Micropipette Manipulation Technique for the Monitoring of pH-Dependent Membrane Lysis as Induced by the Fusion Peptide of Influenza Virus

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ABSTRACT We have assembled a micropipette aspiration assay to measure membrane destabilization events in which large (20–30 μm diameter) unilamellar vesicles are manipulated and exposed to membrane destabilizing agents. Single events can be seen with a light microscope and are recorded using both a video camera and a photomultiplier tube. We have performed experiments with a wild-type fusion peptide from influenza virus (X31) and found that it induces pH-dependent, stochastic lysis of large unilamellar vesicles. The rate and extent of lysis are both maximum at pH 5; the maximum rate of lysis is 0.018 s^{-1} at pH 5. An analysis of our data indicates that the lysis is not correlated either to the size of the vesicles or to the tension created in the vesicle membranes by aspiration.

INTRODUCTION

Membrane fusion is an important and widespread phenomenon in cell biology. The entry of viruses into cells, endocytosis, exocytosis, cell division, fertilization, polykaryon formation in bone and muscle, and communication between intracellular compartments all involve the fusion of two membranes (Blumenthal, 1987). In addition, membrane fusion has potential for use in directed drug delivery, where pharmaceutical agents are targeted to a specific organ in the body (Szoka, 1991; Mayhew, 1988); it is possible that the pharmaceutical agent could be more effectively delivered to the target cells by having them fuse specifically with the liposomes. Many books have been published about membrane fusion, including those edited by Düzgünes (1993), Bentz (1993), Wilschut and Hoekstra (1991), Ohki et al. (1988), Düzgünes and Bronner (1988), and Sowers (1987).

The entry of enveloped viruses into cells is perhaps the most widely studied membrane fusion event (for recent reviews, see Bentz, 1993; Zimmerberg et al., 1993; White, 1990, 1992; Hoekstra, 1990; Stegmann et al., 1989). Almost all enveloped viruses have a protein on their surface that mediates the fusion of the viral membrane with either the plasma membrane or endosome membrane of the target cell. Many fusion proteins have in common a hydrophobic sequence of amino acids that is called the "fusion peptide." In some cases, as with influenza virus, the fusion peptide is at the N-terminus of the fusion protein; in other cases, as with Semliki Forest virus, the fusion peptide is in the middle of the sequence.

The mechanism by which influenza virus infects a cell has been particularly widely studied. The virus binds to sialic acid on the target cell surface, and the entire virus is endocytosed into the cell. This endocytosed vesicle combines with an endosome, and the pH of the compartment is reduced. At the lower pH, influenza virus hemagglutinin undergoes a conformational change, exposing the fusion peptide. There are many proposed models for the mechanism by which influenza hemagglutinin causes fusion (Kemble et al., 1994;Carr and Kim, 1993; Tse et al., 1993; Zimmerberg et al., 1993; Guy et al., 1992; Bentz et al., 1990; Stegmann et al., 1990), but the details have been difficult to elucidate experimentally.

It is known, for example, that the fusion peptide is necessary for fusion. Mutants of influenza hemagglutinin that have altered or missing fusion peptides have altered (Daniels et al., 1985) or no (Gethig et al., 1986; Schoch and Blumenthal, 1993) fusion activity. What is less clear is exactly how much of the fusion protein is needed for fusion activity, and whether the fusion peptide alone is sufficient to cause membrane fusion. There is mounting evidence that the entire hemagglutinin molecule may be necessary to stabilize the membrane fusion site (White, 1992). In addition, there is evidence that the fusion peptide also serves to stabilize the structure of the hemagglutinin protein at neutral pH (Carr and Kim, 1993; Yewdell et al., 1993).

The fusion peptide from influenza virus hemagglutinin, which is found at the N-terminus of the protein, has also been widely studied. Wharton et al. (1988) found that an X31 wild-type peptide 20 amino acids long induced fusion of POPC small unilamellar vesicles (SUV) in a pH-dependent manner; fusion was much more extensive at pH 5 than pH 7. Düzgünes and Gambale (1988), however, found that a 17 amino acid analog of X31 induced lysis, but not fusion, of PS/PC large unilamellar vesicles made by reverse phase evaporation. Rafalski et al. (1991) also found that a 20 amino acid analog of X31 induced extensive contents leakage of

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Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MES, 2--(*N*-morpholino)ethane sulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine; SUV, small unilamellar vesicle.

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POPC large unilamellar vesicles in a pH-dependent manner. In a recent report, Düzgünes and Shavnin (1992) again reported destabilization, but no fusion, of large PC liposomes by a 17 amino acid analog of the fusion peptide from the X31 strain of influenza virus.

Previous fusion studies using fluorescence have been largely done in one of two ways. In the first method, vesicles or cells labeled with fluorescent probes are placed in a well mixed fluorometer cuvette at the desired experimental conditions. The fluorescent probes are often donor-acceptor pairs that can be used in a variety of configurations. Lipid soluble dyes are used to monitor membrane mixing; aqueous dyes are used to monitor contents mixing. By monitoring the fluorescence as a function of time, the rate of fusion can be inferred from the extent of fusion by invoking a mass-action model (Düzgünes and Bentz, 1988). In this model, two vesicles are first assumed to adhere reversibly, and then they irreversibly fuse in a second step. Only bulk properties from the entire population of vesicles can be inferred from these experiments and such a model, and much information about individual fusion events is lost. This is because individual events are overcome by the heterogeneous behavior of the population. Also, fusion and lysis are often seen in the same experiment; it is not always easy to observe which comes first in bulk experiments.

The other method that has been used widely is the fusion of vesicles with a planar bilayer. In this method, a planar bilayer is constructed in a hole in a Teflon substrate (Mueller et al., 1962; White, 1978). The bilayer is formed by spreading a solution of lipid in some organic solvent, usually decane or squalene, across the hole. Alternately, the bilayer can be formed by the apposition of monolayers (Montal and Mueller, 1972; Tancrède et al., 1983). With time, the solvent is excluded from the bilayer and the membrane thins. After the bilayer is formed, vesicles containing membrane mixing or, more commonly, contents mixing dyes are either injected toward the bilayer from below or are allowed to settle onto the membrane from above by gravity. The fusion is monitored with a microscope focused at the bilayer surface. Fusion events thus appear as a bright flash (Melikyan et al., 1993; Perin and MacDonald, 1989; Woodbury and Hall, 1988; Niles and Cohen, 1987). Although this method has the advantage of being able to distinguish single fusion events, it very difficult to construct a planar membrane without some solvent remaining in the bilayer. Because such solvents are known to stabilize inverted membrane phases (Siegel et al., 1989), it is possible that they would also have an effect on membrane fusion, because many proposed fusion intermediates contain structures similar to those found in inverted phases.

