Lysolipids Do Not Inhibit Influenza Virus Fusion by Interaction with Hemagglutinin*

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The interaction of a spin-labeled lysophosphatidylcholine analog with intact and bromelain-treated influenza viruses as well as with the bromelain-solubilized hemagglutinin ectodomain has been studied. The inhibition of fusion of influenza viruses with erythrocytes by the lysophosphatidylcholine analog was similar to that observed for non-labeled lysophosphatidylcholine. Only a weak interaction of the lysophosphatidylcholine analog with the hemagglutinin ectodomain was observed even upon triggering the conformational change of the ectodomain at a low pH. A significant interaction of spin-labeled lysophosphatidylcholine with the hemagglutinin ectodomain of intact viruses was observed neither at neutral nor at low pH, whereas a strong interaction of the lipid analog with the viral lipid bilayer was evident. We suggest that the high number of lipid binding sites of the virus bilayer and their affinity compete efficiently with binding sites of the hemagglutinin ectodomain. We conclude that the inhibition of influenza virus fusion by lysolipids is not mediated by binding to the hemagglutinin ectodomain, preventing its interaction with the target membrane. The results unambiguously argue for an inhibition mechanism based on the action of lysolipid inserted into the lipid bilayer.

Influenza virus enters host cells via receptor-mediated endocytosis. Both the attachment of the viral envelope to cell surface receptors and fusion with the endosomal membrane are mediated by the influenza glycoprotein hemagglutinin (HA)1 (1–3). HA, which is composed of two disulfide-linked subunits, HA1 and HA2, is organized as a homotrimer. The N terminus of HA2 resembles a highly conserved hydrophobic stretch of ~20 amino acids, the so-called fusion sequence (3, 4). The HA-mediated fusion is triggered by the acidic pH milieu of the endosomal lumen, which causes the HA ectodomain to convert into a fusogenic conformation. An extended trimeric coiled coil in the HA2 subunit is formed (5–8). The reconstruction of the three-dimensional structure of the complete HA ectodomain revealed that the preserved trimeric shape of the ectodomain may direct the orientation of the coiled coil with the fusion peptides at its tip toward the target membrane (9). As a consequence of the conformational change, finally the fusion sequence inserts into the target bilayer and/or viral membrane and promotes fusion (10–12). Although many details of the molecular rearrangement of HA at low pH have been described, the molecular mechanism of the subsequent membrane merger is still unknown.

Chernomordik et al. (13) identified an early lipid-sensitive stage of HA-mediated cell-cell fusion. This intermediate, subsequent to the low pH-triggered change in HA conformation but preceding actual membrane merger and fusion pore formation, was sensitive to the presence of specific lipids. It was inhibited by lysophosphatidylcholine (LPC) and promoted by oleic acid. These results were interpreted in the frame of the “stalk model” (13–18). Referred to as “stalk” is a necklike structure of net negative curvature continuously connecting the contacting monolayers of the fusing membranes. According to the model, such a stalk is formed upon bringing membrane lipid bilayers into close contact, for example, by viral fusion proteins (14, 15, 17, 19). If LPC is present in the outer leaflet of a membrane, it inhibits the formation of the stalk because of its inverted cone molecular shape acting against the net negative curvature. In contrast, the unsaturated fatty acid oleic acid enhanced fusion (13, 15, 16, 20). Because of its cone-shaped structure, oleic acid promotes net negative curvature and thus lowers the energy of the stalk intermediate.

However, although many data are consistent with the stalk model (for a review see Ref. 16), the mechanism of LPC-mediated inhibition of fusion is still under discussion. Alternatively, it is suggested that LPC interacts directly with the viral fusion protein. Gething et al. (2) proposed that LPC inhibits the HA-mediated membrane fusion at an earlier stage upstream of the stalk intermediate. The authors suggest that free monomeric or micellar LPC could bind directly to the fusion peptide and thus prevent its insertion into the target membrane (21).

Although many studies using lysolipids for trapping the fusion process at an intermediate step rely on the hypothesis that lysolipids act on the fusion process according to the stalk model, conclusive evidence for this model is lacking. In particular, hitherto existing approaches to elucidate the mechanism of lysolipid-mediated inhibition of fusion did not allow strict differentiation between the stalk model and the action through binding to fusion proteins.

