Initial size and dynamics of viral fusion pores are a function of the fusion protein mediating membrane fusion

Ilya Plonsky1, David H. Kingsley2, Afshin Rashtian, Paul S. Blank and Joshua Zimmerberg3

Laboratory of Cellular and Molecular Biophysics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1855, U.S.A.

Background information. Protein-mediated merger of biological membranes, membrane fusion, is an important process. To investigate the role of fusogenic proteins in the initial size and dynamics of the fusion pore (a narrow aqueous pathway, which widens to finalize membrane fusion), two different fusion proteins expressed in the same cell line were investigated: the major glycoprotein of baculovirus Autographa californica (GP64) and the HA (haemagglutinin) of influenza X31.

Results. The host Sf9 cells expressing these viral proteins, irrespective of protein species, fused to human RBCs (red blood cells) upon acidification of the medium. A high-time-resolution electrophysiological study of fusion pore conductance revealed fundamental differences in (i) the initial pore conductance; pores created by HA were smaller than those created by GP64; (ii) the ability of pores to flicker; only HA-mediated pores flickered; and (iii) the time required for pore formation; HA-mediated pores took much longer to form after acidification.

Conclusion. HA and GP64 have divergent electrophysiological phenotypes even when they fuse identical membranes, and fusion proteins play a crucial role in determining initial fusion pore characteristics. The structure of the initial fusion pore detected by electrical conductance measurements is sensitive to the nature of the fusion protein.

Introduction
In biology, membrane fusion is the protein-mediated rearrangement of lipid structure that combines two surfaces into one surface; the process is crucial for intracellular membrane trafficking, fertilization, synaptic transmission and enveloped virus infection. Enveloped viruses infect target cells by either directly fusing with the plasma membrane or, after endocytosis, the endosomal membrane. The latter process occurs after exposure of viral fusion proteins to the acidic endosomal environment that triggers their transition to fusogenic conformations (Weisshorn et al., 1999). Since viral proteins are the best-characterized fusogenic macromolecules, they are extensively used in studies on membrane fusion (White, 1992; Hernandez et al., 1996).

The envelope glycoproteins of baculovirus and influenza, GP64 and HA (haemagglutinin) respectively, are both necessary and sufficient for ‘low pH’-triggered membrane fusion (White et al., 1982; Blissard and Wenz, 1992; Monsma et al., 1996). Current models postulate that to enable fusion, homotrimeric of both proteins aggregate in supramolecular structures of higher order (Blumenthal et al., 1996; Danielli et al., 1996; Plonsky and Zimmerberg, 1996; Chernomordik et al., 1998;
Kozlov and Chernomordik, 1998; Markovic et al., 1998; Chernomordik and Kozlov, 2005). When a conformational change is triggered in HA, an N-terminal hydrophobic sequence (the fusion peptide) is exposed and inserted into merging membranes (Weber et al., 1994). GP64 is known to undergo a low pH conformational change and an internal hydrophobic segment of GP64 (a putative fusion peptide) has been identified and was found to be crucial for fusion (Monsma and Blissard, 1995). Despite substantial progress in biochemistry, molecular biology and crystallography of fusion proteins, the mechanism of membrane merger upon protein activation has not been elucidated. Further progress requires a combination of approaches.

Currently, electrophysiological investigation provides the highest temporal and signal-to-noise resolution of membrane fusion pores. Dynamic changes in the electrical admittance allow calculation of the capacitance of fusing entities, such as granules, vesicles and cells (Neher and Marty, 1982). At early stages of membrane merger, two fusing objects are connected through a narrow aqueous pathway, defined as the fusion pore (Breckenridge and Almers, 1987) or exocytotic pore (Zimmerberg et al., 1987). The initial electrical conductance through this aqueous pathway can be modelled as a structure with a diameter on the order of nanometres. This initial defect may close, flicker or expand to a stable structure with a diameter in the order of 10–100 nm or larger. Structures of this size have been observed under the electron microscope (Chandler and Heuser, 1980; Ornberg and Reese, 1981) and the atomic force microscope (Schneider et al., 1997).

