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

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Alphaviruses are small enveloped viruses that have been used extensively as model enveloped viruses. During infection, virus particles are taken up into endosomes, where a low pH activates the viral fusion protein, E1. Fusion of the viral and the endosomal membranes releases the viral core into the cytoplasm where cores are disassembled by interaction with 60S ribosomal subunits. Recently, we have shown that in vitro this disassembly is strongly stimulated by low pH. We have proposed that after entry of the core into the cytoplasm, the viral membrane proteins that have been transferred to the endosomal membrane form an ion-permeable pore in the endosome. The resulting flow of protons from the endosome into the cytoplasm through this pore could generate a low-pH environment for core disassembly in vivo. Here we report two types of analysis aimed at the identification of such pores. First, the release of [3H]choline from the interior of liposomes was analysed in the presence of virus particles and viral proteins. Secondly, cells were infected with Sindbis or Semliki Forest alphaviruses at the plasma membrane and the possible generation of ion-permeable pores during this process was analysed by whole-cell voltage clamp analysis of the membrane current. The results obtained indicated that the proposed pores are in fact generated and allowed us to identify the formation of individual pores. Available evidence indicates that the alphavirus E1 protein probably forms these pores. Proteins homologous to the alphavirus E1 protein are present in flaviviruses and hepatitis C virus.

INTRODUCTION

Alphaviruses are small, enveloped, plus-strand RNA viruses that form a genus in the family Togaviridae (Van Regenmortel et al., 2000). The molecular biology of alphaviruses has been studied in detail [for reviews see Strauss & Strauss (1994); Schlesinger & Schlesinger (2001)]. Studies of the early processes involved in alphavirus infection have led to the identification of the endocytotic pathway of virus entry (Helenius et al., 1980). In this process, virus particles bound to receptors on the cell surface are taken up into endosomes. The low pH present in this compartment activates a fusion activity in the viral surface proteins. The ensuing fusion between the viral and the endosomal membranes allows the penetration of the viral core into the cytoplasm through the so-called fusion pore. A large number of viruses infect cells by this route [see Marsh & Helenius (1989) for a review]. The alphavirus surface proteins have been studied using biochemical, genetic and morphological techniques [for reviews see Harrison (1986); Schlesinger & Schlesinger (1986); Garoff et al. (1994); Helenius (1995)]. The virus surface contains 80 glycoprotein spikes arranged in a $T=4$ lattice. Each spike is composed of three heterodimeric complexes of the viral membrane proteins E1 ($\sim 50$ kDa) and E2 ($\sim 50$ kDa). The E1 protein of alphaviruses is responsible for fusion, and studies involving site-directed mutagenesis have indicated that the amino acid residues 80–96 of the Semliki Forest (SF) virus E1 protein constitute the fusion peptide segment [see Kielian (1995) for a review].

The core of alphaviruses is an icosahedral complex of 240 molecules of core protein and 42S viral genome RNA. During virus multiplication, newly synthesized cores accumulate in the cytoplasm prior to budding. A special process must therefore account for the disassembly of cores that are released into the cellular cytoplasm after fusion of the viral into the endosomal membrane. Core disassembly involves an interaction of cores with 60S ribosomal subunits (Wengler & Wengler, 1984; Singh & Helenius, 1992).
Experiments have been reported indicating that this interaction is regulated by an unidentified process (Singh et al., 1997). Recently, we have analysed the disassembly of alphavirus cores in vitro in order to identify this process (Wengler & Wengler, 2002). In these studies, we discovered that a low-pH environment strongly stimulated the disassembly. At first sight this situation seemed to be unphysiological but it has been shown that the accumulation of viral structural proteins in the cell membrane that occurs during virus multiplication alters the permeability of the membrane at low pH late in infection (Lanzrein et al., 1993; Dick et al., 1996). These data led us to propose that the viral surface proteins that are transferred to the endosomal membrane after fusion of the viral and the endosomal membranes in the early stage of virus infection form a pore (Wengler & Wengler, 2002). The resulting flow of protons from the endosome into the cytoplasm through this pore would lead to a region of low pH at the correct time and place to allow the efficient disassembly of alphavirus cores. The molecules and processes involved in the fusion of the viral and the cellular membranes have been studied intensively [for reviews see Garoff et al. (1994); Kielian (1995)]. It is important to note that the data presented below did not analyse the fusion process or the formation or structure of the fusion pore, which is rather large and allows the passage of the core into the cytoplasm. The aim of the experiments was to analyse whether the membrane patch, generated after insertion of the viral membrane into the target membrane, contained ion-permeable pores, which might support the above-mentioned proton flow.