Another assay in which membrane fusion has been studied is that of Liu et al. (1988), in which large fluorescent vesicles are imaged as pixels on a video screen. The relative brightness of the pixels is related to their size and the amount of dye loaded within them; coupled with measurements of the size distribution of vesicles, membrane fusion can be monitored with this system. In this assay, individual vesicles are imaged, but fusion can only be inferred by a shift in the population fluorescence profile. Direct information about individual fusion events is lost.

To overcome some of the problems associated with the assays mentioned above, we use a micropipette aspiration technique in which two large vesicles are brought into close contact with micropipette manipulators. The vesicles are large enough to be seen with a light microscope and, thus, the progress of a single membrane interaction event can be monitored. Micropipette aspiration has been used previously to study adhesion of cells and vesicles (Tözeren et al., 1989; Evans and Metcalfe, 1984a, b; Evans, 1980), the mechanical properties of membranes (Needham and Evans, 1988; Evans and Needham, 1987), and electrofusion of adherent vesicles (Needham, 1993). In addition, micropipette aspiration has been used to study exocytosis in single cells by monitoring the membrane capacitance of whole cells, as reviewed by Lindau (1991). In this study, we use micropipette aspiration to measure the pH-dependent lysis caused by the fusion peptide from the X31 strain of influenza virus.

MATERIALS AND METHODS

Materials

Egg PC and NBD-PE were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). ANTS was purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals were reagent grade or better.

Experimental buffer

All experiments were conducted in a mixed buffer containing 0.1 M glucose, 1 mM HEPES, 1 mM MES, and 1 mM sodium citrate. This combination of buffers was chosen because it would buffer the solution over the entire desired experimental pH range of 4–7. Also, it was slightly more hyperosmolar (106 vs. 100 mOsm/kg) than the solutions the vesicles were stored in, allowing them to become slightly flaccid for micromanipulation.

Peptide synthesis

Peptide X31, the fusion peptide from the X31F/68 strain of influenza virus, was synthesized for us by Star Biochemicals, Inc. (Torrance, CA). The molecular weight of the peptide was confirmed by mass spectrometry to be 2036. The molecular weight determination was performed for us by Multiple Peptide Systems, Inc. (San Diego, CA). The sequence of the peptide is NH_2-GLFGAIAGFIENGWEGMIDG-COOH.

Peptide concentration was verified for each trial using the Pierce Coomassie Plus Protein Assay Reagent (Pierce Chemical Company, Rockford, IL). After each trial, the contents of the experimental chamber were removed with a pipettor and placed in a microfuge tube. These tubes were frozen until the Coomassie assay was performed within several days; in this way, we were able to confirm that we had the desired concentration of peptide in the chamber at the *end* of every trial. Thus, we were able to rule out potential artifacts such as peptide absorption to tubing or chamber walls.

Vesicle synthesis

Vesicles are synthesized using the dehydration/rehydration technique originally developed by Reeves and Dowben (1969) and modified by Needham and Evans (1988). Briefly, a solution of lipid in chloroform is spread on a roughened Teflon substrate. The substrate, placed in a beaker, is allowed to

dry overnight under vacuum in nitrogen at 40°C. The lipid is then prehydrated in water-saturated nitrogen for several hours. After prehydration, the aqueous solution of interest at 40°C is carefully pipetted over the substrate. Vesicles appear as a "cloud" overnight and are carefully harvested with a pipettor.

Generally, vesicles are made in a solution containing 0.1 M sucrose plus 2 mM ANTS. Vesicles are separated from unencapsulated fluorophore as follows. First, the vesicle suspension is diluted with an equal volume of 0.1 M glucose. Because glucose solutions are less dense than sucrose solutions, the vesicles can be pelleted by centrifuging at 210 × *g* for 10 min. Vesicles can then be resuspended in 0.1 M sucrose.

Vesicles were determined to be stable for up to 5 days after harvesting. Vesicles used in experiments were used within 48 h after they were harvested and had diameters of 15–30 μm, the most typical diameter being 22 μm. Vesicle diameters were determined using an eyepiece reticle at 600X and phase contrast optics.

Pipettes

Pipettes are manufactured using 1 mm inner diameter thick-walled capillary tubes made by Narishige (Greenvale, NY; type GD-1). First, pipettes with needle tips are made using a PB-7 vertical gravity puller (Narishige, Greenvale, NY). The needle tip is broken off using a microforge (Technical Products International, Inc., St. Louis, MO), and the resulting larger tip is fire-polished using the microforge. Pipettes are sized using an eyepiece reticle at 600X and bright-field optics. Pipettes used in experiments have inner diameters of 6–10 μm, the most typical diameter being 8 μm.

Pipettes are filled with the same glucose buffer in which the experiment is performed. The tips are filled by capillary action by immersing them in glucose buffer; the thinned portion of the pipette fills in less than an hour. The remainder of the pipette is backfilled using a syringe with a 31 gauge needle.

Chamber

The experimental chamber is essentially a channel that has 0.125 inch high walls. It is 0.25 inch wide and 1 inch long at the bottom. The side walls are sloped, allowing for the entry of micropipettes at an angle. The bottom of the chamber is a glass microscope slide that allows for viewing with an inverted microscope. One side of the chamber has a 0.03 inch hole bored into it through which solutions can be injected.

Fluorescence assays

For lysis experiments, a single population of vesicles is synthesized, with 2 mol% NBD-PE in the membrane and 2 mM ANTS in the aqueous contents. Because the vesicles are held in the micropipettes with a small suction pressure (0.5 cm $H_2O \approx 5 \times 10^{-4}$ atm), lysis results in the vesicle being sucked into the pipette and out of the field of view of the microscope objective, because the membrane is no longer continuous. When using the fluorescence assays, then, a lysis event is seen as a decrease in fluorescence of both NBD-PE and ANTS. Using video microscopy only, the vesicle is seen to deflate abruptly and is sucked into the pipette. This type of rapid disruption is consistent with previous micropipette experiments such as those by Zhelev and Needham (1993) when aspiration pressures such as ours (0.5 cm H_2O) are used.

These fluorophores (NBD and ANTS) were chosen because they have different excitation wavelengths but similar emission wavelengths. NBD excites at 460 nm and emits at 534 nm; ANTS excites at 370 nm and emits at 515 nm. In this way, we can alternately excite the two fluorophores while monitoring their emission with one set of equipment. We use 355 and 460 nm bandpass filters to excite ANTS and NBD, respectively. A dichroic mirror with extended UV region allows the excitation wavelengths to be reflected up to the sample while allowing emitted light to pass through; the emitted light is passed through a 535 nm bandpass filter. All of these filters were purchased from Omega Optical, Inc. (Brattleboro, VT).

