Influenza Virus−Liposome Lipid Mixing Is Leaky and Largely Insensitive to the Material Properties of the Target Membrane†

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ABSTRACT: Monolayer intrinsic curvature, void stabilization, and membrane rupture tension have been suggested as important factors determining the rate of membrane fusion. Here, we have studied the kinetics of fusion between influenza virus and target liposomes as a function of various target membrane material properties. In order to examine the fusion process directly, a simple prebinding step is used and proven to be adequate to achieve fusion-rate-limiting kinetics. To test the hypothesis about membrane curvature and void stabilization, we studied the lipid mixing kinetics with dioleoylphosphatidylcholine (DOPC)/ganglioside GD1a (GD1a) liposomes containing lysooleoylphosphatidylcholine (LPC, positive curvature), dioleoylglycerol (DOG, negative curvature), arachidonic acid (AA, negative curvature), and hexadecane (HD, void stabilization). DOG, AA, and HD (at 4 mol %) showed no significant effect on the fusion kinetics, while LPC reversibly inhibited influenza HA mediated fusion only at very high concentrations. Using target liposomes with different membrane rupture tension values, no obvious correlation between membrane rupture tension and the rate of lipid mixing was observed. Moreover, a reported potential antiviral compound, *tert*-butylhydroquinone (t-b-HQ) (Bodian et al., 1993), showed no significant effect on the kinetics of influenza fusion. Finally, leakage of liposome contents was detected during lipid mixing. For encapsulated molecules smaller than 450 MW, the kinetics of leakage is very similar to the kinetics of lipid mixing. In fact, leakage was also detected for encapsulated molecules up to 10 000 MW, suggesting that HA mediated lipid mixing is a very leaky process. Since "nonleaky fusion" has been the foundation of influenza fusion models, our work suggests the need for a major revision in the modeling of this process.

For two closely apposed liposome bilayers to fuse, nonbilayer intermediates have to be formed. In these intermediate structures, monolayers are highly curved (Siegel, 1993a), and this curvature creates hydrophobic voids that must be filled by stretching out acyl chains of lipids (Gruner, 1989). It has been proposed that the free energy of a putative liposome−liposome fusion intermediate can be estimated by summing the monolayer curvature elastic energy and the free energy to stabilize the hydrophobic voids (Siegel, 1993a; Zimmerberg et al., 1993; Chernomordik et al., 1995). The curvature elastic energy of the intermediate depends on the intrinsic curvature of the lipid monolayer, which is determined by the lipid composition of the monolayer (Keller et al., 1993; Siegel, 1993a). Incorporation of low concentrations of nonlamellar lipids into the membrane(s) is thus expected to alter the free energy of the intermediates and either increase or decrease the rate of fusion. On the other hand, addition of apolar lipids, such as long-chain alkanes, has been found to destabilize the lamellar phase, lower the free energy for void stabilization by intercalating into the hydrophobic voids (Tate & Gruner, 1987; Siegel et al., 1989), and accelerate liposome−liposome fusion (Walter et al., 1994).

Despite extensive research on the role of lipid composition in liposome−liposome fusion, little is known about the lipid requirements for biological fusion. Recently, high concentrations of lysolipids, which increase the intrinsic curvature of the monolayer, have been found to inhibit several biological membrane fusion processes, including Sendai virus fusion (Yeagle et al., 1994), pH-dependent baculovirus fusion, Ca^{2+}-dependent exocytotic fusion of sea urchin cortical granule (Vogel et al., 1993), and GTP-dependent rat microsome fusion (Chernomordik et al., 1993) as well as phospholipase C-induced fusion (Nieva et al., 1993). Based on the diversity of these fusion processes, it is proposed that lysolipids may inhibit a common stalk-like intermediate step in all fusion processes (Vogel et al., 1993; Chernomordik et al., 1993; Yeagle et al., 1994).

On the other hand, Alford et al. (1994) found that influenza virus fusion with DOPC/DOPE[1] (2:1) liposomes was just as fast as its fusion with DOPE liposomes, whereas its fusion with DOPC liposomes was much slower. Since all these liposomes are composed of lipids with the same acyl chains and thus should have roughly the same void stabilization energies as discussed by Gruner (1989), this difference suggested that void stabilization may not be a determining factor in influenza HA mediated fusion. Moreover, because the spontaneous curvature of a mixed monolayer equals the mole fraction weighted sum of the curvatures of the pure components (Keller et al., 1993), the curvature of DOPC/DOPE (2:1) liposomes is expected to be closer to that of DOPC than that of DOPE liposomes. The difference that sets DOPC/DOPE (2:1) and DOPE liposomes apart from DOPC liposomes suggested that monolayer intrinsic curva-

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ture may not be an important factor in influenza fusion either. Recently, Günther-Ausborn et al. (1995) also reported that the inhibition of influenza virus fusion by LPC may be due to direct inhibition on HA itself.

To resolve these questions, we have examined the correlation between fusion kinetics of influenza PR/8 virus and the material properties of the target membrane. In particular, we have focused on additives which alter bilayer intrinsic curvature (Chernomordik et al., 1993; Siegel, 1993; Yeagle et al., 1994; Vogel et al., 1993; Keller et al., 1993), which stabilize hydrophobic voids (Tate & Gruner, 1987; Siegel, 1989; Walter et al., 1994), which alter membrane rupture tension (Evans & Needham , 1987), and which are predicted to bind to HA and stabilize its neutral pH conformation so as to inhibit its fusogenic capacity (Bodian et al., 1993). Our results show that the fusogenic capacity of HA is largely unaffected by any of the above minor alterations in the target membrane or the putative HA binding agent. Moreover, extensive leakage of liposome contents is detected during fusion with influenza virus. The leakage kinetics of encapsulated small molecular weight molecules correlates with the kinetics of fusion, as measured by fluorescence lipid mixing assay. These results are consistent with a fusion mechanism which requires specific lipid−protein interaction and extensive disruption of the lipids at the fusion site (Bentz et al., 1990, 1993a).

MATERIALS AND METHODS

Materials. Arachidonic acid (AA), cholesterol (CHOL), dioleoylglycerol (DOG), dioleoylphosphatidylcholine (DOPC), 1-stearoyl-2-oleoylphosphatidylcholine (SOPC), and lysooleoylphosphatidylcholine (LPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ganglioside GD1a (GD1a), ultrol grade *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, tetrasodium salt (HEPES), and octaethylene glycol monododecyl ether ($C_{12}E_8$) were purchased from Calbiochem (La Jolla, CA). 1-Aminonaphthalene-3,6,8,-trisulfonic acid, sodium salt (ANTS), *N*,*N*′-*p*-xylylenebis-(pyridinium bromide) (DPX), *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE), *N*-(7-nitro-2,1,3,-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), tetrametylrhodamine B Dextran 3000 MW (TMR-D3), and tetrametylrhodamine B Dextran 10,000 MW (TMR-D10) were purchased from Molecular Probes (Eugene, OR). *N*′-[[4-[7-(Diethylamino)-4-methylcoumarin-3-yl]phenyl]-*N*-methylthioureidyl]phosphatidylcholine (CPT-PC) and [[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]phosphatidylcholine (DABS-PC) were generous gifts from Dr. John R. Silvius (McGill University, Quebec, Canada). Hexadecane (HD) were purchased from EM Science (Gibbstown, NJ). Dibucaine, hydroquinone (HQ), and *tert*-butylhydroquinone (t-b-HQ) were purchased from Fluka (Roukonkona, NY). The influenza A/PR/8/34 (H1N1) (catalog no. VR-95) inoculum was purchased from American Type Culture Collection (ATCC) (Rockville, MD), thawed, subaliquoted, and stored at −80 °C. Ten-day-old fertilized eggs were purchased from SPAFAS (Norwich, CT).

