Lysolipids have been reported to inhibit various membrane fusion events, and it was suggested that inhibition was due to their “inverted cone” shape, which hinders the formation of intermediate lipid structures required for fusion (Chernomordik, L. V., Vogel, S. S., Sokoloff, A., Onaran, H. O., Leikina, E. A., and Zimmerberg, J. (1993) FEBS Lett. 318, 71–76). Here, the effect of lysophosphatidylcholine (LPC) on fusion mediated by the hemagglutinin (HA) of influenza virus was investigated. Virus-liposome fusion was inhibited by LPC if the lysolipid was added to the membranes from an aqueous stock solution but not if LPC was symmetrically distributed over both leaflets of the liposomal bilayer. These findings would be consistent with an effect of LPC on lipid intermediate formation, but inhibition increased with increasing acyl chain length and thus a less pronounced inverted cone shape of the lysolipids suggesting that the mechanism of inhibition might be different.

At low pH, due to the exposure of the fusion peptide of HA, followed by its insertion into the liposomal membrane, virus acquires the ability to bind to zwitterionic liposomes lacking receptors for HA. This type of binding was inhibited by LPC. Moreover, leakage of calcein from receptor-containing liposomes, induced by purified HA at low pH, was inhibited by LPC. Therefore, the inhibition of influenza-induced fusion by LPC was caused by the binding of LPC to fusion peptides, thereby preventing their interaction with the target membrane rather than an effect on intermediate lipid structures.

Specialized proteins mediate the fusion of biological membranes (Stegmann et al., 1989). The mechanism of fusion is best understood for the HA glycoprotein of influenza virus (for reviews, see Wiley and Skehel (1987), White (1992), Stegmann and Helenius (1993), Wilschut and Bron (1993), and Clague et al. (1993)). HA-mediated fusion is triggered by low pH, which induces a conformational change in the protein, leading to the exposure of the hydrophobic N terminus of the membrane-anchored subunit, HA2. The N terminus, often referred to as the “fusion peptide,” is then inserted into the target membrane for fusion (Stegmann et al., 1991; Tsurudome et al., 1992), making HA2 an integral membrane protein in both membranes at the same time. How HA then achieves the merger of the lipids of the membranes is not clear. The formation of non-bilayer structures, at least transiently and locally at the site of fusion, is a necessary prerequisite for the fusion of any two lipid bilayers (Wilschut and Hoekstra, 1986). Little is known about these intermediate lipid structures or the role of HA in their formation. One intermediate that was proposed for influenza fusion is a stalk, a semitoroidal structure composed of fused outer leaflets of the viral and target membranes (Markin et al., 1984; Chernomordik et al., 1985, 1987; Koziol et al., 1989; Siegel, 1993a, 1993b).

Lysophosphatidylcholine (LPC) was recently found to inhibit a variety of widely different fusion events, such as microsome-microsome fusion, exocytotic fusion, and baculovirus fusion (Chernomordik et al., 1993). Therefore, it was suggested that a step common to many fusion mechanisms was inhibited by LPC, and this step could be the formation of stalks (Chernomordik et al., 1993, 1995a, 1995b; Vogel et al., 1993). In one version of the stalk model, stalks have negative curvature (Markin et al., 1984; Chernomordik et al., 1985, 1987; Koziol et al., 1989). In this model, LPC should inhibit stalk formation if present in the outer leaflet of the membrane because the headgroup area of LPC is larger than the tail diameter, and this “inverted cone” (Cullis and de Kruijff, 1979) shape of the molecule would block the formation of structures with a negative curvature. In another version, the effect of LPC on stalks themselves can vary, but they develop into trans monolayer contacts and then into fusion pores, both of which have negative curvature, and therefore fusion would be inhibited by LPC in a similar fashion (Siegel, 1993a).

Alternatively, LPC could interact directly with fusion proteins. Fusion between liposomes, induced by synthetic simian immunodeficiency virus fusion peptides, was inhibited by LPC because the lysolipid changed the orientation of these peptides in the membrane (Martin et al., 1993). In contrast, Sendai virus fusion with N-methyl-dioleoylphosphatidylethanolamine (N-methyl-DOPE) liposomes was inhibited by LPC, but the lysolipid also affected fusion between N-methyl-DOPE liposomes, indicating that the formation of lipid intermediates common to both fusion events was inhibited (Yeagle et al., 1994).

