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Receptor-Mediated Entry by Equine Infectious Anemia Virus Utilizes a pH-Dependent Endocytic Pathway

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Previous studies of human and nonhuman primate lentiviral entry mechanisms indicate a predominant use of pH-independent pathways, although more recent studies of human immunodeficiency virus type 1 entry appear to reveal the use of a low-pH-dependent entry pathway in certain target cells. To expand the characterization of the specificity of lentiviral entry mechanisms, we have in the current study examined the entry pathway of equine infectious anemia virus (EIAV) during infection of its natural target, equine macrophages, permissive equine fibroblastic cell lines, and an engineered mouse cell line expressing the recently defined equine lentivirus receptor-1. The specificity of EIAV entry into these various cells was determined by assaying the effects of specific drug treatments on the level of virus entry as measured by quantitative real-time PCR assay of early reverse transcripts or by measurements of virion production. The results of these studies demonstrated that EIAV entry into all cell types was substantially inhibited in a dose-dependent manner by treatment with the vacuolar H^+-ATPase inhibitors concanamycin A and bafilomycin A1 or the lysosomotropic weak base ammonium chloride. In contrast, treatments with sucrose to block clathrin-mediated endocytosis or with chloroquine to block organelle acidification failed to inhibit EIAV entry into the same target cells. The observed inhibition of EIAV entry was shown not to be related to cytotoxicity. Taken together, these experiments reveal for the first time that EIAV receptor-mediated entry into target cells is via a low-pH-dependent endocytic pathway.

Productive infection of target cells by animal viruses requires access to highly specific entry pathways that introduce the critical virion components into the cell cytoplasm for subsequent replication processes, including uncoating and genome expression and replication. Nonspecific entry or entry by an incorrect pathway typically results in inappropriate processing and degradation of the virion, leading to a nonproductive infection. The observed specificity of individual virus entry mechanisms indicates critical virus-cell interactions that, if defined in detail, can provide novel targets for antiviral drug development. Thus, a focus in virus research has been to elucidate the cellular pathways utilized by viruses to infect target cells.

The results of these studies to date have revealed two predominant post-receptor-binding entry pathways accessed by enveloped animal viruses. These distinct pathways are designated pH-independent and pH-dependent entry. In the pH-independent entry pathway, enveloped virus binding to specific receptor triggers a direct fusion of the viral and cellular membrane at the extracellular pH, without a requirement for an acidic environment. This pH-independent pathway is utilized by enveloped viruses such as hepatitis B virus (26), Sendai virus (50), and the human and simian immunodeficiency viruses (15, 41). In the pH-dependent pathway, the receptor binding directs the virus into an intracellular compartment in which an acidic environment is required for fusion of the viral and cellular membranes (21, 28, 54). The pH-dependent pathway is utilized by enveloped viruses such as Semliki Forest virus (29), West Nile virus (11), Hantaan virus (32), vesicular stomatitis virus (41), influenza virus (25), avian leukosis virus (16), and salmon anemia virus (19). Viral entry of amphotropic and ecotropic murine leukemia viruses can be both pH dependent and pH independent, conditional on the virus strain (34, 41, 43). Interestingly, some enveloped viruses, like herpes simplex virus, may enter target cells via more than one pathway (3).

As demonstrated by the summary above, individual members of the retrovirus family apparently can utilize different entry pathways. Among the oncoviruses examined, the murine leukemia viruses enter by a pH-independent pathway, while the avian leukosis viruses enter by pH-dependent endocytosis. To date, examination of lentivirus entry mechanisms has indicated that human and simian immunodeficiency viruses enter target cells by pH-independent pathways. However, more recent studies have reported that human immunodeficiency virus type 1 (HIV-1) entry specificity may be dependent on the specific target cell, as HIV-1 evidently can establish productive infection in certain cell types by receptor-specific, clathrin-mediated endocytosis (15). In addition, other studies indicate that HIV-1 entry may or may not require internalization of CD4 receptor (36–38, 46) and that HIV-1 can infect certain cultured CD4-negative human fibroblast cells, suggesting alternatives to CD4-mediated entry (8, 48, 56). These apparent variations reported from independent studies of HIV-1 entry pathways naturally raise the question of the type of entry mechanisms used by other animal lentiviruses, especially those with defined cell receptors.

Equine infectious anemia virus (EIAV), an exclusively macrophage-tropic lentivirus, causes a uniquely dynamic disease in

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horses that provides a novel model for examining the diverse pathologies associated with lentivirus infection of monocytes and macrophages (42). We recently cloned and characterized a functional cellular receptor for EIAV, designated as equine lentivirus receptor-1 (ELR1) (60). The ELR1 protein is a member of the tumor necrosis receptor protein family and appears to be sufficient for mediating productive virus infection in the absence of any coreceptor, in contrast to human, simian, and feline lentiviruses, which typically require coreceptors. Thus, it was of interest to examine the entry pathway utilized by EIAV in naturally susceptible cells and in nonsusceptible cells transduced with the ELR1 protein.

In the present study, we describe a series of experiments in which we examine the ability of selective drug treatments to inhibit EIAV entry, leading to productive infection of equine macrophages, permissive equine fibroblastic cells, and ELR1-transduced mouse cells. The results of these studies demonstrate for the first time that EIAV receptor-mediated infection occurs via a pH-dependent endocytic pathway. These results are in general agreement with the conclusions drawn by Brindley and Maury in independent experiments with different strains of EIAV, as described in the companion paper (7).

