# The Hemifusion Intermediate and its Conversion to Complete Fusion: Regulation by Membrane Composition

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ABSTRACT To fuse, membranes must bend. The energy of each lipid monolayer with respect to bending is minimized at the spontaneous curvature of the monolayer. Two lipids known to promote opposite spontaneous curvatures, lysophosphatidylcholine and arachidonic acid, were added to different sides of planar phospholipid membranes. Lysophosphatidylcholine added to the contacting monolayers of fusing membranes inhibited the hemifusion we observed between lipid vesicles and planar membranes. In contrast, fusion pore formation depended upon the distal monolayer of the planar membrane; lysophosphatidylcholine promoted and arachidonic acid inhibited. Thus, the intermediates of hemifusion and fusion pores in phospholipid membranes involve different membrane monolayers and may have opposite net curvatures. Biological fusion may proceed through similar intermediates.

## INTRODUCTION

Fusion of two phospholipid bilayers involves the formation of bent, non-bilayer intermediates. The curvature elastic energy of any bent intermediate depends upon the spontaneous (intrinsic) curvature of the lipid monolayer in its unstressed state (Helfrich, 1973; Chung and Caffrey, 1994; Kozlov et al. 1994; Siegel, 1993), which for any given temperature and hydration is a sensitive function of system composition. The striking differences in this spontaneous curvature for naturally occurring lipids reveal themselves in a variety of liquid crystalline phases, within which lipid molecules have effective shapes (Luzzati et al., 1968; Israelachvili et al., 1976; Tate et al., 1991; Hui and Sen, 1989). When lipids are constrained to curvatures other than spontaneous, there is an elastic force to curve the lipid monolayer. Low concentrations of nonlamellar lipids, when added to lamellar phases, can drive those systems into phases with either positive or negative curvature (Cullis et al., 1991; Epand, 1985; Kumar et al., 1989; Hope and Cullis, 1981; Epand et al., 1991).

To test the possibility that membrane fusion may be controlled by specific alteration of the propensity of the monolayers to bend, we have chosen two lipids known to promote opposite spontaneous curvatures. They are lysophosphatidylcholine, which promotes a micellar positive curvature (Epand, 1985; Kumar et al., 1989); and arachidonic acid, which promotes an inverted hexagonal negative curvature (Hope and Cullis, 1981; Epand et al., 1991). Two pairs of membrane monolayers, contacting and distal, must each bend in fusion. By adding our two nonlamellar lipids to each of these pairs of monolayers, we can determine which lipid monolayers form fusion intermediates, and the likely net curvature of each intermediate. Depending upon this curvature, the work of monolayer bending will be increased by one lipid and decreased by the other.

To have access to both sides of the membrane, we studied the fusion of phospholipid vesicles to planar phospholipid bilayer membranes (Zimmerberg et al., 1980; Cohen et al., 1980, 1982, 1984; Cohen and Niles, 1993; Woodbury and Hall, 1988; Perin and MacDonald, 1989), which reproduces the geometry of sphere-to-plane fusion in exocytosis. To distinguish different stages of fusion, vesicles were labeled by porin ion channels and fluorescent lipid at self-quenching concentrations. Membrane interaction was assayed by conductance measurements and fluorescence microscopy.

## MATERIALS AND METHODS

All lipids were purchased from Avanti Polar Lipids (Birmingham, AL), except for ergosterol (Fluka Chemika-BioChemika, Buchs, Switzerland); and arachidonic acid (AA), which, along with fatty acid free bovine serum albumin (BSA), was purchased from Sigma Chemical Company (St. Louis, MO). A stock solution of AA (50 mM) was freshly prepared in ethanol. Stock solutions of lauroyl lysophosphatidylcholine (LPC) were freshly prepared as a 0.5% (w/w) aqueous dispersion. Decane, heptane, squalene, salts, and EGTA were purchased from Fluka Chemika-BioChemika; standard buffer (100 mM KCl, 10 mM 2-[*N*-morpholino]ethanesulfonic acid/(MES), 1 mM EDTA, pH 6.0) and other solutions were prepared in house-distilled, then deionized, water.

If not stated otherwise, phospholipid vesicles from a mixture of egg phosphatidylcholine (PC) with the given percentage of egg phosphatidylethanolamine (PE) and *N*-(lissamine rhodamine B sulfonyl)diacyl phosphatidylethanolamine (R-PE) were prepared daily by a sonication-freeze-thaw technique (Cohen et al., 1984). 5 mg of the desired lipid in chloroform was dried by rotary evaporation. Standard buffer (0.475 ml) with or without 10 $\mu$g of porin from *Bordetella pertussis* (Armstrong et al., 1986) were added. The mixture was vortexed for 30 s and sealed under argon. The suspension was sonicated for ~30 min in a bath sonicator (model G1128P1G; Laboratory Supplies, Hicksville, NY). Then, after three freeze-thaw cycles (dry ice/ethanol bath for 70 s followed by thawing at room temperature), the cloudy suspension was again sonicated for 15 s to disperse the vesicles. Vesicles were stored on ice until use. The vesicles

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obtained by this technique have diameters ranging from 20 to several hundred nanometers with the average vesicle diameter estimated to be ~70 nm (Cohen et al., 1984).

