pH-dependent Fusion of Vesicular Stomatitis Virus with Vero Cells

MEASUREMENT BY DEQUENCHING OF OCTADECYL RHODAMINE FLUORESCENCE

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We have studied fusion between membranes of vesicular stomatitis virus (VSV) and Vero cells using an assay for lipid mixing based on the relief of self-quenching of octadecylrhodamine (R18) fluorescence. We could identify the two pathways of fusion by the kinetics of R18 dequenching, effects of inhibitors, temperature dependence, and dependence on osmotic pressure. Fusion at the plasma membrane began immediately after lowering the pH below 6 and showed an approximately exponential time course, whereas fusion via the endocytic pathway (pH 7.4) became apparent after a time delay of about 2 min. Fusion via the endocytic pathway was attenuated by treating cells with metabolic inhibitors and agents that raise the pH of the endocytic vesicles. A 10-fold excess of unlabeled virus arrested R18VSV entry via the endocytic pathway, whereas R18 dequenching below pH 6 (fusion at the plasma membrane) was not affected by the presence of unlabeled virus. The temperature dependence for fusion at pH 7.4 (in the endosome) was much steeper than that for fusion at pH 5.9 (with the plasma membrane). Fusion via the endocytic pathway was attenuated at hypo-osmotic pressures, whereas fusion at the plasma membrane was not affected by this treatment. The pH profile of Vero-VSV fusion at the plasma membrane, as measured by the dequenching method, paralleled that observed for VSV-induced cell-cell fusion. Fusion was blocked by adding neutralizing antibody to the Vero-VSV complexes. Activation of the fusion process by lowering the pH was reversible, in that the rate of fusion was arrested by raising the pH back to 7.4. The observation that pH-dependent fusion occurred at similar rates with fragments and with intact cells indicates that pH, voltage, or osmotic gradients are not required for viral fusion.

Vesicular stomatitis virus (VSV) is an enveloped virus whose nucleocapsid finds its way to the cytoplasm of the host cell by receptor-mediated endocytosis, followed by fusion with the membrane of the endocytic vesicle (1-4). This fusion is pH-dependent and is inhibited by treating cells with compounds that raise the pH of the endocytic vesicle (3, 5). The fusion activity can be shown to take place on the plasma membrane if cells with VSV attached to their surfaces are placed in a low pH medium (3). Since the fused viral spike glycoproteins induce subsequent cell-cell fusion, viral fusion is measured indirectly by polykaryon formation (6, 7).

In order to study mechanisms of viral fusion it is necessary to directly monitor the kinetics and extent of fusion between viral membrane and cellular target membranes. A number of biophysical techniques based on electron spin resonance and fluorescence probes have been developed to directly monitor fusion between viral and cell membranes (8, 9). An assay based upon incorporation of the fatty acid probe octadecyl rhodamine (R18) into virions has recently been described (9). The probe is quenched in the labeled virion, and is associated with biological membranes or liposomes it becomes dequenched. Studies with R18-labeled Sendai virus indicated, that the probe does not exchange spontaneously between membranes even after binding of virions to the cell (10).

In the present study we show that the R18-dequenching method can be used to study pH-dependent fusion of VSV with a monkey kidney cell line (Vero cells). We studied the kinetics, extent, temperature dependence, effect of neutralizing antibodies, and reversibility of the fusion reaction.

EXPERIMENTAL PROCEDURES

Materials—Octadecyl rhodamine B chloride (R18) was obtained from Molecular Probes (Junction City, OR); methylamine, monensin, amantadine, and chloroquine from Sigma; Triton X-100 from Aldrich; octyl-β-glucopyranoside from Behring Diagnostics. Cell culture media and trypsin-EDTA were obtained from GIBCO, [3H]Leucine and [3H]glucosamine were obtained from Amersham Corp.