The structure observed in live-cell atomic force microscopy is called the porosome (reviewed in Jena, 2006). Ultrastructural imaging of the initial, nanometre-size defect remains an elusive goal; 4 nm structures have been identified in viral–liposome fusion using quick-freezing electron microscopy (Kanaseki et al., 1997). The relationship between these structures and the expanding and dynamic pore revealed through conductance measurements remains to be determined; the initial fusion pore is defined currently by its electrical properties. Fusion pore conductance can be derived from admittance data. Analysis of fusion pore properties yields information about the ionic permeability of the initial pore intermediate and the kinetics and reversibility of its formation and growth. These properties are determined by many factors, including the conformational structure of the fusion protein in the membrane, the composition of the membrane, the forces between fusing membranes and the membrane fusion pathway (Almers and Tse, 1990; Zimmerberg et al., 1991; Nanavati et al., 1992).

To begin to elucidate the contributions that each of these factors make in determining fusion pore ‘phenotype’, we have attempted to constrain the number of variables. Studying the fusion of GP64-bearing Sf9 cells with naïve Sf9 cells or human RBCs (red blood cells), the target membrane was found not to dictate fusion pore characteristics (Plonsky et al., 1999). In the present study, the role of the host membrane is examined, as the electrophysiological features of fusion pores created by acidification of either HA or GP64 were explored in one cellular system. Both glycoproteins were expressed in Sf9 cells with the same promoter, and RBCs were used as target cells. Initial fusion pore conductances induced by HA and GP64 were different and kinetics induced by HA and GP64 were different. These results support the hypothesis that the initial fusion pore conductance and kinetics are determined primarily by the fusion protein.

**Results**

Time-resolved admittance measurements were used to characterize fusion (Figure 1). Previously, the electrophysiology of GP64-mediated fusion to both naïve Sf9 cells and RBCs was described in detail (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999) with a membrane capacitance of $16.6 \pm 5.2 \mu F (n = 33)$, an input resistance of $1.8 \pm 0.6 \mu \Omega (n = 21)$ and series resistance of $4.8 \pm 2.6 \mu \Omega (n = 30)$ (mean ± S.D.; Plonsky and Zimmerberg, 1996). As demonstrated previously, the delivery of the acidic solution to GP64-expressing Sf9 cells with bound RBCs induced characteristic changes in cell admittance (Figure 2A). Theoretically, widening of the fusion pore leads to the reversible alteration in the $R_e$ (real) component and increase in the $I_m$ (imaginary) component to a plateau level. Indeed, $R_e$ and $I_m$ are calculated using the components of the membrane current, $I_m$, that are in-phase and out-of-phase with the command potential, $V_m$, respectively. $V_m$ is applied to $G_p$ (pore conductance) and $C_{m2}$ (RBC membrane capacitance).
Fusion pores depend on fusion proteins

Figure 1 | Principles of time-resolved admittance measurements

(A) A sketch of the Sf9–RBC aggregate with the low-pH-buffer-containing delivery pipette and the recording pipette, connecting cells to the patch-clamp amplifier. The amplifier applies the command sine wave potential, $V_m$, to the cells; the resulting current, $I_m$, is transferred to the phase-sensitive detector. The latter device computes the cell(s)–pipette admittance vector characterized by its co-ordinates in orthogonal axes, Re and Im (real and imaginary respectively). (B) Electrical equivalent circuit of fusing cells. Before fusion (the switch is off), the circuit consists of Sf9 cell elements: $G_{m1}$ (membrane conductance), $C_{m1}$ (membrane capacitance) and $E_r$ (resting potential). After fusion has occurred (the switch is on), the RBC subcircuitry with the elements $G_{m2}$ and $C_{m2}$ (RBC membrane conductance and capacitance respectively) becomes connected to the voltage-clamped Sf9 cells through the fusion pore. The related alterations in pipette–cells admittance allow us to calculate the $G_p$ (pore conductance), $C_{m2}$ and $G_{DC}$ (d.c. conductance).