In some experiments, the same technique was used to prepare the liposomes of PC/R-PE (86/14 mol %) loaded with calcein. In this case, standard buffer was replaced by the following: 200 mM calcein, 10 mM MES, 5 mM *n*-propyl gallate, pH 6.5. The liposomes were separated from external calcein by filtration through a column of Sephadex G-25 and elution with isotonic buffer: 400 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.5. The calcein fluorescence of 1 ml of the liposome suspension, (16 $\mu$g of lipid/ml), with 60 $\mu$g/ml of asolectin (soybean L-$\alpha$-phosphatidylcholine 20%, Avanti Polar Lipids) added to mimic conditions of our planar bilayer experiments, was measured before and after addition of 90 $\mu$M LPC using an Aminco Luminescence spectrometer (SLM, Urbana, IL) at $\lambda_{excitation}$ = 480 nm, $\lambda_{emission}$ = 520 nm. (Asolectin contains 31% PC, 28% PE, 22% of phosphatidylinositol and phosphatidic acid (not separated in this analysis), 12% neutral lipids, 3% lysolipids and 5% other lipids (Walter Shaw, personal communication).) The increase in fluorescence was normalized by maximum dequenching determined after lysing liposomes with 0.5% Triton X-100.

To roughly estimate the incorporation of exogenously added lipids (LPC and AA) into liposome membranes we used rhodamine fluorescence dequenching (MacDonald, 1990). To get a calibration curve of R-PE quenching as a function of R-PE concentration we measured the fluorescence of PC/R-PE liposomes with different percentages of R-PE at $\lambda_{excitation}$ = 540 nm, $\lambda_{emission}$ = 597 nm before ($F$) and after 0.5% Triton X-100 addition ($F_0$). Then we measured the decrease in R-PE quenching (increase in $F/F_0$ ratio) caused by adding 90 $\mu$M LPC or 130 $\mu$M AA to 1 ml of the suspension of PC/R-PE (86/14 mol %) liposomes (16 $\mu$g lipid/ml) with 60 $\mu$g/ml asolectin, the mixture mimicking conditions of our planar bilayer experiments. The incorporation of LPC and AA into liposome membranes was estimated using the calibration curve described.

To evaluate the possible effects of vesicle size, two other techniques of vesicle preparation were used in a few experiments. Large unilamellar vesicles (2–10 $\mu$m diameter) loaded with 200 mM calcein, 5 mM *n*-propyl gallate and 10 mM MES, pH 6.7 were formed from the mixture of asolectin, ergosterol, and R-PE (70:20:10, w/w %) by the technique described in Niles and Cohen (1987). Significantly smaller PC vesicles containing R-PE (14 mol %) were formed by extrusion 19 times in standard buffer through two 100 nm pore polycarbonate filters (Nucleopore, Pleasanton, CA) for 19 times in the Lipofast device (Avestin, Inc., Ottawa, Ontario, Canada).

If not stated otherwise, planar lipid bilayers were formed from asolectin. In some experiments we formed planar bilayers from diphytanoylphosphatidylcholine (DPhC). Two routine methods of planar phospholipid membrane formation were used in the present study. Mueller-Rudin type planar phospholipid membranes were formed by applying a small amount of a lipid solution in *n*-decane (20 mg/ml) onto a 1 mm diameter hole in a Teflon film separating two buffer-filled compartments. Solvent-free bilayers were formed by the Montal-Mueller technique across the ~200 $\mu$m diameter hole of a Teflon partition in a Lucite chamber. 6 $\mu$l of a lipid solution in hexane (10 mg/ml) were added to 1 ml of aqueous buffer in the cell, and the solution was allowed to dry for 10 min. Vesicles were added into one of the two chamber compartments ("*cis* compartment") to a final concentration of 16 $\mu$g of lipid/ml with continuous stirring.

Electrical and surface tension measurements on planar bilayers were performed as described previously (Sukharev et al., 1982). The potential across membranes was clamped at 20 mV, with the *cis* compartment positive. All experiments were performed at room temperature (20–22°C). In preliminary experiments we measured the conductance of a single channel of porin from *B. pertussis* to be ~120 pS, consistent with previously reported data for this porin (Armstrong et al., 1986).

A special chamber for fluorescence microscopy similar to that described (Niles and Cohen, 1987) was placed between an objective and condenser lens mounted on a microbench system (Spindler and Hoyer, Germany). Small portions of the lipid vesicle suspension were pressure-injected toward the planar bilayer so that a number of vesicles became attached to the membrane. Stirring of the *cis* compartment removed unbound liposomes. A 10×, 0.21 numerical aperture (NA) lens was used for observation of the planar bilayer under bright field illumination; and a 40×, 0.5 NA lens (Nikon) was used as an objective for fluorescence. Fluorescent images were obtained with two sets of interference filters (Omega Optical, Brattleboro, VT), 545 nm excitation and 590 nm emission filters for rhodamine fluorescence and 485 nm excitation and 520 nm emission for calcein fluorescence. Images were projected onto a Silicon-intensifier target tube camera (Hamamatsu, Hamamatsu City, Japan) connected to a VCR, videoprocessor, monitor, and computer.

