb/dipicolinate fluorescence intensity corrected for leakage of vesicle contents. Vesicles were injected into the medium in the cuvette, containing 5 mM $CaCl_2$, to the final concentrations (micromolar lipid phosphorus) indicated. The corresponding theoretical simulations are given by the solid or dashed lines. ●———●, PS/cholesterol (2:1); ▲------▲, PS.

cholesterol at a PS/cholesterol molar ratio of 2 : 1. It is evident that at relatively high vesicle concentrations the presence of cholesterol results in an increase of the overall rate of fusion, whereas at lower vesicle concentrations the rate of fusion is slightly reduced. Fig. 2 shows the effect of Ca^{2+} on the overall rate of fusion of PS/cholesterol (2 : 1) vesicles at a vesicle concentration of 20 μM (lipid phosphorus).

By analyzing the data obtained at the three different vesicle concentrations studied, for each particular vesicle composition and Ca^{2+} concentration, we determined the effects of cholesterol on the separate stages of the fusion process, i.e., the initial vesicle aggregation and the subsequent fusion reaction. The results for each condition could be well simulated by the model. Examples of the fit are given in Figs. 1 and 2. Table I presents a survey of the rate constants of the aggregation and fusion steps for vesicles consisting of pure PS or PS/cholesterol mixtures at molar ratios of 4 : 1 and 2 : 1. The results indicate that the c_{11} values decrease and that the f_{11} values increase with increasing cholesterol content of the vesicles. Both effects are rather moderate. The table also illustrates the increase in c_{11} and f_{11} values with increasing Ca^{2+} concentration.

It should be noted that the values of the rate constants in Table I correspond to a value of the deaggregation rate constant, d_{11}, of zero. A setting of $d_{11} = 0$ produced an optimal fit of the data to the model. However, we can not completely rule out the possibility that d_{11} equals f_{11} or $1/2\ f_{11}$, in which case the variation in the rate constants c_{11} and f_{11} as a function of the cholesterol content of the vesicles would become even smaller than shown in Table I.

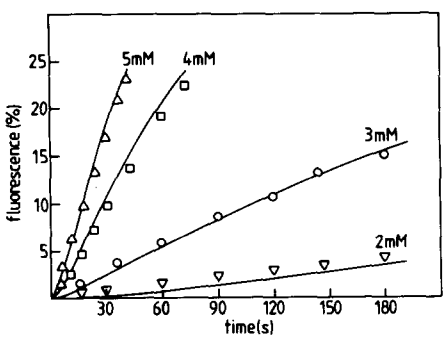

Fig. 2. Effect of the Ca^{2+} concentration on the fusion of PS/cholesterol (2:1) LUV. Data points represent the experimental time-course of Tb/dipicolinate fluorescence intensity, corrected for leakage of vesicle contents. Vesicles were injected to a final concentration of 20 μM (lipid phosphorus) into the medium in the cuvette containing the millimolar $CaCl_2$ concentrations indicated. The lines represent the corresponding theoretical simulations, in which the rate constants presented in Table I were employed.

Cholesterol has been reported to drastically reduce the passive diffusion of water-soluble molecules through lipid bilayer membranes [1], including those consisting of PS [28]. By contrast, in the present study we observed that incorporation of cholesterol in PS vesicles has no significant inhibitory effect on the release of vesicle contents that occurs as a result of fusion of the vesicles in the presence of Ca^{2+}. Table II shows the relative kinetics of mixing of aqueous vesicle contents and of release for pure PS vesicles and PS/cholesterol (2 : 1) vesicles in the presence of 5 mM Ca^{2+}. Clearly, the leakage per fusion, given by the $D/(F + 0.5D)$ values in the table, is not appreciably reduced by the presence of cholesterol in the vesicles.

It has been noted previously that in the early stages of the fusion process the release of internal contents from PS LUV is marginal, particularly at higher vesicle concentrations [10,16,17,19,20,23, 24,27]. This relative nonleakiness of the initial fusion events can also be seen from the results in Table II.