capacitance) in series in such a way that, if the pore has low conductance (narrow), $V_m$ drops across $G_p$ and $I_m$ is not phase-shifted (in-phase). If the pore is wide, $V_m$ drops across $C_{m2}$. Because capacitors are phase-shifting elements, in this situation $I_m$ is mostly out-of-phase. Also, if membrane potentials of Sf9 cells and RBCs are not equal, the amplifier generates a current spike to clamp the RBC membrane to the holding potential applied to the Sf9 cell. This spike is displayed in the $G_{DC}$ (input conductance) trace (Breckenridge and Almers, 1987). GP64-mediated Sf9–RBC fusion was efficient; pores developed in all of the experiments ($N = 12$). Although fusion of cell pairs (transfected Sf9 cell with a single RBC) was studied, Sf9 cells were able to fuse with several RBCs (Figure 2); only the properties of the first pore were included in the analysis. GP64-induced pores formed rapidly; the waiting time between the delivery of the acidic solution and the formation of the pore, $t_w$, varied between 0.18 and 1.91 s with a median of 0.27 s. Initial pore conductance ($G_p$) was calculated as shown in the inset of Figure 2(B); GP64-induced pores had a mean ± S.D. of $1.4 ± 0.8$ nS (range: 0.6–2.9 nS, median 1.2 nS, $N = 12$). Flickering was not observed in any GP64-mediated fusion events. Pores always expanded beyond 10 nS (our arbitrary cut-off); the rapid increase in pore conductance, $G_p$, is depicted in Figure 2(B). The characteristics of GP64-induced pores matched those reported previously (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999).

HA-expressing Sf9 cells fused with RBCs in 34 out of 47 experiments. HA-induced pores had a mean ± S.D. of $0.8 ± 0.5$ nS (range: 0.2–2.1 nS, median 0.7 nS, $N = 31$). Flickering was observed in 29% of experiments. Further widening of the HA pore was
Figure 2 | GP64-induced fusion of a transfected Sf9 cell with two RBCs

(A) Pressure pulse delivery of the low-pH buffer (arrows) induced fusion pore formation with a short lag (∼0.2 s). Fusion was revealed as characteristic changes in real and imaginary components of the pipette–cells admittance. Transients in $G_{DC}$ are indicative of Sf9 cell fusion with two RBCs. Note that during the experiment, $G_{DC}$ did not change drastically, signifying that the recording was ‘tight’ (i.e. no corruptive changes in pipette–cells conductance occurred). (B) Alterations in the cells–pipette admittance, shown in (A), were converted into $G_p$ and $C_{m2}$. $G_p$ has risen beyond 10 nS in 0.04 s. The background noise in $C_{m2}$ trace was defined as zero. The total increment of the Sf9 cell capacitance was equal to 2.1 pF, as expected for two RBCs; calculations of $C_{m2}$ became more reliable with pore growing. Inset: the conductance of the first fusion pore ($G_p$) is displayed in a ms time scale. The abrupt opening of the pore was followed by its slower widening. Here and elsewhere, the initial conductance (2.8 nS) was calculated using linear regression of the recording segment adjacent to the abrupt pore opening.

Figure 3 | HA-induced fusion of a Sf9 cell with a bound RBC

Fusion was triggered 4.5 s before the pore opening (results not shown). $G_p$ has risen to 10 nS in 0.9 s. $C_{m2}$ was equal to 1.0 pF. Inset: the flickering initial pore ($C$) with concurrent changes in $G_{DC}$ (solid line) is shown in greater detail. Background $G_{DC}$ was subtracted. Pore closure (arrows) interrupted the electrical connection between fusing cells, making the $G_{DC}$ trace return to baseline. The conductance of the RBC exceeded the noise level and equalled 0.1 nS. The initial conductance of the pore was 0.8 nS.

