Hemifusion between Cells Expressing Hemagglutinin of Influenza Virus and Planar Membranes Can Precede the Formation of Fusion Pores that Subsequently Fully Enlarge

Vladimir I. Razinkov, Grigory B. Melikyan, and Fredric S. Cohen
Rush Medical College, Department of Molecular Biophysics and Physiology, 1653 W. Congress Parkway, Chicago, Illinois 60612

ABSTRACT The chronological relation between the establishment of lipid continuity and fusion pore formation has been investigated for fusion of cells expressing hemagglutinin (HA) of influenza virus to planar bilayer membranes. Self-quenching concentrations of lipid dye were placed in the planar membrane to monitor lipid mixing, and time-resolved admittance measurements were used to measure fusion pores. For rhodamine-PE, fusion pores always occurred before a detectable amount of dye moved into an HA-expressing cell. However, with DiI in the planar membrane, the relationship was reversed: the spread of dye preceded formation of small pores. In other words, by using DiI as probe, hemifusion was clearly observed to occur before pore formation. For hemifused cells, a small pore could form and subsequently fully enlarge. In contrast, for cells that express a glycosylphosphatidylinositol-anchored ectodomain of HA, hemifusion occurred, but no fully enlarged pores were observed. Therefore, the transmembrane domain of HA is required for the formation of fully enlarging pores. Thus, with the planar bilayer membranes as target, hemifusion can precede pore formation, and the occurrence of lipid dye spread does not preclude formation of pores that can enlarge fully.

INTRODUCTION

Fusion between membranes is a critical process for many cellular functions. The fusion mechanisms have been most extensively studied on molecular, structural, and biophysical levels for viral proteins. The hemagglutinin (HA) of influenza virus, in particular, has received great attention (Bullough et al., 1994; Hernandez et al., 1996). When membranes fuse, two continuities are established: both formerly distinct aqueous spaces and membranes become connected. If it could be determined which of these continuities precedes the other, the mechanism by which fusion occurs would be better defined. In particular, if it were shown that lipid continuity preceded subsequent aqueous continuity, this would provide strong evidence that fusion proceeds through hemifusion. If hemifusion occurs, contacting monolayer leaflets merge and then clear from the site of contact. This clearing allows inner leaflets to come into contact and form a new single bilayer, known as a hemifusion diaphragm, that continues to separate aqueous compartments. Formation of a pore within this new bilayer diaphragm completes fusion. In contrast, if a pore composed solely of protein were to connect the two membranes, in a manner analogous to gap junctions, aqueous continuity occurs before lipid continuity.

Determination of the temporal sequence of continuities has been attempted by simultaneously measuring formation of fusion pores and spread of lipid dye between membranes. When HA-expressing cells are fused to red blood cells (RBCs), small fusion pores are clearly observed by electrical techniques before lipid dye spread. In fact, lipid dye is not observed to pass through the initial small pore (Tse et al., 1993; Zimmerberg et al., 1994). This would appear to support the position that aqueous continuity is established first (Tse et al., 1993; Lindau and Almers, 1995). But it is possible that lipid merger does precede pore formation, yet lipid cannot pass either through the site of local hemifusion or through the small pore. This would be the case if a ring of protein resides at the site of hemifusion (incorporating into the wall of the pore) and lipid is excluded from this ring (Chernomordik et al., 1998; Hernandez et al., 1996).

It is known that, after fusion of intact influenza virus with RBCs, movement of lipid dye between the two membranes is anomalously slow; the diffusion constant of lipid through the fusion pore is two orders of magnitude smaller than for diffusion in biological membranes (Georgiou et al., 1989; Lowy et al., 1990). That is, protein of the RBC, unrelated to fusion, may well be hindering lipid flux. In contrast, movement of lipid dye from virus to a planar phospholipid bilayer membrane is, after fusion, unimpeded, with a diffusion constant characteristic of that of membranes (Niles and Cohen, 1991). Because RBC membranes–but not phospholipid membranes–impede lipid flux for reasons that appear to be unrelated to fusion itself, we determined the temporal relation between lipid continuity and pore formation when cells expressing HA are fused to planar bilayer membranes. Even though detecting pores by electrical means is intrinsically more sensitive than is an observation of dye spread by video fluorescence microscopy, we found that lipid mixing occurs first.

Received for publication 31 March 1999 and in final form 6 September 1999.

Address reprint requests to Dr. Fredric S. Cohen, Department of Molecular Biophysics and Physiology, Rush Medical College, 1653 W. Congress Parkway, Chicago, Illinois 60612.

0006-3495/99/12/3144/08 $2.00

MATERIALS AND METHODS

Cell culture and treatment

HAb2 cells, a subclone of stably transfected National Institutes of Health-3T3 fibroblasts expressing the A/Japan/305/57 strain of influenza virus HA

(Doxsey et al., 1985), were provided by J. M. White (University of Virginia, Charlottesville, VA) and maintained as described (Melikyan et al., 1995a). CHO-K1 cells expressing either X:31 influenza HA (referred to as HA300a) or expressing the GPI-linked ectodomain of X:31 HA (BHA-PI cells) were maintained as described (Kemble et al., 1993; Kemble et al., 1994). For fusion experiments, cells were lifted from culture dishes and incubated with 0.1 mg/ml trypsin (TPCK treated, Sigma Chemical Co., St. Louis, MO), 0.2 mg/ml EDTA in phosphate buffered saline (PBS) for 4 min at 37°C. This cleaved the HA0 expressed on the cell surface into its fusion-capable HA1–HA2 form and enzymatically treated the cells, thereby increasing fusion activity (Melikyan et al., 1995a). The reaction was stopped by adding Dulbecco's minimal eagle's medium supplemented with serum. Fusion efficiency was further increased by incubating cells with 0.2 mg/ml neuraminidase (from *Clostridium perfringence*, type V; Sigma Chemical Co.) in PBS for 7 min at room temperature. Cells were washed three times, concentrated, and stored in PBS on ice and used for experiments within 6 h.