Experimental equipment

Fig. 1 shows an overall schematic of our experiment. The Nikon Diaphot TMD inverted microscope is equipped for epifluorescence. The fluorescent light source is a Xenon lamp equipped with a chopper wheel that is used to excite alternately the two fluorophores. The emitted light can be sent to a photomultiplier tube and/or an intensified video camera. The chopper wheel and photomultiplier tube are coordinated with an NEC PowerMate 286 Plus computer (NEC Information Systems, Inc., Boxborough, MA). The computer, xenon lamp, chopper wheel, and photomultiplier tube collectively are the Photoscan-2 System from Nikon, Inc. (Melville, NY). The model CCD-200E camera and model KS-1381 image intensifier are from Video Scope International, Ltd. (Washington, D.C.). Video images are recorded using a JVC S-VHS VCR with time base corrector and time code reader/generator boards installed (JVC, Elmwood Park, NJ). In addition, we have the capability to do image analysis with a Sun workstation equipped with Inovisions image analysis software (Inovisions Corporation, Durham, NC).

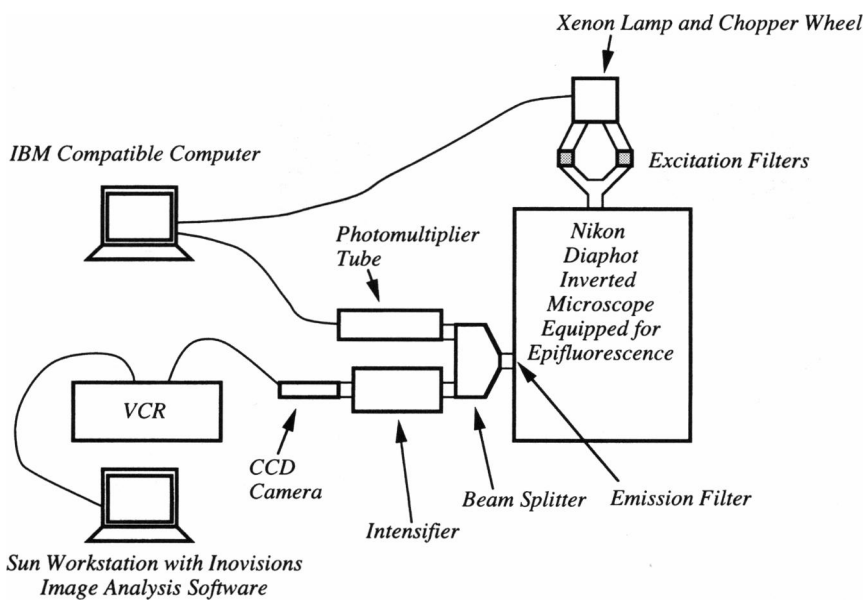

FIGURE 1 Schematic of experimental equipment.

The microscope, xenon lamp, and manometers sit on a Research Series Plus isolation table from Newport Corporation (Irvine, CA).

With the photomultiplier tube and video camera combination, we can obtain several types of data. The photomultiplier tube gives information on a very short time scale (up to 500 points per second) but does not provide any spatial information. This is useful for careful kinetic measurements. The intensified video camera, although providing information on a much longer time scale (30 frames per second), does give spatial information. This could be used, for example, to follow the diffusion of fluorophores from one vesicle to the other.

Fig. 2 shows a schematic of the microscope stage and the equipment used to control the position of and the pressure within the micropipettes. Translation stages from Newport Corporation (Irvine, CA) allow for control of motion of the micropipettes on the order of centimeters along all three axes. Precise control of motion on the order of microns is possible using stage-mounted micromanipulators from Technical Products, Inc. (St. Louis, MO); the operator performs the fine motion using pneumatic joystick controllers connected to the manipulators by tubing.

The pressure within the pipettes is controlled using micrometer manometers machined for us by Research Instruments, Inc. (Durham, NC); the pipettes are connected to the manometers through the wet chuck as shown in Fig. 2. Essentially, the pressure is controlled by adjusting the relative heights of two water-filled reservoirs on the manometer. We can control pressure down to increments of 5 μm of water ($\approx 5 \times 10^{-7}$ atm). The pressure is monitored using sensitive differential pressure transducers (Models DP15 and DP103) manufactured by Validyne Engineering Corporation (Northridge, CA).

Experimental protocol

100 μl of vesicle solution is diluted with 600 μl of glucose buffer and, after vortexing, this solution is placed in the chamber. The vesicles are allowed to settle for 10 min; the vesicles are more dense than the glucose buffer because they contain sucrose. The pipettes are zeroed, and then one or two vesicles are aspirated, depending upon the configuration used. The pipettes holding the vesicles are then aligned and the size of the vesicle(s) is measured. The VCR is then started, and 100 μl of peptide solution is injected into the chamber. Alternately, for fluorescence measurements, the lights are turned off, the photomultiplier tube scan is started, and peptide is injected into the chamber. Videotaped experiments are allowed to progress until the vesicles lyse or until a period of 15 min has passed. Generally, photomultiplier traces were 2 min long.

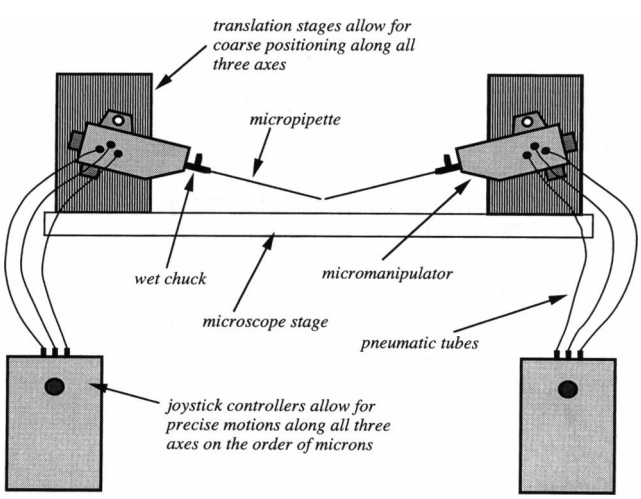

FIGURE 2 Schematic of equipment used to control position of micropipettes on microscope stage. Manometers are connected to the micropipettes through the wet chuck.

