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Role of the Vacuolar-ATPase in Sindbis Virus Infection[▽]

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Bafilomycin A_1 is a specific inhibitor of the vacuolar-ATPase (V-ATPase), which is responsible for pH homeostasis of the cell and for the acidification of endosomes. Bafilomycin A_1 has been commonly used as a method of inhibition of infection by viruses known or suspected to follow the path of receptor-mediated endocytosis and low-pH-mediated membrane fusion. The exact method of entry for Sindbis virus, the prototype alphavirus, remains undetermined. To further investigate the role of the V-ATPase in Sindbis virus infection, the effects of bafilomycin A_1 on the infection of BHK and insect cells by Sindbis virus were studied. Bafilomycin A_1 was found to block the expression of a virus-encoded reporter gene in both infection and transfection of BHK cells. The inhibitory effects of bafilomycin A_1 were found to be reversible. The results suggest that in BHK cells in the presence of bafilomycin A_1, virus RNA enters the cell and is translated, but replication and proper folding of the product proteins requires the function of the V-ATPase. Bafilomycin A_1 had no significant effect on the outcome of infection in insect cells.

Sindbis virus (SV) is the prototype virus of the genus *Alphavirus* in the family *Togaviridae*. It consists of two nested protein shells with T=4 icosahedral symmetry. The inner shell consists of 240 copies of the capsid protein (C) and encloses the single-stranded positive-sense RNA genome of the virus. The outer protein shell is a lattice of 240 copies of each of the two structural proteins E1 and E2 (1, 40). Evidence suggests E1 forms the base of the structural lattice, and E2 is responsible for receptor recognition and binding (33). Between the two protein shells exists a host-derived lipid bilayer (1, 40).

When SV infects a cell, the positive-sense RNA must first be translated and transcribed into a negative-sense RNA template. Translation of the positive-sense RNA results in the four nonstructural proteins (47). The uncleaved P123 and nsP4 form the minus-strand RNA replicase, which translates the positive-sense RNA genome into a negative-sense RNA template. P123 is cleaved into three separate proteins (nsP1, nsP2, and nsP3) that form the plus-strand RNA replicase with nsP4. The nonstructural proteins nsP1, nsP2, nsP3, and nsP4 have methyltransferase, helicase, protease, and RNA polymerase activities, respectively (47). The four nonstructural proteins transcribe the subgenomic 26S RNA, which encodes the structural proteins. Translation of the open reading frame results in the following proteins: E1, PE2, E3, 6K, and C, of which only E1, E2 (processed PE2), and C are present in the mature virion (47).

The method of entry for SV is still being investigated. Many previous studies have used virus RNA or protein synthesis as an indication of successful virus infection, but these events take place after the virus has attached to and penetrated the cell and require translation of the infecting RNA (22). The interaction between the virus and the cell can also be studied by using electron microscopy, but under a microscope one cannot distinguish between an infectious and a noninfectious virus particle. If this ratio of total particles to infectious particles (the particle/PFU ratio) is high, it makes observations by microscopy very difficult because only a fraction of the observed particles are capable of initiating infection. To eliminate this problem, our studies use the heat-resistant strain of SV (SVHR), which after careful purification has a known particle/PFU ratio of 1 (20).

One proposed route of entry is low-pH-mediated fusion occurring after virus uptake through receptor-mediated endocytosis (16, 48). It has been shown that SV and Semliki Forest virus (SFV) are capable of fusion from without in mammalian cells (22), but it is a two-step process (8, 41) requiring exposure to low pH, followed by a return to neutral pH. In contrast in liposome models, SV and SFV fuse with the membrane upon exposure to low pH (44) without a return to neutral pH (8). In the model proposing entry by fusion, a virus particle binds to its receptor and enters the cell by endocytosis. Upon acidification of the endosome, the lower pH causes conformational changes in the virus structural proteins that result in fusion of the virus membrane with the vacuolar membrane releasing the virus RNA (22, 23). A common method for assaying receptor-mediated endocytosis as the pathway of infection has been to use chemicals that inhibit acidification of endosomes, for example, the lysosomotropic weak bases chloroquine and ammonium chloride (NH_4Cl). These weak bases cause the pH of lysosomes to rise from acidic to near neutral (17). The initial accepted model was that these chemicals inhibited SV infection by preventing the release of the virus genome into the cell. Further investigation showed that in NH_4Cl-treated cells, SV infection was inhibited at the step of nonstructural protein processing and not virus penetration (19). Chloroquine was found to reduce virus yield in mammalian cells, but virus production increased slightly in treated mosquito cells (7, 19), even though these cells were protected from diphtheria intoxication, which requires an acid environment (19).

Many studies proposed to support the fusion model have been done using artificial membranes, liposomes, which do not appropriately represent the cell membrane. In order for effi-

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cient fusion to occur in a liposome model, the liposome must contain a molar fraction of cholesterol of ≥0.3 (24). It has been shown that SV does not require cholesterol for infectivity; even mosquito cells containing 0.04% (wt/wt) cholesterol could be infected and produce high titers of SV (13). The liposomes also lack membrane proteins, which would include the alphavirus receptor(s). Fusion of the virus with a liposome is a nonleaky process (45); however, it has been shown that during alphavirus infection, pores in the cell membrane are produced that leak ions (28). A fusion model for entry does not explain the creation of these pores. The results obtained from the liposome studies may not be entirely applicable to biological systems.

An alternate method of entry is that SV attaches to the cell surface receptor and directly injects its RNA by forming a pore in the cell membrane (15, 41). The nucleocapsid core is responsible for maintaining the rigid structure of the virus (9), so after release of the genome the virus is no longer stable and can be released from the cell surface (41), leaving the pore in the membrane temporarily intact (28, 31). This route of entry is independent of endosome acidification and membrane fusion. This method is supported by experiments that have shown that SV is capable of infection at low temperatures that do not allow endocytosis or membrane fusion to occur (51). Direct observation by electron microscopy also provides evidence that virus penetration occurs at the cell surface via formation of a pore (15, 41).