In this study, we have investigated directly the interaction of spin-labeled LPC (SL-LPC) with the influenza virus membrane as well as with the bromelain-solubilized ectodomain of HA (BHA) by electron spin resonance (ESR) spectroscopy (Scheme 1). SL-LPC resembles a reliable analog, because its capacity to
inhibit fusion of influenza virus with red blood cell membranes was very similar to that of non-labeled LPC. Although the binding of SL-LPC to BHA did occur, we found that this was weak in comparison to its interaction with a typical lipid-binding protein, bovine serum albumin (BSA). When labeling intact influenza virus with SL-LPC, we observed an ESR spectrum typical for a lipid bilayer. We could not detect any spectral component reflecting the interaction of SL-LPC with the ectodomain of HA of intact viruses as observed for BHA. Consistent with this finding, no differences among the ESR spectra of SL-LPC added to intact influenza virus or bromelain-treated virus lacking the HA ectodomain were observed. We conclude that the binding sites of the lipid bilayer of the viral envelope compete efficiently for SL-LPC with the weak binding sites of the HA ectodomain. Thus, our data do not support the notion that LPC suppresses fusion by interaction with the N terminus of HA2, the fusion peptide. The results unambiguously point to an inhibition mechanism based on the action of lysolipid inserted into the lipid bilayer.

MATERIALS AND METHODS

Chemicals—Lysophosphatidylcholine ([myristoyl (LPC, 14:0)], palmityl (LPC, 16:0), and stearoyl (18:0)], egg phosphatidylcholine (eggPC)), agaro-immobilized Ricinus communis agglutinin, and d(+)-galactose were obtained from Sigma. Octadecylrhodamine B chloride (R18) and 1,1'-bis[4-aminophthaldehyde-5,5'-disulfonic acid (bis-ANS) was purchased from Molecular Probes. Bromelain and Triton X-100 were from Fluka (Buchs, Switzerland). Fresh blood from healthy donors was obtained from the local blood bank (Berlin, Germany). 1-(15-Doxylpentanoyl)lysophosphatidylcholine (SL-LPC, C18) was kindly provided by Dr. Devaux (Paris, France).

Buffers—Phosphate-buffered saline (PBS), pH 7.4 (5.8 mmol/liter phosphate, 145 mmol/liter NaCl), and sodium acetate buffer, pH 7.4 (20 mmol/liter sodium acetate, 130 mmol/liter NaCl) were used.

Virus Preparation—Influenza virus strain X31 was grown for 48 h in 10-day-old embryonated chicken eggs. The allantois fluid was collected, and cell debris was removed by a low speed spin. The virus was pelleted by spinning the allantois fluid with 95,000 × g for 1 h (4 °C).

The pellet was washed, resuspended, and stored in PBS. This result corresponds to a molar ratio of endogenous lipids to virus lacking the HA ectodomain were observed. We conclude that the binding sites of the lipid bilayer of the viral envelope compete efficiently for SL-LPC with the weak binding sites of the HA ectodomain. Thus, our data do not support the notion that LPC suppresses fusion by interaction with the N terminus of HA2, the fusion peptide. The results unambiguously point to an inhibition mechanism based on the action of lysolipid inserted into the lipid bilayer.

Interaction of Lysolipids with Influenza Virus

Binding of bis-ANS to Influenza Virus and BHA—A 1 ml stock solution of bis-ANS in methanol was prepared. BHA (final concentration = 5 µg/ml) or influenza virus (final concentration of HA = 5 µg/ml, assuming that 25% of virus protein content correspond to HA (30), was transferred to 2 ml of sodium acetate buffer containing 1 nmol of bis-ANS/ml, pH 7.4, at 37 °C. At a given time, the appropriate volume of 0.25 M citric acid was added to the buffer to decrease the pH to 5.0, causing HA/BHA to undergo a conformational change. The conformational change was measured by the increase of bis-ANS fluorescence intensity of intact influenza virus or BHA at pH 7.4.

Preparation of Small Unilamellar Vesicles (SUVs)—3 mol of eggPC dissolved in chloroform were dried under nitrogen and resuspended in 500 µl of PBS. SUVs were made by sonicating the resulting phospholipid dispersion for 10 min on ice with the microtip of a Branson Sonifier model W250 (Carouge-Geneve, Switzerland) at an output control setting between 2 and 50% duty cycle.