Most of the results presented in this paper were obtained using phase contrast optics and the video camera (unintensified) only, because it was found that the lysis events induced by X31 often did not start for several min. Because there is a limitation on the number of data points that can be collected in a single trace with the Photoscan system (10,000 points), it cannot be run for very long at a high rate of data collection. For lysis events that occur on the order of many minutes, the video images give better time resolution. All experiments were performed at room temperature (22–24°C).

Vesicles selected for use in experiments were the ones that appeared the most transparent, had the thinnest membranes, and had no visible internal structures. Determinations of lysis pressures of typical vesicles agree within our experimental error with those of Evans and Needham (1987) for unilamellar vesicles made of egg PC. Our measured lysis tensions were several dynes/cm, well above the tensions exerted by our small aspiration pressures.

RESULTS

With our assay, we can obtain more than one type of data. Fig. 3 shows fluorescence traces for one lysis event induced by peptide X31 at pH 5 and a concentration of 10 μM. Data were collected at a rate of 20 points per second. As expected, lysis results in a decrease in fluorescence as recorded by both assays. Both fluorophore traces show that the vesicle lysed at a time of 32.95 s, or ~3 s after injection. (Peptide was injected at 30 s.) The exact lysis time is determined by narrowing the x axis range to the region of interest (in this case 32–34 s) and locating the point at which the fluorescence begins to decrease.

At most of the conditions tested, and in contrast to the lysis event shown in Fig. 3, most (but not all) of the lysis events occurred very slowly, on a time scale of minutes. Therefore, most of the data reported in this paper were obtained using video microscopy. Fig. 4 shows a sequence of video frames obtained in such an experiment. Fig. 4 *A* shows the two vesicles manipulated into the vicinity of each other at the time of injection. The right vesicle begins to lyse 3 s after

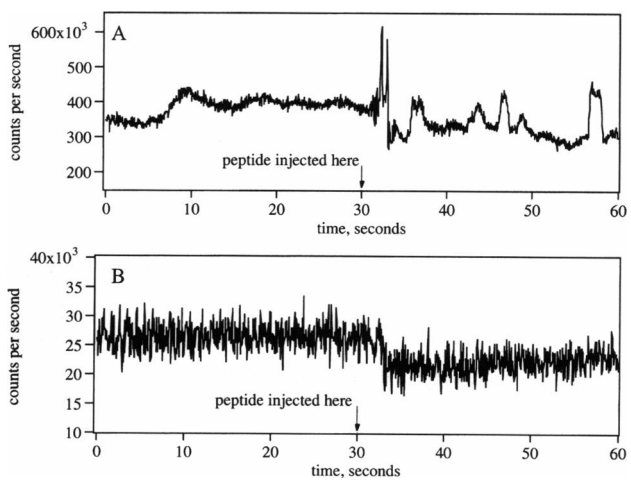

FIGURE 3 Fluorescence traces of vesicle lysis caused by peptide X31. Data were collected at a rate of 20 points per second. Peptide X31 at 10 μM and pH 5 was injected into the chamber at t = 30 s. (*A*) Membrane fluorophore (NBD-PE) trace. (*B*) Aqueous fluorophore (ANTS) trace. Both traces show that lysis occurred at a time between 32 and 33 s. The bumps at the end of the membrane fluorophore trace result from bright, multilamellar vesicles floating by the field of view of the microscope objective.