METHODS

Preparation of viruses and viral proteins. Growth of the alphaviruses Sindbis (SIN) virus and SF virus in BHK cells, purification of these viruses and generation of the SF virus-derived surface protein 30S and (E1–E2)ΔS complexes was performed as described previously (Wengler et al., 1999).

Preparation of [3H]choline-loaded liposomes. Liposomes were prepared from phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SPM) and cholesterol. Thirty-five mg of a dry mixture of these lipids in the proportions indicated in the individual experiments was solubilized in 1 ml 1-propanol at 50 °C and injected rapidly through a 0.45×12 mm needle into 20 ml 50 mM NaCl, 20 mM Tris/HCl, pH 8.1, prewarmed to 50 °C under strong stirring. After a 2 min incubation at 50 °C, the opalescent solution was cooled to 0 °C, filtered through a 0.45 μm Millipore (Durapore) membrane and liposomes were pelleted by centrifugation of the filtrate. Liposome pellets were stored at 0 °C. For loading with [3H]choline, liposomes containing 5 mg lipid were suspended in 300 μl water, and 25 μCi [3H]choline in 12.5 μl ethanol was added. The opalescent solution was sonicated at room temperature 20 times for 0.1 s each and incubated at 37 °C for 30 min to allow rescaling. The material was then adjusted to 50 mM NaCl, 10 mM Tris/HCl, pH 8.1, and subjected to gel filtration on a Superose 6 HR 10/30 column in 50 mM NaCl, 10 mM Tris/HCl, pH 8.1. Approximately 1% of the radioactivity was eluted in the excluded volume together with the liposomes. The liposomes were stored on ice and used within 5 days.

Assay of [3H]choline release from liposomes. Virus was stored at −70 °C as pellets, each containing 50 μg protein. A fresh pellet was used for each experiment. For a single release reaction, 25 μl 50 mM NaCl, 10 mM Tris/HCl, pH 8.1, containing about 2 μg of the protein or 10 μg of the virus to be assayed and 5 μl [3H]choline-containing liposomes (25 μg lipid) were transferred into a 10 K Nanosep ultrafiltration vial (Pall; 500 μl total volume) at 30 °C. After the addition of 10 μl 300 mM MES buffer of appropriate pH (7.0, 6.6, 6.2, 5.8, 5.4, or 5.0), the sample was incubated at 30 °C for 20 min. After this incubation, 300 μl 50 mM NaCl, 10 mM Tris/HCl, pH 8.1, was added and in order to separate the buffer solution from the liposomes, the vials were subjected to centrifugation at 12 000 g at 6 °C until the buffer solution was present in the filtrate reservoir. The radioactivity present in the filtrate was determined by liquid scintillation counting. Control reactions contained 2 μg BSA.

Analysis of binding of protein to liposomes by flotation. For a typical binding analysis, a standard release reaction containing liposomes and protein was scaled up by a factor of four, resulting in a final reaction volume of 160 μl. After 20 min incubation at 30 °C, 80 μg sucrose was dissolved in the reaction fluid and the material was loaded into a 0.8 ml cellulose nitrate vial in an SW 55 Beckman rotor. The material was overlaid with approximately 200 μl each of 30% (w/w) and 20% (w/w) sucrose in 50 mM NaCl, 10 mM Tris/HCl, pH 8.1, and centrifuged at 48 000 r.p.m. at 4 °C for 3 h. The gradients were divided into four fractions by removing the solutions from the top. Fraction 1 contained the top 300 μl of the gradient and fraction 4 contained the bottom 200 μl containing the original reaction. The fractions were transferred into 10 K Nanosep (Pall) concentration vials. The proteins were recovered on the ultrafiltration membrane of the vials by centrifugation, solubilized in sample buffer and subjected to PAGE. Proteins were visualized by Coomassie blue staining.