Planar membrane formation, cell–bilayer fusion, and time-resolved admittance measurement

Solvent-free horizontal lipid bilayers were formed from a solution of dioleoylphosphatidylcholine/dioleoylphosphatidylethanolamine (Avanti Polar Lipids, Inc., Pelham, AL) 2:1 (wt/wt) and 5 mol% of ganglioside G_{D1a} (Sigma Chemical Co.) with addition of either 5 mol% rhodamine-labeled phosphatidylethanolamine (Rho-PE, Avanti Polar Lipids) or 1,1′-dioctadecyl-3,3,3′3′-tetramethyl indocarbocyanin iodide (DiI, Molecular Probes, Eugene, OR) in squalene (Aldrich Chemical Co., Milwaukee, WI) that had been passed through an alumina column. The ganglioside G_{D1a} functioned as a binding receptor for HA (Suzuki et al., 1986). Planar membranes were formed in a 180-μm diameter hole in a Teflon film with a brush technique and were bathed in symmetrical 140 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1mM HEPES, pH = 7.0 maintained at 35–37°C. A small amount (~10 μl) of a concentrated cell suspension (~10^7 cells/ml) in PBS were added above the planar membrane and, within 30–50 sec, 15–20 cells rested upon the membrane. Four minutes after the cells established contact with the lipid membrane, fusion was triggered by lowering the pH of the top, cell-containing, solution to 4.9 by injecting 25 μl of concentrated isotonic succinate buffer directly over the bilayer. Fusion was monitored by changes in the bilayer admittance. The bilayer was voltage-clamped and a sine wave, superimposed on a holding potential of −20 mV, was applied as a command voltage to the bottom solution bathing the membrane. The in-phase (Y_0), out-of-phase (Y_{90}), and DC (Y_{DC}) components of admittance were calculated on-line with a software-based phase detector (Ratinov et al., 1998).

Fluorescence measurements and analysis

The horizontal bilayer chamber was mounted on a stage of an inverted microscope with a fluorescence attachment (Diaphot; Nikon, Garden City, NY). A neutral density filter attenuated the excitation light to minimize photobleaching. A standard filter set was used to monitor the fluorescence of DiI (ex 510–560 nm, dichroic mirror 580 nm, em > 590 nm). Fluorescence was monitored with a video camera (SIT-66; Dage MTI, Indianapolis, IN) set at maximum gain and was recorded on videotape.

Spread of dye from the planar membrane to cells was analyzed by digitizing images off-line from the tape recorder with a frame grabber (Meteor; Matrox Electronic Systems, Dorval, QC, Canada). Areas of interest that corresponded to particular cells on the bilayer were drawn; the average fluorescence intensities within the area were obtained with locally developed software (Qiao et al., 1999) based on a commercial C library (Matrox Imaging Library, Matrox) and plotted against time (Sigma Plot, Jandel Scientific, San Rafael, CA). Fluorescence and electrical signals were synchronized through a computer: upon acidification, the stroke of a keyboard set time = 0 for both the admittance measurements and a time–date generator (Video Timer VTG-33, Four-A Corp., West Newton, MA) that stamped the time on each video frame.

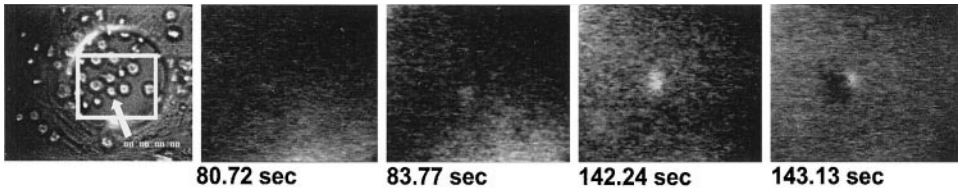
RESULTS AND DISCUSSION

Lipid continuity is established before fusion pore formation

Between 15 and 20 HA-expressing (HAb2) cells were settled on voltage-clamped horizontal planar membranes (Fig. 1, *first image*) and fusion was triggered by lowering (to 4.9) the pH of the solution that contained the cells. A high concentration of fluorescent lipid dye (5 mol%) was included in planar membranes and its spread to cells as a result of hemifusion or fusion was monitored by video fluorescence microscopy. The planar membrane exhibited the background fluorescence of its lipid dye. When dye moved into a cell, its brightness would eventually exceed that of the planar membrane upon which it rested. Formation and enlargement of fusion pores were simultaneously measured by time-resolved electrical admittance measurements. As previously observed (Melikyan et al., 1995b), for Rho-PE as the fluorescent lipid probe, a fusion pore always appeared before observation of spread of the fluorescent dye into any of the adhered cells. We now report that, when DiI was included in the planar membrane as the lipid probe, the DiI was always observed ($n = 30$) to spread into at least one cell before any pores were electrically detected (see Note 1). In control experiments, we did not cleave HA0 with trypsin into disulfide-bonded HA1–HA2 subunits, because this cleavage is well known to be required for fusion activity. Neither fluorescence nor electrical activity were observed for 20 min after acidification ($n = 3$). All observed phenomena required HA0 cleavage.

