Short-Chain Alcohols Promote an Early Stage of Membrane Hemifusion

Alexandr Chanturiya, Eugenia Leikina, Joshua Zimmerberg, and Leonid V. Chernomordik
Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892 USA

ABSTRACT Hemifusion, the linkage of contacting lipid monolayers of two membranes before the opening of a fusion pore, is hypothesized to proceed through the formation of a stalk intermediate, a local and strongly bent connection between membranes. When the monolayers' propensity to bend does not support the stalk (e.g., as it is when lysophosphatidylcholine is added), hemifusion is inhibited. In contrast, short-chain alcohols, reported to affect monolayer bending in a manner similar to that of lysophosphatidylcholine, were here found to promote hemifusion between fluorescently labeled liposomes and planar lipid bilayers. Single hemifusion events were detected by fluorescence microscopy. Methanol or ethanol (1.2–1.6 w/w %) added to the same compartment of the planar bilayer chamber as liposomes caused a 5–50 times increase in the number of hemifusion events. Alcohol-induced hemifusion was inhibited by lysophosphatidylcholine. Promotion of membrane hemifusion by short-chain alcohol was also observed for cell-cell fusion mediated by influenza virus hemagglutinin (HA). Alcohol promoted a fusion stage subsequent to the low pH-dependent activation of HA. We propose that binding of short-chain alcohol to the surface of membranes promotes hemifusion by facilitating the transient breakage of the continuity of each of the contacting monolayers, which is required for their subsequent merger in the stalk intermediate.

INTRODUCTION

Protein-mediated fusion of biological membranes and fusion of purely lipid bilayers appear to involve similar membrane rearrangements and key structural intermediates (Chernomordik et al., 1995b; Monck and Fernandez, 1996). Influenza virus hemagglutinin (HA), the protein that mediates the best characterized biological fusion reaction (White, 1996), was recently found to mediate hemifusion, where the inner monolayers of the two membranes come into close contact but remain distinct (Kemble et al., 1994; Melikyan et al., 1995b; Chernomordik et al., 1998). Breakage of some local and transient hemifusion structure is hypothesized to lead to the opening of a fusion pore, the ionic pathway between aqueous volumes initially separated by membranes (Zimmerberg et al., 1987). As in biological fusion, fusion of protein-free lipid bilayers also involves hemifusion and subsequent formation of relatively small flickering fusion pores (Chernomordik et al., 1995a; Lee and Lentz, 1997a, 1998; Chanturiya et al., 1997). While final expansion of a fusion pore for liposomes fusing to planar bilayers is driven by osmotic tension (Zimmerberg et al., 1980; Cohen et al., 1980, 1984; Niles and Cohen, 1987), membrane hemifusion is observed in the absence of any osmotic gradient.

The spontaneous hemifusion of protein-free lipid bilayers (Chernomordik et al., 1995a) and HA-mediated hemifusion (Chernomordik et al., 1997) similarly depend upon the lipid composition of the contacting membrane monolayers. These lipid effects are consistent with the hypothesis that hemifusion proceeds through the formation of a local connection between membranes referred to as a stalk intermediate (Kozlov and Markin, 1983; Siegel, 1993; Chernomordik et al., 1995b). Because the lipid monolayer in a stalk is strongly bent, the energy of this intermediate depends on the propensity of membrane lipids to support the net negative curvature of a stalk. Lysophosphatidylcholine (LPC), which favors a micelle-like curvature of lipid monolayer, and thus possesses a positive spontaneous curvature (Epand, 1985), should inhibit bending of the lipid monolayers to form a stalk, and indeed inhibits hemifusion. Lipids like unsaturated phosphatidylethanolamine (PE) (negative spontaneous curvature) support the monolayer curvature required in a stalk and promote hemifusion (Chernomordik et al., 1995a).

While the effects of PE, LPC, and some other lipids on fusion are completely consistent with this stalk hypothesis, altering the membrane composition can also modify other properties of membranes relevant to fusion. For instance, lipid composition affects the repulsive and attractive forces between bilayers in contact and, thus, the equilibrium distance between these bilayers (Leikin et al., 1993; McIntosh et al., 1999). The energy of hypothetical bent fusion intermediates depends not only on the spontaneous curvature of the lipid monolayer but also on its bending elasticity (Siegel, 1993; Chernomordik et al., 1995b). In addition, the continuity of the contacting monolayers of two fusing membranes should be disrupted locally to allow these monolayers to bend toward each other and to merge in a new way (Fig. 1). Any breaking of the continuity of a lipid monolayer exposes the hydrophobic interior of the membrane to the aqueous phase and can be a very energy-intensive step (Fig. 1 *B*). Overcoming this energy barrier can be facilitated at close enough distances between membranes (Leikin et al.,

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Address reprint requests to Dr. Leonid V. Chernomordik, Laboratory of Cellular and Molecular Biophysics, NICHD, National Institutes of Health, Bldg. 10, Rm. 10D-04, 10 Center Drive, Bethesda, MD 20892-1855. Tel.: 301-594-1128; Fax: 301-594-0813; E-mail: lchern@helix.nih.gov.

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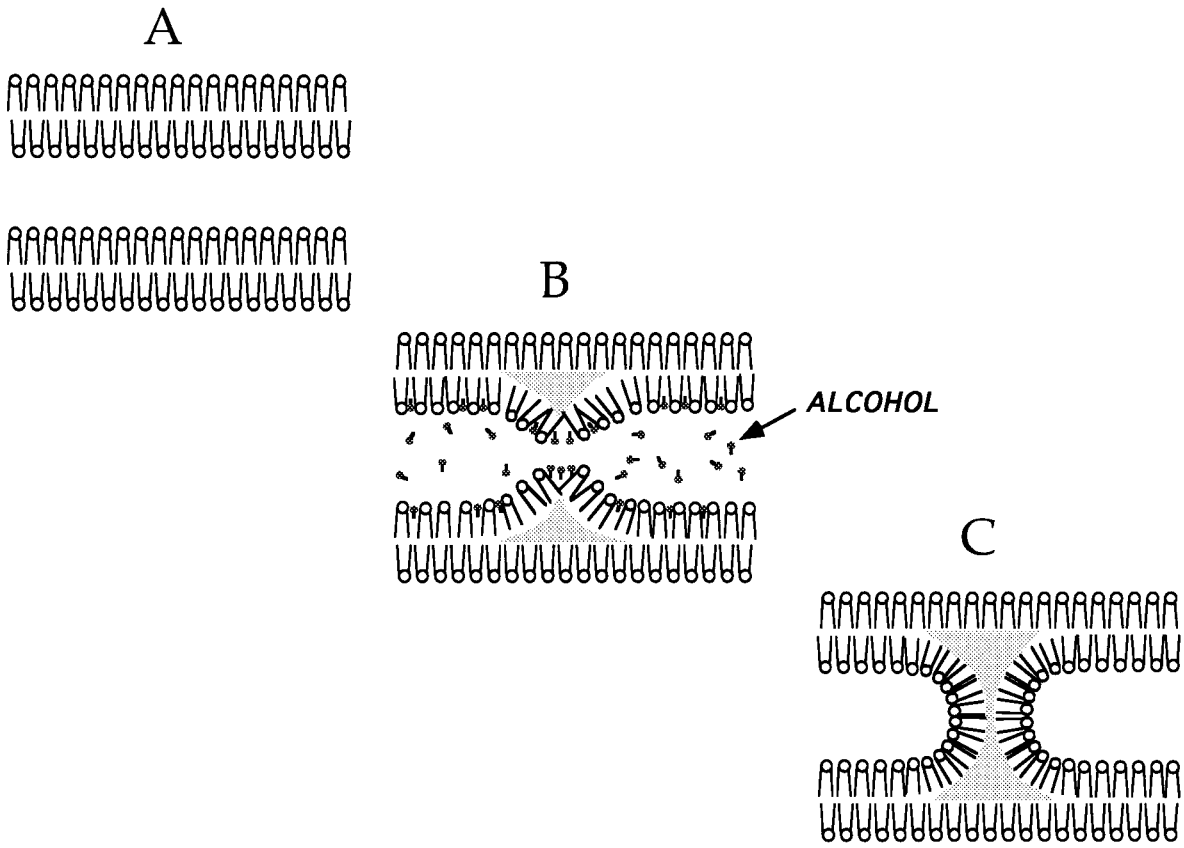

FIGURE 1 Hypothetical stages of lipid bilayer hemifusion. (*A*) Two opposed membranes. (*B*) Rise of the bulging defects (semistalks) in the contacting monolayers. Note that hydrophobic interior of both bilayers is exposed to aqueous medium at the tops of both semistalks. (*C*) Joining of two adjacent semistalks into a stalk.

