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Cholesterol stabilizes hemifused phospholipid bilayer vesicles

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Abstract

Cholesterol was found to inhibit full fusion of oppositely charged phospholipid bilayer vesicles by stabilizing the contacting membranes at the stage of the hemifused intermediate. Vesicles of opposite charge containing different amounts of cholesterol were prepared using cationic (1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine) and anionic (dioleoylphosphatidylglycerol) phospholipids. Pairwise interactions between such vesicles were observed by fluorescence video microscopy in real time after electrophoretically maneuvering the vesicles into contact. Hemifusion accounted for more than 80% of the observed events when the vesicles contained 33–50 mole% cholesterol. In contrast, vesicles containing only a small proportion of cholesterol (≤10 mole%), underwent full fusion in approx. 70% of the interactions monitored. The role of cholesterol is explained both as favoring the formation of the hemifused intermediate according to the adhesion-condensation mechanism of bilayer fusion and as disfavoring the transition from hemifusion to full fusion on the basis of reduced tension in the vesicle bilayers.

Keywords: Hemifusion; Membrane fusion; Adhesion-condensation; Lipid bilayer

1. Introduction

Membrane fusion is fundamental to all eukaryotic cells. Considerable efforts have therefore been devoted to unraveling the molecular and biophysical determinants of the fusion event. Despite these efforts, surprisingly little is known about the mechanisms underlying membrane fusion. What is known is that fusion requires two lipid membranes to come into contact, resulting in the destabilization of the bilayers at the contact region and the exchange of inner contents. The sequence of events leading to membrane fusion remains obscure, but hemifusion has been regarded as a likely intermediate stage. Hemifusion represents the process in which the initially contacting (*cis*) monolayers fuse and retract from the fusion zone, allowing the remaining two (*trans*) monolayers to form a single bilayer separating the aqueous phases of the fusing bodies. Indeed, hemifusion was postulated long ago on the basis of electron microscope evidence to be an intermediate in the fusion of natural membranes [1]. More recently, lipid bilayer vesicles have been studied as model systems for membrane fusion and in several situations evidence consistent with hemifused vesicles as a stable state has been obtained, i.e., the lipids of the outer (*cis*) monolayers of the two apposed bilayers mixed without destabilizing the inner (*trans*) monolayers of the membranes [2–4].

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Most mechanisms proposed for membrane fusion of lipid bilayers include hemifused membranes as an intermediate, followed by full fusion which occurs when the remaining bilayer septum breaks. For example, hemifused intermediates are predicted by the popular stalk-pore model of membrane fusion [5]. The adhesion-condensation mechanism [6], which appears to account particularly well for the fusion of oppositely charged bilayers, has an obligatory hemifused intermediate [7]. Another proposed mechanism, the sequential model proposed the for polyethyleneglycol-mediated fusion of small unilamellar vesicles, also involves hemifused intermediates [8].

Cholesterol has been found to have diverse effects on fusion in both natural and bilayer model membranes, as well as hybrid systems. Cholesterol is generally recognized as being required for fusion of Semliki Forest virus with lipid bilayer vesicles [9]. While the natural cell membrane target of this virus contains cholesterol, some strains of the virus fuse better with membranes depleted of cholesterol. Cholesterol has been reported to be required for fusion of Sendai virus with artificial host membranes [10], although other investigators did not observe such a requirement [11]. Similarly, bilayer-bilayer fusion is either stimulated or reduced by the presence of cholesterol in the participating membranes, depending upon the conditions (for a review that covers most of the important model membrane literature, see [12]). For example, phosphatidylglycerol vesicle fusion induced by calcium ion is reduced by cholesterol [13], adhesion rates of phosphatidylserine vesicles are reduced but fusion rates are increased by cholesterol [14], and phosphatidylserine vesicle fusion in the presence of magnesium ion is increased by cholesterol [12].