After acidification, a variable time elapsed before an adhered cell started to become fluorescent (Fig. 1: 83.77 sec, the cell marked by the arrow in the bright-field image). The onset of DiI spread was determined by marking out a region of an image that contained the cell that became fluorescent, and piecewise fitting straight lines to the fluorescence within this region of interest before and after dye spread (Fig. 1, average intensity). The intersection of these two lines was taken as the moment for initiation of lipid dye spread (Fig. 1, the time until cell brightening, t_b, marked by an arrow). No fusion pore had yet formed by this initiation time, as shown by the pore conductance remaining at baseline. Shortly after the cell started to acquire dye, pore flickering began (Fig. 1, pore conductance; the flickering is shown with finer resolution in the inset). The time from acidification until the appearance of the first pore is denoted t_f. After opening, the conductances of a flickering pore remained in the range of 0.5–5 nS before pore closure. After several flickers, a pore fully enlarged (Fig. 1, full pore enlargement), to a conductance on the order of 1 μS (Melikyan et al., 1995a). We refer to a pore that fully enlarges as successful.

We established that lipid dye spread into at least one cell before pore formation by comparing, for each experiment,



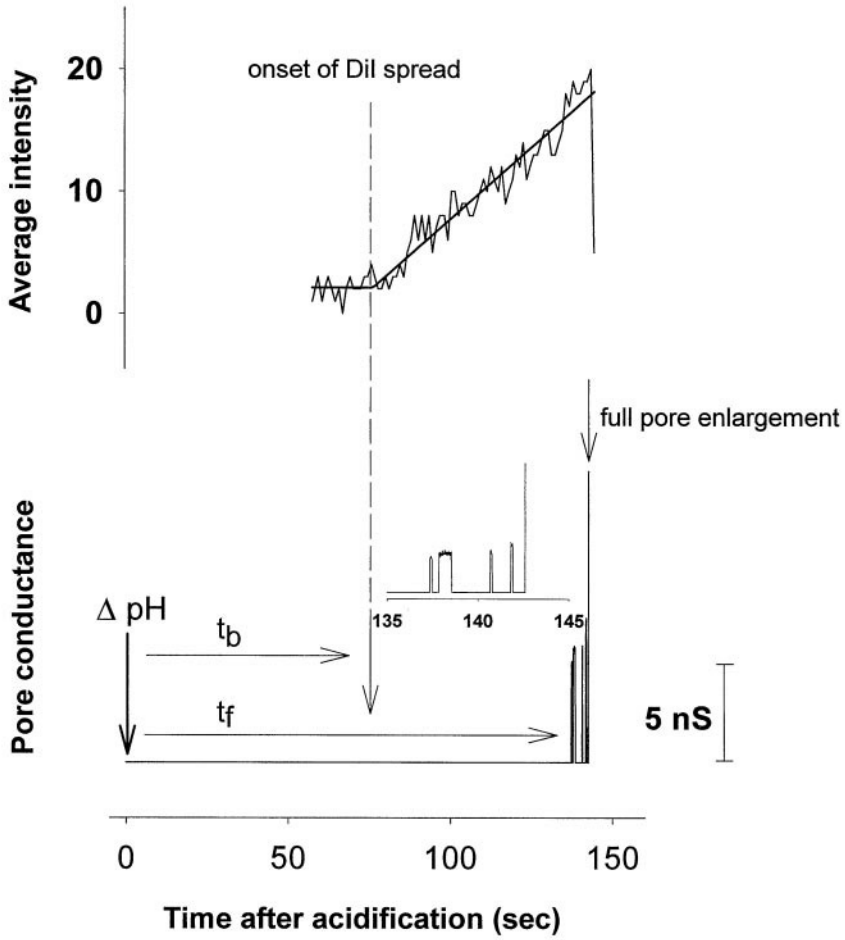


FIGURE 1 DiI spreads from a planar bilayer to a cell before formation of a fusion pore. Top panel, from left to right: Bright field image of HAb2 cells resting on a horizontal planar bilayer. The cell that became stained by DiI before pore formation is marked by an arrow. The region within the rectangle is shown in the following single video frames of fluorescence at the indicated times (as recorded by a date–time generator) after acidification: 80.72 sec, fluorescence before dye spread; 83.77 sec, brightening of the cell is first observed; 142.24 sec, the cell has continued to brighten; 143.13 sec, after full pore enlargement is observed electrically, the membrane under the cell is observed to darken. This may indicate flow of unlabeled lipid from the cell into the planar membrane. *Middle*, average intensity of fluorescence: Straight lines were fit piecewise to fluorescence intensity before and after dye spread. The intersection was taken as the moment dye spread was initiated. The darkening shortly after full pore enlargement results in a decrease in average intensity. *Bottom*, pore conductance: The cell became bright, at time t_b after acidification, before fusion pore formation, t_f. The pore flickers, between 135 and 145 sec, are shown with greater time resolution. The conductance scale bar refers to this inset.

the time from acidification until a cell started to brighten against the time from acidification until flickering (Fig. 2 *A*). The condition that $t_b = t_f$ is indicated by the dotted line. Clearly, for DiI, a cell always brightened before or simultaneously with any pore formation (Fig. 2 *A*, *filled circles*). Electrical detection of pores is much more sensitive than fluorescent assays of dye spread. There is considerable lag time between the moment lipid continuity is established and our ability to detect it by video microscopy: the spread of the dye is relatively slow, and a sufficient amount of dye must move into the cell to be detected by the video camera. Thus, at least one cell hemifused before the formation of any pore. In contrast, when Rho-PE was the fluorescent lipid probe, pore formation always occurred before dye was observed to spread (Fig. 2 *A*, *open circles*). This indicates that the movement of Rho-PE is considerably more restricted than is the movement of DiI.