Initiation of infection by SV first involves attachment to the cell by a receptor or receptor complex. The identity of the SV receptor(s) remains elusive despite many previous attempts and claims of identification. These include the major histocompatibility receptor (18, 30, 38), the major laminin receptor (52), DC sign L sign (25), heparan sulfate (26), the heat shock 70 protein (42), and an unidentified 110-kDa nerve cell-associated protein (49). It is possible that the virus associates nonspecifically with various proteins before finding and binding to its specific receptor or receptor complex. The alphaviruses are capable of infecting a wide variety of cell lines derived from different phyla (e.g., mosquito, *Drosophila*, *Spodoptera*, fish, dog, monkey, and human). Although the alphaviruses may use different receptors in these various cell types, it would simplify matters significantly if the receptor complex possessed essential similarities in the diverse cell populations.

SV experiences dramatic structural changes upon exposure to acid pH. Cryoelectron microscopy reconstructions show that at pH 5.3, the pH required for SV to initiate the fusion event, a protruding structure appears in the center of the 5-fold axis. Upon return to neutral pH, the virus returns to its previous, though not exactly the same, structure (41). It is possible that the protruding structure seen at acid pH is responsible for tight binding of the virus to the receptor and participates in the formation of the putative pore (41). If the receptor complex provided a microenvironment of low pH, which in turn would cause the change in structure at the point of contact, it may create the pore for entry of the virus RNA and bring together some of the conflicting observations regarding alphavirus entry.

Hydrogen ion pumps control the pH of a cell. One such pump, the vacuolar-ATPase (V-ATPase), is a universal hydrogen ion pump present in the membranes of all eukaryotic cells (10, 11, 36, 37). The V-ATPase is responsible for maintaining pH homeostasis of the cell and for endosome acidification during endocytosis. Bafilomycin A_1 (BAF) is a commonly used inhibitor of the V-ATPase. BAF has been used to inhibit V-ATPases from *Neurospora crassa* (4), *Manduca sexta* (55), yeast (53), and bovine chromaffin granules (14). It has also been used in cell culture with a variety of cell lines to look at infection with alphaviruses (12, 27, 46), flaviviruses (6, 35, 43), and other viruses (2, 29). It has been used as a tool for inhibiting alphavirus infection, assuming alphaviruses use receptor-mediated endocytosis, followed by exposure to low pH to establish infection. If BAF was able to inhibit infection, as determined by the induction of virus-specific protein or RNA synthesis, it was considered evidence of a low pH-dependent endocytosis pathway of infection (6, 12, 46). The problem with using BAF and other chemical inhibitors of acidification arises if the primary and secondary effects of the drugs are not well characterized. This problem was addressed previously (7, 19) with chloroquine and NH_4Cl effects on SV infection described above. An inhibitor used to look solely at penetration should not affect transcription, translation, or protein processing and assembly.

In the experiments described below, a clone of SVHR that includes a gene encoding green fluorescent protein (GFP) was used to examine the effects of BAF on SV infection in baby hamster kidney (BHK) cells. The GFP reporter gene is part of the 26S RNA with the structural proteins, but it is under the control of a separate promoter. GFP expression causes the cells to fluoresce and indicates that virus RNA has entered the cell and has been translated.

MATERIALS AND METHODS

Tissue culture. BHK-21 cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, 2 mM glutamine, 50 μg of gentamicin/ml, and 10 mM HEPES (pH 7.4). BHK-21 cells used for cycloheximide experiments were not cultured in gentamicin. Cells were maintained at 37°C with 5% CO_2. C7-10 cells (*Aedes albopictus*) were cultured in MEM supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, and 2 mM L-glutamine. C7-10 cells were maintained at 28°C with 5% CO_2.

Modified SV expressing GFP gene. The modified SV was constructed by Gongbo Wang and Raquel Hernandez (50) using the Toto1101 SV cDNA clone. The plasmid pZsGreen (Clontech) contained the GFP protein from *Zoanthus* sp. Two primers (sense [5′-GCTCTAGAACCATGGCTCAGTCAAAGC-3′] and antisense [5′-GCATCCGGATCTGCATTGCCC-3′]) were used to produce the 743-nucleotide GFP product containing two XbaI-cut sites. The product was then inserted into the SV cDNA downstream of the structural gene sequence and a repeated subgenomic promoter, and the product was screened for proper orientation (50). The GFP construct was purposely made using a second subgenomic promoter to drive GFP expression to eliminate any folding problems involved in the construction of a fusion protein. The cDNA was then transcribed, and the RNA was transfected into BHK cells as previously described (20). The GFP gene is translated from the subgenomic RNA separately of the SV structural proteins.

In vitro transcription. Approximately 1 μg of DNA template was combined with 40 U of Sp6 polymerase; 1 mM (each) ATP, UTP, and CTP; 0.5 mM GTP; 1 mM cap analog (m^7GpppG); and 10 U of RNase inhibitor. The reactions were incubated at 40°C for 1 h. The presence of RNA was verified by agarose gel electrophoresis. The DNA template was removed by incubating the reactions with DNase I at 37°C for 30 min.