Much is known about the physical effects of cholesterol on bilayer membranes, although it remains unclear which, if any, of these accounts for the ubiquitous presence of this molecule in animal cell surface membranes. Best known is the effect that cholesterol has in increasing the microviscosity of phospholipid bilayers [15,16] by constraining the upper portions of the lipid acyl chains and reducing conformational variability [17]. Cholesterol also influences the phase behavior of pure lipid membranes, favoring the liquid crystalline phase for lipids that would otherwise adopt the gel phase [18,19]. Cholesterol also has a small head group relative to the cross-sectional area of the ring system and as a result can cause polymorphic transitions from the lamellar phase to the hexagonal phase [20]. This latter effect appears to be most relevant to its effects on the fusion processes we describe here.

Although others have studied the fusion of oppositely charged vesicles, the most common technique has involved whole populations of vesicles [21–23], a procedure that does not allow examination of the stages of the process. Adhesion of such vesicles, one of which was held with a micropipette, has been examined by phase microscopy [24,25]. A system has recently been developed for the direct visualization of vesicle-vesicle interactions in which both vesicles are free-floating [7]. Based on an electrophoretic fluid cell that allows precise two-dimensional movement of chosen pairs of oppositely charged giant bilayer vesicles, one can observe with fluorescence microscopy the consequences of interactions following vesicle contact. Using these methods, we found that cholesterol stabilizes vesicle pairs in the hemifused state when present in the participating membrane at concentrations of 33–50 mole%. In the absence of cholesterol, or at significantly lower concentrations, complete vesicle fusion occurs with coalescence of the aqueous compartments of both vesicles. The cholesterol content of simple membranes can thus determine the extent of their fusion.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycerophosphoglycerol (DOPG) was obtained from Avanti Polar Lipids (Alabaster, AL) and cholesterol from Sigma (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC) was either purchased from Avanti as the chloride salt or prepared in the laboratory as the trifluoromethylsulfonate salt from dioleoylphosphatidylcholine by alkylating the unsubstituted phosphate oxygen as previously described [26,27]. *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine (RhPE) was obtained from Molecular Probes (Eugene, OR) and *N*,*N*-dioctadecyloxacarbocyanine (DOC) was from Eastman Kodak (Rochester, NY). Calcein was from GFS Chemical Co. (Powell, OH).

2.2. Vesicle preparation

Vesicle samples (typically tens of micrograms per batch) were prepared by mixing the desired molar ratios of lipids in chloroform (typically at about 1 mg/ml) at room temperature. The bulk of the chloroform was evaporated by blowing a stream of argon gas over the sample, followed by 15–30 min of high vacuum to remove residual solvent. The dried lipids were hydrated with 200 or 320 mM sucrose to a final lipid concentration of 0.1 mg/ml and gently swirled. Samples were usually observed within an hour of preparation, but if necessary were stored at 4°C.

In the case of experiments to determine if hemifusion led to any transfer from the aqueous compartment of one vesicle to that of the other, the cationic lipids were hydrated in 320 mM sucrose and the anionic lipids were hydrated in 320 mM sucrose containing 1 mM calcein. After hydration for 1 h, 0.75 vol. of 320 mM sucrose and 2 mM cobalt chloride were added to eliminate the background fluorescence from calcein outside the vesicles.

2.3. Fluorescence video microscopy

Interactions were monitored by fluorescence microscopy. Images of phenomena of interest were recorded on video tape from a color camera attached to the microscope. Electrophoresis of vesicles was performed in a laboratory-constructed fluid cell, similar to that described [7]. The cell consisted of an 18 mm square coverslip attached to the center of a 75×50 mm slide with 0.1 mm spacers at each corner of the coverslip such that two mutually perpendicular channels were created. Thin platinum electrodes were inserted into the ends of the channels. Opposing horizontal channels (as seen in the microscope) were filled with oppositely charged vesicle suspensions, as described [7]. Potentials of 5 V in either polarity could be applied to opposite pairs of electrodes to control the motion of the vesicles along both the x and y axes. Typically, pairs of vesicles 4 μm or larger in diameter were chosen for study. These were brought close to one another and then allowed to contact by Brownian motion, in order to preclude an influence of the electric field on the behavior of the vesicles. Interactions recorded on video tape were subsequently analyzed and tallied according to whether the outcome was complete fusion, hemifusion, rupture, or transient contact.