Full pore expansion detected by the fluorescence signal

Because there are many cells adhered to the planar membrane, fusion pores could have formed in the cell that previously hemifused to the planar membrane or formed in another cell, one that had not hemifused. It cannot be

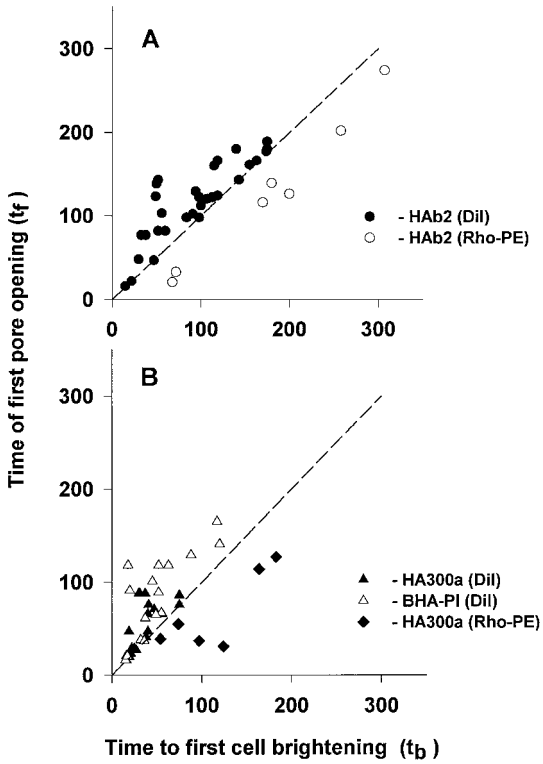


FIGURE 2 The temporal relation between spread of lipid dye (t_b) and formation of a fusion pore (t_f). (*A*) HAb2 cells. With DiI in the planar membrane (*closed circles*), dye was always observed to spread before or coincident with pore formation. In contrast, for Rho-PE as the fluorescent lipid probe (*open circles*) pore formation always occurred first. The ability of a lipid to pass through a hemifusion diaphragm depends on the probe used. (*B*) HA300a and BHA-PI cells. The time of initiation of dye spread caused by full length (HA300a) and GPI-linked ectodomain of HA (BHA-PI) were compared to the time of the first electrical change. HA300a and BHA-PI cells express the same strain of HA (subtype H3, strain X31) in the same cell line (CHO cells). For BHA-PI cells (*open triangles*), DiI spread before or coincident with the appearance of any changes in electrical admittance. The pattern of admittance changes for BHA-PI cells was characteristic of membrane leaks rather than fusion pores. For HA300a cells, DiI spread before, but Rho-PE after, pore formation.

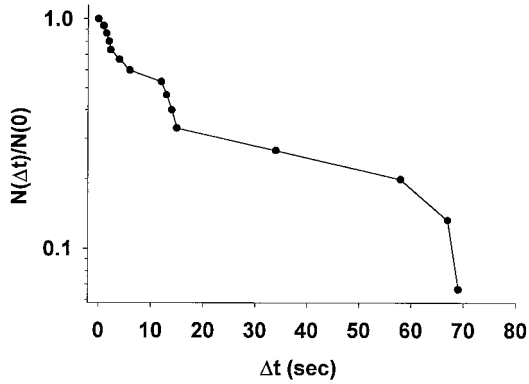


FIGURE 3 The distribution of differences in time, Δt, between the onset of flickering and the brightening of a second cell. A cell always brightened before any flickering. In $N(0) = 15$ experiments, after a cell brightened, pore flickering was observed electrically, and then a second cell was observed to brighten. The number of experiments, $N(\Delta t)$, in which the time difference exceeded the indicated Δt was determined and normalized by $N(0)$. When the time difference Δt was long, flickering was probably associated with the first hemifused cell.

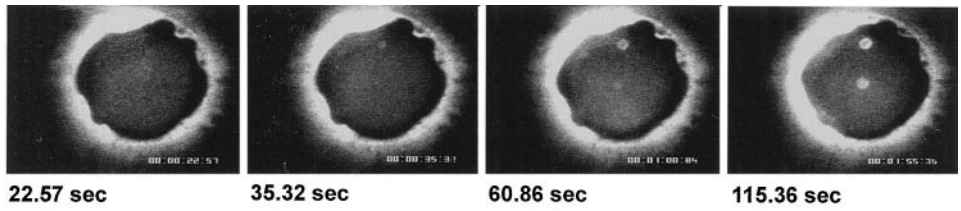
rigorously established that pore flickering occurred in a previously hemifused cell. However, if the flickering pores formed in a different cell, one would expect that the new cell would become bright soon after the onset of flickering. But this was not, in general, observed. In 15 of the 30 experiments performed, one cell was observed to brighten, flickering followed, and then a second cell brightened. In about half of these 15 cases, the delay (Fig. 3, Δt) between the onset of flickering and brightening of the second cell exceeded 10 sec, and, in some cases, this time difference was on the order of a minute (Fig. 3). In these latter cases, it is likely that a flickering fusion pore formed between the first cell that brightened and the planar membrane.