MATERIALS AND METHODS

Cells. The EIAV permissive equine dermal (ED) cell line (ATCC CCL-57) and primary fetal equine kidney (FEK) cells (45) were grown in minimal essential medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Ogden, UT), 2 mM glutamine, nonessential amino acids, 100 U of penicillin, and 100 μg of streptomycin per ml (Gibco BRL). NIH 3T3 (ATCC CRL-1658) and its derivate 3T3-RHA cells (60) stably expressing hemagglutinin (HA)-tagged EIAV receptor were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, 100 U of penicillin, and 100 μg of streptomycin per ml. These cell lines were maintained at 37°C in a humidified incubator at 5% CO_2. Procedures for the isolation and culture of monocyte-derived equine macrophages were essentially as described elsewhere (49).

Cell viability assay. A 5-mg/ml solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was freshly prepared and filtered. Cells were incubated with MTT for 4 h at 37°C, washed, and resuspended in isopropanol-HCl solution. The level of color production was assayed on an enzyme-linked immunosorbent assay plate reader by measuring optical density at 570 nm. Concentrations of drugs used in inhibition studies were limited to those levels maintaining at least 90% of the viability observed in untreated cell cultures; drug concentrations further reducing cell viability were excluded from additional consideration.

Virus stocks. The parental pathogenic proviral molecular clone $EIAV_{UK}$ has been described in detail by Cook et al. (13). ED cells chronically infected with $EIAV_{UK}$ were cultured, and the supernatants from these cells were harvested every 3 days, clarified by centrifugation, aliquoted, and frozen at −80°C. The viral titer on ED cells was 1.66×10^5 IU/ml, as titrated by a standard viral infectious center assay (27).

Measurement of EIAV entry into target cells. To detect virus entry, we initially employed a quantitative real-time PCR assay designed to specifically amplify early reverse transcriptase (early RT) DNA products produced soon after virus internalization. As previously shown in our lab, this assay is a sensitive and specific indicator of EIAV receptor-mediated entry (33, 60).

Pharmacological inhibition of EIAV entry. Specific drug treatments included the use of lysosomotropic agents (ammonium chloride and chloroquine) and vacuolar H^+-ATPase (V-ATPase) inhibitors (bafilomycin A1 [BafA1] and concanamycin A [CA]) to inhibit acidification of endosomal compartments (5, 6, 12, 53, 57–59) and sucrose to block clathrin-coated endocytosis (11, 32, 44). Unless otherwise noted, all of the lysosomotropic agents and V-ATPase inhibitors were purchased from Sigma (St. Louis, MO). Working solutions of BafA1 and CA were prepared in dimethyl sulfoxide and stored at −20°C. Stock solutions of ammonium chloride, chloroquine, and sucrose were prepared daily in distilled water and sterilized through a 0.2-μm filter.

To assess the effects of specific drug treatments on EIAV entry, approximately 4×10^5 cells per well were seeded in six-well plates and incubated overnight. Virus infection was performed in duplicate wells. Cells were infected with $EIAV_{UK}$ at a multiplicity of infection of 0.08 for 2 h at 37°C in the presence of various concentrations of V-ATPase inhibitors, lysosomotropic agents, and dimethyl sulfoxide or medium (diluent controls). After infection, unabsorbed virus was removed by washing three times with phosphate-buffered saline (PBS), and the cells were cultured in fresh drug-free medium for an additional 4 h. Controls included mock-infected cells in the presence of the respective drug or inoculated cells with either medium or dimethyl sulfoxide in place of specific drug treatment. In some cases, cells were incubated with agents for 30 min at 37°C prior to infection.

At 6 h postinfection (hpi), the total DNA was extracted from the infected and treated cells using a DNeasy tissue kit (QIAGEN, Valencia, CA) and subjected to quantitative real-time PCR. Individual samples were evaluated in duplicate in at least two separate experiments. Virus infectivity was determined based on production of early reverse-transcribed viral DNA copies using quantitative real-time PCR. The copy number was normalized for copies of the *gapdh* gene, as described elsewhere (33). The relative infectivity for the culture treated with different agents was normalized against the same cells infected in the absence of agents.

Assays of selective drug inhibition on virus production. To examine the effect of specific agents on virus production, drug treatments were performed as described above, except that the postinfection period was extended to 1 to 6 days. To prevent sequential rounds of virus infection from infected cells, the reverse transcription inhibitor 3′-azido-3′-deoxythymidine (AZT; Sigma), was added at a final concentration of 1 μM to selected cultures at 24 hpi. The culture supernatants were collected, clarified by centrifugation to remove cell debris, and stored at −80°C prior to measuring RT activity as described previously (10).

Assays of EIAV penetration of target cells. To assess the effects of drug treatments specifically on the penetration step in virus infection, infectious $EIAV_{UK}$ virus particles were prebound to target cells for 1 h at 4°C in the absence of drugs. The infectious supernatant was then removed, and the cells were fed with fresh medium in the presence or absence of agents and shifted to 37°C for 2 h. The medium was removed, and the cells were washed three times with cool PBS and cultured in fresh medium without drugs for an additional 4 h. At 6 hpi, the cells were harvested and total cellular DNA was extracted and subjected to quantitative real-time PCR to measure early RT products.