Virus Purification. Influenza A/PR/8/34 (H1N1) was purified by modified procedures of Alford et al. (1994). Briefly, an aliquot of the inoculum was quickly thawed and diluted 1:10000 with sterile 10 mM HBS buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.5). Virus was grown in the allantoic cavity of 10-day-old fertilized chicken eggs. Each egg was inoculated with 0.1 mL of diluted inoculum and incubated at 35 °C for 65 h with periodic turning. After at least 1 h at 4 °C, the allantoic fluid was harvested and centrifuged at 1000*g* for 30 min to remove debris. The virus was pelleted at 25000*g* for 20 h. The pellet was resuspended in 10 mM HBS buffer for 1 h at 4 °C, homogenized, incubated at 20 °C for 45 min, and centrifuged at 2000*g* for 15 min to remove aggregated virus. The supernatant was placed atop a 0/20/40/60% sucrose density step gradient and centrifuged at 85000*g* for 2 h. The purified virus was collected from the 20/40% sucrose interface, assayed for protein, quickly frozen, and stored at −80 °C.

The viral phospholipid−protein ratio was determined by modification of the total lipid extraction procedures described by Bligh and Dyer (1959). Briefly, 1 mL of purified virus was mixed with 1.25 mL of $CHCl_3$ and 2.5 mL of MeOH and bath sonicated for 30 s. $CHCl_3$ (1.25 mL) and 1.25 mL of H_2O were added subsequently followed by bath sonication after addition of each solvent. The mixture was quickly frozen in dry ice/EtOH and spun at 100*g* at room temperature to form a biphase system. The top aqueous layer was removed and extracted again following the same procedure. The $CHCl_3$ layers from both extraction were pooled, dried under argon stream, and redissolved in 2 mL of $CHCl_3$. The phosphate content was determined according to Bartlett (1959). The phospholipid−protein ratio of our purified PR/8 virus is 0.322 μmol/mg.

Liposome Preparation. Large unilamellar vesicles (LUVs) were prepared by reverse phase evaporation procedures (Szoka & Papahadjopoulos, 1980) and extruded 5−10 times through 0.1 μm polycarbonate membrane filter (Poretics Corp., Livermore, CA). All liposome preparations for lipid mixing experiments contained 10 mol % GD1a and 0.6 mol % each of CPT-PC/DABS-PC or NBD-PE/Rh-PE. The remaining 90% consisted of DOPC, SOPC, SOPC/CHOL (1:1), DOPC/AA (86:4), DOPC/DOG (86:4), DOPC/HD (86:4), or DOPC/LPC (86:4). Unless otherwise specified, all above LUVs contained no soluble fluorophores inside and were made in 10 mM HBS buffer.

ANTS/DPX encapsulated LUVs used in the leakage assay contained no fluorescent lipids. They were made in ANTS/DPX buffer (25 mM ANTS, 90 mM DPX, 10 mM HEPES, 0.1 mM EDTA, pH 7.5). The LUVs were separated from unencapsulated ANTS and DPX by passing through a 20 ×

[1] Abbreviations: HA, hemagglutinin; DOPC, dioleoylphosphatidylcholine; SOPC, 1-stearoyl-2-oleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; AA, arachidonic acid; DOG, dioleoylglycerol; CHOL, cholesterol; LPC, lysooleoylphosphatidylcholine; HD, hexadecane; GD1a, ganglioside GD1a; ANTS, 1-aminonaphthalene-3,6,8,-trisulfonic acid, sodium salt; DPX, *N*,*N*′-*p*-xylylenebis(pyridinium bromide); CPT-PC, *N*′-[[4-[7-(diethylamino)-4-methylcoumarin-3-yl]-phenyl]-*N*-methylthioureidyl]phosphatidylcholine; DABS-PC, [[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]phosphatidylcholine; NBD-PE, *N*-(7-nitro-2,1,3,-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; HQ, hydroquinone; t-b-HQ, *tert*-butylhydroquinone; TMR, tetrametylrhodamine B; TMR-D3, tetrametylrhodamine B conjugated dextran 3000 MW; TMR-D10, tetrametylrhodamine B conjugated dextran 10 000 MW; $C_{12}E_8$, octaethylene glycol monododecyl ether; EDTA, (ethylenedinitrilo)tetraacetic acid, tetrasodium salt; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, sodium; HBS, HEPES buffer saline; RET, resonance energy transfer; LUV, large unilamellar vesicles; ODR, octyldecyl rhodamine B, chloride salt.

1.3 cm Sephadex G-75 gel filtration column (Pharmacia, Piscataway, NJ). LUVs eluted after the void volume (4 mL), while the free ANTS and DPX eluted at 15 mL.

Dextran encapsulated DOPC LUVs were double labeled with CPT/DABS in the membrane and TMR in the aqueous content. They were made in dextran buffer (20 mg/mL TMR-conjugated dextran, 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.5). TMR-D3 LUVs were separated from unencapsulated TMR-D3 using a 45 × 1.3 cm Sephadex G-75 gel filtration column. TMR-D3 LUVs eluted after the void volume (9 mL), and unencapsulated TMR-D3 eluted at 19 mL. TMR-D10 LUVs were separated from unencapsulated TMR-D10 using a 45 × 1.3 cm Sephadex G-200 (Pharmacia, Piscataway, NJ) gel filtration column. TMR-D10 LUVs eluted after the void volume (8 mL), and unencapsulated TMR-D10 eluted at 20 mL. No interference between the fluorescence of CPT/DABS and TMR was detected (data not shown).

The lipid concentration of each liposome prep was determined by phosphate assay (Bartlett, 1959) and corrected for GD1a and other nonphospholipids, such as CHOL, AA, HD, and DOG.

Fluorescence Lipid Mixing Assay. Lipid mixing between unlabeled influenza virus and CPT/DABS or NBD/Rh labeled liposomes was measured in 10 mM HBS buffer by the resonance energy transfer (RET) assay (Silvius et al., 1987; Struck et al., 1981). CPT or NBD fluorescence was recorded on a PTI Alphascan fluorometer (South Brunswick, NJ) in a thermostated cuvette with continuous stirring. The excitation and emission wavelengths were 395 and 477 nm for CPT/DABS assay and 450 and 520 nm for NBD/Rh assay. The extent of dequenching measured by CPT/DABS assay is always higher than that obtained using NBD/Rh assay, although the overall lipid mixing kinetics is the same for both assays after the curves are rescaled to the same final extent (Shangguan, 1995).

The lipid mixing assays were performed as described before (Alford et al., 1994). Briefly, in both assays, a concentrated stock of virus and liposomes (25×) was preincubated on ice for at least 30 min to allow prebinding before dilution into a preequilibrated cuvette. The final concentration in the cuvette was 10 μM viral phospholipids and 10 μM liposomal lipids. This fluorescence level was set as 0% lipid mixing. Fusion was initiated after 30 s by injecting 1.67 M acetic acid/acetate solution to reach pH 4.9. The fluorescence level at infinite probe dilution obtained by $C_{12}E_8$ (0.75 mM) lysis was considered as 100% lipid mixing.