We were able to clearly establish, however, that a fully enlarging fusion pore could occur between the planar membrane and a cell that previously hemifused. For about 25% of the cells that hemifuse before the occurrence of any pores (7 out of 30 for HAb2 cells and 5 out of 19 for HA300a cells), a pore results that fully expands. We showed this by noting that, when a pore fully enlarges, as measured by electrical admittance, a darkening of the planar membrane is immediately observable at the site of a cell (Melikyan et al., 1995b). For example, in Fig. 1, the fluorescence of a cell increased before the occurrence of any pores, demonstrating hemifusion. Soon after a pore was electrically observed to fully enlarge, a dark spot formed at the site of the hemifused cell (Fig. 1, 143.13 sec) and this dark spot quickly expanded, reaching its maximal size with a half-time of about a second. This darkening can be accounted for by the tension of the planar membrane as it pulls on the membrane of the fused cell. This pulling causes unlabeled lipid of the cell membrane to flow—not simply diffuse—into the planar membrane, thereby quickly displacing fluorescent lipid molecules with nonfluorescent ones (see Note 2). Thus, cells that have hemifused to bilayer membranes and exhibit lipid dye transfer can support subsequent formation of a successful pore. We do not, however, presently have a means to determine whether the successful pore formed within the previously formed hemifusion diaphragm that supported movement of lipid between membranes, or whether the successful pore formed at a site outside the diaphragm.

The ability of a successful pore to form between an HAb2 cell and phospholipid membranes after lipid dye has spread is distinctly different for the case in which an RBC is the target. In the latter case, when lipid dye movement is observed (without pore formation), fusion pores do not subsequently form; it has been proposed that lipid dye spreads in the absence of pores only if the hemifusion diaphragm expands (Chernomordik et al., 1998). That is, a state of hemifusion that permits mixing of lipids between RBCs and cells expressing HA does not lead to successful pores.

Formation of fully enlarging pores for hemifused cells requires the transmembrane domain of HA

The hemifusion diaphragm should be unusually structured and subjected to an uncommon array of forces: the leaflet derived from the planar bilayer would be under the tension of the planar membrane, whereas the leaflet derived from the cell would not (in fact, it should be compressed). It was therefore possible that successful pores occurred for hemifused cells because of instabilities of the hemifusion diaphragm. The chosen lipid composition or inclusion of DiI could have led to a diaphragm prone to breakage, which would be electrically recorded as full pore enlargement. If this were the case, hemifusion would require HA, but a lipidic pore could form within an extended diaphragm, with pore formation per se thus independent of HA. We tested for this possibility by using cells that express the glycosylphosphatidylinositol (GPI)-linked ectodomain of HA, referred to as BHA-PI cells (Kemble et al., 1993). These cells induce hemifusion without the appearance of successful pores when planar bilayers are the target (Melikyan et al., 1995b). We found that DiI spread from the planar bilayer membrane to BHA-PI cells (Fig. 4, *upper panel*), but successful pores did not form, as determined by both electrical measurements (Fig. 4, *admittance traces*) and video fluorescence microscopy (Fig. 4, *fluorescence traces*). For BHA-PI cells, there was never clear evidence of pore formation before hemifusion—fluorescence always increased before changes in electrical admittance. DiI spread more rapidly into hemi-



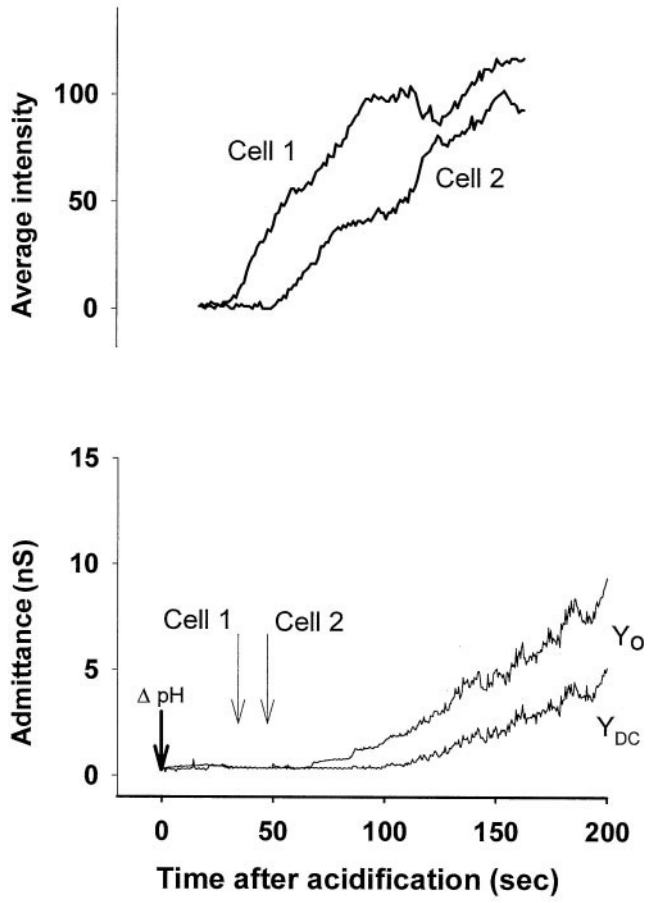


FIGURE 4 DiI spread and electrical changes with BHA-PI cells. *Top panel*, single-frame fluorescence images at the indicated times after acidification: 22.57 sec, fluorescence before dye spread; 35.32 sec, cell 1 begins to brighten; 60.86 sec, cell 1 has become brighter and cell 2 starts to brighten; 115.36 sec, the fluorescence of DiI in cells 1 and 2 has become quite bright. The thick supporting Gibbs-Plateau border (the torus) always contains a large amount of dye and is therefore very bright. *Upper traces*, average intensity—the time course of fluorescence increases in cells 1 and 2. About 115 sec, the average intensity assigned to cell 1 decreased slightly and temporarily due to cell movement somewhat out of the area of interest used to calculate average intensity. *Lower traces*, the admittance of the bilayer increased after cells 1 and 2 started to brighten. $Y_0 - Y_{DC} > 0$, showing that current flows into the cells. Y_0 increased in a smooth fashion, without exhibiting the stepwise increases that characterize the formation of fusion pores. The onset of dye spread into cells 1 and 2 are indicated with arrows.

fused BHA-PI cells than into HAb2 cells that hemifused before pore formation. This can be seen from the higher rate of increase of brightness intensity for BHA-PI cells (Fig. 4, *average intensity*) than HAb2 cells (Fig. 1). Thus, the barriers against DiI movement through the hemifusion diaphragms are different for the two cells.