Effect of drug treatment on ELR1 expression. To examine the effect of drug treatments on EIAV receptor protein expression, 3T3-RHA cells expressing HA-tagged ELR1 were infected with virus in the presence of the indicated concentrations of CA for 2 h at 37°C. Cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Subsequently, cells were stained with rat anti-HA antibody (clone 3F10; Roche) followed by staining with anti-rat immunoglobulin G–fluorescein isothiocyanate–conjugated antibody (Roche) at 4°C. The level of protein expression was analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences, San Jose, Calif.).

RESULTS

EIAV entry into equine cells is inhibited by treatment with ammonium chloride and V-ATPase inhibitors. To identify the entry pathways for EIAV infection, we investigated whether EIAV infection of diverse equine target cells could be modulated by a panel of agents that specifically inhibit distinct endocytic pathways. The drugs tested included the lysosomotropic agents ammonium chloride and chloroquine, the V-ATPase inhibitors BafA1 and CA to inhibit acidification, and sucrose to inhibit clathrin-coated endocytosis. These initial screenings employed single drug concentrations previously reported to inhibit virus entry without significant cytotoxicity (1, 4, 11, 17, 18, 22, 31, 35). Each compound was added to cultures of FEK, ED, or equine macrophage cells and incubated for 30 min prior to infection with $EIAV_{UK}$. The virus infection was then allowed to proceed for 2 h at 37°C in the presence of drug, at which time the supernatants were removed and replaced with drug-free medium. At 6 hpi, the cells were harvested and the total cellular DNA isolated and assayed by real-time quantitative PCR for early RT products as a measure of virus entry. As controls for these drug treatments, parallel cultures of each

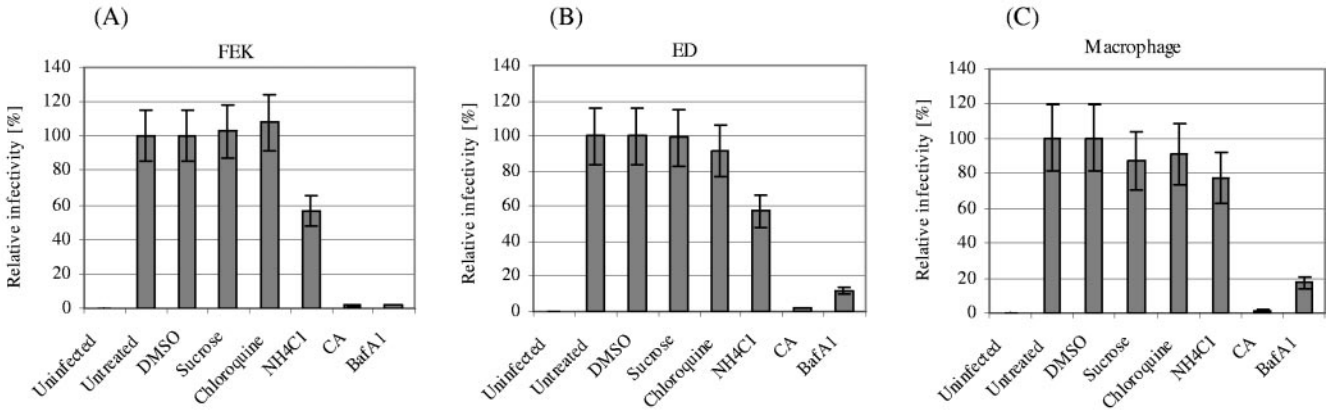

FIG. 1. Effect of endocytosis inhibitors on $EIAV_{UK}$ infectivity in FEK (A), ED (B), or equine macrophages (C). The specific inhibitors at the indicated concentrations were added to target cells at the indicated concentrations during the initial 2-h incubation with infectious virus. FEK and ED cells were treated with 0.3 M sucrose, 200 μM chloroquine, 50 mM NH_4Cl, 5 nM CA, or 0.3 μM BafA1. Equine macrophages were treated with 150 mM sucrose, 10 μM chloroquine, 20 mM NH_4Cl, 50 nM CA, or 1 μM BafA1. At 6 hpi, the cells were harvested, and the total cellular DNA was isolated and subjected to quantitative real-time PCR to assay EIAV early RT products. The data are representative of several independent experiments. Individual treatments were performed in triplicate, and the error bars show deviations from the means. The level of early RT DNA observed in untreated control cells infected with $EIAV_{UK}$ was set to 100% and used for comparison to early RT levels in cells infected in the presence of drugs to calculate a measure of relative infectivity.

cell type were infected with $EIAV_{UK}$ in the absence of any agent and processed identically to the treated cells to measure the respective levels of virus entry.

As summarized in Fig. 1, the results of these inhibition assays revealed substantial reduction of early RT products, and thus virus entry, in all cell types by treatments with BafA1, CA, or ammonium chloride. In contrast, no significant reduction in early RT levels was produced by the presence of either chloroquine or sucrose during virus infection of the same target cells. Specifically, a 97% reduction in early RT products relative to untreated cell infections resulted from treatment of the ED and FEK cells (Fig. 1A and B) with either 5 nM CA or 0.3 μM BafA1, while virus infection in the presence of 50 mM ammonium chloride reduced early RT product levels by about 43% relative to control cell infections. Importantly, a similar pattern of inhibition of virus entry was observed in equine macrophages (Fig. 1C), the natural target cell for EIAV, although relatively higher drug concentrations were required to achieve similar levels of inhibition observed in the ED or FEK cells. For example, 50 nM CA produced a 99% reduction in virus entry, 1 μM BafA1 produced an 83% reduction, and 20 mM ammonium chloride produced a 25% reduction (Fig. 1C). Cell viability measurements using MTT assays (data not shown) confirmed that the cells incubated with the indicated drug concentrations were at least 95% viable, as observed with untreated cells.