In this paper, we show that influenza HA-induced fusion is also inhibited by LPC. It was found that although some of the results appeared compatible with a mechanism of inhibition through an effect on the formation of intermediate lipid structures, LPC did in fact inhibit fusion at an earlier stage. Inhibition seemed to be mediated by the direct binding of free monomeric or micellar LPC to fusion peptides, hindering their...
interactions with the target membrane, and thereby inhibiting fusion. These findings confirm the crucial role of insertion of the fusion peptide into the target membrane in influenza-induced fusion.

**Experimental Procedures**

Materials—Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), egg lysophosphatidylcholine (egg LPC), lyso-1-palmitoyl-sn-3-phosphatidylcholine (LPPC), N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE), and N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) were purchased from Avanti Polar Lipids (Alaska). 1-Lauryl-sn-3-phosphatidylcholine (LLPC), 1-myristoyl-sn-gliosides (type III), Triton X-100, Sepharose-coupled (LMPC), 1-oleoyl-3-phosphatidylcholine (LOPC), bovine brain gangliosides (type III), Triton X-100, Sepharose-coupled Ricinus communis agglutinin, and d-\(^{-}\)galactose were obtained from Sigma. \(^{14}\)C-LPC was from Amersham (Little Chalfont, Buckinghamshire, United Kingdom), calcein was from Molecular Probes, octylglucoside was from Fluka (Buchs, Switzerland), and Sephadex G-100 as well as Sepaheryl 5400 were from Pharmacia (Upssala, Sweden).

Liposomes and Virus—Multilamellar vesicles were prepared by re-suspending a dry lipid film in 145 mM NaCl and 2.5 mM HEPES, pH 7.4. Subsequently, the suspension was frozen and thawed five times, and large unilamellar vesicles were made from the multilamellar liposomes by extrusion through a 0.1-μm-defined pore polycarbonate filter (Nucleopore, Pleasanton, CA) (Mayer et al., 1986). After extrusion, residual multilamellar vesicles were removed by centrifugation at 16,000 × g for 20 min. Liposomes were stored at 4°C under argon. Phospholipid phosphate was determined according to Böttcher et al. (1961).

The X-31 recombinant strain of influenza virus was grown for us by the Schweizerische-und-1mpfstoßnstitut (Bern, Switzerland) in the allantoic cavity of embryonated eggs and purified and stored as described (Nebel et al., 1995).

Fusion Measurements—Fusion between virus and labeled liposomes was measured with the resonance energy transfer assay (Struck et al., 1981). Briefly, 0.6 mol% each of N-NBD-PE and N-Rh-PE were incorporated into liposomes. Fluorescence measurements were made on a Schoeffel RRS 1000 fluorimeter with excitation and emission wavelengths of 465 and 530 nm, respectively. A 515-nm-long pass filter was used in the detection channel. A 515-nm-long pass filter was used in the detection channel.

RESULTS

**LPC Inhibits Influenza-induced Fusion**—To determine if LPC affects the fusion of influenza virus, large unilamellar vesicles were made containing PC, PE, and gangliosides (6:3:1 molar ratio) plus trace amounts of N-NBD-PE and N-Rh-PE (0.6 mol% each) as described under “Experimental Procedures.” Fusion of the virus with these liposomes was measured by the resonance energy transfer assay (Struck et al., 1981). Upon addition of virus to liposomes at pH 5.1, 37°C, there was an increase in fluorescence caused by membrane fusion (Stegmann et al., 1986) (Fig. 1). If LPC (mixed acyl chains, from egg yolk) was added to liposomes from an aqueous stock solution, there was a small jump in fluorescence followed by a decrease. After about 2–3 min, the fluorescence stabilized close to the original level (Fig. 1). Although we do not know precisely what caused these changes in fluorescence, they were most likely the result of the interaction between LPC and the liposomal membranes. In any case, at the concentrations used throughout this paper, LPC did not lyse the membranes, which would have given rise to a permanent increase in fluorescence. If virus was added to a mixture of LPC and liposomes, which had been equilibrated together for 3 min, fusion was much reduced compared to that seen in the absence of LPC, indicating that LPC inhibits the fusion of influenza virus with liposomes (Fig. 1). No increase in fluorescence was seen when virus was added to liposomes at pH 7.4 in the absence or presence of LPC, indicating that LPC did not promote the molecular exchange of probe between the membranes.