After the dye spread, there was a slow increase in the in-phase component (Y_0) of admittance that was greater than increases in Y_{DC} (Fig. 4, *lower traces*). As described previously (Melikyan et al., 1995b), when $Y_0 > Y_{DC}$, there must be an electrical connection between the *trans* solution bathing the bilayer and the interior of the cell. But the increases in Y_0 and the out-of-phase component, Y_{90}, were generally smooth, without the stepwise increases that mark the opening of fusion pores. (Although successful pores did not form, there were isolated instances of admittance changes indicative of flickering pores.) The admittance patterns were characteristic of leaks, but not of fusion pores, in both the planar membrane and the hemifusion diaphragm. Also, darkening did not occur with BHA-PI cells and the large stepwise increases in Y_{90} that reveal the entire cell capacitance did not occur for BHA-PI cells. That is, fully enlarged pores did not form in BHA-PI cells that nevertheless allowed DiI spread (see Note 3). (But such pores did occur for wild type HA expressing HAb2 and HA300a cells that supported lipid dye spread, Fig. 2.) This shows that the entire HA trimer, including the transmembrane domain, is required for successful pores to form subsequent to lipid dye transfer.

The HAb2 cells express the A/Japan 57 strain of HA, an H2 subtype, whereas BHA-PI cells express the ectodomain of X31, an H3 subtype. To be certain that differences in subtypes was not the basis for the functional differences, we used cells that express on their surfaces full-length X31 HA, HA300a cells. As with HAb2 cells, DiI always spread into at least one HA300a cells before formation of any pores (Fig. 2 *B*, *closed triangles*). Also, as was the case with HAb2 cells, if Rho-PE was used as the fluorescent lipid probe, fusion pores always occurred before dye mixing (Fig. 2 *B*, *closed diamonds*). The difference in subtypes was immaterial to the appearance of fully enlarging pores after dye spread.

A greater propensity of dye to spread may be related to its ability to flip-flop

The question naturally arises as to why DiI mixing is observed before pore formation, whereas the opposite is observed for Rho-PE. There are many potential answers. A particularly important one that must be ruled out is that DiI spontaneously transferred from the planar bilayer to cell membrane as a result of close contact rather than membrane merger. In control experiments, we determined that DiI did not spontaneously transfer: We added palmitoyl-lysophosphatidylcholine (LPC) to the *cis* chamber at a final concentration of 20 μg/ml and waited 15 min to ensure complete equilibration before adding HAb2 cells. Out of 10 such experiments, fusion was blocked in seven of them for up to 6 min after lowering pH. (It proved difficult to wait longer than 6 min because the LPC tended to destabilize the bilayer. In the absence of LPC, fusion always occurred by this time [Razinkov et al., 1998].) In the three cases that fusion occurred in the presence of LPC, DiI spread before pore formation, as occurred when LPC had not been added. In the seven cases in which pore formation was absent, spread of lipid dye was also prevented (data not shown). Because LPC blocks HA-mediated fusion at a step before hemifusion without preventing the conformational changes of HA—including the insertion of its fusion peptide into target membranes—(Chernomordik et al., 1997), the planar and cell membranes should have come into molecularly close apposition. The results of these experiments strongly imply that DiI does not move from the bilayer to cell by spontaneous transfer through either the aqueous phase or as a result of direct membrane contact; true membrane merger is required.

The explanation we presently favor for DiI's more rapid movement than Rho-PE from the planar bilayer to the cell membrane is that DiI readily flip-flops between monolayers of a bilayer membrane, whereas Rho-PE does not (Melikyan et al., 1996). Therefore, when hemifusion is established, Rho-PE can only move from the bilayer to the cell by passage along the outer (connecting) monolayers. DiI, in contrast, can also (and should) flip-flop across the hemifusion diaphragm from the inner, noncontacting (nonmerged) monolayer of the planar bilayer to the inner cell monolayer. If this is what happens in practice, the DiI will continue to spread throughout the inner cell monolayer from which it can flip-flop to the outer cell monolayer. If fusion proteins restrict probe transfer through the outer but not the inner monolayer, probe flip-flop would provide the dominant route of transfer upon hemifusion. The fluorescent probe octadecylrhodamine B (R18) also flip-flops (Melikyan et al., 1996). Preliminary experiments indicate that dye transfer can precede pore formation for R18 as well (data not shown).

Is hemifusion a true intermediate of fusion pore formation?

It is now well established that HA can induce hemifusion. But it is still not established that hemifusion in fact occurs as a stage before fusion pore formation. In the case of fusion of HAb2 cells to RBCs, lipid dye cannot pass through the small fusion pores that form initially (Tse et al., 1993; Zimmerberg et al., 1994). This has been taken as evidence that lipid merger is not an obligatory step before pore formation (Tse et al., 1993; Lindau and Almers, 1995). Also, if lipid dye is observed to spread before pore formation, pores do not subsequently form (Chernomordik et al., 1998).

The present study demonstrates that, for planar phospholipid membranes as targets, lipid dye spread does not pre-

clude the subsequent formation of HA-mediated pores that fully enlarge. However, it is possible that these pores originated outside of the previously formed diaphragm. For this reason, simultaneous dye spread and electrophysiological measurements can never definitively establish, for any target membrane, that hemifusion is a bona fide intermediate of full fusion. New experimental techniques that can directly record whether or not the pore forms inside the diaphragm will be required.