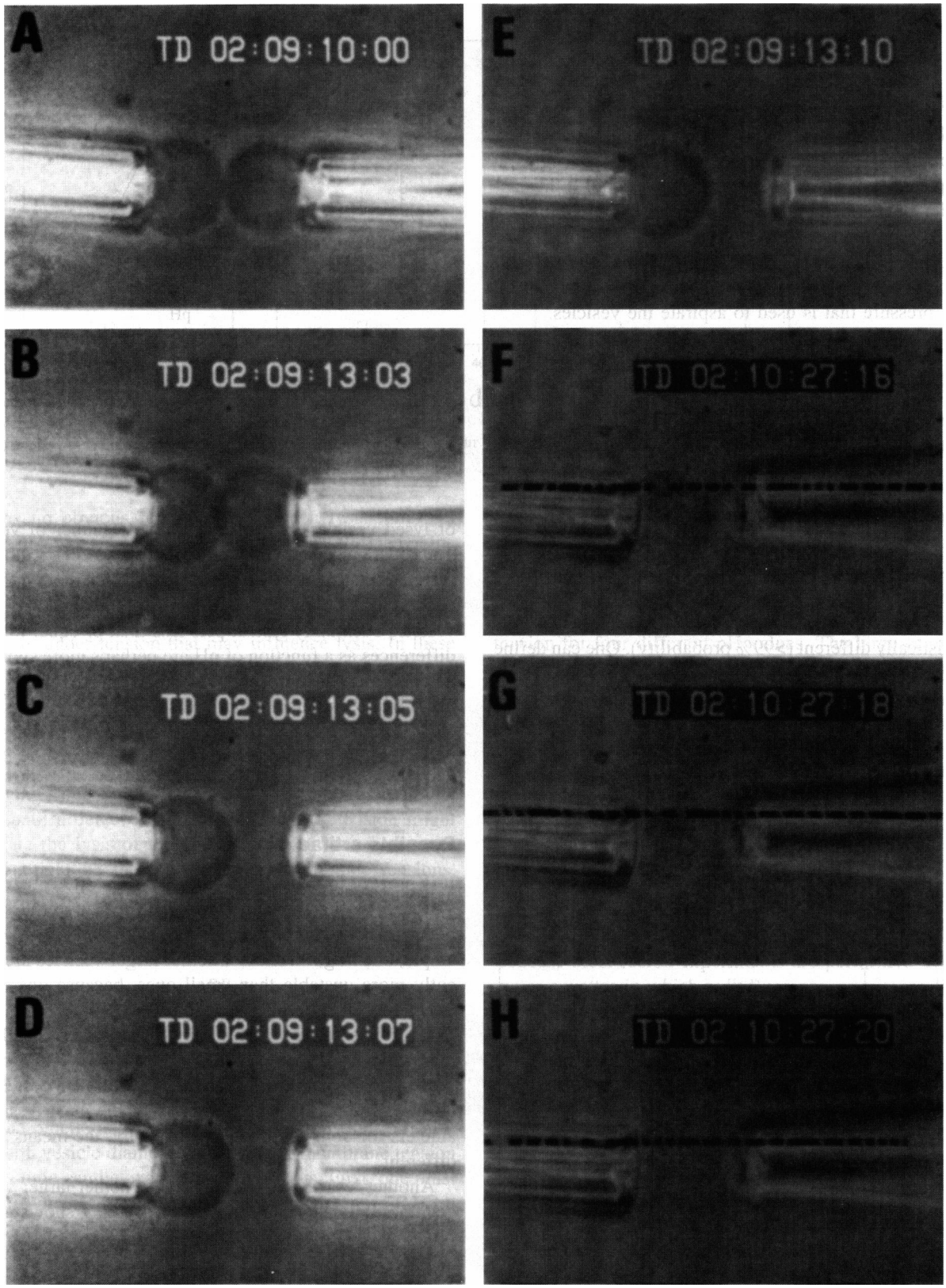

FIGURE 4 Video time sequence showing lysis induced by peptide X31. Numbers at the top of each frame indicate the time code for that frame (h: min:s:frames). Peptide X31 at 10 μM and pH 5 was injected at time 2:09:10:00. The dashed line seen in F, G, and H is an artifact produced by our VCR. (*A*) Vesicles in contact at the time of injection. (*B*) Right vesicle begins to lyse. (*C–E*) Right vesicle shrinks and disappears, characteristic of lysis. (*F*) Left vesicle begins to lyse. (*G–H*) Left vesicle shrinks and disappears..

injection (Fig. 4 *B*). In 7 frames (7/30 second), the right vesicle shrinks and disappears, characteristic of lysis (Fig. 4 *C–E*). About one and one-half min after injection, the left vesicle begins to lyse (Fig. 4 *F*). In 4 frames (2/15 second), the left vesicle shrinks and disappears (Fig. 4, *G* and *H*). This type of behavior, where the vesicles lyse separately and independently of one another, was seen repeatedly.

Both types of experiments show that once lysis begins, it is completed very quickly, in less than a second. It thus appears that once a defect forms in the vesicle membrane, the vesicle is drawn into the pipette. This holds true for all conditions tested (data not shown). It is likely that the constant time for completion of lysis is due to the constant small suction pressure that is used to aspirate the vesicles.

Results of X31 lysis experiments

Fig. 5 shows the time for lysis to start as a function of pH. All of these experiments were performed at a concentration of 10 μM. Peptide X31 has maximum activity at pH of 5, inducing lysis much more quickly at pH 5 than at any other pH. Although one cannot easily compare the lysis mean times at pHs 6 and 7 to the others because of the low number of lysis events seen at those pHs, there are statistical differences in mean lysis time as a function of pH. For example, between pH 4 and 5, an unpaired t-test shows that the means are statistically different (>99% probability). One can define a rate of lysis by taking the inverse of the mean lysis time at each condition. As an example, the lysis rate at pH 5 is 0.018 s^{-1}, which is the highest rate we observed for peptide X31 at 10 μM. The ability to report absolute rates of lysis is one of the advantages of our system.

As can be seen in Fig. 5, more vesicles lyse at pH 5 and below than at higher pH; in fact, not all vesicles exposed to peptide actually lyse. Fig. 6 shows a graph of percent lysis as a function of pH. Percent lysis is defined as the number

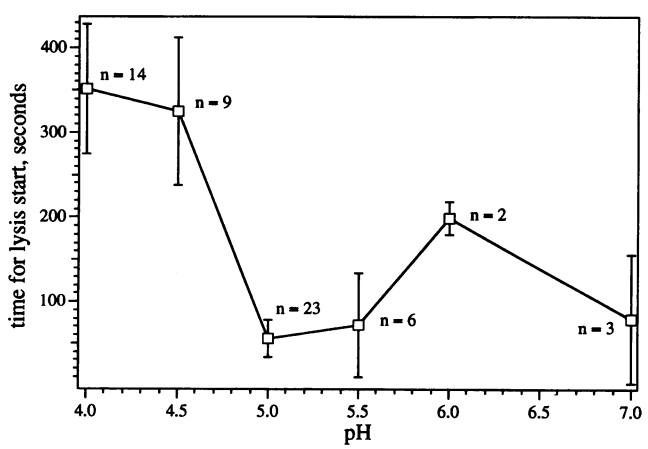

FIGURE 5 Plot of lysis start time versus pH for peptide X31. Data at pH 5 were taken both with fluorescence and video microscopies; data at all other pHs were taken using video microscopy only. Error bars are SEM; n = number of vesicles that lysed. Vesicles that did not lyse are not accounted for in this graph.

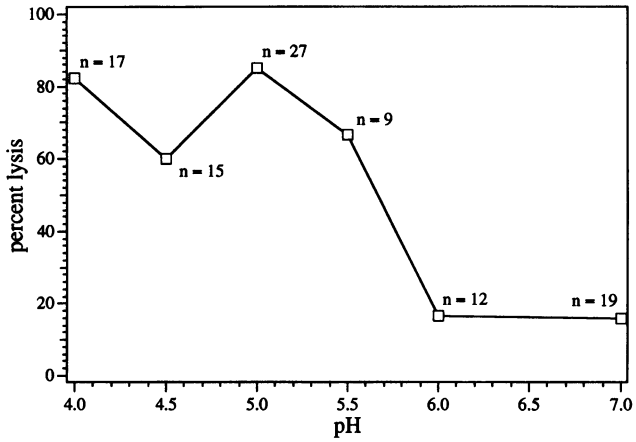

FIGURE 6 Plot of percent lysis versus pH for peptide X31. Data at pH 5 were taken both with fluorescence and video microscopies; data at all other pHs were taken using video microscopy only. Percent lysis is defined as the number of vesicles that lysed divided by the total number of vesicles tested at a given condition. Here, n represents the total number of vesicles (vesicles that lysed plus vesicles that did not lyse).

of lysed vesicles divided by the total number of vesicles. These data also show maximum activity at pH 5; however, there is a plateau at lower pHs. That is, even though the lysis occurs very slowly, as shown in Fig. 5, a majority of the vesicles eventually lyse at pHs less than or equal to 5. The differences as a function of pH are perhaps more significant here, because the numbers of events is approximately equal. So, if one considers activity measured by percent lysis, it is clear that there is an effect of pH on lysis activity. As discussed below, it is the percent lysis data that can most directly be compared with fluorometer experiments.

Possible artifacts

It is prudent to consider if our results are an artifact of our system. The first concern is whether the size of the vesicles being used has any effect on the results obtained. For example, one might wonder whether larger vesicles are inherently more unstable than small ones, because one obtains only a few large vesicles (20–30 μm) compared with a large number of smaller vesicles using our synthesis method. If this were true, the expected observation would be that vesicles that do not lyse are smaller overall than vesicles that do lyse. Table 1 shows a summary of the mean vesicle diameters for both conditions; statistically, the means are not different.

Another test of this hypothesis is whether larger vesicles lyse more quickly than smaller ones. Fig. 7 shows plots of lysis time versus vesicle diameter at four different pH values.

