JOURNAL OF VIROLOGY, May 1995, p. 3049–3058 Vol. 69, No. 5
0022-538X/95/$04.00+0

Control of Baculovirus gp64-Induced Syncytium Formation by Membrane Lipid Composition

LEONID CHERNOMORDIK,* EUGENIA LEIKINA, MYOUNG-SOON CHO, AND JOSHUA ZIMMERBERG

Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, Bethesda, Maryland 20892

Received 8 November 1994/Accepted 10 February 1995

We have investigated the effects of membrane lipid composition on biological membrane fusion triggered by low pH and mediated by the baculovirus envelope glycoprotein gp64. Lysolipids, either added exogenously or produced in situ by phospholipase A2 treatment of cell membranes, reversibly inhibited syncytium formation. Lysolipids also decreased the baculovirus infection rate. In contrast, oleic and arachidonic acids and monoolein promoted cell-cell fusion. Membrane lipid composition affected pH-independent processes which followed the low-pH-induced change in fusion protein conformation. Inhibition and promotion of membrane fusion by a number of lipids could not be explained by mere binding or incorporation into membranes, but rather was correlated with the effective molecular shape of exogenous lipids. Our data are consistent with the hypothesis that membrane fusion proceeds through highly bent membrane intermediates (stalks) having a net negative curvature. Consequently, inverted cone-shaped lysolipids inhibit and cone-shaped *cis*-unsaturated fatty acids promote stalk formation and, ultimately, membrane fusion.

Fusion mediated by specific viral envelope glycoproteins (fusion proteins) is the best-characterized type of biological fusion (5, 50, 60, 63). However, even in the case of influenza virus, whose fusion protein structure has been studied most extensively (6, 61), the mechanism of membrane fusion is still not known. In order to depict the key fusion intermediates, in addition to studying the involved proteins, it is also important to find out whether properties or composition of membrane lipid bilayers can inhibit or promote the biological fusion reaction. In particular, rearrangement of two lipid bilayers into a single bilayer inevitably requires membrane bending. Different lipids bend lipid monolayers in different directions (12, 28, 35), and studies on the effects of lipid composition on membrane fusion may provide important insight into the structure of bent fusion intermediates.

Recently we have found that lysolipids, a single class of tightly regulated membrane components, reversibly inhibit membrane fusion in diverse biological systems at a step between triggering and membrane merger (9, 55). These results suggest that all membrane merger reactions have a common mechanistic step, independent of further action by triggers but sensitive to membrane lipid composition.

To further elucidate the effects of exogenously added lipids on membrane fusion, we concentrated on baculovirus-mediated membrane fusion. Baculoviruses are a diverse group of insect pathogenic DNA viruses, switching at different stages of their infection cycle between two very different phenotypes of the virus, i.e., the budded and occluded forms (3). As with many other enveloped viruses, the budded form (produced by baculovirus nucleocapsid budding from the plasma membrane) is thought to enter host cells by low-pH-triggered fusion of the viral membrane to the endocytotic vesicle membrane of host cells (3, 56, 57). This assumption was recently substantiated by cell-cell fusion experiments. Insect cells infected by baculovirus or transfected to express the viral envelope glycoprotein gp64 (thought to be responsible for virus entry into host cells [56]) are shown to fuse and form syncytia when treated with low-pH medium (4, 34).

In the present study we found that some specific alterations of membrane lipid composition dramatically affect the membrane fusion mediated by gp64. The addition of lysolipids and *cis*-unsaturated fatty acids, respectively, reversibly inhibited and promoted a pH-independent stage of fusion which follows the low-pH-induced change in fusion protein conformation. Whether a given lipid inhibited or promoted fusion was determined by the direction of membrane monolayer bending known to be induced by that lipid. The results are consistent with a specific mechanism of membrane fusion involving the formation of the highly bent (net negative curvature) local and transient lipidic connections between fusing membranes.

MATERIALS AND METHODS

Materials. All lipids and membrane dyes were purchased from Avanti Polar Lipids (Birmingham, Ala.). Tween 80 and monoolein were purchased from Pierce (Rockford, Ill.) and Nu Chek Prep (Elysian, Minn.), respectively. Fatty acid free bovine serum albumin (BSA), arachidonic acid (AA), oleic acid (OA), and phospholipase A2 (PLA2) from bovine pancreas were purchased from Sigma (St. Louis, Mo.). Trypsin and *N*-ethylmaleimide (NEM) were obtained from Fluka (Buchs, Switzerland). Ethidium homodimer and octadecyl rhodamine B (R18) were purchased from Molecular Probes (Eugene, Oreg.). TNM-FH and Grace's media and wild-type *Autographa californica* nuclear polyhedrosts virus (AcNPV) baculovirus were purchased from Invitrogen (San Diego, Calif.).

Three radioactively labeled lysolipids, ^{14}C-palmitoyl lysophosphatidylcholine (LPC) (Amersham, Arlington Heights, Ill.) and ^{3}H-myristoyl and ^{3}H-stearoyl LPCs (SibTech, Novosibirsk, Russia), were used to characterize lysolipid binding to cell membranes.

Stock solutions of lysolipids and Tween 80 were freshly prepared as a 0.5% (wt/wt) aqueous dispersion and intensively vortexed to clear dispersion. We found no difference in fusion extent and kinetics when in some experiments stock solutions of lysolipids in ethanol (50 mg/ml) were used instead of aqueous dispersions. Stock solutions of R18 (100 μM) and *N*-(lissamine rhodamine B sulfonyl) diacylphosphatidylethanolamine prepared from egg (R-PE; 0.5 mg/ml), *N*-(lissamine rhodamine B sulfonyl) lysophosphatidylethanolamine (R-LPE; 0.5 mg/ml), and arachidonic and oleic acids (50 to 100 μM) were freshly made in ethanol.

Cell growth and infection. *Spodoptera frugiperda* (Sf9) insect cells were grown in 24-well plates (Costar) in the TNM-FH medium (10^5 per well) and inoculated with 10^6 PFU of the wild-type baculovirus AcNPV per ml as described previously (34). In some experiments, the number of infected cells at the later stages of

* Corresponding author. Phone: (301) 594-1128. Fax: (301) 594-0813. Electronic mail address: chernomordik@nih.gov.

infection was estimated 48 h after inoculation by counting cells with polyhedral inclusions, using light microscopy.

Quantification of cell-cell fusion. Thirty-six hours after infection with baculovirus, Sf9 insect cells were triggered to fuse by application of a low-pH medium (pH 5.1, if not stated otherwise). To adjust pH to any specific value, we titrated Grace's medium with citrate. The cells were incubated at a low pH for a specific time interval in the range of 20 s to 5 min (2 min, if not stated otherwise) and then returned to their normal pH of 6.4. The percentage of cells in syncytia (ratio of nuclei within syncytia to the total number of cell nuclei in the same field) was determined by light microscopy.

In other experiments, cells were labeled with R-PE by injection of 5 to 8 μl of the dye stock solution into the 0.5 ml of culture medium per dish. Nonincorporated dye was removed by three 2-ml washes of fresh medium, and then cells were resuspended and mixed with unlabeled cells. Fusion, as defined by membrane dye redistribution between labeled and unlabeled cells, was quantified by fluorescent and light microscopy as the ratio of cell-cell contacts where dye redistribution was observed to the total number of the contacts (55). In order to replate labeled and unlabeled cells together for the dye redistribution assay, we first had to resuspend them, since lower fusion rates were generally observed when a low-pH pulse was applied to cells less than 2 h after replating of resuspended cells.

These two fusion assays gave qualitatively consistent results. However, the extents of fusion measured by the dye redistribution assay were higher than those obtained by counting cells in syncytia (e.g., 88.0% ± 9.2% and 35.2% ± 5.4%, respectively). Also, fusion defined as membrane dye redistribution was detected much faster than syncytium formation. Five minutes after application of the low-pH medium, the dye redistribution assay exhibited the maximal (i.e., final) extent of cell-cell fusion, but maximal syncytium formation took 2 h to be established.

Treatment of cells. We used Grace's medium supplemented with lipids, enzymes, and other compounds to replace the medium in the plate wells containing cells. Viability of cells treated with exogenous lipids was controlled 2 h later by using trypan blue or ethidium homodimer exclusion tests. Some of the compounds tested in this study were toxic for cells at some concentrations. However, no correlation was found between the toxicity of these compounds and their ability to affect low-pH-induced cell-cell fusion (see also reference 9). Even cells which were dead according to the ethidium homodimer exclusion test were able to fuse upon exposure to the low-pH medium (see below). All data presented in the paper were obtained at nontoxic concentrations of tested compounds if not stated otherwise.

Exogenous lipids. If not stated otherwise, 5 min prior to exposure to the low-pH medium, the cell medium was replaced by 0.5 ml of Grace's medium freshly supplemented with exogenous lipids. Low-pH medium (used to trigger fusion) and normal-pH medium (pH 6.4) (used to terminate the low-pH treatment) were supplemented with the same concentration of the same lipid. Added lipids did not change the pH of the medium.