## RESULTS AND DISCUSSION

### LPC and AA differentially inhibit or promote fusion depending upon their location

Complete fusion of phospholipid vesicles with planar lipid bilayer membranes (merging of both monolayers and the aqueous volumes they initially separated) is driven by osmotic swelling of vesicles tightly bound to planar membranes in the presence of divalent cations (Zimmerberg et al., 1980; Cohen et al., 1980, 1982, 1984; Cohen and Niles, 1993; Woodbury and Hall, 1988). Accordingly, when phospholipid vesicles containing porin were introduced into one of the compartments (defined as *cis*), followed by addition of $CaCl_2$ to 10 mM, *cis*, and an osmoticant (urea) to 260 mM, *cis*, we observed a succession of fusion events. To avoid scoring mere membrane marker transfer as fusion, we routinely checked to make sure that the conductance jumps were caused by simultaneous insertion of multiple channels. Macroscopically, this sequence of fusion events gave a quasilinear time course of conductance increase for the first 5–10 min. The kinetics of the conductance increase characterized the rate of complete fusion. To obtain good rates of fusion, we tested the effect of adding exogenous lipids on the vesicle fusion to the solvent-containing planar bilayers (Cohen et al., 1984).

Addition of AA (final concentration 130 $\mu$M) into the vesicle-containing *cis* compartment caused no observable effect on the fusion rate (7 out of 9 experiments, Fig. 1 *A*) or slightly promoted fusion (2 out of 9 experiments). In contrast, LPC (90 $\mu$M) in 13 out of 13 experiments dramatically inhibited vesicle-planar bilayer fusion within 5–30 s after addition (Fig. 1, *B* and *C*). Without LPC, increasing the applied osmotic gradient increases the rate of fusion (Cohen et al., 1984). With LPC, we could not obtain fusion by increasing the osmotic gradient even when up to 1.5 M urea was added into the *cis* compartment (4 out of 4 experiments, Fig. 1 *B*). However, when BSA (final concentration 50 mg/ml) was added to the *cis* compartment to extract LPC, fusion ensued with a rate close to the initial one (5 out of 5 experiments, Fig. 1 *C*). In the absence of the exogenous lipids (LPC and AA), addition of BSA to either the *cis* or *trans* compartment caused no conductance changes and thus did not initiate fusion when no osmotic gradient was applied (transmembrane potential 20 mV, 4 out of 4 experiments). BSA also had no effect on the fusion rate