2.4. Video image analysis

The identification of hemifusion was based on the observation of the transfer of dye (RhPE at 8 mole%) from one vesicle to the other [7]. Upon contact, the surfaces of the vesicles adhere and flatten against one another. This process occurs quickly and within a few video frames (30 ms each), the fluorescent probe is observed to diffuse from the anionic to the cationic vesicle. Since the onset of hemifusion is when outer (*cis*) monolayers fuse and the lipids intermix, it is expected that hemifusion corresponds to the onset of dye diffusion across the vesicle junction. More than a dozen instances of hemifusion,

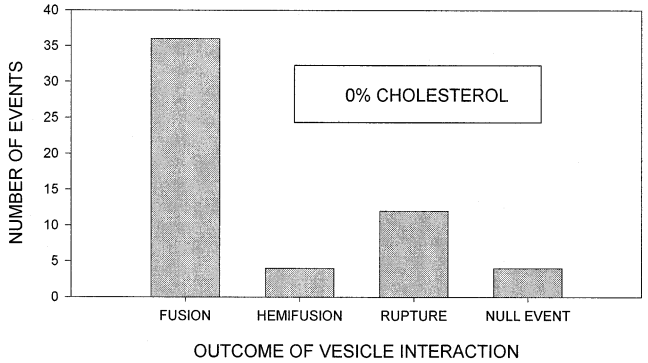

Fig. 1. Distribution of outcomes of 56 pairwise encounters between EDOPC and DOPG vesicles. The cholesterol-free vesicle membranes contained 1.5 mole% fluorophore for visualization. The events occurring after contact were categorized as: fusion, hemifusion, rupture, and 'null event', where no interaction occurred. Aqueous medium was 200 mM sucrose.

both with and without cholesterol, were observed on the basis of RhPE diffusion, and because the process of contact, adhesion and flattening was always the same and flattening was never seen without concomitant transfer of dye, for routine assessment of vesicle interaction we reduced the RhPE concentration to 2 mole% or less and tallied hemifusion as those events leading to the characteristic state of two vesicles in contact through a flat adhesion zone.

3. Results

We first examined the interactions of charged vesicles in the absence of cholesterol. Cationic EDOPC and anionic DOPG vesicles were labeled with 1.5 mole% DOC and 1.5 mole% RhPE, respectively. These vesicles were electrophoretically maneuvered into contact and the outcomes of their interactions are given in Fig. 1. Of the 56 events tabulated, 64% of the interactions represented complete fusion. Complete fusion was always immediate, occurring within a second after contact, and the fusion product was unmistakable. In 21% of the cases, one or both of the vesicles ruptured upon contact. The remaining eight events were evenly distributed between hemifusion (7%) and a 'null event' (8%), in which case the vesicles did not adhere.

Corresponding experiments were performed with vesicles containing 10 mole% cholesterol. Fig. 2A shows the distribution of events obtained with EDOPC/cholesterol/DOC (88.5:10:1.5) and DOPG/cholesterol/RhPE vesicles (88.5:10:1.5). The data show the same general trend as those obtained without cholesterol; complete fusion was the predominant event, constituting 70% of the outcomes of 56 encounters. The extent of hemifusion increased from about 7% to 18%, predominantly at the expense of rupture and null events.

The effect of cholesterol on vesicle fusion was dramatically different when its concentration was increased to 50 mole%. Fig. 2B shows the results obtained from 56 events involving membranes containing the higher concentration of cholesterol. Full fusion comprised only 16% of the observed interactions. The decrease in fusion is highly significant ($P < 0.001$) in comparison to the amount of complete fusion observed in the absence of cholesterol or in the presence of only 10 mole% (Figs. 1 and 2A). A comparably large change was observed in the extent of hemifusion, which became the dominant outcome, amounting to 82% of the events. For the interactions