ESR Measurements—SL-LPC dissolved in chloroform was transferred to a glass tube, dried under nitrogen, and vortexed with the desired volume of PBS. For measuring the interaction of SL-LPC with BHA, 100 µl of BHA (1 mg/ml) were added to 0.3 nmol of SL-LPC in PBS corresponding to a molar ratio of BHA (monomer) to SL-LPC of ~1:5. For SL-LPC-virus interaction (100 µl of virus or bromelain-treated virus) each 1 mg of protein/ml was transferred to 3 nmol of SL-LPC in PBS. This result corresponds to a molar ratio of endogenous lipids to SL-LPC and of HA (monomer) to SL-LPC of 10:1 and 1.5, respectively, by taking into account that 75 and 20% dry weight of influenza virus are proteins and lipids, respectively (2.5 × 10^12 virions/mg virus protein, 500 trimers/virion (28, 30)). For studies with liposomes, 5.25 µl of eggPC-SUVs were added to 3.15 nmol of SL-LPC (eggPC:SL-LPC = 3:5 mol of eggPC).
Interaction of Lysolipids with Influenza Virus

RESULTS

Inhibition of Virus Fusion by SL-LPC—First, we studied whether the SL-LPC analog inhibits influenza virus-induced fusion in a similar manner as non-labeled LPC. Human erythrocyte ghost membranes were used as targets for influenza virus, and fusion was assessed by an FDQ assay using the lipid-like fluorophore R18 initially incorporated into the viral membrane at self-quenching concentrations as initially described by Hoekstra et al. (27) and commonly used thereafter. After the binding of labeled virus to erythrocyte ghosts at neutral pH, fusion was triggered at 37 °C by lowering the pH to 5.0 (Figure 1). In the absence of SL-LPC, the final fusion extent was ~50% (Fig. 1A, control).

To study the influence of SL-LPC on influenza virus fusion, initially we determined whether the fluorescence intensity of R18 is partially quenched by the nitroxide group of SL-LPC. To this end, the analog (13 μM final concentration) was added to the control after the fusion extent had reached its plateau (Fig. 1A). As shown, only a small decrease in fluorescence was observed. From the fluorescence decline, it can be deduced that SL-LPC was incorporated into the membrane and that incorporation was fast taking less than 1 min at 37 °C.

Upon preincubation of virus-ghost complexes with SL-LPC (13 μM final concentration corresponding to 10 mol % endogenous lipids), pH 7.4, at 37 °C for 240 s, a significant decrease of the fusion extent by approximately one-third was found (Figs. 1A and 2). Note that fluorescence dequenching was corrected for quenching caused by the nitroxide group. To compare the inhibitory effect of SL-LPC with that of non-labeled LPC, we measured the extent of fusion upon preincubation of virus-ghost complexes with various LPC differing in the length of the fatty acid chain. In Fig. 1B, a typical example is shown for the effect of LPC (14:0) on fusion. For a concentration of 10 mol % lysolipid with respect to endogenous lipids, we found a similar decrease of the fusion extent as with SL-LPC (Figs. 1A and 2) from ~60 (control) to 40% (with LPC at a molar ratio of 14:0). At this concentration of 10 mol %, we found only a rather shallow dependence of fusion on the acyl chain length (Fig. 2). From these results, we conclude that SL-LPC behaves very similar to non-labeled LPC with respect to inhibition of influenza virus fusion. We noted a variation of the fusion extent among independent experiments (cf. controls in Fig. 1, A and B).

The inhibition of influenza virus fusion by LPC was dose-dependent (data not shown) as known from the inhibition of Sendai virus fusion with vesicles (31), baculovirus-infected cell-cell fusion, cortical granule exocytosis (32), and HA-mediated cell fusion (33). To preserve membrane stability, we selected for our study a maximum concentration of 10 mol % lysolipids with respect to endogenous lipids.