Transfection. BHK cells were treated with trypsin and pelleted by low-speed centrifugation. The cell pellet was washed three times with RNase-free 1× PBS-D (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8 mM Na_2HPO_4) in diethyl pyrocarbonate-treated water. The cells were resuspended in 1× PBS-D to a final concentration of 1×10^7 to 2×10^7 cells/ml. Then, 20 μl of RNA from the

TABLE 1. Effect of BAF on virus-encoded GFP production

Condition (BAF presence or absence)[a]			Effect (% infected)[b]			
Pretreatment	During infection or transfection	Postinfection or posttransfection	Infection		Transfection	
			RT	37°C	RT	37°C
–	–	–	95–100	90–95	95–100	95–100
–	–	X	20–30	50–60	20–30	NA
–	X	–	95–100	5–10	95–100	NA
X	–	–	95–100	20–30	95–100	40–50
X	X	–	95–100	1–5	95–100	40–50
X	–	X	5–10	5–10	10–20	20–30
–	X	X	1–5	1–5	10–20	NA
X	X	X	1–5	1	1	10–20

[a] X, BAF present; –, no BAF present.
[b] The 37°C temperature only refers to pretreatment and posttransfection treatments. NA, not applicable (experiment not performed).

in vitro transcription and 400 μl of cells were combined and added to a 0.5-cm gap-length cuvette. Transfection was done by electroporation on a Bio-Rad Gene Pulser II under the following conditions: 1.5 kV, 25 μF, and ∞ resistance, with a resulting time constant of ~0.7. The cells were pulsed once and allowed to rest for 10 min at room temperature (RT) (25°C) before transfer to a 25-cm^2 flask with MEM. The cells were incubated at 37°C and 5% CO_2 until GFP expression could be visualized, after which images were captured at approximately 24 and 48 h postinfection (hpi).

Infection. BHK cells were cultured in 25-cm^2 flasks until ~90% confluent. Virus was diluted in MEM in the presence or absence of BAF to give a final multiplicity of infection (MOI) of 10 unless otherwise stated. Cells were infected for 1 h at RT (25°C). Inoculum was removed after infection and replaced with MEM either containing or lacking BAF.

An unsuccessful attempt was made to overlay the BAF-treated cells with agarose to differentiate primary and secondary infection; however, the entire monolayer was dead 48 hpi when viewed by neutral red staining (20).

Chemical inhibitors. BAF from *Streptomyces griseus* (Sigma, catalog no. B1793) was used to make a 10-μg/ml stock of BAF in dimethyl sulfoxide (DMSO). The preparations of BAF vary in purity depending on the source. Sigma guarantees greater than 90% purity, while other sources state 95% purity. The stock was used to make a final concentration of 100 nM BAF in MEM, which was filtered with 0.45-μm-pore-size polyvinylidene difluoride syringe filter before use. The MEM containing BAF was used to pretreat cells (30 min at RT), as diluent for virus for infection (60 min at RT), and as medium for postinfection incubation at 37°C until images were captured with a digital camera. For cells transitioning from treatment to absence of BAF, the monolayer was washed once with 1× PBS-D. Previous studies showed that the DMSO used to dissolve the BAF would not adversely affect transfections (32).

Cycloheximide (CHX; Calbiochem) was used to make a stock solution of 100 mg/ml in DMSO. The CHX was diluted to a final concentration of 50 μg/ml in MEM before use.

Microscopy and relative fluorescence values. All microscopy was performed on a Zeiss inverted microscope equipped with a mercury lamp. Fluorescence was observed using a 450- to 490-nm excitation filter and a 510- to 520-nm emissions filter. Images were digitally captured 24 h postinfection (unless noted otherwise) with a Canon EOS 50D camera with a 1-s exposure time for cell monolayers and either a 4-s or a 10-s exposure time to capture the GFP fluorescence. The brightness and contrast were adjusted equally in all fluorescence images using Adobe Photoshop CS4. For quantification of GFP fluorescence, the percentage of cells fluorescing in the monolayer was determined based on an average of observed fields from three separate experiments. Values were found to vary over a range of 5 to 10%. This situation was exacerbated by the fact that some monolayers were well spread and easy to visualize (Fig. 1A), while prolonged drug treatment caused significant loss of cells (Fig. 3C) or clumping of cells (Fig. 3E). The data obtained were not satisfactory for strict statistical analysis. For this reason, a range of values is presented that encompasses the values determined for each set of experiments (Tables 1 and 2), and the data should be viewed as qualitative, with clear trends demonstrated. If no cells were found to express GFP, the sample was labeled as "0" (Fig. 3A), and if the experiment was not performed, the abbreviation "NA" was used.

RESULTS

Effects of BAF on the infection of BHK cells with SV. The effects of BAF on SV infection were first investigated by treating BHK cells with 100 nM BAF as shown previously (56) prior to (preinfection), during (infection), and postinfection (Table 1 and Fig. 1). This was done in an attempt to determine the conditions during which SV infection was most inhibited by observing the relative levels of GFP fluorescence at 24 hpi produced by the GFP virus construct described in Materials and Methods. Between 24 and 48 hpi, the number of cells expressing GFP did not increase, but the intensity of the fluorescence in individual cells increased. The concentration of BAF was chosen based on earlier studies that showed complete inhibition of the V-ATPase in cell culture at 100 nM (56). BHK cells that were treated with BAF at RT prior to and during infection with SV displayed high levels of GFP expression, as seen in untreated cells. Similar results were seen if the cells were only pretreated with BAF at RT or if BAF was only present during infection (Table 1). These results showed that the effects of treating BHK cells with BAF at RT before and/or during infection were reversible. BAF had the most inhibition when it was present in the medium postinfection (Table 1). Although the experiments presented here specifically examine entry, the results appear contrary to previous publications, which suggest that BAF is capable of inhibiting SV and Semliki

TABLE 2. Effect of temperature on BAF reversibility[a]

Time (min) p.i.	Effect (% infected)	
	Infection	Transfection
0	90–95	95–100
15	1	95–100
30	1	95–100
45	1	90–95
60	1	90–95
90	1	90–95
120	1	80–85
180	1	75–80

[a] BHK cells were pretreated with and infected or transfected in the presence of BAF at RT. After infection, the cells were incubated with BAF at 37°C for 0, 15, 30, 45, 60, 90, 120, 150, or 180 min p.i., after which BAF was removed by washing the cells once. The percent GFP-positive cells were determined 24 h later.

