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Requirement for Vacuolar Proton-ATPase Activity during Entry of Influenza Virus into Cells

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The role that endosomal acidification plays during influenza virus entry into MDCK cells has been analyzed by using the macrolide antibiotics bafilomycin A1 and concanamycin A as selective inhibitors of vacuolar proton-ATPase (v-[H$^+$]ATPase), the enzyme responsible for the acidification of endosomes. Bafilomycin A1 and concanamycin A, present at the low concentrations of 5×10^{-7} and 5×10^{-9} M, respectively, prevented the entry of influenza virus into cells when added during the first minutes of infection. Attachment of virion particles to the cell surface was not the target for the action of bafilomycin A1. *N,N'*-Dicyclohexylcarbodiimide, a nonspecific inhibitor of proton-ATPases, also blocked virus entry, whereas elaiophylin, an inhibitor of the plasma-proton ATPase, had no effect. The inhibitory actions of bafilomycin A1 and concanamycin A were tested in culture medium at different pHs. Both antibiotics powerfully prevented influenza virus infection when the virus was added under low-pH conditions. This inhibition was reduced if the virus was bound to cells at 4°C prior to the addition of warm low-pH medium. Moreover, incubation of cells at acidic pH potently blocked influenza virus infection, even in the absence of antibiotics. These results indicate that a pH gradient, rather than low pH, is necessary for efficient entry of influenza virus into cells.

Infection of animal cells by influenza virus commences with the interaction of the virus particle with cell surface receptors as mediated by hemagglutinin (HA) (25, 57), a virus-encoded glycoprotein embedded in the lipid bilayer that surrounds the virus nucleocapsid (52, 57). After this interaction, influenza virus particles are internalized in endosomes following the route used by other viruses and macromolecules (55, 58, 59). Acidification of endosomes is achieved by the activity of the vacuolar proton ATPase (v-[H$^+$]ATPase). This enzyme pumps protons to the endosome interior at the expense of ATP hydrolysis (14, 37, 46), causing endosome acidification within a few minutes (29, 33). Acidification induces conformational changes in several animal virus glycoproteins, including HA (20), so that hydrophobic portions of the proteins become more exposed, thereby increasing the tendency of the protein to interact with membranes (43, 50, 51, 53–55, 61). The interaction of HA molecules with the endosomal membrane leads to fusion of the latter with the viral envelope, allowing release of the viral nucleocapsid into the cellular cytoplasm, where it is ready to start virus replication (9, 51).

Direct acidification of the culture medium induces influenza virus particles to fuse directly with the plasma membrane (56). Hence, the virion particles do not need to become internalized within endosomes for infection to occur. Evidence from electron microscopy indicates that not only influenza virus but also other enveloped viruses, which are usually internalized by endocytosis, can fuse directly with the plasma membrane in an acidic culture medium (32). Further support for this model of virus entry comes from the use of inhibitors of endosome function, such as lysosomotropic agents that accumulate in endosomes and raise the pH (19, 44) and ionophore molecules, such as monensin, that break down the proton gradient generated by the v-[H$^+$]ATPase (19, 30, 45). Although knowledge gained on virus entry from employing these inhibitors has been of crucial importance, their use and the interpretation of certain results are sometimes restricted by the lack of specificity of some drugs and the side effects that they have on other cellular functions (5, 7, 47).

The recent discovery of selective and potent inhibitors of the v-[H$^+$]ATPase, such as bafilomycin A1 (BFLA1) and concanamycin A (1, 10, 36), prompted us to assay their effects on the entry of animal viruses. Recent studies on the actions of BFLA1 indicate that micromolar concentrations of this antibiotic inhibit the entry of Semliki Forest virus and vesicular stomatitis virus, whereas poliovirus entry is not affected (4, 17, 40). The use of BFLA1 to interfere with virus entry represents a new approach to blocking directly the functioning of the v-[H$^+$]ATPase without affecting other cellular enzymes (4, 40). In addition, this compound provides an improved tool over previously described inhibitors of endosome function, which can be used to provide further insight into the pathway of animal virus entry and to test the validity of current models that describe this step of virus infection (2). Accordingly, in the present work, we have carried out a detailed analysis of the action of BFLA1 on the entry of influenza virus, one of the most paradigmatic viruses in this kind of study.