Interaction of SL-LPC with the HA Ectodomain—To explore the interaction of SL-LPC with the HA ectodomain, we isolated the BHA as described under “Materials and Methods.” Isolated intact HA could not be used for these studies, because the interaction of lysolipids with the hydrophobic transmembrane domain would not allow to characterize the lipid interaction of the ectodomain in a well defined manner. The low pH-triggered conformational change of BHA was similar to that of intact virus-embedded HA as probed by its sensitivity toward proteinase K (data not shown, see Ref. 24) and as well by the binding of bis-ANS. We and others (28, 29, 34) have shown previously that structural alterations of the HA ectodomain and the accompanied exposure of hydrophobic sequences at acidic pH could be monitored continuously by means of the hydrophobicity-sensitive dye bis-ANS. In Fig. 3, the kinetics of bis-ANS fluorescence in the presence of influenza virus and BHA at pH 5.0 is shown. BHA or intact virus was added to prewarmed buffer (37 °C) at pH 7.4 containing bis-ANS. After lowering the pH, a rapid increase of the fluorescence intensity of bis-ANS was observed indicating the conformational change of the HA ectodomain. The fluorescence increase is caused by an enhanced binding of bis-ANS to hydrophobic sequences of the HA.
Interaction of Lysolipids with Influenza Virus

Fig. 2. Influence of lysolipids on the extent of influenza virus fusion with erythrocyte ghosts at 37 °C. A comparison between SL-LPC and LPC of various chain length. The fusion extent is expressed as the percentage of the control (no lysolipids). The concentration of lysolipids was 10 mol % with respect of the endogenous lipids of the virus-ghost complexes. The average and the mean ± S.D. (n = 3) is given.

Fig. 3. Increase of bis-ANS fluorescence in the presence of intact virus or BHA at pH 5.0 and 37 °C. BHA (5 g/ml) or influenza virus (5 μg of HA/ml assuming 2.5 × 10^12 virus particle/mg virus protein, 500 trimers/virion (28, 30), and a molecular mass of HA of 75–80 kDa) was added to 2 ml of sodium acetate buffer containing 1 nmol of bis-ANS/ml at pH 7.4 and 37 °C. At time zero, the pH of the buffer was decreased by the addition of citric acid. Fluorescence was measured at excitation and emission wavelengths of 400 and 490 nm, respectively (time resolution, 0.5 s). The relative fluorescence (I/I_0) was calculated as described under “Materials and Methods.”

Fig. 4. Interaction of SL-LPC with BHA. A, the upper (A1) and middle (A2) SL-LPC spectrum was monitored in the presence of BHA at pH 7.4 and at pH 5.0, respectively (molar ratio of BHA (monomer):SL-LPC = 1:1.5). The scaling is 2-fold (pH 7.4) and 4-fold (pH 5.0). The lower spectrum (A3) reflects SL-LPC in aqueous solution (absence of BHA) with a 1-fold scaling. B, the upper spectrum (B1) corresponds to the spectrum of SL-LPC bound to BHA. It was obtained by subtracting the ESR spectrum in the absence of BHA (A3) from that in the presence of BHA at pH 7.4 (A1) using the standard ESR software. The lower spectrum (B2) is the difference spectrum between SL-LPC in the presence of BHA at pH 5.0 (A2) and SL-LPC in aqueous solution (A3). The bound analogs are more strongly immobilized (arrows) in comparison to neutral pH. Note that the narrow spectrum of SL-LPC monomers in aqueous buffer could not be subtracted completely. C, the spectrum of SL-LPC in the presence of BSA (molar ratio of BSA:SL-LPC = 1:1.8). The ESR spectrum reveals a very strong immobilization (arrows) of analogs. All spectra were recorded at 37 °C with a modulation amplitude of 4 G and a scan width of 100 G. The pH of respective BHA samples was lowered to 5.0 in the presence of SL-LPC.

The interaction of SL-LPC with BHA was investigated by ESR spectroscopy at 37 °C. The ESR spectrum of SL-LPC (63 μM final concentration) in aqueous solution in the absence of BHA is shown in Fig. 4A3. The very narrow triplet reflects SL-LPC monomers rapidly tumbling in buffer. No indication for the presence of SL-LPC micelles was observed. The latter can be identified by a broadened line caused by spin-spin interaction of SL-LPC in micelles. Thus, the concentration of SL-LPC was either below or only close to the critical micellar concentration.

