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The membrane proteins of flaviviruses form ion-permeable pores in the target membrane after fusion: identification of the pores and analysis of their possible role in virus infection

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Recently, we presented evidence that the E1 fusion protein of the alphavirus Semliki Forest virus forms ion-permeable pores in the target membrane after fusion. We proposed that the homologous fusion proteins of flaviviruses and hepatitis C virus form similar pores. To test this hypothesis for the E fusion protein of flaviviruses, the release of [^{3}H]choline from liposomes by the flavivirus West Nile (WN) virus was determined. [^{3}H]Choline was released at mildly acid pH. The pH threshold depended on the lipid composition. Release from certain liposomes was activated even at neutral pH. To identify the generation of individual pores, single cells were investigated with the patch-clamp technique. The formation of individual pores during low pH-induced WN virus entry at the plasma membrane occurred within seconds. These experiments were performed in parallel with Semliki Forest virus. The results indicated that, similar to alphavirus infection, infection with flaviviruses via endosomes leads to the formation of ion-permeable pores in the endosome after fusion, which allows the flow of protons from the endosome into the cytoplasm during virus entry. However, *in vitro* translation experiments of viral cores showed that, in contrast to alphaviruses, which probably need this proton flow for core disassembly, the genome RNA of WN virus present in the viral core is directly accessible for translation. For entry of flaviviruses, therefore, a second pathway for productive infection may exist, in which fusion of the viral membrane is activated at neutral pH by contact with a plasma membrane of appropriate lipid composition.

INTRODUCTION

An early step during infection by enveloped viruses is the fusion of the viral envelope with a cellular target membrane. This fusion is thought to be a non-leaky process and results in the formation of a large fusion pore, which allows the interior structure of the virus but no further ions or molecules to enter the cytoplasm. The reaction is regulated by virus-encoded fusion proteins, which can be classified according to the protein structure, the signal that triggers fusion, the presence of a terminal fusion peptide and other properties (for a review, see Hernandez *et al.*, 1996). Recently, we have studied the early processes of infection by the alphaviruses Semliki Forest (SF) virus and Sindbis (SIN) virus. Alphaviruses are small, enveloped, plus-strand RNA viruses (for reviews, see Strauss & Strauss, 1994; Schlesinger & Schlesinger, 2001). These viruses contain on their surface 80 spikes, each composed of three heterodimers of the viral proteins E1 and E2, and it has been shown that the E1 protein is the viral fusion protein (for a review, see Kielian, 1995). By using the plasma membrane as the site of virus entry and by patch-clamp analyses of membrane currents occurring during this process, we have obtained evidence that the viral membrane proteins form, after fusion, an ion-permeable pore in the target membrane (Wengler *et al.*, 2003) that is different from the fusion pore. *In vitro* analyses of the disassembly of alphavirus cores indicated that a flow of protons through this pore from the endosome into the cytoplasm may be involved in the regulation of disassembly of alphavirus cores *in vivo* (Wengler & Wengler, 2002). These results and important earlier experiments (Lanzrein *et al.*, 1993; Dick *et al.*, 1996; Nyfeler *et al.*, 2001) led us to conclude that the E1 protein of alphaviruses is not only responsible for fusion but probably has an additional function, namely the ability to form an ion-permeable pore in the target membrane after fusion (Wengler *et al.*, 2003).

The atomic structure of the E1 fusion protein of SF virus has recently been determined (Lescar *et al.*, 2001). Unexpectedly, these data showed that this protein is structurally homologous to the E fusion protein of flaviviruses (Rey *et al.*, 1995). Furthermore, sequence homology analyses have indicated that the E protein of hepatitis C virus is structurally homologous to these proteins (Yagnik *et al.*, 2000). These data suggest that the fusion proteins of flaviviruses and hepatitis C virus may also

form ion-permeable pores in the target membrane after fusion. The experiments reported herein analyse this question for the E protein of flaviviruses using the flavivirus West Nile (WN) virus. The experiments identify the formation of ion-permeable pores during WN virus entry at the plasma membrane and analyse the possible role of these pores during multiplication of alpha- and flaviviruses.

METHODS

Growth of cells and preparation of viruses. WN virus was grown in BHK-21 cells and purified by centrifugation as described previously (Castle *et al.*, 1985). Growth and purification of alphaviruses were as described previously (Wengler *et al.*, 1999). *Aedes albopictus* C2 insect cells (Singh, 1967) were grown at 28 °C and were adapted in our laboratory to Dulbecco's modified Eagle's medium (DMEM) with 4 % foetal bovine serum and 2 % Yeastolate (Wengler *et al.*, 1978). Vero cells were grown in DMEM with 5 % foetal bovine serum.

Preparation of [^{3}H]choline-loaded liposomes. Liposomes were prepared as described previously (Wengler *et al.*, 2003), but the procedure was scaled down and liposomes were loaded with [^{3}H]choline during their preparation. Liposomes were prepared from phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SPM) and cholesterol. These lipids (350 μg) in the proportions indicated in the experiments were solubilized in 10 μl 1-propanol. [^{3}H]Choline (40 μCi) was dried down and solubilized in 150 μl buffer containing 50 mM NaCl, 10 mM Tris/HCl, pH 8·1. This buffer and the lipid solution were kept at 50 °C and 7·5 μl of lipid was injected through a microinjection loading pipette (Eppendorf) into the buffer containing [^{3}H]choline under stirring. After incubation at 50 °C for 1 min and at 22 °C for 5 min, the opalescent solution was loaded on to a column containing 9 ml Sephadex G-25 in 50 mM NaCl, 10 mM Tris/HCl, pH 8·1. The liposomes eluted in the excluded volume and contained about 0·1 % of the radioactivity. Liposomes were stored on ice and used within 5 days.

Assay of [^{3}H]choline release from liposomes. The assay was performed as described previously (Wengler *et al.*, 2003). In short, for each reaction 30 μl 50 mM NaCl, 10 mM Tris/HCl, pH 8·1, containing about 20 μg UV-inactivated virus and 5 μg liposomes was incubated at 30 °C in a 10K Nanosep ultrafiltration vial. The reaction was adjusted to the desired pH by addition of 10 μl MES buffer. After 15 min incubation, the reaction vials were centrifuged, and radioactivity released from liposomes was recovered in the filtrate.

Patch-clamp analysis of membrane permeability. Patch-clamp measurements (Hamill *et al.*, 1981) were performed as described previously (Wengler *et al.*, 2003). In short, for whole-cell patch-clamp analysis, cells were incubated for 5–10 min in extracellular solution (140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7·4, adjusted with NaOH). The recording pipette contained an intracellular solution composed of 140 mM potassium glutamate, 10 mM NaCl, 2 mM $MgCl_2$ and 10 mM HEPES, pH 7·3, adjusted with KOH. Pellets containing UV-inactivated WN virus particles, which had been stored at −80 °C, were suspended in extracellular solution containing 2 % Ficoll 400 to a concentration of 100 μg E protein ml^{-1} and used for adsorption. Virus had been inactivated by UV illumination at 254 nm for 8 min at 4 °C. The same suspension and inactivation were performed for SF virus particles, which contained 100 μg E1 protein ml^{-1}. No infectivity was detected in 50 μl of virus suspension by ID_{50} measurements using the cytolytic effect of SF and WN virus in BHK cells. Virus suspension (40 μl) was transferred to the surrounding of the patch-clamped cell. This suspension contained about 4 μg of the viral fusion protein. After 3 min incubation at 20 °C for virus adsorption, 100 μl of low pH solution consisting of extracellular solution with 3 % Ficoll 400, buffered either with 10 mM HEPES at pH values between 8·0 and 6·2, or with 10 mM MES at lower pH, was applied to the surrounding of the patch-clamped cell.