The $G_p$ induced by HA and that induced by GP64 were different (Figure 4A). The CDFs (cumulative distribution functions) of $G_p$ were both described using a cumulative logarithmic normal distribution (where erfc is complementary error function):

$$CDF = 0.5 \times \text{erfc} \left[ \ln \left( \frac{G_p}{M} \right) / (W \times 2^{0.5}) \right]$$

with distribution midpoints ($M$) and width parameters ($W$), $M_{HA} = 0.67 \pm 0.01$ nS, $M_{GP64} = 1.11 \pm 0.02$ nS, $W_{HA} = 0.60 \pm 0.02$, $W_{GP64} = 0.53 \pm 0.04$, $N_{HA} = 31$ and $N_{GP64} = 12$. Using three measures describing $G_p$ statistics, HA-induced initial pores were significantly smaller than those induced by GP64: (i) $M_{HA}$ compared with $M_{GP64}$, $P < 0.01$, Figure 4(B); (ii) single factor ANOVA, $P < 0.004$; and (iii) $t$ test (equal variance) of log($G_p$), $P < 0.005$. The non-Gaussian distribution (long tail) of $G_p$ required logarithmic transformation in order to use the $t$ test; this property is reflected in the ability to characterize the distribution of $G_p$ with a logarithmic normal CDF.
Fusion pores depend on fusion proteins

Figure 4 | Initial fusion pores induced by different viral fusion proteins in the Sf9–RBC experimental system

(A) GP64-induced non-flickering, highly conductive initial pores (panels 1–4), whereas HA pores often flickered and had lower initial conductance (panels 5–7). Gp (C) is co-plotted with GDC (solid lines) for reasons explained in the legend of Figure 2. A lack of GDC transients in panels 2 and 6 signifies that in these experiments Sf9 cells fused with RBCs having resting potentials approx. −20 mV. Gradual closure and reopening of an HA pore is shown in panel 5. Repetitive opening of an HA pore with a small conductance (0.4 nS) is shown in panel 8. (B) CDF functions of the initial conductance of GP64- (○) and HA- (●) induced fusion pores and the cumulative logarithmic normal fits to the data. The median conductance values were 1.2 and 0.7 nS for GP64 and HA respectively.

Figure 5 | Kinetics of initial pore formation

Waiting times between triggering of fusion and initial pore formation are displayed as a survival probability semi-logarithmic plot (see the Materials and methods section for details of data handling). Compared with HA (○), the waiting times for GP64-induced fusion (●) were drastically shorter. Both datasets were fitted to the MPM (solid lines) with the number of elements, n, fixed at 11 and 3 for GP64 and HA respectively.

HA-induced fusion between Sf9 cells and RBCs had a substantial lag; the median waiting time between triggering and pore formation was 11.3 s (range: 1.6–94.7 s). The distribution of waiting times, t_w, was plotted as survival probability, P_t (Figure 5, see the Materials and methods section for details). The P_t curve, reflecting the kinetics of pore formation, had a quasi-sigmoidal shape; this shape was observed in earlier studies (Spruce et al., 1989). P_t was fit to the theoretical probability function, P(t), derived from the MPM (multi-element parallel model), with the multi-element parameter n = 3. The results of the fitting are shown in Figure 5. The following parameter values were obtained (+– S.E.M. of the estimate): n = 3 (fixed), k_1 = 0.33 ± 0.04 s⁻¹, k_−1 = 0.04 ± 0.00 s⁻¹, k_2 = 0.06 ± 0.00 s⁻¹. The value n = 3 that best describes HA-mediated fusion was smaller than the value obtained for GP64-induced pores (n≈11) following correction for the lag in triggering (Plonsky and Zimmerberg, 1996; and the Materials and methods section, the present paper). In the present study, with n = 11 fixed, the following parameter values were obtained for GP64-induced fusion: n = 11 (fixed), k_1 = 16.71 ± 1.36 s⁻¹, k_−1 = 0.08 ± 0.06 s⁻¹ and k_2 = 3.89 ± 0.62 s⁻¹. The forward rate constants, k_1 and k_2, for HA-induced fusion are significantly smaller than those observed for GP64-induced fusion.