MATERIALS AND METHODS

Cells, viruses, and media. Influenza virus, Victoria strain, was grown and titrated in Madin-Darby canine kidney (MDCK) cells. MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum.

Radioactive virus. MDCK cells were infected with influenza virus at a multiplicity of infection (MOI) of 10 PFU/cell. At 2.5 h postinfection, the medium was removed, and the cell monolayer was washed twice with methionine-free medium and finally incubated in this medium plus [^{35}S]methionine (6 μCi/ml). Infected cells were incubated at 37°C until a complete cytopathic effect was observed. Cells and cellular debris were then removed by low-speed centrifugation, and the virus-containing supernatant was centrifuged through a 30% sucrose layer for 90 min at 25,000 rpm. The viral pellet was resuspended overnight at 4°C.

Inhibitors. BFLA1 and concanamycin A were provided by K. Altendorf (University of Osnabruck, Osnabruck, Germany). Monensin was purchased from Sigma.

Analysis of proteins by electrophoresis. Cells grown in 24-well plates were infected at an MOI of 50 PFU/cell. After virus adsorption (1 h at 37°C), the cells were incubated in DMEM. Protein labeling was performed with 20 μCi of

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[35S]methionine per ml (1.45 Ci/mmol; Amersham International, Amersham, United Kingdom) added to methionine-free medium for the indicated period of time at 37°C. The radiolabeled cell monolayers were dissolved in sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue). Samples were heated at 90°C for 5 min and electrophoresed on a 15% acrylamide gel overnight at 80 V. Fluorography was carried out in 1 M sodium salicylate. Finally, gels were dried and exposed to Agfa X-ray films.

Binding to cells and uptake assays of influenza virus. To measure virus binding, MDCK cells were grown in 35-mm-diameter dishes. The cell monolayers were washed with DMEM and cooled for 10 to 15 min at 4°C. Approximately 200,000 cpm of radioactive influenza virus preparation was added to each dish, followed by incubation at 4°C for the time indicated. After incubation, free virus was removed, and cell monolayers were washed twice with cold phosphate-buffered saline (PBS). Cells were scraped from the dish, and the radioactivity precipitable by trichloroacetic acid (TCA) was measured. The measurement of virus uptake was performed under the conditions of the binding assays but at 37°C.

Low-pH treatment during virus entry. MDCK cells were grown in DMEM supplemented with 5% fetal calf serum. Cell monolayers were washed, and virus was allowed to adsorb at 4°C for 1 h in DMEM. The medium was then removed, and fresh DMEM, without bicarbonate and buffered with 20 mM MES (morpholineethanesulfonic acid) or HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at the indicated pH, was added. Cells were incubated for 15 min at 37°C in an atmosphere free of CO_2. The medium was then replaced by fresh DMEM, and the cells were incubated at 37°C until the labeling period. When indicated, the virus was added at the same time as the medium buffered with MES or HEPES but without preadsorption at 4°C.

Electron microscopy. MDCK cells grown on 35-mm-diameter dishes were infected with influenza virus at an MOI of 100 PFU/cell and incubated at 4°C for 1 h. The samples were immediately or after 15 min of incubation at 37°C fixed with 2% (vol/vol) glutaraldehyde and 2% (wt/vol) tannic acid in PBS at room temperature for 30 min and washed three times with PBS. Postfixation was carried out with 1% (wt/vol) OsO_4 in PBS at 4°C for 1 h. Samples were then dehydrated through a 30 to 100% (vol/vol) ethanol series and embedded in Eppon 812 (Fluka Chemie AG) after a graded mixing in ethanol-Epon 812 (3:1, 1:1, and 1:3, vol/vol). En bloc staining was performed during the 70% ethanol step with 2% (wt/vol) uranyl acetate. Ultrathin sections were obtained with an Ultracut E ultramicrotome (Reichert-Jung) using a diamond knife, stained with lead citrate, and washed with 20 mM NaOH and distilled water. Electron micrographs were taken with a JEOL 1010 microscope working at 80 kV.