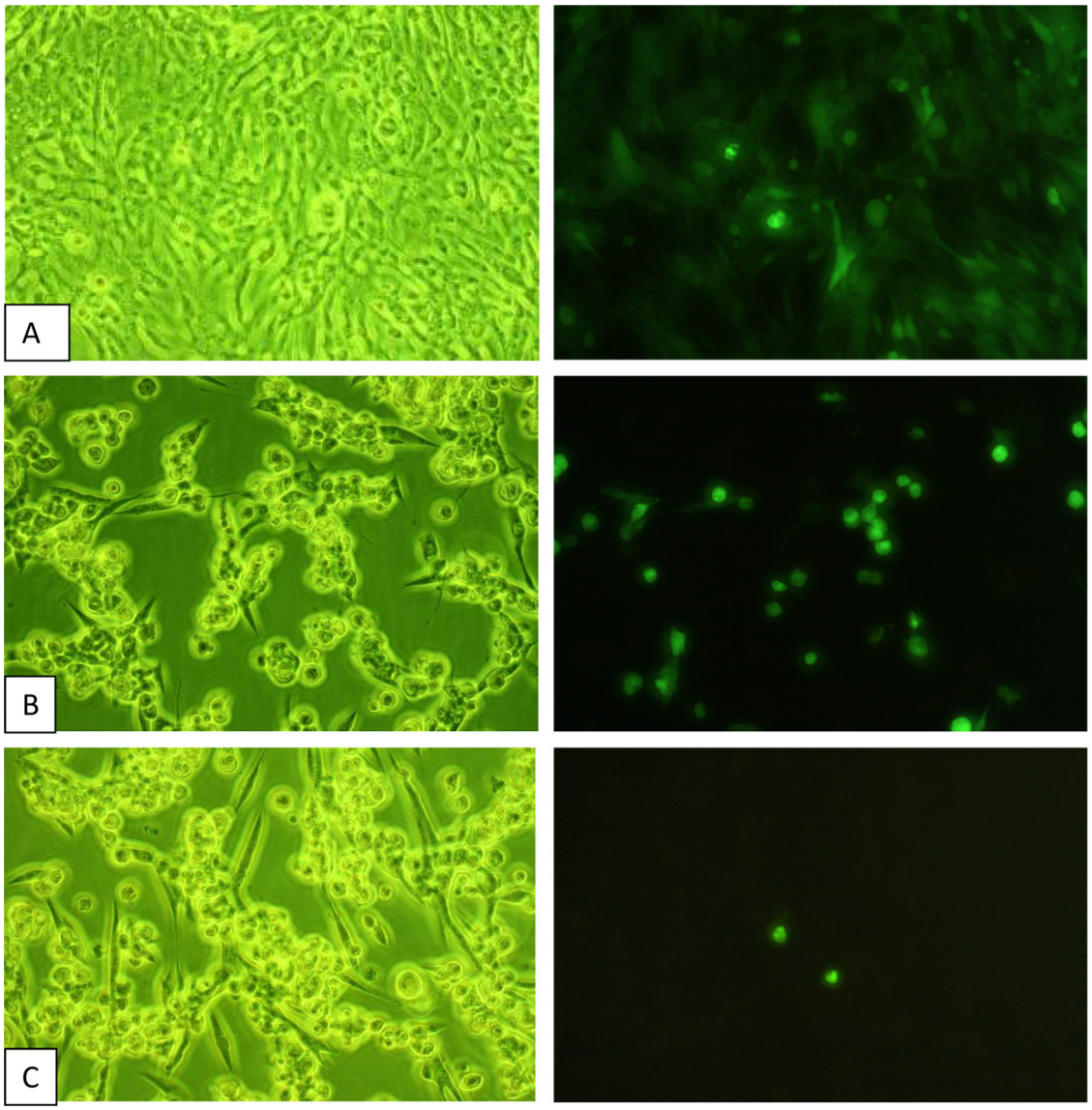

FIG. 1. Effect of BAF on GFP expression. The BHK cell monolayer (left) and the same cells upon exposure to blue light to excite GFP (right) 24 hpi are shown. (A) Cells infected with SV at RT. (B) Cells infected with SV at RT and treated after infection with BAF for 24 h at 37°C. (C) Cells pretreated with BAF, infected with SV in the presence of BAF, and treated after infection with BAF for 24 h at 37°C (see also Table 1).

Forest virus production if it is present before or during infection but has no effect when added after infection (12). Titration by plaque assay showed that cells infected or transfected with SV maintained in BAF after infection (as in Fig. 1C) produced virus titers of 10^5 PFU/ml compared to infected or transfected untreated cells, which produced 10^{10} PFU/ml.

The degree to which GFP was expressed in BAF-treated cells at 37°C was dependent on the MOI of the virus. At an MOI of 1, if BAF was present prior to, during, and after infection, it caused the greatest inhibition of GFP expression (Fig. 2A). At an MOI of 10, a few more fluorescent cells were seen (Fig. 2B). An MOI of 100 displayed even more fluorescent cells (Fig. 2C), and an MOI of 1,000 showed still more cells expressing GFP (Fig. 2D). In the presence of BAF, the amount of fluorescence depended on the number of virus particles adsorbed to the monolayer during infection. This suggested that replication of the input RNA was greatly restricted in the BAF-treated cells and that the amount of virus RNA that entered the cell during the initial infection determined the level of GFP fluorescence.