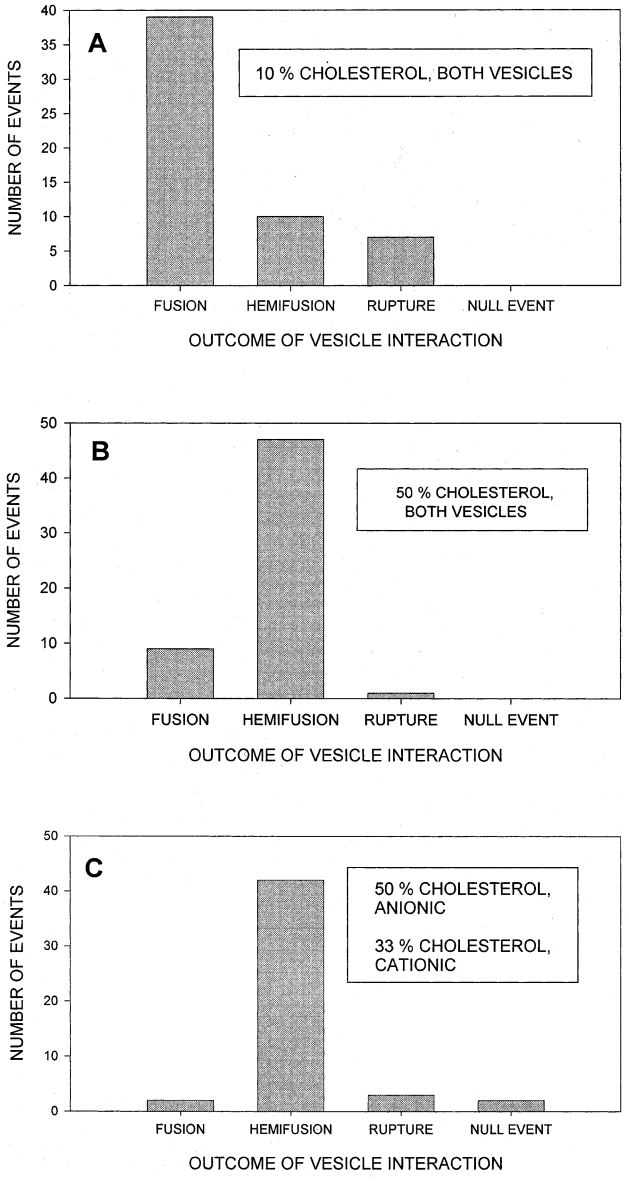

Fig. 2. Distributions of outcomes of contacts between charged vesicles containing cholesterol. Procedures were as in Fig. 1 except that vesicles contained cholesterol. (A) The results of 56 interactions between EDOPC/cholesterol/DOC (88.5:10:1.5) and DOPG/cholesterol/RhPE vesicles (88.5:10:1.5) are shown. (B) The results of 57 interactions between EDOPC/cholesterol/DOC (48.5:50:1.5) and DOPG/cholesterol/RhPE vesicles (48.5:50:1.5) are shown. (C) The results of 49 interactions between DOPG/cholesterol/RhPE (48:48:5) and EDOPC/cholesterol/DOC (66:32:2) vesicles. The sucrose concentration in this case was 320 mM.