The reason the chronological relation between pore formation and lipid dye spread is different for planar bilayer membranes than for RBCs is not clear. The HA trimers probably assemble into a ring and act cooperatively (Danieli et al., 1996; Blumenthal et al., 1996). If hemifusion is an intermediate stage of fusion, the trimers would create a hemifusion diaphragm within the ring's enclosure and then a fusion pore would form within the diaphragm (Nanavati et al., 1992; Hernandez et al., 1996; Chernomordik et al., 1998; Melikyan et al., 1999). It has been proposed that a tight association between the HA trimers of the ring would prevent lipid transfer even after the initial lipid merger of hemifusion has been achieved; lipid dye would spread in the absence of pores only if the trimers dissociated from each other, eliminating the trimer's ability to act cooperatively to form a pore within the diaphragm (Chernomordik et al., 1998). Alternatively, protein of the RBC unrelated to fusion may occlude the lipid passageways between HA trimers at the initial point of hemifusion. For planar bilayers as target, extraneous proteins are not present and lipid dye may consequently move more freely and/or the hemifusion diaphragm may be able to enlarge sufficiently to allow passage of lipid while still allowing HA trimers to form fusion pores.

A central question for understanding the mechanism of membrane fusion is whether hemifusion is an intermediate of fusion or whether the initial pore is formed solely by proteins, in analogy to gap junctions. The evidence in favor of a hemifusion mechanism is extensive: Lipid bilayer membranes can hemifuse with each other (Chanturiya et al., 1997; Lee and Lentz, 1997; Pantazatos and MacDonald, 1999). GPI-HA induces hemifusion (Kemble et al., 1994; Melikyan et al., 1995b; Blumenthal et al., 1996; Nüssler et al., 1997; Chernomordik et al., 1998). The ability of biological membranes to fuse depends on their lipid composition (Chernomordik et al., 1997). The initial conductance of fusion pores exhibit a broad distribution (Lanzrein et al., 1993; Plonsky and Zimmerberg, 1996), whereas gap junctions have narrow distributions (Wang and Veenstra, 1997). The transmembrane domain of HA can be substituted by those of nonviral proteins unrelated to fusion and pores still form, showing that this domain does not form a pore wall within the HA-expressing membrane as would be expected in a gap junction mechanism (Melikyan et al., 1999). The main evidence in favor of a gap junction type mechanism of fusion is that, with RBCs as target, lipid dye is not observed to spread until the fusion pore enlarges (Tse et al., 1993; Zimmerberg et al., 1994). The fact that, with planar bilayers as target, lipid dye spread can be observed before formation of a fusion pore that fully enlarges (shown in the present study) adds to the evidence in favor of a hemifusion mechanism.

We thank Ms. Sofya Brener for steady technical assistance, and Dr. Barry Lentz for suggesting the control experiments with LPC. Supported by National Institutes of Health grants GM 27367 and GM 54787.

NOTES

1. The fluorescence of DiI in the membrane is not strongly quenched (Melikyan et al., 1996). It was therefore somewhat surprising that the fluorescence increased as much as it did as a result of dye transfer. The increase occurs, at least in part, because the newly-acquired fluorescence of the cell superimposes on the background fluorescence of the bilayer below it. It appears that, for unknown reasons, fluorescence is greater when DiI is within the cell membrane than within the planar membrane.

2. The maximal diameter of the dark spot was usually somewhat larger than the cell diameter. The tension of the cell membrane equilibrates toward that of the planar membrane as the cell membrane is pulled into the planar membrane. Diffusion of lipid dye from the surrounding bilayer into the dark spot causes the spot to brighten. Because diffusive movement is slower than convective movement, brightening of the cell occurs with an appreciably slower time course than darkening, on the order of about one minute. The time course of darkening and the subsequent brightening of the cell is as expected theoretically for known membrane viscosity (Chizmadzhev et al., 1999).

3. When Rho-PE was used as the lipid dye, darkening would sometimes occur for BHA-PI cells (Melikyan et al., 1995b) concomitantly with a large step increase in Y_{90}. This indicates that the hemifusion diaphragm ruptured. We do not understand why this phenomenon occurs with Rho-PE but not DiI in the planar membrane.

REFERENCES

Blumenthal, R., D. P. Sarkar, S. Durell, D. E. Howard, and S. J. Morris. 1996. Dilation of the influenza hemagglutinin fusion pore revealed by the kinetics of individual cell–cell fusion events. *J. Cell Biol.* 135: 63–71.

Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley. 1994. Structure of influenza hemagglutinin at the pH of membrane fusion. *Nature.* 371:37–43.

Chanturiya, A., L. V. Chernomordik, and J. Zimmerberg. 1997. Flickering fusion pores comparable with initial exocytotic pores occur in protein-free phospholipid bilayers. *Proc. Natl. Acad. Sci. USA.* 94: 14423–14428.

Chernomordik, L. V., E. Leikina, V. Frolov, P. Bronk, and J. Zimmerberg. 1997. An early stage of membrane fusion mediated by the low pH conformation of influenza hemagglutinin depends upon membrane lipids. *J. Cell Biol.* 136:81–93.

Chernomordik, L. V., V. A. Frolov, E. Leikina, P. Bronk, and J. Zimmerberg. 1998. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. *J. Cell Biol.* 140:1369–1382.

Chizmadzhev, Y. A., D. A. Kumenko, P. I. Kuzmin, L. V. Chernomordik, J. Zimmerberg, and F. S. Cohen. 1999. Lipid flow through fusion pores connecting membranes of different tensions. *Biophys. J.* 76:2951–2965.