Cell Cultures—Vero cells were grown to confluency in Dulbecco's minimal essential medium supplemented with 10% calf serum in 75 cm² plastic dishes. The fusion experiments were carried out with the cells in suspension. Incubation for 10 min at 37 °C in 1 ml of phosphate-buffered saline containing 25 μg of trypsin and 10 μg of EDTA yielded 2 × 10⁶ cells/ml. Before the experiments the cells were washed three times by centrifugation in a solution containing 145 mM NaCl and 10 mM Hepes, pH 7.4 (NaCl-Hepes).

Virus—Purified VSV (Indiana) was obtained from J. Brown and B. Newcomb at the University of Virginia. The virus was grown on monolayer cultures of baby hamster kidney (BHK-21) cells (11) and purified by sucrose velocity and density gradients, to approximately 1 mg of VSV protein/ml (12). [3H]Leucinated VSV virus was prepared by infecting baby hamster kidney cells in the presence of [3H]leucine or [3H]glucosamine (5 μCi/ml growth medium). The specific activity ranged from 200 to 2000 cpm/pg of VSV protein.

Antibodies to the VSV spike glycoprotein (G protein) were prepared as described previously (13). G protein was extracted from purified virus with 30 mM octyl-β-glucopyranoside and purified by sucrose density centrifugation (14). About 1 mg of purified G protein in 2 ml of NaCl-hibarconate buffer with 60 mM octyl-β-glucopyranoside was mixed with Freund's adjuvant and injected into a rabbit.

Labeling of VSV with R18. The fluorescent probe was inserted into the viral bilayer by injecting 10 μl of a 1.4 mM R18 solution in ethanol under vigorous vortexing into 1 ml of NaCl-Hepes containing 1 mg of virus protein. After incubation for 1 h on a rotary shaker (300 rpm) at room temperature, unbound probe was removed by centrifuging 0.5 ml of the virus suspension through a small (6 × 1 cm) column containing Sephadex G-75. This procedure resulted in

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† The abbreviations used are: VSV, vesicular stomatitis virus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholinolinsulfanilsulfonic acid; R18, octadecyl rhodamine.
incorporation of 1500 molecules of R18 per virion, which is 2% of total VSV lipid (12). Fluorescence intensity of intact R18VSV was about 5% that of labeled virus treated with Triton X-100. Labeling with R18 did not alter the hemolytic activity of VSV (15) against duck erythrocytes (data not shown).

Binding to Cells—R-H-Labeled VSV was added to 2 × 10⁷ Vero cells in 1 ml of NaCl-Hepes buffer, pH 7.4, and incubated at 4 °C for 1 h to form VSV-Vero complexes. The cells were washed in the same buffer and centrifuged at 300 × g. The amount of virus in the pellet and supernatant was determined by radioactivity measurements with a liquid scintillation counter.

Binding of both R18VSV and unlabeled VSV to Vero cells was linear with dose of virus up to 60 µg of virus, and 20–40% of the added dose of R18VSV bound to Vero cells after 1 h at 4 °C. Labeling with R18 enhanced binding of virus about 2-fold.

Envelope Fusion Assay—R18VSV (20 µg of protein) was incubated with 3 × 10⁷ Vero cells in 1 ml of buffer at 4 °C for 1 h as in the binding assay. After washing, about 5 µg remained bound, giving rise to 750 virions per cell (protein mass of VSV is 2 × 10⁶ daltons (12)). For kinetic experiments 50 µl of the R18VSV-Vero suspension was pipetted into a cuvette containing pH 7.4 buffer prewarmed to 37 °C. After about 1 min, sufficient to warm up the cells, the pH was changed by injecting 20-100 µl of 1 M MES. The pH was always measured at the end of the experiment. Fluorescence was measured using an SLM 8000 spectrofluorometer with 1-s time resolution at 560 and 585 nm excitation and emission wavelengths, respectively. A 570 cutoff filter was used at the emission to reduce scatter contributions. We express the fusion rate as the percent fusion after a given period (usually 15 min) of incubation, calculated from the fluorescence changes according to:

\[
\% \text{fusion} = 100 \times \frac{(F_0 - F_f)}{(F_d - F_f)} (1)
\]