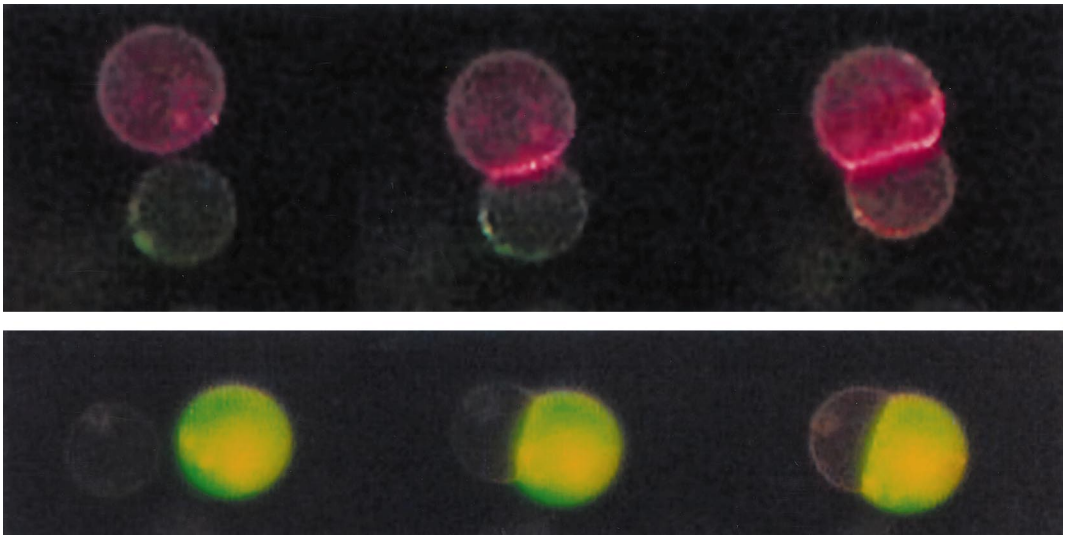

Fig. 3. Visualization of hemifusion. (Upper panel) Transfer of dye from anionic (red) to cationic (green) vesicle. The middle frame is immediately after contact and before hemifusion. A few frames later, the two vesicles underwent additional mutual flattening and the rhodamine probe rapidly diffused to the cationic vesicle (third frame). (Lower panel) Lack of transfer of aqueous phase fluorophore. The left panel shows the vesicles before contact: the vesicle on the left side of the frame was positively charged and is barely visible next to the anionic vesicle that was loaded with calcein, which fluoresces bright green. The middle frame shows hemifusion in progress, with the rhodamine probe just beginning to enter the cationic vesicle membrane. The right frame was taken seconds after hemifusion. Although the outer membrane leaflets have mixed, as is apparent from the increased fluorescence of the membrane of the cationic vesicle on the left, the interior of this vesicle remains non-fluorescent. The interior of the anionic vesicle on the right remains brightly fluorescent, showing that the inner compartments of the vesicles have not mixed and the anionic vesicle contents have not leaked into the external phase. Bilayer composition for both panels was the same as for Fig. 2C.

represented in Fig. 2B, hemifused vesicles did not undergo subsequent fusion during the time of observation (about 1 min). To determine whether or not fusion occurred at later time points, several hemifused vesicles were monitored for times of up to 5 min; no changes were observed. These results clearly indicate that cholesterol can inhibit complete fusion while stabilizing the hemifused state.

A second set of measurements were made on vesicle pairs in which the cationic vesicles contained 1/3 mole fraction cholesterol. These were done to provide data that would be directly comparable to those on contents mixing (see below) which were performed with cationic vesicles of this composition. These results (Fig. 2C) were very similar to those from vesicles containing 1/2 mole fraction cholesterol, with hemifusion amounting to 86% of the events observed.

When hemifusion occurs, the two outer membranes merge and dye in one vesicle is able to diffuse to the other. This process is shown in the upper panel of Fig. 3 for vesicles having the same composition as those of Fig. 2C. Upon contact, the vesicles flattened upon each other and dye flowed from one vesicle to the other (Fig. 3, middle frame of upper panel). Typically, within about a second, dye transfer was complete (right frame of upper panel).

Hemifusion is normally assumed to involve intact and entirely separate aqueous phases in the two participating vesicles. It is possible, however, that the hemifusion process could lead to some breach in the integrity of the single bilayer septum separating the aqueous compartments. We tested this possibility by loading one of the vesicles (the anionic one) with calcein, so as to be able to observe the fluorescence of this intense dye if it diffused from one vesicle to the other. The anionic vesicle membrane also contained RhPE to allow easy visualization of its transfer to the cationic vesicle. Fig. 3 (lower panel) shows a typical result of at least six such experiments involving anionic membranes that were half cholesterol and cationic membranes that were one third cholesterol (same composition as those of Fig. 2A and the upper panel of Fig. 3). It is clear from the images

that, following extensive adhesion of the two vesicles, the membrane probe has transferred abruptly and the calcein fluorescence remained in the anionic vesicle, neither passing to the cationic vesicle nor leaking to the external phase. We conclude, therefore, that hemifusion has occurred and the septum remains intact during the process.