TABLE 1 Comparison of vesicle diameters

	n*	mean (μm)	SD (μm)
No lysis	42	22.1	4.1
Lysis	57	22.4	5.1

*n, number of vesicles.

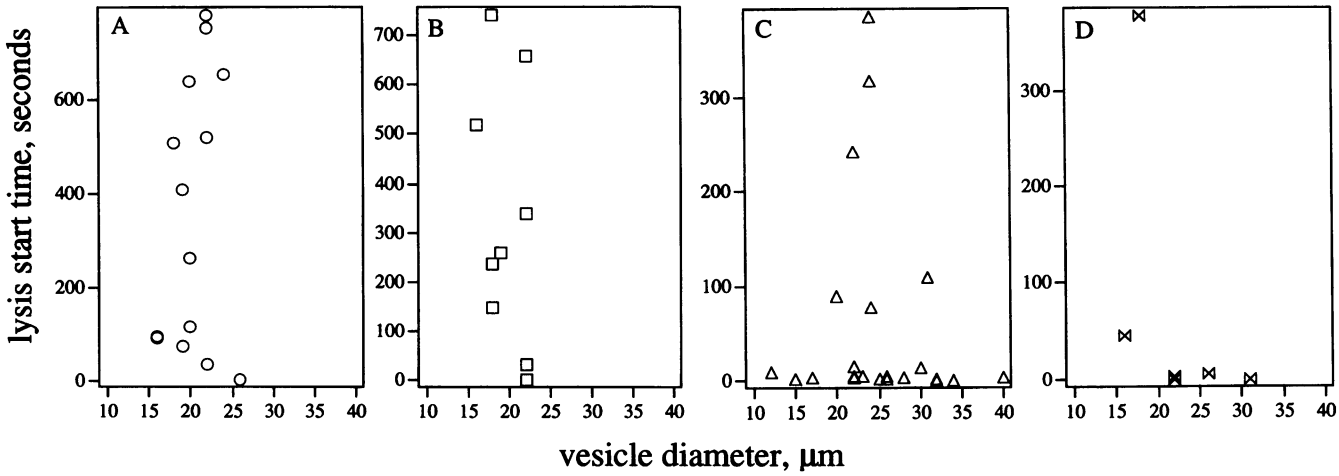

FIGURE 7 Plots of lysis start time versus vesicle diameter at four different pH values. (*A*) pH 4. (*B*) pH 4.5. (*C*) pH 5. (*D*) pH 5.5.

There is no obvious relationship between lysis time and vesicle diameter. Thus, it does not appear that the size of the vesicles is the cause of the behavior seen.

Another concern is that, because the vesicles are held in the micropipettes with a small suction pressure, the membranes are under tension that may influence lysis. In these experiments, it was our desire to exert sufficiently small pressures, giving rise to sufficiently small tensions, such that the effect of tension on lysis was negligible. However, because of differences in vesicle and pipette diameters, this tension varies from trial to trial and might have inadvertently been the driving force behind the behavior we see. For example, when small a defect forms in a membrane, tension might accelerate the lysis of the vesicle; vesicles that lyse more quickly might be those with higher membrane tensions. Kwok and Evans (1981) have shown that the mean tension in the membrane of a vesicle held in a micropipette can be approximated as follows:

$$\bar{\tau} = \frac{PR_p}{2(1 - R_p/R_o)} \quad (1)$$

where $\bar{\tau}$ = mean tension, P = suction pressure in pipette, R_p = inner radius of pipette, and R_o = radius of vesicle. The pressure within the pipette is always 0.5 cm H_2O ($\approx 5 \times 10^{-4}$ atm); the pipette and vesicle radii vary from trial to trial.

As with vesicle diameter, the effect of membrane tension on lysis time can be examined in two ways. First, one must consider whether vesicles that lyse are under more tension than vesicles that do not lyse. Table 2 gives the means and SDs of membrane tension for both vesicles that lysed and vesicles that did not lyse. The two means are not statistically different, and so it appears that vesicles that lyse are not under greater tension than those that do not lyse, at least within our experimental error.

TABLE 2 Comparison of membrane mean tension

	n*	τ (dyn/cm)	SD (dyn/cm)
No lysis	42	0.17	0.05
Lysis	56	0.19	0.09

*n = number of vesicles.

Second, do greater tensions lead to faster lysis? Fig. 8 shows plots of lysis time as a function of mean membrane tension for four different pH values. The large error in the estimates of membrane tension result from inaccuracies in our measurements of pipette diameter, the parameter in Eq.1 that we know with the least accuracy. Within the measurement error, however, there is no effect of membrane tension on lysis time at these conditions. We conclude, then, that within our experimental error, membrane tension is not the source of the lysis behavior seen. In addition, Figs. 7 and 8 demonstrate that vesicle failure was quite variable under identical conditions.

DISCUSSION

Because most fusion experiments are performed in fluorometer cuvettes in which bulk fluorescence is measured as a function of time, care must be taken in distinguishing between true fusion and other processes, such as lysis. This is particularly true in cases where membrane mixing, aqueous contents mixing, and contents leakage are not all measured (Burgess et al., 1991). When a large amount of contents leakage is seen along with membrane fusion, it can be unclear whether the fusion and leakage occurred simultaneously, or if the leakage occurred after membrane fusion. Another possibility, which is hard to measure using bulk fluorometer experiments, is that some fraction of the vesicles actually fuse while another fraction of the vesicles lyse. One of the true advantages of a system such as ours, in which we can observe single membrane interaction events, is that we should be able to determine unambiguously whether fusion or some other process is occurring for each vesicle pair tested. In addition, because we should be able to separate fusion from lysis, we can determine rates of lysis (and fusion) directly from our data, without resorting to a mass-action model.