Discussion

In the present study, we explored how two different viral envelope proteins fuse, on average, identical membranes. For this purpose, using the same promoter, the HA of the influenza virus and GP64 of
the baculovirus were expressed in the Sf9 system by transfecting cells with plasmids encoding these fusion proteins. Using admittance measurements, the admittance changes during membrane fusion mediated by HA of the influenza Japan strain (Spruce et al., 1989, 1991; Tse et al., 1993; Zimmerberg et al., 1994) and by GP64 of the Autograph californica multinuclear polyhedrosis virus baculovirus (Leikina et al., 1992; Plonsky and Zimmerberg, 1996; Plonsky et al., 1999) were recorded. HA-induced fusion pores had features resembling those previously described for the fusion of RBCs to HA-expressing fibroblasts, whereas GP64-induced pores had features closely resembling those described for the fusion of baculovirus-infected Sf9 cells. Essential features of HA-induced fusion pores, measured using the time-resolved admittance measurement technique, include smaller initial conductance ($M_{HA} \approx 0.7 \text{nS}$), pore opening and closure before final expansion (flicker), formed and dilated slowly or not at all, and long waiting times (median $t_w = 11.3 \text{s}$) after triggering. GP64-induced pores reveal different characteristics; their initial conductance is greater ($M_{GP64} \approx 1.1 \text{nS}$), they never flicker, they widen rapidly, and, compared with HA, they have much shorter waiting times (median $t_w = 0.27 \text{s}$) (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999; the present study). Prior to the present study, conceivable reasons for the observed divergence in pore phenotypes may have been due to membrane composition and/or fusion protein since different membranes were fused in previous HA and GP64 studies. However, in the present study, HA- and GP64-induced membrane fusion revealed pores with distinct characteristics while fusing, on average, identical membranes; this result is consistent with the hypothesis that the pore phenotype is a function of the protein mediating membrane fusion.

These data extend earlier findings that the target membrane has little impact on the GP64 pore phenotype (Plonsky et al., 1999). Based on the present study, we suggest that (i) the molecular dimensions of the initial fusion site are determined by the fusion protein, and not by the fusing membranes; (ii) different fusion proteins induce pores with distinct characteristics; and (iii) the kinetics of HA pore formation can be described using the MPM, with $n \approx 3$ elements and, compared with GP64, smaller forward rate constants, $k_1$ and $k_2$. Thus the properties of the initial fusion pore depend on its initiator, the fusion protein. These findings rule out the so-called proximity hypothesis, which suggests that proteins bring membranes into contact and the ensuing fusion is a spontaneous reaction of contacting membranes, the same for all proteins capable of bringing membranes together. However, these findings do support studies showing that the transmembrane domain of fusogenic proteins and the amino acid sequence of the fusion peptide play an important role in determining fusion pore formation (Kemble et al., 1996; Melikyan et al., 1995a; Han et al., 2004; Vaccaro et al., 2005). Thus fusogenic proteins play a more intimate role throughout the pathway of protein-mediated biological fusion (Almers and Tse, 1990; Lindau and Almers, 1995; Chernomordik et al., 1998; Kozlov and Chernomordik, 1998).

**Electrophysiological phenotype is determined by fusion proteins**

Identification and characterization of the initial fusion pore conductance are dependent on the temporal resolution of the technique used to record the electrophysiological events and the cell membrane. For example, the initial conductance of GP64-bearing Sf9 cells fusing with naive Sf9 cells measured using the double whole-cell recording (0.1 ms resolution) was significantly smaller compared with admittance measurements (1.0 ms resolution) ($1.0 \pm 0.3 \text{nS}$ versus $1.3 \pm 0.6 \text{nS}$; Plonsky and Zimmerberg, 1996). This difference in conductance, postulated to arise from differences in temporal resolution, suggests that the initial mean conductance of the HA-induced fusion pores measured using admittance, $\sim 0.7 \text{nS}$, may actually be $\sim 0.5 \text{nS}$ or less. This resolution-adjusted value is comparable with the $G_p$ reported for the HA-induced fusion pores between fibroblasts and RBCs [temporal resolution $\sim 1–10 \text{ms}$; Spruce et al., 1989 (HA-b2 and RBCs); Zimmerberg et al., 1994 (GP4F and RBCs)] but is larger than that reported by Spruce et al. (1991) ($\sim 0.15 \text{nS}$ using capacitance discharge, temporal resolution 0.1 ms, but $\sim 0.6 \text{nS}$ using admittance measurements with a temporal resolution of 3.0 ms) in the same cell pair, HA-b2 and RBCs. Thus the initial mean conductance of HA-expressing fibroblasts and Sf9 cells fusing with RBCs may differ by approximately a factor of 3. The difference in initial mean conductance and apparent sensitivity to temporal resolution may reflect the
Fusion pores depend on fusion proteins