Trypsin and NEM. Treatment of cells with trypsin (5 mg/ml; 20 min; freshly replated cells) or NEM (1 mM; 10 min; plated cells) in TNM-FH medium (pH 6.4) at 27°C was followed by three 2-ml washes of cells with fresh TNM-FH medium.

PLA2. R-PE-labeled and unlabeled cells were separately treated with PLA2 (1.2 mg/ml) from bovine pancreas (~20% protein, 25 to 75 U/mg of protein) for 10 min in 0.5 ml of Grace's medium at 37°C. After treatment, the cells were washed with 2 ml of TNM-FH medium three times, resuspended, and coplated onto the cover glass. Thirty minutes after replating, when the cells had attached and had established contacts, the medium was replaced with the pH 5.1 medium.

Antibodies and immunofluorescence microscopy. The tissue culture supernatants of monoclonal antibodies to baculovirus gp64, AcV1 and AcV5 (26), were generously provided by G. Blissard (Cornell University, Ithaca, N.Y.). Judging by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the concentrations of immunoglobulin G in AcV1 and AcV5 supernatants were equivalent. To study the effects of anti-gp64 antibodies on cell-cell fusion, cells were incubated in antibody-containing medium for 20 min. All solutions used for further treatments of cells contained antibodies at the same concentration.

For immunofluorescence microscopy, cells were fixed for 10 min at room temperature with 4% paraformaldehyde in Ca- and Mg-free phosphate-buffered saline (PBS), washed four times with PBS, and incubated for 5 min with 0.2% bovine fetal serum in PBS. Then the cells were incubated with the appropriate primary antibodies with 0.2% bovine fetal serum in PBS; this was followed by three washes and a 5-min incubation with 0.2% bovine fetal serum in PBS. Finally, rhodamine-conjugated goat anti-mouse immunoglobulin G (Pierce) was applied; this was followed by two washes with 0.2% bovine fetal serum in PBS and two washes with plain PBS.

Electron microscopy. Cells were washed with PBS before fixing them in the mixture of 3% glutaraldehyde–1% paraformaldehyde in 0.131 M sodium phosphate buffer for 30 min at room temperature and overnight at 4°C. They were postfixed in 1% OsO_4 for 1 h and stained with saturated uranyl acetate in 25% methanol for 15 min. After rinsing of the samples, followed by alcohol-acetone dehydration and epon-araldite infiltration steps, the blocks were cured in a 60°C oven and thin sectioned with LKB Ultrotome V. After staining with uranyl acetate and lead-citrate, the sections were analyzed with a Philips CM-10 transmission electron microscope.

TABLE 1. Action of antibodies, trypsin, and NEM on an intermediate of membrane fusion induced by low pH and arrested by LPC

Inhibitor	% of infected cells in syncytia under the following conditions[a]:			
	+Inhibitor, pH pulse[b]	+LPC, pH pulse[c]	+LPC, pH pulse, −LPC[d]	+LPC, pH pulse, +inhibitor, −LPC[e]
AcV1 antibodies	26 ± 7	4 ± 1	86 ± 7	86 ± 14
Trypsin	22 ± 4	0 ± 0	80 ± 7	26 ± 17
NEM	2 ± 1	11 ± 3	90 ± 18	36 ± 6

[a] The percentage of baculovirus-infected Sf9 cells found in syncytia was determined 2 h after treatment with a low-pH medium (pH 5.1, for 1 min). The fusion extents normalized to those observed in control experiments in which neither LPC nor other inhibiting treatments were applied (31.0% ± 3.1%). All values are means and SE (n = 4). +, substance was added; −, substance was washed out.

[b] Cells were treated with the antibodies to gp64 or with trypsin or NEM, and a low-pH pulse was applied, as described in Materials and Methods. No LPC was used.

[c] Lauroyl LPC (100 μM) was added to cells 5 min prior to application of a low-pH pulse and was not removed until counting of cells in syncytia was performed. No other inhibitors were used. Pretreatment of cells with LPC for 2 h did not affect the ability of these cells to fuse when a low-pH pulse was applied after removing the LPC (data not shown).

[d] LPC was added to cells 5 min prior to application of a low-pH pulse and washed out 20 min after the end of the low-pH pulse. No other inhibiting treatments were used.

[e] LPC was added to cells 5 min prior to application of a low-pH pulse. Just after the pulse, cells were treated for 20 min with antibodies to gp64 or with trypsin or NEM, and then, 20 min after the end of the low-pH pulse, LPC was washed out.

Binding and incorporation of exogenously added lipids. After a 10-min incubation of cells (10^5 cells per 0.5 ml of Grace's medium) with a mixture of radioactively labeled and unlabeled LPC, LPC binding was determined by pelleting cells at 1,000 × g for 5 min and measuring the distribution of ^{14}C- or ^{3}H-labeled LPCs between cell pellet and supernatant.

The incorporation of exogenous rhodamine-labeled lipids into cell membranes was detected by rhodamine fluorescence dequenching (25). The fluorescence of 180 μl of medium with exogenous lipids was measured before and after the addition of cells (10^5 cells per 20 μl of the medium) with Fluoroscan II (LabSystems, Helsinki, Finland) at $\lambda_{excitation}$ = 554 nm and $\lambda_{emission}$ = 590 nm. The incorporation of initially self-quenched fluorescent lipids into cell membranes caused some relief of self-quenching and an increase in fluorescence. A larger fluorescence increase corresponds to more incorporation. The increase in fluorescence was normalized by maximum dequenching, determined after solubilizing membranes with 0.5% Triton X-100 (25).

To roughly estimate the ratio of bound or incorporated exogenous lipids to plasma membrane lipids, we used the total number of lipids in cell plasma membranes, N_0, which we estimated by using the number of Sf9 cells in the well and their average size. For instance, for 10^5 cells having a mean diameter of ~30 μm, assuming an area of 6×10^{-15} cm^2 for an average lipid molecule, we had a total of 9.4×10^{14} lipid molecules in plasma membranes. This is an apparent underestimate of N_0, since we are not taking into account the possible roughness of cell surfaces and the inevitable presence of some fraction of dead cells, where not only plasma membrane but also a much greater lipid surface within each dead cell was exposed to exogenous lipids.

RESULTS

Baculovirus-mediated syncytium formation is driven by specific proteins. As was reported earlier (4, 34) Sf9 cells infected by baculovirus fuse with each other when exposed to a low-pH medium. Fusion, assayed as syncytium formation, was inhibited by treating cells with neutralizing AcV1 antibodies to the baculovirus envelope protein gp64, prior to application of the low-pH medium (Table 1). No inhibition was observed when cells were incubated with AcV5 antibodies, which recognize only the denatured form of gp64. Immunofluorescence experiments confirmed the specific binding of AcV1 antibodies to the membranes of infected cells (Fig. 1). We found no binding of antibodies to noninfected cells (not shown) or to infected cells treated for 5 min with the pH 5.1 medium (Fig. 1D) or

