Characterization of Junin arenavirus cell entry

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Junin virus (JUNV) entry is conducted by receptor-mediated endocytosis. To explore the cellular entry mechanism of JUNV, inhibitory effects of drugs affecting the main endocytic pathways on JUNV entry into Vero cells were analysed. Compounds that impair clathrin-mediated endocytosis were shown to reduce virus internalization without affecting virion binding. In contrast, drugs that alter lipid-raft microdomains, impairing caveola-mediated endocytosis, were not able to block virus entry. To show direct evidence of JUNV entry, transmission electron microscopy was performed; it showed JUNV particles of about 60–100 nm in membrane depressions that had an electron-dense coating. In addition, JUNV particles were found within invaginations of the plasma membrane and vesicles that resembled those of pits and clathrin-coated vesicles. Taken together, these results demonstrate that clathrin-mediated endocytosis is the main JUNV entry pathway into Vero cells and represent an important contribution to the characterization of the arenavirus multiplication cycle.

INTRODUCTION

The initial steps in virus infection involve the specific attachment of the virus particle to a receptor(s) on the cell surface, followed by internalization of the virus into the cell and subsequent uncoating of the virion to release the active transcription complex. These events are essential for the successful initiation of the virus replication cycle and play an important role in the tissue tropism and pathogenesis of viruses.

In general, viruses can enter cells by fusion between the viral and the cellular membranes at the plasma-membrane level, in endocytic vesicles or, more rarely, and only in cases of non-enveloped viruses, by a direct mechanism at the cell surface by which the virus particles are translocated directly into the cytoplasm. So far, two distinct internalization pathways for receptor-mediated endocytosis have been well studied. Clathrin-mediated endocytosis is the best characterized of these pathways and is considered the primary route of endocytic entry into cells (Brodsky et al., 2001). This process is mediated by the formation of characteristic membrane invaginations, known as clathrin-coated pits (CCPs). Assembly of clathrin into a CCP occurs at the inside face of the plasma membrane in response to a receptor-mediated internalization signal. This kind of endocytosis has been defined in molecular terms as the internalization pathway for Semliki Forest virus, Sindbis virus, rubella virus and Hantaan virus among others (DeTulleo & Kirchhausen, 1998; Helenius et al., 1980; Jin et al., 2002; Kee et al., 2004).

As an alternative to clathrin, caveolae have been described as mediating the entry of many viruses. Caveolae are small, flask-shaped invaginations of the plasma membrane, characterized both by high levels of cholesterol and glycosphingolipids and by the presence of caveolin, a 20–24 kDa integral membrane protein. It has been described that simian virus 40 (Pelkmans et al., 2001) and some human enteroviruses (Stuart et al., 2002) enter cells via caveolae, by means of internalization in the so-called caveosomes before being transported to the endoplasmic reticulum.

Macropinocytosis is considered to be a non-specific and non-receptor-dependent mechanism for viral internalization (Bishop, 1997). Some stimuli, such as growth factors or phorbol ester, induce ruffling of the membrane and its subsequent closure, which forms large, cytoplasmic vacuoles up to several micrometres in diameter, called macropinosomes. Several types of evidence have implicated macropinocytic mechanisms in the entry of Epstein–Barr virus and human immunodeficiency virus type 1 in some cell cultures (Marechal et al., 2001; Miller & Hutt-Fletcher, 1992). An additional route of entry, yet ill-defined, is known as the clathrin- and caveola-independent internalization pathway (Nichols & Lippincott-Schwartz, 2001). This pathway has been implicated in the entry of many viruses, including some picornaviruses and influenza virus (Madshus et al., 1987; Matlin et al., 1981).

The family Arenaviridae is divided geographically and phylogenetically into two major complexes, the New World and the Old World complexes (Bowen et al., 1997; Charrel...
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& de Lamballe, 2003). Five of the known arenaviruses can cause viral haemorrhagic fever (VHF) in humans, and four of them, namely Junin virus (JUNV), Guanarito, Machupo and Sabia viruses, belong to the phylogenetic clade B of New World arenaviruses. This clade also contains Tacaribe virus (TACV) and Cupixi and Amapari viruses (Bowen et al., 1996; Charrel et al., 2002), which are not implicated in human disease.

