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# Equine arteritis virus is delivered to an acidic compartment of host cells via clathrin-dependent endocytosis

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ABSTRACT

Equine arteritis virus (EAV) is an enveloped, positive-stranded RNA virus belonging to the family Arteriviridae. Infection by EAV requires the release of the viral genome by fusion with the respective target membrane of the host cell. We have investigated the entry pathway of EAV into Baby Hamster Kindey cells (BHK). Infection of cells assessed by the plaque reduction assay was strongly inhibited by substances which interfere with clathrin-dependent endocytosis and by lysosomotropic compounds. Furthermore, infection of BHK cells was suppressed when clathrin-dependent endocytosis was inhibited by expression of antisense RNA of the clathrin-heavy chain before infection. These results strongly suggest that EAV is taken up via clathrin-dependent endocytosis and is delivered to acidic endosomal compartments.



## Introduction

Equine arteritis virus (EAV) is an enveloped positive-stranded RNA virus (van Dinten et al., 1997) of the family Arteriviridae which belongs together with Coronaviruses to the order Nidovirales. Horses are the typical target of EAV. Consequences of infection range from an asymptomatic, persistent carrier state to abortion or even to lethal haemorrhagic fever (Snijder and Meulenberg, 1998). EAV buds from the rough endoplasmic reticulum and the outer nuclear membrane, but not from the cell surface (Wada et al., 1995).

EAV contains seven structural proteins which are essential for virus infectivity and maturation. The nucleocapsid protein N forms an isometric core particle (de Vries et al., 1992), which is surrounded by the lipid envelope. N and the complex of the non-glycosylated membrane protein M and the major glycoprotein Gp5 are required for virus assembly (Wieringa et al., 2004). The putative envelope heterotrimer of the glycosylated proteins Gp2b, Gp3, Gp4 (ratio: 1:1:1) is a prime candidate for receptor-binding and for fusion of EAV envelope with the cellular target membrane (Wieringa et al., 2003, 2004). The function of the non-glycosylated envelope protein E is not yet known.

Two major cell entry pathways, the endosomal and the non-endosomal pathway, are known for enveloped virus to deliver the viral genome into the target cell. In both cases the release of the viral genome requires fusion of the viral envelope with the respective target membrane of the host cell, the endosomal or the plasma membrane. Membrane fusion is mediated by a conformational change of viral proteins. For viruses utilizing the non-endosomal pathway (Marsh and Helenius, 2006), like Retroviruses (HIV-1) and Paramyxoviruses (e.g. Sendai virus) the conformational change of the viral glycoproteins is induced by interaction with the receptor of the host cell surface, thereby triggering fusion between the viral envelope and the plasma membrane. In the case of viruses which are taken up by cells via the endosomal pathway (Marsh and Helenius, 2006), acidification of late endosomal lumen by the proton pump activity of V-type ATPases (Perez and Carrasco, 1994) is essential for activating the fusion competence of viral glycoproteins. The low pH triggers the conformational change either directly as for example for the glycoprotein hemagglutinin of influenza virus (Orthomyxoviruses) or indirectly by activation of endosomal proteases cleaving the ectodomain of the viral glycoprotein to enable the structural transition into a fusion competent state. The latter mechanism has been described for the fusion mediating S-protein of SARS virus (Coronaviruses) (Simmons et al.,

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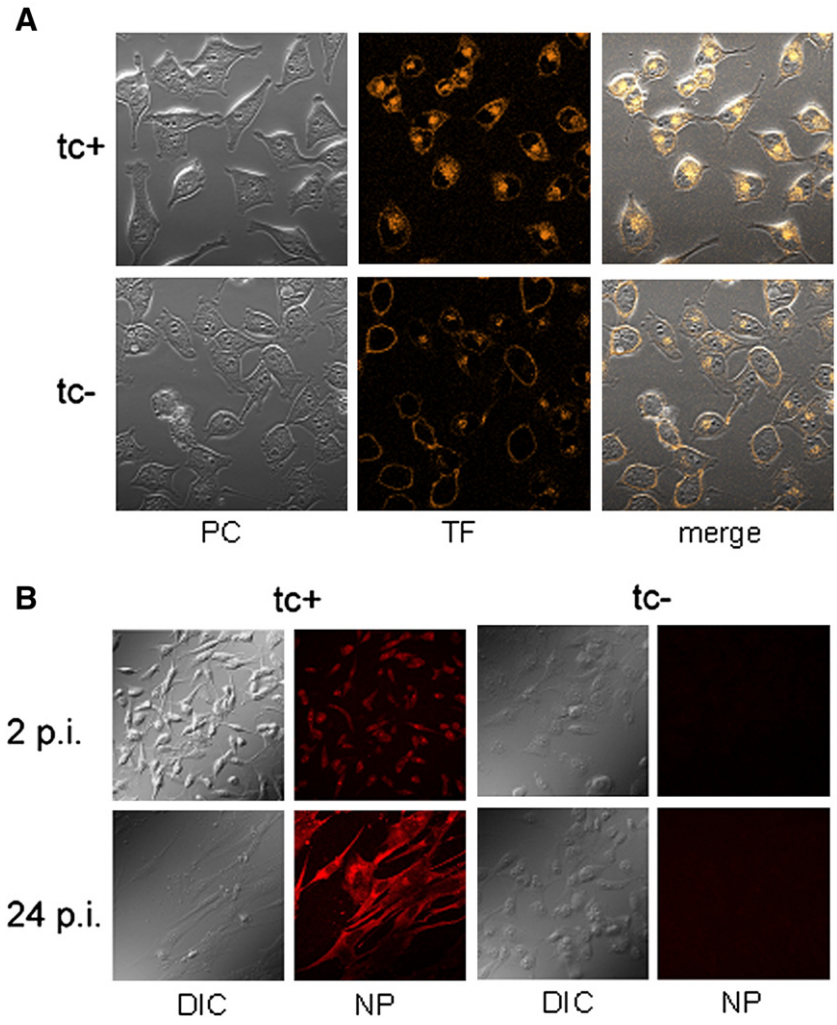


