Sodium Hydrogen Exchangers Contribute to Arenavirus Cell Entry

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Several arenaviruses, chiefly Lassa virus (LASV), cause hemorrhagic fever (HF) disease in humans and pose a great public health concern in the regions in which they are endemic. Moreover, evidence indicates that the worldwide-distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical significance. The limited existing armamentarium to combat human-pathogenic arenaviruses underscores the importance of developing novel antiarenaviral drugs, a task that would be facilitated by the identification and characterization of virus-host cell factor interactions that contribute to the arenavirus life cycle. A genome-wide small interfering RNA (siRNA) screen identified sodium hydrogen exchanger 3 (NHE3) as required for efficient multiplication of LCMV in HeLa cells, but the mechanisms by which NHE activity contributed to the life cycle of LCMV remain unknown. Here we show that treatment with the NHE inhibitor 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) resulted in a robust inhibition of LCMV multiplication in both rodent (BHK-21) and human (A549) cells. EIPA-mediated inhibition was due not to interference with virus RNA replication, gene expression, or budding but rather to a blockade of virus cell entry. EIPA also inhibited cell entry mediated by the glycoproteins of the HF arenaviruses LASV and Junin virus (JUNV). Pharmacological and genetic studies revealed that cell entry of LCMV in A549 cells depended on actin remodeling and Pak1, suggesting a macropinocytosis-like cell entry pathway. Finally, zoniporide, an NHE inhibitor being explored as a therapeutic agent to treat myocardial infarction, inhibited LCMV propagation in culture cells. Our findings indicate that targeting NHEs could be a novel strategy to combat human-pathogenic arenaviruses.

Arenaviruses cause chronic infections of rodents with a worldwide distribution (1). Invasion of human dwellings by infected rodents can result in human infections through mucosal exposure to aerosols or by direct contact of abraded skin with infectious material. Several arenaviruses cause hemorrhagic fever (HF) disease in humans and pose significant public health concerns in the regions in which they are endemic (2–6). Arenaviruses are classified into two main groups, Old (OW) and New (NW) World (1). The OW Lassa virus (LASV), the causative agent of Lassa fever (LF), is the most significant pathogen among arenaviruses. LASV is estimated to infect several hundred thousand individuals annually in West Africa, and these infections are associated with high morbidity and significant mortality. Likewise, the NW Junin virus (JUNV) is the causative agent of Argentine HF, a disease associated with high mortality (7). On the other hand, the worldwide-distributed prototypic arenavirus, lymphocytic choriomeningitis virus (LCMV), is considered to be a neglected human pathogen of clinical significance, especially in congenital infection (8–12). Moreover, LCMV poses a special threat to immunocompromised individuals, as has been illustrated by fatal cases of LCMV infection associated with organ transplants in humans (13, 14). Despite the significant impact of arenaviruses on human health, there are no FDA-licensed vaccines, although a live attenuated strain of JUNV, Candid#1, has been licensed in Argentina. However, Candid#1 does not protect against LASV or LCMV infections. Likewise, current antiarenaviral therapies are limited to an off-label use of the nucleoside analogue ribavirin that is only partially effective and can cause significant side effects (15–17). Therefore, it is important to develop novel antiviral strategies to combat human-pathogenic arenaviruses, a task that would be facilitated by a detailed understanding of the molecular and cell biology of arenaviruses.

Arenaviruses are enveloped viruses with a bisegmented, negative-strand RNA genome and a life cycle restricted to the cell cy-

toplasm. Each genome RNA segment, S and L, uses an ambisense coding strategy to direct the expression of two viral polypeptides in opposite orientation, separated by a noncoding intergenic region. The S segment encodes the viral nucleoprotein (NP) and the glycoprotein precursor (GPC) that is processed by the cellular site 1 protease to generate the mature virion surface glycoproteins, GP1 and GP2. Trimers of GP1 and GP2 form the spikes that decorate the virus surface and mediate virus cell entry via receptor-mediated endocytosis. α -Dystroglycan (α DG) has been identified as a primary receptor of OW arenaviruses, including LCMV and LASV (18), whereas human-pathogenic clade B NW arenaviruses, including JUNV, use human transferrin receptor to enter cells (19). The L segment encodes the viral RNA-dependent RNA polymerase, L protein, and the small RING finger protein, Z, which is the counterpart of the matrix protein found in many enveloped negative-strand RNA viruses (20, 21). The identification and characterization of arenavirus-host cell factor interactions required to complete the virus life cycle may uncover novel targets and facilitate the discovery of drugs to combat human-pathogenic arenaviruses. In this regard, a recent genome-wide small interfering RNA (siRNA) screen identified a variety of host genes involved in multiplication of both vesicular stomatitis virus (VSV) and LCMV (22). One of the candidates identified was sodium hydrogen exchanger 3 (NHE3), a molecule implicated in receptor-mediated endocytosis (23, 24). In this study, we have investigated the role of

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NHEs in LCMV multiplication. We document that 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), a potent inhibitor of NHE activity (25) as well as of macropinocytosis (23, 24), exerted a robust inhibitory effect on LCMV multiplication, and this effect was due to a blockade of LCMV cell entry, rather than an effect of EIPA on viral RNA replication, gene expression, or budding. OW and NW arenaviruses have been shown to follow different endocytic pathways (26). Thus, endocytic vesicles of the OW arenavirus LCMV are noncoated, and the LCMV cell entry process was reported to be cholesterol dependent but clathrin, dynamin, caveolin, ARF6, flotillin, and actin independent (27–32). In contrast, the NW arenavirus JUNV is internalized via coated vesicles using a clathrin- and dynamin-dependent pathway (3, 29, 30, 33). However, we found that NHE activity was required for efficient cell entry mediated by the GPs of both OW (LASV) and NW (JUNV) HF arenaviruses. Moreover, Pak1 and actin were involved in LCMV entry in human cells, supporting a role for macropinocytosis in this process. Finally, we document that the NHE1 inhibitor zoniporide (34), used in clinical trials for ischemia (35), inhibited LCMV propagation in cultured cells. Our findings suggest that targeting NHEs could represent a novel antiviral strategy to combat human-pathogenic arenaviruses.