where \(F_0\) is the fluorescence of R18VSV-Vero complex after incubation for 15 min at 37 °C; \(F_f\) is the fluorescence of the complex after incubation for 15 min at 0 °C and \(F_d\) the total fluorescence measured by disrupting the cells in Triton X-100. Since fluorescence of R18 is temperature-dependent, the measurements of \(F_0\), \(F_d\) and \(F_f\) were always performed at the same temperature (usually 10 °C). In the kinetic experiments shown in Figs. 1 and 5, all the fluorescence measurements were done at 37 °C, and \(F_f\) was the fluorescence measurement at zero time. Consistent values for \(F_d\) were obtained by incubating the tubes for 10 min at 37 °C with Triton X-100 (final concentration 0.05%) resulting in complete dissolution of R18 by detergent.

If all of the bound R18VSV were to fuse with the cells, the maximum surface density of the probe in the cell membrane is calculated to be 0.02%. The value of the fluorescence in Triton X-100 should represent infinite dilution of the probe in the cell membrane. However, due to possible incomplete dilution and differences in the probe environment, the fluorescence of R18 in the Triton micelle is not equal to that in the cell membrane at infinite dilution. In order to measure this difference, we labeled Vero cells with R18 at different concentrations, similar to those obtained after fusion with R18VSV. After dissolution with Triton X-100, we found that the fluorescence of R18 in the Triton micelle is about 1.56 times that of R18 in the cell membrane. We correct \(F_d\) by that factor in our calculations.

RESULTS

Kinetics—Fig. 1 shows the kinetics of Vero-VSV fusion. As described under "Experimental Procedures," the R18VSV-Vero complexes formed at 4 °C, pH 7.4, were warmed to 37 °C for 1 min, and then the pH was lowered by adding 20–50 µl of 1 M MES. At pH 5.8, fluorescence increased with an approximately exponential time course reaching, after 15 min, a value which corresponds to 25% fusion calculated according to Equation 1. The fusion continued to increase gradually after those 15 min and reached about 75% after 2 h (data not shown).

The pattern of increase in fluorescence at pH 7.4 was very different from that at pH 5.8, although the fluorescence reached about the same level after 15 min. After a delay of about 100 s, the fluorescence increased linearly with time. We interpret this pattern of dequenching as fusion of VSV with the membrane of endocytic vesicles after endocytosis of the virion. The 1–2-min time lag corresponds to the time required for initial entry of ligands, toxins, and virions into an acidic compartment after receptor-mediated endocytosis (16). Consistent with this interpretation was the observation shown in Fig. 1a that the rate of fusion at pH 7.2 (i.e. via the endocytic pathway) was inhibited about 50% by addition of 5 mM methylamine, an agent that raises the pH in endocytic vesicles. The delay in increase of fluorescence in the presence of methylamine was very similar to that without inhibitor. The time lag indicates that dequenching in the presence of methylamine was due to incomplete inhibition of the endocytic pathway rather than to residual fusion at the plasma membrane at pH 7.2.

Methylamine inhibited VSV entry at pH 6.2 by 40% (see Fig. 2), indicating that viral entry via the endocytic pathway occurs at this pH. This observation is consistent with a study by Sandvig and Olsnes (17) showing that NH₄Cl inhibits entry of diphtheria toxin into Vero cells down to pH 6. As shown in Fig. 1a, in the presence of methylamine the rate of fusion was slightly higher at pH 6.1 than that at pH 7.2. At pH 5.8 methylamine had only a slight effect on the rate of dequenching (see Fig. 2).
Inhibition by Unlabeled VSV—To further characterize the two pathways of viral entry, we performed competition experiments with unlabeled virus. Since binding of R18VSV to Vero cells was not inhibited by a 10-fold excess of unlabeled virus (data not shown), we would not expect fusion at the plasma membrane to be inhibited by adding the unlabeled VSV. On the other hand, since unlabeled virus might compete with R18VSV for entry via the limited number of coated pits, we would expect an attenuation of the rate of R18 dequenching at pH 7.4 (i.e. via the endocytic pathway).