**Fig. 1.** Role of clathrin-dependent endocytosis in infection of BHK cells by EAV. BHKasc cells were preincubated in the presence (tc+) and absence (tc−) of tetracycline. In the latter case antisense RNA against clathrin heavy chain is expressed suppressing clathrin-dependent endocytosis. A. Alexa Fluor 633 transferrin uptake. CHC expressing cells (tc+) display fluorescent transferrin (Tf) with high intensities in intracellular vesicles, whereas cells (tc−) with suppressed CHC expression show no or less intense intracellular internalisation of transferrin. Uptake was measured one min after addition of transferrin (37 °C). B.Infection of BHKasc cells by EAV. At 2h p.i. and 24h p.i. cells were fixed and examined, stained with a primary antibody against nucleocapsid protein and TRITC labelled secondary antibodies, and examined by confocal microscopy. Fluorescence images were recorded at identical gain settings. PC — phase contrast microscopy. DIC — differential interference microscopy.

2005). Recently, it has been shown for Avian Sarcoma/Leucosis virus (ASLV) that the conformational change of fusion mediating protein requires both receptor priming and low pH (Mothes et al., 2000).

In this study we have investigated the entry pathway of EAV using BHK cells as a target. The influence of substances interfering either with the endosomal pathways or with the acidification of late endosomal compartments, such as lysosomotropic agents and inhibitors of the vacuolar $H^+$-ATPase (V-type ATPases) (Perez and Carrasco, 1994), was studied by the plaque assay. To assess more specifically the role of clathrin-dependent endocytosis in EAV infection we utilized a BHK-21 cell line with inducible expression of clathrin heavy chain (CHC) antisense RNA (Iversen et al., 2001). Activation of the antisense RNA causes a block of the clathrin-mediated endocytosis. Our data provide evidence that the clathrin-dependent endocytic pathway is the major route of EAV cell entry and that infection by EAV requires a low pH trigger.

## Results and discussion

### *EAV enters BHK cells via clathrin-dependent endocytosis*

To investigate whether EAV cell entry proceeds via endocytic uptake we first studied the involvement of clathrin-coated pits in EAV internalization using a BHK cell line that can be induced to express antisense RNA of the clathrin heavy chain (CHC) causing a selective block in clathrin-dependent endocytosis (Iversen et al., 2001). For expression of antisense CHC (anti-CHC) cells (tc−) were incubated in the absence of tetracycline (see Materials and methods). To demonstrate the repression of CHC-expression clathrin-dependent endocytosis of fluorescent transferrin was measured. While efficient transferrin uptake was observed for CHC expressing cells (tc+), internalisation of transferrin was strongly inhibited for tc− cells (Fig. 1A). The results confirm previous studies that in tc− cells cathrin-dependent endocytosis is abolished (Iversen et al., 2001).