The survey of rate constants in Table I does not include values on vesicles consisting of PS/cholesterol at a 1 : 1 molar ratio. As shown in Fig. 3, with vesicles of this composition we observed a considerable decrease of the overall rate of fusion relative to the results obtained with PS/cholesterol (2 : 1) vesicles. Due to the large degree of release, relative to mixing of vesicle contents (Fig. 3), kinetic analysis of the cases involving PS/cholesterol (1 : 1) vesicles became less reliable. It can be concluded with certainty that the rate of aggregation of PS/cholesterol (1 : 1) vesicles is significantly lower than that of PS/cholesterol (2 : 1) vesicles. However, it is difficult to determine whether or not the rate of fusion is also reduced.

In order to evaluate the effect of cholesterol on PS vesicle bilayers, possibly underlying its effect on the fusion of the vesicles, we examined the

TABLE I

EFFECT OF CHOLESTEROL ON THE Ca^{2+}-INDUCED AGGREGATION AND FUSION OF PS LUV: SURVEY OF RATE CONSTANTS

The relative uncertainty in the c_{11} values is ±20%. For the f_{11} values the uncertainty is ±40%, unless specified by the range of values indicated, which is somewhat overestimated.

Ca^{2+} concn. (mM)	PS		PS/cholesterol (4:1) [a]		PS/cholesterol (2:1) [a]	
	c_{11} ($M^{-1}\cdot s^{-1}$)	f_{11} (s^{-1})	c_{11} ($M^{-1}\cdot s^{-1}$)	f_{11} (s^{-1})	c_{11} ($M^{-1}\cdot s^{-1}$)	f_{11} (s^{-1})
2	$2.3\cdot10^6$	0.004–0.01	$1.6\cdot10^6$	0.004–0.01	$1.0\cdot10^6$	0.01–0.05
3	$1.2\cdot10^7$	0.02 –0.03	$5.0\cdot10^6$	0.07	$3.4\cdot10^6$	0.12
4	$3.3\cdot10^7$	0.05	$1.7\cdot10^7$	0.08	$1.6\cdot10^7$	0.16
5	$6.0\cdot10^7$	0.08	$3.3\cdot10^7$	0.12	$3.0\cdot10^7$	0.20

[a] Molar ratio.

occurrence of pre-existing lipid phase separations in the vesicles. Phase separations have been reported for cholesterol-containing vesicles [21,29–32]. Hoekstra [21] has shown that the fluorescence self-quenching of *N*-NBD-PE can be utilized to measure the extent of lipid phase separation in cholesterol-containing vesicles. In the present study we used the same technique. Fig. 4 shows that cholesterol does not produce any detectable phase separation in PS LUV up to a PS/cholesterol molar ratio of 2:1. At higher cholesterol contents the self-quenching of *N*-NBD-PE increases significantly, indicating the coexistence of cholesterol-rich and cholesterol-poor domains in the vesicle bilayer. This result is in agreement with observations of Bach [32] on PS/cholesterol multibilayer systems.

TABLE II

EFFECT OF CHOLESTEROL ON THE RELATIVE KINETICS OF MIXING AND RELEASE OF VESICLE CONTENTS DURING Ca^{2+}-INDUCED FUSION OF PS LUV

Mixing of vesicle contents (F) and release of pre-encapsulated Tb/dipicolinate complex, resulting in its dissociation (D), were measured at the vesicle concentrations indicated in the presence of 5 mM Ca^{2+}. Relative release is given by $D/(F+0.5D)$, in which $F+0.5D$ represents the mixing of contents corrected for release (Refs. 16,24).

Vesicle concn. (μM PS)	Time (s)	PS			PS/cholesterol (2:1) [a]		
		F (%)	D (%)	$D/(F+0.5D)$	F (%)	D (%)	$D/(F+0.5D)$
5	30	5.1	0.3	0.06	6.9	0	0
	45	9.1	0.8	0.08	9.3	0.4	0.04
	60	12.8	1.3	0.10	11.5	1.5	0.12
	90	19.0	4.0	0.19	14.7	4.6	0.27
	120	23.0	7.1	0.27	16.2	8.8	0.43
20	12	4.8	0	0	6.6	0	0
	24	12.6	0	0	13.1	0.2	0.02
	36	20.1	1.0	0.05	19.5	2.4	0.12
	48	26.5	4.2	0.15	22.7	8.4	0.31
100	6	7.3	0	0	8.7	0	0
	12	14.5	0.9	0.06	21.2	0.4	0.02
	18	20.7	4.2	0.18	43.8	6.3	0.13

[a] Molar ratio.