JUNV is the aetiological agent of Argentine haemorrhagic fever (Parodi et al., 1958), an endemo-epidemic disease affecting the population of the most fertile farming land of Argentina (Weissenbacher et al., 1987). To date, thousands of cases have been reported for these infections in the endemic regions (Charrel & de Lamballe, 2003; Peters, 2002). South American VHF’s initially show unspecific, flu-like symptoms. Haemorrhagic and neurological complications may occur during the further course of disease, with high mortality rates in humans (Peters, 2002). Although a live-attenuated vaccine is available against Argentine haemorrhagic fever (Maiztegui et al., 1998), there is growing concern that these viruses could be used as agents of bioterrorism. Therefore, arenaviruses remain a serious threat to global public health. Complete knowledge of the mechanisms of arenavirus multiplication would facilitate and improve design of new strategies to combat or avoid these dangerous microbial agents.

Virions of members of the family Arenaviridae are enveloped and contain two segments of ambisense, single-stranded RNA. The small segment encodes the nucleocapsid-associated protein NP, which is the first protein to be translated during the arenavirus life cycle, and the glycoprotein precursor GPC. This precursor is processed proteolytically into a peripheral membrane glycoprotein (GP1), which is implicated in receptor binding (Borrow & Oldstone, 1992), an integral glycoprotein (GP2) (Candurra & Damonte, 1997) and the signal peptide (SP) that may serve in envelope glycoprotein structure and trafficking (York et al., 2004). Several members of the family Arenaviridae, including the Old World arenaviruses, use α-dystroglycan as a cellular receptor (Spiropoulou et al., 2002). Previous studies have shown that events following lymphocytic choriomeningitis virus (LCMV) receptor binding involve viropexis in large, smooth-walled vesicles, followed by a pH-dependent fusion event inside cells (Borrow & Oldstone, 1994). Likewise, studies concerning early steps of the JUNV replication cycle have also shown that JUNV enters the cell by receptor-mediated endocytosis and that a low pH is necessary for virus internalization through fusion activity (Castilla et al., 1994). However, the entry mechanism used preferentially by JUNV to enter cells is not yet known.

In the present study, we used different approaches to investigate the main internalization mechanism of JUNV into Vero cells. Agents that disrupt clathrin-dependent receptor-mediated endocytosis inhibited infection of cells by JUNV. In contrast, agents that disrupt lipid rafts, inhibiting caveola-dependent endocytosis, had no significant effect on the entry of JUNV. Nevertheless, to confirm this result, transmission electron microscopy (EM) was performed and the virus was only found in electron-dense vesicles.

METHODS

Cells and virus. Monolayers of Vero cells were grown in minimum essential medium (MEM) containing 10% heat-inactivated fetal calf serum and supplemented with 50 μg gentamicin ml⁻¹. Maintenance medium consisted of MEM containing 2% fetal calf serum. The naturally attenuated IVS5 strain, used in all experiments, was propagated in Vero cells. The titer of the JUNV stock suspensions were between 2 × 10⁶ and 8 × 10⁶ p.f.u. ml⁻¹. Virus yields were then determined by plaque-formation (p.f.u.) assays in Vero cells. The TRLV 11573 strain of TACV was used.

Reagents and antibodies. Nystatin (NT), methyl-β-cyclodextrin (MjCD), sucrose, chlorpromazine (CPZ), fluorescein isothiocyanate (FITC)–goat anti-mouse, FITC–transferrin (TF) and FITC–cholera toxin B subunit (CTx) were purchased from Sigma.

Anti-JUNV monoclonal antibodies (mAbs) IC06-BE10 for indirect immunofluorescence and NA05-AG12 for Western blot, reactive against NP, were kindly donated by Dr A. Sanchez (CDC, Atlanta, GA, USA) (Sanchez et al., 1989). In some cases, anti-JUNV rabbit polyclonal serum was used (Damonte et al., 1986).