BHKasc tc+ and tc− cells were infected with EAV. Infection was assessed by immunofluorescence detection of the nucleocapsid protein N (see Materials and methods). For control cells (tc+), N was detected after 2h p.i. and much stronger after 24h p.i. (Fig. 1B). Similar observations were made for BHK-21 cells (not shown). We surmise that N after 2h p.i. originated from uncoated, disassembled viruses in infected cells rather than from expression of new proteins, while N detected after 24h p.i. corresponded to newly expressed proteins. For cells expressing anti-CHC (tc−) N protein was observed neither 2h p.i nor 24h p.i. (Fig. 1B) showing that inhibition of clathrin-dependent endocytosis prevents cell infection. To confirm the result we performed an infectivity assay. The

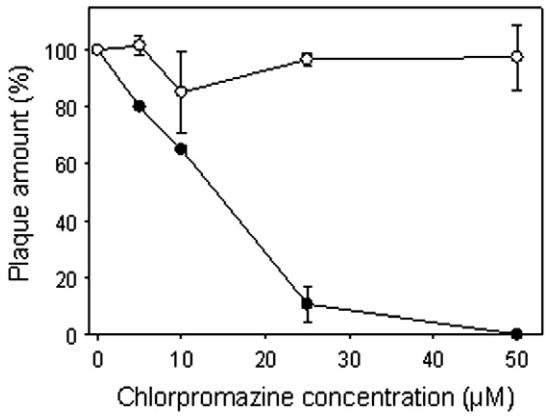


**Fig. 2.** Influence of chlorpromazine on EAV infection of BHK-21 cells. BHK-21 cells were incubated with chlorpromazine and infection by EAV (filled circles) or by SV5 (open circles) was assessed by the plaque assay. Data present mean±standard error of estimate of three independent experiments.

supernatant of cell monolayers incubated for 20–22h after virus adsorption was harvested and probed for infectivity by the plaque assay (see Materials and methods). While we observed strong infectivity of the supernatant from tc+ cells, we could not find any plaque formation when supernatants from tc− cells were used (data not shown).

Next we studied the influence of chlorpromazine known to abolish the formation of clathrin-coated endocytic vesicles by interfering with the interaction between the adapter protein AP-2 and the clathrin-coated pit lattice (Wang, Rothberg, and Anderson, 1993). Chlorpromazine as well as all other drugs used (see below) were present shortly before and during virus adsorption, but not during the subsequent plaque assay. As shown in Fig. 2 a strong inhibition of infection of BHK-21 cells was observed upon treatment of cells with chlorpromazine. In agreement with this observation, we could detect the nucleocapsid protein N neither at 2h p.i nor at 24h p.i of chlorpromazine treated BHK-21 cells (60 μM chlorpromazine) by immunofluorescence (images not shown).

The absence of any detectable intracellular N protein after 2h p.i. in chlorpromazine treated cells supports strongly that the early phase of virus infection is inhibited, and that it is unlikely, that suppression of virus production is due to inhibition of transport of viral proteins or assembly and budding of virions. In contrast to EAV, chlorpromazine treatment of BHK-21 cells did not reduce infection by SV5 (Fig. 2) belonging to Parainfluenza viruses which are known to infect cells via a non-endocytic mechanism (Lamb and Kolakofsky, 2001). This result confirms that treatment with chlorpromazine does not interfere with other cellular processes at least those which are important for virus maturation.

Taken together these results strongly argue for a clathrin-dependent endocytic uptake as the main cell entry route for EAV.

### *EAV infection requires a low pH compartment*

Based on the observation that EAV enters via endocytosis the host cell we asked whether EAV infection requires the acidic environment of the endosomal compartment. To this end, we incubated cells with substances preventing endosomal acidification. We have used lysosomotropic agents as (i) *ammonium chloride*, a relatively weak base accumulating inside endosomal vesicles (Brindley and Maury, 2005; Jin et al., 2005; Ohkuma and Poole, 1978), (ii) bafilomycin A1