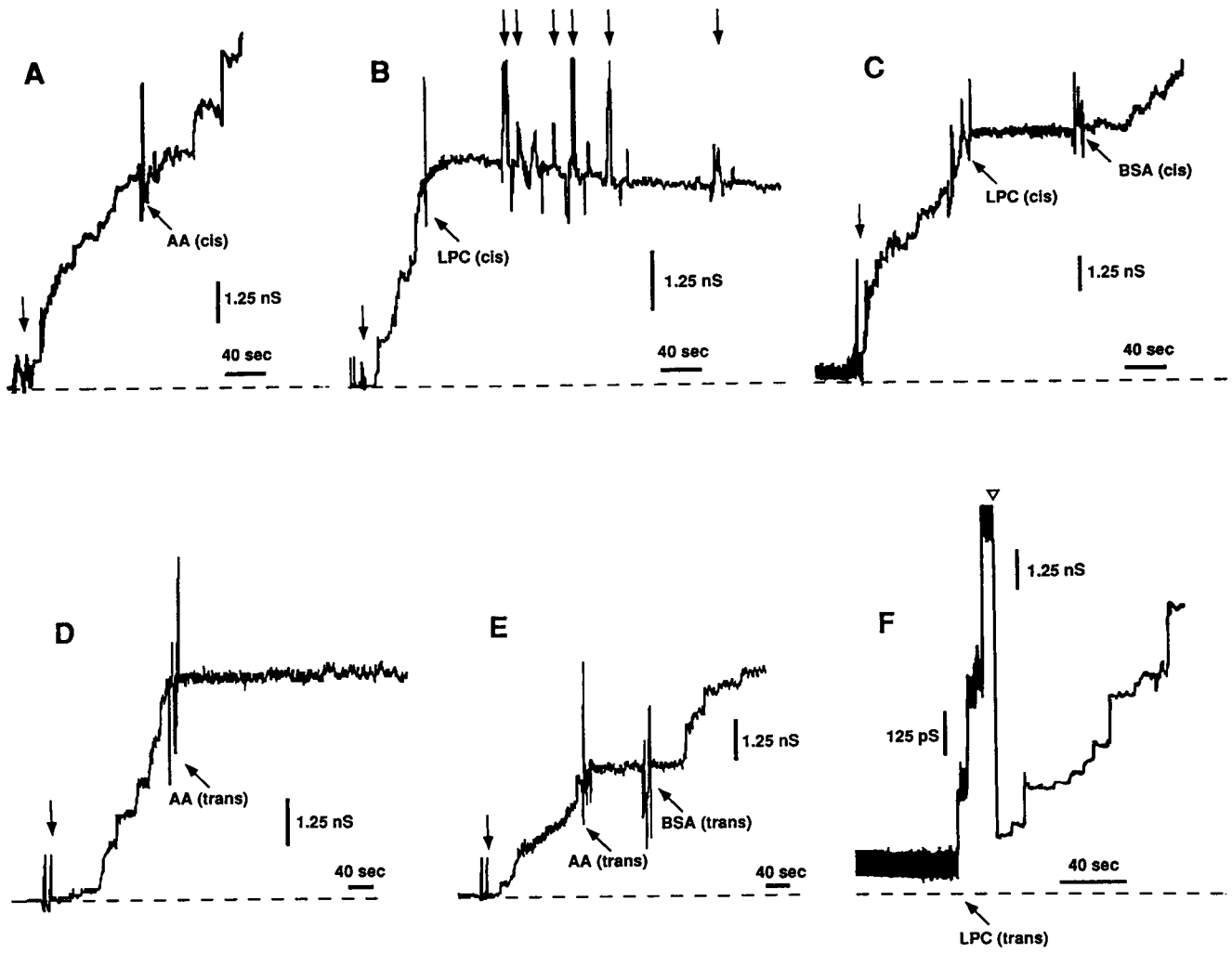


FIGURE 1 Effect of asymmetrically added lipids on the rates of phospholipid vesicle-planar lipid bilayer fusion. Vesicles containing porin channels, calcium (20 mM) and, finally, urea (270 mM, vertical arrows in *A–E* were added sequentially at one side of planar lipid bilayer into the same compartment of the chamber (*cis*) to start vesicle-planar bilayer fusion (*A*, *B*, and *C*). Because each event of complete fusion is seen as an increase in current due to porin channels inserting into the planar bilayer, an increase in conductance characterized the fusion rate. Zero conductance levels are shown by dashed horizontal lines. In the six independent experiments shown, arachidonic acid (130 $\mu$M; *A*, *D* and *E*) or lysophosphatidylcholine (90 $\mu$M; *B* and *C*) were added to either *cis* (*A*, *B*, and *C*) or *trans* (*D* and *E*) compartments of the chamber. (*F*) In this experiment vesicles were added to the *cis* compartment but no urea was added and calcium was added to both compartments of the cell, symmetrically, to avoid any osmotic gradient. Fusion, seen as the stepwise increase in current, was triggered by addition of 270 $\mu$M LPC to the *trans* compartment. The arrowhead indicates a 10-fold decrease of measurement sensitivity.

when added after application of the osmotic gradient (3 out of 3 experiments).

Neither planar bilayers nor vesicle membranes were permeabilized by LPC in our experiments. In the absence of liposomes, 20 min incubation of planar membranes with 270 $\mu$M LPC added to one of the two chamber compartments caused no conductance changes (transmembrane potential 20 mV, 4 out of 4 experiments). To test the possibility that LPC in the *cis* compartment causes lysis of liposomes, we used liposomes loaded with calcein at a self-quenching concentration. A small increase of calcein fluorescence was observed when 90 $\mu$M LPC was added to a suspension of liposomes in isotonic buffer containing the same concentration of asolectin (60 $\mu$g/ml) that we used to form solvent-free bilayers. This small fluorescence increase was only ~3.3 ± 0.5% ($n$ = 3) of that observed at complete lysis of the vesicles by 0.5% Triton ×100. Thus the inhibition of vesicle fusion by adding LPC to the *cis* compartment is not secondary to LPC-induced lysis of liposomes.

Vesicle-planar bilayer fusion was affected in a very different way when the same lipids were added to the other side (*trans* compartment) of a planar bilayer to incorporate into the distal monolayer of solvent-containing planar bilayers. AA (130 $\mu$M) abruptly stopped fusion within ~30 s of addition in 12 of 12 experiments (Fig. 1, *D* and *E*). In contrast, LPC (270 $\mu$M) in 11 out of 14 experiments promoted fusion when added to the *trans* compartment, with no osmotic gradient applied (Fig. 1 *F*). Both AA inhibition and LPC promotion of fusion were reversible: addition of the lipid-absorbing BSA *trans* caused resumption of the fusion that had been blocked by AA and it stopped LPC-induced

fusion (7 out of 7 and 4 out of 4 experiments, respectively; Fig. 1 *E*).

The different effects of LPC and AA observed when these lipids were added to different membrane monolayers could not be explained by changes in the surface tension of planar lipid bilayers in the presence of these exogenous lipids. Adding lipids lowered the surface tension, but no significant differences were seen between LPC (0.40 ± 0.15 mN/m in 90 μM *cis* and *trans*, $n = 8$) and AA (0.44 ± 0.16 mN/m in 130 μM *cis* and *trans*, $n = 8$), compared with 0.78 ± 0.1 mN/m without added lipids ($n = 6$). A rough estimate of exogenous lipid incorporation into vesicle membranes (based on the extent of the dequenching of rhodamine fluorescence) gave ~13 and 9.5 mol % for 90 μM LPC and 130 μM AA, respectively. These estimates of the LPC concentration in membranes are consistent with the estimate, based on the data on lauroyl LPC binding to erythrocyte membranes (Weltzien, 1979), which gives ~16 mol % LPC (1.5 nmol LPC bound to 8 nmol PC in the outer monolayer of the added liposomes).

## Hemifusion occurs in the absence of osmotic gradients, in solvent-free membranes, and depends upon membrane composition

Our electrical assay detects complete fusion. To test for lipid mixing during fusion, we used fluorescence microscopy. Vesicles containing both porin channels and 14 mol % R-PE were injected close to planar lipid bilayers. Initially, a number of vesicles were seen bound to the planar bilayer as bright points on a dark background. In time, bright flashes associated with increased circular fluorescent disks appeared to grow radially from originally fixed vesicles (Fig. 2 *a–d*) indicating fast diffusion of fluorescent lipid from the vesicle to the planar bilayer. The fast, flashlike character of the dye transfer to the planar bilayer from one vesicle at a time argues against the exchange of the membrane dye through an aqueous gap between membranes; it rather suggests single merging events of labeled and unlabeled membranes. The flashes took place randomly and were distributed in a time interval of ~10 min. After a number of flashes, the whole planar bilayer became fluorescent. In other experiments, we decreased the R-PE content of the vesicle membrane from 14 mol % to 5 mol %. This resulted in higher vesicle fluorescence due to the lower degree of dye self-quenching. We still observed flashes of fast dye redistribution, and as expected, they were less bright due to the lower dye concentration (not shown).