To resolve well the spectrum of SL-LPC bound to BHA, experiments were done at a rather high BHA to SL-LPC ratio in comparison to the respective ratio at conditions of fusion inhibition by SL-LPC (see below). In the presence of BHA (molar ratio BHA (monomer) to SL-LPC = 1:1.5), the spectrum of SL-LPC (63 μM final concentration) was composed of a narrow triplet as well as an immobilized component. This immobilized component, which was caused by the binding of SL-LPC to BHA, was more pronounced at pH 5.0 (Fig. 4A2) than at neutral pH (Fig. 4A1). Note that to prevent the shielding of possible hydrophobic binding sites for SL-LPC by protein aggregation, the pH of respective BHA samples was lowered to 5.0 in the presence of SL-LPC (see “Discussion”). To determine the amount of SL-LPC bound to BHA, the spectrum of SL-LPC in aqueous solution was subtracted from the spectra of SL-LPC in the presence of BHA at the given pH using standard Bruker ESR software. The resulting spectra are shown in Fig. 4B. Unfortunately, we were not able to subtract the narrow spectrum completely. Neglecting this minor component at neutral pH, 30% SL-LPC was bound to BHA, whereas at low pH, 64% SL-LPC was associated with BHA. The spectrum of bound SL-LPC was more strongly immobilized at low pH (see arrows) with respect to neutral pH and was almost unaffected upon renaturalization of the suspension (data not shown).

However, when recording the strong binding of SL-LPC to the fatty acid and lipid-binding protein BSA (Fig. 4C), it has become obvious that the association of SL-LPC with BHA is comparatively weak even at low pH. As shown at a molar ratio of BSA:SL-LPC of 1:1.8, a strongly immobilized spectrum of SL-LPC was found (Fig. 4C, arrows). Because no narrow component was detected, almost all the SL-LPC was bound to BSA.

Interaction of SL-LPC with Intact and Bromelain-treated Virus—The interaction of SL-LPC with intact viruses as well as with bromelain-treated viruses was studied at the same molar ratio of SL-LPC to endogenous phospholipid (1:10) used for fusion measurements (see above). Based on the data given under “Material and Methods,” this ratio corresponds to a SL-LPC:HA (monomer) ratio of 5:1. In the presence of intact ectodomain that is accompanied by an increased quantum efficiency of the fluorophore.

The influence of lysolipids on the extent of influenza virus fusion with erythrocyte ghosts at 37 °C. A comparison between SL-LPC and LPC of various chain length. The fusion extent is expressed as the percentage of the control (no lysolipids). The concentration of lysolipids was 10 mol % with respect of the endogenous lipids of the virus-ghost complexes. The average and the mean ± S.D. (n = 3) is given.
virus, the ESR spectrum of SL-LPC was composed of two components, a small narrow component arising from free tumbling SL-LPC monomers (see above) and a component typical for a membrane spectrum. In Fig. 5, only the membrane spectrum obtained after the subtraction of the narrow component is shown for intact and bromelain-treated viruses for pH 7.4 and 5.0 (Fig. 5, A and B, C and D), respectively. Again, the pH of respective samples was lowered to 5.0 in the presence of SL-LPC (see “Discussion”). The subtraction revealed that the contribution of the narrow component to total spectrum is <4%. No significant difference of the membrane spectrum was found between intact viruses (Fig. 5, A and B) and bromelain-treated viruses (Fig. 5, C and D) and between pH 7.4 (Fig. 5, A and C) and pH 5.0 (Fig. 5, B and D). We note a small more immobilized component in the spectra (Fig. 5, arrows). However, this component was similar for intact viruses and viruses lacking the HA ectodomain (bromelain-treated). For liposomes consisting of lipids extracted from influenza virus, this component was not observed (data not shown). By additional experiments, we could show that SL-LPC is located in the outer leaflet of the viral envelope. Upon the mixing of labeled viruses with BSA, we found the same immobilized spectrum that was obtained when mixing BSA with SL-LPC without a membrane present (Fig. 4C). Thus, all SL-LPC were extracted from liposomes and bound to BSA. It has been previously shown that BSA can extract those analogs from the exposed outer leaflet but not from the inner leaflet (35).