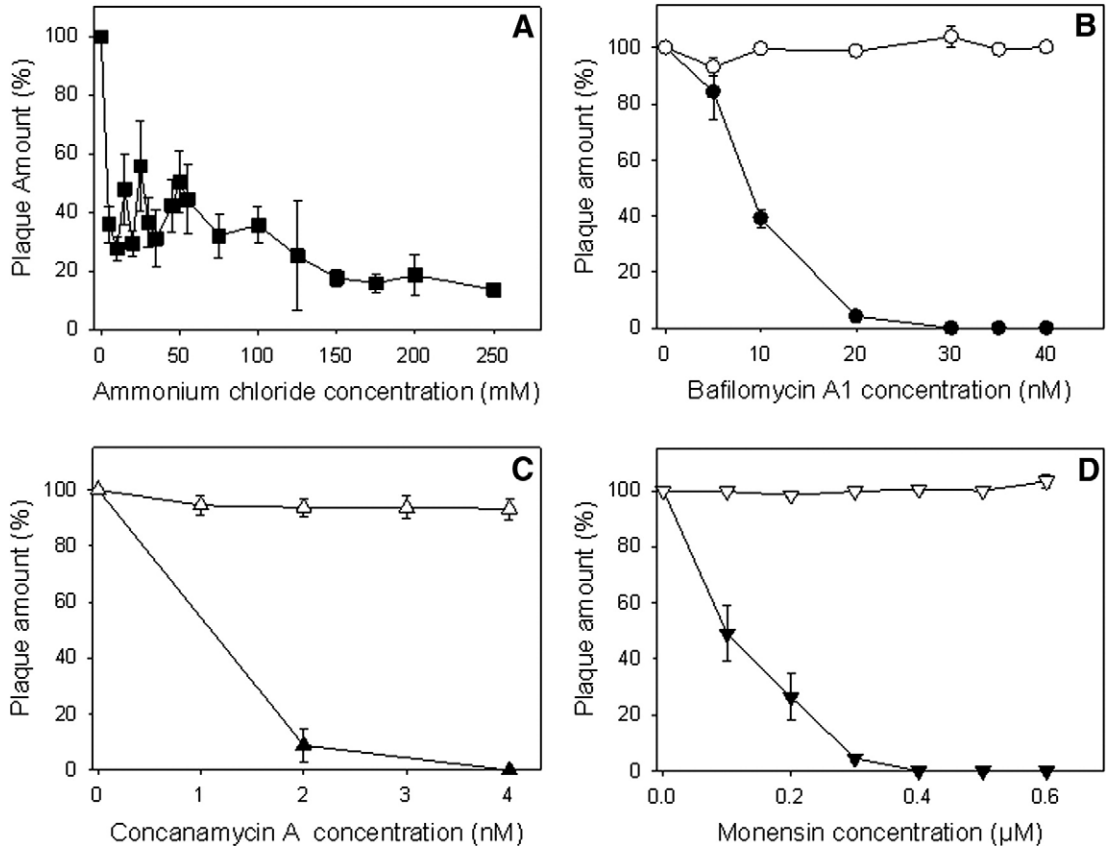


**Fig. 3.** Influence of the lysosomotropic agents on EAV infection of BHK-21 cells. BHK-21 cells were incubated with ammonium chloride (squares) (A), with the vacuolar type $H^+$-ATPase inhibitors (B) bafilomycin A1 (circles) and (C) concanamycin A (triangles up), and (D) with monensin (triangles down), and infection by EAV (filled symbols) or by SV5 (open symbols) was assessed by the plaque assay. Data present mean±standard error of estimate of twelve (A) and at least three (B–D) and independent experiments.

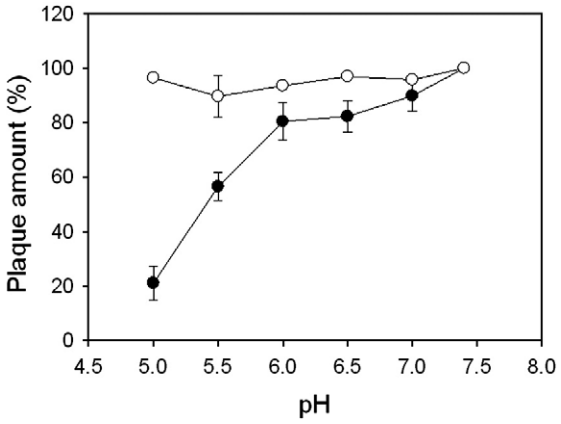


**Fig. 4.** Influence of preincubation at acidic pH. EAV (filled circles) or SV5 (open circles) were incubated at the indicated pH for 1h at 37 °C. After pH neutralization of virus suspension, viruses were adsorbed to BHK cells and the plaque assay was performed (see Materials and methods). Data present mean ± standard error of estimate of three independent experiments.

and concanamycin A, specific inhibitors of the vacuolar $H^+$-ATPase (V-ATPase) in animal and other eukaryotic cells belonging to macrolide lactone ring antibiotics (Drose and Altendorf, 1997), and (iii) *monensin*, an ionophore that disrupts the proton gradient across vesicular membranes (Perez and Carrasco, 1994).

Preincubation of BHK-21 cells with ammonium chloride revealed an inhibitory effect on virus reproduction (Fig. 3A).

Incubation of cells with the vacuolar $H^+$-ATPase inhibitors bafilomycin A1 and concanamycin A caused also a suppression of EAV infection (Figs. 3B, C). Even at 2nM of concanamycin A infection was completely abolished. A similar extent of infection suppression was observed for bafilomycin A1 at 20nM. It is known that concanamycin A has a higher inhibitory activity in comparison to bafilomycin A1 (Drose and Altendorf, 1997).

Finally, EAV infection was abolished in the presence of monensin (Figs. 3D). At 100nM plaque reduction was diminished by about 50% with respect to the control. In agreement with this observation, we could detect the nucleocapsid protein N neither at 2h p.i nor at 24h p.i of monensin treated BHK-21 cells (400nM monensin) by immunofluorescence (images not shown).