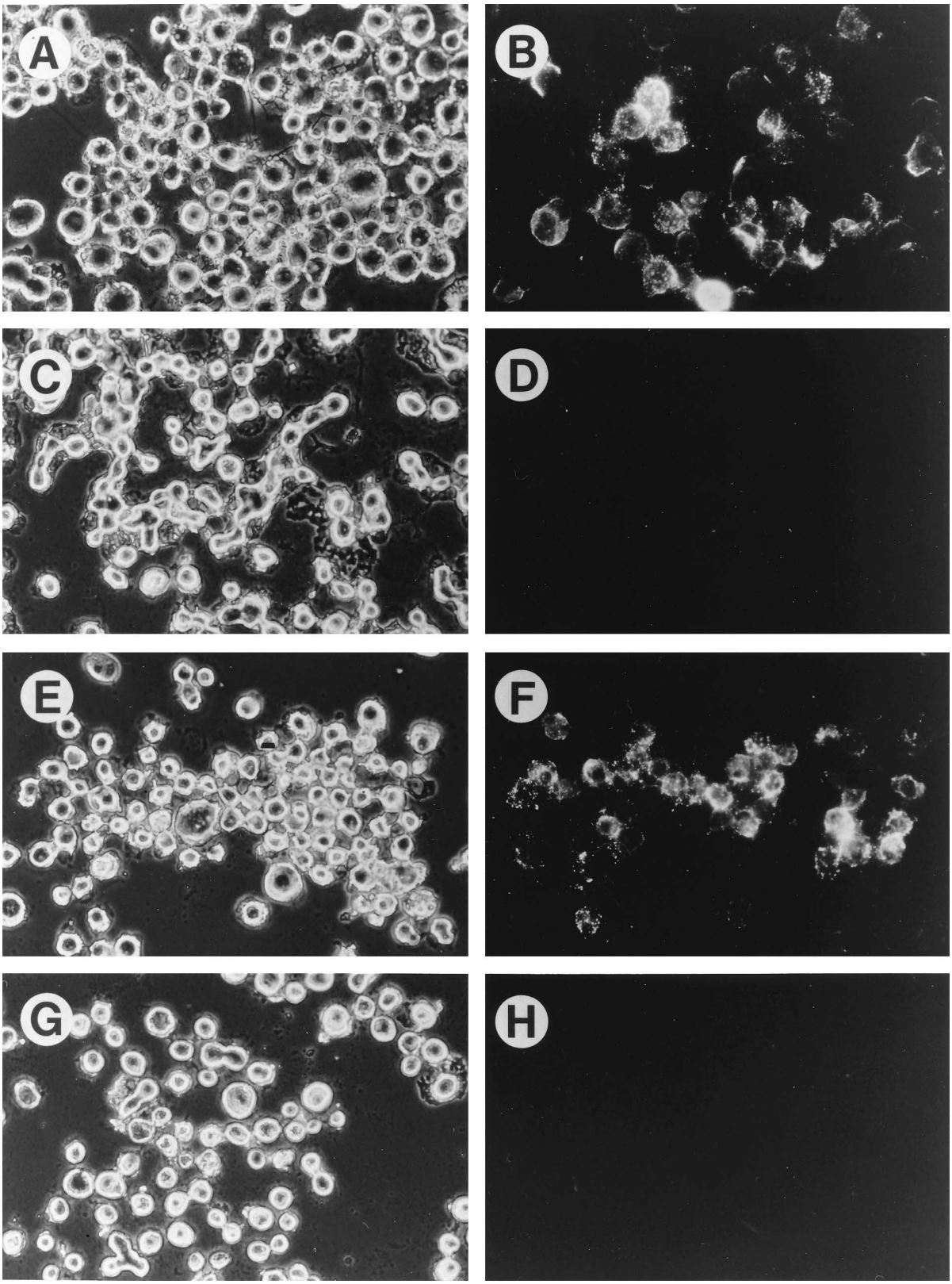

FIG. 1. Low-pH-dependent change of the gp64 conformation detected by immunofluorescence analysis. (A and B) Infected cells stained with AcV1 monoclonal antibodies against gp64. Same field of view photographed by phase-contrast (A) and fluorescence microscopy (B). (C and D) After a 5-min incubation in pH 5.1 medium, infected cells were stained with AcV1 antibodies. Phase-contrast (C) and fluorescence microscopy (D). (E and F) After a 5-min incubation in pH 6.4 medium containing 95 μM oleoyl LPC, infected cells were stained with AcV1 antibodies. Phase-contrast (E) and fluorescence microscopy (F). (G and H) After a 5-min incubation in pH 5.1 medium containing 95 μM oleoyl LPC, infected cells were incubated for 20 min in the pH 6.4 medium containing the same concentration of LPC and then stained with AcV1 antibodies. Phase-contrast (G) and fluorescence microscopy (H).

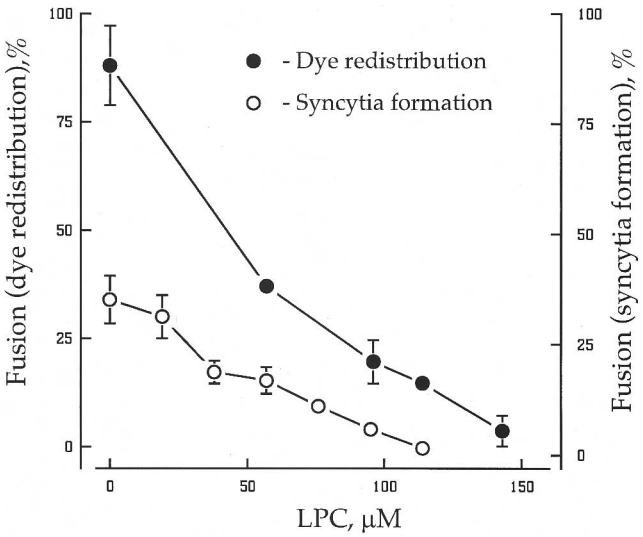

FIG. 2. Inhibition of cell-cell fusion by LPC. Fusion of baculovirus-infected cells in the presence of different concentrations of oleoyl LPC was quantified by a membrane dye redistribution assay and by counting nuclei in syncytia. Fusion was triggered by a 1-min application of pH 5.1 medium. Shown are means ± standard errors (SE), $n = 3$.

when infected cells were incubated with AcV5 antibodies (not shown).

Treatment of membranes to modify proteins also inhibited cell-cell fusion. Treatment of cells with trypsin, a proteolytic enzyme, or with NEM, modifying free sulfhydryl groups of membrane proteins, dramatically suppressed syncytium formation (Table 1). Membrane fusion inhibition by AcV1 antibodies, trypsin, and NEM was also confirmed by a fluorescent membrane dye redistribution assay (data not shown).

Interestingly, cells with permeabilized membranes were still fusion competent. Cells were treated with staphylococcus α toxin, and ethidium homodimer was added to label nuclei of cells with permeabilized membranes. Using light and fluorescent microscopy, we then observed fusion of these permeabilized cells with each other after application of the low-pH medium (not shown).

Addition of exogenous lipids affects cell-cell fusion. Addition of LPC dramatically inhibited low-pH-induced cell-cell fusion, as determined by membrane dye redistribution between labeled and unlabeled cells (Fig. 2), syncytium formation (Fig. 2), and electron microscopy (data not shown). Although addition of 95 μM oleoyl LPC caused an approximately fourfold inhibition of syncytium formation, no obvious changes in cell morphology or in the morphology of cell contacts were observed by thin-section electron microscopy (Fig. 3).

The dose-response curves of lysolipid inhibition for three different lipids with the same phosphatidylcholine polar head but different hydrocarbon chains, varying in the number of CH_2 groups, i.e., myristoyl, palmitoyl, and stearoyl LPCs, show that the shorter the hydrocarbon chain, the higher the inhibitory activity of the lipid (Fig. 4). Since with stearoyl LPC the extent of inhibition did not depend on the time of cell incubation within a 5- to 15-min range (data not shown), this difference was not related to the slower incorporation of lipids with longer hydrocarbon chains into cell membranes (18, 47).

Not only naturally occurring lysolipids inhibited cell-cell fusion; synthetic detergent Tween 80 did also (Fig. 5). The inhi-

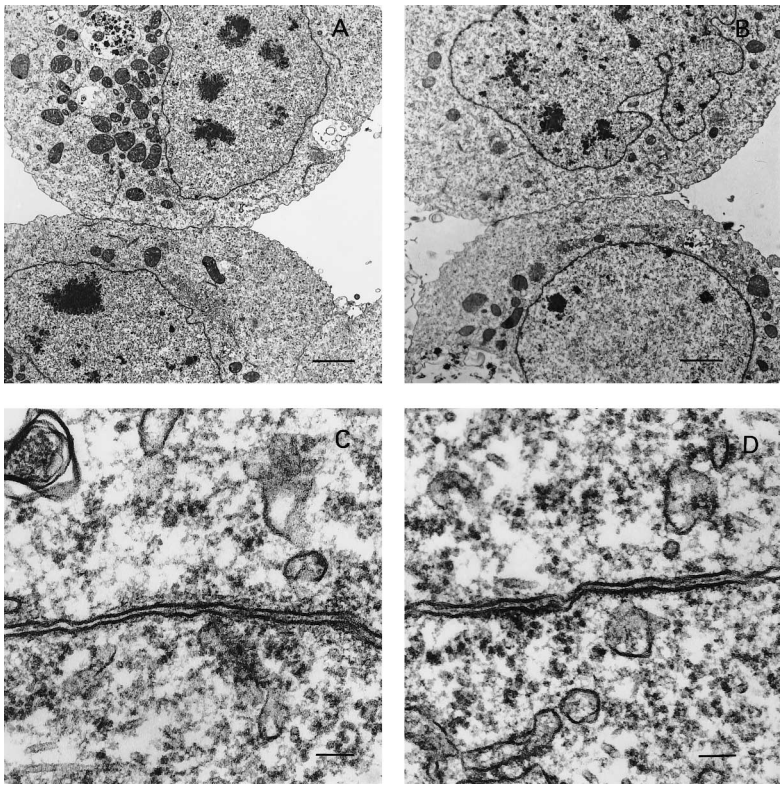

FIG. 3. Morphology of the cell-cell contact. Electron micrographs of the cell-cell contact at pH 6.4 with (B and D, different magnification, same contact area) and without (A and C, different blowup, same contact area) 95 μM oleoyl LPC. Scale bars in panels A and B and in panels C and D, 1 and 0.1 μm, respectively.