Determination of intracellular Ca^{2+} concentration by fluorescence microscopy. The intracellular concentration of Ca^{2+} was determined ratiometrically using the fluorescence of Fura-2 (Silver, 1998). BHK cells grown on glass coverslips were loaded with Fura-2 by incubation in extracellular solution containing Fura-2-AM (5 μg ml^{-1}) and Pluronic F-127 (10 μg ml^{-1}) for 20 min at 37 °C. Cells were then observed by fluorescence microscopy in the presence of extracellular solution. Excitation was at 340 and 380 nm. Emission was determined at 510 nm. The intracellular Ca^{2+} concentration was determined ratiometrically from these data using the Metafluor imaging software.

***In vitro* translation.** A master mix containing 100 μl micrococcal nuclease-treated rabbit reticulocyte lysate (Promega), 3 μl unlabelled amino acids (1 mM) without methionine, 5 μl 10 % (v/v) NP-40 in water and 50 μCi [^{35}S]methionine was divided into five identical aliquots. To these aliquots on ice was added either 5 μl water, 5 μl 42S WN genome RNA (2·5 μg), 5 μl WN virus suspended in water (containing about 20 μg E protein), 5 μl SF genome RNA (2·5 μg) or 5 μl SF virus suspended in water (containing about 20 μg E1 protein). The reactions were incubated at 30 °C and aliquots of 4 μl were removed after incubation for 20, 40, 60 and 80 min and subjected to SDS-PAGE. The gel was stained with Coomassie blue and subjected to autoradiography.

RESULTS

Release of [^{3}H]choline from liposomes by WN virus

A reasonable starting point for the development of an assay of the ability of the membrane proteins of flaviviruses to form a pore in the target membrane after fusion was to develop a liposome preparation that would release [^{3}H]choline from the liposomal interior after fusion with virus particles. Recently, we have used this approach with alphaviruses (Wengler *et al.*, 2003). The fusion of flaviviruses with liposomes has been studied previously (Gollins & Porterfield, 1986; Corver *et al.*, 2000; Stiasny *et al.*, 2001). Fusion of flaviviruses with liposomes composed of PC, PE, SPM and cholesterol has already been obtained at mildly acid pH. Following these lines, we prepared different liposomes composed of PC, PE, SPM and cholesterol in varying proportions containing [^{3}H]choline in the interior aqueous phase, and analysed the release of [^{3}H]choline in the presence of WN virus as a function of pH. BSA, which should not release [^{3}H]choline, and SF virus, which forms pores in liposomes (Wengler *et al.*, 2003), were also analysed in these assays. A pH-dependent release of [^{3}H]choline in the presence of WN virus was detected. A typical reaction is shown in Fig. 1(A). Maximal release of [^{3}H]choline from liposomes containing all four lipids in equimolar concentrations occurred at pH 6·5 in the case of WN virus particles. At this pH, no release was obtained by SF virus particles, which have a threshold for release of pH 6·0. These data are in accordance with the earlier experimental findings

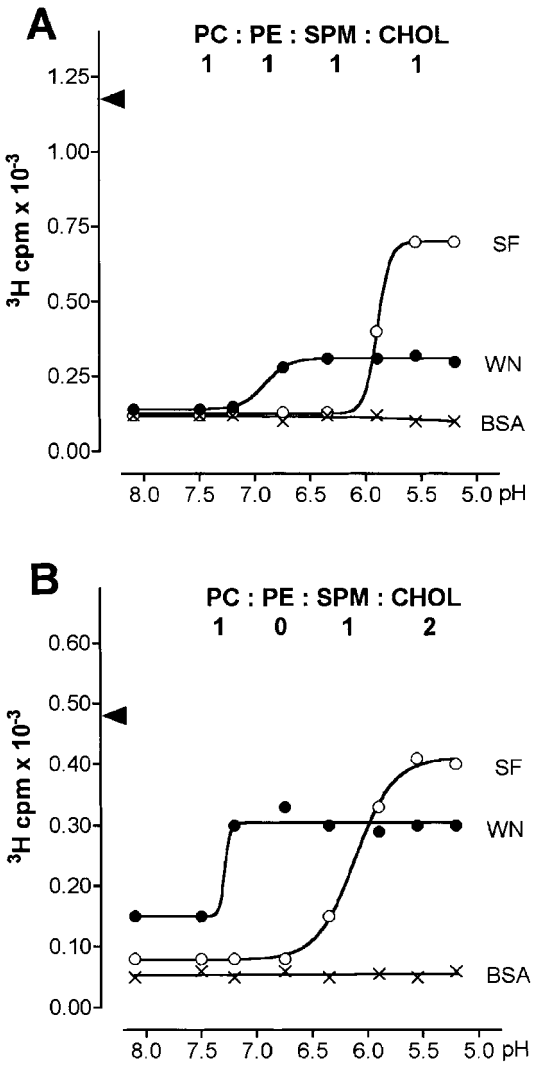


Fig. 1. WN virus particles release soluble molecules from liposomes. Solutions containing [^{3}H]choline-loaded liposomes and either BSA, SF virus or WN virus at pH 8·1 were prepared. Aliquots of these solutions were adjusted to a series of pH values between pH 8·0 and 5·0 and incubated at 30 °C for 15 min. [^{3}H]Choline release from the liposomes during this incubation is shown. The total radioactivity present in each reaction is indicated by the filled triangle on the vertical scale. Liposomes containing PC, PE, SPM and cholesterol in the relative molar proportions of 1 : 1 : 1 : 1 and 1 : 0 : 1 : 2 were present in the reactions shown in (A) and (B), respectively. Experimental details are given in Methods.