Hence, all lysosomotropic agents used, showed a strong inhibitory effect on virus infection. In contrast to EAV, neither concanamycin A nor bafilomycin A1 nor monensin treatment of BHK-21 cells did affect SV5 mediated plaque formation (Fig. 3) consistent with the fact that infection by Parainfluenza viruses does not require a low pH compartment (Lamb and Kolakofsky, 2001).

In conclusion, our results strongly argue that EAV after clathrin-dependent endocytic uptake has to be exposed to the low pH environment of endosomes as presuppositions to release the viral genome into the cytoplasm. The low pH compartment might be required to expose EAV to acid-dependent proteases, i.e. to cathepsins. It has recently been reported that cathepsins cleave the S-protein of SARS (Simmons et al., 2005) and gp1 of Filoviruses (Chandran et al., 2005; Brindley et al., 2007) and that this cleavage is required to facilitate the acid-dependent conformational change required to activate the fusion capacity of those proteins. To explore whether proteases of the late acidic compartments may play a role in fusion activation of EAV, we measured infection of BHK-21 cells upon incubation with leupeptin and E64d, which have been shown to inhibit acidic proteases in the endosomal compartment. Leupeptin is a competitive inhibitor of serine and cysteine proteases, while E64d is an irreversible cysteine protease inhibitor. We did not observe any influence on EAV infection up to concentrations of 100 µM leupeptin and 10 µM E64d appropriate concentrations formerly used for inhibition of those proteases (Qiu et al., 2006; Simmons et al., 2005) (data not shown). However, before conclusive results on this issue can be made further studies are warranted to demonstrate that inhibitors indeed are efficient to suppress endosomal proteases.

Since endosomal protease activity does not seem to play a role in infection by EAV, it is more plausible that the low pH directly induces a conformational change of the (still unknown) glycoproteins of EAV mediating membrane fusion. This is supported by our observation of a strong inhibition of infection of BHK cells when viruses were preincubated at pH<6.0 (Fig. 4). The irreversible loss of infectivity upon low pH treatment resembles the behaviour of other enveloped viruses which requires an acidic pH for infection of host cells such as Influenza viruses (Earp et al., 2005). The fusion mediating glycoprotein hemagglutinin of the Influenza virus A undergoes a conformational change at acidic pH and elevated temperatures (Korte et al., 1999; Puri et al., 1990). This conformational change is irreversible and abolishes the fusion activity and, thus, the infectivity of influenza viruses. Infection of cells by SV5 was not affected by low pH preincubation of viruses (Fig. 4). It is known that the conformational change of the fusion mediating F protein of Parainfluenza viruses is triggered by interaction with cellular receptors but not by acidic pH (Lamb and Kolakofsky, 2001).

To confirm the important role of acidic pH for infection of cells, we have performed 'low pH-bypass' experiments. Those experiments show whether cells treated with inhibitors of virus entry can be infected by triggering fusion of viruses with the plasma membrane at low pH. For this purpose, cells were pretreated as described above either with chlorpromazine (60 µM), concanamycin A (4nM), or monensin (400nM) known to inhibit infection of BHK-21 cells (see Figs. 2 and 3). During virus adsorption, virus–cell complexes were incubated for 15 min at pH 5.0, 37 °C, and subsequently re-neutralised (see Materials and methods). While without low pH incubation infection was almost completely abolished (Fig. 5) as expected (see above) we observed a very strong infection when virus–cell complexes were exposed to low pH. These experiments support that low pH triggers fusion of EAV viruses. Remarkably, infection was four to six folds higher in comparison to infection of cells not treated with inhibitors (control, without low pH incubation). Obviously, the low pH incubation was much more efficient in activating the infection

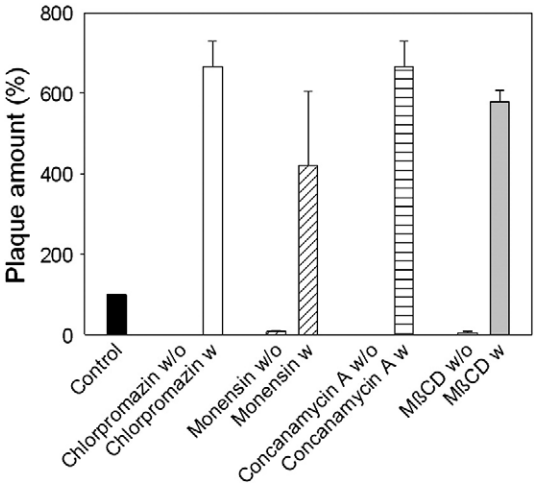


**Fig. 5.** Low pH-bypass of inhibition of virus infection. BHK cells were pretreated as described above either with chlorpromazine (60 µM), concanamycin A (4nM), monensin (400nM), or MβCD. After the first 60 min of virus adsorption at 37 °C, the pH was lowered to 5.0 for 15 min. After neutralization, virus–cell complexes were incubated for further 45 min and the plaque assay was performed as described in Materials and methods. Control — mock pretreatment, no low pH exposure. w/o — without low pH exposure. w — with low pH exposure. Data present mean ± standard error of estimate of three independent experiments.