Inhibition of JUNV infectivity. Confluent Vero cells grown on a 24-well plate were treated with different concentrations of the compound to be evaluated for different times, according to the drug. After the treatment period, the medium was removed and, for NT and MjCD, which demonstrate virucidal activity, cells were washed three times with PBS. When CPZ or sucrose was used, the cells were infected in the presence of the appropriate concentration of the compound. After infection, the inoculum was removed and fresh medium was added. Supernatants were cultured the next day and JUNV yields were determined by p.f.u. assays.

Inhibition of JUNV entry. Vero cells grown on glass coverslips for 48 h were treated with drugs at various concentrations, as shown in the figure legends, for 2 h for CPZ or for 30 min for sucrose, and then infected with JUNV at an m.o.i. of 5 or 0.5. Only when CPZ or sucrose was used, the infection was performed in the presence of the same concentration of drug. When we used NT for 2 h, or MjCD for 30 min, the infection medium did not contain the reagent, so we washed cells exhaustively before infection. Nevertheless, when we performed experiments with sucrose or CPZ, and cells were infected after washing out the drug, as was done for NT and MjCD, the effect of the treatment was not significantly different. Anti-JUNV rabbit polyclonal serum was added to the fresh medium and the cells were incubated for 48 h. After incubation, cells were rinsed three times with PBS, fixed with 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100 and incubated with a mAb against NP (IC06-BE10). After washing, anti-JUNV bound antibody was detected by incubation with FITC–goat anti-mouse antibody. Samples were examined by using an Olympus BX51 microscope and the mean number of positive cells on each coverslip was calculated as the mean of 20 optical fields, chosen randomly. In each of them, we counted positive cells and total cells, using infected and non-treated monolayers as controls.

When we used molecular markers to ensure that the chemical treatment was specifically inhibiting one way of entry, we treated Vero cells with sucrose or CPZ to inhibit CCP endocytosis. Before
incubating with Tf–FITC for 1 h at 37 °C in the presence of the drugs, we washed three times with PBS and lysed with equal amounts of PBS and PAGE buffer (25 mM HEPES, 0.75 M NaCl). After incubation, cells were rinsed three times with cold HEPES buffer/alumnum (10 % v/v) at 4 °C, fixed with 4 % PFA and permeabilized with 0.2 % Triton X-100. When CTx was used as a marker of caveola-dependent entry, cells were treated with NT or MCD for an acute depletion of cholesterol from the plasma membrane. We washed cells three times with cold PBS before incubating them with CTx for 1 h at 37 °C without the drugs. After incubation, we washed the cells three times with PBS and fixed them for fluorescence under the same conditions as were used for Tf.

**Protein expression analysis.** We used Western blot analysis to study the expression of JUNV NP in cultures treated with the different drugs. Briefly, after infection, fresh medium was added to cells and, 4 h later, monolayers were rinsed three times with PBS and lysed with equal amounts of PBS and PAGE 4 × buffer (0.5 M Tris/HCl, pH 6.8; 10 % glycerol; 2 % β-mercaptoethanol; 0.005 % bromophenol blue; 5 % SDS). Each sample was sonicated for 10 min and boiled for 3 min. For protein analysis, 20 μl of each sample was resolved by SDS-PAGE (10 % gel) and proteins were transferred onto PVDF membranes (Polyscreen; PerkinElmer) and then incubated with specific antibodies. Horseradish peroxidase–goat anti-mouse antibodies were added and protein band detection was performed by using the ECL (Enhanced Chemiluminescence) system (Amersham Biosciences).

**Virion binding and internalization assays.** Purified, [35S]methionine-labelled JUNV preparation was performed as described previously (Damonte et al., 1994). Radiolabelled virions (5 × 10⁷ p.f.u. ml⁻¹, 1 × 10⁹ d.p.m. ml⁻¹) were added to Vero cells and incubated at 4 °C for 1 h. Then, cells were washed extensively with PBS and lysed with 0.1 M NaOH solution containing 1 % SDS, and cell-bound radioactivity was quantified by using a liquid scintillation counter. For the internalization assay, after virus adsorption at 4 °C for 1 h, cells were incubated at 37 °C for 1 h to allow virus penetration. Then, cultures were washed with PBS and treated with 1 mg proteinase K ml⁻¹ in PBS to remove external adsorbed virus. Protease treatment was then stopped by adding 1 mM PMSF in PBS containing 3 % BSA. Cells were pelleted and lysed in NaOH/SDS solution as described above, and cell-associated radioactivity was quantified.