Danieli, T., S. L. Pelletier, Y. I. Henis, and J. M. White. 1996. Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. *J. Cell Biol.* 133: 559–569.

Doxsey, S. J., J. Sambrook, A. Helenius, and J. White. 1985. An efficient method for introducing macromolecules into living cells. *J. Cell Biol.* 101:19–27.

Georgiou, G. N., I. E. Morrison, and R. J. Cherry. 1989. Digital fluorescence imaging of fusion of influenza virus with erythrocytes. *FEBS Lett.* 250:487–492.

Hernandez, L. D., L. R. Hoffman, T. G. Wolfberg, and J. M. White. 1996. Virus–cell and cell–cell fusion. *Annu. Rev. Cell Dev. Biol.* 12:627–661.

Kemble, G. W., T. Danieli, and J. M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell.* 76:383–391.

Kemble, G. W., Y. I. Henis, and J. M. White. 1993. GPI- and transmembrane-anchored influenza hemagglutinin differ in structure and receptor binding activity. *J. Cell Biol.* 122:1253–1265.

Lanzrein, M., N. K. Esermann, R. Weingart, and C. Kempf. 1993. Early events of Semliki Forest virus-induced cell–cell fusion. *Virology.* 196: 541–547.

Lee, J., and B. R. Lentz. 1997. Evolution of lipidic structures during model membrane fusion and the relation of this process to cell membrane fusion. *Biochemistry.* 36:6251–6259.

Lindau, M., and W. Almers. 1995. Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr. Opin. Cell Biol.* 7:509–517.

Lowy, R. J., D. P. Sarkar, Y. Chen, and R. Blumenthal. 1990. Observation of single influenza virus–cell fusion and measurement by fluorescence video microscopy. *Proc. Natl. Acad. Sci. USA.* 87:1850–1854.

Melikyan, G. B., W. D. Niles, V. A. Ratinov, M. Karhanek, J. Zimmerberg, and F. S. Cohen. 1995a. Comparison of transient and successful fusion pores connecting influenza hemagglutinin expressing cells to planar membranes. *J. Gen. Physiol.* 106:803–819.

Melikyan, G. B., J. M. White, and F. S. Cohen. 1995b. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* 131:679–691.

Melikyan, G. B., B. N. Deriy, D. C. Ok, and F. S. Cohen. 1996. Voltage-dependent translocation of R18 and DiI across lipid bilayers leads to fluorescence changes. *Biophys. J.* 71:2680–2691.

Melikyan, G. B., S. Lin, M. G. Roth, and F. S. Cohen. 1999. Amino acid sequence requirements of the transmembrane and cytoplasmic domains of influenza virus hemagglutinin for viable membrane fusion. *Molec. Biol. Cell.* 10:1821–1836.

Nanavati, C., V. S. Markin, A. F. Oberhauser, and J. M. Fernandez. 1992. The exocytotic fusion pore modeled as a lipidic pore. *Biophys. J.* 63: 1118–1132.

Niles, W. D., and F. S. Cohen. 1991. Fusion of influenza virions with a planar lipid membrane detected by video fluorescence microscopy. *J. Gen. Physiol.* 97:1101–1119.

Nüssler, F., M. J. Clague, and A. Herrmann. 1997. Meta-stability of the hemifusion intermediate induced by glycosylphosphatidylinositol-anchored influenza hemagglutinin. *Biophys. J.* 73:2280–2291.

Pantazatos, D. P., and R. C. MacDonald. 1999. Directly observed membrane fusion between oppositely charged phospholipid bilayers. *J. Membrane Biol.* 170:27–38.

Plonsky, I., and J. Zimmerberg. 1996. The initial fusion pore induced by baculovirus GP64 is large and forms quickly. *J. Cell Biol.* 135: 1831–1839.

Qiao, H., R. T. Armstrong, G. B. Melikyan, F. S. Cohen, and J. M. White. 1999. A specific point mutant at position 1 of the influenza hemagglutinin fusion peptide displays a hemifusion phenotype. *Mol. Biol. Cell.* 10:2759–2769.

Ratinov, V., I. Plonsky, and J. Zimmerberg. 1998. Fusion pore conductance: experimental approaches and theoretical algorithms. *Biophys. J.* 74:2374–2387.

Razinkov, V. I., G. B. Melikyan, R. M. Epand, R. F. Epand, and F. S. Cohen. 1998. Effects of spontaneous bilayer curvature on influenza virus-mediated fusion pores. *J. Gen. Physiol.* 112:409–422.

Suzuki, Y., Y. Nagao, H. Kato, M. Matsumoto, K. Nerome, K. Nakajima, and E. Nobusawa. 1986. Human influenza A virus hemagglutinin distinguishes sialyloligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. Specificity for oligosaccharides and sialic acids and the sequence to which sialic acid is attached. *J. Biol. Chem.* 261: 17057–17061.

Tse, F. W., A. Iwata, and W. Almers. 1993. Membrane flux through the pore formed by a fusogenic viral envelope protein during cell fusion. *J. Cell Biol.* 121:543–552.

Wang, H. Z., and R. D. Veenstra. 1997. Monovalent ion selectivity sequences of the rat connexin43 gap junction channel. *J. Gen. Physiol.* 109:491–507.

Zimmerberg, J., R. Blumenthal, D. P. Sarkar, M. Curran, and S. J. Morris. 1994. Restricted movement of lipid and aqueous dyes through pores formed by influenza hemagglutinin during cell fusion. *J. Cell Biol.* 127:1885–1894.