In cases where antiviral compounds were tested, the compounds were present in both the preincubation mixture and the diluted fusion reaction mixture in the cuvette at the indicated concentration. Titration of LPC was carried out by injecting $CHCl_3$ dissolved LPC stock solutions into the cuvette containing prebound virus and liposomes. Addition of LPC caused a dose-dependent increase in initial fluorescence. After the fluorescence level restabilized, lipid mixing was initiated as described above. The restabilized fluorescence level was set as 0% lipid mixing in this case. Different concentrations of LPC stock solutions were used to keep the added volume of $CHCl_3$ constant, 0.34% of total volume inside the cuvette.

Leakage of ANTS/DPX. Leakage of ANTS/DPX encapsulated liposomes was measured as the dequenching of ANTS fluorescence. Measurements were made on the PTI fluorometer as described above. The excitation and emission wavelengths were 360 and 514 nm, respectively. The emission fluorescence was further refined with an KV470 filter (Schott Glass Technologies, Inc., PA). Prebinding and fusion steps were performed as described above. The fluorescence level before acidification was set as 0% leakage, and the fluorescence level after $C_{12}E_8$ lysis was used as 100% leakage.

Leakage of Dextran. Tetramethylrhodamine B (TMR) on both 3000 and 10 000 MW dextran is self-quenched at our encapsulation concentration (20 mg/mL). Thus, it is possible to detect leakage of dextran by monitoring the dequenching of TMR. The assay followed the same procedure as ANTS/DPX leakage assay. The excitation and emission wavelengths were 555 and 580 nm, respectively.

Dextran leakage was also confirmed by column chromatography. Three hundred fifty nanomoles of viral phospholipids and 350 nmol of liposomal lipid from dextran containing liposomes were prebound as described above. After the mixture was shifted to 37 °C, 1.67 M acetic acid/acetate solution was added to acidify the mixture to pH 4.9. After 10 min of incubation at 37 °C to allow fusion to complete, the mixture was neutralized and loaded on a 45 × 1.3 cm Sephadex G-75 column in the case of TMR-D3 liposomes or Sephadex G-200 column in the case of TMR-D10 liposomes. One milliliter fractions were collected using a Foxy fraction collector (ISCO, Lincoln, NE). The concentration of dextran in each fraction was assayed by measuring the fluorescence of TMR in the presence of 0.75 mM $C_{12}E_8$ on the PTI fluorometer.

When the TMR fluorescence level of each fraction was plotted versus the elution volume, two peaks were detected for each fusion reaction, one for liposome encapsulated dextran, the other for the free dextran that had leaked out. The relative amount of dextran in each peak was measured by printing out the elution profile, cutting out the peaks, and weighing them separately. The percentage of dextran in each peak was determined as 100% × (the weight of this peak)/(the total weight of two peaks).

RESULTS

Effect of Prebinding Step. Studying fusion kinetics requires, under the experimental conditions used, that fusion rather than virus−liposome aggregation is rate-limiting to the overall lipid mixing kinetics being measured. To reach fusion-rate-limiting conditions, we preincubated unlabeled virus with DOPC/GD1a (90:10) liposomes at 4 °C for 30 min at a concentration that is 25 times the final concentration for the fusion reaction. The lipid mixing is measured by NBD/Rh resonance energy transfer (RET) assay (Struck et al., 1981). Lipid mixing leads to the dilution of the fluorophores into the viral envelop and dequenching of the NBD fluorescence. The virus−liposome aggregation step is completed during the preincubation period, judged by the fact that prolonged incubation does not change the lipid mixing kinetics (data not shown). This is consistent with published data (Alford et al., 1994).

To determine whether significant dissociation occurs after an aliquot of the prebound virus−liposomes is delivered to the preequilibrated cuvette, the prebound virus−liposome were incubated in the cuvette at 37 °C for up to 30 min before acidification (Figure 1). The lipid mixing kinetics

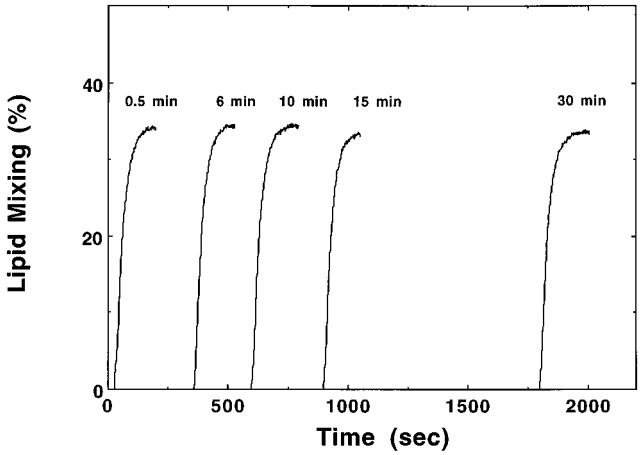

FIGURE 1: Lipid mixing kinetics of prebound virus-liposomes as a function of incubation time at 37 °C. Unlabeled PR/8 virus and NBD/Rh labeled DOPC/GD1a (90:10) liposomes were prebound at 4 °C for 30 min at 25× the final concentration in the cuvette. After a small aliquot was transferred into the cuvette pre-equilibrated at 37 °C, the prebound virus−liposomes were incubated for different periods of time (as noted on the figure) before acidified to pH 4.9. The final concentration in the cuvette were 10 μM viral phospholipids and 10 μM liposomal lipids. All subsequent experiments followed the same prebinding/fusion procedures, except that the prebound virus−liposomes were incubated at 37 °C for 30 s before acidification.

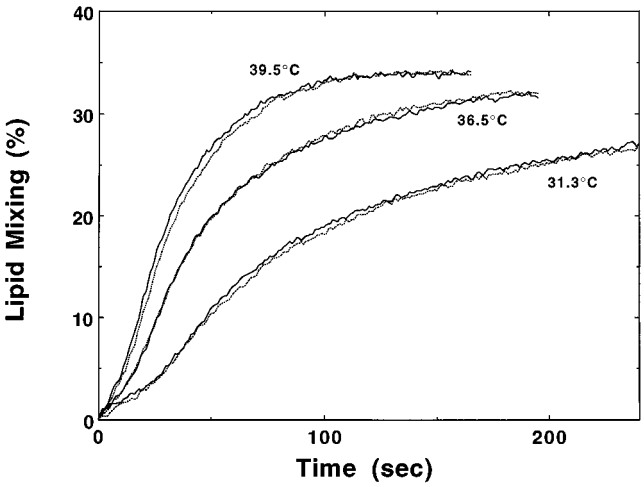

FIGURE 2: Lipid mixing kinetics between PR/8 virus and DOPC/GD1a (90:10) (solid line) or DOPC/HD/GD1a (86:4:10) (dotted line) liposomes at three temperatures (as noted on the figure) measured by NBD/Rh RET assay.

are essentially identical for different incubation periods. As discussed later, this shows that this prebinding step ensures all the experiments described in this paper are performed under fusion-rate-limiting conditions.