**Transmission EM.** Vero cell cultures were infected with JUNV (m.o.i. of 50). After 1 h at 4 °C, cell cultures were exposed to 37 °C for 15 min. Then, the cell layer was washed and fixed for 4 h by the addition of ice-cold 1.5 % glutaraldehyde in 0.2 M phosphate buffer, pH 7.2. After fixation, cell cultures were washed overnight in a 0.32 M sucrose solution. Then, they were post-fixed in 1.5 % osmium tetroxide for 2 h at 4 °C, dehydrated in graded ethanol solutions, stained with uranyl acetate and embedded in an epoxy resin. After embedding, ultrathin sections were taken by using a diamond knife; they were stained with uranyl acetate and then with Reynolds solution. Electron micrographs were taken with a C10 Zeiss electron microscope, using Kodak 4489 film.

**RESULTS**

**Inhibition of JUNV infection by endocytosis-disrupting drugs.**

In order to investigate the early steps of JUNV infection in Vero cells, we studied the effects of compounds that specifically block different entry pathways. We first evaluated the role of clathrin-dependent endocytosis by treating cells with CPZ, which causes clathrin lattices to assemble on endosomal membranes and, at the same time, prevents the assembly of coated pits at the cell surface (Wang et al., 1993). Virus replication was inhibited by CPZ in a dose-dependent manner after 24 h infection. As can be seen in Fig. 1(a), virus yield was inhibited by up to 90 % in the presence of the drug. The lack of toxicity of the compound was determined in the cultures by MTT assay, under the same treatments conditions. Concentrations showing cell viability above 90 % were used in further experiments.

![Fig. 1](image-url). Dose–response curves showing the effect of various drugs on JUNV infection. The percentage of inhibition of infectivity was plotted against drug concentration. Vero cell cultures were treated 2 h with (a) CPZ or (b) NT. When cells were treated with NT, the solution was made in MEM without serum, to avoid the presence of exogenous cholesterol. Drug-treated cells were infected with JUNV (m.o.i. of 1) in the presence of the appropriate concentration of CPZ, or they were washed before infection in the case of NT. Virus infection was detected by plaque assay. Cell viability was measured by using an MTT assay.
Afterwards, to study the role of the caveola-dependent pathway in virus entry, cells were treated with NT or MβCD (see Methods), as it has been shown that the sequestration of cholesterol with sterol-binding drugs or depletion from the membrane impairs caveola-mediated endocytosis (Sieczkarski & Whittaker, 2002a). Then, the cultures were infected with JUNV. Results from representative assays with NT are shown in Fig. 1(b). Inhibition values of JUNV production by NT (Fig. 1b) were notably lower than those obtained with clathrin-disrupting compounds. Maximum values of inhibition with NT were around 40% (Fig. 1b), with 90% cell viability. The same experiments were performed with TACV and similar results were obtained (see Supplementary Fig. S1, available in JGV Online).

Role of clathrin-mediated endocytosis in JUNV entry

For a more accurate study, the specific effect of CPZ and sucrose was monitored by using Tf, which is known to be internalized by a clathrin-dependent pathway. Tf was visualized by a direct fluorescence assay under the same treatment conditions as those shown in Fig. 1. As can be seen in mock-treated cells, Tf appeared concentrated in the cytoplasm (Fig. 2a), whereas the uptake of Tf in cells treated with 40 μM CPZ appeared as a diffuse fluorescence around the cells, indicating that internalization of Tf was inhibited by this compound (Fig. 2).