Importantly, flashes were observed even if no osmotic gradient was applied across the vesicle membrane or planar bilayer. In agreement with other findings (Zimmerberg et al., 1980; Cohen et al., 1980, 1982, 1984; Cohen and Niles, 1993), no conductance jumps manifesting insertion of porin channels were seen under these conditions. Thus, flashes of membrane dye redistribution took place even in the absence of complete fusion. This demonstrates the existence of an osmotically independent stage of membrane fusion: hemifusion, the merger of contacting monolayers which allows rapid lipid diffusion. To further characterize this stage of the fusion process, the subsequent experiments were performed with isotonic media in the *cis* and *trans* compartments (no osmotic gradient applied).

Neither porin nor decane are required for hemifusion. The probability of hemifusion (number of flashes of fluorescence divided by the number of bound vesicles) was the same for vesicles formed with or without porin channels (0.24 ± 0.08 ($n = 4$) and 0.23 ± 0.07 ($n = 3$), respectively). Spontaneous hemifusion of vesicles to planar bilayers was

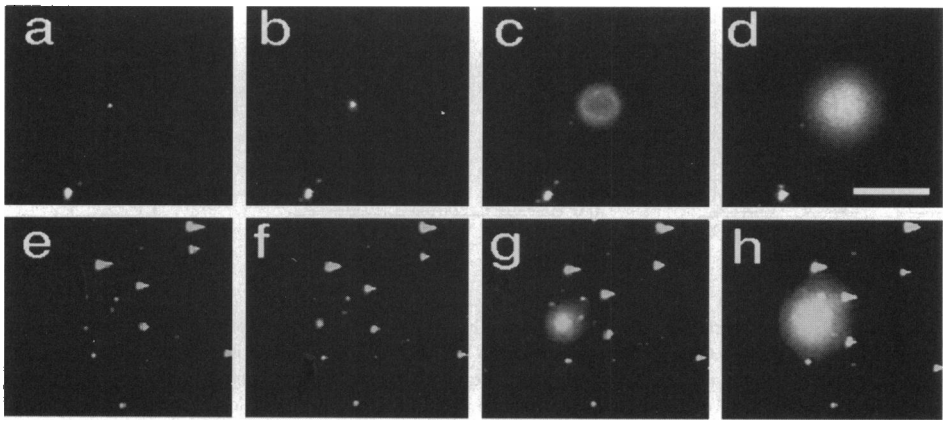


FIGURE 2 Light flash due to dilution of self-quenched fluorescent lipids of a vesicle upon its hemifusion to a planar bilayer. Calcium (20 mM) was present in both compartments of the chamber, and PC vesicles containing both porin channels and R-PE (14 mol %) were injected into the immediate proximity of a decane-containing planar bilayer (*a–d*) or a solvent-free planar bilayer (*e–h*). No conductance changes were observed under these conditions, indicating the absence of complete fusion events without osmotic gradients (data not shown). However, flashes of a fast dequenching and spatially concentric distribution of R-PE from the vesicle membrane into the decane-containing planar bilayer (*a–d*) and into the solvent-free planar bilayer (*e–h*) demonstrated the establishment of lipid continuity between the vesicle membrane and the planar bilayer. Individual frames were selected from the videotaped record of these flashes. *b*, *c*, and *d* were taken 1, 2, and 5 s after *a*. *f*, *g*, and *h* were taken 2, 7, and 14 s after *e*, respectively. The scale bar denotes 50 μm.

observed not only for decane-containing planar lipid membranes but also for "solvent-free" bilayers (Fig. 2, *e–h*). Qualitatively, the results were the same, but with fewer flashes and slower redistribution of dye.

Hemifusion probability depends upon membrane composition increasing with increasing PE concentration in the vesicle membrane (Fig. 3 *A*). When LPC was added to the *cis* compartment, almost no flashes were observed (Fig. 3 *B*). Extraction of lysolipid by BSA restored the initial probability of flashes. In contrast, AA (both *cis* and *trans*) had no significant effect on hemifusion probability (not shown). Note the significantly higher probability of flashes for decane-containing planar bilayers (Fig. 3 *B*) than for solvent-free bilayers (Fig. 3 *A*).

The time course of hemifusion also depended upon the lipid composition of membranes. To characterize the kinetic rates of bilayer hemifusion we measured the period of time elapsing between vesicle application and each hemifusion event observed. Addition of LPC to the *cis* compartment increased the median delay for hemifusion of PC/R-PE vesicles (86/14 mol %) to solvent-free bilayers (Fig. 3 *C*).

In the experiments presented above we used the planar lipid bilayers formed from asolectin, a natural mixture of soybean lipids. Vesicles will also spontaneously hemifuse with solvent-free bilayers formed from one synthetic phospholipid: DPhC. The median delay for hemifusion of PC vesicles, containing 14 mol % of R-PE, to DPhC planar bilayers in the presence of 20 mM $CaCl_2$ was 57 ± 16 s (mean ± SD, total of 93 flashes, 4 experiments).