While both ammonium chloride and chloroquine are lysosomotropic agents, it is interesting that the latter drug failed to inhibit virus entry in all cell types tested at nontoxic concentrations. While it is not clear why chloroquine failed to inhibit EIAV entry, it has previously been reported that chloroquine does not inhibit pH-dependent entry by human foamy virus or by Sindbis virus, both of which are sensitive to treatment with ammonium chloride or V-ATPase inhibitors (14, 30, 47).

Taken together, these initial observations of substantial inhibition of virus entry by a lysosomotropic agent and two V-ATPase inhibitors suggest that EIAV entry is by a pH-dependent endocytic pathway. In addition, the absence of inhibition by sucrose is consistent with EIAV entry being independent of clathrin-mediated uptake.

Inhibition of EIAV entry is dose dependent. Having identified specific inhibitors of EIAV entry in the preceding screening assays, we next examined the dose dependence of the observed inhibitory effects by BafA1, CA, and ammonium chloride. The data in Fig. 2 demonstrate that all three agents inhibited virus entry in a dose-dependent manner, and they also reveal the relative potency of the various drugs in suppressing EIAV entry into FEK or ED cells. For example, from Fig. 2, it can be estimated that a greater-than-90% inhibition of virus entry into either the FEK or ED cells required at least 100 nM BafA1, but only about 3 to 4 nM CA. In contrast, only about 75% virus entry was inhibited in both cell types by 50 mM ammonium chloride, the maximum concentration that could be used without significant cytotoxicity. Based on the relative specific activities of the various agents evaluated, we chose the V-ATPase inhibitors for subsequent mechanistic studies of the inhibition of EIAV entry into target cells.

V-ATPase inhibitors act at early stages of virus infection. In the preceding experiments, target cells were pretreated with the selected agents prior to virus exposure. To confirm that the observed inhibition of virus entry was due to a specific blockage of early events in virus infection, we next examined the level of inhibition of EIAV entry by BafA1 and CA when added concurrently with the virus inoculum, or at 2 h and 4 h after the addition of virus to target cells. As shown in Fig. 3, a delay in the addition of the agents after exposure of the cells to the virus inoculum resulted in marked decreases in the net inhibition of virus entry compared to that observed with concurrent addition of the drug and virus to the cells. In the case of the BafA1 (Fig. 3A), delaying the addition of drug by 2 h after virus exposure produced a 60% inhibition of virus entry into FEK cells, compared to the nearly 100% inhibition ob-