MATERIALS AND METHODS

Plasmids. pol1MG-CAT, pCAGGS-NP, and pCAGGS-L (36, 37), as well as pCAGGS-LCMV-ZFlag (38), have been described previously. Expression plasmids for wild-type and dominant negative (DN) forms of Arf1, Cdc42, and GRAF1 fused to enhanced green fluorescent protein (eGFP) were kindly provided by T. Weber (39). Expression plasmids for Myc-tagged wild-type and dominant negative forms of Pak1 (pCMV6M-Pak1 and pCMV6M-Pak1 K299R) (40) were purchased from AddGene. The β -galactosidase (β -Gal)-expressing vector pCMV- β -gal was kindly provided by Y. Cho.

Cells and viruses. BHK-21, A549, and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/ml of streptomycin, and 100 U/ml of penicillin. Recombinant LCMVs, Armstrong (rARM) and clone 13 (rCl-13) strains, and trisegmented LCMVs expressing GFP (r3ARM/GFP and r3Cl-13/GFP) or chloramphenicol acetyltransferase (CAT) (r3ARM/CAT) as well as rLCMV expressing GPC from vesicular stomatitis virus (VSV) (rARM/VSVG) and LASV (rARM/LASVGPC) were generated as described previously (30, 41–43). A recombinant LCMV expressing GPC from the live attenuated Candid#1 vaccine strain of Junin virus (rARM/JUNVGPC) was generated by reverse genetics using procedures similar to those used to generate rARM/LASVGPC. Wild-type rVSV (rVSV-WT) and rVSV expressing GPC of LCMV Cl-13 (rVSV/Cl-13GPC) have been described previously (29). Recombinant adeno-associated virus 2 expressing dsRed (rAAV2-dsRed) was obtained from the Viral Vector Core Facility, Salk Institute for Biological Studies.

Chemical inhibitors. 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA), cytochalasin D (CytD), and zoniporide were purchased from Sigma-Aldrich (product numbers A3085, C8273, and SML0076, respectively). p21-activated kinase inhibitor III (IPA-3) was purchased from Calbiochem (product number 506106). EIPA, zoniporide, and IPA-3 were added at the concentrations and times indicated below and were present throughout the end of the experiment. Conditions for cytochalasin D treatment are indicated in the corresponding figure legend.

Virus titration. LCMV titers were determined using an immunofocus assay as described previously (44). Briefly, 10-fold serial virus dilutions were used to infect Vero cell monolayers in a 96-well plate, and at 20 h postinfection (p.i.), cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). After cell permeabilization by treatment with 0.3% Triton X-100 in PBS containing 3% bovine serum albumin

(BSA), cells were stained by using an anti-NP rat monoclonal antibody (VL-4) and an Alexa Fluor 568-labeled anti-rat second antibody. VSV titers were determined by a plaque assay.

Growth kinetics. Cells were infected with rLCMVs for 1 h at the multiplicities of infection (MOI) indicated below. At the indicated times p.i., cell culture supernatants were collected and viral titers determined using an immunofocus assay. In the indicated cases, 20 mM ammonium chloride (NH_4Cl) was added at 4 h p.i. to prevent secondary infections.

Cytotoxicity assay. The effect of compounds on cell viability was assessed using the CellTiter 96 AQueous One Solution reagent (Promega; product number G3580). This method determines the number of viable cells based on the level of formazan product converted from MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] by NADPH or NADH generated in living cells. Briefly, 5×10^4 cells were plated per 96-well plate and cultured overnight. Cells were treated with the indicated concentrations of EIPA, IPA-3, and zoniporide for 24 h or cytochalasin D for 4 h and cultured with fresh medium for 5 h before the CellTiter 96 AQueous One Solution reagent was added. Thereafter the assay was performed according to the manufacturer's recommendations and the absorbance (490 nm) was obtained using an enzyme-linked immunosorbent assay (ELISA) reader (Spectra-Max Plus384; Molecular Devices). Mean values obtained with dimethyl sulfoxide (DMSO)-treated cells were set to 100%.

LCMV minigenome assay. LCMV MG assay was done as described previously (37, 45). BHK-21 cells seeded on 12-well plates were transfected with the minigenome-encoding plasmid (pol1MG-CAT) together with *trans*-acting elements expressing plasmids pCAGGS-NP and pCAGGS-L using Lipofectamine 2000 reagent (Invitrogen). At 5 h posttransfection, the transfection medium was replaced with fresh medium containing the compound and concentrations indicated below. At 72 h posttransfection, cell lysates were prepared to determine levels of CAT protein by ELISA using a CAT ELISA kit (Roche; product number 11363727001) as described previously (46).

Budding assay. Budding assay was done as described previously (46). BHK-21 cells in 12-well plates were transfected with 0.5 μg of pCAGGS-LCMV-ZFlag using Lipofectamine 2000. At 5 h posttransfection, the medium was replaced with fresh medium and incubated at 37°C for 19 h. Then the medium was replaced with fresh medium containing DMSO or EIPA (5 to 10 μM), and 24 h later, virus-like particle (VLP)-containing tissue culture supernatants (TCS) and cells were collected. After clarification from cell debris by centrifugation at $1,500 \times g$ and 4°C for 5 min, VLPs were collected by ultracentrifugation at $100,000 \times g$ and 4°C for 30 min through a 20% sucrose cushion. Cells and VLPs were resuspended in lysis buffer (1% NP-40, 50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA, 0.4% sodium deoxycholate) and analyzed by Western blotting.