The inhibition of fusion by LPC in various biological systems was found to depend on the acyl chain length of LPC (Cherno-
than 12 carbon atoms (not shown), we found that inhibition increased with increasing hydrocarbon molecules were tested for their inhibitory activity in our system, in concentrations up to 50 molar. 

**Fig. 2.** The inhibitory effect of LPC depends on the chain length. Liposomes were equilibrated with different concentrations of LLPC, LMPC, LPPC, or LOPC, at pH 5.1, 37 °C, for 3 min before virus was added. Numbers in brackets denote the number of carbon atoms in the acyl chain. The initial rate of fusion was measured as described under "Experimental Procedures", and expressed relative to the rate of fusion in the absence of LPC. Error bars are ± 1 S.D. Other conditions as in Fig. 1.

Remarkably, the chain length dependence that Chernomordik et al., (1993, 1995b) found for some other biological membranes, such as the low pH-induced fusion mediated by a baculovirus protein. These authors suggested that if LPCs inhibit fusion because they inhibit the formation of lipid intermediates with negative curvature, then LPCs with shorter hydrocarbon tails, which have a more pronounced "inverted cone" shape, should be better inhibitors. In our case, for LPCs with 14-18 carbon atoms, inhibition seemed to correlate better with the partition coefficients of LPCs between the viral and liposomal membrane, such as the low pH-induced fusion mediated by a baculovirus protein. These authors suggested that if LPCs inhibit fusion because they inhibit the formation of lipid intermediates with negative curvature, then LPCs with shorter hydrocarbon tails, which have a more pronounced "inverted cone" shape, should be better inhibitors. In our case, for LPCs with 14-18 carbon atoms, inhibition seemed to correlate better with the partition coefficients of LPCs between water and membranes and therefore with the concentration of LPC present in the membrane or possibly another hydrophobic compartment (see below). Partition coefficients can be derived from the critical micellar concentrations (Stafford et al., 1989) of the different LPCs, and affinity constants of binding of different LPCs to erythrocytes have been reported (Weltzien et al., 1977). Thus, based on our results, LPC might inhibit influenza fusion by a different mechanism. To investigate this possibility, we tested other predictions of the stalk theory and considered direct effects of LPC on the fusion activity of HA. According to the theory, LPC should inhibit fusion when it is present only in the outer leaflet of a bilayer (Chernomordik et al., 1995a; Siegel, 1993a). Symmetric incorporation in both leaflets should not affect fusion. The effect of the presence of LPC in the inner leaflet is more difficult to estimate. Based on data from monolayer-liposome fusion experiments, it could promote fusion (Chernomordik et al., 1987), but in one version of the stalk model it could inhibit fusion (Siegel, 1993a).

To obtain liposomes containing LPC symmetrically distributed over both leaflets, the liposomal lipids were mixed with LPPC in chloroform/methanol. Subsequently, the mixture was dried by the evaporation of solvent, and large unilamellar vesicles were prepared by extrusion as described under "Experimental Procedures." LPC incorporated into vesicles of this size should be symmetrically distributed (Van den Besselaar et al., 1979). Since Chernomordik et al. (1995a) found that, at inhibiting concentrations, LPC comprises 5-10% of membrane lipids, fusion of influenza virus with liposomes containing 5 or 10 mol% LPPC in both leaflets of the bilayer, and fusion is shown with liposomes that were incubated with 5 μM of LOPC (d) or LPPC (e) in one-twentieth of the final volume of buffer for 10 min at pH 7.4, 25 °C before their dilution into the pH 5.1 buffer in the cuvette. Fusion is shown of virus with that had been preincubated in one-twentieth of the final volume of buffer for 10 min in the absence (f, h) or presence of 5 μM LLPC (g, i) at pH 5.0, 0 °C (f, g) or at pH 7.4 (h, i).