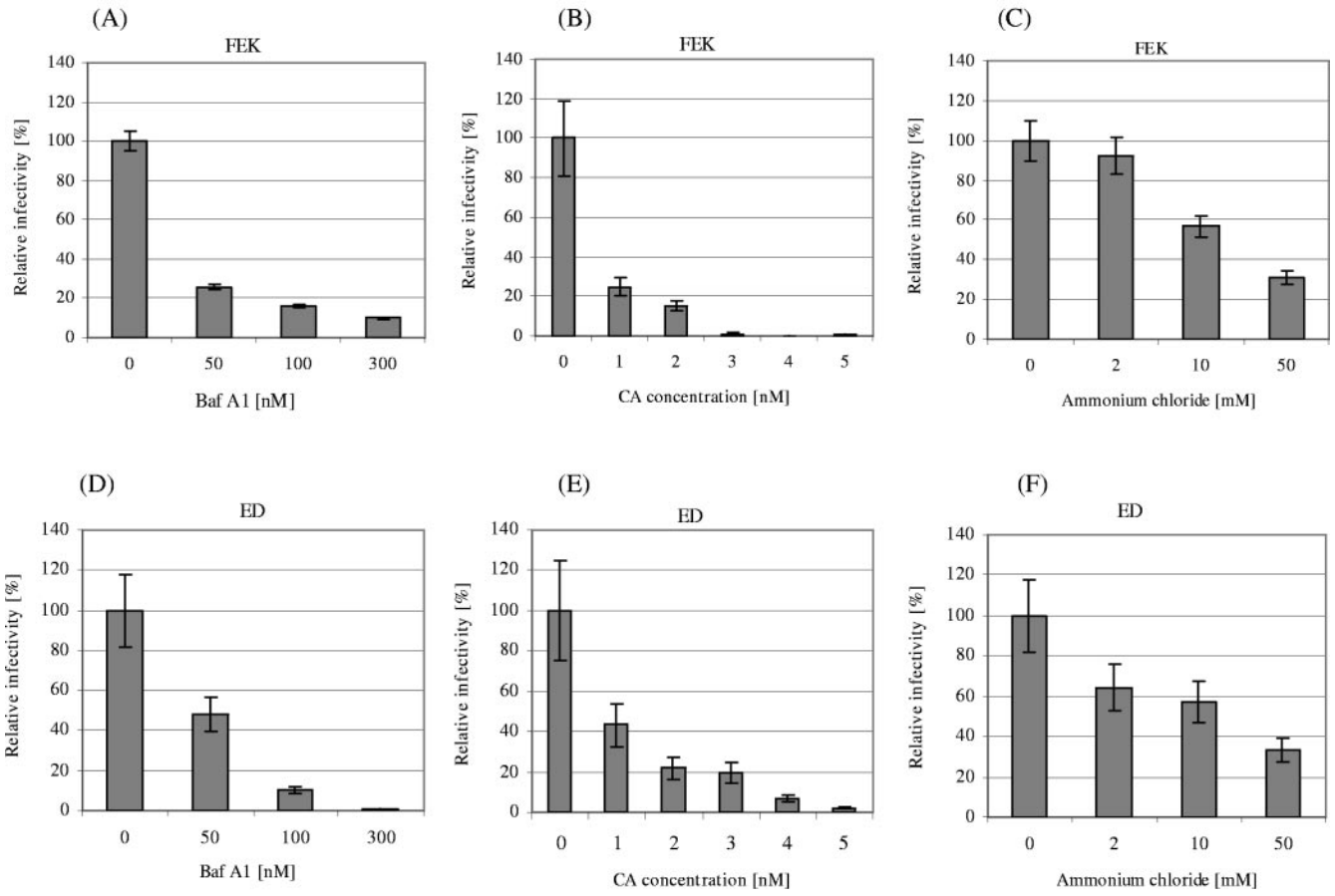

FIG. 2. Dose dependence of inhibition of $EIAV_{UK}$ entry into FEK or ED cells by treatments with different concentrations of endocytosis inhibitors. Infections of FEK or ED cells with $EIAV_{UK}$ were performed as described in the legend for Fig. 1 in the presence of increasing concentrations of BafA1 (A and D), CA (B and E), or NH_4Cl (C and F). After 2 h of infection at 37°C in the presence of drug, the cells were washed with PBS and fed with drug-free medium. At 6 hpi, total cellular DNA was isolated for determining early RT products as a measure of virus infectivity as described in Materials and Methods. The results represent the averages of two independent experiments, with the standard deviations indicated as error bars.

served when the agent was added concurrently with the infectious virus. By 4 h post-virus exposure, addition of BafA1 had no evident effect on EIAV entry. As summarized in Fig. 3B, CA effectively blocked EIAV infection in FEK cells when the drug was present during the initial virus exposure, but no inhibition of virus entry was observed when the drug treatment was delayed by 2 h or 4 h post-virus exposure. Similar kinetics of inhibition by the two V-ATPase inhibitors were also observed in ED cells (data not shown). Taken together, these data indicate that the suppression of virus entry by the V-ATPase inhibitors was associated with early steps in EIAV infection that take place within about 2 h after exposure of the cells to virus.

V-ATPase inhibitors specifically block EIAV penetration. In light of the preceding experiments indicating that the V-ATPase inhibitors were blocking an early step in virus infection, we next sought to determine if these agents specifically blocked entry of the virus after binding to target cells. To this end, FEK and ED cells were incubated with an $EIAV_{UK}$ inoculum at 4°C for 1 h to allow virus binding to cells but not entry. The supernatant containing unbound virus was removed, and the cells were fed with fresh medium in the presence or absence of CA or BafA1 and shifted to 37°C for 2 h to initiate virus infection. The cells were then washed three times with PBS and cultured for another 4 h at 37°C, at which time they were assayed for virus entry by measurement of intracellular early EIAV-specific RT products. As summarized in Fig. 4, the data demonstrate that treatment with either CA or BafA1 during virus penetration inhibited virus entry into either FEK (Fig. 4A) and ED (Fig. 4B) cells by an average of 99% and 90%, respectively. Thus, these results indicate that V-ATPase inhibitors are evidently able to specifically block infection of target cells by bound virions, presumably due to an inhibition of early pH-dependent penetration steps that follow receptor binding.

Inhibition of pH-dependent entry mediated by the equine lentivirus receptor-1. To examine specifically the inhibition of EIAV infection mediated by the recently characterized ELR1 receptor, we next assayed the ability of CA and ammonium chloride to block infection of our engineered NIH 3T3 mouse cells stably expressing an HA-tagged ELR1 receptor, designated 3T3-RHA (60). As summarized in Fig. 5, both CA (Fig. 5A) and ammonium chloride (Fig. 5B) inhibited virus entry in EIAV receptor-transduced NIH 3T3 cells, although relatively higher drug concentrations were required compared to FEK