Flow cytometry analysis. 293T cells cultured in 6-well plates were transfected with 2.0 μg of expression plasmids expressing dominant negative forms of Arf1, Cdc42, and GRAF1 fused with eGFP or pEGFP-C1 using Lipofectamine 2000. At 5 h posttransfection, the transfection medium was replaced with fresh medium. At 24 h posttransfection, the cells were infected with rCl-13 at an MOI of 1.0 for 1 h at 37°C, then washed with PBS, and cultured with fresh medium. At 20 h p.i., the infected cells were fixed with 4% PFA, permeabilized, and stained with rat monoclonal anti-LCMV-NP antibody (VL-4) conjugated with Alexa Fluor 647. Cell analysis was performed on an LSR II flow cytometer (Becton, Dickinson).

Infection of cells with r3ARM/CAT. Cells in 24-well plates were transfected or treated with cytochalasin D and then were infected 24 h or 1 h later, respectively, with r3ARM/CAT (MOI = 1). At 8 h p.i., cell lysates were prepared and CAT protein expression levels measured by a CAT ELISA kit (Roche). Equal amounts of each cell lysate were also analyzed by Western blotting to detect plasmid-expressed and host cell proteins.

AAV2 infection. 293T cells cultured on coverslips in 24-well plates were transfected (0.5 μg /well) with plasmids expressing eGFP-tagged versions of wild-type or dominant negative forms of GRAF1, or with the control plasmid expressing eGFP, using Lipofectamine 2000. At 5 h post-

FIG 1 Effect of EIPA on LCMV multiplication. (A) BHK-21 and A549 cells were pretreated with EIPA (10 μ M) or DMSO for 30 min at 37°C and then infected with r3ARM/GFP at an MOI of 0.01. At 24 and 48 h p.i., GFP expression was examined by epifluorescence. Bars, 200 μ m. (B) BHK-21 cells were pretreated with 5 μ M or 10 μ M EIPA or DMSO for 30 min before infection with rLCMV ARM-WT (MOI = 0.1) or with rVSV-WT (MOI = 0.01). Tissue culture supernatants were collected at the indicated h p.i. and virus titers determined by an immune focus-forming assay. (C) BHK-21 and A549 cells (5×10^4 /well) were seeded into 96-well plates and incubated overnight. Cell culture medium was replaced with 100 μ l of DMEM–2% FBS containing EIPA at the indicated concentrations. After 24 h of incubation, cell viability was determined by CellTiter 96 Aqueous One Solution reagent. DMSO-treated samples were set to 100%. Data represents means $\pm$ standard deviations (SD) of triplicate (B) or quadruplicate (C) samples. EIPA was present in cell culture medium throughout the experiment.

transfection, the transfection medium was replaced with fresh medium. At 24 h posttransfection, cells were transduced with rAAV2-dsRed using 5×10^4 genome copies per cell, and 48 h later, cells were fixed with 4% PFA. Numbers of transfected (GFP⁺) and transduced (dsRed⁺) cells were determined by epifluorescence microscopy. For each sample the fraction of transfected cells that were also transduced (GFP⁺ and dsRed⁺) was determined based on inspection of three different fields (30 to 100 cells/field), and values were normalized (percent) with respect to cells transfected with the control plasmid expressing GFP.

Western blot analysis. Cell lysates or VLP samples were mixed with sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Clarified protein samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) using 4 to 20% gradient polyacrylamide gels (Novex 4 to 20% Tris-glycine minigels; Life Technologies) and electroblotted onto polyvinylidene difluoride membranes (Immobilon transfer membranes; Millipore). To detect GFP- and Myc-tagged proteins or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), membranes were incubated with a mouse monoclonal antibody (MAb) to GFP (Clontech), Myc (9E10), or GAPDH (Millipore), followed by incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (Jackson ImmunoResearch Laboratories). For the detection of actin, we used a rabbit polyclonal Ab to actin (Santa Cruz) as a primary antibody and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Pierce) as a secondary antibody. SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was used to elicit chemiluminescent signals that were visualized using ImageQuant LAS 4000 (GE Healthcare Life Science).

RESULTS

Effect of EIPA on LCMV multiplication. To investigate the roles played by NHEs in LCMV infection, we examined the consequences of chemical inhibition of NHE activity on LCMV multiplication. For this, we treated both BHK-21 (rodent) and A549 (human) cells with EIPA and then infected them with a recombi-

nant LCMV (ARM strain) expressing GFP (r3ARM/GFP). EIPA treatment resulted in significantly reduced numbers of GFP-expressing cells at 24 and 48 h p.i. (Fig. 1A). Consistent with this finding, growth of wild-type (WT) ARM was also inhibited in a dose-dependent manner by EIPA (Fig. 1B). In contrast, and consistent with published observations (47), infection with rVSV-WT was not affected significantly by EIPA treatment (Fig. 1B). EIPA inhibited LCMV multiplication at concentrations that did not affect cell viability (Fig. 1C). These results suggested that NHEs are required for efficient multiplication of LCMV.