Effect of Void Stabilization. A low concentration (6 mol %) of hexadecane (HD) has been reported to increase the rate of divalent cation-induced lipid mixing of acidic phospholipid vesicles (Walter et al., 1994). Here we have investigated the effect of 4 mol % HD in the target liposome membrane on the kinetics of influenza HA mediated fusion. Lipid mixing of prebound unlabeled virus and NBD-Rh labeled liposomes was measured by the NBD/Rh RET assay. Figure 2 shows the lipid mixing kinetics of both DOPC/GD1a and DOPC/HD/GD1a liposomes at three temperatures, 35, 25, and 15 °C. Obviously, 4 mol % HD in the target membrane has no effect on fusion kinetics. Higher concentrations of HD were not attempted since it would not be "physiologically relevant" (as a model molecule for larger hydrophobic molecules found in membrane) and miscibility problems would occur (Walter et al., 1994).

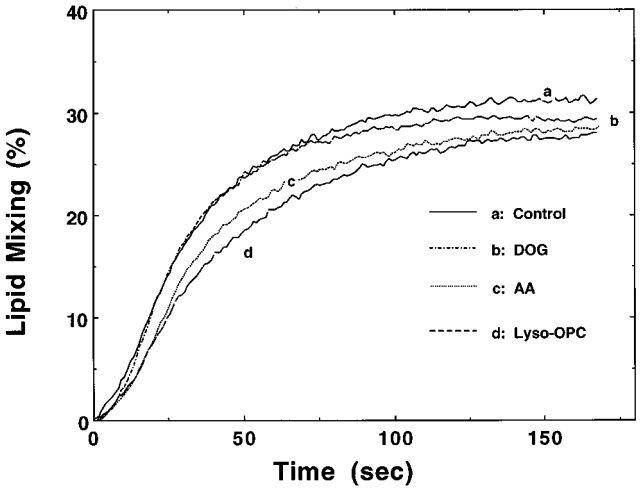

FIGURE 3: Lipid mixing kinetics between PR/8 virus and DOPC/GD1a (90:10) (solid line, curve a), DOPC/DOG/GD1a (86:4:10) (dash/dot line, curve b), DOPC/AA/GD1a (86:4:10) (dotted line, curve c), or DOPC/LPC/GD1a (86:4:10) (dashed line, curve d) liposomes at 37 °C, measured by NBD/Rh RET assay.

Effect of Monolayer Intrinsic Curvature. To test the effect of target membrane intrinsic curvature, we measured the lipid mixing of prebound unlabeled virus and NBD/Rh labeled DOPC/GD1a (86:10) liposomes made with 4 mol % of LPC (positive curvature), or DOG (negative curvature), or AA (negative curvature). These lipid additives were incorporated into both leaflets of the target liposomes. Figure 3 shows the lipid mixing kinetics of unlabeled virus and NBD/Rh labeled liposomes at 37 °C, as measured by RET assay. Lipid mixing kinetics were largely insensitive to these membrane additives.

Lysolipids have been reported to inhibit fusion in a wide variety of systems, when added to the fusion reaction mixture at high concentrations (Yeagle et al., 1994; Chernomordik et al., 1993; Vogel et al., 1993; Nieva et al., 1993). To test whether the same is true for influenza HA mediated fusion, we monitored lipid mixing of prebound unlabeled virus and NBD/Rh labeled DOPC/GD1a (90:10) liposomes in the presence of various concentrations of exogenously added LPC. Figure 4 shows the lipid mixing kinetics obtained at 37 °C using the NBD/Rh assay. The final concentrations of both viral phospholipids and liposomal lipids are 10 μM. Since LPC can rapidly incorporate only into the outer leaflets of both membranes when added exogenously, 0.5 μM LPC is approximately 5 mol % of the total outer monolayer lipids, if all is incorporated. At this concentration, LPC had no significant effect. However, as the LPC concentration increased, the extent of lipid mixing decreased. Eventually, the presence of 50 μM LPC completely abolished lipid mixing. In this set of experiments, LPC was added from $CHCl_3$ solutions. The volume of $CHCl_3$ added (6.8 μL) was constant for all concentrations. The same volume of $CHCl_3$ was used to generate the control curve, which has the same initial kinetics and a slightly lower fusion extent than that obtained in the absence of $CHCl_3$ (data not shown). These results are similar to those published by Günther-Ausborn et al. (1996), given the difference of preincubation conditions.

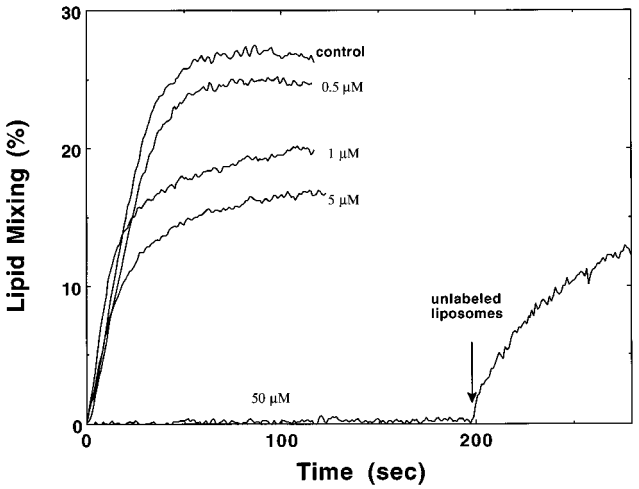

FIGURE 4: Lipid mixing kinetics between PR/8 virus and DOPC/GD1a (90:10) liposomes in the presence of various concentrations of LPC at 37 °C, measured by NBD/Rh RET assay. LPC was added to the fusion reaction buffer from $CHCl_3$ solutions. The volume of $CHCl_3$ was constant for all the curves, about 0.34% of the total volume inside the cuvette. The same volume of $CHCl_3$ was used to generate the control curve. For the 50 μM LPC curve, 200 s after acidification, 100 μM unlabeled DOPC/GD1a (90:10) liposomes were added (arrow).

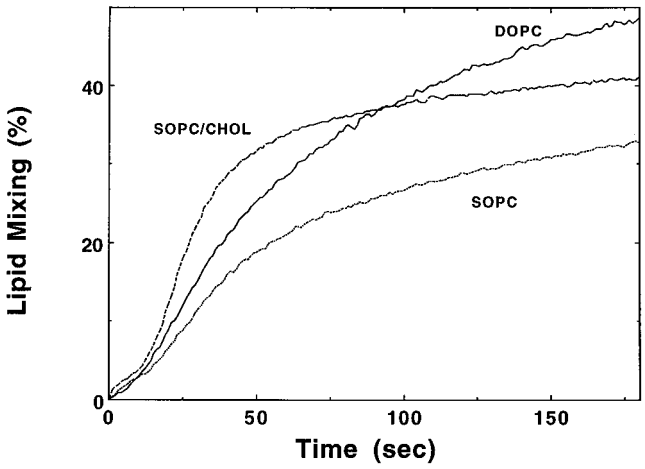

FIGURE 5: Lipid mixing kinetics between PR/8 virus and DOPC/GD1a (90:10, solid line), SOPC/GD1a (90:10, dashed line), or SOPC/CHOL/GD1a (45:45:10, dotted line) liposomes at 37 °C, measured by NBD/Rh assay.