These results suggest that the affinity of SL-LPC to the viral membrane is more pronounced in comparison with the HA ectodomain. To test this notion, we have labeled eggPC-SUV with SL-LPC at same molar ratio as virus-ghost complexes (SL-LPC:eggPC = 1:10). Again, we obtained a two-component spectrum, a small narrow component (see above) as well as an immobilized component corresponding to SL-LPC incorporated into the outer leaflet of SUV probed by BSA extraction (see below). The membrane component was not affected upon the addition of BHA, neither at neutral nor at low pH (data not shown). The molar ratio SL-LPC:BHA (monomer) was similar to that of SL-LPC to HA for intact viruses (5:1). This finding demonstrated that BHA is not able to extract SL-LPC from membranes in a detectable amount confirming the higher affinity of SL-LPC to lipid bilayers in comparison with BHA. This low affinity to BHA was in remarkable contrast to the high binding capacity of BSA removing essentially all analogs from the bilayer. After the addition of BSA to eggPC/BHA liposomes, we obtained a spectrum as shown in Fig. 4C.

**DISCUSSION**

As reported earlier, LPC was shown to inhibit not only HA-mediated membrane merger (13, 21) but also Sendai virus fusion with vesicles (31), baculovirus-infected cell-cell fusion, cortical granule exocytosis (32), fusion of cells expressing the envelope glycoprotein of HIV-1 (gp120/gp41) with cells expressing the receptor for this virus CD4 (36), and microsome-microsome fusion (33). Essentially, two alternative mechanisms for LPC-mediated suppression of influenza virus fusion have been proposed. Originally, Chernomordik and co-workers (13–17, 20) suggested that upon membrane incorporation of LPC, its positive spontaneous curvature prevents the bending of the outer monolayer toward each other to form a stalk (see Introduction). In contrast, Günther-Ausborn et al. (21) proposed that the direct interaction of LPC with the HA ectodomain inhibits the interaction of the fusion sequence with the target membrane. Although the prevailing numbers of studies on this topic are in line with the stalk model, the final experimental verification for this model is still lacking. A consequence of this situation is that studies employing lysolipids for studying intermediates of membrane fusion must always consider the alternative mode of lysolipid action suggested by Günther-Ausborn et al. (21) by respective control experiments (for example see Ref. 37).

In this study, we have addressed the interaction of lysolipids like LPC with the influenza virus membrane, particularly with the ectodomain of HA. To monitor directly the interaction of LPC with the viral envelope, we have used a spin-labeled lysophosphatidylcholine. For the spin-labeled derivative, the inverted cone shape, which is considered to be typical for LPC, is somehow perturbed by the nitric oxide moiety on the hydrophobic terminus of the C18:0 acyl chain. Nevertheless, we found that this spin-labeled lysolipid resembled a reliable analog, because it inhibited influenza virus fusion similar to non-labeled LPC (see Fig. 2).

SL-LPC interacted with BHA in a pH-dependent manner. Although in the non-fusogenic conformation of HA, hydrophobic stretches of the ectodomain are not exposed, we observed a binding of SL-LPC to BHA. An increase of SL-LPC binding to BHA was found when lowering the pH to 5.0. This observation can be readily explained by hydrophobic sequences becoming exposed by the low pH-triggered conformational change of the HA ectodomain. This finding is supported by the observation that enhanced binding of SL-LPC at low pH was almost preserved upon reneutralization. It is known that the conformational change of HA is irreversible (29, 38). Very likely, the fusion sequence of the N terminus of HA2 resembles one of the binding sites for SL-LPC. The hydrophobicity of those sites could cause a tighter binding of the lysolipid analog to the ectodomain. This possibility is supported by an enhanced immobilization of SL-LPC associated with BHA. However, when comparing the association of SL-LPC with the fatty acid and lipid-binding protein BSA, we conclude that the interaction of SL-LPC with BHA is comparatively weak. Indeed, to resolve
the interaction of SL-LPC with the HA ectodomain, we had to choose a rather high molar ratio of BHA to SL-LPC.