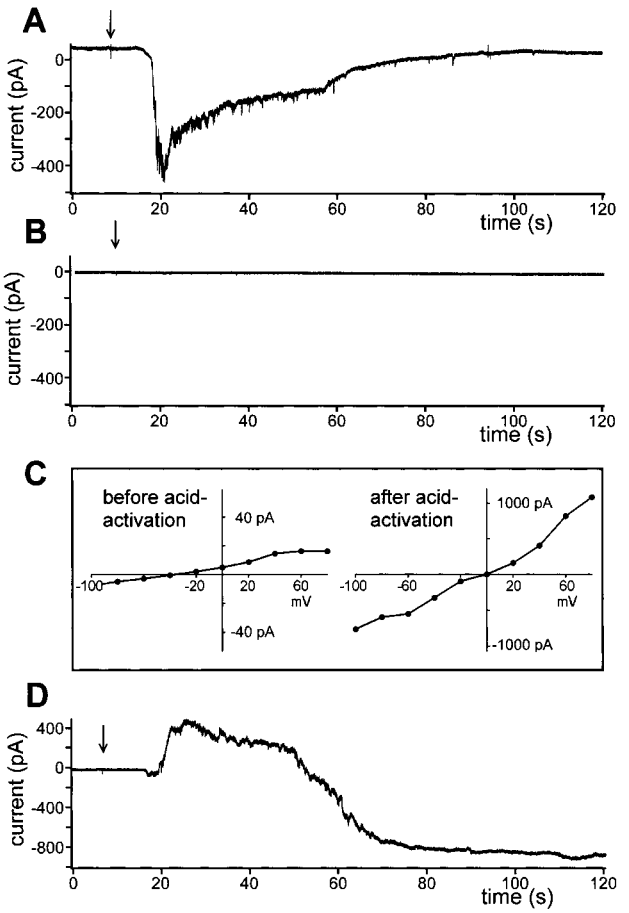


Fig. 2. Infection of vertebrate cells by WN virus at the plasma membrane is accompanied by opening and closure of ion-permeable pores. Original membrane current recordings are shown in (A), (B) and (D). In all experiments, cells were subjected to the whole-cell configuration of the patch-clamp technique, followed by adsorption of virus and treatment with pH 5·4 solution, as described in Methods. The time of addition of the pH 5·4 solution is indicated by an arrow. Holding membrane potential was −40 mV. (A) Membrane current of a Vero cell after addition of WN virus containing 4 μg E protein and low pH treatment. (B) Membrane current recorded in a control experiment. WN virus particles were pretreated with pH 5·4 solution for 2 min at room temperature prior to the addition to Vero cells. (C) Current–voltage relationships measured in an experimental setting as shown in (A). Data were recorded before acid activation and 10 s after low pH activation. Membrane current amplitudes were measured at the end of voltage pulses from −100 to +80 mV in 20 mV steps lasting for 200 ms. Between every step the potential was switched back for 100 ms to the holding potential of −40 mV. (D) Membrane current of a BHK-21 cell after addition of WN virus containing 4 μg E protein and low pH treatment.

cited above. The release of [^{3}H]choline by WN virus was always less efficient than the release generated by SF virus. Furthermore, the analysis showed that the threshold for [^{3}H]choline release by WN virus was dependent on the lipid composition, in contrast to the situation for alphaviruses (data not shown). These results led to the question of whether WN virus might release [^{3}H]choline from appropriate liposomes that were already at neutral pH. The data presented in Fig. 1(B) showed that this was the case for liposomes containing PC, PE, SPM and cholesterol in the molar proportions 1 : 0 : 1 : 2. In our analysis of [^{3}H]choline

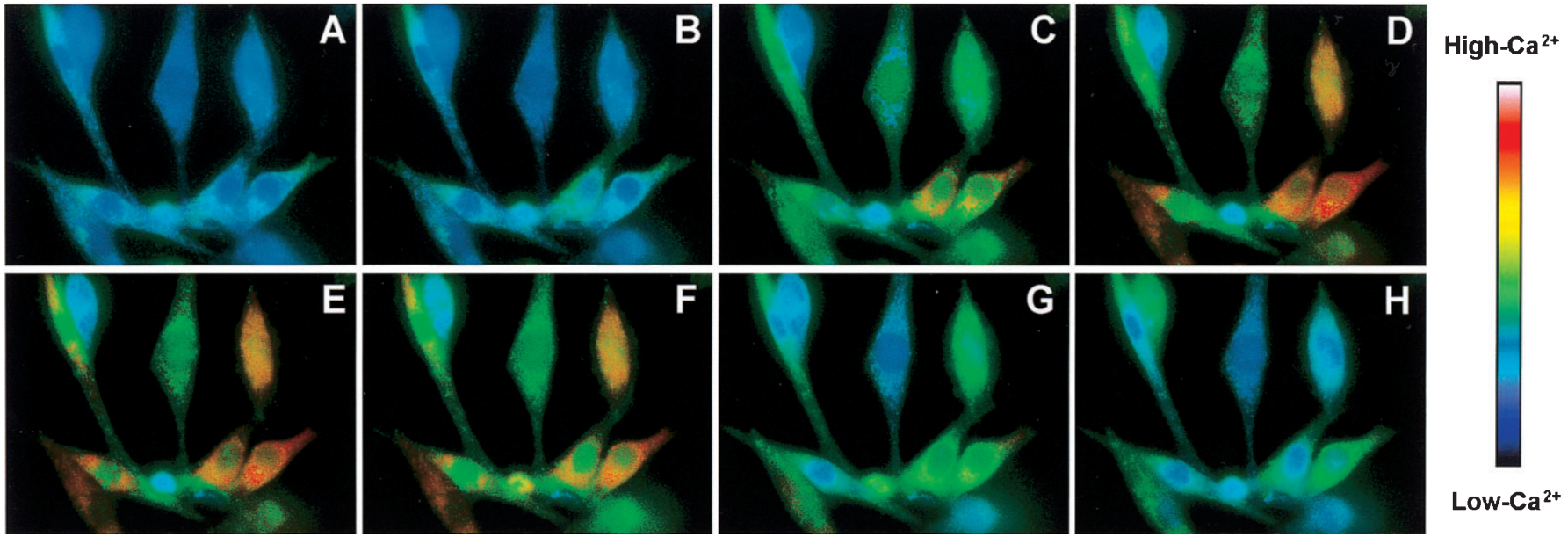


Fig. 3. Identification of Ca^{2+} entry during infection of BHK-21 cells by WN virus at the plasma membrane. Cells were preloaded with Fura-2, as described in Methods. WN virus (4 μg E protein) was suspended in 40 μl extracellular solution, adsorbed to the cell surface during microscopic observation and fusion was induced by the addition of extracellular solution of pH 5·4, exactly as described for patch-clamp experiments. The intracellular Ca^{2+} concentration was determined by ratiometric fluorescence measurements as described in Methods. Measurements were made every 2 s throughout the experiment, which comprised 2 min incubation prior to virus adsorption and virus adsorption for 3 min, followed by low pH-activated virus entry at the plasma membrane and a further incubation of variable time without further treatment. For each measurement, two photographs were taken using the fluorescence emission at 510 nm after excitation at either 340 or 380 nm. The ratio of emission intensities determined for each point of the picture after these excitations, which reflects the concentration of Ca^{2+} in the cytoplasm, is shown in false colour representation in this figure, as indicated by the colour scale. The measurements shown were made at the following times: (A) prior to virus adsorption; (B) after 3 min virus adsorption prior to low pH treatment; (C–H) 10, 20, 30, 60, 120 and 240 s after low pH-activated virus entry, respectively.

release, a pH of 6·2 or lower was always necessary for the alphaviruses SF or SIN, whereas in the case of WN virus release occurred at pH values between 7·2 and 6·5, depending on the composition of the liposomes.