Effect of EIPA on LCMV cell entry. We next asked what step of the LCMV life cycle was blocked by EIPA. To examine this, we performed a time-of-addition experiment in which we added EIPA at several times prior to and after infection with either r3ARM/GFP or r3Cl-13/GFP and monitored GFP expression at 24 h p.i. We used both r3ARM/GFP and r3Cl-13/GFP to examine whether high (Cl-13) or low (ARM) viral GP1 affinity for α DG, a bona fide receptor for LASV and most immunosuppressive strains of LCMV (48), could influence NHE-dependent virus cell entry. Addition of EIPA 60 min prior to infection strongly inhibited spread of both r3ARM/GFP and r3Cl-13/GFP, whereas addition of EIPA at 60 min, or later, p.i. did not significantly affect virus multiplication (Fig. 2A). Because by 60 min p.i. LCMV cell entry is completed, our finding suggested that EIPA blocked an early step of LCMV cell entry. To further investigate this issue, we examined the effect of EIPA on cell entry of a recombinant VSV expressing either its homologous surface glycoprotein G (rVSV-WT) or the GPC from the Cl-13 strain of LCMV (rVSV/Cl-13GPC). Pretreatment with EIPA inhibited multiplication of rVSV/Cl-13GPC in a dose-dependent manner (Fig. 2B), whereas multiplication of rVSV-WT was hardly affected (20% reduction in titer) by EIPA pretreatment. These results suggested that EIPA inhibited cell en-

FIG 2 EIPA blocks an early step of LCMV cell entry. (A) BHK-21 cells were infected (MOI = 0.1) with either r3ARM/GFP or r3CI-13/GFP. EIPA (10 μ M) or a DMSO vehicle control was added to culture medium at the indicated time points throughout the end of the experiment. At 24 h p.i., GFP expression was assessed by epifluorescence. Bars, 200 μ m. (B) BHK-21 cells were pretreated with EIPA at the indicated concentration for 30 min prior to infection (MOI = 0.01) with wild-type rVSV (rVSV-WT) or an rVSV expressing CI-13GPC instead of VSVG (rVSV/CI-13GPC). At 24 h p.i., culture medium was collected and viral titers were determined by a plaque assay. EIPA was included throughout the experiment. (C) BHK-21 cells were seeded (4×10^4 /well) in a 96-well plate and pretreated with EIPA (10 μ M) for 60 min prior to infection with 200 PFU/well of either rARM or rCI-13. At 4 h p.i., NH_4Cl (final concentration, 20 mM) was added to the medium to prevent secondary infections, and at 16 h p.i., cells were fixed and infected cells detected by immunofluorescence. (D) BHK-21 cells were transfected with 0.5 μ g of PolIMG-CAT and 0.4 μ g of pCAGGS-NP together with 0.6 μ g of pCAGGS-L using Lipofectamine 2000 reagent. As a negative control, 0.6 μ g of empty pCAGGS was used instead of pCAGGS-L [L (-)]. At 5 h posttransfection, the culture medium was replaced with DMEM-2% FBS supplemented with EIPA (10 μ M) or a DMSO vehicle control. The culture medium was changed to a fresh one containing EIPA or DMSO every 24 h. At 72 h posttransfection, CAT expression was determined by ELISA. Data represent means $\pm$ SD of triplicate samples. CAT expression levels of L transfected [L (+)] and DMSO-treated cells was set to 100%. (E) BHK-21 cells were transfected with 0.5 μ g of pCAGGS-LCMV-ZFlag or pCAGGS empty vector. At 5 h posttransfection, the medium was replaced with fresh medium, and 19 h later, DMSO or EIPA (10 μ M) was added. After 24 h in the presence of EIPA, VLPs present in tissue culture supernatants were collected by ultracentrifugation and total cell lysates prepared. Levels of Z in VLPs and cell lysates were determined by Western blotting using an antibody to Flag. Representative Western blotting data from three independent experiments are shown. Budding activity for each sample was estimated by dividing the signal intensity of Z-Flag associated with VLPs by the signal intensity of Z-Flag detected in the cell lysate normalized for actin expression levels. Budding activity of DMSO-treated samples was set to 100%. Data in panels B to E represent means $\pm$ SD of triplicate samples.

try mediated by LCMV GP. Production of infectious LCMV progeny can be detected about 6 to 8 h p.i., and therefore, inhibition of a postentry event could have contributed to a decrease in virus titers at 12 and 24 h p.i. in EIPA-treated cells (Fig. 1). However, the same EIPA inhibitory effect was observed in infected cells when 20 mM NH_4Cl was added at 4 h p.i. to prevent secondary infections (Fig. 2C). To further confirm a specific effect of EIPA on LCMV cell entry, we tested whether EIPA interfered with the activity of an LCMV minigenome (MG), which would reflect an effect on RNA biosynthetic processes, both replication and transcription, directed by the virus polymerase complex, in isolation from other steps of the virus life cycle. EIPA did not affect LCMV MG activity (Fig. 2D). Likewise, we also examined whether EIPA could inhibit Z-mediated budding. For this, we transfected BHK-21 cells with pCAGGS-LCMV-ZFlag, and 5 h later, cells were washed and cultured in fresh medium. At 24 h posttransfection, the medium was replaced with fresh medium containing EIPA at the indicated concentration, and 24 h later, VLP-containing tissue culture supernatants were isolated and their levels determined by Western blotting. EIPA treatment did not affect the efficiency of Z budding (Fig. 2E).

Effect of EIPA on cell entry mediated by HF arenavirus GPs.

We next examined whether the inhibitory effect of EIPA on LCMV cell entry could be extended to HF arenaviruses. To this end, we took advantage of rLCMV expressing GPC from the two most relevant HF arenaviruses, LASV and JUNV, referred to as rARM/LASVGPC and rARM/JUNVGPC, respectively. As a control, we used an rLCMV that expressed VSV G instead of its own GPC (rARM/VSVG). Consistent with results shown in Fig. 2B, EIPA did not inhibit multiplication of rARM/VSVG, whereas EIPA inhibited very robustly multiplication of rARM/LASVGPC in BHK-21 cells (Fig. 3A). Human transferrin receptor was identified as a cellular receptor for NW arenaviruses, including JUNV (19); however, JUNV can infect and multiply in BHK-21 cells, suggesting that JUNV can enter rodent cells using a transferrin receptor-independent mechanism. Notably, EIPA inhibited also very efficiently multiplication of rARM/JUNVGPC in BHK-21 cells (Fig. 3A). However, EIPA did not inhibit significantly multiplication of rARM/JUNVGPC in A549 human cells expressing human transferrin receptor 1 (Fig. 3B).