Patch-clamp analysis of membrane permeability. Patch-clamp measurements were performed as described by Hamill et al. (1981). HEK 293 cells were grown on 35 mm Petri dishes in growth medium consisting of DMEM and Ham’s F12 (1:1) with 10% (v/v) foetal bovine serum and used at a density of approximately 4×104 cells per dish.

The following solutions were used. Cells were incubated in extracellular solution composed of 140 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM glucose and 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 MgCl2 and 10 mM HEPES, pH 7.3, adjusted with KOH. A free intracellular Ca2+ concentration of 100 nM was obtained using 100 μM of the Ca2+ chelator BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid] and a total Ca2+ concentration of 29.69 μM, assuming an apparent dissociation constant, Ki, of 0.24 μM (pH 7.3) for the Ca2+-BAPTA complex. Differences in osmolarity between extra- and intracellular solutions were compensated for by adding sorbitol in the range of 10–20 mM. Bath and pipette solutions were filtered through 0.2 μm pore filters.

The following materials were used for the recordings. Recording pipettes were pulled from borosilicate glass capillaries ( Hilgenberg) with an outer diameter of 1.5 mm and a wall thickness of 0.3 mm. After fire polishing, they had a resistance of 5–10 MΩ when filled with pipette solution. The currents were recorded and filtered with an EPC-8 patch-clamp amplifier (HEKA GmbH) with sampling frequencies of up to 30 kHz, according to the particular experiment. All signals were filtered at one-third of the sampling frequency. Membrane potentials were measured at zero current in the current-clamp mode of the whole-cell recording configuration. Data acquisition and off-line analysis were performed with Pulse and Pulsefit software (HEKA GmbH). The data were corrected for the liquid-junction potential between the
pipette and bath solutions, which was \(-10\) mV for the standard potassium glutamate internal solution. Data were expressed as means \(\pm\) SEM unless stated otherwise.

Whole-cell patch-clamp analysis was performed as follows. Growth medium was removed and cells were incubated for 5–10 min in 2 ml extracellular solution. Thereafter a tight seal between a cell and the recording pipette was established. The pipette was slowly withdrawn from the cell, combined with a 10 ms electrical pulse with an amplitude of \(-0.9\) V. This usually ruptured the membrane, leading to a direct electrical and ionic access to the cytoplasm without loosening the tight seal between the membrane and recording pipette, so that the recording of membrane current could be started. A pellet of UV-inactivated virus particles containing 50 \(\mu\)g viral protein had been suspended in 250 \(\mu\)l extracellular solution containing 3 \%(v/v) Ficoll 400, and 50 \(\mu\)l of this material was transferred with a pipette into the immediate surroundings of the cell. This virus suspension contained 10 \(\mu\)g viral protein, which corresponds to about 4 \(\mu\)g E1, 4 \(\mu\)g E2 and 2 \(\mu\)g C protein in the virus particles. After 5 min incubation at 20 °C to allow adsorption of virus, 100 \(\mu\)l low-pH buffer solution consisting of Earle’s buffered salt solution containing 20 mM MES buffer adjusted to pH 5–0 with NaOH and containing 5 \% Ficoll was applied to the immediate surrounding of the cell. The osmolarity of this solution had been adjusted to the osmolarity of the extracellular solution. Thereafter a tight seal between a cell and the recording pipette was obtained as described by Hamill et al. (1981). The outside-out configuration of the patch-clamp technique was performed as described by Hamill et al. (1981). After establishing a whole-cell recording configuration, the pipette was slowly withdrawn from the cell, leading to a long small-diameter sand-glass-shaped tube of cell membrane, connecting the interior of the cell to the interior of the measuring pipette. Merging of the membrane walls of this tube at the isthmus often generates a small bubble of cell membrane of about 4 \(\mu\)m, the so-called ‘patch’. This membrane patch is still tightly sealed with the measuring pipette and has a direct electrical and ionic access to the former cytoplasmic side of the membrane, but has no other connection with the cell. The external side of this membrane patch represents the physiological outside of the former cell membrane and is exposed to the bath solution. Virus adsorption to this membrane and low-pH treatment were performed as described above.