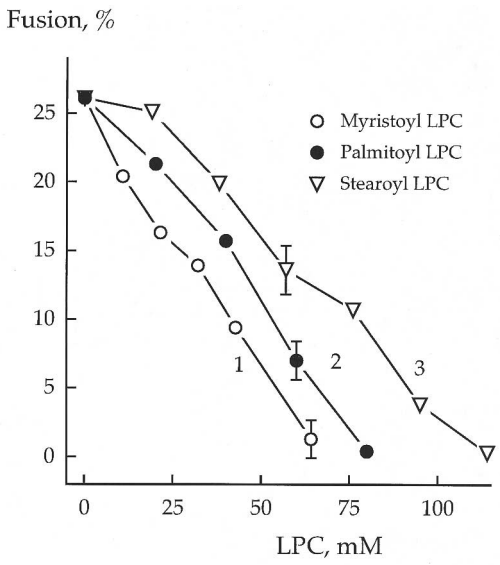

FIG. 4. Inhibiting activity of the LPCs with different hydrocarbon chains. Cell-cell fusion was triggered by a 1-min application of pH 5.1 medium in the presence of different concentrations of myristoyl LPC, palmitoyl LPC, and stearoyl LPC. The final extent of fusion was quantified by counting cells in syncytia. Each point is a mean (n = 3), and the representative SE are shown for one point on each curve.

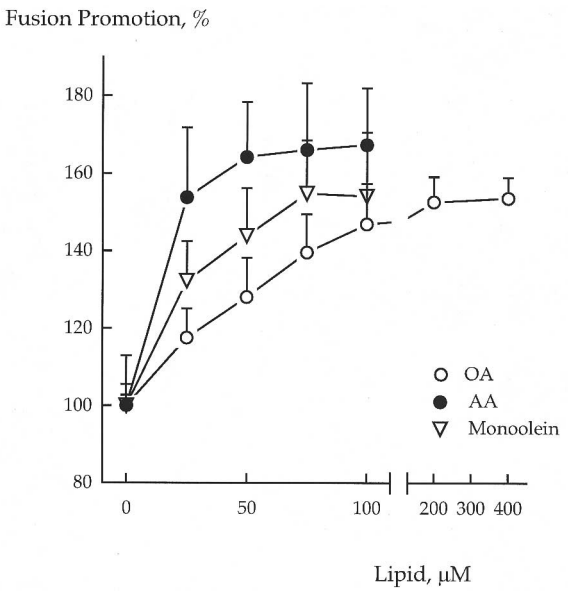

FIG. 6. Promotion of the cell-cell fusion by OA and AA and by monoolein. To trigger fusion, cells were exposed to pH 5.35 medium for 1 min in the presence of different concentrations of OA, AA, and monoolein. The suboptimal pH was chosen to accentuate fusion promotion by these lipids (see the text). The extent of fusion, quantified by counting cells in syncytia, was normalized to the maximum extent of fusion observed in the experiment presented in the particular curve with no lipids added (38.2, 38.3, and 14.6% for the OA, AA, and monoolein experiments, respectively). Shown are means ± SE, n = 3.

bition of baculovirus-mediated fusion by LPC and Tween 80 had an additive character: lower concentrations of Tween 80 were required to reach the same extent of fusion inhibition when added together with LPC.

Not all exogenously added lipids inhibited cell-cell fusion. Some had no visible effects (see below and Fig. 7B) (9). AA and OA actually promoted syncytium formation (Fig. 6). The surface charge of these fatty acids was not required for fusion promotion, since nonionic monoolein also promoted fusion. No fusion was observed in control experiments when noninfected cells were treated with 200 μM arachidonic acid and then incubated in a low-pH medium (data not shown).

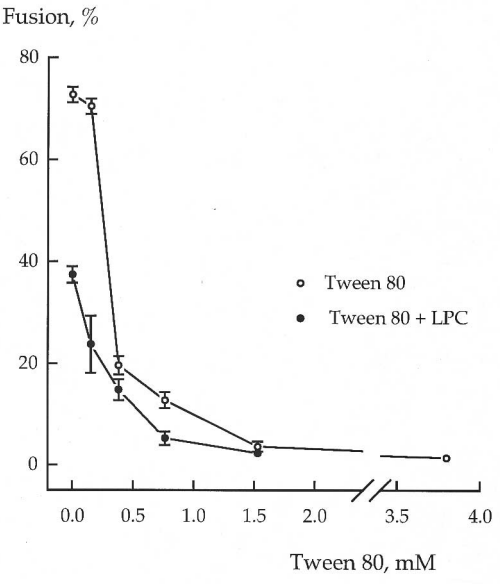

FIG. 5. Synthetic amphiphile Tween 80 and LPC inhibit fusion additively. Cell-cell fusion was triggered by a 2-min application of pH 4.9 medium in the presence of different concentrations of Tween 80 with (●) and without (○) 75 μM oleoyl LPC. The final extent of fusion was quantified by counting cells in syncytia. Each point is a mean (n = 3), and the representative SE are shown for one point on each curve.

Fusion promotion was observed only for moderately low pH values, under which conditions fusion was submaximal. In general, baculovirus-mediated fusion reached a maximum at pH 5.1 and started at pH 5.5 (4, 34). The basal fraction of multinucleated cells (≤2%) found for both infected cells at pH ≥5.6 and for noninfected cells for any pH value in the range from 5.1 to 6.6 was apparently not related to cell-cell fusion. The effects of AA, OA, and monoolein were statistically reliable in the pH range from 5.2 to 5.4, with the maximum, almost twofold, promotion observed at pH 5.3 and 5.35. However, these lipids caused no significant fusion promotion when the pH applied was low enough to achieve the maximum extent of fusion observed for these experimental conditions (multiplicity of infection and hours after infection). The maximum extents of fusion observed at pH < 5.2 (~30 and ~80% for syncytium formation and the membrane dye redistribution assay, respectively) may correspond to the percentage of cell-cell contacts which have fusion proteins (or some functional complexes of fusion proteins) present.

Inhibition and promotion of fusion by exogenous amphiphiles were completely reversible. Washing of cells treated with LPC, Tween 80, and OA with fresh medium containing 50 mg of BSA per ml to extract those amphiphiles prior to the low-pH pulse application completely restored the original fusion competence of cells (data not shown) (see also references 9 and 55).

Lipid incorporation into membrane and inhibition of fusion. Using radioactively labeled lysolipids, we found no correlation between the inhibition of fusion by different lipids and their binding to cell membranes. The LPC bound-to-LPC

added ratios were nearly constant in the 15 to 80 μM range of LPC concentration at 0.66 ± 0.18, 0.7 ± 0.15, and 0.62 ± 0.14 (n = 3) for myristoyl, palmitoyl, and stearoyl LPCs, respectively. So these three lysolipids, although inhibiting fusion to different extents (Fig. 4), had quite similar binding characteristics. Bound palmitoyl LPC was estimated to constitute ~6.5% of the total plasma membrane lipids under 50% fusion inhibition conditions.

Because binding does not necessarily equal incorporation of exogenous lipid into membranes but rather gives an upper limit, an additional approach was applied to test the relationship between incorporation of and inhibition by some amphiphilic compounds. We added a concentrated suspension of Sf9 cells to the dispersions of different rhodamine-labeled lipids (R18, R-PE, and R-LPE) and measured the increase in fluorescence caused by dilution of each membrane dye upon incorporation into the cell membranes from micelles (Fig. 7A). The range of concentrations of the fluorescent lipids was limited to avoid contributions of absorbance, leading to a nonlinear dependence of fluorescence on dye concentration, seen even after the addition of Triton X-100. In parallel experiments, we studied the effects of the same lipids on baculovirus-mediated fusion (Fig. 7B). We found no clear correlation between the ability of these compounds to incorporate into membranes and their ability to inhibit membrane fusion. R-PE barely incorporates into membranes and has no inhibiting activity. However, incorporation alone is not sufficient for inhibition. Both R18 and R-LPE readily incorporated into membranes, but only the latter inhibited fusion. We estimated the incorporated R-LPE to constitute ~5.5% of the total plasma membrane lipids under conditions of 50% fusion inhibition.

PLA2 treatment of cell membranes inhibited syncytium formation. Not only exogenously added lysolipids but also those produced in situ by PLA2 treatment of cell membranes inhibited baculovirus-mediated fusion (Fig. 8). When treated cells were incubated with BSA to extract hydrolysis products, partial restoration of fusion competence was observed. No inhibition was found when cells were incubated with PLA2 in the absence of Ca^{2+} ions, required for PLA2 enzymatic activity (54).

Exogenous lipids affect fusion even when added after a low-pH pulse. The final extent of fusion depended on the duration of the low-pH pulse (Fig. 9A, curve 1). For short pH pulses (less than 60 s), the longer the pulse, the higher the fusion extent. In another series of experiments (Fig. 9A, curve 2), the normal-pH medium (pH 6.4), used to replace the pH 5.1 medium at the end of the low-pH pulse, was supplemented with LPC and less fusion was seen. Finally, we gave a constant 20-s low-pH pulse, returned cells to a normal pH, and later added LPC. We varied the interval between the end of the low-pH pulse and LPC addition and found that there is a limited time interval after treatment with a low-pH medium during which the addition of lysolipid inhibited fusion (Fig. 9B). Similarly, the addition of arachidonic and oleic acids still promotes fusion if added immediately after a low-pH pulse (not shown). Thus, alterations of membrane lipid composition affected pH-independent processes which follow the triggering of fusion.