4. Discussion

The results presented here demonstrate that cholesterol inhibits full fusion of oppositely charged lipid bilayers but strongly potentiates hemifusion. The effect was just becoming evident at 10 mole% cholesterol relative to the charged phospholipids and became very pronounced at 50 mole%. At the latter concentration, complete fusion was rare and the majority of vesicle-vesicle contacts led to hemifused intermediates. The hemifused state was evidently stable; it formed in fractions of a second and could be observed for minutes (or until the fluorophore became bleached).

The effects of cholesterol that we observed are explicable on the basis of the adhesion-condensation mechanism of membrane fusion. Originally proposed by Kozlov and Markin [6], this mechanism was put forward to explain the effect of divalent ions like calcium on anionic lipid vesicles, which was being intensely studied at the time as a model of cellular membrane fusion. A related mechanism was subsequently and independently proposed by MacDonald [28]. In both cases, the initial stages of the interaction are postulated to comprise hemifusion and it is these stages that are relevant here. Although these two proposals differ somewhat in detail, the essential elements are: (1) When the external or *cis* monolayers of two vesicles come into contact under conditions leading to charge neutralization, the reduction in electrostatic repulsion in the monolayers causes head group condensation. (2) Condensation creates a tension in the external monolayers which acts uniformly over the surface. (3) The tension can be relieved by formation of a rend or opening in the *cis* monolayers within the contact zone. The opening can only occur at the contact zone, because, in that region, it can be filled by the alkyl chains of the *trans* monolayers and there is essentially no energy cost for two such hydrophobic surfaces to come together. The result is hemifusion. A somewhat related mechanism is the inverted micelle model proposed by Verklei et al. [29] and analyzed by Siegel [30]. That model does not postulate formation of holes per se in the *cis* monolayers, but it does involve condensation of the head groups such that inverted micelles form between the *trans* monolayers.

The adhesion-condensation mechanism was found to be even more apt for fusion of oppositely charged bilayers than for calcium-induced fusion of anionic bilayers. In the former case, the area condensation is due to the mutual electrostatic neutralization of the two oppositely charged vesicles, rather than to the binding of calcium ion by each anionic surface as in the latter. Dioleoylphosphatidylethanolamine (DOPE) also promotes hemifusion [7], and it is significant that both unsaturated phosphatidylethanolamines and cholesterol stabilize the inverted hexagonal phase, a phase that requires smaller head groups than hydrocarbon tail cross-sectional areas [31]. Stabilization of the edges of the hole in the *cis* monolayers can be accomplished by cholesterol given its small head group and propensity for negative curvature in lipid bilayers. It is therefore consistent with the adhesion-condensation mechanism that cholesterol, like DOPE, stabilizes the hemifused intermediate.

The stalk-pore mechanism [5] also predicts that molecules with intrinsic negative curvature should promote hemifusion, and on this basis, might also be considered to provide a satisfactory explanation of our findings; however in the present situation of oppositely charged membranes that flatten against each other with great avidity, there would seem to be little provocation or opportunity for stalks to be involved. In contrast the adhesion-condensation mechanism is consistent with all of our observations and thus seems the preferred mechanism for the situation described here.

For complete fusion, another effect must operate in addition to the formation of openings in and annealing of the edges of the *cis* monolayers; the new bilayer that forms upon hemifusion must also be broken. In model systems, this was postulated [7,28] to be due to the tension developed in the walls (*both* monolayers of the bilayer) from the adhesion and flattening of the vesicles against each other [32,33]. Since the tension is proportional to the ad-

hesion energy, reducing the charge density in oppositely charged vesicles must reduce that tension. Clearly, the higher the proportion of the uncharged cholesterol molecule in a bilayer, the lower the surface charge density would be. In addition, cholesterol could well play a role in inhibiting full fusion by its unfavorable effect on positive curvature, which is needed for fusion pore formation. It therefore appears that cholesterol not only promotes hemifusion, but also inhibits full fusion, both of which increase the probability of the hemifused state.

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