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**RESULTS**

**Analysis involving the release of radioactive molecules from liposomes**

A reasonable starting point for the development of an assay of the pore-forming ability of structural proteins was to develop a liposome preparation that would release a radioactive water-soluble molecule from the interior after fusion with virus particles. We used \([\text{\(^3\)H}]\)choline as the water-soluble molecule (Nyfeler et al., 2001). Fusion of alphaviruses with membranes has a special requirement for cholesterol (White & Helenius, 1980; Ahn et al., 2002) and sphingolipid (Nieva et al., 1994). The low-pH-dependent fusion of alphaviruses with liposomes has been analysed in detail (White & Helenius, 1980; Smit et al., 1999). Following these lines we prepared a series of liposomes containing PC, PE, SPM and cholesterol in various proportions, loaded these liposomes with \([\text{\(^3\)H}]\)choline and measured the release of \([\text{\(^3\)H}]\)choline in the presence of SIN virus and SF virus (Fig. 1A, B). Efficient release was obtained if cholesterol-rich liposomes containing total phospholipid and cholesterol in equimolar amounts were incubated at pH 5.5 with virus particles (Fig. 1B). Almost no radioactivity was released by SIN virus particles from liposomes that contained all individual lipids in equimolar proportions (Fig. 1A). The data indicated that a specific interaction of virus particles and appropriate liposomes at acid pH is necessary for the release of the radioactive tracer molecule and support the hypothesis that a pore is formed in the liposomal membrane after fusion with virus particles.

The membrane proteins of SF virus can be recovered as a 30S protein complex (Helenius & Bonsdorff, 1976; Morein et al., 1978; Wengler et al., 1999). This complex is obtained if membrane proteins, extracted by NP-40 from virus particles, are separated from lipid and detergent by centrifugation. The protein complex cannot be dissociated by NP-40 or other non-ionic detergents, is stable in 6 M urea, and has a characteristic morphology in negative-stained preparations in the electron microscope and represents a very active haemagglutinin at low pH (Wengler et al., 1999). The 30S complex led to the release of \([\text{\(^3\)H}]\)choline from liposomes with a pH dependence very similar to that of intact virus (Fig. 2A). Treatment of the 30S complex with subtilisin removes C-terminal sequences from both the E1 and E2 protein molecules. The shortened proteins, called E1AS and E2AS, respectively, can be recovered by gel filtration as a soluble heterodimer, which has been called (E1–E2)AS in order to indicate its dimeric structure and the loss of sequences as a result of subtilisin digestion (Wengler et al., 1999).
The residues Y (390) and T (343) probably represent the C-terminal residues of the proteins E1ΔS and E2ΔS, respectively. The atomic structure of the E1ΔS protein, obtained from the (E1–E2)ΔS complex, has been determined (Lescar et al., 2001). An analysis of the activity of the (E1–E2)ΔS complex in releasing [3H]choline is shown in Fig. 2(B). Since no release was observed, the binding of proteins to the liposomes was determined by flotation. It can be seen from the data presented in Fig. 2(C) that the binding of protein to liposomes was dependent on pH. In all reactions, the E2ΔS protein remained in the bottom compartment fraction 4, into which the samples were loaded. At pH 5-5, E1ΔS protein floated with liposomes into fraction 2. These data indicated that at pH 5-5, the (E1–E2)ΔS complex dissociates and that the E1ΔS protein binds to the liposomes without the formation of a pore.