In this set of experiments, LPC was added to the prebound virus and liposomes inside the cuvette. Addition of LPC resulted in a dose-dependent dequenching of NBD fluorescence (data not show), which generally stabilized within 2 min, similar to the observation of Günther-Ausborn et al. (1996). During this period, dose-dependent contents leakage was also detected using ANTS/DPX encapsulated DOPC/GD1a (90:10) liposomes (data not shown). This LPC induced leakage has the same kinetics as the LPC induced dequenching of NBD (data not shown). Apparently, the disturbed membranes resealed. Only 9% NBD dequenching and 15% ANTS leakage were detected at the highest LPC concentration tested, 50 μM. On the other hand, addition of 50 μM $C_{12}E_8$ resulted in both complete dequenching of NBD and complete leakage of ANTS (data not shown), suggesting that the inhibitory effect of LPC was not due to lysis of liposomes.

Inhibition of fusion by lysolipids is reversible in many systems tested (Chernomordik et al., 1993; Vogel et al., 1993). Our results prove the same is true for influenza fusion. Lipid mixing could be restored by addition of 100 μM unlabeled DOPC/GD1a liposomes even after it was completely inhibited by 50 μM LPC (Figure 4, bottom curve). The concentration of unlabeled liposomes required to restore lipid mixing depended on the concentration of LPC (data not shown).

Effect of Membrane Rupture Tension. To test the effect of membrane rupture tension, we compared the lipid mixing kinetics, measured by NBD/Rh RET assay, of influenza fusion with DOPC/GD1a (90:10), SOPC/GD1a (90:10), and SOPC/CHOL/GD1a (45:45:10) liposomes. The membrane rupture tension values of pure SOPC liposomes and SOPC/CHOL (1:1) liposomes are 6 dyn/cm and 20 dyn/cm, respectively (Evans & Needham, 1987). The membrane rupture tension of DOPC was not reported; however, it is probably similar to that of egg PC, which is 3.5 dyn/cm. If membrane rupture tension is a determining factor for influenza fusion and the addition of 10% GD1a does not alter the order of the membrane rupture tension values, then we would expect that SOPC/CHOL/GD1a liposomes should have the slowest fusion rate, whereas DOPC/GD1a liposomes should have the fastest fusion rate. Figure 5 shows the lipid mixing kinetics of these liposomes at 37 °C. The maximal rates of lipid mixing for the DOPC/GD1a and SOPC/GD1a liposomes are roughly similar. However, the presence of 45 mol % CHOL increased the maximal rate about 2-fold, consistent with data published by Stegmann et al. (1989). Similar results were also obtained at 25 and 15 °C (data not shown). Evidently, there is no direct correlation between the membrane rupture tension value and the rate of fusion induced by influenza.

Effect of Putative Antiviral Compounds. Bodian et al. (1993) reported that several derivatives of 4A,5,8,8A-tetrahydro-5,8-methano-1,4-naphthoquinone inhibited influenza virus-induced syncytia formation, viral infectivity *in vitro*, and HA-mediated hemolysis. They speculated that this inhibition was achieved by binding to HA and stabilizing it at the neutral pH conformation which is incapable of mediating membrane fusion, a common step shared by all these processes. However, data on the direct inhibition of influenza fusion were not obtained for these compounds due to their interference with the octyldecyl rhodamine B (ODR) dequenching fusion assay which they used.

With more than one lipid mixing assay available, we tested the effect of some of these compounds on influenza lipid mixing. From these compounds, we chose hydroquinone (HQ), which showed no inhibition on any of the processes mentioned above, and *tert*-butylhydroquinone (t-b-HQ), which was the most effective inhibitor which was not toxic to cells (Bodian et al., 1993). Dibucaine was chosen to serve as a negative control. When the compounds were added to the prebound unlabeled virus and NBD-Rh labeled DOPC/GD1a liposomes, the fluorescence of NBD was quenched by the compounds to a variable extent, which made it impossible to obtain reliable results.

Thus, another RET assay using CPT/DABS (Silvius et al., 1987) was tested. When the compounds were added to the prebound unlabeled virus and DOPC/GD1a liposomes containing 0.6 mol % CPT-PC and DABS-PC, no effect on the CPT fluorescence was detected. After acidification, lipid mixing leads to dilution of the fluorophores into the viral

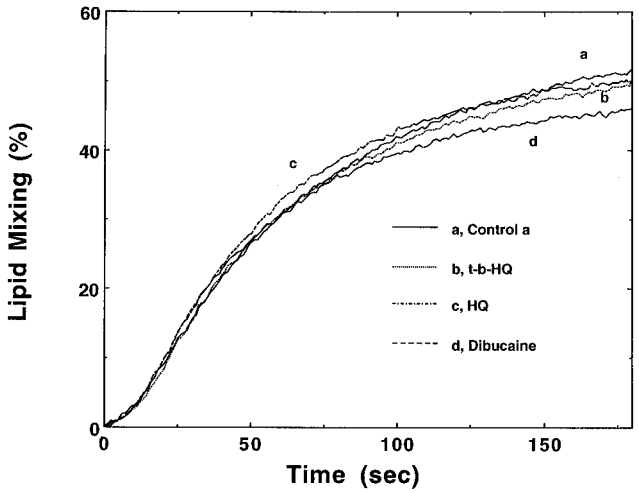

FIGURE 6: Lipid mixing kinetics of PR/8 virus and DOPC/GD1a (90:10, curve a, solid line) liposomes at 37 °C, measured by CPT/DABS RET assay in the presence of following compounds: t-b-HQ (curve b, dotted line), HQ (curve c, dash/dot line), and dibucaine (curve d, dashed line).

envelop and dequenching of the CPT fluorescence. Using this assay, we observed no significant inhibition on lipid mixing by any of these compounds at 100 μM concentration (Figure 6). Similar results were also obtained at 20 μM concentration (data not shown), at which t-b-HQ (Bodian et al., 1993) showed 50% inhibition on viral infectivity. From these, we conclude that the inhibitory effect of t-b-HQ on syncytia formation, hemolysis, and infectivity is not a result of its inhibition of fusion.

Leakage During Lipid Mixing. The contents leakage during influenza fusion was monitored using ANTS/DPX encapsulated DOPC/GD1a (90:10) liposomes and unlabeled virus. Leakage leads to the dilution of the fluorophores into the reaction buffer and subsequent dequenching of ANTS fluorescence. Figure 7A shows, at 37 °C, the kinetics of leakage detected using ANTS/DPX assay and the kinetics of lipid mixing measured by CPT/DABS assay, using CPT/DABS labeled DOPC/GD1a (90:10) liposomes. Obviously, leakage occurred simultaneously with lipid mixing.

Eventually the leakage reached more than 90% dequenching, while the lipid mixing reached about 50% dequenching when using the $C_{12}E_8$ lysis level as 100%. In both experiments, the stoichiometry was roughly one virus particle per liposome and the sizes of virus and liposomes were about the same. Since virions cannot fuse with a virus−liposome fusion product (Nir et al., 1986), the level of about 50% dequenching in lipid mixing assay is due to one round fusion between one virus with one liposome. However, if the leakage is complete, one round fusion can cause 100% dequenching in leakage assay. If we rescale the leakage curve such that it reaches the same extent of dequenching as the lipid mixing curve at 125 s, then the two curves are essentially identical over the entire time range (Figure 7B). This suggests that the lipid mixing reaction is completely lytic with respect to encapsulated molecules with molecular weights below those of ANTS (445 MW) and DPX (422 MW).