1987; Helm et al., 1992). At the same distance the height of the energy barrier under consideration is expected to depend on the properties of the monolayer surface. Indeed, the bilayer propensity to fuse was reported to correlate with some dehydration of the bilayer surface, its elevated hydrophobicity, and the appearance of some hydrophobic lipid packing defects (Duzgunes et al., 1981; Aldwinckle et al., 1982; Wilschut and Hoekstra, 1986; Ohki, 1988; Lentz, 1994).

Note that different properties of the membranes are interconnected, and it appears to be impossible to alter only one of them. For instance, adding Ca^{2+} ions to cardiolipin-containing membrane apparently changes both the spontaneous curvature and surface properties of lipid monolayers (Lee and Lentz, 1997b; Powell and Marsh, 1985; Smaal et al., 1987).

In this work we found that spontaneous curvature of the monolayers is not the only property of the lipid bilayer relevant to fusion. We studied the effects of short-chain alcohols (methanol, ethanol, and *n*-butanol) on liposome-planar bilayer and HA-mediated cell hemifusion. While these alcohols, in a manner similar to that of LPC, inhibit formation of an inverted hexagonal phase (negative curvature) (Hornby and Cullis, 1981; Veiro et al., 1989), their presence, in contrast to LPC, promotes rather than inhibits hemifusion. We hypothesize that the insertion of alcohol into the lipid-water interface (Barry and Gawrisch, 1994; Ho and Stubbs, 1997; Holte and Gawrisch, 1997) may promote stalk formation by facilitating the local breakage of the monolayer required for stalk formation.

MATERIALS AND METHODS

Liposome-planar bilayer fusion

All lipids were purchased from Avanti Polar Lipids (Birmingham, AL), except for ergosterol (Fluka Chemika-Biochemica, Buchs, Switzerland). Hexane was purchased from Fluka Chemika-Biochemica. Planar lipid membranes were formed by the Montal-Mueller technique across a hole in a Teflon partition separating two aqueous compartments. We used soybean L-α-phosphatidylcholine 20% (asolectin) or dioleoylphosphatidylcholine (DOPC)/cholesterol mixture (82/18 mol%) or 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhC) in hexane solution (10 mg/ml). The chamber, milled from Teflon, was similar to that described previously (Chernomordik et al., 1995a). It was placed between an objective and a condenser lens of a custom-made dual-wavelength fluorescence microscope (Chanturiya, 1996).

Ag/AgCl electrodes, placed in saturated KCl, were connected to the planar bilayer-bathing solution through 2% agarose bridges. An electrode placed in the compartment to which the liposomes were added (*cis* compartment) was used as a virtual ground. Another electrode placed in the

trans compartment was connected to the input of an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Membrane capacitance was determined from the capacitive current in response to the application of a 100 V/s linear voltage sweep with an amplitude of +20 mV. The specific capacitance of planar bilayers was estimated by dividing the capacitance by the area of the aperture in the Teflon partition.

Two types of liposomes were used in experiments. Giant unilamellar vesicles (GUVs) (2–10-μm diameter) were formed, using a modified Kim and Martin method (Kim and Martin, 1981; Chernomordik et al., 1995a), from asolectin, ergosterol, and *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine (RhPE) (70:20:10, w/w %). GUVs were formed as described by Chanturiya et al. (1997), either in buffer A (200 mM calcein, 10 mM MES, 5 mM *n*-propylgallate, pH 6.5, 835 mOsm (osmotically stressed vesicles)) or in buffer A diluted to 780 mOsm (osmotically balanced vesicles). The internal solution of GUV was supplemented with 10 μg/ml of channel-forming antibiotic nystatin (Chanturiya et al., 1997).

Because this method of liposome preparation did not allow significant variation of the lipid composition, we also used vesicles prepared by the sonication-freeze-thaw technique (SFTVs) (Cohen et al., 1984; Chernomordik et al., 1995a). SFTVs were formed from different mixtures of egg phosphatidylcholine (PC) or DOPC, egg PE, and cholesterol. In the preliminary experiments with SFTVs loaded with calcein, we found that these liposomes were too small (20 to several hundred nanometers; Cohen et al., 1984) to allow fluorescence microscopic detection of the calcein fluorescence in the liposomes. Thus in the subsequent experiments SFTVs were labeled only by RhPE in self-quenching concentration (14 mol%) or by RhPE and by the porin ion channel from *Bordetella pertussis*, as described by Chernomordik et al. (1995a).

Experiments with GUVs were conducted in buffer B containing 400 mM KCl, 10 mM HEPES, 1 mM EDTA (pH 7.5, 780 mOsm). If not stated otherwise, buffer B was supplemented with 20 mM $CaCl_2$. Experiments with SFTVs were conducted in the buffer C containing 100 mM KCl, 10 mM MES, 1 mM EDTA (pH 6.0, 237 mOsm).

A glass pipette filled with the liposome suspension was fixed to a hydraulic micromanipulator. A small portion of the liposome suspension was pressure injected toward the planar bilayer, so that a number of liposomes became attached to the membrane while others moved out of the field of view.

HA-mediated fusion

Fusion of HA-expressing cells with human red blood cells (RBCs) was studied as described earlier (Chernomordik et al., 1998). Briefly, HAb2 cells, a line of stably transfected National Institutes of Health-3T3 fibroblasts expressing the A/Japan/305/57 strain of influenza virus HA, were cultured as described (Melikyan et al., 1995a). RBCs, freshly isolated from whole blood, were labeled with the fluorescent lipid PKH26 (Sigma, St. Louis, MO) and the water-soluble fluorescent dye carboxyfluorescein (CF) (Molecular Probes, Eugene, OR), as done by Chernomordik et al. (1998).

HAb2 cells were treated with 5 μg/ml trypsin (Fluka, Buchs, Switzerland) for 10 min at room temperature to cleave HA0 into its fusion-competent HA1-S-S-HA2 form.

To terminate the reaction, HA-expressing cells were washed twice with complete medium containing 10% fetal serum. After two washings with phosphate-buffered solution (PBS), cells were incubated for 10 min with a 1-ml suspension of RBCs (0.05% hematocrit). HA-expressing cells with 0–2 bound RBCs per cell were washed three times with PBS to remove unbound RBCs.

All fusion experiments were performed at room temperature (20–22°C). Fusion was triggered by replacing PBS with an isoosmotic buffer titrated by citrate to acidic pH. After a 5-min incubation of cells at low pH, the acidic solution was replaced by PBS. Lipid (PKH26) mixing and content (CF) mixing were assayed by fluorescence microscopy more than 20 min after low-pH application, as the ratio of dye-redistributed bound RBCs to the total number of bound RBCs.

Addition of alcohol, LPC, and tetradecane

In experiments on liposome-planar bilayer fusion, methanol (Mallinckrodt Baker, Paris, KY), ethanol (Warner Graham Co., Cockeysville, MD), *n*-butanol (Baker Chemical, Phillipsburg, NJ), or lauroyl LPC (Avanti Polar Lipids) were added to the buffer in the *cis* compartment of the planar bilayer chamber under constant stirring. In some experiments, the chloroform solution of the lipid mixture used to prepare SFTVs was supplemented with 5 mol% of tetradecane (Sigma). In cell fusion experiments the medium bathing the plastic- or glass-attached HA-expressing cells with bound RBC was replaced by PBS, which was supplemented with alcohol or lauroyl LPC and vortexed intensively.