Analysis of membrane permeability during early steps of virus infection

The experiments described above support the hypothesis that the membrane proteins of the WN virus form an ion-permeable pore in the target membrane after fusion. A standard procedure for the identification of such pores is the measurement of membrane permeability by the patch-clamp technique (Hamill *et al.*, 1981). It has been shown that WN virus particles fuse with the plasma membrane at pH 6·4 (Kimura *et al.*, 1986). We therefore used the plasma membrane as the target membrane for WN virus infection and pore formation in an analysis involving three steps: (i) membrane current of a single cell was monitored in the whole-cell configuration of the patch-clamp technique; (ii) virus was applied to the surroundings of the cell and allowed to adsorb to the cell membrane; and (iii) fusion of virus particles with the plasma membrane was induced by addition of buffer of low pH. The experiments were performed at 21 °C. Recently, we successfully performed this experimental approach using the SF alphavirus (Wengler *et al.*, 2003). Original traces obtained in such experiments are shown in Fig. 2. In the experiment shown in Fig. 2(A), WN virus particles containing 4 μg E protein were added to Vero cells and fusion was induced by incubation at pH 5·4. Within a few seconds of low pH treatment, a distinct transient increase in membrane current, caused by the opening and closure of a number of single pores, could be detected (refer to Fig. 5A for a detailed depiction of single pore events). The number of these events was reduced if the amount of virus used during adsorption was reduced (data not shown). In the experiment reported in Fig. 2(B), virus particles were mixed with low pH (pH 5·4) buffer prior to adsorption to the patch-clamped cell. No pores were formed under these conditions. Virus particles pretreated at low pH, or the low pH itself, therefore had no effect on membrane permeability. Fig. 2(C) shows current–voltage relationships of the membrane currents of Vero cells in the presence of WN virus prior to and after addition of low pH solution. The membrane potential shifted from $-25{\cdot}3 \pm 2$ mV (SEM, $n=11$) to nearly 0. Under the physiological ionic conditions used in the patch-clamp experiments, the reversal potential was +58 mV for an Na^+-selective current and −98·7 mV for a K^+-selective current. The finding that the reversal potential of the virus-induced, fast-activating, voltage-independent current was $-2{\cdot}9 \pm 3{\cdot}1$ mV (SEM, $n=8$) indicated that the pores were non-selective, at least for cations such as Na^+ and K^+. The current–voltage relationship after acid activation shown in Fig. 2(C) did not exactly match a straight line, although a linear curve would be expected for non-specific ionic currents of ohmic (=voltage-independent) behaviour. The determination of the current–voltage relationships takes about 3 s (see Fig. 2 legend), whereas the open times of the pores are mostly less than 0·1 s (see Fig. 5). Therefore, different groups of pores were open during the ten individual membrane current measurements at the different membrane potentials. This led to the observed mismatches of the current–voltage relationship. Further current–voltage analyses showed that the virus-induced current was transient and that about 3 min after acid treatment the membrane regained its original permeability properties ($n=3$). Analyses involving BHK-21 cells (Fig. 2D), a cell line that is used in our laboratory to propagate WN virus, indicated that the pores were also permeable to Ca^{2+}. In these cells, inflow of Ca^{2+} through the pores led to rapid opening of Ca^{2+}-activated K^+ channels, which generated an outward K^+ current with a positive current amplitude. This K^+ current inactivated within about 30 s after its maximal activation and was followed by the development of distinct pore-induced non-selective membrane currents ($n=3$).

These data show that during low pH-induced infection of BHK cells, Ca^{2+}-permeable pores are formed in the plasma membrane. A further experiment that identifies these pores is shown in Fig. 3. Low pH-induced virus entry was performed using cells that were not subjected to patch-clamp conditions, but were preloaded with the Ca^{2+}-sensitive fluorescent dye Fura-2. Since the extracellular

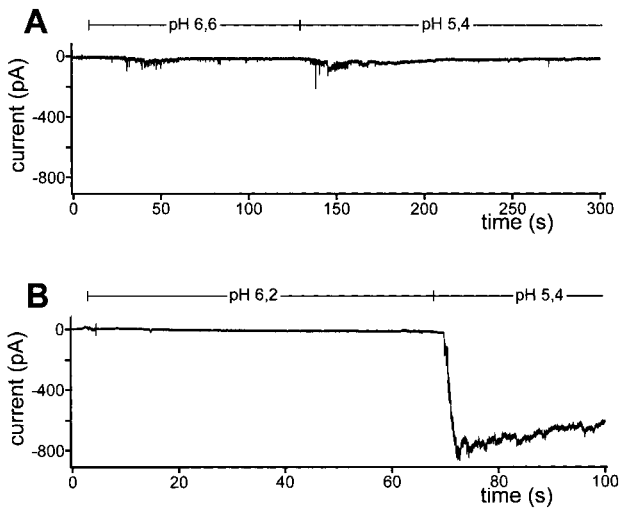
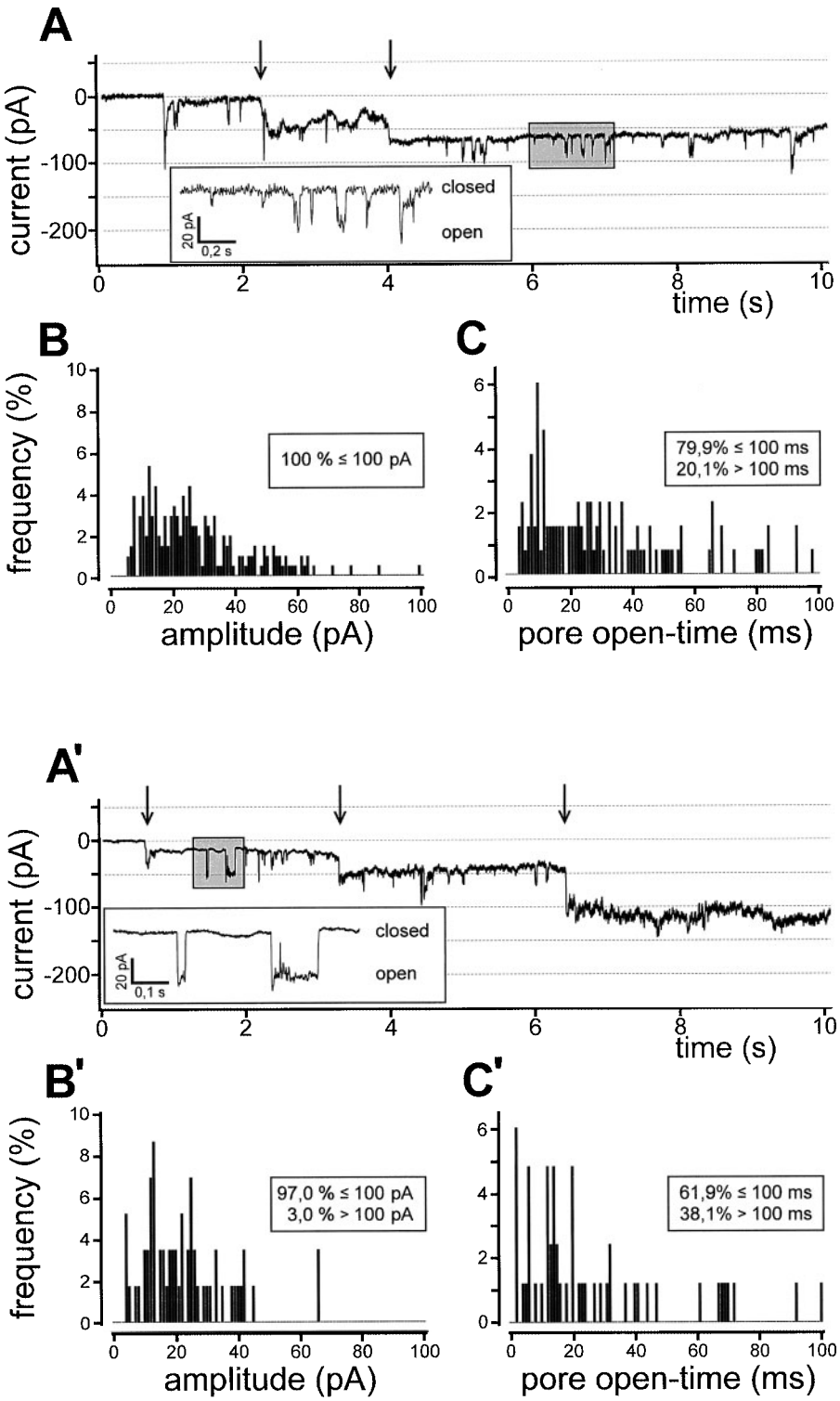