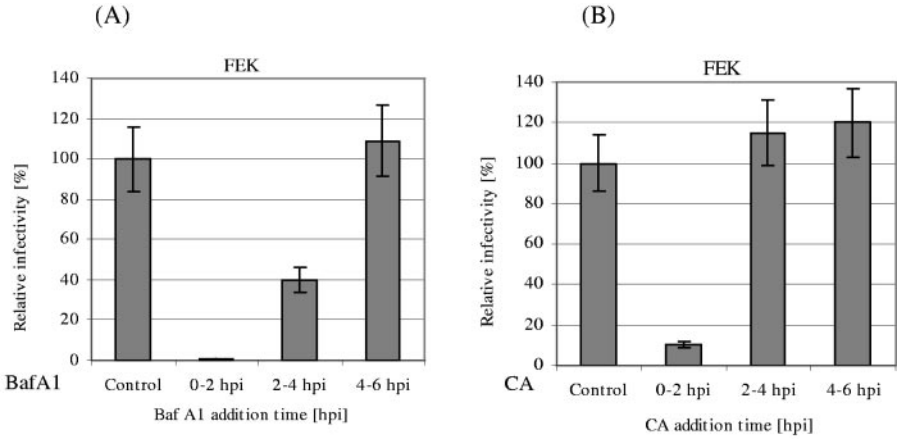

FIG. 3. Kinetics of inhibition of $EIAV_{UK}$ infection by endocytosis inhibitors. Cultures of FEK cells were infected with $EIAV_{UK}$ at 37°C and treated with either 300 nM BafA1 or 5 nM CA at the following times relative to virus inoculation: 0 to 2, 2 to 4, or 4 to 6 hpi. At 6 hpi, cells were harvested and assayed for early RT products as a measure of virus infectivity as described in the preceding figure legends. Early RT levels observed in untreated control cells at 6 hpi were set as 100% and compared to the respective early RT levels observed in cells treated with drugs at the indicated times to calculate a measure of relative infectivity. Data represent the averages of at least two independent experiments with treatments performed in duplicate wells, with standard deviations indicated.

and ED cells. For example, a concentration of about 200 nM CA was required to achieve about 90% inhibition of virus infection in the 3T3-RHA cells (Fig. 5A), compared to about 5 nM CA to produce a similar level of inhibition of virus infection in FEK or ED cells (c.f., Fig. 2). Similarly, treatment with 50 mM ammonium chloride produced a 60% inhibition of infection of the 3T3-RHA cells (Fig. 5B), whereas the same concentration of ammonium chloride resulted in a 75% inhibition of virus infection in FEK and ED cells. To examine whether CA treatment of 3T3-RHA cells interferes with the level of cell surface expression of ELR1 receptor, 3T3-RHA cells were infected in the absence or presence of various doses of CA at 37°C for 2 h. The cells were then fixed, permeabilized, stained with HA tag-specific antibodies, and subjected to flow cytometry analysis to quantify ELR1 expression levels. The data summarized in Fig. 5C demonstrate that ELR1 expression levels in 3T3-RHA cells treated with 5, 50, or 200 nM CA were indistinguishable from those in untreated cells. Thus, virus infection mediated by the ELR1 protein is specifically blocked by CA and ammonium chloride, indicating that receptor-mediated virus infection is via a pH-dependent pathway.

Inhibition of pH-dependent endocytosis suppresses virus production. The preceding experiments examined virus infection of target cells by measuring the production of early RT products by quantitative real-time PCR. To demonstrate that these measurements of early virus infection of target cells correlated with the levels of virus production, we compared the effects of CA treatment on the production of extracellular virus as measured by supernatant RT activity. In these experiments, duplicate cultures of ED or FEK cells were incubated with

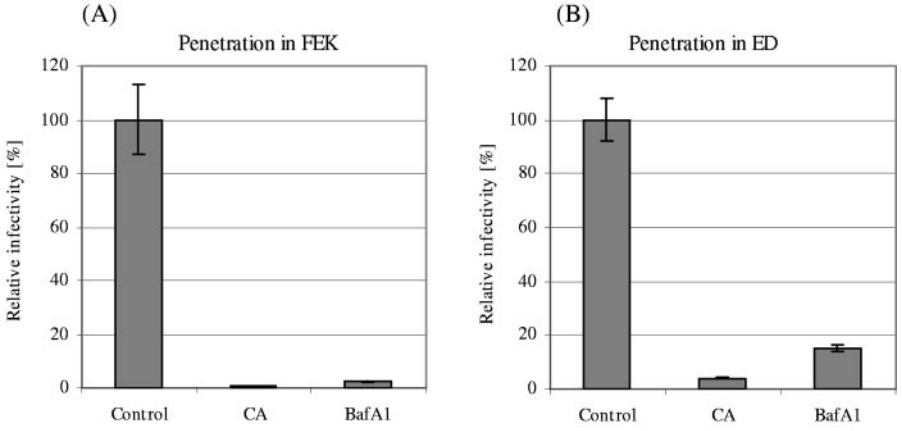

FIG. 4. Effect of V-ATPase inhibitors on $EIAV_{UK}$ penetration into target cells. FEK (A) or ED (B) cells were incubated with $EIAV_{UK}$ for 1 h at 4°C to allow binding but not penetration of virus into target cells. Unbound virus was then removed by washing, and the cultures were shifted to 37°C to initiate virus entry in the presence or absence of the indicated V-ATPase inhibitors (5 nM CA or 300 nM BafA1). At 6 hpi, the cells were harvested, and the total DNA was isolated and assayed for early RT as a measure of infectivity. The early RT levels observed in untreated control cells were set as 100% and compared to early RT levels observed in treated cells as a measure of relative infectivity. The data presented here represent the averages of two independent experiments.