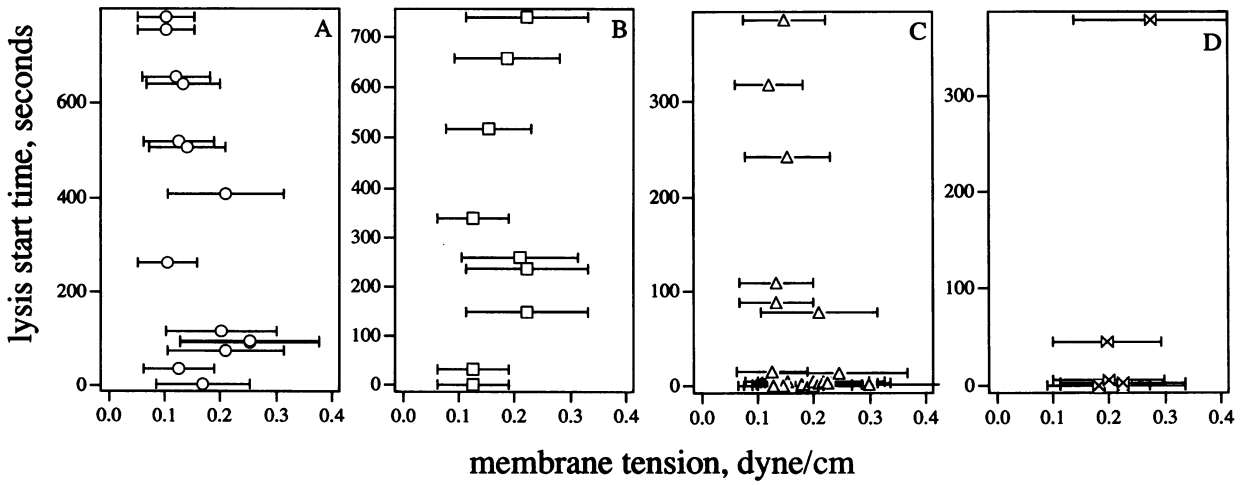

FIGURE 8 Plots of lysis start time versus mean membrane tension at four different pH values. (*A*) pH 4. (*B*) pH 4.5. (*C*) pH 5. (*D*) pH 5.5. Horizontal error bars are 50% of value, giving an estimate of the error in the measurements.

The major drawback to the micropipette method is the large amount of time that is required to obtain statistically significant amounts of data; in most cases, only one set of experimental conditions can be tested on a given day. This assay, therefore, is not suited to screening large numbers of experimental conditions. In general, we feel that a combination of assays would be most useful: fluorometer experiments could be used to screen large numbers of experimental conditions, and then specific rate and extent information could be obtained with the micropipette assay.

At each of the pHs reported in this paper, at least one experiment (consisting of many trials) was performed in a two-vesicle configuration. In all cases, the vesicles were seen to lyse separately and independently of one another; the times between lysis of two neighboring vesicles may be very different. This behavior does not appear to be the result of a transient fusion event that results in the eventual lysis of the two vesicles; in such a case, the vesicles would likely be seen to lyse at the same or close to the same time.

Although we cannot rule out the possibility that peptide X31 causes fusion at some experimental conditions, using our system and our buffer, we did not see fusion. Rather, we found that X31 induces membrane lysis in a pH-dependent manner. This agrees with the results of Düzgünes and Shavnin (1992), Düzgünes and Gambale (1988), and Rafalski et al. (1991), who also found that X31 induced extensive leakage of vesicle contents. Although Wharton et al. (1988) report membrane fusion induced by X31, these experiments were performed with SUV made by sonication; such vesicles are known to have highly strained bilayers because of their extremely small radii (Gruner, 1987). Thus, they are much more prone to fusion than vesicles with larger radii. It is interesting to note, however, that Wharton et al. report that about 90% of the contents of their SUVs leak during fusion. In addition, Wharton et al. (1988) report that peptide X31 is active in low electrolyte media, which further supports our results.

Both Figs. 7 and 8 show the scatter of lysis times at a given pH. That is, at constant conditions, there is a range of lysis times seen. It does not appear that this scatter is caused by either vesicle diameter or membrane tension, however. This large scatter in data from single events has also been observed by Kaplan et al. (1991), who observed broad heterogeneity in both the lag times and completion times for membrane mixing between cells expressing influenza hemagglutinin on their surfaces.

Qualitatively, our results agree very well with the studies mentioned above. It is important to consider how one defines lysis activity. For example, the lysis rates are very slow at pHs 4 and 4.5, but the percent lysis in both cases is very high. We see a maximum in lysis activity measured by lysis rate at pH 5; the lysis rate decreases at other pHs. There is also a maximum in percent lysis at pH 5 or below. This maximum at pH 5 is expected, because the charged residues on the peptide have pK_as of 4–5. Also, the extent of lysis data, which peaks at pH 5 but appears to plateau at lower pH, agrees qualitatively with the plot of fusion efficiency versus pH of Wharton et al. (1988, Fig. 2). Data at other concentrations have been taken, and will be published separately in a more thorough comparison of the activity of X31 and other peptides (S. A. Soltesz and D. A. Hammer, unpublished data).

One of the other advantages of our assay is that we can present actual rates of lysis (or fusion) because we derive rates directly from individual events. We have expressed rate as 1/time and report here that lysis occurs most rapidly at pH 5, with a rate of 0.018 s^{-1}. Although absolute rates cannot be derived from fluorometer experiments without invoking a model like that of Düzgünes and Bentz (1988), it is commonly seen that under similar conditions, the fusion or leakage process takes several min to occur (Düzgünes and Shavnin, 1992; Rafalski et al., 1991; Wharton et al., 1988). Thus, it seems that our numbers are consistent with previous fluorometer results.

Because micropipette aspiration has not been widely used to study membrane lysis, we have explored the possibilities of artifacts in our system. First, we explored whether the size of the vesicles themselves was responsible for the lysis behavior seen. One might expect that larger vesicles are inherently more unstable than smaller ones; one line of evidence supporting this is that there are fewer numbers of large vesicles relative to small ones after synthesis.

However, an examination of the data showed no difference in vesicle diameter between vesicles that lysed and vesicles that did no lyse (Table 1). Further, by plotting lysis time versus vesicle diameter at a given pH, one sees no detectable relationship between lysis time and vesicle diameter (Fig. 7). We have concluded, then, that the difference in vesicle diameters between trials is not the cause of the observed lysis.

Second, we have examined the effect of the membrane tension created by vesicle aspiration on lysis time. We have estimated that our measurements of mean membrane tension are correct within about a factor of two, and the average tensions were <0.2 dyn/cm. This is an order of magnitude below the tensions required to cause membrane lysis (Evans and Needham, 1987). The parameters in Eq. 1 that are measured experimentally are the aspiration pressure, the vesicle radius, and the pipette inner radius. The aspiration pressure is constant from trial to trial at 0.5 cm H_2O; the actual value varies only by a few percent. Larger errors are introduced in the measurement of vesicle and pipette radii. This is because of the measurement method, using an eyepiece reticle at 600X magnification, which introduces errors of 1–2 μm in diameter. Although this is not an excessive error on the scale of a 30 μm vesicle, the percent error introduced for an 8 μm inner diameter pipette can be as large as 25%. Thus, the measurement of pipette diameter is the largest source of error. We have plans in the near future to use a more sophisticated method to measure pipette diameter and improve our measurements of membrane tension accordingly.