Fig. 4. Comparison of low pH activation of pore formation in insect cells by WN virus and SF virus. *Aedes albopictus* cells were subjected to the whole-cell configuration of the patch-clamp technique, followed by adsorption of either WN virus (4 μg E protein) (A) or SF virus (4 μg E1 protein) (B). Virus was activated by pH 6·6 solution (A) or pH 6·2 solution (B) for the times indicated. A further treatment with pH 5·4 solution was performed as shown in order to obtain complete activation of all virus particles. Original membrane current recordings are shown. The scales of current measurements are identical in both experiments in order to show differences in the induced membrane current amplitudes between the two viruses.

medium and the cellular cytoplasm contain Ca^{2+} concentrations of 2 mM and about 0·1 μM, respectively, a strong inflow of Ca^{2+} into the cytoplasm should occur during virus entry, which increases the cytoplasmic Ca^{2+} concentration directly and possibly by the release of intracellular Ca^{2+}. Changes in intracellular Ca^{2+} concentration are determined from the fluorescence intensity of the Fura-2 dye using fluorescence microscopy. False colour representations of intracellular Ca^{2+} concentration are shown in Fig. 3. It can be seen that entry of WN virus led to a rapid increase in



the cytoplasmic Ca^{2+} concentration. Most of the cells restored their original Ca^{2+} concentration within 4 min. A similar process occurred during entry of SF virus and was not observed in control experiments in the absence of virus particles (data not shown).

Flaviviruses and alphaviruses multiply in insect cells. Therefore, we compared the formation of pores in the cell membrane of *A. albopictus* C2 insect cells by WN virus and SF virus as shown in Fig. 4. In this experiment, two consecutive steps of acid activation were performed. WN virus particles had already led to pore formation at pH 6·6 (Fig. 4A), whereas SF virus particles were inactive even at pH 6·2 and needed a lower pH to induce pores (Fig. 4B). The total membrane current in the presence of SF virus was about four times greater than the current in the presence of WN virus (882 ± 229 pA, SEM, $n=8$ for SF; 217 ± 66 pA, SEM, $n=28$ for WN) and persisted for about three times longer. During pore formation by WN virus particles in C2 cells, a voltage-independent membrane current was induced with a reversal potential of $-2{\cdot}9 \pm 2{\cdot}6$ mV (SEM, $n=6$) and a normal membrane potential was restored during the recovery process ($n=12$, data not shown).

Analyses of individual pores generated in the plasma membrane of C2 insect cells are presented in Fig. 5. Fig. 5(A) shows a 10 s time interval from a standard experiment involving WN virus particles containing 4 μg E protein. Individual pores are easily recognized. The analysis of the individual current amplitudes (Fig. 5B) showed a heterogeneous distribution with a first peak of about 12 pA, corresponding to a pore conductance of about 300 pS. Heterogeneity also existed in the distribution of the open times of the pores (Fig. 5C). Although about 80 % of the pores showed open times shorter than 0·1 s, some events lasted longer than 2 s. The arrows in Fig. 5(A) indicate the opening of pores that persisted for several seconds. For comparison, corresponding analyses of pores generated in the membrane of C2 cells during entry of SF virus are presented in Fig. 5(A′–C′). In a standard experiment involving SF virus particles containing 4 μg of E1 fusion protein, a rather large current flowed through the membrane of C2 cells (Fig. 4B) and individual pores could rarely be differentiated. In order to generate a number of pores that allowed differentiation of single events, comparable with the experiment presented in Fig. 5(A), the number of SF virus particles used during adsorption had to be reduced 100-fold to about 0·04 μg E1 protein (Fig. 5A′). The distributions of pore currents and of opening times are shown in Fig. 5(B′) and (C′). The pores that were generated by WN virus and SF virus were rather similar in the distribution patterns of pore currents and opening times. A peak for SF virus-induced single pore amplitudes existed at about 12 pA and about 62 % of the pores showed open times of less than 0·1 s. Identical analyses as presented in Fig. 5 were also performed using Vero cells. The results obtained matched those presented in Fig. 5, with a similar distribution of pore amplitudes and pore open times (data not shown).

Analysis of a possible role of pores in endosomes during virus infection

A major pathway for infection of cells by alphaviruses and flaviviruses is the endosomal pathway. The data described above indicate that ion-permeable pores are formed in the endosomal membrane after fusion. Data supporting this interpretation are presented in Fig. 6. In this experiment, the co-entry of virus and the membrane-impermeable protein synthesis inhibitor hygromycin B was analysed. It has been shown by Carrasco (1981) that during the entry of virus particles in a number of virus systems, including the SF virus, co-entry of hygromycin B into the cytoplasm occurs (for a review, see Carrasco, 1995). The data presented in Fig. 6 show a comparison of this effect for WN and SF virus. Co-entry of hygromycin B occurred during entry of both viruses, but WN virus was much less effective.