Fig. 1b shows that the rate and extent of fusion at pH 5.2 in the presence of a 10-fold excess of unlabeled virus was approximately equal to fusion at the plasma membrane in the absence of unlabeled VSV. On the other hand, presence of a 10-fold excess of unlabeled virus resulted in 60% inhibition of fusion at pH 7.4 (via the endocytic pathway). This experiment confirms the notion that we are observing the two pathways of envelope virus entry by fusion using the R18-dequenching method.

pH Dependence of Fusion and Endocytosis—We measured single time points and calculated relative fusion rates according to Equation 1 in the next series of experiments. Fig. 2 shows that fusion of VSV with Vero cells is highly pH-dependent with a sharp transition between pH 6.1 and 5.8. In the absence of inhibitors there was 18% fusion at pH 7.4 in 15 min. As discussed above, we interpret the dequenching at pH 7.4 as fusion in the endocytic vesicles. This fusion rate decreased by lowering the pH, presumably due to inhibition of endocytosis at pH levels lower than 7. However, below pH 6.2, fusion at the plasma membrane took over with a steep pH dependence. The pH dependence shown in Fig. 2 parallels that of VSV-induced cell-cell fusion and VSV-cell fusion as measured by electron spin probes (8).

In the experiments shown in Fig. 2, fusion at pH 7.4 (i.e. the endocytic pathway) was about 60% of that at pH 5.8 or below, whereas in the experiments shown in Fig. 1 and Table I the fusion rates at those pH values were about the same. This could be due to the fact that, in the experiment shown in Fig. 2, the cells were less active.

Effects of Inhibitors—In Fig. 1 we showed that the fusion rate at pH 7.4 (i.e. via the endocytic pathway) was inhibited about 50% by addition of 5 mM methylamine, an agent which raises the pH in endocytic vesicles. On the other hand, pHdependent fusion of VSV at the plasma membrane was not affected by the presence of methylamine (Fig. 2). These observations are consistent with the notion that the dequenching at pH 7.4 represents fusion from within the endocytic vesicles.

In order to test this hypothesis further, we examined the effects of agents that either block the endocytic pathway or change the pH in the endocytic vesicles. Table I shows a variety of inhibitors that we have used. Methylamine, NH4Cl, amantadine, chloroquine, and monensin affect the pH of the endocytic compartment, and azide blocks metabolic energy production required for endocytosis. Except for chloroquine, all the agents listed in Table I significantly inhibited fusion via the endocytic pathway (i.e. at pH 7.4). On the other hand, except for monensin, those agents had no significant effect on fusion at the plasma membrane (i.e. at pH 5.9).

Although the degree to which fusion was blocked in the endosome was surprisingly low, our data are in agreement with effects of lysosomotropic agents on rates of internalization of ligands via receptor-mediated endocytosis. For instance, Dickson et al. (18) found that the dose of methylamine needed for half-maximal inhibition of internalization of α2-macroglobulin was 50 mM in Swiss 3T3 cells and 100 mM in NRK-2T cells. We used only 5 mM methylamine. Most of the agents listed in Table I inhibit infectivity (3, 5) to a greater extent than internalization. Perhaps those agents have additional effects on cellular metabolism that result in inhibition of viral replication.

Inhibition by Neutralizing Antibody—The pH dependence of fusion and the characteristics of the two pathways (fusion at the plasma membrane and in the endocytic vesicles) (Figs. 1 and 2 and Table I) indicate that R18 dequenching reflects the biological activity of the viral spike glycoprotein in inducing membrane fusion.

In order to confirm this notion, we used antibody raised against the VSV G protein ("neutralizing antibody"). The antibody was added to the R18VSV-Vero complex formed at 4 °C. It did not remove bound VSV from Vero cells (data not shown). The R18VSV-Vero-antibody complex was then added to a cuvette prewarmed to 37 °C at different pH values in the same way as the R18VSV-Vero complexes were treated to induce fusion. Fig. 2 shows that fusion was inhibited up to pH 5.2, indicating that R18 dequenching was mediated by the VSV G protein.