The effects of these treatments on JUNV internalization were then determined. Vero cells were exposed to CPZ and,
after infection, NP expression of JUNV was monitored, considering that this is the earliest protein expressed in the JUNV cell cycle. Two different m.o.i.s were used (5 and 0.5), as it has been suggested that infection by viruses at an m.o.i. of <1 reflects the use of a more specific infection route. Results shown in Fig. 2(d, f) indicate that CPZ at a dose of 40 μM reduced NP expression. Fig. 2(g) shows quantification of NP expression in various drug treatments on cells infected at m.o.i.s of 5 and 0.5. CPZ reduced JUNV infection notably at the concentrations tested, in a dose-dependent manner at both m.o.i.s. However, at an m.o.i. of 5, protein expression was only reduced by 50%, indicating that, at higher m.o.i.s, another way of entry is being used. When the same experiments were carried out with TACV, similar results were seen (see Supplementary Fig. S2, available in JGV Online). When cells were treated with the compound and processed for Western blot analysis, the same results were obtained: the expression of JUNV NP was not inhibited by the presence of CPZ (Fig. 2h) when it was added in early steps. Endogenous β-actin was included as an internal loading control for the Western blot (Fig. 2i).

Assays with [35S]methionine-labelled JUNV were performed to determine the kinetics of binding and internalization under the same drug and infection conditions as those used before. As can be seen in Fig. 2(j), when clathrin-dependent endocytosis was being disrupted by CPZ, virion binding did not seem to be altered. In contrast, radioactivity levels, representing internalization of JUNV into cells, showed a significant reduction compared with control cells (Fig. 2k). These results provide additional evidence that this is the method being used by the virus to penetrate into cells and that the adsorption of the virus is not being affected by the compounds.

When all of these experiments were performed with a hypertonic sucrose medium, which results in the dissociation of clathrin vesicles from the plasma membrane (Heuser & Anderson, 1989), similar results were obtained (data not shown).

**Role of caveolae in JUNV entry**

To study the involvement of caveola-dependent endocytosis in JUNV entry, we used compounds that are known to alter lipid rafts. To verify the effect of NT and MβCD on this way of entry, we measured the uptake of CTx, as this toxin is targeted to caveolae through its receptor, the ganglioside GM1 (Torgersen et al., 2001). As can be seen in Fig. 3, mock-treated cells internalized CTx, with the fluorescence concentrated around the nuclei. In contrast, when cells were treated with NT under the same conditions as those shown in Fig. 1(b), they internalized CTx poorly, with the label appearing as diffuse fluorescence in the cytoplasm, demonstrating the effectiveness of this drug for caveola-mediated uptake.

Vero cells were then treated with 70 μM NT and, after infection, protein expression was monitored. As for CPZ treatment, two different m.o.i.s were used (5 and 0.5). The results shown in Fig. 3(d, f) indicate that a 70 μM dose of NT did not reduce protein expression. As can be seen in Fig. 3(g), showing the quantification of NP expression after various drug treatments on cells infected at m.o.i.s of 5 and 0.5, NT did not reduce JUNV infection significantly at the concentration tested. This would indicate that, at these m.o.i.s, another way of entry that does not depend on lipid rafts is being used. Similar results were obtained with TACV (see Supplementary Fig. S2, available in JGV Online). Simultaneously, cells were treated with NT (Fig. 3h) and then processed for Western blot. The same results were obtained: expression of JUNV NP was not inhibited by inhibitors of lipid raft-dependent endocytosis (Fig. 3h).

To confirm this result, we performed assays to determine the kinetics of binding and internalization of [35S]methioninelabelled JUNV under the same conditions of drug treatment. As can be seen in Fig. 3, radioactive levels representative of binding (Fig. 3j) and internalization (Fig. 3k) of JUNV in the presence of the compound did not show a significant reduction respective to the control. This result would indicate that lipid rafts are not essential for any of the early steps of the JUNV cell cycle.

Similar results were obtained when we used MβCD, which depletes cholesterol from the membrane, impairing lipid raft-mediated endocytosis (Sieczkarski & Whittaker, 2002a) (data not shown).