**Electrophysiological analysis of membrane permeability during early steps of virus infection**

The experiments described above support the hypothesis that complete virus particles or intact viral structural proteins may be able to form an ion-permeable pore in the target membrane after fusion. A standard procedure for the identification of such pores is the measurement of the membrane permeability by the patch-clamp technique (Hamill et al., 1981). The endosome cannot be subjected to this technique, but it has been shown that infection by alphaviruses can occur at the plasma membrane if virus adsorbed to this membrane is subjected to low-pH treatment (White et al., 1980). We therefore used the plasma membrane as the target membrane in an analysis involving three steps. First, the membrane current of a single cell was monitored in the whole-cell configuration of the patch-clamp technique. Secondly, virus was applied to the surroundings of the cell and allowed to bind for 5 min. Lastly, fusion with the plasma membrane was induced by the addition of buffer of low pH. The experiments were performed at 20 °C. Original traces from such experiments using SF virus and HEK 293 cells are shown in Fig. 3. It is important to note that a strong cytopathic effect developed in HEK 293 cells infected with SF or SIN virus (data not shown). In the experiment reported in Fig. 3(A), a mock-adsorption (with no virus) was performed. Due to the change in pH, about 1 s after addition of the low-pH solution a transient inward current occurred in these cells. In a further control experiment, mock-adsorption was followed by addition of low-pH solution containing virus particles (10 μg total protein). The same membrane permeability changes as described in Fig. 3(A) were observed (data not shown). These results showed that virus particles, pretreated at low pH, have no effect on the permeability. In a complete assay in which virus particles containing 10 μg total protein were present during the adsorption step (Fig. 3B), the transient inward current was followed by a rapid stepwise increase in inward current, which reached 800 pA at about 13 s after addition of the low-pH solution. These data showed that the stepwise increase depends on the presence of native virus particles. They suggested that these steps might correspond to the fusion of single virus particles with the plasma membrane and represent pores formed by the viral surface proteins. If this interpretation is correct, the number of steps and the resulting maximal value of the inward current should decrease if decreasing amounts of virus are added during the adsorption step. This effect was indeed found (Fig. 3B, C). Maximal inward currents of 1408±422 pA (n=6), 447±101 pA (n=3) and 214±28 pA (n=3) were determined at a holding potential of −40 mV, if virus particles containing 10, 1 and 0.1 μg protein, respectively, were...
present during the adsorption step. Current–voltage relationships prior to addition of low-pH buffer and during virus-induced membrane current are presented in Fig. 3(D). Prior to acid treatment, the cell showed a non-linear current–voltage relationship, typical for HEK 293 cells. Small currents flowed through the cell membrane at negative potentials and a voltage-dependent K\(^+\) current was recorded at positive potentials. The membrane potential was \(-29.7 \pm 3.2\) mV (n=42). It can be seen that the electrical properties of this membrane were completely altered after acid activation of virus. The virus-induced current had a linear current–voltage relationship and the reversal potential was shifted from \(-29.7\) mV to 0 (n=24). These data indicated that a pore allowing the flow of at least K\(^+\) and Na\(^+\) ions is involved in the generation of this current. If, during adsorption, virus containing 1 \(\mu\)g protein or less was