RESULTS

Liposome-planar bilayer fusion

As reported earlier (Chanturiya et al., 1997; Chernomordik et al., 1995a), combining fluorescence microscopy and electrical measurements allows us to dissect fusion between planar lipid bilayers and single giant cell-size liposomes into distinct stages such as membrane contact, hemifusion, formation of small flickering fusion pores, and their expansion. After injection of GUVs toward a planar lipid bilayer, liposomes in close proximity to the planar bilayer moved across the field of view much more slowly than free-floating liposomes. Then some of these liposomes came to a standstill upon the establishment of the liposome-planar bilayer contact. Over the next several minutes, a flash-like spread of RhPE was observed for ~20% of the liposomes in contact with the planar bilayer (Chanturiya et al., 1997). As this fast redistribution of membrane dye from a single liposome to the planar bilayer was not accompanied by any evidence of fusion pore formation (such as a decrease in calcein fluorescence and an increase in membrane conductance), we interpret the flashes of rhodamine fluorescence as hemifusion events. For osmotically stressed GUVs hemifusion was followed by opening and expansion of a fusion pore (a decrease in calcein fluorescence and an increase in membrane conductance in Fig. 2, *B* and *C* (see also Chanturiya et al., 1997). Osmotically balanced GUVs demonstrated only hemifusion or hemifusion with flickering nonexpanding fusion pores. Similarly, for osmotically balanced SFTVs, flashes of rhodamine fluorescence (Fig. 2 *E*) were not accompanied by any changes in planar bilayer conductance (Fig. 2 *F*) and thus correspond to hemifusion events (Chernomordik et al., 1995a). To focus on the hemifusion stage, only osmotically balanced liposomes were used in the experiments presented below.

Both the total number of the observed hemifusion events and the rate of hemifusion were promoted by adding short-chain alcohols (e.g., methanol, ethanol, and butanol). The number of hemifusion events increased with the concentration of methanol added to the *cis* compartment of the planar bilayer chamber (or to both compartments; not shown), reaching a plateau at ~1.5 w/w % or ~0.5 M methanol (Fig. 3 *A*). The same concentration of ethanol caused a similar promotion of hemifusion. Butanol also increased the frequency of hemifusion events (data not shown). Adding 0.5

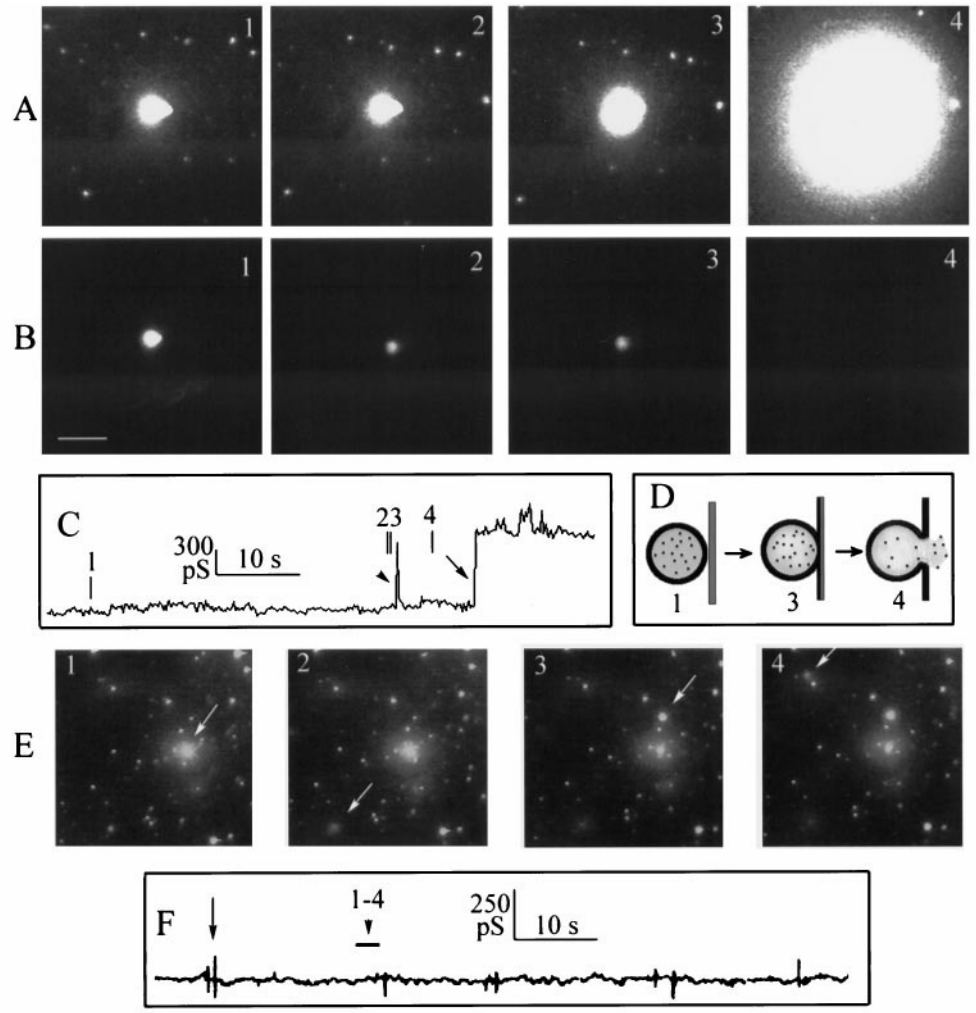

FIGURE 2 Vesicle-planar bilayer interaction assayed by combined fluorescence microscopy and conductance measurements. (*A–D*) Fusion of a single osmotically stressed GUV to a planar bilayer of asolectin. (*A*) Membrane dye (RhPE) fluorescence. (*B*) Fluorescence of vesicle aqueous content (calcein). The time interval between the corresponding images in *A* and *B* (e.g., *A1* and *B1*) is 0.2 s. (*C*) Electrical recording. As both the GUV and the *cis* compartment of the chamber contained 10 μg/ml of nystatin, the vesicular membrane is permeabilized by nystatin channels. After opening of a fusion pore, the high resistance of the planar bilayer is shunted by conductive permeabilized vesicular membrane. Thus the fusion of a vesicle with the planar bilayer produces a steplike increase in conductance. Vertical bars with numbers in the conductance record indicate the position of the video frames corresponding to *A1–4*. (*D*) Diagram of the distinct stages of lipid bilayer interaction detected in the experiment presented in *A–C*. The image of the vesicle in the center of panels *A* (observed with a rhodamine filter set) remains unchanged over a relatively long period of time (*A1, A2*). The decrease in the brightness of this vesicle between the *B1* and *B2* panels (calcein filter set) is apparently due to calcein bleaching. A transient opening of the fusion pore and subsequent complete fusion of the vesicle are reflected in changes in membrane conductance (conductance spike and the steplike increase, respectively, *C*), a decrease in the calcein fluorescence (*B3, B4*), and in a flash-like lipid dye transfer from the vesicle to the planar bilayer (*A3, A4*). Vesicle-planar bilayer hemifusion was detected here as lipid mixing in the absence of conductance changes. Lipid bilayers were hemifused from the onset of lipid mixing (*A3*) until complete fusion (*arrow in C*), except for the short time interval corresponding to the transient opening of a fusion pore (*arrowhead in C*). (*E, F*) Hemifusion of osmotically balanced SFT vesicles of egg PC/RhPE (86/14 mol%), containing porin channels, to a planar bilayer of asolectin. (*E*) Sequential flashes of RhPE redistribution from four different SFT vesicles (marked by *arrows*) with the same planar bilayer. *E1* was taken ~15 s after liposome injection. *E2–4* were taken 0.73, 2.1, and 2.7 s later. (*F*) A series of flashes of rhodamine fluorescence, reflecting lipid mixing between liposome and planar bilayer, was not accompanied by any change in planar bilayer conductance, evidence of hemifusion. An arrowhead marks the time interval during which the images *E1–4* were taken. A 20-μm scale bar for all video images is shown in *B1*.

M methanol only to the *trans* compartment had no effect on hemifusion.

With no alcohol added, hemifusion of GUV was observed only in the presence of millimolar concentrations of Ca^{2+} or Mg^{2+} (Fig. 3 *B*). In contrast, methanol-induced hemifusion was independent of the presence of divalent ions and was several times more effective than that induced by 20 mM $CaCl_2$. Methanol, whether added before or after vesicular binding to the planar bilayer, promoted hemifusion (data not shown).