Measurement technique and/or differences between fibroblasts and SF9 cells. Further uncertainty is raised by the current limitation on surface expression level, which may also affect pore phenotype (Clague et al., 1991; Melikyan et al., 1995b). What is important in the present study is that the same method (admittance measurement), having the same temporal resolution and other experimental properties, was used to evaluate two different viral envelope fusion proteins.

One difference between the membranes of insect and mammalian cells is their lipid composition. Yeh et al. (1997) indicate that insect SF9 cell membranes contain a high proportion of diacyl phosphatidylethanolamine and fatty acid chains, with high proportions of 16:1 and 18:1 unsaturated fatty acids and low proportions of polyunsaturated fatty acids, making them considerably different from mammalian cell membranes that contain high proportions of phosphatidylethanolamine composed of both the diacyl and alkenylacyl forms. In addition, SF9 cells are very low in cholesterol. Another compositional difference is that SF9 insect cells generally do not produce complex glycoproteins containing penultimate galactose or terminal sialic acid (Jarvis and Finn, 1996; Hollister et al., 1998). Studies with HA from an avian influenza A strain expressed in SF9 cells have confirmed this altered glycosylation pattern (Wagner et al., 1996). Membrane compositional differences and terminal sugar modifications of HA appear not to be dominant determinants of the electrophysiological phenotypes (initial conductance, ability to flicker, formation time and pore expansion) in HA-mediated cell fusion when compared with the electrophysiological phenotype in GP64-mediated cell fusion.

**Initial fusion pore formation: co-operative versus parallel kinetic models**

The process of viral fusion is believed to be enabled by aggregates consisting of a few fusion-active protein trimers (Bundo-Morita et al., 1988; Bentz et al., 1990; Ellens et al., 1990; Stegmann et al., 1990; Gauden et al., 1996; Plonsky and Zimmerberg, 1996; Markovic et al., 1998; Gibbons et al., 2004). In addition, the reaction lag-phase dependence on fusion protein surface density suggested a co-operative component in the mechanism for HA-mediated fusion (Morris et al., 1989; Ellens et al., 1990). Assuming that (i) all three fusion peptides in the HA trimer are activated together, and (ii) their successful insertion in the target membrane facilitates incorporation of additional trimers as components of the same fusion mechanism, Danieli et al. (1996) suggested that three to four HA trimers form the initial fusion site. It is important to note that the analysis performed by Danieli et al. (1996) did not preclude non-co-operative aggregation of HA trimers as a rate-limiting stage of fusion. Since the present study lacks manipulation of HA surface density, co-operativity was not tested.

The sigmoidal distribution of times between small and partially open HA-induced pores led Blumenthal et al. (1996) to use another approach that yielded the stoichiometry of fusion aggregates. Since small pores are not permeable to cytoplasmic dyes, trimers in the aggregate must move apart before dye transfers through fusing membranes (from the labelled to the unlabelled cell). Assuming that all trimers move independently (i.e. in parallel), these authors fit their data to a model first introduced for ionic currents (Hodgkin and Huxley, 1952). This approach yields six trimers for the initial fusion site at the beginning of its disassembly. The apparent controversy between conclusions on HA stoichiometry (three to four co-operative versus six parallel-acting trimers in the fusion mechanism) could indicate that additional trimers are recruited into the circumference of the fusion site, while the fusion pore begins to widen.