**JUNV is internalized via CCPs**

To further trace the fate of the virus particle, transmission EM was used. Vero cells were first incubated with JUNV particles prepared by concentration procedures (see Methods) (m.o.i. of 50) at 4 °C for 60 min. Low-temperature treatment was performed to allow binding of virions to cell-surface receptors, but not internalization of virus particles into the cells. Subsequently, the cells were warmed to 37 °C and the virus-infected cells were processed for transmission EM 15 min after warming. Virions were identified by their morphology; transmission EM showed oval JUNV particles with a mean diameter of about 80–100 nm, comparable to the reported size of JUNV and other arenavirus particles (Fig. 4a) (Borrow & Oldstone, 1994; Murphy et al., 1970). It was seen that entry was an asynchronous process: all of the images in Fig. 4, which illustrate the virus at different stages in the entry process, were taken 15 min after incubation at 37 °C. Attachment of JUNV particles along the surface of Vero cells was observed, making contact with electron-dense plasma-membrane depressions. These typical electron-dense membrane structures, as seen in Fig. 4(b), are assigned to clathrin-coated membranes (Marsh & Helenius, 1980; Matlin et al., 1982). Moreover, JUNV particles were found within CCPs (Fig. 4c) and, as can be seen in Fig. 4(d), internalization occurred into clathrin-coated vesicles (CCVs).
DISCUSSION

We have shown that the main process by which JUNV enters Vero cells is mediated by CCVs. We examined the entry route of JUNV to infect Vero cells, initially by using compounds that have been shown to impair different entry pathways. These compounds inhibited clathrin-mediated endocytosis (CPZ and sucrose) or lipid raft-dependent endocytosis (NT and MβCD). These compounds were first tested at a range of concentrations to identify the optimum dose to inhibit the uptake of Tf and CTx, and were shown not to have any cytotoxic effect on cells. We demonstrated that an early step in JUNV infection was inhibited by CPZ and sucrose. Likewise, for Semliki Forest virus, clathrin has been well established to be required for internalization (Helenius et al., 1980; Sieczkarski & Whittaker, 2002b).

JUNV infection has previously been found to be sensitive to agents that interfere with the acidic pH of endosomes (Castilla et al., 1994). This is consistent with entry by clathrin-mediated endocytosis, as CCVs deliver their cargo into endosomes with an acidic content. The sensitivity to lysosomotropic agents has often been considered evidence for entry by endocytosis. However, viruses insensitive to lysosomotropic agents, such as mouse polyomavirus, may also enter cells this way (Liebl et al., 2006). Actually, several endocytic pathways in addition to the well-established clathrin entry pathway have recently been identified.

Fig. 3. Effect of caveola-dependent endocytosis-disrupting drugs on JUNV NP expression. (a–f) Vero monolayers were treated with NT (70 μM) (b) for 2 h or with MEM as a control (a). Then, they were incubated with CTx–FITC in the presence of the compound to corroborate the specific effect of the drug on caveola-mediated endocytosis. Vero cell monolayers grown on coverslips for 48 h were treated with 70 μM NT (d, f) for 2 h or with MEM only in control cells (c, e). Treated cells were then infected with JUNV at an m.o.i. of 5 (c, d) or 0.5 (e, f). Infection was detected by immunofluorescence of formaldehyde-fixed cells. Magnification, ×400. (g) Data were plotted as a percentage of virus-positive cells over the number of total cells as indicated in Methods (C, control; NT60, NT at 60 μM, etc.). (h) Western blot analysis of treated cells; total proteins were extracted 5 h after infection and subjected to immunoblot analysis using anti-NP antibodies. (i) The amount of protein in each lane was verified by actin immunoblot analysis. (j, k) Binding and internalization of radiolabelled, concentrated virions of JUNV. Vero cells were treated with 70 μM NT for 2 h and incubated with JUNV for 1 h at 4 °C, and radioactivity levels were then measured (j). For the internalization assay (f), following adsorption of virus at 4 °C, the cells were raised to 37 °C before quantifying radiolabelled virus every 10 min for the next hour. Control infected cells in the absence of the drug and treated samples were processed to quantify cell-associated radioactivity, as described in Methods. Each value is the mean ± SD of triplicate measurements in two independent experiments.
(Conner & Schmid, 2003; Pelkmans & Helenius, 2003). These newly discovered pathways differ in the type of vesicular carrier formed at the plasma membrane to carry out the internalization step and also in the type of intracellular compartment to which the internalized material is transported. Some of them carry ligands to the classical acidic early and late endosomes, which are also reached by endocytic ligands originating from CCVs, whereas others deliver their cargo to compartments with neutral pH, such as caveosomes, endoplasmic reticulum or the Golgi complex (Nichols, 2003; Pelkmans & Helenius, 2003).