Effect of BAF on the transfection of BHK cells with SV RNA. To investigate possible secondary effects of BAF on events not related to virus entry, BHK cells were transfected with SV RNA in the presence of BAF, which would effectively eliminate the process of penetration. The GFP variant used in these experiments (pZsGreen; Clontech) is a soluble protein, and the gene encoding it is under the control of its own promoter (50); therefore, the inhibitory effects BAF has on protein trafficking (3, 39) should not affect the expression of GFP. BHK cells were treated with BAF before, during, and after transfec-

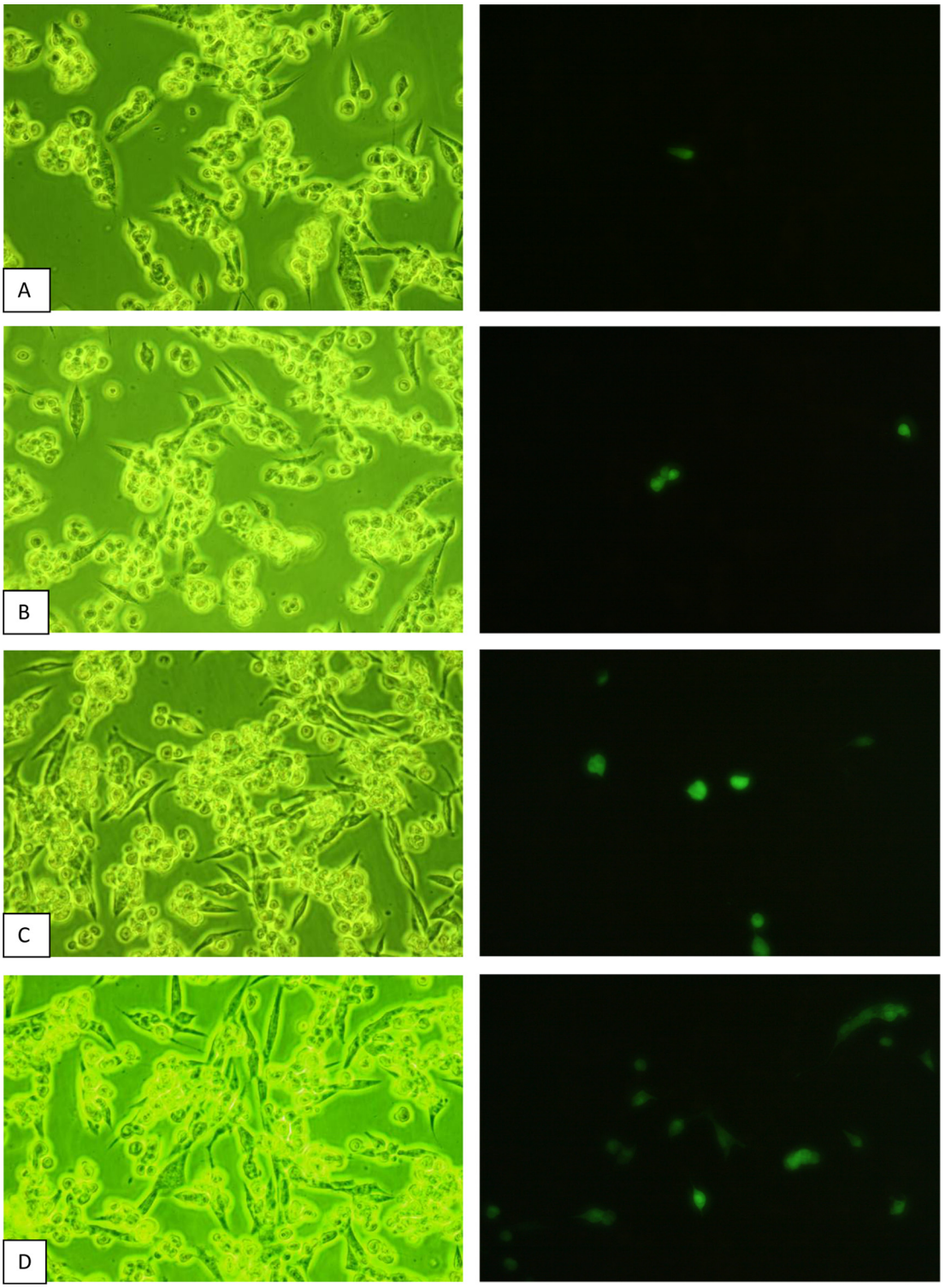

FIG. 2. Effect of MOI on the expression of GFP in the presence of BAF. The BHK cell monolayer (left) and the same cells upon exposure to blue light to excite GFP (right) 24 hpi are shown. Cells were pretreated with BAF, infected with SV in the presence of BAF at RT, and treated with BAF for 24 h at 37°C at the following multiplicities: MOI = 1 (A), MOI = 10 (B), MOI = 100 (C), and MOI = 1,000 (D).

tion according to the protocol described above for infections. Cells pretreated and/or transfected in the presence of BAF displayed a high level of fluorescence (Table 1). Inhibition was seen when BAF was present in the medium after transfection, during which it was maximal. These results were similar to those obtained with SV infection and indicated that BAF was having secondary effects on the cells other than the direct effect of raising endosomal pH.

Effect of temperature on BAF reversibility. We found that the inhibitory effects of BAF at RT were reversible (Table 1). Preliminary experiments indicated that higher temperatures might render the inhibition more permanent. To test the effects of temperature on the reversibility of BAF, BHK cells were pretreated with and infected in the presence of BAF at RT as described above; after infection, the cells were incubated with BAF at 37°C for 0, 15, 30, 45, 60, 90, 120, 150, 180, or 210 min postinfection, after which the BAF was removed by washing the cells once (Table 2). The cells were then incubated at 37°C in fresh medium without BAF for 24 hpi. If BAF was removed after infection but before incubation at 37°C, the cell monolayer recovered, and the majority of cells expressed GFP at 24 hpi. If BAF remained in the medium at 37°C for at least 15 min postinfection, the effects of BAF were irreversible at 24 hpi: the cells appeared rounded and detached from the flask, and there was substantial inhibition of GFP expression. When the experiment was repeated with transfected cells, only those that had BAF present for at least 3 h posttransfection (hpt) showed a decrease in GFP fluorescence at 24 hpt (Table 2); this effect was likely the result of cell death in the prolonged presence of BAF. These results suggest that at higher temperatures, BAF has a more significant effect on inhibition of GFP fluorescence in infected cells.