To make sure vesicle-planar bilayer hemifusion is not dependent on the particular technique of vesicle preparation or vesicle size, in some experiments R-PE-containing vesicles of quite different sizes were formed by two techniques in addition to the sonication-freeze-thaw method used in the experiments described above. Fluorescent lipid redistribution indicative of hemifusion was observed both for large unilamellar vesicles (diameters ~0.1 μm) formed by extrusion (MacDonald et al., 1991) (data not shown), and for "cell-size" unilamellar vesicles (diameters ~2–10 μm) formed by modification of the Kim and Martin technique (Niles and Cohen, 1987) (Fig. 4). Flashes of membrane dye redistribution (Fig. 4, *a–d*) with no visible release of the water soluble dye, loaded into giant vesicles, (Fig. 4, *e–h*) further confirmed the existence of the vesicle-planar bilayer hemifusion.

Incorporation of LPC into the contacting monolayers of membranes reversibly arrested hemifusion and thus inhibited complete fusion. On the other hand, modification of the distal monolayer had no obvious effect on hemifusion, but promoted complete fusion. To our knowledge, promotion of complete fusion by incorporation of lysophosphatidylcholine into the distal monolayer of a planar bilayer is the only known example of phospholipid vesicle-planar bilayer fusion without osmotic swelling of vesicles.

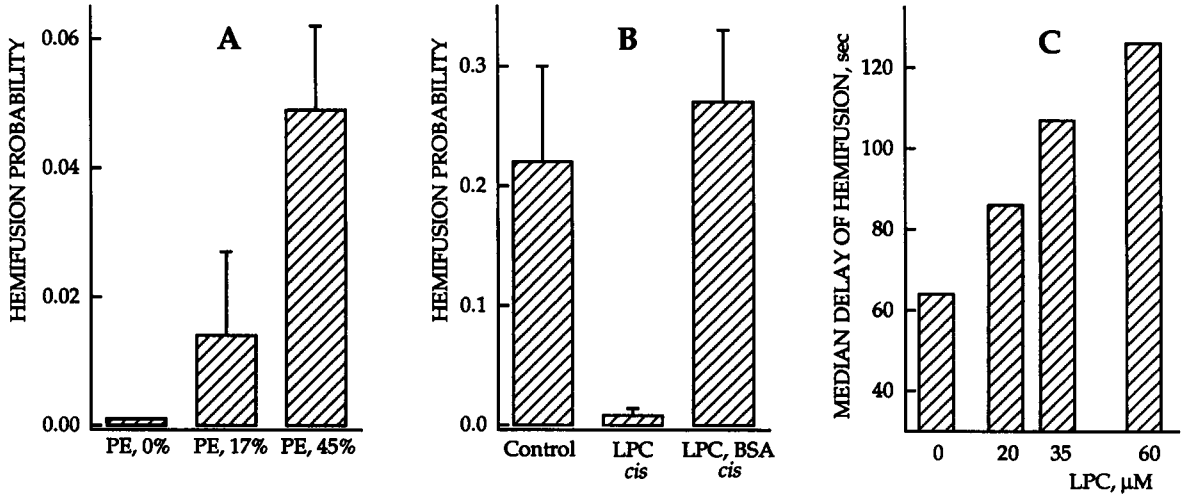


FIGURE 3 Dependence of hemifusion on membrane lipid composition. The number of light flashes of vesicle-planar bilayer hemifusion observed as in Fig. 2 was counted. Vesicles contained no porin. Other conditions were as in Fig. 2. (*A* and *B*) The total number of light flashes was divided by the number of bound vesicles to estimate probabilities of hemifusion. Values shown are the mean ± SE for at least three independent experiments. (*A*) Hemifusion probabilities for PC/PE vesicles containing 5 mol % R-PE and solvent-free planar bilayers, as a function of the PE percentage in vesicles, i.e., the ratio of the molar concentration of PE to the sum of the molar concentrations of PE, PC, and R-PE. (*B*) Hemifusion probabilities for PC/R-PE (86/14 mol %) vesicles and decane-containing planar bilayers with no exogenous lipid added; with 90 μM LPC added *cis*; and with 90 μM LPC added *cis* followed by BSA addition (final concentration 50 mg/ml) to the same compartment. (*C*) The time course of hemifusion for the vesicles formed of PC/RPE (86/14 mol%) and solvent-free planar bilayers in the presence of different concentrations of LPC in the *cis* compartment. The ordinate indicates the periods of time elapsing between vesicle application and the moment when half the flashes had occurred (median delay of hemifusion). The distributions of the hemifusion waiting times were built up in the absence of LPC (73 flashes, 9 experiments); and in the presence of 20 μM LPC (74 flashes, 8 experiments); 35 μM LPC (60 flashes, 9 experiments); and 60 μM LPC (50 flashes, 14 experiments). Because of overloading of the camera we were not able to observe fluorescence flashes for the first ~ 15 s after ejection of the vesicles. However, even when we were ready to record flashes, none were observed during the next 15–20 s until ~30 s after vesicle application. Because of the lower probability of hemifusion with LPC added *cis*, to accumulate a comparable number of flashes more vesicles were applied to planar bilayers in the presence of LPC.