Fusion is also shown with liposomes before (k) and after (l) they were separated from LPC on a column. 1 μM liposomes were incubated with 1 μM of LLPC in 1 ml of buffer for 30 min at 25 °C and subsequently passed over a Sephacryl S400 column. Fusion of virus with aliquots of the mixture was tested. Error bars are ± 1 S.D. The final concentrations of liposomes and virus were 5 μM each of viral and liposomal phospholipid. Fusion was measured in 2 ml of buffer at pH 5.1, 37 °C.

It should be noted that in these experiments some of the LPC was rapidly transferred from the liposomal to the viral membrane. To estimate the transfer, symmetric liposomes were made, consisting of PC and PE (2:1 ratio), 10 mol% of LOPC, and trace amounts of [14C]LPPC, and incubated with virus at neutral pH for various periods of time. Virus does not bind to these zwitterionic liposomes at pH 7, allowing their separation by centrifugation as described under "Experimental Procedures." It was found that within seconds after incubation, approximately 10% of the outer membrane leaflet [14C]LPPC was transferred to the viral membrane. The concentration in the viral membrane did not increase significantly over the course of 1 h. Similar data were reported for the transfer of fluorescently labeled LPCs between liposomal membranes; in these studies, the half-time of transfer of the C16 analogue was about 8 s, and equilibrium was reached within minutes (Zhang and Nichols, 1994).

In the experiment presented in Fig. 2, in which LPC was added from an aqueous stock solution, there were several different populations of LPC. LPC became incorporated into the outer leaflet of the liposomal and viral membranes (De Kruijff et al., 1977), and there were monomers and micelles of it in solution. To test if the inhibition of fusion was due to membrane-associated LPC, liposomes were preincubated with LPPC or LOPC in a small volume at concentrations that would inhibit fusion by more than 50%. Subsequently, the vesicles were diluted 20-fold by their injection into the cuvette of the fluorimeter and mixed with virus. No inhibition of fusion was observed (Fig. 3), suggesting that incorporation of LPC into membranes was either readily reversible or did not occur, or...
that LPC present in the membrane did not inhibit fusion. However, although transfer of LPC from liposomal to viral membranes does occur (see above), membrane-incorporated LOPC and LPPC should be almost non-extractable into buffer by dilution (Weltzien, 1979). Likewise, if virus was preincubated with LPPC at pH 7.4 before 20-fold dilution by addition to liposomes in the cuvette, fusion was not inhibited (Fig. 3; fusion appears slower due to incomplete recovery of the sample). If virus was preincubated at pH 5, 0°C, for 10 min in the absence of LPC, fusion was somewhat faster than after preincubation at pH 7.4, as reported previously (Stegmann et al., 1987), probably because the conformational change that leads to the exposure of the fusion peptide had already taken place. If the low pH preincubation was carried out in the presence of LPPC, fusion was slower than without LPPC, indicating an inhibitory effect of LPPC under these circumstances (cf. Fig. 3, f and g). The amount of membrane-associated LPC would be the same in these latter cases. The above results suggested that inhibition of fusion was not caused by membrane-associated LPC and might be specific for the low pH form of HA.

In an attempt to measure how much LPC was associated with membranes compared to that present in solution, a mixture of [14C]LPPC and LPC was added to liposomes and incubated for 30 min at 25°C. Fusion of virus with aliquots of this mixture of liposomes and LPC was inhibited. Subsequently, the mixture was passed over a Sephacryl S400 column to remove unbound LPC. We found that LPC stuck irreversibly to the column, and only about 1 mol% remained associated with the liposomes. Fusion of virus with these liposomes was not inhibited (Fig. 3).

In view of the minute amount of radioactivity remaining associated with the liposomes and the strength of the binding of LPC to the column, it seemed unlikely that the column only separated free from associated LPC by molecular sieve filtration; it probably also extracted LPC from liposomal membranes by hydrophobic interactions. This property of the column was exploited to obtain liposomes with LPC confined predominantly to the inner leaflet. Liposomes containing traces of [14C]LPPC and 5 mol% LPC, symmetrically distributed over both leaflets, were prepared as described above and passed repeatedly over a Sephacryl column. As shown in Fig. 4, each subsequent run of the material over the column removed more LPC from the liposomes. After the first run, 18% of the LPC was no longer associated with the vesicles. After the third, 57% of radioactivity was left. If liposomes were prepared containing 25 mol% LPC in both leaflets, 56% of the radioactivity was recovered also (not shown), indicating that the partial removal was not due to saturation of the column. Since the translocation of LPPC across membranes (flip-flop) is very slow (de Kruijff et al., 1977), these data indicate that, with 2.8 mol% remaining, LPPC was nearly completely removed from the outer leaflet of the liposomes that originally contained 5 mol% of LPC. Thus, with every passage over the column, the vesicles became more asymmetric, having more LPPC in the inside leaflet. However, fusion was not affected (Fig. 4).