Role of the CLIC/GEEC endocytotic pathway in LCMV infection. Arenaviruses enter cells via receptor-mediated endocytosis,

FIG 3 Effect of EIPA on cell entry mediated by GPC of HF arenaviruses. (A) BHK-21 cells pretreated with DMSO or EIPA (10 μ M) for 60 min were infected with rARM expressing the indicated GPC at an MOI of 0.1 (rARM and rARM/VSVG), 0.01 (rARM/LASVGPC), or 0.35 (rARM/JUNVGPC). At the indicated time points, virus titers in culture medium were determined by an immunofocus assay. Data represent means $\pm$ SD of triplicate samples. (B) A549 cells pretreated with DMSO or EIPA (10 μ M) for 60 min were infected with rARM (MOI = 0.01) or rARM/JUNVGPC (MOI = 0.1). At the indicated time points, virus titers in culture medium were determined by an immunofocus-forming assay. Data represent means $\pm$ SD of triplicate samples.

but the specific endocytotic components and pathways involved have not been entirely elucidated and appear to differ between OW and NW arenaviruses (26). The clathrin-independent carrier (CLIC) and GPI-enriched endocytic compartment (GEEC) pathway plays a key role in cell internalization of a large variety of cargoes independently of the canonical coat protein clathrin (39, 49). Arf1, GRAF1, and Cdc42 have been identified as the three main effectors of the CLIC/GEEC pathway (50–52), and EIPA has been shown to block the CLIC/GEEC pathway via inhibition of

Cdc42. We therefore investigated whether LCMV uses the CLIC/GEEC endocytotic pathway. To this end, we examined the effect on LCMV infection of expression of dominant negative (DN) forms of Arf1, Cdc42, and GRAF1. For this we transfected 293T cells with WT or DN forms of Arf1, Cdc42, and GRAF1, all of them fused to GFP, as well as with GFP alone as a control, and 24 h later, cells were infected (MOI = 1) with rCI-13. At 20 h p.i., infected cells were analyzed by flow cytometry. To assess whether expression of a DN form of Arf1, Cdc42, or GRAF1 resulted in reduced levels of LCMV multiplication, we determined in each case the numbers of NP⁺ cells among transfected cells expressing high levels of GFP (GFP^{hi}) and normalized (percent) values with respect to the one obtained for cells transfected with GFP alone, which was assigned as 100% (Fig. 4A and B). Expression of DN forms of Arf1 and Cdc42 resulted in modest reductions of the numbers of NP⁺ cells (25 and 20% reduction, respectively), while expression of DN GRAF1 did not affect the numbers of NP⁺ cells (Fig. 4B). Similar observations were made in cells transfected with WT versions of Arf1, Cdc42, or GRAF1 (data not shown). The Rho-GAP domain-containing protein GRAF1 plays a critical role in coordinating small G protein signaling and membrane remodeling to enable internalization of CLIC/GEEC pathway cargoes (51, 53). Therefore, our observation that LCMV cell entry was not affected by overexpression of a DN form of GRAF1 indicates that it is highly unlikely that the CLIC/GEEC pathway plays a significant role in cell entry of LCMV. To rule out the possibility that DN forms of Arf1, Cdc42, and GRAF1 might have a direct effect on viral protein expression at early times p.i., we transfected 293T cells with plasmids expressing either wild-type or DN forms of Arf1, Cdc42, or GRAF1, and 24 h later cells were infected with rLCMV expressing the CAT protein. At 8 h p.i., CAT protein expression levels were determined by ELISA. Consistent with results shown in Fig. 4A and B, expression of DN forms of Arf1 and Cdc42 resulted in modest reductions in CAT expression levels compared to those of cells expressing their WT counterparts (Fig. 4C). WT and DN forms of Arf1 and Cdc42 were expressed to similar levels (Fig. 4C). In contrast, DN GRAF1 was expressed at significantly higher levels than its WT version, but WT and DN forms of GRAF1 exhibited the same very modest effect on CAT expression levels (Fig. 4C). To confirm that under our experimental conditions the DN form of GRAF1 was able to inhibit GRAF1-dependent cell entry of molecules, we examined the effect of WT and DN forms of GRAF1 on AVV2 cell entry, which was shown to be GRAF1 dependent (39). Consistent with published results, the DN form of GRAF1 inhibited cell entry of AAV2 (Fig. 4D).

Role of actin dynamics in cell entry of LCMV. EIPA has been also shown to block macropinocytosis (23, 24) through inhibition of NHEs. Macropinocytosis requires actin remodeling, and previous studies have documented that chemical inhibitors of actin remodeling did not affect LCMV entry in Vero or CV-1 cells (28–30), a finding that would make it unlikely that macropinocytosis contributes to arenavirus cell entry. However, our finding that EIPA inhibited LCMV entry in BHK-21 and A549 cells without the contribution of the CLIC/GEEC endocytotic pathway led us to explore whether macropinocytosis could contribute to arenavirus cell entry in a cell-type-specific manner. To examine this issue, we tested the effect of cytochalasin D (CytD), an inhibitor of actin multimerization, on early viral gene expression in r3ARM/CAT-infected A549 and BHK-21 cells (Fig. 5A). In contrast to previous reports, CytD inhibited very strongly viral gene expression, as de-