Application of other inhibitors of fusion at the stage arrested by lysolipids. AcV1 antibodies to gp64, trypsin, and NEM, applied prior to the pH pulse, inhibited baculovirus-mediated fusion (see above). We have also studied the effects of these inhibitors at the stage of fusion reversibly arrested by LPC. In these experiments, a low-pH pulse was applied in the presence of lysolipid and then, still in the presence of LPC but already at a normal pH, cell membranes were treated with the agents listed above. Then LPC was washed out, and the extent of fusion was assayed 2 h later (Table 1). We found that trypsin and NEM still inhibited fusion if applied after a low-pH pulse (Table 1), acting at a fusion stage after triggering but prior to the actual membrane merger.

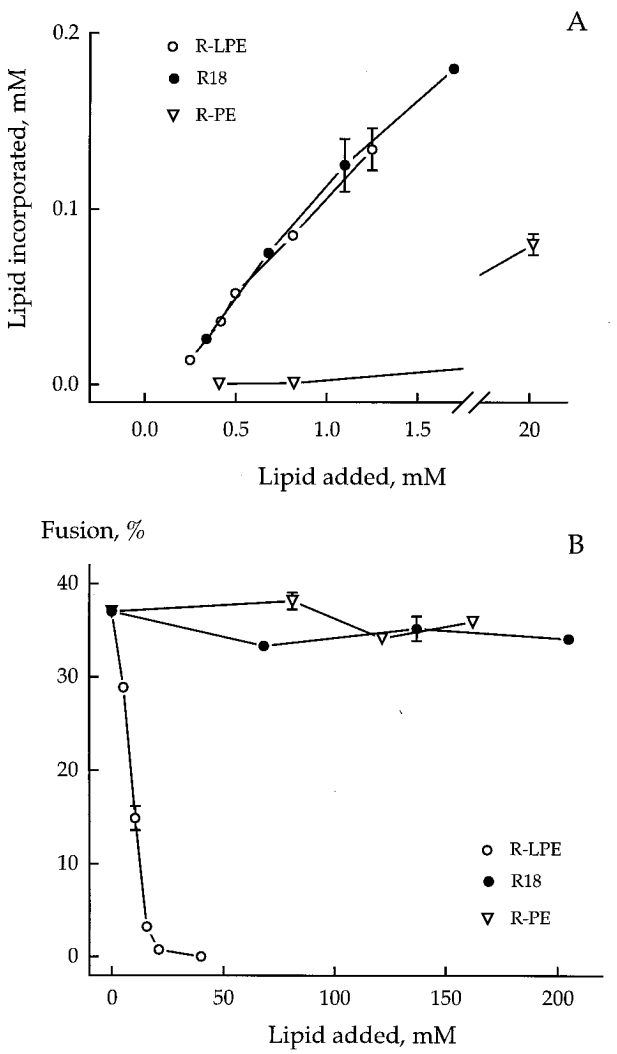

FIG. 7. Incorporation of rhodamine-labeled lipids into cell membranes and the effects of these lipids on membrane fusion. (A) Dequenching of the fluorescent lipid derivatives R-LPE, R18, and R-PE as a measure of their incorporation into cell membranes. Only relatively low concentrations of fluorescent lipids were used in these experiments, since at higher lipid concentrations a decrease of the fluorescence was observed, because of inner filtering effects. (B) Final fusion extent as a function of the R-LPE, R18, and R-PE concentrations was found by counting cells in syncytia. Fusion was triggered by a 2-min pulse of pH 5.1. Each point in panels A and B is a mean (n = 3), and the representative SE are shown for one point on each curve.

According to immunofluorescence experiments, AcV1 antibodies do not bind to membranes of infected cells treated with a pulse of low pH (Fig. 1C and D), suggesting that these antibodies recognize gp64 in its neutral pH conformation but do not bind to the low-pH conformation of this protein. We found that LPC affects fusion at a stage following a pH-dependent change of gp64 conformation, since AcV1 antibodies do not bind to cell membranes (Fig. 1G and H) and do not inhibit fusion (Table 1) if added at the stage reversibly arrested by LPC. In control experiments, we verified that the presence of LPC did not interfere with antibody binding to infected cells

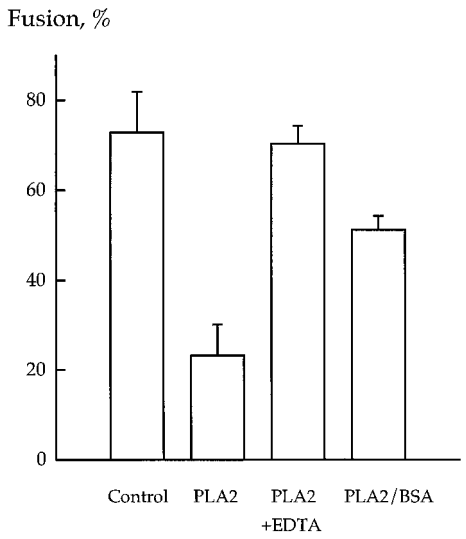

FIG. 8. Fusion inhibition by PLA2 treatment of cell membranes. Syncytium formation was triggered by a 2-min pulse of pH 5.2 applied to cells not treated with PLA2 (control), cells treated with PLA2, cells incubated with PLA2 in the presence of EDTA, and cells first treated by PLA2 and then incubated with fatty acid-free BSA. The final fusion extent was calculated as the ratio of cells in syncytia to the total number of cells. Shown are means ± SE, $n = 3$.

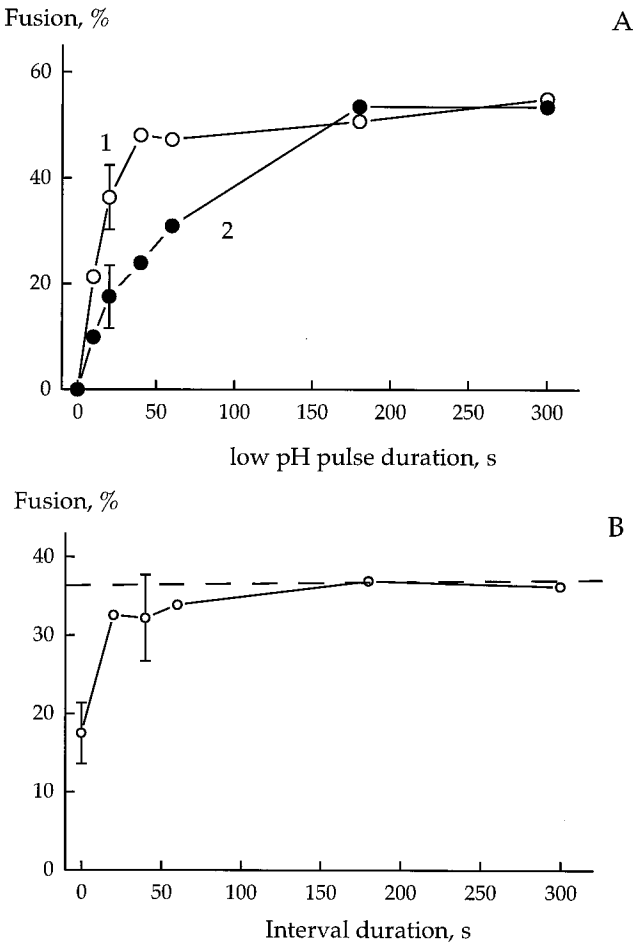

FIG. 9. Inhibition of syncytium formation by LPC applied after a low-pH pulse. (A) A pH 5.1 pulse of various durations was applied to the infected cells. At the end of the pulse, the low-pH medium was replaced by a pH 6.4 medium without (curve 1) or with (curve 2) 90 μM oleoyl LPC. (B) A 20-s pulse of pH 5.1 applied to cells to trigger fusion and the addition of 90 μM oleoyl LPC were separated by a time interval of various durations when cells were incubated in normal medium (Grace's medium, pH 6.4, with no LPC). The extent of fusion with no LPC added is indicated by the dashed line (see the corresponding point in panel A, curve 1).

which had not been treated with a low-pH medium (Fig. 1E and F).

LPC slows baculovirus infection. Membrane fusion is thought to play an important role in the entry of the budded form of baculovirus into host cells (56, 57). We tested whether alteration of membrane lipid composition before viral entry would affect the infection process. Addition of exogenous LPC to cells 10 min before the inoculation of viral particles significantly slowed the development of infection, as quantified 48 h later by counting cells with polyhedral inclusions characteristic for the later stages of baculovirus infection (Fig. 10A and B). The inhibition is not related to cytotoxic and virucidal effects of the lysolipids. Treatment of cells with the same concentrations of lysolipid after inoculation and washing out the unbound virus causes no suppression of the infection process. No inhibition was observed when viral particles (but not cells) were treated with LPC for 10 min before the addition of viral particles to the cell suspension (Fig. 10A).