Promotion of hemifusion by short-chain alcohols was also observed for SFTVs and for planar bilayers, each of different lipid composition (Figs. 3 *C* and 4). Fig. 3 *C* illustrates the effects of alcohol on the time course of

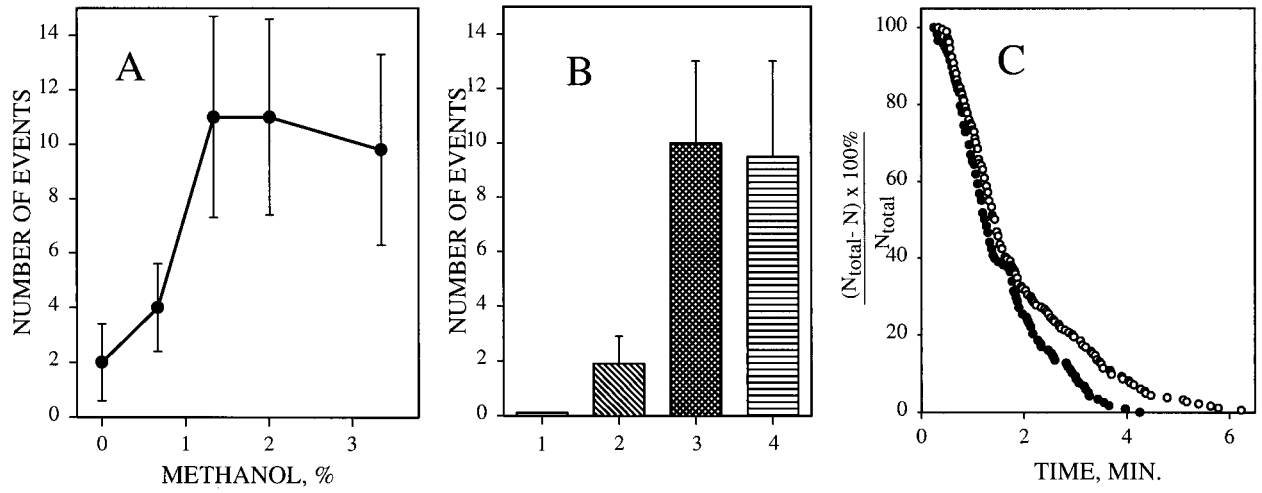

FIGURE 3 Promotion of vesicle/planar bilayer hemifusion by methanol and ethanol. (*A*) The dependence of vesicle/planar bilayer hemifusion and fusion on methanol concentration. Methanol was added to the *cis* compartment of the chamber under stirring. Osmotically balanced GUVs were injected toward the asolectin planar bilayer, and the total number of RhPE flashes per injection was counted (for all liposomes including small and calcein-free). Values shown are the mean ± SD for three to five independent experiments; in each liposomes were injected two to three times. (*B*) Relative effectiveness of methanol and calcium in hemifusion promotion for osmotically balanced GUVs and a planar bilayer formed from asolectin. *1*: Control (neither $CaCl_2$ nor methanol was added). *2*: 20 mM $CaCl_2$ added *cis*. *3*: 1.7 w/w % methanol added *cis*. *4*: Both $CaCl_2$ (20 mM) and methanol (1.7 w/w %) are present in the *cis* compartment. Values shown are the mean ± SD. (*C*) Time course of vesicle-planar bilayer hemifusion in the presence of ethanol. SFTVs were formed from 59% egg PC, 28% egg PE, and 13% RhPE (mol%). 1.1 w/w % (○, eight experiments) or 4.1 w/w % (●, six experiments) of ethanol were added to the *cis* compartment of the chamber. Vesicles were injected one or two times during each experiment, and flashes were recorded on a VCR over a period of up to 8 min. The time distribution of all registered RhPE fluorescence flashes (total of 184 and 118 flashes in the presence of 1.1 and 4.1 w/w % ethanol, respectively) is presented here as a "survival plot," i.e., the number of vesicles that will eventually hemifuse but have not yet hemifused by a given time. The number of flashes observed before any given time (N) is subtracted from the total number of flashes observed in the experiment (N_{total}) and normalized by it.

hemifusion of SFTVs with planar bilayers. The more ethanol added, the higher the rate of hemifusion.

The character of vesicle-planar bilayer interactions is known to depend dramatically on the presence of osmotic gradients across the vesicular membrane (Zimmerberg et al., 1980; Cohen et al., 1980, 1984; Niles and Cohen, 1987). However, the promotion of hemifusion by short-chain alcohol did not appear to be mediated by any osmotic effects. As mentioned above, the rates of methanol-induced hemifusion were statistically identical when alcohol was added to both compartments of the planar bilayer chamber or only to the same compartment as the liposomes. To verify that altering

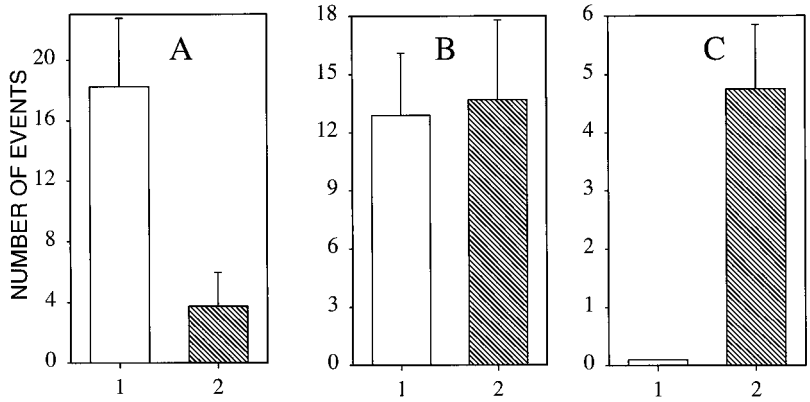

FIGURE 4 Effects of LPC (*A*), tetradecane (*B*), and cholesterol (*C*) on methanol-induced vesicle/planar bilayer hemifusion. (*A*) Numbers of rhodamine flashes observed upon hemifusion of SFTV (86% of PC and 14% of RhPE (mol%)) with planar bilayers formed from asolectin in the absence or presence of 90 μM LPC. Other details are as described in Materials and Methods. The number of hemifusion flashes in the presence of 3.5 w/w % methanol observed with (*2*) and without (*1*) LPC is shown as the mean ± SD, $n = 3$. (*B*) Vesicles were formed from *1*: 59% egg PC, 28% egg PE, and 13% RhPE (mol %); or *2*: 5 mol% of tetradecane, 54% egg PC, 28% egg PE, and 13% RhPE (mol%). The number of hemifusion flashes in the presence of 3.5 w/w % methanol is shown as the mean ± SD, where n equals 10 and 11, with and without tetradecane. (*C*) SFTV formed from DOPC/cholesterol/RhPE (68/20/12 mol%) and planar bilayers formed from DOPC/cholesterol (82/18 mol%). The number of hemifusion flashes (shown as mean ± SD; $n = 4$) was determined in the absence (*1*) and in the presence (*2*) of 3.5 w/w % methanol.

water activity did not cause the promotion, we compared the rate of hemifusion in the presence of two isoforms of propanediol: 1,2-propanediol or 1,3-propanediol, of which 1,2-propanediol interacts more strongly with lipids (McIntosh et al., 1989). In our experiments 7.4 w/w % (970 mM) of either one of the two isoforms was added to the *cis* compartment of the chamber before liposome injection. It was found that 1,2-propanediol induced seven to nine times more RhPE flashes than the 1,3 isomer (Table 1). At the concentrations used, 1,2-propanediol and 1,3-propanediol increased the osmolarity of buffer C from 237 ± 1 mOsm to 765 ± 9 mOsm and 1079 ± 2 mOsm, respectively. The difference between the osmotic activities of the two propanediol isomers can be caused by some aggregation of 1,2-propanediol molecules in aqueous medium. Thus the promotion of hemifusion by the diols appears to correlate with their interaction with membranes rather than with their effects on water activity.

The rates of hemifusion and fusion of lipid bilayers can be altered by adding nonbilayer lipids (Chernomordik et al., 1985, 1995b) or small apolar molecules (Basanez et al., 1998; Walter et al., 1994). As in other hemifusion reactions (Chernomordik et al., 1995b), short-chain alcohol-induced hemifusion involves an LPC-arrested stage. Lauroyl LPC inhibited methanol-induced hemifusion when both methanol and LPC were added to the same compartment of the chamber as liposomes (Fig. 4 *A*). In contrast, while hydrocarbons are known to promote liposome-liposome fusion, tetradecane did not affect liposome-planar bilayer hemifusion in the presence of methanol. The number of rhodamine flashes observed for PC/PE/RhPE liposomes formed with or without 5 mol% of tetradecane was statistically identical (Fig. 4 *B*). No hemifusion was observed for tetradecane-containing liposomes in the absence of methanol (not shown).