FIG 4 Effect of expression of DN forms of Arf1, Cdc42, and GRAF1 on LCMV infection. (A and B) 293T cells were transfected with 2 μ g of plasmids expressing GFP-tagged DN forms of Arf1, Cdc42, and GRAF1, or GFP as a control. At 24 h posttransfection, cells were infected with rCl-13 (MOI = 1), and at 20 h p.i., cells were fixed and stained with VL-4 (MAB to LCMV NP) conjugated with Alexa Fluor 647. Expression of LCMV NP and GFP was analyzed by flow cytometry. (B) Quantitation of results from flow cytometry in panel A. (C) 293T cells were transfected with 1.0 μ g of expression plasmid for WT or DN forms of Arf1, Cdc42, and GRAF1. At 24 h posttransfection, cells were infected with r3ARM/CAT (MOI = 1), and at 8 h p.i., CAT expression levels were determined by ELISA. Expression levels of GFP-tagged proteins were assessed by Western blotting. Data represent means $\pm$ SD of triplicate samples. (D) Expression of a DN form of GRAF1 inhibited cell entry of AAV2. 293T cells were transfected with GFP-tagged versions of WT or DN forms of GRAF1, as well as GFP alone. At 24 h posttransfection, cells were transduced with rAAV2-dsRed (50,000 genome-containing particles [gcp]/cell), and 24 h later, cells were fixed (4% PFA–PBS) and examined by epifluorescence microscopy. For each sample we determined the fraction of transfected cells (GFP⁺) that were also AAV2 transduced (dsRed⁺), and values were normalized (percent) with respect to cells transfected with the control vector expressing GFP.

terminated by levels of CAT protein expression, at early time points (8 h) p.i. in both A549 and BHK-21 cells (Fig. 5B). At the lowest (1 μ M) concentration used, CytD resulted in 85% and 40% reduction of CAT expression in A549 and BHK-21 cells, respectively, whereas within the range (1 to 10 μ M) of concentrations tested, CytD did not affect expression of the housekeeping GAPDH gene (Fig. 5B) or cell viability (Fig. 5D). To determine whether this CytD-mediated inhibitory effect on viral gene expression was mainly due to disruption of the virus cell entry step, we compared the effect of CytD on viral gene expression when added prior to and after initiation of virus infection. We treated A549 cells with CytD (1 μ M) before or after infection with r3ARM/CAT and determined CAT expression levels at 8 h p.i. CytD treatment prior to, but not 1 h after, addition of virus inoculum resulted in an 80% reduction of CAT expression levels (Fig. 5C).

Role of Pak1 in cell entry of LCMV. Since we found that actin remodeling was required for entry of LCMV in BHK-21 and A549 cells, we further investigated a potential role for macropinocytosis in LCMV cell entry. Macropinocytosis is a complex pathway that involves a large number of components, with many of them having also roles in other endocytic pathways (54). However, Pak1 plays an important and rather specific regulatory role in the process of macropinocytosis. We therefore assessed whether Pak1 played a significant role in LCMV cell entry. Treatment with IPA-3 (10 μ M), a chemical inhibitor of Pak1, prior to infection resulted in inhibition of multiplication of both the ARM and

Cl-13 strains of LCMV (Fig. 6A) without affecting cell viability (Fig. 6B). To gain additional insights about the mechanisms by which IPA-3 interfered with LCMV infection, we infected IPA-3-treated (10 μ M) cells with rVSV-WT or rVSV/Cl-13GPC. IPA-3 caused about a 95% reduction in production of infectious rVSV/Cl-13GPC, whereas it affected production of infectious rVSV-WT by less than 25% (Fig. 6A). We also examined whether expression of a DN form of Pak1 inhibited viral gene expression at early times of infection. For this, we transfected 293T cells with expression plasmids for either the WT or DN form of Pak1, and β -galactosidase (β -Gal) as a control, and 24 h later infected the cells with r3ARM/CAT. At 8 h p.i., we prepared cell lysates and determined expression levels of CAT protein. CAT protein expression levels were significantly reduced (33%) in cells expressing the DN form of Pak1 compared to those in cells expressing either WT Pak1 or the control, β -Gal (Fig. 6C). WT and DN Pak1 proteins were expressed to similar levels in transfected cells. These data suggested that Pak1 is involved in cell entry of LCMV and further supported a contribution of macropinocytosis to cell entry of LCMV.

Role of TIM and TAM proteins on EIPA-mediated inhibition of LCMV cell entry. Viral envelopes usually contain lipids that are enriched in the cellular membranes where viral budding takes place. One of these lipids, phosphatidylserine (PS), and its receptors, T-cell immunoglobulin and mucin domain-containing proteins (TIM) and receptor tyrosine kinases, Tyro-3, Axl, and Mer,

FIG 5 Effect of CytD on early steps of LCMV infection. (A) Schematic diagrams of experimental conditions used. Durations of virus absorption (open square) and CytD treatment (filled square) are shown. Diagrams i, ii, and iii correspond to conditions used for experiments in panels B, C, and D, respectively. (B) A549 or BHK-21 cells were treated with DMSO or CytD at the indicated concentrations for 1 h and infected with r3ARM/CAT (MOI = 1.0). At 3 h p.i., cells were washed and fresh medium was added without CytD, and 5 h later (8 h p.i.), CAT expression levels were determined by ELISA. Expression levels of GAPDH were determined by Western blotting to rule out CytD-mediated cell toxicity. (C) A549 cells infected with r3ARM/CAT (MOI = 1.0) were cultured in the presence of 1 μM CytD from -1 to 3 h p.i. (CytD-pre) or from 1 to 4 h p.i. (CytD-post) or DMSO from -1 to 4 h p.i. as a control. At 8 h p.i., CAT expression levels were determined by ELISA. (D) A549 or BHK-21 cells seeded on a 96-well plate (5.0×10^4 cells/well) were treated with CytD or DMSO. Cell viability was measured by CellTiter 96 AQueous One Solution reagent (Promega). Data represent means $\pm$ SD of triplicate (B and C) or quadruplicate (D) samples.

collectively designated TAM, have been recently documented to play a role in cell entry of several viruses, and macropinocytosis contributes to this PS-mediated cell entry (55–59). We therefore examined the possibility that the inhibitory effect of EIPA on LCMV cell entry was due to its ability to block PS-mediated virus cell entry. For this, we tested the effect of EIPA on multiplication of LCMV in 293T cells, which have been shown to express very low levels of TIM and TAM proteins, and therefore, the interaction between PS present in the virus envelope and cellular PS receptors makes a negligible contribution to virus cell entry. Treatment of 293T cells with EIPA (10 μM) significantly reduced levels of production of infectious LCMV-ARM (Fig. 7A) without affecting cell viability (Fig. 7C). Likewise, production of rVSV/CI-13GPC, but not rVSV-WT, was significantly inhibited by EIPA treatment (Fig. 7B). These results suggested that the inhibitory effect of EIPA on cell entry of LCMV was highly unlikely due to a blockade of PS-mediated cell entry.