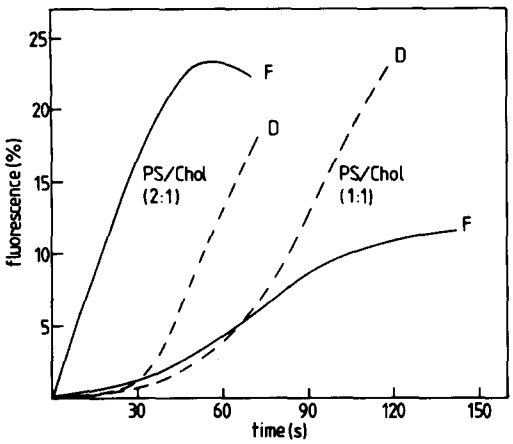

Fig. 3. Relative kinetics of mixing and release of aqueous vesicle contents during fusion of PS/cholesterol (2:1) or PS/cholesterol (1:1) LUV in the presence of 5 mM Ca^{2+}. The solid lines present the time course of Tb/dipicolinate fluorescence intensity, F, after injection of a 1:1 mixture of Tb/ and dipicolinate-vesicles into the cuvette to a final concentration of 20 μM (lipid phosphorus). The dashed lines show the corresponding release experiments, in which the decrease of Tb/dipicolinate fluorescence, due to dissociation (D) of the Tb/dipicolinate complex, was monitored after injection of Tb/dipicolinate-vesicles into the cuvette to the same final lipid concentration as that in the fusion experiment. The PS/cholesterol molar ratio in the vesicles is indicated.

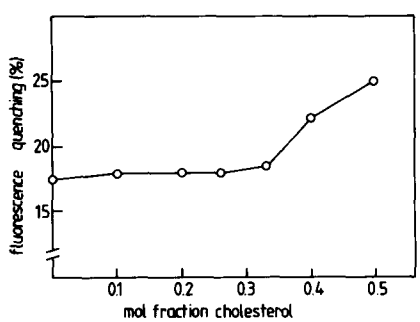

Fig. 4. Lipid phase separations in PS/cholesterol LUV: dependence of the self-quenching of *N*-NBD-PE on the mole fraction of cholesterol. *N*-NBD-PE was incorporated in PS/cholesterol LUV, containing increasing amounts of cholesterol, and the fluorescence self-quenching of the fluorophore was determined, as described in Materials and Methods.

Discussion

The results presented in this paper demonstrate that incorporation of cholesterol in PS LUV results in a moderate reduction of the rate of Ca^{2+}-induced vesicle aggregation, while at the same time the rate of the actual fusion reaction is enhanced to an equally limited extent. This conclusion is in agreement with the results of a previous investigation of the effects of cholesterol on the Ca^{2+}-induced fusion of PS SUV [14].

The observation that cholesterol enhances the tendency of PS vesicles to fuse, while it slows down the rate of vesicle aggregation, provides an explanation for the opposite effects of cholesterol on the overall rate of fusion, depending on the vesicle concentration. In dilute vesicle suspensions, where $c_{11}V_0 \ll f_{11}$, the overall rate of fusion is mainly determined by the rate of vesicle aggregation, whereas in more concentrated suspensions the rate of the fusion reaction itself becomes increasingly important. This is illustrated in Fig. 1, which shows that the stimulatory effect of cholesterol on the fusion reaction per se becomes apparent only at the higher vesicle concentrations.

The rate of vesicle aggregation is proportional to $\exp(-V^*/kT)$, where V^* is the height of the potential barrier for close approach of the vesicles. According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory [33] the total intervesicle potential energy can be considered as the sum of an attractive Van der Waals term and a repulsive electrostatic term [10,34]. In PS vesicle systems the presence of Ca^{2+} reduces the electrostatic repulsion, mainly as a result of binding of the ions to the negatively charged lipid molecules [10,15,16, 23,24]. This explains the dramatic increase of the c_{11} values for PS vesicles in the presence of increasing Ca^{2+} concentrations (Table II, Fig. 2). However, the decrease of the c_{11} values with increasing cholesterol content of the vesicles is less easily understood. Electrostatic repulsion is expected to be reduced for vesicles consisting of PS/cholesterol mixtures, relative to those consisting of pure PS, due to a decreased average surface charge density in the presence of the sterol. Furthermore, Van der Waals attraction is expected to increase slightly in the presence of cholesterol [35,36]. That nonetheless the rate of Ca^{2+}-induced vesicle aggregation is reduced in the presence of cholesterol, albeit to a limited extent, may be due to a moderately decreased binding of Ca^{2+} to the vesicles. Such an explanation would be consistent

with observations of Gregory and Ginsberg [37], who reported a slight reduction of Ca^+ binding to PS in monolayer systems due to the presence of up to 33 mol% of cholesterol in the monolayer. Alternatively, short-range repulsive hydration forces [38] may be enhanced by the presence of cholesterol in the vesicle bilayer [39,40], thus interfering with the formation of stable vesicle aggregates.