Short-chain alcohols are known to induce an interdigitated state where lipid acyl chains extend across the bilayer to the polar headgroups of the opposite leaflet (Simon and McIntosh, 1984; Slater and Huang, 1988; Zeng et al., 1993; Mou et al., 1994). Interdigitation of lipid bilayers is accompanied by a significant decrease in their thickness. Thinning of the planar bilayer can be detected as an inversely proportional increase in the specific capacitance of the bilayer. We found no significant difference in the specific capacitance of the planar lipid bilayer (DPhC) in the presence or absence of a maximally hemifusion-promoting concentration of methanol (7.7 ± 0.5 mF/m^2 (n = 13) versus 7.3 ± 0.8 mF/m^2 (n = 15), with and without 2.6 w/w % methanol). The absence of any measurable thinning of the lipid bilayer, and our finding that alcohols promoted hemifusion of lipid bilayers formed from mixtures of natural lipids (e.g., asolectin) that are unlikely to form interdigitated structures (Komatsu and Okada, 1995) indicated that hemifusion promotion is not mediated by the formation of completely interdigitated bilayers. However, there was still a possibility that some minor components of the lipid mixture can form interdigitated domains. Cholesterol and unsaturated lipid acyl chains stabilize bilayers against interdigitation (Slater and Huang, 1988; Komatsu and Rowe, 1991; Cunningham et al., 1995). Thus, to test whether the promotion of hemifusion by alcohol is mediated by interdigitation, we used liposomes and planar bilayers formed from a DOPC/cholesterol mixture. As with other liposomes, no rhodamine flashes were observed when vesicles were applied to the planar bilayer in the absence of divalent ions and alcohols. However, upon methanol addition, flashes were observed at a rate comparable to the rate observed for planar bilayers formed from asolectin (Fig. 4 *C*).

TABLE 1 Promotion of hemifusion by isomeric propanediols

	Hemifusion flashes per injection*	SE	Hemifusion probability# (%)	No. of experiments§
Control¶	0	—	0	(3)
1,2-Propanediol	3.4	2	7.68	(8)
1,3-Propanediol	0.5	0.8	0.85	(9)
1,2/1,3 ratio	6.8	—	9.0	—

*Average number of RhPE flashes observed per injection of SFTVs formed from 55 wt. % egg PC, 25% PE, 20% RhPE. Planar bilayers were formed from DPhC.

#Hemifusion probability was defined here as a ratio of the number of hemifusion (= RhPE) flashes observed after vesicle injection, and the total number of vesicles visible on the planar bilayer after unbound vesicles were washed out.

§The number in parentheses indicates the total number of experiments for these conditions.

¶No flashes were observed in the control experiments with no propanediol added.

HA-mediated fusion

As reported earlier (Sarkar et al., 1989; Chernomordik et al., 1998), under optimal conditions (e.g., pH 4.9, 37°C), low pH application to HAb2 cells with bound RBCs for most cell pairs resulted in complete fusion, seen as a fast transfer of both PKH26 and CF from RBCs to HAb2 cells. In contrast, for suboptimal conditions (e.g., pH 5.3, 22°C) we observed no CF transfer and a low percentage of PKH26-transferred cells (Fig. 5 *A*). Lipid mixing (PKH26 transfer) with no content mixing (CF transfer) is referred to as unrestricted hemifusion (Chernomordik et al., 1998). Note, however, that our assay would not distinguish hemifusion from small nonexpanding fusion pores.

HA-mediated fusion is a multistep reaction, which starts with a low pH-induced refolding of HA to a fusion-competent conformation. Merger of the contacting monolayers of fusing membranes is followed by the opening and expansion of a fusion pore and lipid mixing, or in the case of unrestricted hemifusion, only by lipid mixing (Chernomordik et al., 1998). To focus on the stage of actual membrane merger, we first incubated HAb2 cells with bound RBCs in low pH medium for 5 min, then returned the pH to neutral and applied alcohol. Low pH-induced refolding of HA was presumably completed before the application of the

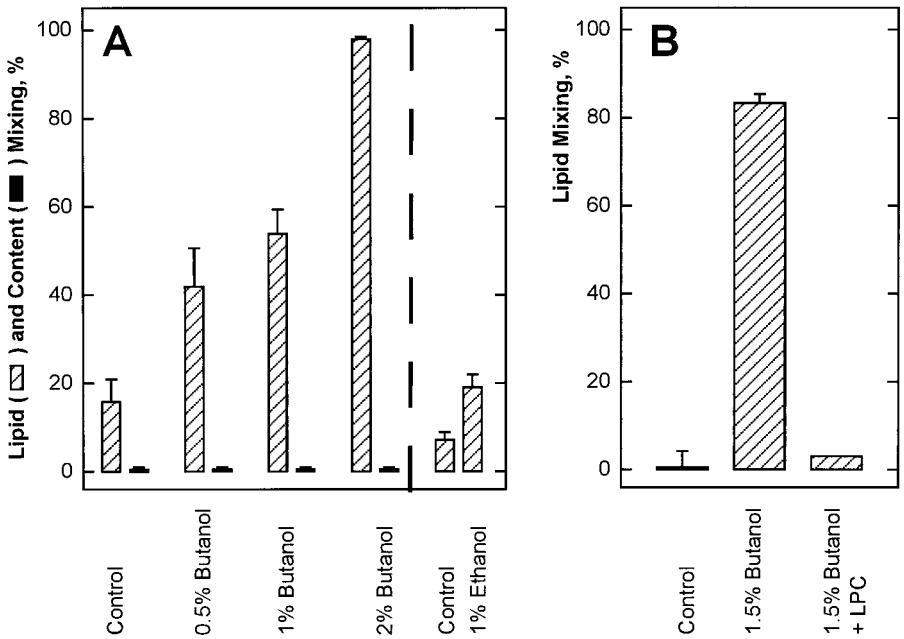

FIGURE 5 Butanol and ethanol promote HA-mediated hemifusion. (*A*) HAb2 cells with bound PKH26- and CF-labeled RBC were triggered to fuse by a 5-min application of pH 5.3 medium. Cells were incubated at neutral pH for an additional 20 min, and then hemifusion (= lipid mixing in the absence of content mixing) and complete fusion (= mixing of both lipids and contents) were assayed by fluorescence microscopy. Note the absence of content mixing, and, thus, no complete fusion at all alcohol concentrations, and the rather low extent of hemifusion in the control experiments (no alcohols added) characteristic for suboptimal conditions of fusion triggering (Chernomordik et al., 1998). Butanol and ethanol effects were studied in the independent experiments and presented each with its own control. Butanol (final concentration 0.5–2 w/w %) or ethanol (1 w/w %) were applied for 2 min immediately after the end of a low-pH pulse. Each bar represents mean hemifusion/fusion extent ± SE for 200 cells counted. (*B*) Hemifusion promotion by butanol was inhibited by LPC. HAb2-RBC fusion was triggered by a 5-min pH 5.35 pulse. Butanol (1.5 w/w %) was applied for 2 min immediately after the end of a low-pH pulse. Butanol-containing medium was supplemented or not with 170 μM lauroyl LPC. Hemifusion was assayed as in *A*.

alcohol (White and Wilson, 1987; Korte et al., 1997). (The addition of 1 w/w % ethanol before low-pH application caused a significant inhibition of lipid and content mixing (data not shown), suggesting that ethanol did affect low pH-dependent stages of fusion if present at the time of low pH application.)

Application of butanol or ethanol after the end of low-pH application resulted in a profound promotion of hemifusion (Fig. 5 *A*). Butanol was more effective in hemifusion promotion than ethanol, which can be explained by the difference between their partition coefficients, with the larger fraction of butanol molecules bound to the bilayer interface than that for ethanol. Neither hemifusion nor complete fusion was observed in the control experiments when the same concentration of alcohols was applied to HAb2 cells with bound RBCs not treated by low pH, or if HAb2 cells were not pretreated with trypsin to cleave fusion-incompetent HA0 into the fusion-competent HA1-HA2 form.

As lipid mixing between merged membranes at early fusion stages can be restricted by HA molecules surrounding the fusion site (Chernomordik et al., 1998), alcohol could promote either actual membrane merger or lipid mixing through existing hemifusion connections. We found that LPC, known to inhibit membrane merger, prevented hemifusion promotion by butanol (Fig. 5 *B*), suggesting that butanol facilitates hemifusion rather than lipid mixing.