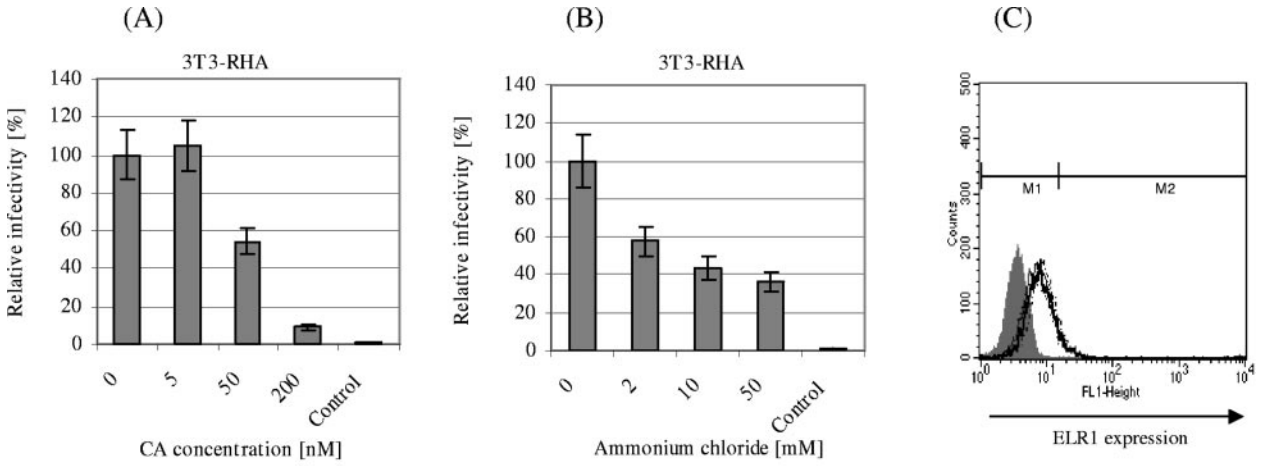

FIG. 5. Inhibition of $EAIV_{UK}$ entry into murine cells transduced to express the ELR1 receptor. (A and B) Murine 3T3-RHA cells were infected with $EIAV_{UK}$ in the presence or absence of either of the indicated doses of CA (A) or ammonium chloride (B) and assayed for early RT products as a measure of virus infectivity, as described in Materials and Methods. Control, nontransduced NIH 3T3 cells were incubated with infectious EIAV to demonstrate lack of significant early RT product production indicative of entry in the absence of the ELR1 receptor, as reported by Zhang et al. (60). (C) To examine the effect of inhibitor treatments on ELR1 expression levels, flow cytometry was used to analyze the levels of ELR1 expression on untreated 3T3-RHA cells compared to cells treated with the indicated concentration of CA for 2 h at 37°C. Following this incubation, the cells were then stained with anti-HA and anti-immunoglobulin G–fluorescein isothiocyanate at 4°C to label the HA-tagged ELR1 protein. NIH 3T3 cells treated with drugs and stained with the above antibodies were measured to determine nonspecific binding by the anti-HA antibody (gray peak). Thin line, 0 nM CA; dark solid line, 5 nM CA; dotted line, 50 nM CA; dashed line, 200 nM CA.

virus in the absence or presence of CA for 2 h at 37°C. After the 2-h infection, the cells were washed with PBS three times and cultured in drug-free medium. At 24 hpi, 1 μM AZT was added to half of the ED or FEK cultures to prevent further rounds of infection. At various times, the levels of virus production were quantified by measurements of supernatant RT activity. As shown in Fig. 6A, CA treatment reduced the levels of virus production in FEK cells in the absence of AZT by about 75% compared to untreated cells at 6 days postinfection. In the presence of AZT to limit infection to a single round, the CA treatment reduced virus production by about 90% compared to untreated cells. Similar results were also observed in ED cells (Fig. 6B) for the effects of CA treatment on virus production levels during single- and multiple-round infections. Specifically, CA treatment reduced virus production by about 90% in ED cells in the presence of multiple-round infections (without AZT) and by about 67% in a single round of infection (with AZT). Thus, these data demonstrate that CA specifically blocks virion production in single- and multiple-round infections of ED and FEK cells, correlating with the inhibition of virus entry by agents that block pH-dependent entry.

DISCUSSION

Several important advances in our EIAV research program provided a unique foundation for the current studies on the mechanism of EIAV entry into target cells. First, we recently

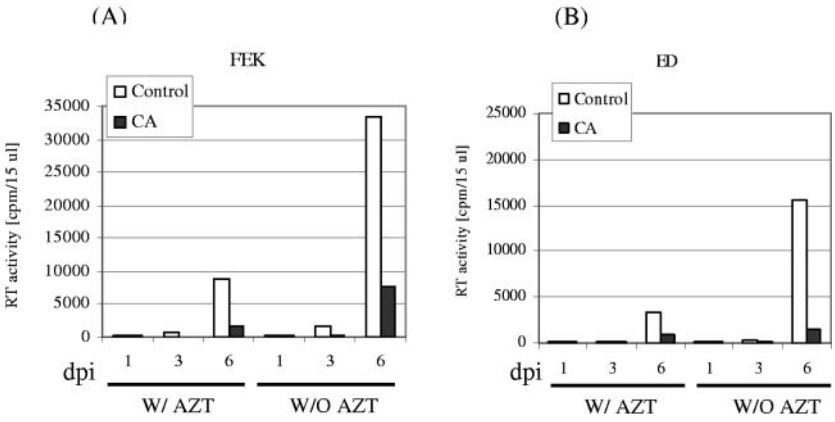

FIG. 6. Effect of CA on virion production in single- and multiple-cycle infections by $EIAV_{UK}$. Cultures of FEK (A) or ED (B) cells were infected by $EIAV_{UK}$ at 37°C in the presence or absence of 5 nM CA. At 24 hpi, 1 μM AZT was added to one-half of the cell cultures to inhibit subsequent cycles of virus infection, while the remaining cell cultures were continued in the absence of AZT to allow multiple cycles of virus infection. At the indicated days postinfection, culture supernatants were analyzed for RT activity as a measure of virion production. The data presented represent the mean values from duplicate experiments.