In the presence of influenza virus, essentially all SL-LPC interacted with the viral membrane. At an analog concentration of 10 mol % of endogenous viral lipids, only <4% SL-LPC remained in the aqueous buffer. This finding suggests a high affinity of the lysolipid analog to membranes. The spectrum measured was typical for a membrane spectrum. We did not find any indication that a significant amount of SL-LPC bound to the ectodomain of HA of intact viruses, neither at neutral or low pH. A spectral component observed for the interaction of SL-LPC with BHA (see above) was not detected. Furthermore, the spectrum of SL-LPC bound to intact influenza viruses was similar to that of viruses lacking the HA ectodomain. We suggest that the high number of lipid bindings sites of the virus bilayer and their affinity compete with binding sites of the HA ectodomain and prevent a significant binding of the lysolipid analog to the ectodomain. Additional support for this suggestion was given by investigating the interaction of BHA with liposomes labeled with SL-LPC. We did not find any extraction of analogs from membranes by BHA. This was in contrast to the high affinity of SL-LPC to BSA leading to a complete analog extraction from liposomes.

For membrane spectra of SL-LPC, we observed a more immobilized component of low intensity. This component was similar for intact viruses and for viruses lacking the ectodomain. Therefore, we conclude that the spectrum originated from SL-LPC incorporated into the viral membrane but not from binding to the HA ectodomain. Furthermore, this component was different from that found for SL-LPC bound to BHA. Presumably, the more immobilized component of the membrane spectra was caused by the interaction of SL-LPC with the transmembrane domain of proteins. Indeed, in liposomes consisting of lipids extracted from influenza virus, we did not observe this component (data not shown).

In conclusion, under conditions in which SL-LPC inhibits influenza virus fusion similar to non-labeled LPC, no detectable binding of the spin-labeled analog to the HA ectodomain with the exception of a strong interaction with the lipid bilayer of the viral envelope is observed. This observation provides strong evidence for an inhibition mechanism based on the action of lysolipids incorporated into the lipid bilayer as proposed in the frame of the stalk hypothesis (16). Our data do not support the hypothesis that inhibition of influenza virus fusion is caused by a direct interaction of SL-LPC with the HA ectodomain preventing the insertion of the fusion sequence into the target membrane (21). However, the situation and respective conclusions might be different when influenza viruses are added to an acidic suspension of target membranes preincubated with lysolipids (for review see Ref. 21). Because the conformational change and exposure of hydrophobic sequences of HA is very rapid, particularly at optimal conditions of pH 5.0 at 37 °C (4, 39, 40), viruses can interact with remaining lysolipid micelles via those hydrophobic sequences before or instead of binding to target membranes. Even if the interaction is reversible, it may be responsible for a conformational transition of HA to a fusion-inactivated state by interaction with lysolipids and/or be due to the delayed contact with the target membrane. It is known that the fusion activity of influenza viruses X31 used in our work and that of Günther-Ausborn et al. (21) is rapidly lost with a half-time in the order of 3 min at pH 5.0 at 37 °C (29). This type of interaction with lysolipids can be circumvented by the prebinding of viruses or HA-containing membranes to the target membrane before incubation with lysolipids and subsequent acidification as done here as well as in previous studies (13, 16).

However, although the recording of ESR spectra of respective samples was started immediately after acidification, inactivation cannot be neglected in our study because of time-consuming accumulation of ESR spectra. Inactivation has been associated with an irreversible conformational change of the ectodomain and the subsequent aggregation of HA trimers via hydrophobic interaction of the ectodomains (41). Thus, one may wonder whether in the time course of measurements inactivation could have been accompanied by a decrease of possible bindings sites of the ectodomain for SL-LPC because of aggregation of ectodomains. To prevent such a loss of possible hydrophobic binding sites, the pH of respective samples of BHA and of virions was lowered in the presence of SL-LPC. From our previous study (42), we surmise that the half-time of binding of SL-LPC to hydrophobic sites should be in the order of ≤5 s. This fast binding of SL-LPC allows to compete efficiently for hydrophobic binding sites. Therefore, we conclude that the low binding of SL-LPC to HA is not because of a loss or shielding but because of the absence of hydrophobic sites with high affinity for SL-LPC.

Finally, although our results argue for an inhibition of influenza virus fusion by lysolipids incorporated into the membrane, our data do not preclude that a preferred interaction of the fusion sequence with lysolipids occurs upon insertion of that sequence into the membrane. This would lead to a local enrichment of lysolipids blocking the formation of structures with a negative curvature. Thus, in this case, the formation of a stalk with a hemifusion intermediate would be even more energetically unfavorable because of the local enrichment of lysolipids.

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Interaction of Lysolipids with Influenza Virus

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