DISCUSSION

Short-chain alcohols are found here to promote the spontaneous hemifusion of protein-free lipid bilayers and HA-mediated hemifusion of biological membranes. Either in the presence or in the absence of short-chain alcohols, the same inhibitor, LPC, can block hemifusion. These data suggest that the mechanism by which alcohol promotes hemifusion is through a pathway that passes through the LPC-sensitive fusion stage, rather than a new pathway.

What determines the membrane propensity to fuse? The shift from the initial fusion-incompetent state of the bilayer to a fusion-competent one is accompanied by changes in numerous interconnected properties of membrane bilayers. One of these "fusogenic" changes involves a shift of the spontaneous curvature of the contacting membrane monolayers toward negative values, which is thought to be favorable for the net negative curvature of the monolayer in the stalk fusion intermediate (Kozlov and Markin, 1983; Chernomordik et al., 1995b). Changes in the spontaneous curvature are always accompanied by changes in other properties of lipid bilayers such as surface hydrophobicity, packing defects, the degree of hydration of lipid headgroups, etc. (Ohki, 1984; Epand, 1993; Leckband et al., 1993; Lentz, 1994; Lee and Lentz, 1997b), which have also been hypothesized to be of consequence for fusion. It ap-

pears very unlikely that short-chain alcohols promote hemifusion by lowering the spontaneous curvature of lipid monolayer. Because ethanol and methanol apparently support positive rather than negative spontaneous curvature of lipid monolayers (Hornby and Cullis, 1981; Veiro et al., 1989), short-chain alcohols increase hemifusion rates by altering lipid bilayer properties other than the spontaneous curvature. While it remains possible that alcohol promoted protein-free liposome-planar bilayer hemifusion and HA-mediated hemifusion of biological membranes by different mechanisms, below we will focus on the possible common mechanisms of this promotion.

Alcohol effects on hydrophobic voids, membrane binding, bending rigidity, and lipid flip-flop

Hemifusion requires peeling apart the hydrophobic sides of the monolayers of fusing membranes, creating hydrophobic interstices, the hydrophobic voids that form between the bilayer leaflets (Siegel, 1993). Hydrocarbons such as hexadecane and tetradecane, which can fill these voids, facilitate their formation (Rand et al., 1990; Chen and Rand, 1998) and promote liposome-liposome fusion (Basanez et al., 1998; Walter et al., 1994). Since along with its binding to the lipid-water interface of lipid bilayers, ethanol disorders the entire length of the hydrocarbon chains (Ho and Stubbs, 1997; Holte and Gawrisch, 1997), one may hypothesize that alcohol-induced change in lipid hydrocarbon chain packing promotes hemifusion by decreasing the energy of the hydrophobic voids. However, the rate of methanol-induced hemifusion in our experimental system was not sensitive to tetradecane, a known modifier of interstitial energy. In addition, the lack of hemifusion for tetradecane-containing liposomes in the absence of alcohol argues against the possibility that while short-chain alcohol promotes hemifusion by lowering the energy of hydrophobic voids, this effect was already saturated at the alcohol concentrations used. Our results suggest that the energy of hydrophobic interstices is not a major contribution to the total energy of rate-limiting intermediates in liposome-planar bilayer hemifusion, and thus it is very unlikely that ethanol addition promotes hemifusion by lowering the energy of the interstices. Note that the contribution of the void energy and, thus, the effects of tetradecane could be downplayed in our system because planar bilayers (even so-called solvent-free bilayers used in this work) contain some traces of the hydrocarbon solvent, which can fill the hydrophobic voids in hemifusion and fusion intermediates.

Hemifusion is affected by alcohol present between fusing membranes. Methanol added to the *trans* compartment of the planar bilayer/liposome chamber did not promote hemifusion. Methanol permeation through the membrane is very fast. For the case of methanol added to the *trans* compartment, the ratio between alcohol concentration c_{cis} at the *cis* surface (the one in contact with liposomes) and c_{tr} at the *trans* surfaces of planar bilayer is determined by three parameters: 1) the permeability of the membrane to methanol, P; 2) a diffusion coefficient of methanol in bulk water, $D \approx 1.5 \times 10^{-5}$ cm^2/s (Hao and Leaist, 1996); and 3) the thickness of the unstirred layer at the surface of the planar bilayer, d. For stationary diffusion conditions that correspond to equal flows of methanol to the bilayer, through it, and from the bilayer, $P(c_{tr} - c_{cis}) = Dc_{cis}/d$, and thus $c_{tr}/c_{cis} = (p + D/d)/P$. Because, to the best of our knowledge, P has never been measured for planar bilayers, we used $P \approx 3.7 \times 10^{-3}$ cm/s, measured for RBC membrane (Brahm, 1983). The value of d varies with the geometry of the system (for instance, the partition thickness in the planar bilayer chamber) and stirring conditions. d is at best 1×10^{-2} cm for 1-mm-diameter decane-containing planar bilayer (Finkelstein, 1987), but in RBCs much smaller unstirred layer thicknesses of 1.7×10^{-4} cm are measured (Brahm, 1983). Depending on the value of d, c_{cis} can be 1.4–30 times lower than c_{tr} (estimated for $d \approx 1 \times 10^{-2}$ and 1.7×10^{-4} cm, respectively). So the lack of hemifusion promotion by methanol added to the *trans* compartment may reflect insufficient concentration of methanol in the zone of liposome/planar bilayer contact. However, it is also possible that another, unknown mechanism is at work.

Short-chain alcohols affect numerous properties of lipid bilayers, which can be relevant for hemifusion and fusion. For instance, ethanol is reported to induce the aggregation of liposomes formed from dipalmitoyl PC (Zeng et al., 1993; Komatsu and Okada, 1995). In our experiments, hemifusion was promoted by alcohols added to prebound membranes (i.e., liposomes bound to planar bilayers, and HA-expressing cells with bound RBCs). However, it remains possible that this promotion was mediated by altering some properties of the binding, such as the distance between membranes and the area of the contact zone. Ethanol is also known to accelerate phospholipid flip-flop in the erythrocyte membrane (Schwichtenhovel et al., 1992). The growth of a hemifusion diaphragm can be limited by the tension that develops in the inner monolayers of the membranes along with lipid compression in the outer membrane monolayers (Chernomordik et al., 1987). Alcohol-induced acceleration of the lipid redistribution between the two monolayers of the membrane bilayer can release these stresses and thus promote hemifusion. The concentration of ethanol required to significantly accelerate flip-flop in the work of Schwichtenhovel et al. (1992) was two to three times higher than that causing 10-fold promotion of liposome-planar bilayer hemifusion. However, the possibility that hemifusion promotion by short-chain alcohol is mediated by the effects of these alcohols on lipid bilayer binding and phospholipid flip-flop still needs to be addressed experimentally.

Adding short-chain surfactants to long-chain surfactants is known to decrease thickness and bending rigidity (= bending modulus) of the bilayer (Safinya et al., 1989). As the energy of monolayer bending is proportional to the bending rigidity (Helfrich, 1973; Kozlov et al., 1994), this would result in promotion of bilayer undulations and facilitate stalk formation. Note, however, that even maximally

hemifusion-promoting concentrations of ethanol do not cause any measurable change in the bilayer thickness as detected by measuring the specific capacitance of the planar bilayers, which is inversely proportional to the thickness. Assuming a 2% thinning of the bilayers (within a standard deviation of our capacitance measurements), one may expect only a ~6% decrease in the bending rigidity, which is inversely proportional to the cube of the thickness (Safinya et al., 1989). While this effect does not appear to be very profound for the planar bilayer, it could be stronger for liposomal and cellular membranes, and we cannot exclude such a decrease in the bending rigidity and a proportional decrease in the energy barrier of stalk formation as a possible mechanism of hemifusion promotion.

Alcohol can facilitate a local breaking of lipid monolayer continuity required for stalk formation

Our results are consistent with the hypothesis that membrane rearrangement in hemifusion starts with a transient and local breaking of the continuity of the lipid monolayers in the contact zone. Such a breakage of monolayers allows them to form a semistalk (Fig. 1 *B*) and leads to an energy-intensive exposure of the hydrophobic interior of the membrane. Stalk formation upon the merger of two semistalks present on the contacting monolayers completes hemifusion (Fig. 1 *C*).