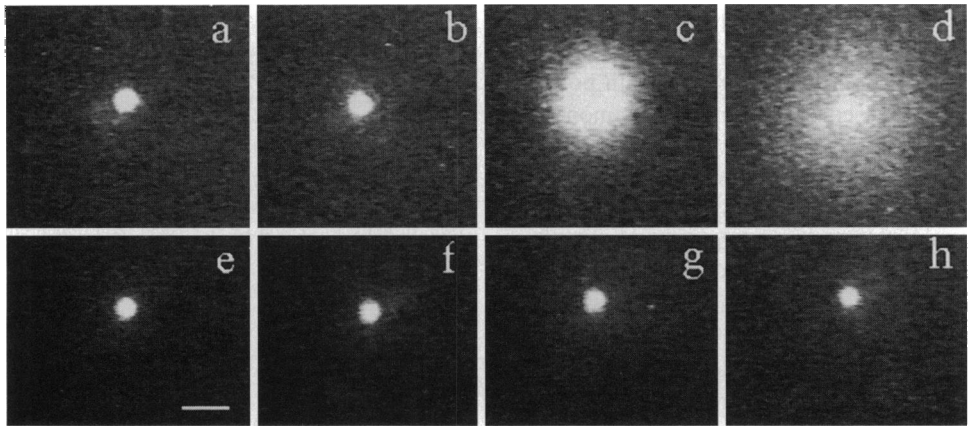


FIGURE 4 Membrane dye redistribution upon vesicle-planar bilayer hemifusion is not accompanied by the release of vesicular water-soluble dye trapped within. Although the membrane dye, R-PE, dilutes and dequenches to give a flash (*a–d*), the fluorescence of the trapped aqueous dye, calcein, remains unchanged (*e–h*). Frames were taken sequentially with a 1 s interval between corresponding rhodamine fluorescence and calcein fluorescence video images. Individual pairs of frames were selected from the videotaped record. *b*, *c*, and *d* were taken 24, 26, and 31 s after *a*. *h*, *f*, *g*, and *h* were taken 1, 25, 27, and 32 s after *a*. The scale bar denotes 10 $\mu$m. Membrane bathing solution: 400 mM KCl, 20 mM $CaCl_2$; 10 mM HEPES; 1 mM EDTA, pH 7.5.

The existence of a hemifusion stage of the fusion process for vesicle-planar lipid bilayers was hypothesized (Chernomordik et al., 1987) and confirmed by dye redistribution measurements for decane-containing membranes (Perin and MacDonald, 1989). In contrast, no hemifusion was found for solvent-free planar bilayers by the same approach (Perin and MacDonald, 1989) nor by an assay where hemifusion was supposed to be revealed by consequent vesicle lysis mediated by formation of nystatin channels in the developed hemifusion zone (Niles and Cohen, 1987). In the present study we have applied a dequenching assay more direct than the nystatin approach (Niles and Cohen, 1987) and more sensitive than an approach based on mere redistribution of the membrane fluorescent lipid (Perin and MacDonald, 1989). Vesicles were found to hemifuse even with solvent-free planar bilayers (Fig. 2, *e–h*; Fig. 4) although with a lower probability than with decane-containing membranes (Fig. 3, *A* and *B*). Hemifusion of lipid bilayers has been documented for planar bilayer-planar bilayer (Chernomordik et al., 1985, 1987) and vesicle-vesicle fusion (Bentz et al., 1985; Duzgunes et al., 1985; Ellens et al., 1986). One can never exclude the possible presence of the local inclusions of solvent even in so-called solvent-free membranes (Niles et al., 1988; Chanturiya, 1995). However, our finding of hemifusion for solvent-free membranes, together with the dependence of hemifusion on vesicle lipid composition, argues that planar membrane microlenses are not involved. Increase of the fusion rates in the presence of hydrocarbon solvents (e.g., decane or hexadecane) was reported earlier for vesicle-planar bilayer fusion (Cohen et al., 1984), planar bilayer-planar bilayer fusion (Chernomordik et al., 1987), and vesicle-vesicle fusion (Walter et al., 1994).

## The stalk-pore hypothesis for membrane fusion

Spontaneous hemifusion of vesicles to planar lipid bilayers, its blocking by LPC, promotion by PE, and the reciprocal effects of LPC and AA on different membrane monolayers are consistent with the hypothesis that different stages of membrane fusion may be controlled (promoted or inhibited) by specific alterations of spontaneous curvature of lipid monolayers. In particular, our results agree with the predictions of the stalk-pore model of fusion (Fig. 5 and (Chernomordik et al., 1987; Leikin et al., 1987; Kozlov et al., 1989; Chernomordik et al., 1995a). The stalk is a hypothetical lipidic connection between contacting monolayers that leads to hemifusion (Kozlov and Markin, 1983; Chernomordik et al., 1985; Siegel, 1993). Similar structures, "contact points" between bilayers, were observed under fusion conditions by freeze fracture electron microscopy and phosphorus-31 nuclear magnetic resonance (Hui et al., 1981).

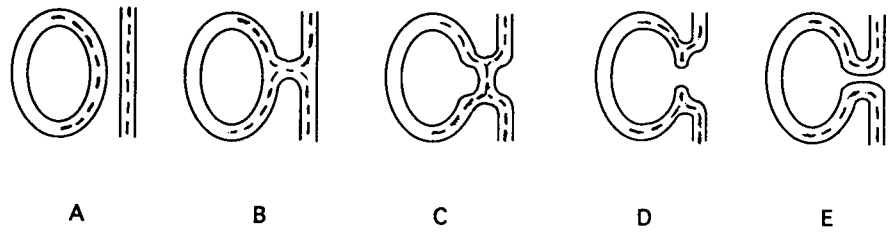


FIGURE 5 Stalk-pore hypothesis of membrane fusion. (*A*) Membranes in contact; (*B*) a stalk intermediate; (*C*) extended hemifusion structure (*D*) formation of a fusion pore; (*E*) completion of the fusion process. Dashed lines show the boundaries of the hydrophobic surfaces of monolayers.