The increase of f_{11} with an increasing cholesterol content of the vesicles up to a PS/cholesterol molar ratio of 2:1 (Table I) demonstrates that, once the vesicles are aggregated, they have a greater tendency to fuse in the presence of cholesterol than in its absence. This enhanced tendency to fuse is also indicated by the capacity of PS/cholesterol (2:1) LUV to fuse in the presence of Mg^{2+} (results not shown), whereas PS LUV merely aggregate under these conditions [19]. The fusion-promoting influence of cholesterol may be related to the presence of cholesterol-rich and cholesterol-poor microdomains in the vesicle bilayer prior to the initiation of the aggregation and fusion process. Even though the fluorescence data in Fig. 4, and the calorimetric data of Bach [32], do not reveal any lipid phase separation in PS/cholesterol mixtures up to molar ratios of 2:1, the presence of microdomains, that go unnoticed with these techniques, can not be excluded. In fact, there is evidence indicating the presence of microdomains in cholesterol-containing phosphatidylcholine or sphingomyelin bilayers, even at relatively low cholesterol contents [29,30]. Imperfections in lipid packing at microdomain boundaries can be expected to render the apposed vesicle bilayers susceptible to direct hydrophobic interactions and, thus, could serve as nucleation points for fusion.

The decrease of c_{11} with increasing cholesterol contents of the vesicles includes the case of the PS/cholesterol (1:1) mixture. However, as indicated in Results, we could not determine unambiguously whether f_{11} continues to increase up to cholesterol contents of the vesicles of 50 mol%. At such high cholesterol levels there is an extensive phase separation in the system (Fig. 4, Refs. 29–32). In contrast to the situation at lower cholesterol levels, where presumably microdomains coexist, under these conditions domain boundaries are likely to be less abundant. This may result in a reduction of the rate of fusion for vesicles with a high cholesterol content.

A surprising outcome of the present study is the marginal extent of the effect of cholesterol on the fusion of PS vesicles. It has been demonstrated previously [41] that the Ca^{2+}-induced fusion of PS LUV is dependent on the fluidity of the vesicle bilayer: vesicles that are in the gel state do not fuse, but merely aggregate in the presence of Ca^{2+}. In the light of this observation we did not expect cholesterol, which presumably creates a state of substantially reduced fluidity in PS bilayer systems [1], to have such a small effect on the Ca^{2+}-induced fusion of PS vesicles. Equally surprising is the observation that cholesterol does not reduce the leakage of internal contents during fusion (Table II). This result supports the proposal made earlier [17,19,20] that the leakage of vesicle contents occurring as a result of vesicle fusion, does not proceed via a mechanism of passive diffusion but rather by collapse and rupture of fused vesicles. Incorporation of cholesterol in PS vesicles does reduce the release of vesicle contents associated with fusion induced by calcium phosphate [13]. Indeed, in this case there is evidence indicating that the passive permeability of the vesicles is affected, since the induced permeability changes appear to result from alterations in the crystal structure of calcium phosphate complexes at the vesicle surface [13].

The marginal effect of cholesterol on PS vesicle fusion indicates that in cases where cholesterol is a prerequisite for fusion, its role is of a different character than in the PS vesicle system. For example, the cholesterol requirement in the fusion of Semliki Forest virus [3,4], rather than representing an overall effect of the fluidity of the target membrane lipids, is likely to reflect a specific interaction of cholesterol with one of the viral spike glycoproteins, possibly leading to local fluctuations in lipid packing and bilayer destabilization.