Leakage was also detected during influenza fusion with ANTS/DPX encapsulated SOPC/GD1a (90:10) and SOPC/CHOL/GD1a (45:45:10) liposomes at 37 °C (data not shown). Complete leakage was observed in both cases and

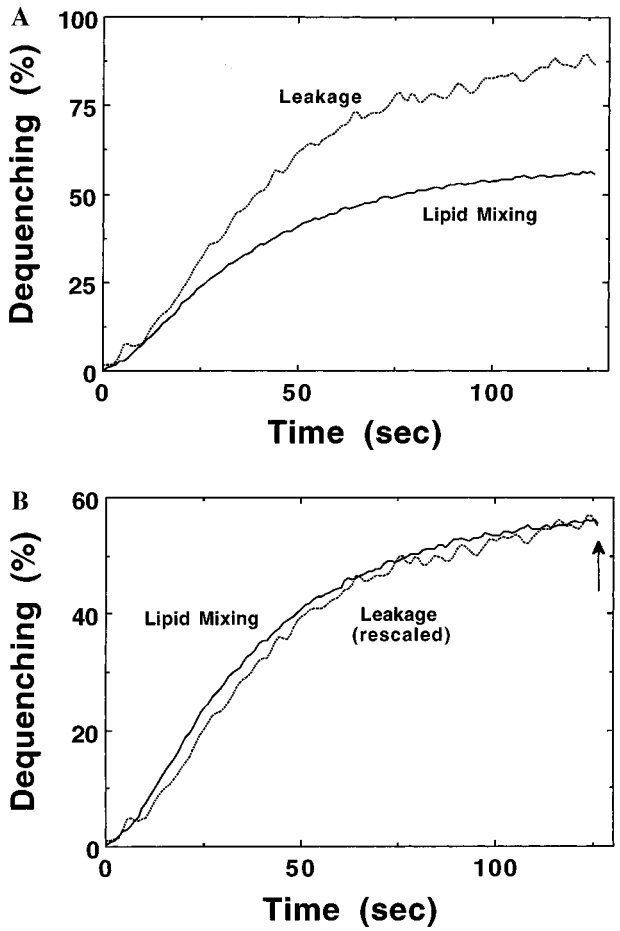

FIGURE 7: (A) Leakage (dotted line) and lipid mixing (solid line) kinetics of DOPC/GD1a (90:10) liposomes during fusion with PR/8 virus at 37 °C. Leakage is measured by ANTS/DPX assay, whereas lipid mixing is measured by CPT/DABS assay. (B) The leakage curve in A is rescaled (dotted line) to the same final extent as the lipid mixing curve (solid line) at time 125 s (arrow). The lipid mixing curve is not changed from panel A.

the kinetics of leakage corresponded with the kinetics of lipid mixing the same way as shown in Figure 7B with DOPC/GD1a liposomes. The presence of 45 mol % CHOL in the target membrane increased the leakage rate constant by 2-fold, the same observed in lipid mixing (Figure 5). The similar correlation between leakage kinetics and lipid mixing kinetics was also obtained at three other temperatures, 35, 25, and 15 °C for each kind of liposomes (data not shown).

In order to characterize the size of the membrane lesion caused by fusion, we monitored leakage of high molecular weight tetrametylrhodamine B (TMR) conjugated dextrans, encapsulated in DOPC/GD1a (90:10) liposomes. At the encapsulated concentration (20 mg/mL), the fluorescence of TMR is self-quenched. Leakage leads to the dilution of dextran into the relatively large volume of reaction buffer and dequenching of TMR fluorescence. Thus, it is possible to monitor the kinetics of dextran leakage directly. Figure 8 shows the 37 °C leakage curves of 3000 and 10 000 MW dextran and the lipid mixing curve of these dextran encapsulated liposomes as measured by CPT/DABS assay. Essentially, leakage of dextran happens at the same time as lipid mixing, and there is no significant difference between different size dextrans. Thus, the membrane lesion generated during fusion is big enough to allow 10 000 MW dextran to leak out quite rapidly.

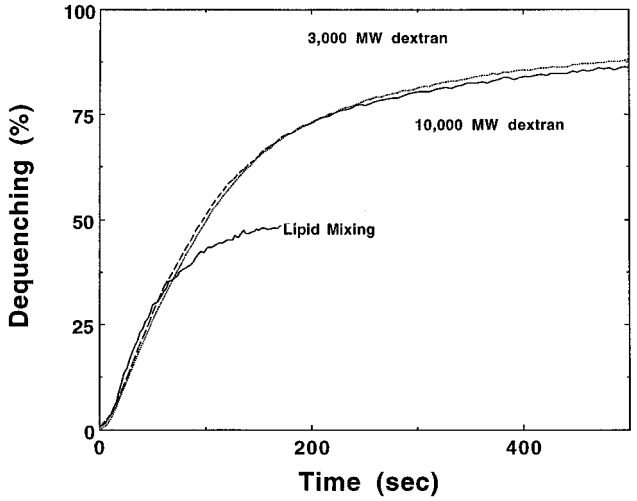

FIGURE 8: Leakage kinetics of encapsulated 3000 MW (dotted line) and 10 000 MW (dashed line) dextran from DOPC/GD1a (90:10) liposomes during fusion with PR/8 virus at 37 °C, measured by TMR dequenching assay. Lipid mixing kinetics between these liposomes and PR/8 virus at 37 °C, measured by CPT/DABS assay, is shown for comparison (solid line).

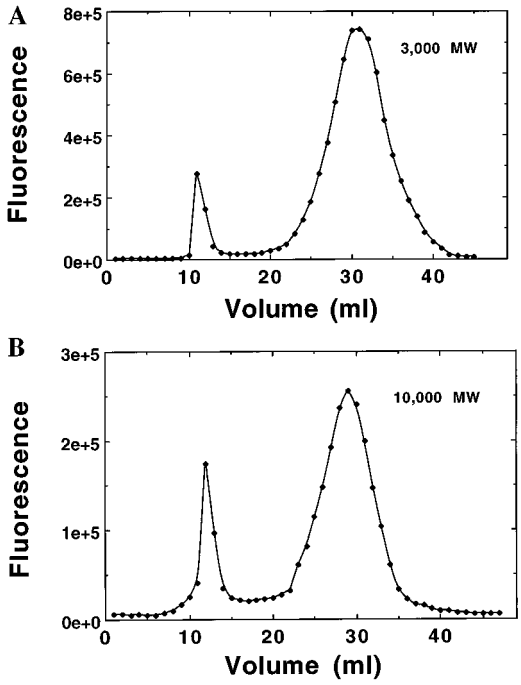

FIGURE 9: (A) Elution profile of 3000 MW dextran on Sephadex G-75 column after fusion. (B) Elution profile of 10 000 MW dextran on Sephadex G-200 column after fusion. The liposome peak is on the left, and the free dextran peak is on the left. Unlabeled virus (350 μM) and 350 μM TMR conjugated dextran encapsulated DOPC/GD1a (90:10) liposomes were prebound, incubated at 37 °C, pH 4.9, for 10 min, neutralized, and passed through the gel filtration column. The concentration of dextran in each fraction was determined by measuring the fluorescence of TMR in the presence of 0.75 mM $C_{12}E_8$.