Studying GP64-induced fusion, we have found that the Hodgkin–Huxley formalism describes the quasi-sigmoidal distribution of the time required for pore formation (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999; the present study). In the present study, we suggest that for GP64-mediated fusion, (i) the time interval between acidification and pore formation reflects processes occurring after the formation of the aggregate; (ii) all GP64 molecules in the aggregate are activated independently; and (iii) yet another unknown process coincides with the peptides’ volley for fusion to occur. This unknown process is modeled as a transition of the model element b with rate constant $k_2$ and may reflect a lipid-dependent stage of fusion (Plonsky and Zimmerberg, 1996). Re-analysis of previous GP64 kinetics suggests that $n \approx 11$ elements (perhaps reflecting two to five trimers of GP64) assemble the initial fusion site. Compared with HA with $n = 3$ elements, the higher $G_p$ of GP64-induced fusion may be due to a difference in the stoichiometry of the initial fusion site: the GP64 fusion...
mechanism has more participating elements than that of HA.

Compared with GP64, a smaller number of participating elements or aggregates of HA may apply a weaker bending force to the fusing membranes. Therefore HA pore formation requires either a favourable thermal fluctuation(s) of the opposite membrane(s) or a local, permissive lipid composition where fusion peptides are inserted. These conditions are reflected in the $k_2$ value, which is much lower in HA-mediated fusion compared with GP64-mediated fusion [0.06 compared with 2.07 (Plonsky and Zimmelberg, 1996 re-analysis) or 3.89 s$^{-1}$ (the present study)]. Also, since the bending force provided by the HA aggregate is assumed to be weak, the fusion pore formation would be reversible (i.e. the pore flickers).

In summary, a kinetic model featuring multiplicity and independence of participating elements can describe the distribution of waiting times for HA- and GP64-mediated cell fusion. The differences in electrophysiological phenotype observed in HA- and GP64-mediated cell fusion may reflect the differences in the number of permissive elements in the fusion mechanism and the kinetic rate constants. Most importantly, the electrophysiological phenotypes observed are apparently dependent on the fusion protein and not upon the fusing membranes themselves.

Materials and methods

Cells, expression plasmids and transfection

Sf9 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured at 28°C in Grace’s insect cell medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were used only until their 25th passage. The expression plasmid pATH-1 was described previously by Kingsley et al. (1999). This plasmid utilized the OpMNPV (Orgyia pseudotsugata multinuclear polyhedrosis virus) GP64 early promoter to express the A:MNPV GP64 gene within Sf9 cells. In order to express HA in Sf9 cells, plasmid pHA-rhd was engineered by deletion of the GP64 coding sequences and insertion of HA sequences from the X31 strain of HA (Kingsley et al., 1999). Supercoiled plasmids were double purified by cesium chloride ultracentrifugation. Transfection of Sf9 cells was performed by the calcium phosphate method as described by Monsma and ultracentrifugation. Transfection of Sf9 cells was performed by Supercoiled plasmids were double purified by cesium chloride

Electrophysiological measurements

Fusion pore conductance and RBC capacitance were calculated using time-resolved admittance measurements as described previously (Ratinov et al., 1998; Plonsky et al., 1999), with modifications. A temperature that was permissive for fusion of cells mediated by both HA and GP64, 30 ± 1°C, was determined and used in all experiments. A 2 kHz, 50 mV sine wave was superimposed on the holding potential (~20 mV); the resulting signal ($V_m$) was applied to the patch-clamped Sf9 cell. The related current ($I_{p}$) was acquired and separated into 'in-phase', 'out-of-phase' and d.c. (direct current) components. The input conductance ($G_{Di}$), the real (Re) and the imaginary (Im) components of the cell-pipette admittance were calculated using a digital phase-sensitive detector (Figure 1A). The phase was adjusted using the automated Neher–Marty technique; fusion pore conductance ($G_p$) and RBC capacitance ($C_{meg}$) were calculated according to equations (55) and (56) respectively, derived in Ratinov et al. (1998) and introduced in Lindau (1991). Multiple RBCs were sometimes bound to the Sf9 cell. However, only the appearance of the first pore was evaluated; the properties of these other pores were not included in the reported results. For whole-cell recordings, internal and external solutions had the following composition: 130 mM potassium glutamate, 16 mM KCl, 2 mM MgCl$_2$, 10 mM Hepes and 5 mM EGTA (pCa = 8, pH 7.0) (internal); and 55 mM NaCl, 10 mM KCl, 4 mM CaCl$_2$, 5 mM MgCl$_2$, 5 mM glucose, 2 mM Hepes and 140 mM sucrose (pH 7.0) (external). Fusion was triggered by an acidic solution: 50 mM sodium citrate, 50 mM NaCl, 10 mM KCl, 4 mM CaCl$_2$, 5 mM MgCl$_2$, 5 mM glucose and 20 mM sucrose (pH 5.0). This solution was ejected from a micropipette by a pressure pulse. The pipette was placed approximately one cell diameter from the transfected Sf9 cell.