Fig. 2 shows that, in the control experiment, fluorescence
dequenching continued to increase as the pH was lowered below 5.2. However, at pH levels lower than pH 5.2, the inhibition of fusion by the antibody was incomplete. We surmise from this experiment that, at pH levels below 5.2, either dequenching occurs in an artifactual way by mechanisms unrelated to viral spike protein-mediated fusion, or that the antibody rapidly dissociated from the virus at the lower pH values.

An interesting aspect of the protocol is that the antibody was added after viral protein-target interaction had taken place. Hence, the antibody binds to the viral spike glycoprotein at sites distinct from the virus-cell binding locus. Consequently, fusion seems to require interaction between the viral protein and the target membrane at sites distinct from the original binding locus.

Temperature Dependence—Fig. 3a shows the temperature dependence of fusion at pH 5.9 and 7.4. The fusion rate at pH 7.4 (i.e. via the endocytic pathway) was low below 27 °C, but increased very steeply above that temperature. At pH 5.9, the temperature dependence was more linear from 10 to 37 °C. Fig. 3b shows a separate set of data at pH 7.4 (i.e. via the endocytic pathway) and pH 5.5, presented as an Arrhenius plot. From the Arrhenius plots we calculated activation energies of 14.8 ± 3.0 kcal/mol at pH 5.5-5.9 and 32.8 ± 2.8 at pH 7.4 (i.e. via the endocytic pathway) above 20 °C. The 2-fold higher activation energy for pH 7.4 is consistent with fusion via the endocytic pathway, which requires metabolic energy. The activation energy for VSV fusion at the plasma membrane (pH 5.5-5.9) was about the same as that for hemolysis of duck erythrocytes (data not shown).

Effect of Cell Disruption—Osmotic swelling has been proposed as an important determinant of membrane fusion (19). In order to test this hypothesis, we monitored fusion as a function of osmotic strength of the bathing medium. Fig. 4 shows percent fusion at pH 7.4 (via the endocytic pathway) and at pH 5.5 (with the plasma membrane) as a function of osmotic pressure of the extracellular solutions. Fusion via the endocytic pathway (pH 7.4) seemed to be resistant to hypotonic extracellular solutions up to 600 mosm, but was inhibited at hypo-osmotic pressures below 200 mosm. At those hypo-osmotic pressures, the cells are completely disrupted and no endocytosis would take place.

On the other hand, the disrupted cells were still good targets for fusion at the plasma membrane (pH 5.5). In fact, fusion at the plasma membrane did not seem to be greatly dependent on osmotic pressure. This experiment indicates that no gradient of osmotic pressure, pH, or membrane potential is required for VSV-cell fusion.

Reversibility of the Process Triggering Viral Fusion—The reversibility of the process triggering viral fusion can be tested by monitoring the kinetics of R18 dequenching. In the experiment shown in Fig. 5 fusion at the plasma membrane was triggered by lowering the pH to 5.2. Bringing the pH back to 7.4 100 s later resulted in arresting the fusion reaction. Lowering the pH to 5.2 200 s later resulted in a similar fusion rate as that seen with the original experiment at pH 5.2 (data not shown). We will offer possible interpretations of these observations under "Discussion."

The time course of change in fluorescence after switching back to pH 7.4 is quite interesting. Initially fusion was arrested, but it recommenced after about 200 s. Our interpretation of the time course is that endocytosis is initially inhibited by lowering the pH to 5.2. However, 2-3 min after switching back to pH 7.4, the cells recovered and fusion via the endocytic pathway resumed.