Taken together, the above data indicate that fusion is inhibited only if LPC is added to liposomes from a stock solution but not when it is symmetrically distributed over both leaflets of the bilayer. Assuming that some of the LPC added to membranes was indeed incorporated into the outer leaflet of liposomes, as was found in other studies (de Kruijff et al., 1977), and if this population of LPC caused the inhibition of fusion, these results would be in line with the predictions of the stalk theory. On the other hand, some of the results described above suggested that it was not the membrane-associated LPC that affected fusion. Also, LPC in the inner leaflet had little effect. Therefore, we investigated if LPC inhibited fusion through a different mechanism than inhibition of the formation of lipid intermediates. LPC does not inactivate HA (Fig. 3), but it could for example inhibit virus-liposome binding or bind to the hydrophobic fusion peptide, interfering with its interaction with the liposomal target membrane.

Binding of the virus to ganglioside-containing liposomes was measured as described under “Experimental Procedures” at neutral pH. At this pH, binding is entirely due to specific interactions between the receptor binding site on HA and the sialic acid moiety of the gangliosides (Stegmann et al., 1995). It was found that binding was reduced by about one-third in the presence of 20 μM LPPC. At that concentration, the rate of fusion was reduced by about 80% (Fig. 2). Therefore, although it could contribute to the inhibition of fusion, reduced binding is not the main cause of it.

To investigate the effect of LPC on the interaction of fusion peptides with the liposomal membrane, the binding of virus to zwitterionic liposomes was measured at low pH. Influenza virus does not bind to these liposomes, which lack sialic acid-containing receptors, at neutral pH. However, after the conformational change in HA at low pH, HA (Doms et al., 1985) and whole virus (Stegmann et al., 1987, 1995) acquire the ability to bind to zwitterionic liposomes through insertion of the hydrophobic fusion peptide into the liposomal membrane (Stegmann et al., 1991; Tsurudome et al., 1992). Liposomes and virus were incubated together in the presence of different concentrations of exogenously added LPPC at pH 5.1, 0°C, for 14 min. During this time at 0°C, the conformational change occurs and there is binding, but little fusion (Stegmann et al., 1990, 1991). Subsequently, the samples were neutralized, and binding was determined as described under “Experimental Procedures.” LPPC inhibited binding completely at 20 μM (Fig. 5). Half-maximal inhibition of binding was found at around 6.7 μM, 50% fusion inhibited by 5 μM LPPC.

To investigate if the inhibition of binding was caused by membrane-associated or free LPPC, the binding of zwitterionic liposomes, containing 5 or 10 mol% LPC symmetrically distributed over both leaflets, to virus was measured. Binding was not inhibited (not shown), indicating that the presence of LPC in the membrane did not prevent binding.

Inhibition of insertion of the fusion peptide by LPPC could be the result of steric inhibition of HA-target membrane interactions by LPPC or be caused by binding of LPPC to the hydrophobic fusion peptides upon their exposure. To investigate the
latter possibility, 15 nmol of virus was incubated with 5 nmol of a mixture of LPPC with [14C]LPPC at pH 5.1 or 7.4 for 10 min at 0°C. Subsequently, the mixture was centrifuged through a 10% sucrose cushion, and the amount of radioactivity pelleted with the virus was determined. At neutral pH, 38 ± 3% of LPPC bound to the virus, whereas at low pH, 54 ± 4% of LPPC was associated with the virus. Taken together, the above data indicate that LPPC binds to the fusion peptide of HA, inhibiting its insertion into the membrane.

LPC Inhibits HA-induced Leakage of Liposomal Contents—

Binding of virus to liposomes is a necessary prerequisite for fusion, and therefore fusion of virus with PC/PE liposomes lacking receptors would have been abolished completely by inhibition of insertion of the fusion peptide. However, insertion of the fusion peptide into the target membrane is facilitated by HA-receptor binding interactions (Stegmann et al., 1995). To investigate if the inhibition of fusion of virus with ganglioside-containing liposomes was also caused by the effect of LPC on the fusion peptide-target membrane interaction, we studied the leakage of liposomal contents which is induced by this interaction.