Within our error, as shown in Table 2, vesicles that lyse do not have significantly higher membrane tensions than those that do not lyse. Further, as shown in Fig. 8, there is no detectable relationship between lysis time and membrane tension. We conclude that, within the error of our measurements, membrane tension is not the driving force behind the lysis behavior that we see.

Another potential concern with our system is whether the chamber is well mixed when peptide is injected. We have three lines of evidence that support our belief that the chamber is well mixed. First, video records clearly show nonaspirated vesicles flowing quickly by the aspirated vesicles and pipettes during injection. Second, one can estimate a characteristic mixing time in the chamber as the length of the chamber divided by the velocity of the fluid injected into the chamber. With 100 μl of fluid being injected through a 0.03 inch hole in 5 s, and a chamber length of 1 inch, one obtains a value of 0.6 s for the characteristic mixing time. Thus, on the time scale of lysis, which in the majority of cases occurs a minute or more after injection, we conclude that the fluid in the chamber is certainly well mixed. Finally, under the proper conditions of pH and peptide concentration, we have observed lysis on the order of a few seconds, such as in the example given in Fig. 3, which again confirms our belief that the chamber is well mixed on a time scale of several seconds.

In addition, one might be concerned that the lysis behavior we have seen is caused by stochastic failure of the vesicles resulting from accumulation of lipid on the inside of our pipettes. Other groups (Needham, 1993; Evans and Needham, 1987) have added small amounts of BSA to their experimental buffers to minimize this effect. Although lysis is rapid and occurs in nearly all trials at some conditions, such as at pH 5, at other conditions, such as at high pH, a large majority of the vesicles never lyse within 15 min. These data all serve essentially as negative controls. Despite the omission of BSA from our buffer, we feel that the lysis we have reported is caused by peptide X31, and not some nonspecific failure.

Another possible concern about our measurements is that the stochastic failure of vesicles is caused by multiple bilayers. In such a case, one would expect that lysis times would be arranged in groups or clusters according to the number of lamellae (Kwok and Evans, 1981). As seen in Figs. 7 and 8, the lysis times do not suggest a grouping of lysis times that would be suggestive of multibilayer structures. As mentioned in Materials and Methods section, our measured lysis tensions agree within our experimental error with those of Evans and Needham (1987) for unilamellar vesicles. Although measurement of the materials properties of the actual vesicles used in lysis experiments could definitively rule out the possibility of multilamellar vesicles, we feel we have measured lysis of sufficiently many vesicles to be confident that our experiments were performed on unilamellar vesicles.

Our intention in these experiments was to determine if the X31 peptide could cause fusion of large unilamellar vesicles. We observed no fusion, even when we manipulated the vesicles into "contact"; however, "contact" is ill-defined in our system. Although there is ample evidence that phosphatidylcholine membranes will adhere in water free of salt (Marra and Israelachvili, 1985), we did not observe a large adhesive region between our vesicles, perhaps because of the tensions we used to hold the vesicles. Furthermore, the 2% (0.1 M) glucose solution we used as a buffer should only have a modest attenuating effect on the van der Waals attraction, based on calculation made of interbilayer forces in 20% sucrose (Nir and Bentz, 1978), the fact that we used only 2% glucose, and that the properties of glucose and sucrose for van der Waals attraction are similar (Nir and Andersen, 1977). We thus believe egg PC membranes have the potential to be adherent in 2% glucose buffer. Nevertheless, we do not have detailed knowledge of the interbilayer separation for our lipid/solvent system, which can be obtained with x-ray diffraction (Simon et al., 1994). We do not know if the membranes were too close to exclude peptide, or too far away to permit fusion. Although our technique holds the potential to

address the mechanism of membrane fusion, the experiments here shed little light on whether X31 is capable of mediating fusion.

In summary, we have combined fluorescence quenching and video microscopy with micropipette aspiration to develop an assay for membrane lysis. With this assay, new questions can be answered about the actions of molecules on biological membranes. For example, there is potential to measure the materials properties of membranes, such as the area expansivity modulus, as a function of peptide concentration. Also, the precise relationship between adhesion and fusion can be assessed, because both can be measured with micropipette aspiration (Needham, 1993). Because we can observe single events, we should able to distinguish between membrane fusion and lysis. Further, by incorporating the appropriate fluorescent dyes, we should be able to detect lysis that followed membrane fusion, as is believed to occur in some systems. We are also able to determine rates of lysis from the average of single events; we do not have to fit our data to a model to determine the rates at which lysis or fusion occurs. We can compare our extent of lysis data, determined from the number of vesicles that lyse versus the number of vesicles tested, with previously published results. Our extents of lysis agree well qualitatively with previously reported values of percent leakage or lysis as induced by peptide X31. Finally, although we did not observe membrane fusion of egg PC vesicles induced by peptide X31, our assay has been designed to monitor many types of membrane interaction phenomena, including fusion, and we believe that it certainly has the potential to do so.

Although we cannot rule out the possibility that peptide X31 is able to cause fusion at some conditions, our results and those of other groups suggest that the fusion peptide alone is not sufficient for membrane fusion to occur. It might be that a specific lipid and solvent composition is required to give optimal fusion activity of X31, perhaps by providing the correct intermembrane separation. It is also possible that the entire hemagglutinin protein is required to stabilize the intermediates that occur during fusion. In such a scenario, the fusion peptide would serve to disrupt one or both apposed bilayers; the entire protein would form a scaffold on which the lipids could diffuse. Such models have been proposed by Stegmann et al. (1990) and Bentz et al. (1990). Although these models differ in the intermediates formed, both hypothesize that the entire fusion protein is needed to stabilize the fusion complex; both models are consistent with our results. We agree with Düzgünes and Shavnin (1992) and Stegmann et al. (1989), however, that studying the ability of the fusion peptide to disrupt membranes does have relevance to fusion induced by the intact hemagglutinin protein, because the results provide important insights into the role that the peptide may play in the overall fusion process.

We are currently pursuing experiments in our laboratory involving fusion molecules reconstituted into model membranes and also virally infected cells to confirm that our assay is actually able to measure membrane fusion. In addition, because we were unable to make detailed measurements of the membrane projections into the pipettes with our current system, which might allow us to learn more about the mechanism of lysis, we will modify the equipment to include measurement of membrane extension length. Several steps are involved in lysis, including transport of the peptide to the vesicle surface, absorption of peptide, and membrane destabilization. Our current measurements do not distinguish between these steps, but more detailed experiments in which area dilation is measured might very well elucidate the mechanism of action of peptides such as X31.

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