The stalk has a net negative curvature (Kozlov and Markin, 1983; Chernomordik et al., 1985), and so was inhibited in our experiments by insertion of LPC, a lipid with positive spontaneous curvature into contacting membrane monolayers. In contrast, PE, a lipid with negative spontaneous curvature (Cullis et al., 1991), promoted hemifusion. Because in a bilayer composed of the distal monolayers of two fusing membranes, the fusion pore is a structure with net positive curvature (Chernomordik et al., 1985; Kozlov et al., 1989), complete fusion was expected to be, and was, promoted by LPC and inhibited by AA, a lipid with negative spontaneous curvature, if inserted into the distal leaflets. Thus, the stalk-pore hypothesis explains why exogenous lipids (which change monolayer spontaneous curvature in opposite directions) affect membrane fusion in opposite ways, and why the same lipids have opposite effects if added to opposing membrane monolayers.

Hemifusion of biological membranes has been shown or inferred in some systems (Pinto da Silva and Nogueira, 1977; Song et al., 1991; Stamatatos and Düzgünes, 1993; Kemble et al., 1994) but not all (Ornberg and Reese, 1981; see also discussion in Tse et al., 1993; Zimmerberg et al., 1994). LPC reversibly inhibits $Ca^{2+}$-, pH- and GTP-dependent biological fusion reactions when added to contacting monolayers of membranes (Chernomordik et al., 1993; Vogel et al., 1993; Chernomordik et al., 1995b). AA added to the outer leaflet of the plasma membrane of sea urchin eggs inhibits exocytotic fusion (Elhai and Scandella, 1983). The qualitative similarity between results presented here for fusion of model lipid membranes and those reported for biological membranes supports the hypothesis that stalk and pore types of fusion intermediates are involved in biological fusion. Specialized "fusion" proteins may modulate the spontaneous curvatures of the biological membrane monolayers to promote formation of these intermediates.

## APPENDIX

In the stalk-pore fusion model (Kozlov and Markin, 1983; Chernomordik et al., 1985, 1987, 1995a; Kozlov et al., 1989) alterations of membrane lipid composition affect fusion by modulation of the propensity of the membrane monolayers to bend. Using a number of assumptions one may roughly estimate the minimal membrane concentrations of the "non-bilayer" lipids, which according to the stalk-pore hypothesis may significantly affect fusion of lipid bilayers. If the thickness of the water gap separating contacting monolayers of fusing membranes is assumed to be equal to the thickness of the monolayer, $h$ (Rand and Parsegian, 1989) and the neutral surface, which keeps its area constant with bending deformation (Kozlov and Winterhalter, 1991), is located at half of the monolayer thickness, the elastic energy of the stalk, $F$, is given by the following expression (see Kozlov and Markin, 1983, Chernomordik et al., 1995a)

$$F = 2\pi\kappa\left\{J_s h[\pi(\rho + 1.5) - 4] + 2\frac{(\rho + 1.5)^2}{[(\rho + 1.5)(\rho + 2.5)]^{1/2}}\arctan\frac{(\rho + 2.5)^{1/2}}{(\rho + 1.5)^{1/2}} - 4\right\} \quad \text{(A1)}$$

where $\kappa$ and $J_s$ are bending rigidity and spontaneous curvature of the monolayer, respectively, and $\rho$ is a dimensionless radius of the contact bilayer ($\rho = R/h$, where $R$ is the radius of the contact bilayer). Suppose lipid monolayers are originally formed from lipids of a given spontaneous curvature, $J_s$, and then we introduce into these monolayers an additional lipid component of spontaneous curvature, $J_s'$, to a mol fraction $N$. If we additionally assume that the spontaneous curvature of a composite monolayer is additive in the spontaneous curvatures of the constituting molecules (Rand et al., 1990; but see Rilfors et al., 1984), then the spontaneous curvature of a composite monolayer, ${}^cJ_s = J_s + N(J_s' - J_s)$. The waiting time of stalk formation ($R = 0$) and, consequentially, that of membrane hemifusion, $t_{hf}$, changes from $t_{hf,0}$, to $t_{hf,1}$, according to the following equation (see (Chernomordik et al., 1985)):

$$\frac{t_{hf,1}}{t_{hf,0}} = \exp\left\{\frac{4.48\kappa h N(J_s' - J_s)}{kT}\right\} \quad \text{(A2)}$$

where $k$ is the Boltzman constant and $T$ is the absolute temperature. For a rough estimate one may substitute $J_s \sim 0$ for PC and asolectin, and $J_s' \sim 1/h$ for LPC and $J_s' \sim -1/h$ for PE. The bending rigidity of lipid monolayer has the same order of magnitude for different lipids and can be estimated as 10 $kT$ (Kozlov and Winterhalter, 1991). Substituting these values into Eq. 2 we get ~5 mol % for the concentrations of "non-bilayer" lipid (e.g., LPC, PE), which can cause an order of magnitude change in the hemifusion rate. Similar concentrations of non-bilayer lipids affected hemifusion in our experiments (see Fig. 3). Analogous estimates can be made for the effects of the monolayer spontaneous curvature on pore formation (Chernomordik et al., 1985). Estimates of this kind are clearly oversimplified and based on a number of assumptions, and although they indicate that the changes of the spontaneous curvature of a monolayer at concentrations of LPC, AA, and PE used in the present study may indeed account for the observed dramatic effects on lipid bilayer fusion, a quantitative test of the stalk-pore model must await further research.

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