Previous experiments with alphavirus infection in BHK cells used BAF at 37°C instead of RT (12). To determine whether the temperature change would cause a difference in the results, the infections in the presence of BAF were repeated with both the 30-min pretreatment and the 60-min infection occurring at 37°C instead of RT (Table 1). The results showed that BAF had a much more pronounced effect on the inhibition of infection, as measured by GFP fluorescence when cells were treated at 37°C. The cells only pretreated with BAF at 37°C showed lasting inhibition after the BAF was removed, so that GFP expression was significantly reduced compared to the same experiment done at RT. The cells treated with BAF only after infection at 37°C showed more GFP expression than cells infected at RT. This may have resulted from a faster virus penetration process at 37°C than at RT, so more virus particles infected the cells. We have demonstrated that infection of cells with Sindbis virus is efficient at RT (25°C), and we have further shown that endocytosis occurs efficiently at RT (51). Thus, the differences seen at 37°C probably result from an interaction of BAF with the V-ATPase and are not an effect of BAF on the penetration process. A similar pattern of fluorescence was seen when transfected cells were pretreated with BAF for 30 min at 37°C (Table 1). Only the pretreatment temperature could be adjusted since electroporation, which can only be done at RT, was used for the transfection.

Production of virus-encoded protein in the presence of BAF. The inhibition of GFP production in transfected cells (Table 1) suggested that BAF was having a secondary effect related to the processing and translation of the virus RNA or the assembly of the functional GFP protein. To investigate this effect, cells infected in the presence of BAF were exposed to 50 μg of cycloheximide (CHX; an antibiotic that is known to stop new protein synthesis by inhibiting the 60S ribosomal subunit)/ml immediately after the BAF was removed. If GFP was translated but not properly assembled in the presence of BAF, then the removal of BAF in the presence of CHX might allow for proper folding of the protein in the absence of *de novo* synthesis of native GFP. To establish the effectiveness of CHX in preventing translation of the SV RNA, CHX was added to the monolayer for 2 h at 37°C prior to infection with SV (Fig. 3A). No fluorescence was seen in the monolayer after 48 h, which suggested that no GFP was translated in the presence of CHX.

BHK cells were pretreated with BAF and infected with various multiplicities of virus at RT in the presence of BAF as described above. Cells were incubated in the presence or absence of BAF for various time points after infection, after which they were treated with CHX and BAF was removed. Incubation continued (37°C) in medium containing CHX until images were captured at 24 and 48 hpi. Cells were also infected in medium lacking BAF for the same time points postinfection until CHX was added for comparison. Any GFP fluorescence was considered evidence for translation in the presence of BAF.

The untreated cells exposed to CHX produced low levels of fluorescence that were both time and MOI dependent. The low levels of fluorescence were likely due to the limited translation of the GFP gene during the short incubation in the presence of BAF. The first signs of fluorescence were seen 24 hpi in cells not treated with BAF but treated at 2 hpi with CHX. Those infected with an MOI of 10 were extremely faint, whereas at an MOI of 100 the fluorescent cells were brighter, and at an MOI of 500 there was a higher number of fluorescent cells than seen in those infected with lower MOIs (data not shown). The untreated cells continued to show more fluorescence as the time between infection and CHX treatment increased, with an MOI of 500 always showing the most fluorescence and an MOI of 10 showing the least.

Surprisingly, BAF-treated cells showed low levels of GFP fluorescence, which indicated that the cells became infected and translated virus RNA in the presence of BAF (Fig. 3C). After 24 h, BAF-treated cells infected with an MOI of 10 showed fluorescence in cells treated with CHX at 5 hpi. Those infected with an MOI of 100 expressed GFP when treated with CHX at 4 hpi, and cells infected with an MOI of 500 first showed fluorescence in those treated with CHX at 3 hpi (data not shown). Overall, the BAF-CHX-treated cells appeared very unhealthy compared to those only treated with CHX, with most of the cells appearing rounded and detached from the flask by the end of the experiment at 48 hpi. This is likely the result of the combined toxic effect of virus infection and treatment with BAF and CHX.

To determine whether SV RNA already present in the cell could be translated in the presence of BAF, transfected cells were treated with CHX, which eliminated the process of virus infection. Transfection was carried out as described above in the presence or absence of BAF, and CHX was added 3 hpt. GFP expression was seen in the transfected cells not treated with BAF (Fig. 3D) and in those treated with BAF (Fig. 3E). Thus, the GFP that was synthesized in the presence or absence of BAF during the 3 hpt before the addition of the CHX could assemble and produce fluorescence. Therefore, if viral RNA were present in the infected cells, BAF would not inhibit translation. These results suggest that although BAF did not prevent virus RNA from entering the cell, the presence of BAF prevented the correct folding of synthesized GFP protein, whereas the presence of only CHX did not.