DISCUSSION

We have studied here the syncytium formation mediated by baculovirus envelope glycoprotein gp64. Although driven by protein, this fusion reaction was found to be significantly modulated by some specific changes in the lipid composition of the cell membrane. Lysolipids inhibited low-pH-triggered cell-cell fusion mediated by the baculovirus fusion protein (9, 55). In contrast, *cis*-unsaturated fatty acids (namely, AA and OA) and monoolein brought about significant promotion of cell-cell fusion. The data presented allow us to identify the trigger-independent but lipid-sensitive stage of viral fusion and to draw some conclusions about the structure of lipid-involving intermediates of this reaction.

Lipid effects on viral fusion are reversible. Lysolipids were found to slow baculovirus infection when present during inoculation of viral particles. No slowing of infection was observed when only virus but not cells was treated with LPC (Fig. 10A). Thus, the impeding of infection cannot be explained by any virucidal effects as proposed in reference 43, and we surmise it is related to the lysolipid inhibition of fusion which is required for viral entry to host cells. However, we cannot rule out the possibility that lysolipids slow infection by acting not just on viral fusion but also on later stages of infection.

Removing lysolipids by washing them out before the low-pH pulse application or after it (Table 1) (9, 55) completely restored fusion competence, including the original pH dependence of fusion. Fusion promotion by *cis*-unsaturated fatty acids was also completely reversible. Thus, both inhibition and promotion of fusion by specific changes in membrane lipid composition cannot be mediated by any irreversible effects such as cell lysis, irreversible inactivation, or solubilization of membrane components.

Many of the lipids tested here affected cell-cell fusion. AA and OA, along with monoolein, promoted syncytium formation at a suboptimal fusion pH (Fig. 6). The synthetic surfactant Tween 80, having a polar group structure dissimilar to that of lipids, inhibited fusion in an additive way with LPC, suggesting a common mechanism of action (Fig. 5). The absence of a

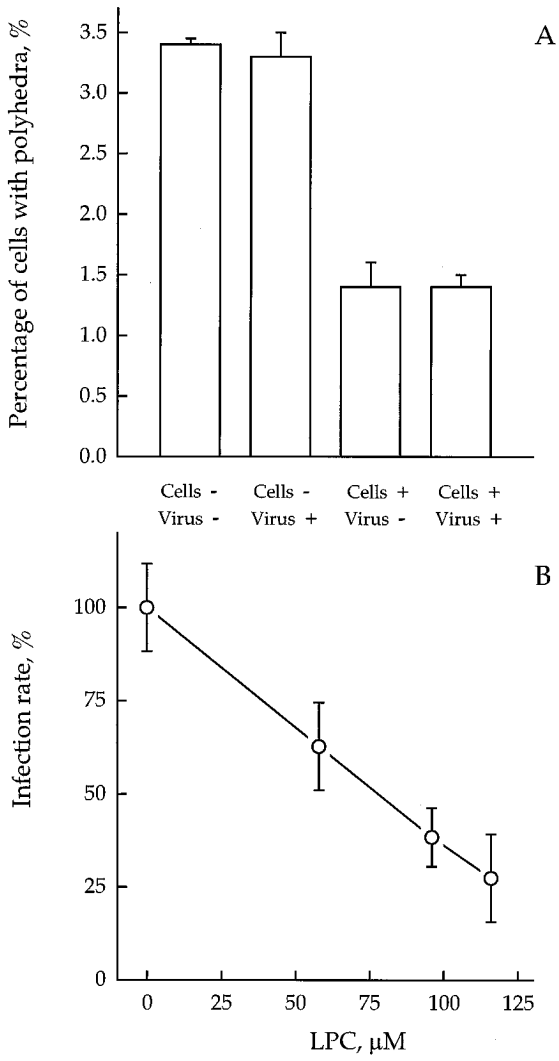

FIG. 10. LPC inhibition of the development of the baculovirus infection. The percent cells with polyhedral inclusions, which are characteristic for the late infection stages, was used as a measure of the infection rate. (A) Percent cells with polyhedral inclusions was determined 48 h after infection of cells with baculovirus for experiments in which neither cells nor virus was treated with LPC (cells−, virus−); virus was treated with 90 μM LPC, but cells were not (cells−, virus+); cells were treated with 90 μM LPC, but virus was not (cells+, virus−); and, finally, both cells and virus were treated with LPC (cells+, virus+). Shown are means ± SE (n = 3). (B) Both cells and virus were treated with LPC at various concentrations. The percent cells with polyhedral inclusions determined 48 h later was normalized to that observed with no LPC. Shown are means ± SE (n = 3).

specific chemical group directly correlating with fusion inhibition (9) or promotion, the relatively short exposure time (seconds to minutes) to lipids needed for maximal effect, and the reversibility of these effects suggest a direct action of these lipids on cell-cell fusion, rather than indirect effects (e.g., mediated by intermediate biochemical reactions).

Asymmetrical incorporation of lipids and fusion rates. In most of the experiments presented, we altered the lipid composition of cell membranes by adding exogenous lipids. Rough estimates based on the data on the binding of radioactively labeled lysolipids and on the incorporation of R-LPE suggest an 11 to 13% increase in the number of lipids in the outer leaflet of cell membranes under 50% fusion inhibition conditions. Taking into account the relatively low rates of transmembrane redistribution of lysolipids (19, 20, 45), asymmetric lipid incorporation into outer but not inner membrane monolayers may ripple contacting leaflets of adjacent membranes and disturb the cell-cell contacts required for fusion. However, our data show that lipid modulation of fusion cannot be explained by mere binding or incorporation of exogenous lipids into cell membranes. First, no visible changes in cell morphology or in the morphology of cell contacts were found by thin-section electron microscopy of cells treated with fusion-inhibiting concentrations of LPC (Fig. 3). Second, three derivatives of LPC, slightly varied in their capacities for binding to cell membranes (19, 59), displayed different potencies as fusion inhibitors (Fig. 4). A 22% decrease in lipid hydrocarbon chain length from 18 to 14 CH_2 groups resulted in an almost twofold decrease of the LPC concentration required for 50% inhibition. Third, OA and AA caused promotion rather than inhibition of membrane fusion (Fig. 6), in spite of the well-documented ability of exogenous fatty acids to incorporate into cell membranes and to cause an erythrocyte shape transformation similar to that induced by lysolipids (52). Fourth, we found that R18 incorporated into cell membranes to the same extent as R-LPE, but only the latter inhibited membrane fusion (Fig. 7). Fifth and finally, not only exogenously added lysolipids but also ones generated in situ by PLA2 were able to inhibit membrane fusion (Fig. 8). Partial reversibility of this inhibition by BSA extraction of the phospholipase hydrolysis products suggests the direct involvement of these products in the inhibition. PLA2 cleaves membrane phospholipids to lysoforms and free fatty acids (54). One could suggest that the release of free fatty acids leaves the membrane enriched by lysolipids (24). Inhibition of biological fusion by PLA2 treatment of cell membranes was reported earlier for myoblasts (44) and vesicular stomatitis virus-mediated fusion (10). Thus, the dissimilarities in the effects of the different lipids on baculovirus-mediated fusion cannot be accounted for by the differences in the lipids' abilities to incorporate into cell membranes.

Actual merger of membranes is lipid sensitive and protein dependent. Viral fusion is known to be a multistep reaction (5, 29, 39, 50, 51). It involves (i) triggering of a conformational change of a fusion-mediating protein by lowering the pH or by receptor binding; (ii) formation of a fusion intermediate(s); and (iii) the fusion event itself, i.e., the actual merger of membranes. We have shown recently that lysolipids reversibly arrested both low-pH-triggered fusion of cells infected by baculovirus and Ca^{2+}-triggered cortical granule exocytosis, at a stage between triggering and the membrane merger (55). The data presented here further substantiate the hypothesis that the alteration of the membrane lipid composition affects fusion at a stage subsequent to the conformational change in fusion-mediating proteins.

Our findings confirm earlier indications that baculovirus-mediated, low-pH-triggered cell-cell fusion is mediated by viral envelope glycoprotein gp64 (4, 34). The AcV1 specific antibodies to gp64 known to suppress viral infection, but not AcV5 antibodies to the denatured form of the same protein, were found in the present study to inhibit fusion. Importantly, AcV1 antibodies were found here to specifically bind only to the original, pH 6.4 form of gp64 and, thus, to inhibit syncytium formation only when added prior to the low-pH pulse application (Table 1). Neither specific binding of AcV1 antibodies nor inhibition of fusion was observed when antibodies were added to cells committed to fuse but arrested in the activated state by LPC (Table 1), indicating that at this stage fusion proteins had already undergone a low-pH-dependent conformational change or were sequestered from the aqueous medium.

The final extent of fusion observed when LPC was added immediately after application of a short low-pH pulse was lower than that in control experiments (no LPC added) or when the addition of LPC was preceded by a short (<20 s) incubation of low-pH-treated cells at a normal pH (Fig. 9). These results suggest that for fusion to occur, the low-pH-triggered conformational change in the fusion protein and, possibly, some subsequent processes also dependent on low pH have to be followed by some further maturation stage(s) that does not require a low pH (5, 40, 55). Lysolipids affect this latter stage and, thus, inhibit fusion only if applied before the end of this trigger-independent stage. Similarly, OA and AA promoted fusion when added immediately after a low-pH pulse.