To further confirm that, after lipid mixing, the dextran is indeed outside of the liposomes, the prebound virus and dextran encapsulated DOPC/GD1a (90:10) liposomes were incubated at 37 °C, pH 4.9, for 10 min. The mixture was neutralized and passed through a gel filtration column to separate free dextran that leaked out from the liposomes. Figure 9 panels A and B show the elution profile of fusion product using 3000 MW dextran liposomes and 10 000 MW dextran liposomes, respectively. While over 90% of 3000 MW dextran was outside of liposomes after 10 min at 37 °C, pH 4.9, about 80% of 10 000 MW dextran leaked under the same conditions.

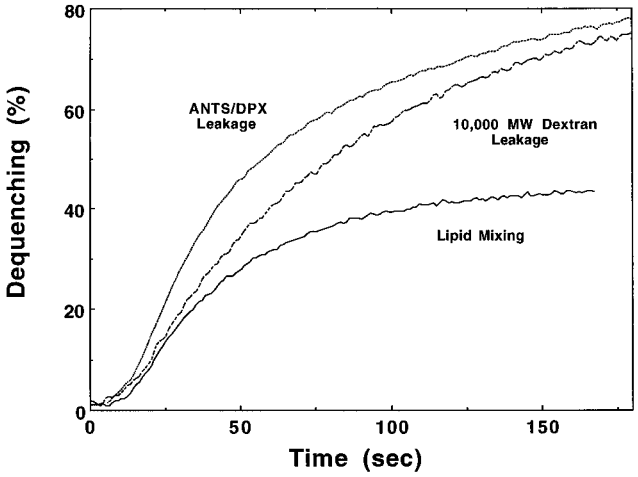

FIGURE 10: Leakage kinetics of encapsulated ANTS/DPX (dotted line) and 10 000 MW (dashed line) dextran from DOPC/GD1a (90:10) liposomes during fusion with freshly purified PR/8 virus at 37 °C, measured by TMR dequenching assay. Lipid mixing kinetics between these liposomes and freshly purified PR/8 virus at 37 °C, measured by CPT/DABS assay, is shown for comparison (solid line).

In all the leakage assays, incubation of liposomes alone at low pH did not result in leakage of any of the encapsulated probes (data not shown). Moreover, no leakage was observed with prebound virus−liposomes at neutral pH (data not shown).

Young et al. (1983) detected an increase in conductance when pretreated influenza virus was incubated with planer bilayers at a fusion associated pH, indicative of leaky fusion. However, when freshly purified virus was used, no increase in conductance was detected. They concluded that the increase in ionic permeability was caused by the incorporation of pretreatment damaged viral membranes into the planer bilayer while the fresh virus underwent nonleaky fusion. The pretreatments used in their experiment were either 10 freeze/thaw cycles, sonication for 10 min, or storage in the cold for 2 weeks. The virus used in our study, on the other hand, was quickly frozen in 40% sucrose after purification, stored at −80 °C, and used within 3 days after thawing. These conditions are far less harsh. In fact, when our leakage experiments were repeated with freshly purified influenza PR/8 virus, i.e., virus never frozen, the leakage during lipid mixing was also observed. Figure 10 shows the kinetics of lipid mixing measured by CPT/DABS assay and the kinetics of both ANTS/DPX and 10 000 MW dextran leakage induced by freshly purified virus at 37 °C. The ANTS/DPX leakage curve is identical to the lipid mixing curve after it is rescaled to the same extent as the lipid mixing curve (data not shown). From this, we conclude that the leakage we have detected is a true phenomenon accompanying influenza fusion.

DISCUSSION

Membrane fusion, in general, involves four separate steps (Bentz, 1992): the close apposition of the two membranes, the destabilization of the outer monolayers, the initial fusion pore formation, and the expansion of the pore. While liposome−liposome fusion has been proposed to proceed

through discrete lipidic intermediate structures that evolve from membrane defects [see reviews in Bentz (1993b) and Zimmerberg et al. (1993)], little is known about how membrane merge occurs in biological systems where fusion proteins are indispensable. This is true even for the so far best understood fusion process, influenza HA mediated fusion. However, recent work (Stegmann et al., 1985; Stegmann, 1993; Alford et al., 1994; Günther-Ausborn et al., 1995) has shown that careful analysis of the fusion kinetics as a function of the material properties of the target membrane can be fruitful.

To obtain useful information from this type of study, it is crucial that the measured kinetics reflects the kinetics of actual fusion instead of virus−liposome aggregation, i.e., fusion instead of aggregation should be rate-limiting for the overall reaction. To achieve this goal, a prebinding step is included in all the experiments described here. Previously, a similar prebinding protocol was used in ODR labeled influenza virus fusion with sialic acid containing liposomes to reach fusion-rate-limiting conditions (Alford et al., 1994). Here, using a different lipid mixing assay, we further tested this prebinding approach (Figure 1).

Before fusion, a small aliquot of prebound virus−liposome was diluted into the 37 °C buffer inside the cuvette. If significant dissociation happens under these conditions and the aggregation rate is slow compared to the fusion rate, then different lipid mixing kinetics should be observed after different incubation time at 37 °C. However, the lipid mixing curves after different incubation periods, from 30 s to 30 min, show no difference (Figure 1). This proves one of the two possibilities. It could be that, after the prebinding step, virus and liposomes stay bound and dissociation is negligible even after dilution and temperature shift. Alternatively, it could also be that the dissociation and the reaggregation of the virus and liposomes happen so fast that within the 30 s incubation in the cuvette (which precedes the reduction in pH) the same equilibrium is reached. If the first possibility is true, then we have further demonstrated that fusion-rate-limiting conditions can be achieved by our prebinding protocol. If the second possibility is true, it means that the aggregation step is fast and is not rate-limiting for lipid mixing. Thus, in either case, after the prebinding, it is certain that the measured kinetics is the kinetics of fusion, not aggregation.

Influenza fusion has been speculated to proceed through similar mechanisms as liposome−liposome fusion. It is proposed that the function of HA is to lower the activation energy of the fusion intermediates by destabilizing membranes, creating membrane defects or stabilizing hydrophobic voids (Siegel, 1993). Although the L_α/H_{II} phase transition, per se, has recently been demonstrated to be irrelevant to influenza fusion (Stegmann, 1993, 1995; Alford et al., 1994; Chernomordik et al., 1993), refined stalk models have been proposed (Stegmann, 1993; Siegel, 1993b; Chernomordik et al., 1993). As in liposome−liposome fusion, monolayer intrinsic curvature and void stabilization are thought to be two important factors determining the rate of this fusion process (Siegel, 1993b). However, we discovered that incorporation of low but physiologically relevant concentrations (i.e., 4% of total target membrane lipids) of LPC (positive curvature), AA (negative curvature), or DOG (negative curvature) into target membrane had no significant effect on the rate of influenza fusion (Figure 3), albeit that these natural lipids can change the spontaneous curvature of the monolayers (Keller et al., 1993). Moreover, addition of 4 mol % HD, which is expected to fill the hydrophobic voids and accelerate fusion, into the target membrane had no significant effect either. This suggests that monolayer curvature and void stabilization are not important in influenza HA mediated fusion, i.e., they do not affect the rate-limiting step(s) of the process.