The energy barrier, F_{HF}, which determines the hemifusion rate, corresponds to the difference between the energy of the initial state of the flat and continuous monolayer, F_0, and the energy of the stalk nucleus, F_N:

$$F_{HF} = F_N - F_0 = F_{O/W} + F_E + F_I - F_0, \qquad (1)$$

where $F_{O/W}$ is the energy of the oil/water interface at the top of stalk nuclei, F_E is the elastic energy of lipid monolayer in the stalk nuclei, and F_I is the energy of hydrophobic interstices (Kozlov and Markin, 1983; Siegel, 1993).

Note that, for simplicity, the possible contributions of hydration repulsion (Leikin et al., 1987, 1993) and hydrophobic attraction (Helm et al., 1992), as well as the possible mechanisms underlying the correlated formation of two semistalks in adjacent sites of contacting monolayers are neglected in this analysis.

Because the exposure of hydrocarbon chains of lipids to water is highly unfavorable, the contribution of the $F_{O/W}$ can be quite significant. Assuming an interfacial tension of 50 mN/m and a 1 nm^2 area of exposure (close to the surface area occupied by one lipid molecule), $F_{O/W}$ is ~12kT. For the semistalk with a hydrophobic patch of ~3 nm diameter (corresponding to that of the narrowest part of the stalk), $F_{O/W}$ can be ~80kT, i.e., it can be comparable to the elastic energy of a stalk (Chernomordik et al., 1995b). At the hemifusion-promoting concentration of 0.5 M, ethanol decreases the water/*n*-heptane interface tension from 50 to 40 dyne/cm (Bartell and Davis, 1941). For a 3-nm hydrophobic patch, the alcohol-induced decrease in interfacial tension lowers $F_{O/W}$ from ~80kT to 64kT and thus might notably facilitate breaking of the lipid monolayer required for semistalk formation.

As mentioned above, it can be impossible to alter just one of the energy contributions in Eq. 1 without changing other contributions. For instance, lowering of F_{HF} in the presence of PE can be explained not only by a decrease in F_E, but also by an increase in F_0. In contrast, LPC can increase the barrier F_{HF} by increasing F_E of the lipid monolayer in a semistalk and in a stalk, and by decreasing F_0 for the situation where initial curvature of contacting lipid monolayers exceeds their spontaneous curvature. If short-chain alcohol, similar to LPC, shifts the spontaneous curvature of lipid monolayers to more positive values and thus increases T_E, this increase may be overcompensated by more profound alcohol-induced decreases in $F_{O/W}$, providing for the net promotion of hemifusion.

Our results suggest that the rate-limiting stage in membrane hemifusion corresponds to the formation of two adjacent semistalks rather than their merger into a stalk. The similar effects of alcohol on liposome-planar bilayers and on HA-mediated cell-cell fusion are consistent with the hypothesis that the merger of membrane lipid bilayers in fusion proceeds through stalk-like intermediates (Chernomordik et al., 1995b; Monck and Fernandez, 1996; Chanturiya et al., 1997; Lee and Lentz, 1997a, 1998; Siegel and Epand, 1997). We hope that better understanding of the energetics of the early fusion intermediates will eventually bring new ideas on how proteins control biological fusion.

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REFERENCES

Aldwinckle, T. J., Q. F. Ahkong, A. D. Bangham, D. Fisher, and J. A. Lucy. 1982. Effects of poly(ethylene glycol) on liposomes and erythrocytes. Permeability changes and membrane fusion. *Biochim. Biophys. Acta.* 689:548–560.

Barry, J. A., and K. Gawrisch. 1994. Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. *Biochemistry.* 33:8082–8088.

Bartell, F. E., and J. K. Davis. 1941. Adsorption at water-air and water-organic liquid interfaces. *J. Phys. Chem.* 45:1321–1336.

Basanez, G., F. M. Goni, and A. Alonso. 1998. Effect of single chain lipids on phospholipase C-promoted vesicle fusion. A test for the stalk hypothesis of membrane fusion. *Biochemistry.* 37:3901–3908.

Brahm, J. 1983. Permeability of human red cells to a homologous series of aliphatic alcohols. Limitations of the continuous flow-tube method. *J. Gen. Physiol.* 81:283–304.

Chanturiya, A. N. 1996. Filter switching device for dual wavelength videoimaging. *J. Fluorescence.* 6:103–106.

Chanturiya, A., L. V. Chernomordik, and J. Zimmerberg. 1997. Flickering fusion pores comparable with initial exocytotic pores occur in protein-free phospholipid bilayers. *Proc. Natl. Acad. Sci. USA.* 94: 14423–14428.

Chen, Z., and R. P. Rand. 1998. Comparative study of the effects of several *n*-alkanes on phospholipid hexagonal phases. *Biophys. J.* 74:944–952.

Chernomordik, L., A. Chanturiya, J. Green, and J. Zimmerberg. 1995a. The hemifusion intermediate and its conversion to complete fusion: regulation by membrane composition. *Biophys. J.* 69:922–929.

Chernomordik, L. V., V. A. Frolov, E. Leikina, P. Bronk, and J. Zimmerberg. 1998. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. *J. Cell Biol.* 140:1369–1382.

Chernomordik, L., M. Kozlov, and J. Zimmerberg. 1995b. Lipids in biological membrane fusion. *J. Membr. Biol.* 146:1–14.

Chernomordik, L. V., E. Leikina, V. Frolov, P. Bronk, and J. Zimmerberg. 1997. An early stage of membrane fusion mediated by the low pH conformation of influenza hemagglutinin depends upon membrane lipids. *J. Cell Biol.* 136:81–94.

Chernomordik, L. V., G. B. Melikyan, I. G. Abidor, V. S. Markin, and Y. A. Chizmadzhev. 1985. The shape of lipid molecules and monolayer membrane fusion. *Biochim. Biophys. Acta.* 812:643–655.

Chernomordik, L. V., G. B. Melikyan, and Y. A. Chizmadzhev. 1987. Biomembrane fusion: a new concept derived from model studies using two interacting planar lipid bilayers. *Biochim. Biophys. Acta.* 906: 309–352.

Cohen, F. S., M. H. Akabas, J. Zimmerberg, and A. Finkelstein. 1984. Parameters affecting the fusion of unilamellar phospholipid vesicles with planar bilayer membranes. *J. Cell Biol.* 98:1054–1062.

Cohen, F. S., J. Zimmerberg, and A. Finkelstein. 1980. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes. II. Incorporation of a vesicular membrane marker into the planar membrane. *J. Gen. Physiol.* 75:251–270.

Cunningham, B. A., L. Midmore, O. Kucuk, L. J. Lis, M. P. Westerman, W. Bras, D. H. Wolfe, P. J. Quinn, and S. B. Qadri. 1995. Sterols stabilize the ripple phase structure in dihexadecylphosphatidylcholine. *Biochim. Biophys. Acta.* 1233:75–83.

Duzgunes, N., J. Wilschut, R. Fraley, and D. Papahadjopoulos. 1981. Studies on the mechanism of membrane fusion. Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochim. Biophys. Acta.* 642:182–195.

Epand, R. M. 1985. Diacylglycerols, lysolecithin, or hydrocarbons markedly alter the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines. *Biochemistry.* 24:7092–7095.

Epand, R. M. 1993. Hydrophobicity curvature and membrane fusion [letter]. *Trends Biochem. Sci.* 18:81.

Finkelstein, A. 1987. Water Movement through Lipid Bilayers, Pores, and Plasma Membranes. John Wiley and Sons, New York.

Hao, L., and D. G. Leaist. 1996. Binary mutual diffusion coefficients of aqueous alcohols. Methanol to 1-heptanol. *J. Chem. Eng. Data.* 41: 210–213.

Helfrich, W. 1973. Elastic properties of lipid bilayers: theory and possible experiments. *Z. Naturforsch.* 28c:693–703.

Helm, C. A., J. N. Israelachvili, and P. M. McGuiggan. 1992. Role of hydrophobic forces in bilayer adhesion and fusion. *Biochemistry.* 31: 1794–1805.

Ho, C., and C. D. Stubbs. 1997. Effect of *n*-alkanols on lipid bilayer hydration. *Biochemistry.* 36:10630–10637.

Holte, L. L., and K. Gawrisch. 1997. Determining ethanol distribution in phospholipid multilayers with MAS-NOESY spectra. *Biochemistry.* 36: 4669–4674.

Hornby, A. P., and P. R. Cullis. 1981. Influence of local and neutral anaesthetics on the polymorphic phase preferences of egg yolk phosphatidylethanolamine. *Biochim. Biophys. Acta.* 647:285–292.