Generally, virus entry into cells at a low m.o.i. may reflect the use of a more specific and physiologically relevant route, whereas at a higher m.o.i., the virus may spill over into normally less productive routes. As another way of entry where at a higher m.o.i., the virus may spill over into generally larger than the diameter of most viruses that enter cells through endocytosis. However, for clathrin-mediated endocytosis to be an essential part of the life cycle of rhabdoviruses, such as vesicular stomatitis virus (VSV), which are bullet-shaped with a diameter of 75 nm and a maximal length of 180 nm, the CCP must undergo some deformation from its typical morphology (Brodsky et al., 1999; Subtil et al., 1999). In addition, NT could stay bound to the cell membrane despite the PBS washes, causing an effect on virus budding, as we have recently found that JUNV exits cells through lipid raft (S. M. Cordo, M. G. Martinez & N. A. Candurra, unpublished results).

Several enveloped and non-enveloped viruses, either sensitive or insensitive to acidic pH, have been reported to use alternative endocytic pathways. Echovirus 1 and rotavirus are internalized by clathrin- and caveola-independent pathways, which require dynamin function and are sensitive to the cholesterol-sequestering drug MβCD. On the other hand, influenza viruses can be endocytosed by both clathrin-independent and clathrin-dependent mechanisms into the same cell (Rust et al., 2004). In order to examine the role of clathrin-mediated endocytosis in JUNV entry more precisely, we performed binding and internalization experiments by using 35S-labelled JUNV. We demonstrated that only CPZ and sucrose inhibited internalization into cells. These data confirm that these compounds were acting at a post-binding early stage during entry. In addition, these results confirm that the NP reduction seen in Fig. 2 was due to inhibition of JUNV entry, rather than of virus adsorption.

To corroborate these results, we performed transmission EM experiments and observed that JUNV was always observed inside CCPs or CCVs (Fig. 4). CCVs are most often found with a barrel-shaped or spherical morphology, 65–75 and 90–100 nm in diameter, respectively, although larger structures (comprising approximately 20% of total CCVs) with diameters of up to 120 nm are also found (Crowther et al., 1976; Ehrlich et al., 2004). Such CCVs are generally larger than the diameter of most viruses that enter cells through endocytosis. However, for clathrin-mediated endocytosis to be an essential part of the life cycle of rhabdoviruses, such as vesicular stomatitis virus (VSV), which are bullet-shaped with a diameter of 75 nm and a maximal length of 180 nm, the CCP must undergo some deformation from its typical morphology (Brodsky et al., 2001). This deformation is indeed noticeable in electron micrographs of VSV in densely stained endocytic vesicles (Matlin et al., 1982). JUNV could use the same strategy to utilize CCPs, despite its documented diameter of between 50 and 300 nm (Murphy et al., 1970). However, previous studies concerning members of the same family, such as LCMV, have shown by transmission EM that it is endocytosed into rodent fibroblast cell lines by non-coated vesicles (Borrow & Oldstone, 1994). On the other hand, when LCMV entry was studied, it was observed that agents that disrupt lipid rafts inhibit virus endocytosis (Shah et al., 2006). In view of the fact that the receptor used by Old World arenaviruses is not the same as that used by clade B
New World arenaviruses (Reignier et al., 2006; Rojek et al., 2006), it should not be surprising that another way of entry is also being used for JUNV. Furthermore, while this work was being revised, a new work about arenaviruses was published, showing that transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses (Radoszitzky et al., 2007). As this receptor is a molecular marker for clathrin-mediated endocytosis, these data support our results.

Understanding the nature of interactions between arenaviruses and their host, both at the cellular and whole-organism levels, is essential for successful development of efficacious prophylactic and therapeutic measures. Therefore, although the initial interaction between JUNV and Vero cells, including virus binding to its receptor(s), is not completely understood, the results in the present study demonstrate that the clathrin-dependent endocytic pathway is the subsequent step of entry.

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