Purified HA is known to cause low pH-induced hemolysis (Maeda et al., 1981; Sato et al., 1983; Wharton et al., 1986) and the leakage of small molecular weight compounds from liposomes (Maeda et al., 1981). The pH dependence and other properties of these processes suggest that they are caused by insertion of the fusion peptide into the target membrane (Maeda et al., 1981; Sato et al., 1983). To measure HA-induced leakage of liposomal contents, liposomes (PC/PE/gangliosides in a 6:3:1 ratio), containing 50 mM of the water-soluble fluorescent dye calcein, were first incubated with virus at pH 5.1. Calcein leakage was measured by monitoring the fluorescence increase resulting from the relief of self-quenching of the dye after dilution into the buffer. Confirming earlier results (Stegmann et al., 1985), calcein was released from liposomes upon their fusion with virus. LPC was found to inhibit this release (Fig. 6).

To investigate if the release was a consequence of fusion or mediated by HA, the protein was purified as described under “Experimental Procedures.” Since HA is an integral membrane protein, it will form micelle-like structures called HA rosettes in solution, with the membrane anchor pointing inward. As shown in Fig. 7, HA rosettes induced the leakage of calcein at pH 5.1, 37°C. Leakage could be inhibited by LPPC (Fig. 7). At the concentrations used, LPPC by itself did not induce leakage. In our hands, HA rosettes did not induce any liposome fusion (not shown). The pH dependence of leakage induced by rosettes...
One attractive model features the formation of stalks, trilaminar structures composed of fused outer leaflets but unfused inner leaflets of the interacting membranes. In the first of two versions of the model, stalks expand radially, and then the inner leaflets rupture at the center, leading to complete fusion between the membranes (Markin et al., 1984; Chernomordik et al., 1985, 1987; Kozlov et al., 1989). In the second, stalks evolve into trans monolayer contacts and finally into fusion pores, which are the first structures in which the lipids of the inner leaflets are seen to merge (Siegel, 1993a, 1993b). The two versions share common features, in that they always contain structures with negative curvature, namely trans monolayer contacts and fusion pores in the second model and stalks themselves in the first. Insertion of molecules with an inverted cone shape, such as LPC, into the outer leaflet of one or both membranes should make the formation of these structures energetically less favorable and therefore decrease the likelihood of fusion between the membranes. Likewise, insertion of molecules with a cone shape, such as unsaturated PEs, into the outer leaflets should promote fusion. LPC is incorporated into the outer leaflets of membranes upon the addition of a stock solution of LPC, and translocation of the molecule to the inner leaflets is slow (De Kruijf et al., 1977). Therefore, in principle, LPC should provide an excellent tool to test the stalk model.

However, if LPC is added to membranes in this fashion, there remains a sizable population of monomeric and micellar LPC in solution. Even if LPC is incorporated into the membrane it may, depending on its chain length and water solubility, redissolve in the buffer surrounding it, as suggested by our finding that within a few days of storage of symmetric liposomes containing 25 mol% of LPC, fusion with virus became slower. The data presented in this paper show that for influenza virus, inhibition of fusion is caused by non-membrane bound LPC. Therefore, direct effects of exogenously added LPC other than those on intermediate lipid structures with a negative curvature should probably be considered in other cases also.