During replication of alphaviruses, viral cores accumulate as stable structures in the cytoplasm of infected cells. A specific process is therefore necessary for disassembly of cores in the early stages of infection. During multiplication of flaviviruses, an accumulation of cores is not observed. Instead, the cores of flaviviruses are apparently generated during the process of virus budding (for a review, see Lindenbach & Rice, 2001). It is therefore possible that the core of flaviviruses, which enters the cytoplasm early in infection, is a labile structure that does not need a specific disassembly process in order to allow translation of the viral genome. An experimental analysis that addressed this question is shown

Fig. 5. Analysis of individual pores formed during entry of WN and SF virus particles at the plasma membrane of C2 cells. (A, A′) Original membrane current traces recorded in the whole-cell configuration of the patch-clamp technique during low pH (pH 5·4)-induced entry of WN virus (A) or SF virus (A′). WN virus particles and SF virus particles containing 4 μg E fusion protein or 0·04 μg E1 fusion protein, respectively, were used for these experiments. The magnified traces shown in the insets correspond to the shaded boxes on the original trace. Arrows indicate the opening of long-lasting pores. (B, B′) Amplitude distributions of pore events of WN virus (B) or SF virus-induced (B′) currents. Each bar represents the frequency of pore events with the noted amplitude. Bin width was 1 pA, bin centre the natural number. The number of analysed events was 210 for WN virus and 101 for SF virus-induced events. (C, C′) Distribution of open times of individual pores generated by WN virus (C) and SF virus (C′). Bars represent the frequency of events with the noted duration. Bin width was 1 ms, bin centre the natural number. The number of analysed events was 134 for WN virus- and 84 for SF virus-induced events. Scales of B, B′, C and C′ were truncated at 100 pA or 100 ms, respectively, for better depiction. The overall percentage of events with greater values are noted in the insets. All data were transformed to percentages for better comparison, due to the different number of events analysed. All data were derived from whole-cell voltage-clamp experiments.

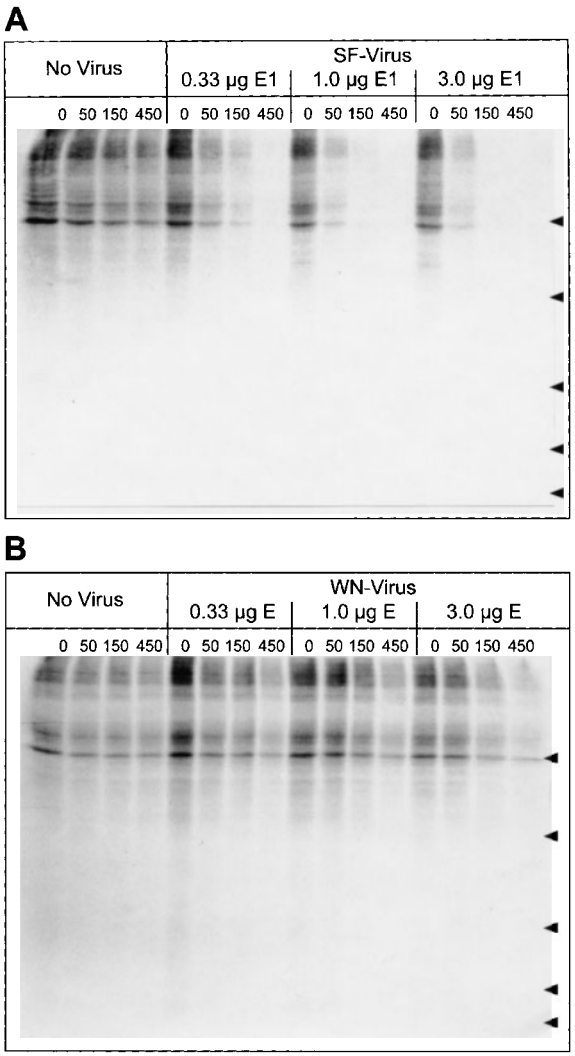


Fig. 6. Analysis of the co-entry of hygromycin B and SF or WN virus. A concentrated suspension of UV-inactivated virus in Earle's buffered salt solution containing 20 μg fusion protein ml^{-1} was diluted to 6·6 μg protein ml^{-1} and 2·2 μg protein ml^{-1}. Each of these three dilutions was split into four identical aliquots of 150 μl. These aliquots contained 3·0 μg fusion protein in the most concentrated solution and 1·0 μg or 0·33 μg fusion protein in the diluted samples. The four aliquots were adjusted to 0, 50, 150 and 450 μg hygromycin B ml^{-1}, respectively. A series of four control samples containing no virus was prepared in parallel and the resulting 16 solutions were used to infect 16 monolayer cultures (1·5 cm diameter) of BHK-21 cells by incubation at 37 °C for 30 min. After washing the cell layers with Earle's salt solution, 150 μl of labelling medium containing 50 μCi [^{35}S]methionine and [^{35}S]cysteine were added to each layer. After incubation at 37 °C for 30 min, the cells were suspended in electrophoresis sample buffer and subjected to 12·5 % SDS-PAGE. The experiment was performed in parallel for both WN and SF virus, and the 16 samples generated from each experiment were analysed on separate gels. Autogradiographs of these gels are shown. The concentration of virus and hygromycin B present in the individual samples during infection is indicated. Triangles indicate the migration of marker proteins of 43, 29, 18, 14 and 6 kDa molecular mass.

in Fig. 7. The autoradiogram presented shows the products generated by *in vitro* translation reactions in rabbit reticulocyte lysates containing as template the genome RNA of WN virus, the core of WN virus, the genome RNA of SF virus or the core of SF virus. The core of flaviviruses aggregates after removal of the viral membrane. Therefore, all *in vitro* translations were performed in the presence of 0·5 % NP-40 detergent, and virus particles were added as templates to study the translation of viral cores. NP-40 solubilizes the membrane of alpha- and flaviviruses and liberates the viral core ribonucleoprotein complex within the reticulocyte lysate. It can be seen that the genome RNA molecules of alpha- and flaviviruses were translated into a series of proteins. On the other hand, it was evident that the core of the SF alphavirus could not be translated, whereas the core of the WN flavivirus had an activity in *in vitro* protein synthesis that was similar to that of purified WN virus genome RNA.

DISCUSSION

Flaviviruses are arboviruses that form a genus in the family *Flaviviridae* (Van Regenmortel *et al.*, 2000). The molecular biology of flaviviruses has been studied in great detail (for

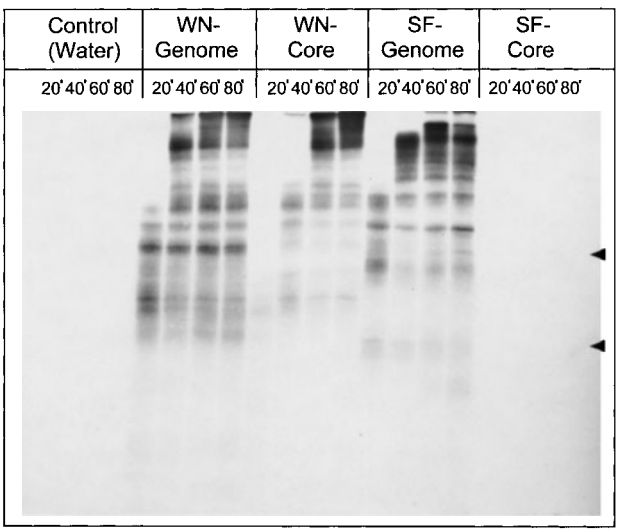


Fig. 7. *In vitro* translation of genome RNA and core ribonucleoprotein complexes of WN virus and SF virus. Five *in vitro* rabbit reticulocyte S30 lysate translation reactions containing 0·5 % NP-40 were incubated at 30 °C with different templates. The following templates were present in the reactions as indicated: H_2O, purified WN virus genome RNA, WN virus core ribonucleoprotein, purified SF virus genome RNA and SF virus core ribonucleoprotein. Experimental details are given in Methods. Aliquots of 4 μl were removed from each reaction at 20, 40, 60 and 80 min and separated by SDS-PAGE using a single 10 % polyacrylamide gel. The gel was stained with Coomassie blue and subjected to autoradiography. The triangles indicate the positions of proteins of 50 and 30 kDa molecular mass.