Fig. 3. Temperature dependence of fusion of R18VSV with Vero cells. Fusion rates were measured as in the legend to Fig. 2. A, pH 7.4 (i.e. via the endocytic pathway); C, pH 5.9. Temperature was controlled in a water bath. b is a separate experiment presented as an Arrhenius plot. A, pH 7.4 (i.e. via the endocytic pathway); Δ, pH 5.5. The activation energies obtained from the slopes of the Arrhenius plots are 14.8 ± 3.0 kcal/mol for pH 5.5-5.9 and 32.8 ± 2.8 kcal/mol for pH 7.4 (i.e. via the endocytic pathway) (average of two experiments).
not spontaneously exchange between membranes. Previously, the R18 assay was used to monitor fusion between Sendai virus and liposomal or cellular targets (9, 10, 20). In the case of Sendai virus, “pharmacological” inhibitors such as diethiolethral and phenylmethylsulfonyl fluoride could be used to block fusion, even in the presence of binding, and virtually no R18 dequenching was observed when virus was treated with those inhibitors (10). However, similar treatment of VSV with those agents did not affect its binding or fusion activity (data not shown). On the other hand, the pH dependence of fusion and the characteristics of the two pathways (fusion at the plasma membrane and in the endocytic vesicle) (Figs. 1 and 2 and Table 1) indicate that R18 dequenching reflects the biological activity of the viral spike glycoprotein in inducing membrane fusion. Inhibition by neutralizing antibody (Fig. 2) provided additional evidence that we are monitoring viral spike glycoprotein-mediated fusion.

Labeling VSV with R18 did enhance binding to Vero cells (see “Experimental Procedures”). R18 is positively charged, and presumably confers positive charge on the viral surface. This enhanced binding is consistent with the observation that the polycation DEAE-dextran increases binding of VSV to baby hamster kidney cells (21).

Kinetics and Extent—In this study we eliminated the adhesion step by preincubation at 4 °C followed by removal of unbound virus. The kinetics followed a first-order reaction. The rate constant at pH 5.8 and below was about 30%/15 min or 3.3 × 10⁻⁴/s, about 2 orders of magnitude slower than Sendai-ghost fusion (20). The rate constant did not depend on the number of virions bound (data not shown). In the case of Sendai-erythrocyte interaction there was considerable dissociation of virus particles when the sample was transferred from 4 to 37 °C (20). We found no R18VSV in the supernatant during the 37 °C incubation period, indicating that there was no dissociation.

Although the data shown in this paper are 15-min records of R18 dequenching, we have observed that the process is continuous, reaching 75–100% after about 2 h. This indicates that nearly all of the bound virus is capable of fusing with the plasma membrane.

Fusion at the Plasma Membrane Versus Fusion in the Endosomes—We interpreted dequenching at pH 7.4 as fusion in the endocytic vesicles after internalization of the virus. Three types of experiments support this interpretation: 1) effects of inhibitors which affect endocytosis or change the pH in the endosomes, 2) temperature dependence, and 3) discriminating effect of hypo-osmotic lysis at pH 5.5 compared to pH 7.4. Moreover, Fig. 1b shows that a 10-fold excess of unlabeled virus inhibited the rate of dequenching of R18VSV at pH 7.4 (i.e. via the endocytic pathway). Presumably the coated pit-vesicle system is only able to take up one virion at a time, and therefore the unlabeled virus will compete with labeled virus for entry into the coated pits. In contrast, no competition was observed between unlabeled and labeled virus for fusion at the plasma membrane.

We measured an activation energy of 32.8 kcal/mol for the endocytic pathway, which is higher than the reported 17–25 kcal/mol for receptor-mediated endocytosis of proteins in single cell preparations (22). The difference may be due to the cell type or to a higher energy requirement for internalization of the larger viral particles compared to that for a smaller ligand, or it may be that both endocytosis and fusion contribute to the measured activation energy. A strong temperature dependence was noted for the endocytic uptake of Semliki Forest virus (23), but no value for the activation energy was reported.
Reversibility of the Triggering of Fusion—One of the features that could be tested in this system was reversibility of the triggering process. It has been hypothesized that, in order to induce membrane fusion, the viral spike glycoproteins undergo a pH- and temperature-dependent conformational change. Such conformational changes have been observed in the extracytoplasmic domain of the influenza virus hemagglutinin protein by development of protease sensitivity and liposome binding capacity (24, 25) and in the isolated extracytoplasmic domain of the VSV G protein by tryptophan quenching by a hydrophobic probe (26). In the case of the influenza protein, the time for conversion of 50% of the molecules to the low pH conformation was 2 min (25). The conformational change of the influenza protein to the low pH form was irreversible, whereas the conformational changes which occurred upon acidification of G protein were reversed when the sample was neutralized (26).