Membrane proteins are still required to support lipid-sensitive and trigger-independent membrane merger reactions. Proteolytic treatment of cell membranes and NEM modification of the free sulfhydryl groups of membrane proteins at the stage arrested by LPC, like the same treatments performed prior to the low-pH pulse application, resulted in significant inhibition of syncytium formation upon the removal of lysolipid (Table 1). Interestingly, trypsin treatment of baculovirus particles caused no reduction of viral infectivity (data not shown). This result agreed with earlier observations that treatment of baculovirus with trypsin cleaved the gp64 into smaller fragments without reducing viral infectivity (58). The apparent contradiction between these results and the trypsin inhibition of pH-triggered fusion of baculovirus-infected cells may be explained by a lower surface density of gp64 in infected cells' membranes in comparison with that in the viral envelope.

Lipid intermediates of baculovirus-mediated membrane fusion. Although for at least some enveloped viruses (e.g., influenza) no single specific lipid is needed to enable the fusion machinery (1, 41, 49), lipids modulate the fusion reaction. These effects may be mediated by lipid interaction with fusion proteins. In particular, lysolipids may hydrophobically interact with the same fusogenic peptides of viral envelope glycoproteins which would otherwise bind lipids of the target membrane and mediate fusion. In fact, activation of the influenza hemagglutinin manifests itself by a significant increase in the binding of amphiphilic compounds (14, 48). Alternatively, exogenous lipids may hinder the mobility of proteins in the cell membrane (21, 30) and, thus, disturb the lateral association of these proteins into some multimolecular complex presumably required to drive the membrane merger (2, 23). Finally, the tested compounds might alter the mode of insertion of the fusogenic peptide into the target membrane (36). We cannot exclude the possible role of protein-lipid interactions in the effects described. However, lysolipids added between fusing membranes are known to inhibit, and *cis*-unsaturated fatty acids to promote, not only protein-mediated fusion (this paper and references 9, 11, 37, and 42) but also fusion of model lipid bilayers (7, 8, 16, 38, 62). This suggests that the lipid effects under consideration are the results of changes in the properties of lipid bilayers of biological membranes.

The lipid effects on baculovirus-mediated cell-cell fusion specifically correlate with the effects of these lipids on the intrinsic (spontaneous) curvature of the lipid monolayer, often referred to as the effective shape of the component lipids (8, 12, 28, 31, 46, 53). This effective shape of lipids reflects their average geometry in a monolayer and can be determined by the analysis of the structures which these lipids form in aqueous dispersion. The inhibitors of membrane fusion, lysolipids and Tween 80, have an inverted cone shape and favor a micelle-like, positive curvature (9, 15, 33). Moreover, at the same phosphatidylcholine polar head, the fusion-inhibiting activity of different lysolipids was higher for the shorter hydrocarbon tails and, therefore, for the compounds having more profound inverted cone shapes (Fig. 4). In contrast, AA, OA, and monoolein, which facilitated cell-cell fusion (Fig. 6), are known to promote an inverted H_{II} phase formation and, thus, are cone shaped (16, 17, 27). Incorporation of noncylindrical lipids into the lipid bilayer of cell membranes modulates the propensity of lipid leaflets of membranes to bend in specific directions. Thus, our results suggest that the membrane merger stage of viral fusion involves the formation of bent intermediates; the energy of these intermediates depends upon the membrane lipid composition.

Fusion of purely lipid bilayers is thought to involve the formation of a stalk-type intermediate, a local connection between contacting leaflets of two membranes (8, 32, 46). Because of the negative net curvature of this bent fusion intermediate (7, 8, 32), its formation and, ultimately, membrane fusion should be facilitated by the presence of cone-shaped lipids and inhibited by inverted cone-shaped ones. The fusion dependence on lipid composition found in this study, and similar dependences reported for purely lipid membranes, is in agreement with the stalk model.

To conclude, our results suggest that biological membrane fusion mediated by the baculovirus envelope glycoprotein includes a low-pH-triggering event, apparently independent of membrane lipid composition. Conformational change in the fusion protein manifests itself by a dramatic increase in the lipid bilayers' capability to fuse. The following stage of the actual membrane merger is still protein dependent and may be inhibited by trypsin and NEM. This stage does not require a low pH and can be reversibly modulated by changes in membrane lipid composition. As in the case of purely lipid bilayers, viral fusion was found to be promoted by cone-shaped unsaturated fatty acids and inhibited by inverted cone-shaped lysolipids. Our results suggest that the membrane merger stage of viral fusion occurs by way of the formation of stalk-type bent intermediates having a net negative curvature.

ACKNOWLEDGMENTS

Our sincere thanks are due to G. Melikian, A. Sokoloff, and S. Vogel for numerous helpful discussions. We are also very grateful to G. Blissard for making available the specific antibodies to gp64 and to Lynn Kelly for her help in preparing the manuscript.

REFERENCES

1. **Alford, D., H. Ellens, and J. Bentz.** 1994. Fusion of influenza virus with sialic acid-bearing target membranes. Biochemistry **33:**1977–1987.
2. **Aroeti, B., and Y. I. Henis.** 1988. Effects of fusion temperature on the lateral mobility of Sendai virus glycoproteins in erythrocyte membranes and on cell fusion indicate that glycoprotein mobilization is required for cell fusion. Biochemistry **27:**5654–5661.
3. **Blissard, G. W., and G. F. Rohrmann.** 1990. Baculovirus diversity and molecular biology. Annu. Rev. Entomol. **35:**127–155.
4. **Blissard, G. W., and J. R. Wenz.** 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J. Virol. **66:**6829–6835.
5. **Blumenthal, R., C. Schoch, A. Puri, and M. J. Clague.** 1991. A dissection of steps leading to viral envelope protein-mediated membrane fusion. Ann. N. Y. Acad. Sci. **635:**285–296.
6. **Carr, C. M., and P. S. Kim.** 1993. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell **73:**823–832.
7. **Chernomordik, L. V., M. M. Kozlov, G. B. Melikyan, I. G. Abidor, V. S. Markin, and Y. A. Chizmadzhev.** 1985. The shape of lipid molecules and monolayer membrane fusion. Biochim. Biophys. Acta **812:**643–655.
8. **Chernomordik, L. V., G. B. Melikyan, and Y. A. Chizmadzhev.** 1987. Biomembrane fusion: a new concept derived from model studies using two interacting planar lipid bilayers. Biochim. Biophys. Acta **906:**309–352.
9. **Chernomordik, L. V., S. S. Vogel, A. Sokoloff, H. O. Onaran, E. A. Leikina, and J. Zimmerberg.** 1993. Lysolipids reversibly inhibit Ca^{2+}-, GTP- and pH-dependent fusion of biological membranes. FEBS Lett. **318:**71–76.
10. **Conti, C., P. Mastromarino, and N. Orsi.** 1991. Role of membrane phos-