reviews, see Lindenbach & Rice, 2001; Mackenzie *et al.*, 2002). The morphology of flaviviruses is very similar to that of alphaviruses. Both viruses contain an infectious single-stranded RNA genome of about 11 000 nucleotides, but the gene organization and expression are quite different. Alphaviruses contain heterodimers of the viral membrane proteins E1 (~50 kDa) and E2 (~50 kDa) on their surface. The surface of flaviviruses contains heterodimers of the proteins E (~50 kDa) and M (~8 kDa). Unexpectedly, the determination of the atomic structure of the E fusion protein of the flavivirus tick-borne encephalitis virus (Rey *et al.*, 1995) and the E1 fusion protein of the alphavirus SF virus (Lescar *et al.*, 2001) has shown that both proteins are structurally homologous. As described in the Introduction, we recently obtained evidence that the E1 fusion protein of alphaviruses forms ion-permeable pores in the target membrane after fusion (Wengler *et al.*, 2003). The experiments reported above indicate that the homologous E fusion protein of flaviviruses forms similar pores and support the hypothesis that all members of this family of fusion proteins, which probably also includes the E protein of hepatitis C virus (Yagnik *et al.*, 2000), form such pores.

The data involving the release of [^{3}H]choline from liposomes are in accordance with earlier experiments in which fusion of flaviviruses with liposomes was analysed by determination of the transfer of [^{3}H]uridine-labelled viral genome RNA into the lumen of liposomes (Gollins & Porterfield, 1986) or by analyses of mixing of fluorescent-labelled viral lipids into the liposomal membrane (Corver *et al.*, 2000). The major new findings reported here are the identification of the release of [^{3}H]choline from liposomes by WN virus and the description of a virus–liposome system in which the threshold of [^{3}H]choline release was pH 7·4 and maximal release was obtained at pH 7·0.

The pores generated during entry of WN virus and SF virus show a number of similarities. The pores generated by WN virus are permeable to Na^+, K^+ and Ca^{2+} and the same permeabilities have been described for the SF virus pores (Wengler *et al.*, 2003). Furthermore, the data presented in Fig. 5 show that WN and SF virus particles generate similar pores during entry into C2 insect cells. The pores are heterogeneous in individual currents and opening times. In both systems a relatively large number of pores that allow currents of 12 pA or multiples of 12 pA are generated and the majority of pores have opening times of less than 100 ms. Similar pores are also formed during entry of both viruses into Vero cells (data not shown).

The structural organization formed after fusion by the alphavirus E1 protein (for reviews, see Garoff *et al.*, 1994; Helenius, 1995; Kielian, 1995) and the flavivirus E protein (for a review, see Heinz & Allison, 2000) have been studied intensively. Both proteins are class II fusion proteins that form an icosahedral shell on the viral surface (Lescar *et al.*, 2001; Pletnev *et al.*, 2001). This assembly is reorganized during fusion and the fusion active state is a short-lived intermediate in this reaction. At the end of this process, both proteins are present in the target membrane as homotrimers. The data discussed above indicate that these homotrimers might during a certain stage of their existence form pores that allow the flow of ions between the lumen of the endosome and the cytoplasm. An individual particle of SF virus or WN virus contains 240 molecules of E1 or 180 molecules of E, respectively (Kuhn *et al.*, 2002). Fusion of an SF or WN virus particle therefore could generate up to 80 or 60 homotrimers, respectively, in the target membrane. These data indicate that the generation of this set of trimers represents the formation of a single pore measured in the patch-clamp analyses. It is not unlikely that during entry of each individual virus particle, the number of trimers that form ion-permeable structures in the target membrane is variable and generally less than the maximal number possible. This fact could explain the heterogeneity in current permeabilities of the pores.

The experiments reported above show that the number of pores and the total current that flows through the plasma membrane in a standard experiment involving virus particles containing 4 μg of fusion protein is much higher for SF virus than for WN virus (see Fig. 4, and data not shown). The small number of pores formed by WN virus particles probably reflects to some extent the small number of infectious particles present in the WN virus preparation. The E and E1 fusion proteins both have a molecular mass of about 50 kDa. Since an individual particle of SF virus and WN virus contains 240 molecules of E1 and 180 molecules of E, respectively, the number of physical particles present in a standard experiment is slightly larger for WN virus than for SF virus. However, for the WN virus preparation the ratio of physical particles to plaque-forming particles is about 600 (Wengler & Wengler, 1989) but is only about 8 for the SF virus preparation (data not shown). Therefore, the number of infectious particles is about 100-fold higher in the standard experiment with SF particles than with WN particles. In the experiments reported in Fig. 5, the concentration of virus particles was adjusted to obtain a similar number of fusion events to allow us to identify the formation of individual pores. In these experiments, WN virus particles and SF virus particles containing 4 μg of E protein and 0·04 μg of E1 protein, respectively, were used. Under these conditions, therefore, the number of infectious particles present in both virus preparations was similar in spite of the ~100-fold difference in the number of physical particles. The low efficiency of co-entry of hygromycin B in the presence of WN virus particles, as compared with SF virus particles, also probably reflects the relatively small number of infectious WN virus particles present in the endosome.

The formation of ion-permeable pores in the target membrane by viral structural proteins during the early stages of infection leads to a significant alteration in the conceptual and practical analysis of this process. Infection would involve three steps: (i) fusion and transfer of the internal complex of the virus into the cytoplasm; (ii)

insertion of ion-permeable pores in the target membrane during or after fusion; and (iii) flow of appropriate ions through these pores, which regulates further processes necessary for virus replication. Spyr *et al.* (1995) have presented evidence that ion-permeable pores may be generated by E1 protein already at low pH in the membrane of SF virus particles. The experiments reported in this and in our previous work (Wengler *et al.*, 2003) do not allow detection of such pores. Further experiments are necessary to determine whether the ion-permeable pores that are detected in our experiments have already been formed before the generation of the fusion pore, as indicated by the work of Spyr *et al.* (1995), and then persist in the target membrane after fusion.