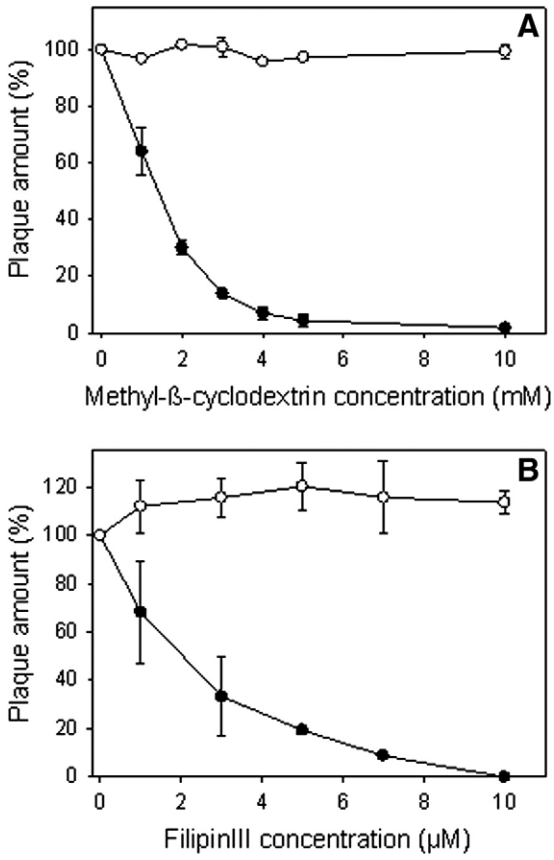


**Fig. 6.** Influence of cholesterol depletion on EAV infection of BHK-21 cells. (A) After pretreatment of BHK cells with MßCD or (B) incubation with filipin III infection of cells by EAV (filled circles) or by VSV (open circles) was assessed by the plaque assay. For treatment of the cells with substances see Materials and methods. Data present mean ± standard error of estimate of three independent experiments.

potential of viruses in comparison to the natural entry pathway of viruses. Infection of BHK-21 cells by SV5 was neither enhanced nor reduced by low pH exposure (data not shown) indicating also that cells were not affected by a temporary acidic environment.

*Cholesterol is a determinant of EAV entry*

Recent studies provide evidence that cell entry of enveloped viruses, for example murine coronavirus (Choi et al., 2005; Eifart et al., 2007), could be associated with lipid domains enriched of cholesterol, so called rafts. To assess the role of cholesterol in EAV entry, BHK-21 cells were depleted of cholesterol by preincubation with methyl-beta-cyclodextrin (MβCD, see Materials and methods). At concentrations >5 mM MβCD EAV infection was completely blocked (Fig. 6A). Furthermore, treatment of cells with filipin III which is known to form complexes with cholesterol in membranes abolished EAV infection (Fig. 6B). As a control BHK-21 cells were infected with VSV, which enters cells independently of cholesterol and/or rafts (Guyader et al., 2002). Indeed, cholesterol depletion of the BHK-21 cells by MβCD (Fig. 6A) or treatment of cells with filipin III (Fig. 6B) did not diminish infection by VSV. Notably, we found a slightly enhanced infectivity of VSV upon filipin III treatment.

These findings strongly indicate an important role of cholesterol for EAV infection. Since the inhibitory effect of cholesterol depletion could be overcome by low pH-bypass (Fig. 5) we conclude that cholesterol depletion inhibits virus uptake but not binding of viruses to cells. Cholesterol is strongly engaged in the clathrin- (Rodal et al., 1999; Subtil et al., 1999) as well as in the caveolae-dependent endocytic pathway (Yumoto et al., 2006). Because inhibition of expression of the clathrin-heavy chain completely blocked infection (see above) the caveolae-dependent endocytic entry plays no role in EAV uptake. Hence, cholesterol depletion may interfere with the clathrin-dependent entry of EAV.