pholipids and glycolipids in cell-to-cell fusion by VSV. Comp. Immunol. Microbiol. Infect. Dis. **14:**303–313.
11. **Creutz, C. E.** 1981. Cis-unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin. J. Cell Biol. **91:**247–256.
12. **Cullis, P. R., C. P. Tilcock, and M. J. Hope.** 1991. Lipid polymorphism, p. 35–64. *In* J. Wilschut and D. Hoekstra (ed.), Membrane fusion. Marcel Dekker, The Netherlands.
13. **Doms, R. W., M.-J. Gething, J. Henneberry, J. White, and A. Helenius.** 1986. Variant influenza virus hemagglutinin that induces fusion at elevated pH. J. Virol. **57:**603–613.
14. **Doms, R. W., A. Helenius, and J. White.** 1985. Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change. J. Biol. Chem. **260:**2973–2981.
15. **Epand, R. M.** 1985. Diacylglycerols, lysolecithin, or hydrocarbons markedly alter the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines. Biochemistry **24:**7092–7095.
16. **Epand, R. M., R. F. Epand, N. Ahmed, and R. Chen.** 1991. Promotion of hexagonal phase formation and lipid mixing by fatty acids with varying degrees of unsaturation. Chem. Phys. Lipids **57:**75–80.
17. **Epand, R. M., R. F. Epand, and C. R. Lancaster.** 1988. Modulation of the bilayer to hexagonal phase transition of phosphatidylethanolamines by acylglycerols. Biochim. Biophys. Acta **945:**161–166.
18. **Fujii, T., and A. Tamura.** 1983. Dynamic behaviour of amphiphilic lipids to penetrate into membrane of intact human erythrocytes and to induce change in the cell shape. Biomed. Biochim. Acta **42:**S81–S85.
19. **Fujii, T., and A. Tamura.** 1984. Shape change of human erythrocytes induced by phosphatidylcholine and lysophosphatidylcholine species with various acyl chain lengths. Cell Biochem. Funct. **2:**171–176.
20. **Fujii, T., A. Tamura, and T. Yamane.** 1985. Trans-bilayer movement of added phosphatidylcholine and lysophosphatidylcholine species with various acyl chain lengths in plasma membrane of intact human erythrocytes. J. Biochem. **98:**1221–1227.
21. **Golan, D. E., S. T. Furlong, C. S. Brown, and J. P. Caulfield.** 1988. Monopalmitoylphosphatidylcholine incorporation into human erythrocyte ghost membranes causes protein and lipid immobilization and cholesterol depletion. Biochemistry **27:**2661–2667.
22. **Guirakhoo, F., A. R. Hunt, J. G. Lewis, and J. T. Roehrig.** 1993. Selection and partial characterization of dengue 2 virus mutants that induce fusion at elevated pH. Virology **194:**219–223.
23. **Gutman, O., T. Danieli, J. M. White, and Y. I. Henis.** 1993. Effects of exposure to low pH on the lateral mobility of influenza hemagglutinin expressed at the cell surface: correlation between mobility inhibition and inactivation. Biochemistry **32:**101–106.
24. **Haest, C. W., G. Plasa, and B. Deuticke.** 1981. Selective removal of lipids from the outer membrane layer of human erythrocytes without hemolysis. Consequences for bilayer stability and cell shape. Biochim. Biophys. Acta **649:**701–708.
25. **Hoekstra, D., T. de Boer, K. Klappe, and J. Wilschut.** 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry **23:**5675–5681.
26. **Hohmann, A. W., and P. Faulkner.** 1983. Monoclonal antibodies to baculovirus structural proteins: determination of specificities by Western blot analysis. Virology **125:**432–444.
27. **Hope, M. J., and P. R. Cullis.** 1981. The role of nonbilayer lipid structures in the fusion of human erythrocytes induced by lipid fusogens. Biochim. Biophys. Acta **640:**82–90.
28. **Israelachvili, J. N., S. Marcelja, and R. G. Horn.** 1980. Physical principles of membrane organization. Q. Rev. Biophys. **13:**121–200.
29. **Kemble, G. W., T. Danieli, and J. M. White.** 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. Cell **76:**383–391.
30. **Klausner, R. D., D. K. Bhalla, P. Dragsten, R. L. Hoover, and M. J. Karnovsky.** 1980. Model for capping derived from inhibition of surface receptor capping by free fatty acids. Proc. Natl. Acad. Sci. USA **77:**437–441.
31. **Kozlov, M., S. Leikin, and R. P. Rand.** 1994. Bending, hydration and void energies quantitatively account for the hexagonal-lamellar-hexagonal reentrant phase transition in dioleoylphosphatidylethanolamine. Biophys. J. **67:**1603–1611.
32. **Kozlov, M. M., and V. S. Markin.** 1983. Possible mechanism of membrane fusion. Biophysics **28:**255–261.
33. **Kumar, V. V., B. Malewicz, and W. J. Baumann.** 1989. Lysophosphatidylcholine stabilizes small unilamellar phosphatidylcholine vesicles. Phosphorus-31 NMR evidence for the wedge effect. Biophys. J. **55:**789–792.
34. **Leikina, E., H. O. Onaran, and J. Zimmerberg.** 1992. Acidic pH induces fusion of cells infected with baculovirus to form syncytia. FEBS Lett. **304:**221–224.
35. **Luzzati, V., T. Gulik-Krzywicki, E. Rivas, F. Reiss-Husson, and R. P. Rand.** 1968. X-ray study of model systems: structure of the lipid-water phases in correlation with the chemical composition of the lipids. J. Gen. Physiol. **51:**37–43.
36. **Martin, I., M. C. Dubois, T. Saermark, R. M. Epand, and J. M. Ruysschaert.** 1993. Lysophosphatidylcholine mediates the mode of insertion of the NH_2-terminal SIV fusion peptide into the lipid bilayer. FEBS Lett. **333:**325–330.
37. **Mayorga, L. S., M. I. Colombo, M. Lennartz, E. J. Brown, K. H. Rahman, R. Weiss, P. J. Lennon, and P. D. Stahl.** 1993. Inhibition of endosome fusion by phospholipase A2 (PLA2) inhibitors points to a role for PLA2 in endocytosis. Proc. Natl. Acad. Sci. USA **90:**10255–10259.
38. **Meers, P., K. Hong, and D. Papahadjopoulos.** 1988. Free fatty acid enhancement of cation-induced fusion of liposomes: synergism with synexin and other promoters of vesicle aggregation. Biochemistry **27:**6784–6794.
39. **Melikyan, G. B., W. D. Niles, and F. S. Cohen.** 1993. Influenza virus hemagglutinin-induced cell-planar bilayer fusion: quantitative dissection of fusion pore kinetics into stages. J. Gen. Physiol. **102:**1151–1170.
40. **Morris, S. J., D. P. Sarkar, J. M. White, and R. Blumenthal.** 1989. Kinetics of pH-dependent fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells. Measurement by dequenching of fluorescence. J. Biol. Chem. **264:**3972–3978.
41. **Nir, S., M. C. Pedroso de Lima, C. E. Larsen, J. Wilschut, D. Hoekstra, and N. Duzgunes.** 1993. Kinetics and extent of virus-cell aggregation and fusion, p. 437–452. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Boca Raton, Fla.
42. **Paiement, J., C. Lavoie, G. R. Gavino, and V. C. Gavino.** 1994. Modulation of GTP-dependent fusion by linoleic and arachidonic acid in derivatives of rough endoplasmic reticulum from rat liver. Biochim. Biophys. Acta **1190:**199–212.
43. **Sandow, D., P. Nuhn, K. Deutschmann, U. Eichhorn, F. Hofmann, and R. Klocking.** 1986. Antiviral action of lysolecithin analogs against human pathogenic viruses. Pharmazie **41:**404–406.
44. **Schudt, C., and D. Pette.** 1976. Influence of monosaccharides, medium factors and enzymatic modification on fusion of myoblasts in vitro. Cytobiologie **13:**74–84.
45. **Schwichtenhovel, C., B. Deuticke, and C. W. Haest.** 1992. Alcohols produce reversible and irreversible acceleration of phospholipid flip-flop in the human erythrocyte membrane. Biochim. Biophys. Acta **1111:**35–44.
46. **Siegel, D. P.** 1993. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. Biophys. J. **65:**2124–2140.
47. **Silvius, J. R., and R. Leventis.** 1993. Spontaneous interbilayer transfer of phospholipids: dependence on acyl chain composition. Biochemistry **32:**13318–13326.
48. **Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA **79:**968–972.
49. **Stegmann, T.** 1993. Influenza hemagglutinin-mediated membrane fusion does not involve inverted phase lipid intermediates. J. Biol. Chem. **268:**1716–1722.
50. **Stegmann, T., R. W. Doms, and A. Helenius.** 1989. Protein-mediated membrane fusion. Annu. Rev. Biophys. Chem. **18:**187–211.
51. **Stegmann, T., J. M. White, and A. Helenius.** 1990. Intermediates in influenza induced membrane fusion. EMBO J. **9:**4231–4241.
52. **Tamura, A., K. Morita, and T. Fujii.** 1982. Interaction of added amphiphilic lipids with the membrane of intact human erythrocytes to induce change in the cell shape. J. Biochem. **91:**73–85.
53. **Tate, M. W., E. F. Eikenberry, D. C. Turner, E. Shyamsunder, and S. M. Gruner.** 1991. Nonbilayer phases of membrane lipids. Chem. Phys. Lipids **57:**147–164.
54. **Verheij, H. M., A. J. Slotboom, and G. H. de Haas.** 1981. Structure and function of phospholipase A2. Rev. Physiol. Biochem. Pharmacol. **91:**91–203.
55. **Vogel, S. S., E. A. Leikina, and L. V. Chernomordik.** 1993. Lysophosphatidylcholine reversibly arrests exocytosis and viral fusion at a stage between triggering and membrane merger. J. Biol. Chem. **268:**25764–25768.
56. **Volkman, L. E.** 1986. The 64K envelope protein of budded Autographa californica nuclear polyhedrosis virus. Curr. Top. Microbiol. Immunol. **131:**103–118.
57. **Volkman, L. E., and P. A. Goldsmith.** 1985. Mechanism of neutralization of budded Autographa californica nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis. Virology **143:**185–195.
58. **Volkman, L. E., and P. A. Goldsmith.** 1988. Resistance of the 64K protein of budded Autographa californica nuclear polyhedrosis virus to functional inactivation by proteolysis. Virology **166:**285–289.
59. **Weltzien, H. U.** 1979. Cytolytic and membrane-perturbing properties of lysophosphatidylcholine. Biochim. Biophys. Acta **559:**259–287.
60. **White, J. M.** 1992. Membrane fusion. Science **258:**917–924.
61. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature (London) **289:**366–373.
62. **Yeagle, P. L., F. T. Smith, J. E. Young, and T. D. Flanagan.** 1994. Inhibition of membrane fusion by lysophosphatidylcholine. Biochemistry **33:**1820–1827.
63. **Zimmerberg, J., S. S. Vogel, and L. V. Chernomordik.** 1993. Mechanisms of membrane fusion. Annu. Rev. Biophys. Biomol. Struct. **22:**433–466.