Kemble, G. W., T. Danieli, and J. M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell.* 76:383–391.

Kim, S., and G. M. Martin. 1981. Preparation of cell-size unilamellar liposomes with high captured volume and defined size distribution. *Biochim. Biophys. Acta.* 646:1–9.

Komatsu, H., and S. Okada. 1995. Ethanol-induced aggregation and fusion of small phosphatidylcholine liposome: participation of interdigitated membrane formation in their processes. *Biochim. Biophys. Acta.* 1235: 270–280.

Komatsu, H., and E. S. Rowe. 1991. Effect of cholesterol on the ethanol-induced interdigitated gel phase in phosphatidylcholine: use of fluorophore pyrene-labeled phosphatidylcholine. *Biochemistry.* 30: 2463–2470.

Korte, T., K. Ludwig, M. Krumbiegel, D. Zirwer, G. Damaschunand, and A. Herrmann. 1997. Transient changes of the conformation of hemagglutinin of influenza virus at low pH detected by time-resolved circular dichroism spectroscopy. *J. Biol. Chem.* 272:9764–9770.

Kozlov, M. M., S. Leikin, and R. P. Rand. 1994. Bending, hydration and void energies quantitatively account for the hexagonal-lamellar-hexagonal reentrant phase transition in dioleoylphosphatidylethanolamine. *Biophys. J.* 67:1603–1611.

Kozlov, M. M., and V. S. Markin. 1983. Possible mechanism of membrane fusion. *Biofizika.* 28:255–261.

Leckband, D. E., C. A. Helm, and J. Israelachvili. 1993. Role of calcium in the adhesion and fusion of bilayers. *Biochemistry.* 32:1127–1140 (erratum 32:4974).

Lee, J., and B. R. Lentz. 1997a. Evolution of lipidic structures during model membrane fusion and the relation of this process to cell membrane fusion. *Biochemistry.* 36:6251–6259.

Lee, J., and B. R. Lentz. 1997b. Outer leaflet-packing defects promote poly(ethylene glycol)-mediated fusion of large unilamellar vesicles. *Biochemistry.* 36:421–431.

Lee, J., and B. R. Lentz. 1998. Secretory and viral fusion may share mechanistic events with fusion between curved lipid bilayers. *Proc. Natl. Acad. Sci. USA.* 95:9274–9279.

Leikin, S. L., M. M. Kozlov, L. V. Chernomordik, V. S. Markin, and Y. A. Chizmadzhev. 1987. Membrane fusion: overcoming of the hydration barrier and local restructuring. *J. Theor. Biol.* 129:411–425.

Leikin, S., V. A. Parsegian, D. C. Rau, and R. P. Rand. 1993. Hydration forces. *Annu. Rev. Phys. Chem.* 44:369–395.

Lentz, B. R. 1994. Polymer-induced membrane fusion: potential mechanism and relation to cell fusion events. *Chem. Phys. Lipids.* 73:91–106.

McIntosh, T. J., K. G. Kulkarni, and S. A. Simon. 1999. Membrane fusion promoters and inhibitors have contrasting effects on lipid bilayer structure and undulations. *Biophys. J.* 76:2090–2098.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1989. Range of the solvation pressure between lipid membranes: dependence on the packing density of solvent molecules. *Biochemistry.* 28:7904–7912.

Melikyan, G. B., W. D. Niles, and F. S. Cohen. 1995a. The fusion kinetics of influenza hemagglutinin expressing cells to planar bilayer membranes is affected by HA density and host cell surface. *J. Gen. Physiol.* 106: 783–802.

Melikyan, G. B., J. M. White, and F. S. Cohen. 1995b. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* 131:679–691.

Monck, J. R., and J. M. Fernandez. 1996. The fusion pore and mechanisms of biological membrane fusion. *Curr. Opin. Cell Biol.* 8:524–33 (erratum 8:890).

Mou, J., J. Yang, C. Huang, and Z. Shao. 1994. Alcohol induces interdigitated domains in unilamellar phosphatidylcholine bilayers. *Biochemistry.* 33:9981–9985.

Niles, W. D., and F. S. Cohen. 1987. Video fluorescence microscopy studies of phospholipid vesicle fusion with a planar phospholipid membrane. Nature of membrane-membrane interactions and detection of release of contents. *J. Gen. Physiol.* 90:703–735.

Ohki, S. 1984. Effects of divalent cations, temperature, osmotic pressure gradient, and vesicle curvature on phosphatidylserine vesicle fusion. *J. Membr. Biol.* 77:265–275.

Ohki, S. 1988. Surface tension, hydration energy and membrane fusion. *In* Molecular Mechanisms of Membrane Fusion. Plenum Press, New York. 123–139.

Powell, G. L., and D. Marsh. 1985. Polymorphic phase behavior of cardiolipin derivatives studied by ^{31}P NMR and x-ray diffraction. *Biochemistry.* 24:2902–2908.

Rand, R. P., N. L. Fuller, S. M. Gruner, and V. A. Parsegian. 1990. Membrane curvature, lipid segregation, and structural transitions for phospholipids under dual-solvent stress. *Biochemistry.* 29:76–87.

Safinya, C., E. Sirota, D. Roux and G. Smith. 1989. Universality in interacting membranes—the effect of cosurfactants on the interfacial rigidity. *Phys. Rev. Lett.* 62:1134–1137.

Sarkar, D. P., S. J. Morris, O. Eidelman, J. Zimmerberg, and R. Blumenthal. 1989. Initial stages of influenza hemagglutinin-induced cell fusion

monitored simultaneously by two fluorescent events: cytoplasmic continuity and lipid mixing. *J. Cell Biol.* 109:113–122.

Schwichtenhovel, C., B. Deuticke, and C. W. Haest. 1992. Alcohols produce reversible and irreversible acceleration of phospholipid flip-flop in the human erythrocyte membrane. *Biochim. Biophys. Acta.* 1111:35–44.

Siegel, D. P. 1993. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* 65:2124–2140.

Siegel, D. P., and R. M. Epand. 1997. The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms. *Biophys. J.* 73:3089–3111.

Simon, S. A., and T. J. McIntosh. 1984. Interdigitated hydrocarbon chain packing causes the biphasic transition behavior in lipid/alcohol suspensions. *Biochim. Biophys. Acta.* 773:169–172.

Slater, J. L., and C. H. Huang. 1988. Interdigitated bilayer membranes. *Prog. Lipid Res.* 27:325–359.

Smaal, E. B., C. Schreuder, J. B. van Baal, P. N. Tijburg, J. G. Mandersloot, B. de Kruijff, and J. de Gier. 1987. Calcium-induced changes in permeability of dioleoylphosphatidylcholine model membranes containing bovine heart cardiolipin. *Biochim. Biophys. Acta.* 897:191–196.

Veiro, J. A., R. G. Khalifah, and E. S. Rowe. 1989. The polymorphic phase behavior of dielaidoylphosphatidylethanolamine. Effect of *n*-alkanols. *Biochim. Biophys. Acta.* 979:251–256.

Walter, A., P. L. Yeagle, and D. P. Siegel. 1994. Diacylglycerol and hexadecane increase divalent cation-induced lipid mixing rates between phosphatidylserine large unilamellar vesicles. *Biophys. J.* 66:366–376.

White, J. 1996. Membrane fusion: the influenza paradigm. *Cold Spring Harb. Symp. Quant. Biol.* 60:581–588.

White, J. M., and I. A. Wilson. 1987. Anti-peptide antibodies detect steps in a protein conformational change: low-pH activation of the influenza virus hemagglutinin. *J. Cell Biol.* 105:2887–2896.

Wilschut, J., and D. Hoekstra. 1986. Membrane fusion: lipid vesicles as a model system. *Chem. Phys.Lipids.* 40:145–166.

Zeng, J., K. E. Smith, and P. L. Chong. 1993. Effects of alcohol-induced lipid interdigitation on proton permeability in L-α-dipalmitoylphosphatidylcholine vesicles. *Biophys. J.* 65:1404–1414.

Zimmerberg, J., F. S. Cohen, and A. Finkelstein. 1980. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes. I. Discharge of vesicular contents across the planar membrane. *J. Gen. Physiol.* 75:241–250.

Zimmerberg, J., M. Curran, F. S. Cohen, and M. Brodwick. 1987. Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. *Proc. Natl. Acad. Sci. USA.* 84:1585–1589.