![Fig. 3. Infection of cells at the plasma membrane is accompanied by a stepwise increase of inward membrane currents through the membrane. Original membrane current recordings obtained during acid-induced SF virus infection or mock infection are shown in (A)–(C) and (E). HEK 293 cells were subjected to the whole-cell configuration of the patch-clamp technique, followed by adsorption of virus and treatment of the cell with pH 5.0 solution, as described in Methods. The time of addition of low-pH solution is indicated by an arrow. Holding membrane potential was \(-40\) mV. (A) Current recorded in an experiment in which a mock-adsorption step in the absence of virus was performed. (B, C) Current recorded in experiments in which virus containing 10 \(\mu\)g protein or 1 \(\mu\)g protein was present during adsorption, respectively. (D) Current–voltage relationships measured 10 s before and 20 s after acid-induced virus infection during an experiment in which SF virus, containing 1 \(\mu\)g of protein, was present. Membrane current amplitudes were measured at the end of voltage pulses from \(-100\) to +80 mM 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. (E) Current recorded in an experiment in which virus containing 10 \(\mu\)g protein was present during adsorption. La\(^{3+}\) (1 mM LaCl\(_3\)) was added to the extracellular solution as indicated. It should be noted that the time scale of this recording is different from the recordings in (A)–(C).]
present, current–voltage measurements showed that the cells regained their original membrane properties within about 5–10 min ($n = 5$, data not shown). The data indicated that the formation of individual pores and not an unspecific disruption of the cytoplasmic membrane was observed. Further evidence for this conclusion came from the finding that addition of La$^{3+}$ to the cells after acid-induced fusion led to a rapid resealing of the cell membrane, as determined by measurements of membrane current (Fig. 3E) and of membrane potential (data not shown). It is well known that a number of ions lead to closure of pores [Lanzrein et al., 1993] and that La$^{3+}$ especially is a potent blocker of non-specific ion channels [see Hescheler et al. (1993) for a review]. Basically the same results were obtained in the presence of SIN virus particles (data not shown).

For a better demonstration of the step change of membrane current, which might correspond to the fusion of single virus particles, the outside-out configuration of the patch-clamp technique was used. Since such an isolated membrane patch comprises about 0.4% of the cell surface, these experiments are possible only if at least $2 \times 10^2$ virus particles fuse into the whole cell membrane. The experiments reported in Fig. 3 indicated that it might possible to obtain such conditions using membrane patches derived from HEK 293 cells and a high concentration of virus particles. The result obtained in such an experiment is shown in Fig. 4(A). A small number of individual steps of current increase could be identified. The data supported the interpretation that each step corresponds to the fusion of a single virus particle. In order to show that pore formation is not unique to HEK 293 cells, whole-cell patch-clamp analysis was also performed using BHK 21 cells and NIH 3T3 cells. BHK 21 cells were used in our earlier experiments on the disassembly of alphavirus cores. Virus-induced current changes observed in a BHK cell are presented in

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**Fig. 4.** Analysis of single steps of change of membrane current during acid-induced virus infection. (A) Current generated in an outside-out membrane patch derived from HEK 293 cells. SF virus containing $10 \mu$g protein was present during the adsorption step. The holding potential was $-40$ mV. The addition of low-pH solution at time zero is not part of this graph. Experimental details are given in Methods. (B) Current generated in a BHK 21 cell subjected to the whole-cell patch-clamp technique. SF virus containing $10 \mu$g protein was present during the adsorption step. The low-pH solution was added at time zero. The distances between horizontal bars of identical length shown in (A) and (B) represent membrane current amplitudes taken for pore current analyses. (C–E) Distributions of single steps of SF virus-induced currents. The strength of the individual currents is shown on the horizontal scale. Each bar represents the number of events in which a step change of current in a 2 pA interval around this value was observed. All data are derived from whole-cell voltage-clamp experiments. The distributions of individual current steps obtained in HEK 293 cells (C), BHK 21 cells (D) and NIH 3T3 cells (E) are shown.
Fig. 4(B). In these cells and in NIH 3T3 cells, the virus-induced stepwise increase of inward current was rapidly followed by activation of Ca\(^{2+}\)-dependent \(K^+\) outward currents, probably due to pore-induced Ca\(^{2+}\) influx. Since the \(K^+\) current limits the number of virus-induced current steps that can be analysed, HEK 293 cells were used in most of our experiments \((n = 29)\). After inactivation of the Ca\(^{2+}\)-activated \(K^+\) current, the amplitudes of the pore-generated membrane currents were similar in BHK 21 and in HEK 293 cells, whereas the current amplitudes of NIH 3T3 cells were significantly lower \((236 \pm 14\) pA, 10 \(\mu\)g protein, \(n = 6\)). The distribution of virus-induced current steps, observed in whole-cell recording configuration of HEK 293, BHK 21 and NIH 3T3 cells, are shown in Fig. 4(C–E). As well as the smallest steps of about 4–5 pA, which corresponded to a conductance of 100–125 pS, a broad range of larger current steps was observed. The patterns of steps, obtained in outside-out patch-clamp experiments in NIH 3T3 and in HEK 293 cells, were similar to those obtained in whole-cell analyses (data not shown).