The alphavirus core is a stable structure that does not allow direct translation of the viral genome. The alphaviruses have probably evolved a process of low pH-induced disassembly of cores (Wengler & Wengler, 2002). A fusion process dependent on low pH is therefore appropriate for these viruses. Only in such a system are fusion and pore formation accompanied by a flow of protons, which generates a region of low pH necessary for core disassembly at the appropriate time and place. Extensive experimental evidence shows that flaviviruses can infect cells by a low pH-dependent endosomal route (for a review, see Heinz & Allison, 2001). The low pH-dependent release of [^{3}H]choline from liposomes, the patch-clamp analyses of membrane current and single pore events, and the analyses of co-entry of hygromycin B reported above indicate that a pore is formed in the endosomal membrane in this route of WN virus infection. However, the entry of flaviviruses may not be restricted to this pathway but may occur also at neutral pH. The processes involved in virus entry at neutral pH have been reviewed recently (Sieczkarski & Whittaker, 2002). Electron microscopic analyses of virus entry indicate that flaviviruses can enter cells at the plasma membrane in a low pH-independent way (Hase *et al.*, 1989a, b). The results concerning the release of [^{3}H]choline from liposomes at neutral pH reported above indicate that the fusion activity of the WN virus surface protein may be activated at neutral pH by contact with a patch of membrane of appropriate lipid composition. Furthermore, the *in vitro* translation experiments show that the WN genome RNA present in the viral core is accessible for translation. These data indicate that a proton flow through the pore formed during virus entry may not be necessary in the case of flaviviruses and that entry at an appropriate plasma membrane at neutral pH may be possible for flaviviruses and may lead to productive infection. Further studies are necessary to clarify this question.

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REFERENCES

Carrasco, L. (1981). Modification of membrane permeability induced by animal viruses early in infection. *Virology* **113**, 623–629.

Carrasco, L. (1995). Modification of membrane permeability by animal viruses. *Adv Virus Res* **45**, 61–112.

Castle, E., Nowak, T., Leidner, U., Wengler, G. & Wengler, G. (1985). Sequence analysis of the viral core protein and the membrane-associated proteins V1 and NV2 of the flavivirus West Nile virus and of the genome sequence for these proteins. *Virology* **145**, 227–236.

Corver, J., Ortiz, A., Allison, S. L., Schalich, J., Heinz, F. X. & Wilschut, J. (2000). Membrane fusion activity of tick-borne encephalitis virus and recombinant subviral particles in a liposomal model system. *Virology* **269**, 37–46.

Dick, M., Barth, B. U. & Kempf, C. (1996). The E1 protein is mandatory for pore formation by Semliki Forest virus spikes. *Virology* **220**, 204–207.

Garoff, H., Wilschut, J., Liljeström, P. & 7 other authors (1994). Assembly and entry mechansims of Semliki Forest virus. *Arch Virol* **9**, 329–338.

Gollins, S. W. & Porterfield, J. S. (1986). pH-dependent fusion between the flavivirus West Nile and liposomal model membranes. *J Gen Virol* **67**, 157–166.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* **391**, 85–100.

Hase, T., Summers, P. L. & Eckels, K. H. (1989a). Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch Virol* **104**, 129–143.

Hase, T., Summers, P. L. & Cohen, W. H. (1989b). A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis viruses. *Arch Virol* **108**, 101–114.

Heinz, F. X. & Allison, S. L. (2000). Structures and mechanisms in flavivirus fusion. *Adv Virus Res* **55**, 231–269.

Heinz, F. X. & Allison, S. L. (2001). The machinery for flavivirus fusion with host cell membranes. *Curr Opin Microbiol* **4**, 450–455.

Helenius, A. (1995). Alphavirus and flavivirus glycoproteins: structures and functions. *Cell* **81**, 651–653.

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

Kielian, M. (1995). Membrane fusion and the alphavirus life cycle. *Adv Virus Res* **45**, 113–151.

Kimura, T., Simon, W., Gollins, S. W. & Porterfield, J. S. (1986). The effect of pH on the early interaction of West Nile virus with P388D1 cells. *J Gen Virol* **67**, 2423–2433.

Kuhn, R. J., Zhang, W., Rossmann, M. G. & 9 other authors (2002). Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108**, 717–725.

Lanzrein, M., Weingart, R. & Kempf, C. (1993). pH-dependent pore formation in Semliki Forest virus-infected *Aedes albopictus* cells. *Virology* **193**, 296–302.

Lescar, J., Roussel, A., Wien, M. W., Navaza, J., Fuller, S. D., Wengler, G., Wengler, G. & Rey, F. A. (2001). The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. *Cell* **105**, 137–148.

Lindenbach, B. D. & Rice, C. M. (2001). *Flaviviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 991–1041. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Mackenzie, J. S., Barrett, A. D. T. & Deubel, V. (2002). *Japanese Encephalitis and West Nile Viruses*. Berlin, Heidelberg & New York: Springer-Verlag.

Nyfeler, S., Senn, K. & Kempf, C. (2001). Expression of Semliki Forest virus E1 protein in *Escherichia coli*. *J Biol Chem* **276**, 15453–15457.

Pletnev, T. S., Zhang, S. V., Mukhopadhyay, W., Fisher, S., Hernandez, B. R., Brown, R., Baker, D. T., Rossmann, M. G. & Kuhn, R. J. (2001). Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. *Cell* **105**, 127–136.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375**, 291–298.

Schlesinger, S. & Schlesinger, M. J. (2001). *Togaviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 895–916. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Sieczkarski, S. B. & Whittaker, G. R. (2002). Dissecting virus entry via endocytosis. *J Gen Virol* **83**, 1535–1545.

Silver, R. B. (1998). Ratio imaging: practical considerations for measuring intracellular calcium and pH in living tissue. *Methods Cell Biol* **56**, 237–251.

Singh, K. R. P. (1967). Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). *Curr Sci* **36**, 505–506.

Spyr, C. A., Kasermann, F. & Kempf, C. (1995). Identification of the pore forming element of Semliki Forest spikes. *FEBS Lett* **375**, 134–136.

Stiasny, K., Allison, S. L., Mandl, C. W. & Heinz, F. X. (2001). Role of metastability and acidic pH in membrane fusion by tick-borne encephalitis virus. *J Virol* **75**, 7392–7398.

Strauss, J. H. & Strauss, E. G. (1994). The alphaviruses: gene expression, replication and evolution. *Microbiol Rev* **58**, 491–562.

van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. & Wickner, R. B. (2000). *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*. San Diego: Academic Press.

Wengler, G. & Wengler, G. (1989). Cell-associated West Nile flavivirus is covered with E+PreM protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J Virol* **63**, 2521–2526.

Wengler, G. & Wengler, G. (2002). *In vitro* analyses of factors involved in the disassembly of Sindbis virus cores by 60S ribosomal subunits identify a possible role of low pH in this process. *J Gen Virol* **83**, 2417–2426.

Wengler, G., Wengler, G. & Gross, H. J. (1978). Studies on virus-specific nucleic acids synthesized in vertebrate and mosquito cells infected with flaviviruses. *Virology* **89**, 423–437.

Wengler, G., Wengler, G. & Rey, F. A. (1999). The isolation of the ectodomain of the alphavirus E1 protein as a soluble hemagglutinin and its crystallization. *Virology* **257**, 472–482.

Wengler, G., Koschinski, A., Wengler, G. & Dreyer, F. (2003). Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents. *J Gen Virol* **84**, 173–181.

Yagnik, A. T., Lahm, A., Meola, A., Roccasecca, R. M., Ercole, B. B., Nicosia, A. & Tramontano, A. (2000). A model for the hepatitis C virus envelope glycoprotein E2. *Proteins Struct Funct Genet* **40**, 355–366.