Effect of zoniporide on LCMV multiplication. Zoniporide is a specific inhibitor for NHE1 and has been used in clinical trials for the treatment of ischemia (35). We investigated the possibility of repurposing zoniporide as an antiarenaviral drug by examining the effect of zoniporide on LCMV infection in BHK-21 cells. Zoniporide inhibited multiplication of both ARM and CI-13 strains of LCMV (Fig. 8A) within a range of concentrations that did not have significant effects on cell viability (Fig. 8C). Consistent with an effect on cell entry mediated by LCMV GP, infection of rVSV/CI-13GPC, but not of rVSV-WT, was affected by zoniporide treatment (Fig. 8B).

DISCUSSION

Genome-wide siRNA screens have been successfully utilized to identify host cell factors required for efficient propagation of a variety of viruses, including HIV-1, West Nile virus, and influenza virus (60–62). The rationale for the studies we have presented in this paper stems from results of a recent genome-wide siRNA screen that identified NHE3 as a host factor required for efficient multiplication of LCMV in HeLa cells (22). However, the mechanisms by which NHEs may contribute to the arenavirus life cycle remain to be elucidated. In the present study, we have demonstrated that treatment of cells with the NHE inhibitor EIPA resulted in strong inhibition of LCMV multiplication. This inhibitory effect of EIPA was due to its ability to interfere with virus cell entry, whereas virus RNA replication and gene expression, as well as budding, were not significantly affected by the EIPA treatment.

Arenavirus cell entry occurs via receptor-mediated endocytosis (27, 63), which involves a pH-dependent fusion event between viral and cellular membranes within the acidic environment of the endosome (18, 48, 64–68). However, a contribution of NHE activity to this fusion events seems highly unlikely because the proton flow directed by NHEs moves in the opposite direction to the one mediated by the vacuolar ATPase (v-ATPase) that plays a central role in acidification of vesicles (69).

EIPA has been shown to block the CLIC/GEEC-dependent endocytic pathway by inhibiting the activity of Cdc42, a key regulator of this pathway (39). To examine whether LCMV could enter host cells via the CLIC/GEEC pathway, we tested the effect on LCMV infection of overexpression of DN mutant forms of Arf1, Cdc42, and GRAF1 (50–52). Overexpression of DN mutants of either Cdc42, a known target of EIPA, or GRAF1, known to be a key regulator of the CLIC/GEEC pathway, had only a very modest

FIG 6 Role of Pak1 in LCMV cell entry. (A and B) A549 cells were pretreated with 10 μ M IPA-3 or DMSO for 2 h and infected with rLCMV (MOI = 0.1) or rVSVs (MOI = 0.01). At 24 h p.i., virus titers in cell culture supernatants were determined. (B) Viability of A549 cells treated with IPA-3 was determined as in Fig. 1C. (C) 293T cells were transfected with 1.0 μ g of pCMV- β -gal (β -gal), pCMV6M-Pak1 (Pak1), or pCMV6M-Pak1 K299R (Pak1 DN) and 24 h later infected with r3ARM/CAT (MOI = 1.0). At 8 h p.i., CAT expression levels were measured by ELISA. CAT expression levels in lysates of cells transfected with β -Gal were set to 100%. Expression levels of Myc-tagged Pak1 proteins were confirmed by Western blotting. Data represent means $\pm$ SD of triplicate samples.

effect on LCMV infection. Moreover, the CLIC/GEEC pathway carries cargo in the range of 40 to 80 nm (70), which could result in a rather inefficient transport of LCMV particles with a typical diameter in the range of 100 nm (1). However, viruses are able to manipulate endocytotic pathways, resulting in vesicles able to accommodate a cargo larger than what they normally carry in non-infected cells. Nevertheless, together these results suggest that the CLIC/GEEC pathway does not significantly contribute to arenavirus cell entry. It should be noted that although expression of

Arf1-DN resulted only in a modest (25%) reduction in early viral gene expression, this finding was consistent with the fact that Arf1 was also identified in the same siRNA screen as a host factor required for efficient LCMV propagation (22). It is possible that Arf1 contributes to LCMV cell entry or multiplication, or both, through a CLIC/GEEC-independent pathway.

EIPA is also a potent inhibitor of macropinocytosis, a distinct

FIG 7 Effect of EIPA on LCMV multiplication in 293T. (A) 293T cells were pretreated with EIPA (10 μ M) and infected with rARM (MOI = 0.01). At the indicated time points, virus titers in the cell culture supernatants were determined by an immunofocus assay. (B) 293T cells were pretreated with DMSO or EIPA (10 μ M) for 1 h and infected with rVSVs (MOI = 0.01). At 24 h p.i., virus titers in cell culture supernatants were determined by a plaque assay. (C) Cell viability of 293T cells treated with EIPA was determined as in Fig. 1C.