**DISCUSSION**

The data reported above give experimental support to our working hypothesis that the membrane patch, generated after insertion of the viral membrane into the target membrane during virus entry, contains ion-permeable pores. A large body of experimental evidence shows that virus infection can lead to changes in membrane permeability [see Carrasco (1995) for a review]. In many experiments, the permeability of uninfected and infected cells has been compared at late stages of virus multiplication, but membrane permeability changes in the early steps of virus infection have also been described. Carrasco (1981) showed that during the early stages of infection by a number of unenveloped and enveloped viruses, including SF virus, cells become permeable to selected inhibitors of protein synthesis. It has been concluded that the virions themselves are responsible for the modification of the cellular membrane, and co-entry of protein toxins together with the entry of viruses has been described (Perez & Carrasco, 1994). The molecular basis of this process has remained elusive. A series of analyses of changes in membrane permeability in cells that had accumulated alphavirus membrane proteins have been made by C. Kempf and coworkers (Kempf et al., 1987; Schlegel et al., 1991; Dick et al., 1996; Käsermann & Kempf, 1996; Nvfeler et al., 2001). These authors studied the permeability of the plasma membrane of cells late in infection. Cells were adjusted to low pH and the permeability of the membrane was determined by the release of \(^{3}H\)choline and by whole-cell patch-clamp measurements. It was found that at low pH the membrane of infected cells became permeable to \(^{3}H\)choline and to protons, and that the intracellular concentrations of Na\(^+\) and K\(^+\) were altered. Furthermore, the whole-cell patch-clamp analysis of SIN virus-infected insect cells at about 20 h post-infection revealed a large increase in membrane conductance on exposure to low pH. These experiments led to the suggestion that viral proteins brought to their fusogenic conformation at low pH form pores in the membrane (Lanzrein et al., 1993).

Our experiments are a continuation of these experiments, but differ from the earlier analyses. In the earlier experiments, the role of low pH on the permeability of cell membranes containing large amounts of virus-specific proteins that had accumulated during virus multiplication or after transfection was analysed. The generation of pores has been postulated, but the formation of individual pores has not been detected, since the time scale of the patch-clamp analysis was not sufficiently rapid. We attempted to identify the formation of individual pores during the early stages of virus infection. Therefore, in our experiments, viral proteins were added to the cell system as part of virus particles, and the proteins were transferred to the cell membrane by low-pH treatment. This meant that permeability changes that accompany the early stages of virus infection were analysed in our experiments. Such experimental settings with appropriate patch-clamp analyses, using a rather short time scale, allowed us to analyse the entry of virus particles and to identify the possible formation of individual pores during this process. The patch-clamp analysis in our experiments is not suitable for detecting membrane currents supported by H\(^+\) ions because of the non-selectivity of the pore. The concentration of H\(^+\), ions, even at pH 5–0, is very low relative to that of other ions, e.g. Na\(^+\) or K\(^+\), present in the system, which mask any currents supported by H\(^+\) ions. Since the pores allow permeation of larger ions, e.g. glutamic acid and arginine, albeit at a reduced rate (data not shown), the small ions cannot be substituted for by larger impermeable ions in order to detect small H\(^+\) currents. These data also indicated that the diameter of the aqueous channel formed is such that the migration of these individual amino acids is significantly restricted.