It has been reported that when LPC is present in the fusion reaction buffer at high but sublytic concentrations, it inhibits membrane fusion in a wide variety of systems (Vogel et al., 1993; Chernomordik et al., 1993; Yeagle et al., 1994; Nieva et al., 1993). When we monitored influenza fusion in the presence of exogenous LPC, no significant inhibition was observed at biologically relevant concentration, i.e., 0.5 μM (Figure 4). However, with increasing LPC concentrations, the inhibitory effect begins to show up. Complete inhibition was achieved with 50 μM LPC, a concentration at which complete lysis of liposomes did not occur, although some leakage of contents was observed. Moreover, as reported in other systems (Vogel et al., 1993; Chernomordik et al., 1993), this inhibition was reversible. Fusion activity could be restored by addition of high concentrations of unlabeled liposomes, further demonstrating that the inhibition is not caused by LPC mediated lysis. However, it must be noted that this LPC concentration is 2.5 times greater than the 20 μM total lipid concentration of our virus−liposome fusion system.

There are two possible explanations for this observed LPC inhibition. First, most of the fusion intermediates proposed so far, such as stalk intermediates, contain surfaces with small negative radii of curvature (Markin et al., 1984; Chernomordik et al., 1993; Siegel, 1993b; Stegmann, 1993). The presence of LPC, a lipid that stabilizes positive curvature, in the outer monolayer may prevent the formation of these intermediate structures. If influenza fusion does proceed through these intermediate structures, failure to form them will diminish fusion. While we do not know how much LPC is actually incorporated into the outer monolayer, the fact that the concentration of LPC required for complete inhibition is 2.5 times the total lipids involved in fusion puts the biological relevance of this inhibition into question. The same problem was also encountered in other systems studied so far (Vogel et al., 1993; Chernomordik et al., 1993; Yeagle et al., 1994; Nieva et al., 1993).

The second possible explanation is that LPC acts directly on HA. It is well known that, under mild acidic pH, HA undergoes low pH induced conformational changes, exposes its fusion peptide, and becomes hydrophobic (White et al., 1982a; Stegmann et al., 1990). Under this condition, virions can bind hydrophobically to the target membrane (Stegmann et al., 1990) or aggregate with each other in the absence of the target membrane (Shangguan, 1995). The exposure of the fusion peptide is essential for influenza fusion (Gething et al., 1986; Sato et al., 1983; White et al., 1982). Since LPC is amphipathic, it may cover the exposed hydrophobic surfaces of HA at low pH, especially when LPC is present in the buffer at very high concentrations. This interaction may prevent the fusion peptide and/or other parts of HA from interacting with the membranes and thus inhibits fusion (Günther-Ausborn et al., 1995).

So far, we are unable to distinguish between these two possibilities, because, theoretically, either one of the inhibi-

tory effects can be reversed by removing LPC with unlabeled liposomes. One could take BHA in 50 μM LPC and see if it still binds to the liposomes hydrophobically at low pH. However, such an experiment would not be conclusive, since BHA alone does not cause fusion (White et al., 1982). Since other lipids we tested that can alter the monolayer intrinsic curvature (AA and DOG), the void stabilization energy (HD), or the membrane rupture tension (see below) show no significant effect on the rate of influenza fusion, we think it is less likely that LPC inhibition is caused by curvature effect. It is more likely that interaction with the low pH form of HA is responsible for LPC inhibition, although more experiments will be needed to reach a definite conclusion.

In order for the two apposed membrane bilayers to merge, monolayers in the intermediate structures have to rupture to form the fusion pore. To determine whether the toughness of the target membrane, as measured by membrane rupture tension (Evans & Needham, 1987), plays a role in influenza fusion, we compared the fusion kinetics of target membranes with dramatically different membrane rupture tension values (Figure 5). To our surprise, there is no direct correlation between the membrane rupture tension value and the fusion rate, assuming that 10% GD1a does not significantly change the order of the membrane rupture tension of the liposomes.

Taken together, all these results suggest that the material properties of the target membrane are not important for influenza fusion except for extreme cases, such as frozen membranes, e.g., the L_β phase (Stegmann, 1993). From an evolutionary perspective, this provides the virus an advantage enabling it to infect cells with very different membrane compositions. Moreover, the infection would then not depend on special lipids, such as LPC, AA, or DOG, the presence of which in the host plasma membrane is regulated by the host cell instead of by the virus.

Influenza fusion has long been believed to be nonleaky (Young et al., 1983; Stegmann et al., 1989; Stegmann & Helenius, 1993). On the other hand, there exists no evolutionary reason for it to be absolutely nonleaky. Young et al. (1983) found an increase in conductance across planer bilayers when harshly treated (as described above) influenza virus was added to one compartment at low pH. On the other hand, no conductance change was detected when freshly purified virus was used. From these data, they concluded that the fusion of undamaged influenza virus was nonleaky. However, using three different assays, we detected leakage of liposome content during fusion with influenza virus, no matter whether the virus was frozen/thawed once in 40% sucrose or freshly purified. The leakage kinetics of molecules smaller than 450 MW is almost identical to the lipid mixing kinetics, suggesting that lipid mixing is completely lytic for these molecules. Leakage of encapsulated molecules as big as 10 000 MW dextran is also detected during lipid mixing, indicating that the membrane lesion generated during the fusion process is rather large. Also, leakage for SOPC and SOPC/cholesterol (1:1) liposomes matched their lipid mixing kinetics.

Despite this extensive leakage induced by HA mediated lipid mixing, a conductance change was not observed by Young et al. (1983) with fresh virus. There are two possible explanations: first, fusion of influenza virus with the planer membrane may be different from the liposomal membrane we used; second, since there was no positive control for fusion in their experiments, it was not proven that the freshly purified virus actually fused with the planer membrane. Thus, these experiments would need to be repeated. Recently, Günther-Ausborn et al. (1995) also reported leakage of calcein from liposomes induced by influenza virus in a similar system.

Our results suggest that, unlike liposome−liposome fusion (Bentz & Ellens, 1988), influenza virus fusion is a very leaky process which does not proceed through the same types of intermediates currently proposed for liposome−liposome fusion. The fact that the membranes at the fusion site are not tightly sealed is consistent with the finding that target membrane material properties are not very important for this fusion process. In fact, influenza fusion may involve a completely different mechanism, a mechanism that requires specific protein−lipid interaction at the fusion site (Bentz et al., 1990; Alford et al., 1994). The simplest picture is that the hydrophobic surfaces of HA, prior to inactivation, facilitate disruption of the apposed bilayers in a disorderly way, leading to leakage of contents and that the bilayer ultimately reseals as the protein and lipid reach equilibrium (Bentz et al., 1990).

Such a process, while somewhat difficult to model at a molecular level, is not inconsistent with other biological fusion systems. In nonleaky eukaryotic fusion systems, e.g., secretion, it is quite possible that the fusion site is concentric, with an inner ring of protein(s) responsible for disrupting the apposed bilayers and an outer ring of protein(s) responsible for sealing the site, in order to make the "fusion" nonleaky.

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