The results presented in this paper do not imply that stalks are not involved in influenza virus-induced fusion or that LPC would not be capable of inhibiting the formation of lipid intermediates in this case, but rather that LPC inhibited fusion at an earlier stage. A mutant HA was recently shown to induce hemifusion, which is the formation of structures with fused outer leaflets but unfused inner leaflets, without subsequent fusion between the inner leaflets (Kemble et al., 1994). Thus, these structures had some of the properties expected for stalks. There is good evidence for an effect of LPC on the formation of lipid intermediates involved in the fusion between N-methyl-DOPE liposomes (Yeagle et al., 1994). This fusion event does not involve any proteins. In the case of fusion driven by baculovirus fusion protein, in some experiments LPC was produced by phospholipase treatment of cell membranes, and the cells were washed with buffer. Fusion between the cells was then induced by low pH (Chernomordik et al., 1995b). In this experimental set-up, there is little free LPC. Besides, although there is a short hydrophobic segment in the fusion protein that is important for fusion (Monsma and Blissard, 1995), it is not clear if baculovirus actually possesses a fusion peptide. Moreover, a good indication that inhibition was caused by the effect of LPC on the formation of lipid intermediates with negative curvature in this case is the chain-length dependence of the inhibition of fusion. Shorter LPCs have a more pronounced inverted cone shape and are less hydrophobic, as is obvious from their critical micelle concentration (Weltzien, 1979; Stafford et al., 1989) and their affinity constant for membranes (Weltzien et al., 1977). Therefore, if inhibition increases with increasing chain length, the effect was most likely by interaction with something hydrophobic, like the fusion peptide, whereas if it decreases with increasing chain length, as with the baculovirus protein (Chernomordik et al., 1995b), it is more likely to be on the formation of lipid intermediates. Considering the partition coefficients of LPCs between buffer and membranes, a clear-cut effect should only be expected within a limited range of chain lengths, e.g. 14–18 carbon atoms (Weltzien, 1979).

In other cases, for example, microsome-microsome fusion (Chernomordik et al., 1993), it seems possible, at least in principle, that LPC could have an effect on fusion proteins rather than lipid intermediates. The data presented in this paper provide additional evidence that insertion of the fusion peptide into the target membrane is a prerequisite for influenza-induced fusion. If homologues of viral fusion peptides were discovered because LPC inhibits fusion in these cases, that would be a key observation toward understanding endoplasmic fusion. So far, viral fusion proteins serve as the paradigm for exoplasmic fusion events, where the initial contact between the membranes involves the externally oriented surfaces of cells or their topological equivalent on viral membranes (Stegmann et al., 1989). Although many proteins involved in endoplasmic fusion events, which take place after initial contact between cytoplasmically oriented surfaces of cellular membranes, have been identified (Rothman, 1994), the mechanism by which these proteins induce fusion is unknown. Since endoplasmic fusion takes place in a controlled and uniform environment with soluble factors that could be used to drive or regulate fusion, the mechanism may differ from that of exoplasmic fusion, where the environment is not regulated and soluble factors are not available. Therefore, the mechanism whereby LPC inhibits endoplasmic fusion events should be elucidated.

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REFERENCES

Böttcher, C. J. F., Van Gent, C. M., and Fries, C. (1961) Anal. Chim. Acta 24, 203–204
Chernomordik, L. V., Kozlov, M. M., Melikyan, G. B., Abidor, I. G., Markin, V. S., and Chizmadzhev, Y. A. (1985) Biochim. Biophys. Acta 812, 643–655
Chernomordik, L. V., Melikyan, G. B., and Chizmadzhev, Y. A. (1987) Biochim. Biophys. Acta 906, 309–352
Chernomordik, L. V., Vogel, S. S., Sokoloff, A., Onaran, H. O., Lekînîna, E. A., and Zimmerberg, J. (1993) FEBS Lett. 318, 71–75
Chernomordik, L. V., Kozlov, M. M., and Zimmerberg, J. (1995a) J. Membr. Biol. 146, 1–14
Chernomordik, L. V., Lekînîna, E. A., Cho, M.-S., and Zimmerberg, J. (1995b) J. Virol. 69, 3040–3051
Clague, M. J., Schoch, C., and Blumenthal, R. (1993) in Viral Fusion Mechanisms

Fig. 8. HA-induced leakage from liposomes as a function of pH. Leakage of calcein induced by purified HA was measured as described in the legend to Fig. 7 in the absence of LPPC, and the initial rate of leakage was determined from the slope of the fluorescence tracing. Error bars are ± 1 S.D.
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(Bentz, J., ed) pp. 113–132. CRC Press, Boca Raton, FL.