In summary, EAV enters host cells via clathrin-dependent endocytosis. After uptake endocytic vesicles are delivered to a late acidic endosomal compartment. Low pH is essential for infection of the cell. Since we have no indication that proteases of this late compartment are essential for infection, we surmise that low pH is required to trigger a conformational change of the structural virus protein mediating fusion. Alternatively, it might be that EAV in principle can fuse at neutral pH, but its cellular receptor is rapidly endocytosed and subsequent acidification in the endosome is required to release virus particles from the receptor. Although the structural proteins of the EAV envelope have been identified, it is not yet known which of the protein(s) is (are) responsible for mediating fusion. The Gp2b/Gp3/Gp4 complex is currently a prime candidate for the membrane-fusion activity of EAV (Wieringa et al., 2003, 2004).

One may wonder whether the clathrin-dependent endocytosis is typical for infection of cells by Arteriviruses. As shown recently mouse hepatitis viruses (MHV) belonging to Coronavirus family may use different routes for cell entry. While MHV-A59 is taken up via clathrin-dependent endocytosis (Eifart et al., 2007), non-endocytic internalisation has been described for MHV-4 (JHM) and MHV-S4 ((Nash and Buchmeier, 1997; Kooi etal., 1991). The entry pathway of EAV shares similarities with that of another Arteriviruses, the Porcine Reproductive and Respiratory Syndrome virus (PRRSV). It has been shown that PRRSV enters cells via receptor-mediated and low pH-dependent endocytic pathway through a pH-dependent mechanism like we have described here for EAV (Kreutz and Ackermann, 1996; Nauwynck et al., 1999). Kreutz and Ackermann (1996) found that infection of a monkey kidney cell line by PRRSV was strongly inhibited in the presence of 20 µM cytochalasin B pointing to an involvement of active transport of PRRSV containing endocytic vesicles by microfilaments. Lysosomotropic agents such as ammonium chloride and bafilomycin A1 suppressed PRRSV infection of those cells at concentrations similar to that used in our study. Taken together, these results suggest that viruses belonging to the family Arteriviridae enter cells via a similar endocytic route.

## Materials and methods

*Cells and viruses*

Cell lines were propagated as adherent monolayer cultures. Baby hamster kidney (BHK-21 C13) (American Type Culture Collection) cells were grown in Dulbecco's modified Eagle medium (DMEM) (Cambrex, Verviers, Belgium) mixed one to one with Leibovitz L-15 medium (Cambrex, Verviers, Belgium) supplemented with 5% heat-inactivated fetal bovine serum (ΔFBS) (HyClone, Logan, UT) called grow media (GM). These cells were maintained at 37 °C in a humidified incubator at 5% $CO_2$.

EAV strain bucyrus (Doll et al., 1957) were grown in roller cell culture flask in roller incubator at 5% $CO_2$, 37 °C. The adherent, confluent BHK-21 monolayers were incubated with virus multiplicity (MOI) of 1 in serum-free grow media at 37 °C and 5% $CO_2$ for 3h (0.25rpm). After adsorption, the inocula were washed and replaced with GM. The cell supernatant was harvested at 20–22h post infection (titer was determined by the plaque assay). After differential centrifugation to remove cellular debris (sequential spins of 15 min at 4 °C at 4000×g, followed by 2 h at 4 °C at 13000×g) the virus preparations were either used immediately or stored at −80 °C in phosphate buffered saline (PBS, Cambrex, Verviers, Belgium). No influence of storage on virus infection was observed.

The BHK-21-tTA clathrin heavy chain/antisense (BHKasc) cell line, kindly provided by K. Sandvig (Institute for Cancer Research at the Norwegian Radium Hospital, Montebello), was grown in complete

DMEM (Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (ΔFBS) (HyClone, Logan, UT), 2 mM L-glutamine (Cambrex, Verviers, Belgium), and 20μg/ml tetracycline in a cell culture flask at 5% $CO_2$, 37 °C. For induction of CHC antisense RNA expression, tetracycline was removed from the medium.

*Titer determination of viral stocks*

The viral titer was determined by the plaque assay using BHK-21 cells as indicator cells in EMEM (Cambrex, Verviers, Belgium) with 1% ΔFBS, 1% L-Glutamine (Sigma, Berlin, Germany) and 0.9% v/v SeaPlaque agarose (Cambrex, Verviers, Belgium).

*Plaque assay*

BHK-21 cells were plated on 6-well plates and grown in GM (2ml/well). The confluent BHK-21 monolayer was washed with PBS. Sequential 10-fold dilutions of virus were prepared and cells were inoculated with a volume of 1ml/well to determine the plaque forming units (pfu) which are plotted as the amount of plaques in%. A decrease of pfu by 10% corresponds to a reduction of plaques by one order of magnitude.

After adsorption for 2h at 37 °C and 5% $CO_2$ the cells were washed again to remove also any lysosomotropic substance or inhibitor (see below). Subsequently, the overlay, EMEM (Cambrex, Verviers, Belgium) with 1% ΔFBS, 1% L-Glutamine (Sigma, Hannover, Germany) and 0.9% v/v SeaPlaque agarose (Cambrex, Verviers, Belgium) was added.