We have proposed that, in the case of alphavirus infection, the formation of these pores has a well-defined function: under physiological conditions the low-pH-induced fusion occurs in the endosome. The pores have a permeability for at least Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions, and it seems reasonable to propose that in the endosome protons will flow into the cytoplasm through these pores, e.g. in exchange for K\(^+\) ions. We have recently shown that disassembly of alphavirus cores by 60S ribosomal subunits is strongly stimulated by a pH of about 6–0 (Wengler & Wengler, 2002). Together these results represent a physiological mechanism that can lead to efficient disassembly of alphavirus cores at low pH early in infection and allows the assembly of stable cores during virus multiplication in the cytoplasm at neutral pH. The finding that the alphavirus replica is located on the cytoplasmic surface of endosomes and lysosomes (Froshauer et al., 1988) is in accordance with the above interpretation that a low-pH region in the vicinity of the endosomes leads to a localized core disassembly and translation of the viral genome.
After the preparation of this paper, Smit et al. (2002) published a paper stating that the fusion of alphaviruses with liposomes is a non-leaky process. This is in contrast to the experiments described in Fig. 1. The techniques used in both analyses are quite different. Only a specific analysis of these differences may possibly allow us to identify why the results obtained are so different. For instance, the liposomes used in the studies were prepared by fundamentally different techniques and different labelled molecules were used in the release assays.

Taken together, the results of our experiments involving liposomes and the results obtained in the patch-clamp analysis indicate that the pores are made up of the viral membrane proteins and are not generated by activation of endogenous membrane channels. Further support for this interpretation comes from published data. SF virus particles from which lipid and the E2 protein were removed by protease treatment in the presence of detergent did retain a residual infectivity (Omar & Koblet, 1988). Furthermore, it has been shown that SF virus E1 protein expressed in E. coli is incorporated into the bacterial plasma membrane and that these membranes become permeable to $^3$H cholane at low pH (Nyfeler et al., 2001). Comparable results have been obtained in similar experiments using eukaryotic cells (Dick et al., 1996). These authors concluded that it was highly likely that the E1 protein formed pores in the membrane in which it accumulated. Recently, the atomic structure of the SF virus-derived E1 protein and its organization within the viral membrane have been characterized (Forsell et al., 2000; Pletnev et al., 2001; Lescar et al., 2001). These experiments have shown that the E1 molecules form an icosahedral lattice on the virus surface, which determines the structure of the particle. Proteins homologous to the SF virus E1 protein are present in other viruses. The atomic structure of the E protein of tick-borne encephalitis (TBE) virus (Rey et al., 1995), which belongs to the Flavivirus genus, is homologous to the structure of the SF virus E1 protein (Lescar et al., 2001). Furthermore, molecular modelling analysis has indicated that the envelope protein E2 of hepatitis C virus is homologous to the E protein of TBE virus (Yagnik et al., 2000). The data discussed above indicate that the E1 protein of alphaviruses and the homologous proteins might constitute a family of viral surface proteins that have three properties: (i) they form a continuous icosahedral protein shell on the virus surface at neutral pH (Forsell et al., 2000; Lescar et al., 2001; Pletnev et al., 2001); (ii) they can be converted into a fusion-active state at low pH; and (iii) after fusion they can form an ion-permeable pore in the target membrane. The last point does not imply that the functional role of this pore is identical in the different virus systems. It may be possible that members of this protein family are also present in viruses that do not have a lipid envelope, since alphaviruses from which lipid had been removed do retain a small residual infectivity (Omar & Koblet, 1988) and the 30S membrane protein complex derived from SF virus led to release of $^3$H cholane from liposomes at low pH. The fusion of the viral lipid membrane into the target membrane brought about by these proteins could then be regarded as a side-effect of the basic function of these proteins: the ability to form an icosahedral closed surface at neutral pH which, in the presence of an appropriate target membrane, is converted at low pH into a planar assembly of proteins in the target membrane that can form ion-permeable pores. We think that this proposal may lead to further attempts to identify additional proteins belonging to this family and to analyze the possible formation of ion-permeable pores by the corresponding virus.

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