Cullis, P. R., and de Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420

De Kruijff, B., Van den Besselaar, A. M. H. P., and Van Deenen, L. L. M. (1977) Biochim. Biophys. Acta 465, 443–453

Doms, R. W., Helenius, A., and White, J. (1985) J. Biol. Chem. 260, 2973–2981

Kerbiri, G. W., Daniell, T., and White, J. M. (1994) Cell 76, 383–391

Kozlov, M. M., Leikin, S. L., Chernomordik, L. V., Markin, V. S., and Chizmadzhev, Y. A. (1989) Eur. J. Biochem. 17, 121–129

Maeda, T., Kawasaki, K., and Onishi, S. I. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4133–4137

Mandersloot, J. G., Reman, F. C., Van Deenen, L. L. M., and De Gier, J. (1975) Biochim. Biophys. Acta 382, 22–26

Markin, V. S., Kozlov, M. M., and Borovjagin, V. L. (1984) Gen. Physiol. Biophys. 3, 363–377

Martin, I., Dubois, M.-C., Saermark, T., Epand, R. M., and Ruysschaert, J. M. (1993) FEBS Lett. 333, 325–330

Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) Biochim. Biophys. Acta 858, 161–168

Monsma, S. A., and Blissard, G. (1995) J. Virol. 69, 2583–2595

Neble, S., Bartoldus, I., and Stegmann, T. (1995) Biochemistry 34, 5705–5711

Peterson, G. L. (1977) Anal. Chem. 83, 346–356

Rothman, J. E. (1994) Nature 372, 55–63

Sato, S. B., Kawasaki, K., and Onishi, S. I. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3153–3157

Siegel, D. P. (1993a) Biophys. J. 65, 2124–2140

Siegel, D. P. (1993b) in Viral Fusion Mechanisms (Bentz, J., ed) pp. 475–512. CRC Press, Boca Raton, FL

Stafford, R. E., Fanin, T., and Dennis, E. A. (1989) Biochemistry 28, 5113–5120

Stegmann, T., Hoekstra, D., Scherpoh, G., and Wilschut, J. (1985) Biochemistry 24, 3107–3113

Stegmann, T., Hoekstra, D., Scherpoh, G., and Wilschut, J. (1986) J. Biol. Chem. 261, 10966–10969

Stegmann, T., Booy, F. P., and Wilschut, J. (1987) J. Biol. Chem. 262, 17744–17749

Stegmann, T., Doms, R. W., and Helenius, A. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 187–211

Stegmann, T., White, J. M., and Helenius, A. (1990) EMBO J. 9, 4231–4241

Stegmann, T., Defino, J. M., Richards, F. M., and Helenius, A. (1991) J. Biol. Chem. 266, 18401–18410

Stegmann, T., and Helenius, A. (1993) in Viral Fusion Mechanisms (Bentz, J., ed) pp. 89–111. CRC Press, Boca Raton, FL

Stegmann, T., Bartoldus, I., and Zumbrunn, J. (1995) Biochemistry 34, 1825–1832

Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Biochemistry 20, 4093–4099

Tsurudome, M., Glück, R., Graf, R., Falchietto, R., Schaller, U., and Brunner, J. (1992) J. Biol. Chem. 267, 20225–20232

Van den Besselaar, A. M. H. P., de Kruijff, B., Van den Bosch, H., and Van Deenen, L. L. M. (1979) Biochim. Biophys. Acta 555, 193–199

Vogel, S. S., Leikina, E. A., and Chernomordik, L. V. (1993) J. Biol. Chem. 268, 25764–25768

Veltzien, H. U. (1979) Biochim. Biophys. Acta 559, 259–287

Weiztien, H. U., Arnold, B., and Reuther, R. (1977) Biochim. Biophys. Acta 466, 411–421

Wharton, S. A., Skehel, J. J., and Wiley, D. C. (1986) Virology 149, 27–35

White, J. M. (1992) Science 258, 917–924

Wiley, D. C., and Skehel, J. J. (1987) Annu. Rev. Biochem. 56, 365–394

Wilschut, J., and Bron, R. (1993) in Viral Fusion Mechanisms (Bentz, J., ed) pp. 133–161. CRC Press, Boca Raton, FL

Wilschut, J., and Hoekstra, D. (1986) Chem. Phys. Lipids 40, 145–166

Yeagle, P. L., Smith, F. T., Young, J. E., and Flanagan, T. D. (1994) Biochemistry 33, 1820–1827

Zhang, Z., and Nichols, J. W. (1994) Am. J. Physiol. 267, G80–G86