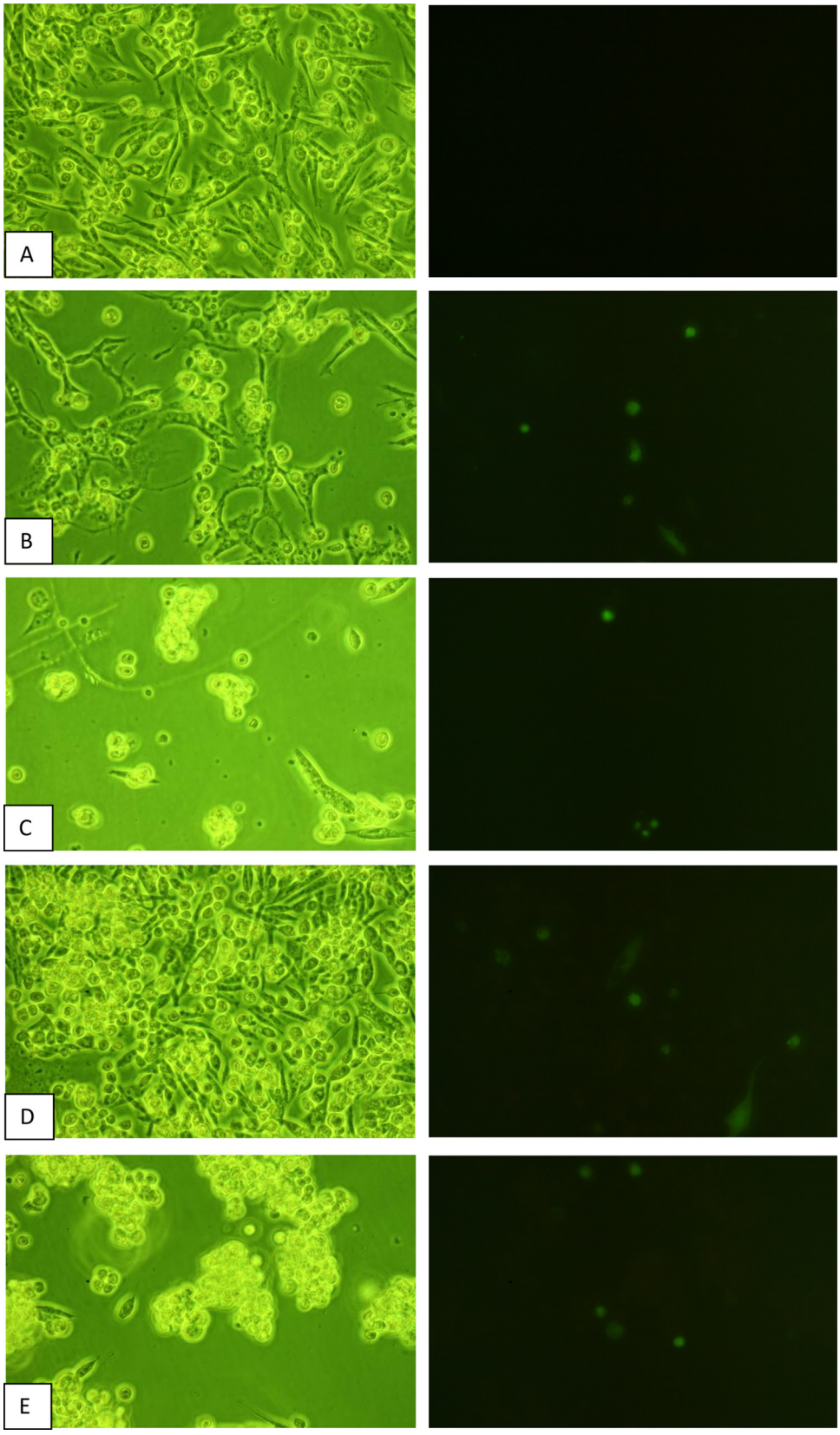

FIG. 3. Expression of virus-encoded GFP in the presence of BAF. The BHK cell monolayer (left) and the same cells upon exposure to blue light to excite GFP (right) 48 hpi or 48 hpt are shown. (A) Cells treated with CHX for 2 h at 37°C, infected with SV in the presence of CHX at RT, and further incubated with CHX at 37°C. (B) Cells infected with SV at RT and treated with CHX 4 hpi at 37°C. (C) Cells pretreated with BAF, infected with SV in the presence of BAF, and treated with BAF for 4 h at 37°C, at which point CHX was added. (D) Cells transfected with SV RNA and treated with CHX 3 hpt at 37°C. (E) Cells pretreated with BAF, transfected with SV RNA in the presence of BAF, and treated with BAF for 3 h at 37°C when CHX was added and BAF was removed.