After 2days of incubation at 37 °C and 5% $CO_2$ the cells were stained with neutral red (Biochrom, Berlin, Germany), fixed with 10% formalin (Roth, Karlsruhe, Germany), and viral plaques were counted. For control experiments Simian Parainfluenza virus 5 (SV5) and Vesicular Stomatitis virus (VSV) (strain Indiana) was used.

*Low pH pretreatment*

In some cases, viruses (EAV or SV5) were pretreated for 1 h at 37 °C in serum-free growth medium adjusted to various acidic pH-values with 0.25 M citric acid. After neutralization of virus suspension, viruses were adsorbed to BHK cells and a plaque assay was performed.

*Influence of lysosomotropic agents, various inhibitors and cholesterol depleting substances*

Bafilomycin A1, chlorpromazine, concanamycin A, E64d, filipin III, leupeptin, methyl-beta-cyclodextrin (MβCD), monensin were obtained from Sigma (Hannover, Germany), and ammonium chloride from Roth (Karlsruhe, Germany). Substances were used within the following concentration range; bafilomycin A1 ranged from 5 to 40 nM, chlorpromazine from 5 to 100 μM, concanamycin A from 2 to 4 nM, E64d from 0.1 to 10 μM, filipin III from 1 to 10 μM, leupeptin from 1 to 100 μM, methyl-beta-cyclodextrin from 1 to 10 mM, monensin from 100 to 600nM, and ammonium chloride from 5 to 30 mM.

Before addition of virus and performing the plaque assay, cells were treated with the substances for 30 min in GM without ΔFBS. Subsequently, cells were infected in the presence of different drug concentrations and incubated for 2 h (see above). Subsequently, lysosomotropic substances and inhibitors were washed out. Only for MβCD and filipin III cells were washed with PBS (with physiological conentrations of $Mg^{2+}$ and $Ca^{2+}$) before infection.

*Low pH-bypass*

To bypass any inhibitory effect of substances, virus was bound to BHK cells pretreated with substances for 2 h at 37 °C and 5% $CO_2$ as described above. After the first 60 min of virus adsorption, the pH was lowered to 5.0 for 15 min. After neutralization, virus–cell complexes were incubated for further 45 min and processed as described above. To account for strong infectivity and, hence, high amounts of plaques sequential 10-fold dilutions of virus were performed.

*Infectivity assay*

The adherent, confluent BHKasc cell monolayers (tc− or tc+) were incubated with virus multiplicity (MOI) of 1 in serum-free growth media at 37 °C and 5% $CO_2$ for 2h. After adsorption, the inocula were washed and replaced with growth medium. The cell supernatant was harvested at 20–22 h post infection and the titer was determined by plaque assay using BHK-21 cells.

*Immunofluorescence*

To induce the expression of antisense CHC RNA (anti-CHC) in BHKasc cells, the cells were incubated for 18 h in the absence of tetracycline (tc−), for control cells tetracycline was present (tc+). The adherent, confluent BHKasc cell monolayers were incubated with virus multiplicity (MOI) of approximately 10 in serum-free grow media at 37 °C and 5% $CO_2$ for 2h. After adsorption, the inocula were washed twice with PBS. Cells were fixed at different time points post infection by PBS containing 3% formaldehyde, subsequently quenched with 100 mM glycin, permeabilized with 0.5% Triton X-100, and then incubated with the primary mouse monoclonal antibody against EAV nucleocapsid protein (VMRD Inc., Pullman USA) (Deregt et al., 1994) for 30 min at 37 °C. Subsequently, cells were incubated with the appropriate TRITC labelled secondary antibody against mouse IgG developed in goat (Sigma, Hannover, Germany). Coverslips were obtained from Marienfeld (Germany). Confocal fluorescence microscopy was done with an Olympus FV-1000.

*Uptake of transferrin*

BHKasc cells with and without removal of tetracycline from culture medium were seeded into 8-well μ-slide observation chambers (Ibidi, Martinsried, Germany) and incubated for 24h at 37 °C at 5% $CO_2$. Prior to the experiment the culture medium was gently removed from the cells and exchanged against transferrin conjugated to Alexa Fluor 633 (Invitrogen, Paisley, UK) (10μg/ml final concentration) diluted medium (GM). Immediately after adding of the marker dilution to the cells were incubated for 1h at 37 °C and images were taken. Confocal and phase contrast images were acquired with a Zeiss laser scanning microscope LSM510 Meta mounted on an Axiovert 200 M inverted microscope using a 63× phase contrast oil immersion plan-apochromat objective NA1.4. Visualization of transferrin uptake was performed under identical conditions for the tc+ and tc− cells.

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