developed quantitative real-time PCR protocols to quantify EIAV-specific early RT products produced immediately after virus entry into target cells (33), providing a sensitive and specific assay for quantifying virus entry. Second, we identified and cloned the ELR1 functional receptor for EIAV (60), permitting the examination of specific receptor-mediated entry into engineered cell lines. Using a combination of these new resources and previously described selective inhibitors of endoctyosis, the results of our studies uniformly support the model that productive EIAV infection of target cells post-receptor binding is via a pH-dependent endocytic pathway, rather than by pH-independent mechanisms. The studies further demonstrate that pH-dependent endocytosis is required for entry into permissive equine fibroblasts and into equine macrophages, the natural target cell in vivo. In this regard, these findings are remarkably similar to the independent observations of Brindley and Maury (7) who used a complementary panel of EIAV strains, cell targets, endocytosis inhibitors, and virus entry assays, as described in detail in their accompanying paper in this issue. The consistency of these independent studies, not often evident in lentivirus research, provides a high level of confidence in the interpretation of our data.

In addition to the major finding that EIAV entry into permissive fibroblastic and macrophage cells utilizes pH-dependent endocytosis, the studies described here also provide new information about important specific details of the mechanism of virus entry. In this regard, the observation that CA and ammonium chloride blocked EIAV entry into 3T3-RHA cells provides conclusive proof that pH-dependent endocytosis is associated with EIAV entry via a defined functional receptor (ELR1) and that this entry pathway is not restricted to nonspecific uptake of virus by target cells. Moreover, the kinetics of inhibition of virus entry (Fig. 3 and 4) demonstrate that the inhibitors specifically block early steps in virus infection that follow binding of the virion to the cell receptor and that are completed by about 2 h postexposure; drug treatments initiated at 2 to 4 h postexposure failed to inhibit virus entry. This time window for effective inhibition is consistent with the time required for the formation and acidification of endocytic vesicles that is necessary for pH-dependent virus entry (9, 23).

Despite the consistency of the data from the current EIAV entry studies, there was an inconsistency in the fact that chloroquine treatment failed to inhibit EIAV entry, in distinct contrast to the substantial inhibition observed with the other lysosomotropic agent, ammonium chloride. Both of these agents are weak bases that in relatively high concentrations enter and block the acidification of intracellular vesicles. It does not appear that the failure of chloroquine to inhibit EIAV entry can be attributed to the concentration used in the current study (10 μM), as chloroquine at 0.6 μM has been shown to raise endosomal pH levels (52). However, it is possible that equine cells are more resistant to chloroquine treatment, as previous studies have indicated a cell type dependency of chloroquine sensitivity (14, 30). We observed substantial cytotoxicity in equine macrophages treated with greater than 10 μM chloroquine, which precluded further evaluations of inhibition of EIAV entry by this agent in the current studies. Although the reason for the lack of inhibition by chloroquine is not certain, it is important to note that discrepancies between ammonium chloride and chloroquine as inhibitors of virus entry have been reported previously (14, 30, 47).

While the inhibition of EIAV entry observed with ammonium chloride and the two V-ATPase inhibitors indicates the importance of pH-dependent endocytosis in virus entry, the lack of inhibition by sucrose treatment suggests that this entry pathway is not clathrin dependent, as high sucrose concentrations inhibit clathrin-dependent endocytosis (11, 31, 32, 35, 44). However, this conclusion should at this time be considered tentative, as the data are currently based on a lack of effect by a single drug treatment. The postentry cellular pathways utilized by infecting viruses may follow diverse and complex pathways, and biochemical and imaging studies using additional specific inhibitors of endocytic pathways are indicated to clarify the role of clathrin-mediated endocytosis in EIAV entry.

The current finding that receptor-mediated EIAV infection is by pH-dependent endocytosis is in contrast to the predominantly pH-independent entry reported for HIV-1, HIV-2, and simian immunodeficiency virus (20, 39–41). This apparent difference may reflect the fact that EIAV infection can be mediated by a single receptor (e.g., ELR1), while productive infection by human and nonhuman primate lentiviruses in general requires a combination of coreceptors (e.g., CD4 and CXCR4 or CCR5). Studies on the mechanisms of HIV-1 receptor interactions indicate that sequential receptor binding is necessary to induce the fusion active form of the viral envelope transmembrane protein to mediate fusion of the viral and cellular lipid bilayers (24, 51, 55). Based on the current data, we would propose that EIAV binding to the ELR1 receptor triggers endocytosis of the virion into vesicles in which subsequent acidification activates viral envelope fusion of the viral and vesicle lipid bilayers. The data reported in the companion paper by Brindley and Maury (7) demonstrate an optimization of EIAV infectivity at pH levels of around 5.0, consistent with the proposed pH dependence of the EIAV envelope in mediating fusion and entry into target cells. In this regard, it is also interesting that Clements and colleagues (2) have identified an apparent complex of a 30-kDa chondroitin sulfate proteoglycan and a 45-kDa protein kinase as a functional receptor for visna virus, a sheep lentivirus. These observations raise the possibility that the ungulate lentiviruses of horses, sheep, and goats that exclusively infect monocytes/macrophages may have a common entry mechanism in which interactions with a single receptor protein induces pH-dependent endocytosis, perhaps reflecting a fundamental difference in the envelope structure-function related to fusogenicity. Thus, future studies are designed to characterize in detail the specificity and mechanisms of EIAV envelope fusion activity.

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