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References

1 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132
2 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207–218
3 White, J. and Helenius, A. (1980) Proc. Natl. Acad. Sci. USA 77, 3273–3277
4 Kielian, M.C. and Helenius, A. (1984) J. Virol. 52, 281–283
5 Kundrot, C.E., Spangler, E.A., Kendall, D.A., MacDonald, R.C. and MacDonald, R.I. (1983) Proc. Natl. Acad. Sci. USA 80, 1608–1612
6 Hsu, M.-C., Scheid, A. and Choppin, P.W. (1983) Virology 126, 361–369
7 Citovsky, V., Blumenthal, R. and Loyter, A. (1985) FEBS Lett. 193, 135–140
8 Hope, M.J., Bruckdorfer, K.R., Hart, C.A. and Lucy, J.A. (1977) Biochem. J. 166, 255–263
9 Horwitz, A.F., Wight, A., Ludwig, P. and Cornell, R. (1978) J. Cell Biol. 77, 334–357
10 Nir, S., Bentz, J., Wilschut, J. and Düzgüneş, N. (1983) Progr. Surf. Sci. 13, 1–124
11 Wilschut, J. and Hoekstra, D. (1984) Trends Biochem. Sci. 9, 479–483
12 Wilschut, J. and Hoekstra, D. (1986) Chem. Phys. Lipids, 40, 145–166
13 Fraley, R., Wilschut, J., Düzgüneş, N., Smith, C. and Papahadjopoulos, D. (1980) Biochemistry 19, 6021–6029
14 Braun, G., Lelkes, P.I. and Nir, S. (1985) Biochim. Biophys. Acta 812, 688–694
15 Nir, S., Düzgüneş, N. and Bentz, J. (1983) Biochim. Biophys. Acta 735, 160–172
16 Bentz, J., Düzgüneş, N. and Nir, S. (1983) Biochemistry 22, 3320–3330
17 Wilschut, J., Düzgüneş, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021
18 Böttcher, C.S.F., Van Gent, C.M. and Fries, C. (1961) Anal. Chim. Acta 24, 203–204
19 Wilschut, J., Düzgüneş, N. and Papahadjopoulos, D. (1981) Biochemistry 20, 3126–3133
20 Wilschut, J., Düzgüneş, N., Hong, K., Hoekstra, D. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 309–318
21 Hoekstra, D. (1982) Biochemistry 21, 1055–1061
22 Papahadjopoulos, D. and Kimelberg, H.K. (1973) Progr. Surf. Sci. 4, 141–232
23 Nir, S., Wilschut, J. and Bentz, J. (1982) Biochim. Biophys. Acta 688, 275–278
24 Bentz, J., Nir, S. and Wilschut, J. (1983) Colloids Surf. 6, 333–363
25 Nir, S., Stegmann, T. and Wilschut, J. (1986) Biochemistry 25, 257–266
26 Wilschut, J., Nir, S., Scholma, J. and Hoekstra, D. (1985) Biochemistry 24, 4630–4636
27 Wilschut, J., Scholma, J., Bental, M., Hoekstra, D. and Nir, S. (1985) Biochim. Biophys. Acta 821, 45–55
28 Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta 266, 501–583
29 Estep, T.N., Mountcastle, D.B., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1979) Biochemistry 18, 2112–1227
30 Snyder, E. and Freire, E. (1980) Proc. Natl. Acad. Sci. USA 77, 4055–4059
31 Hoekstra, D. (1982) Biochemistry 21, 2833–2840
32 Bach, D. (1984) Chem. Phys. Lipids 35, 385–392
33 Verwey, E.J. and Overbeek, J.Th. (1948) Theory of the Stability of Lyophobic Colloids, Elsevier, Amsterdam
34 Bentz, J. and Nir, S. (1981) Proc. Natl. Acad. Sci. USA 78, 1634–1637
35 Nir, S. (1977) Progr. Surf. Sci. 8, 1–58
36 Nir, S. and Andersen, M. (1977) J. Membrane Biol. 31, 1–18
37 Gregory, D.P. and Ginsberg, L. (1984) Biochim. Biophys. Acta 769, 238–244
38 Rand, R.P. (1981) Annu. Rev. Biophys. Bioeng. 10, 277–314
39 Jendrasiak, G.L. and Hasty, J.H. (1974) Biochim. Biophys. Acta 337, 79–91
40 Lis, L.J., McAllister, M., Fuller, N., Rand, R.P. and Parsegian, V.A. (1982) Biophys. J. 37, 657–666
41 Wilschut, J., Düzgüneş, N., Hoekstra, D. and Papahadjopoulos, D. (1985) Biochemistry 24, 8–14