The data shown in Fig. 5 indicate that viral fusion induced by lowering the pH can be arrested by bringing the pH back to neutral. Although the conformational change induced in the isolated extracellular domain of the G protein can be reversed in dilute solution (26), the situation in the intact virion at high surface protein density and attached to the target membrane might be different. The observation shown in Fig. 5 has at least three possible explanations: 1) Fusion requires both a conformational change of the viral protein and the low pH. 2) The conformational change of the viral protein is irreversible and rate-limiting; pH neutralization prevents more viral spike glycoproteins from undergoing a conformational change. 3) The conformational change of the viral protein is reversible and fusion is the rate-limiting step. Further studies are required to distinguish among these possibilities.

Stegmann et al. (27) have shown that the induction of fusion activity of influenza virus with human erythrocyte ghosts was reversible. However, the conformation change of the influenza hemagglutinin is irreversible. In that case fusion either requires both the conformational change of the hemagglutinin and the low pH, or the conformational change of the viral protein is rate-limiting.

Relationship to Virus-Mediated Cell-Cell Fusion—in studies on virus fusion with cells in monolayer culture it was observed that the cells fuse with each other to form giant polykaryons (6, 7). In those experiments virus is allowed to bind to cells in the cold, the cells are warmed to 37 °C, pH 5.5, for 30–60 s, and the medium is replaced with medium at neutral pH. When incubation is continued, massive cell-cell fusion becomes apparent after 2–4 h (6, 7). The most plausible mechanism for virus-mediated cell-cell fusion is that first viral spike glycoproteins are integrated into the cell plasma membrane by virus-cell fusion, and that subsequently cell fusion results from an interaction between an area of the plasma membrane on one cell containing the viral proteins and unmodified plasma membrane on an adjacent cell (28). Consistent with this hypothesis is the fact that viral glycoproteins expressed in eukaryotic cells by transfection of plasmids containing cloned complementary DNAs encoding those viral proteins promote cell-cell fusion according to the above protocol (7, 25).

The question is: How can viral protein-mediated cell fusion occur at neutral pH, whereas the fusogenic activity of the viral proteins is activated only at low pH? One implication of the pH-dependent virus-mediated cell-fusion experiments is that the viral protein, once triggered at low pH, continues its fusogenic activity at neutral pH. However, our reversal experiments shown in Fig. 5 indicate that this is not the case. An alternative explanation is that the insertion of a given number of viral proteins into the cell plasma membrane and exposure at the low pH causes destabilization of the latter membrane, resulting in subsequent plasma membrane fusion at neutral pH.

Mechanism of Fusion—Our study does reveal the following features of fusion of VSV with cell membranes: 1) We have clearly established the kinetics and pharmacology of fusion via the endocytic pathway and contrasted it with fusion at the plasma membrane. 2) Since at low pH the rate of fusion was about the same in hypo-osmotic solutions (where cells are disrupted) as in iso- or hyperosmotic solutions, osmotic swelling does not seem to play a role. This is in contrast with Sendai virus, where it seems that osmotic swelling of the target can enhance fusion (29). 3) The same experiment shows that pH gradients and membrane potential gradients are not required for fusion. 4) We have shown that the process of fusion initiated by lowering the pH can be arrested by bringing the pH back to neutral.

The quantitative real-time spectroscopic assay for viral-cell fusion reported here enables further detailed examination of hypotheses regarding possible mechanisms of viral spike glycoprotein-mediated membrane fusion.

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REFERENCES