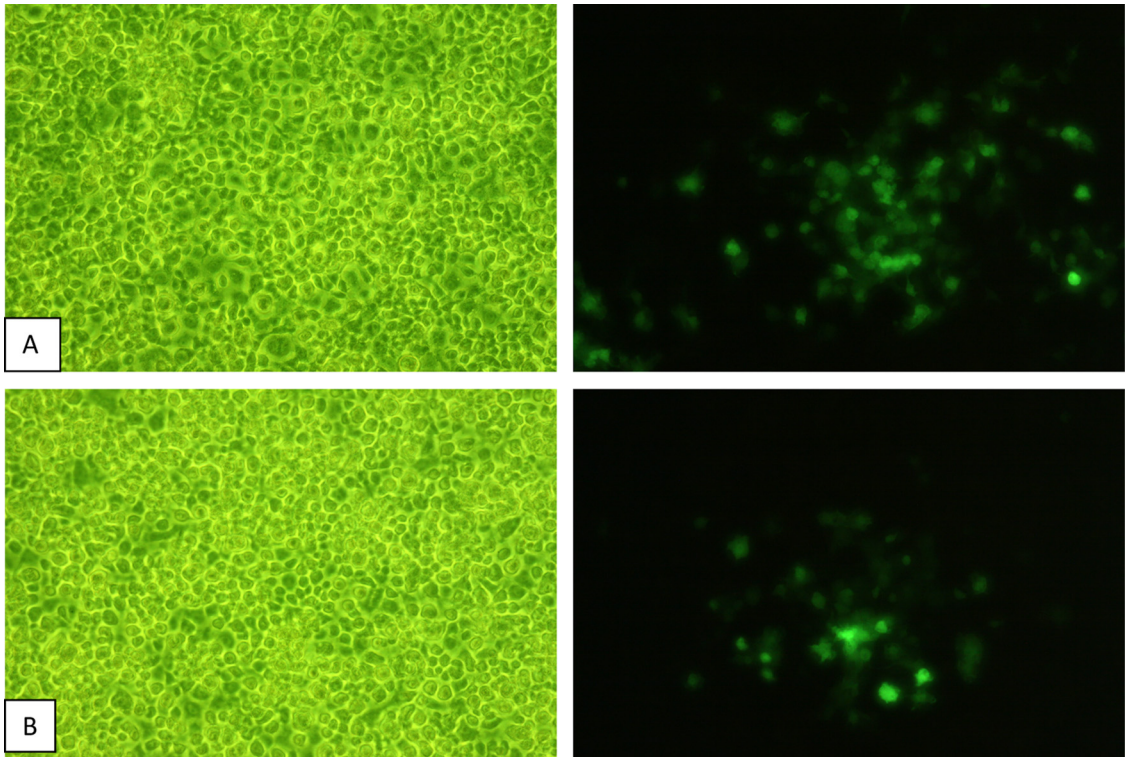

FIG. 4. Effect of BAF on GFP expression in SV-infected *Aedes albopictus* C7-10 cells. C7-10 cells (left) and the same cells upon exposure to blue light to excite GFP are shown. (A) Cells infected with SV 48 hpi. (B) Cells pretreated with 500 nM BAF, infected with SV in the presence of 500 nM BAF, and treated after infection with 500 nM BAF for 48 hpi. The experiment was conducted at 28°C.

Effect of BAF on mosquito cells. In an effort to expand our study to other cell types, the effect of BAF was investigated in *Aedes albopictus* C7-10 cells, which grow at 28°C (Fig. 4). The same protocol for infections was used as described above using 100 nM BAF; however, at 48 hpi with the constant presence of BAF, the cells expressed the same level of GFP fluorescence as the untreated cells (Fig. 4A; see also Materials and Methods). The infections were repeated with increasing concentrations of BAF from 100 nM to 1 μM. The BAF did not have an inhibitory effect on GFP fluorescence in the infected cells at concentrations below 500 nM (data not shown); however, from 500 nM (Fig. 4B) to 1 μM, the GFP fluorescence began showing slight inhibition with increasing concentrations of BAF. BAF has been shown to affect the V-ATPases from a variety of insects at concentrations as low as 10 nM (21, 35, 54, 55). These results agree with previous observations showing that lysosomotropic weak bases do not block the entry of virus RNA into insect cells (7, 19) and further support the idea that a functional V-ATPase, and by extension endosome acidification and endocytosis, is not required for infection of cells by SV.

DISCUSSION

The data presented above reveal some of the effects of BAF on the establishment of infection in mammalian cells by the model alphavirus SV. BAF has been used to inhibit alphavirus infection (12, 27, 46), with the assumption that the acidification of endosomes by the V-ATPase was required for penetration. BAF has also been shown to block stages of endocytosis and intracellular vesicle transport (3). The data presented here show that a functional V-ATPase, and by extension endocytosis, is not required for entry of the virus RNA into the cytoplasm but is required for efficient SV protein production and assembly.

It was shown that during SV infection, the effects of BAF were reversible when used at RT (Table 1). SV infection has been shown to be efficient at RT; SV RNA penetrated the cell and was therefore protected from neutralizing antiserum (51). More importantly these results have shown that addition of BAF after infection, defined as RNA entry, had an inhibitory effect on GFP expression. This suggested that BAF had effects other than inhibiting virus entry. Treating transfected cells with BAF supported this conclusion since the transfected cells showed the same levels of GFP expression as similarly treated infected cells (Table 1). To determine whether the translation of incoming RNA was inhibited by BAF, cells were treated with CHX following infection and transfection in the presence of BAF (Fig. 3) to determine whether the effects of BAF allowed the translation but not the assembly of GFP. Transfected cells showed that BAF did not prevent translation of the RNA; however, infected cells displayed an inhibition of GFP expression related to the multiplicity of virus used for infection. Higher MOIs resulted in earlier GFP expression at 24 hpi in CHX- and BAF-treated cells than did lower MOIs.

It appears that BAF does not prevent the penetration of the cells by SV because the RNA from the virus is able to infect the cells. The difference in the results of the CHX infections and transfections is likely related to the number of copies of the SV

genome that entered the cell. The data presented above suggest that input RNA copy number determines the amount of protein produced, implying that replication of the viral RNA is limited or may not take place. The RNA that enters the cell during the initial infection may be the only viral RNA the cell encounters. By adding a specific MOI to a monolayer during an infection, the number of genome copies theoretically available to enter a cell can be approximated; however, the number of copies of the genome entering a cell during a transfection is unknown. It is possible that the number was much higher in the transfections and that is the reason for the higher level of fluorescence seen in those BAF-treated cells after addition of CHX at the same amount of time postinfection. In addition, BAF has a known effect on cellular trafficking (3), so the cell may be unable to produce infectious virus.

We have suggested that the conformational changes seen in SV upon exposure to low pH may have a role for the V-ATPase in the infection process (above). The data presented here suggest that this hypothesis is invalid. The conformational changes may have less to do with the process of infection than the fact that the SV structural proteins are metastable (5, 34). They may change configuration with changes in environmental conditions. The low pH may result in the protonation of key amino acids that then produce a conformational change, which also inactivates the virus. The conformational changes seen upon exposure to low pH may mimic the changes that occur at a single vertex when the virion interacts with a receptor complex. These structural changes may result in the development of the putative pore through which the viral RNA passes.

The outcome of the BAF-treated transfections and the observation that BAF inhibition was dependent on the MOI of virus adsorbed to a monolayer underscore the reasons for caution in the use of these agents in combination with observation of RNA or protein synthesis as an indication that virus delivery of RNA into a cell has or has not taken place. BAF sensitivity only suggests that the V-ATPase is required for the production of virus components. It does not specify the point (or points) at which it is essential that the V-ATPase be functional. The data presented here also suggest that a functional V-ATPase may be required for postentry events, regardless of whether it is required for entry itself.

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