FIG 8 Effect of zoniporide on LCMV propagation. (A) BHK-21 cells were pretreated with zoniporide at the indicated concentrations or a DMSO vehicle control for 1 h and subsequently infected with rARM, rCI-13, VSV-WT, or VSV/CI-13GPC (MOI = 0.01). At 24 (for rVSVs) or 48 (for rLCMV) h p.i., viral titers in cell culture supernatants were determined by an immunofocus assay (rLCMV) or plaque assay (rVSVs). Zoniporide was present in the cell culture medium throughout the experiment. Data represent means $\pm$ SD of triplicate samples. (B) Cell viability of BHK-21 cells treated with zoniporide was determined as in Fig. 1C.

process for uptake of extracellular fluid that has been shown to contribute to internalization of variety of viruses, including HIV-1 (71, 72), influenza A virus (73, 74), Ebola virus (75), coxsackievirus (76), echovirus 1 (77, 78), vaccinia virus (79), herpes simplex virus 1 (80, 81), and adenovirus 3 (82). Macropinocytosis was considered not to play a role in LCMV cell entry because inhibitors of actin remodeling, which is required for macropinocytosis, did not affect entry of LCMV in Vero or CV-1 cells (28–30). In contrast, we observed that treatment with the actin remodeling inhibitor CytD had a significant effect on LCMV entry in A549 and BHK-21 cells. The reasons for these discrepancies remain to be determined but might be related to cell-type-specific differences in LCMV cell entry. Our finding that either chemical or genetic interference with the activity of Pak1 inhibited specifically cell entry mediated by LCMV GPC provided additional experimental support for a role of macropinocytosis in LCMV cell entry. Macropinocytosis involves a large number of components that participate in a complex network of interactions that could be influenced in a cell-type-dependent manner (54). Our results have shown that several factors known to play important roles in macropinocytosis, including actin remodeling, NHE, and Pak1, contributed significantly to LCMV cell entry, although the underlying molecular mechanisms remain to be solved. On the other hand, expression of a DN form of Cdc42, another important player in macropinocytosis, did not affect LCMV infection, suggesting that a non-classical macropinocytosis-like pathway may contribute to LCMV cell entry. A recent publication showed that upon interacting with its receptor α DG, OW arenavirus cell entry follows an endocytic pathway that bypasses classical EEA1-positive early endosomes but merges into the classical endocytic pathway at the level of the multivesicular body (MVB). Passing through the MVB, the virus is delivered to the late endosome in an endosomal sorting complex required for transport (ESCRT)-dependent process (83). It is conceivable that in some cell types, LCMV enters cells via macropinocytosis and that early macropinosomes are redirected to merge with the MVB for subsequent delivery of the virus-receptor complex to the late endosome in an ESCRT-dependent manner (83). Unlike OW arenaviruses, many NW arenaviruses, including JUNV, use the human transferrin receptor to initiate cell entry via clathrin-mediated endocytosis (19). Intriguingly, cell entry mediated by JUNV GPC, but not by VSV-G, was strongly inhibited by EIPA in BHK-21 cells. In contrast, in A549 cells expressing the human transferrin receptor, treatment with EIPA had only a very minimal effect on cell entry mediated by JUNV GPC. These results would suggest that in BHK-21 cells JUNV uses a different receptor that is internalized by an EIPA-sensitive endocytic pathway that may involve activation of the receptor tyrosine kinases TIM or TAM, or both, as has been proposed for several other enveloped viruses (56). Accordingly, broad-range protein kinase inhibitors like staurosporine and genistein are able to inhibit viral cell entry mediated by macropinocytosis (84), and genistein treatment was shown to inhibit both LASV (85) and Pichinde virus (86) infections.

NHE3 was also identified in the same siRNA screen as a host cell factor required for efficient multiplication of VSV in HeLa cells (22). However, our results indicated that NHEs did not significantly contribute to VSV cell entry and multiplication in A549 or BHK-21 cells, suggesting that the NHE requirement of VSV is cell type dependent. Alternatively, it is possible that in A549 and BHK-21 cells other host factors might complement the activity of

NHE required for VSV multiplication in HeLa cells. These results underscore the need to conduct detailed validation studies of candidates identified by genome-wide siRNA screens, and they also illustrate how the specific cell system used for the experiment could influence the experimental outcome.

Our results showed that EIPA inhibited very robustly multiplication of rARM/LASV-GPC and rARM/JUNV-GPC in BHK-21 cells (Fig. 3A), suggesting that inhibition of NHE function may interfere, at least in certain cell types, with cell entry mediated by GPs of HF arenaviruses. However, we cannot rule out the possibility that rLCMV in which GPC was replaced by GPC of LASV or JUNV may not accurately recreate all the aspects of cell entry associated with infection by bona fide LASV or JUNV. Nevertheless, our findings suggested that inhibitors of NHE function may have potential value as antiarenaviral drugs.

Reduced oxygen supply during ischemia results in increased glycolysis and consequent reduced intracellular pH that activates NHEs. Increased NHE-directed intracellular Na^+ activates the sodium calcium exchanger, which results in an accumulation of intracellular Ca^{2+} , leading to activation of cellular proteases, cardiomyocyte contracture, arrhythmias, and cell death (87). Accordingly, several NHE inhibitors are being investigated as potential therapeutic drugs to treat ischemia (35). We therefore explored the repurposing of NHE inhibitors as potential antiarenaviral drugs. There are nine different NHE isoforms. NHE3 is highly expressed in epithelial cells and was the one identified in the siRNA screen as required for efficient LCMV multiplication in HeLa cells (88). EIPA inhibits to different degrees most NHE isoforms, whereas zoniporide is a rather specific inhibitor of NHE1. We found that similarly to EIPA, zoniporide inhibited multiplication of LCMV in BHK-21 cells at concentrations that did not result in noticeable levels of cell toxicity. This finding would suggest that NHE1 contributes to arenavirus cell entry in BHK-21 cells. It is plausible that different NHE isoforms may contribute in a cell-type-dependent manner to arenavirus cell entry, and therefore, the use of a pan-NHE inhibitor would be the best option for evaluating targeting of NHE function as a novel arenavirus drug target to be used in combination with other known arenavirus inhibitors, like ribavirin, to develop novel antiviral strategies to combat human-pathogenic arenaviruses.

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