Fusion pore kinetics

The waiting time ($t_w$) was measured from the onset of the pressure pulse used to deliver the acidic solution until fusion pore opening. The $t_w$ points were displayed as a semi-logarithmic survival plot. For this purpose, the probability that a fusion pore has not yet appeared at time $t$ ($P(t)$) was defined as $1 − N_t/N_e$, where $N_t$ is the number of pores that have formed by time $t$ and $N_e$ is the total number of experiments (Melikyan et al., 1993). $P(t)$ was fit using the MPM, described previously (Plonsky and Zimmelberg, 1996). Briefly, MPM suggests that fusion occurs when $n$ elements of type $a$ and one element of type $b$, independently enter their permissive states $a^*$, $b^*$:

\[
\begin{align*}
& n \cdot a \quad \xrightarrow{k_{-1}} \quad n \cdot a^* \\
& b \quad \xrightarrow{k_1} \quad b^*
\end{align*}
\]

where $k_1$, $k_{-1}$ and $k_2$ are rate constants. This kinetic scheme leads to the following theoretical probability function, $P(t)$:

\[
P(t) = 1 - \left( k_1 \exp \left[ t (k_1 + k_{-1}) \right] \right) \frac{1 - \exp \left[ -(k_1 + k_{-1}) t \right]}{k_1 + k_{-1}}
\]

\[
\cdot [1 - \exp (-k_2 t)]
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Fusion pores depend on fusion proteins

Experimental data were fitted using TableCurve 2D, v5.00 (SPSS Inc., Chicago, IL, U.S.A.). Goodness of fit was evaluated using the F statistic, parameter standard error, R value and residuals. Since GP64-dependent fusion was examined previously (Plonsky and Zimmerberg, 1996; Plonsky et al., 1999), a limited dataset was collected. However, in the previous analysis, the lag time between the triggering of the pressure pulse and the delivery of the triggering solution to the cell was not considered. A conservative estimate for this lag time is 25 ms; maximum 10 ms valve response time (manufacturer’s specification), 10 ms pressure pulse propagation time, and 5 ms delivery time (of the order of the diffusion time) from pipette tip to cell. The data presented in Plonsky and Zimmerberg (1996; Figure 6) were re-analysed using the four-parameter MPM and a 25 ms lag time; 25 ms was subtracted from each time point. The parameter values obtained are \( k_1 = 5.74 \pm 0.50 \text{s}^{-1}, k_{-1} = 0.06 \pm 0.01 \text{s}^{-1}, k_2 = 2.07 \pm 0.19 \text{s}^{-1}, n = 11.08 \pm 2.17 \); this is in contrast with the previously published values, \( k_1 = 6.70 \pm 0.42 \text{s}^{-1}, k_{-1} = 0.03 \pm 0.001 \text{s}^{-1}, k_2 = 1.78 \pm 0.008 \text{s}^{-1}, n = 19.2 \pm 3.82 \). Sensitivity analysis indicates that \( k_1 \) and \( k_2 \) have a weak dependence on lag-dependent changes in the number of elements, \( n \), and that the lag time would have to be \( \sim 90 \) ms for the data presented in Plonsky and Zimmerberg (1996) to be described by \( n = 3 \). In the present study, the multielement parameter, \( n \), was set to 11, the value obtained from re-analysis of the previous dataset; this parameter reduction was necessary to fit the reduced number of fusion events examined in the present study. HA-dependent fusion was modelled using integer \( n \) values (1–20). The \( n = 3 \) fit maximized the F statistic and was used to characterize the model rate constants.

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References


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