RESULTS

BFLA1 is a powerful inhibitor of influenza virus. The macrolide antibiotic BFLA1 is a powerful and selective inhibitor of the v-[H+]ATPase (1, 10). This enzyme is inhibited by micromolar concentrations of the drug, whereas a 100-fold-higher concentration of BFLA1 is needed to interfere with the function of the plasma proton-ATPase, an enzyme located at the plasma membrane (1, 10). To assay the inhibitory potency of BFLA1, we first tested different concentrations of this antibiotic against the entry of influenza virus (Fig. 1A). For this purpose, influenza virus was added to cells, which were then incubated for 1 h in the presence of BFLA1. Then, excess virus and inhibitor were removed, and the cells were incubated with fresh medium until the synthesis of proteins was analyzed. Concentrations of BFLA1 as low as 0.5 μM almost totally prevented influenza virus infection in MDCK cells, whereas at a 1 μM drug concentration, the inhibition of virus entry, as judged from the appearance of virus proteins, was total. This antibiotic showed no toxic effects on control cells even at a drug concentration of 5 μM under the experimental conditions used (Fig. 1A); in fact, longer incubation times, up to 8 h, with BFLA1 had no adverse effects on the synthesis of cellular proteins.

BFLA1 blocks an early step of influenza virus growth. Influenza virus is blocked by BFLA1 when the compound is present only during the first hour of infection, suggesting that the antibiotic affects an early step during influenza virus growth (Fig. 1A). It is possible that inhibition of influenza virus translation results from the blockade of an event that occurs after virus entry. The experiment shown in Fig. 1B was performed in order to test this possibility. BFLA1 was added either together

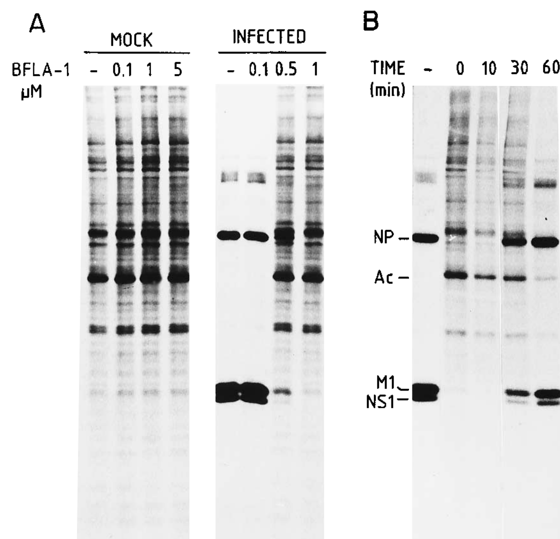

FIG. 1. Effect of BFLA1 on influenza virus infection. (A) MDCK cells were mock infected or infected with influenza virus at an MOI of 50 PFU/cell. At the time of virus addition, different concentrations of BFLA1 were added, and the cells were incubated for 1 h. After that time, cells were washed and incubated in DMEM at 37°C until the labeling period, which was 5.5 to 6.5 h postinfection. (B) MDCK cells were infected with influenza virus (50 PFU/cell), and 5 μM BFLA1 was added together with the virus 10, 30, or 60 min later. After 1 h at 37°C, the cells were washed and further incubated at 37°C until protein labeling was carried out as indicated under Materials and Methods.

with the virus or at different times after virus addition. With the compound present at time zero or added 10 min after virus addition, infection is prevented. However, some influenza virus proteins appear when BFLA1 is administered 30 min postinfection and, no protection is apparent if BFLA1 is added 60 min after infection. The conclusion from this experiment is that the antibiotic needs to be present during the first 10 min of infection to be an effective inhibitor. In addition, this compound has no effect on influenza virus replication if added 1 h after the virus.

Influenza virus infection commences when the virion particles recognize and then attach to cellular receptors. The particles are then internalized into endosomes (29, 57). To test the action of BFLA1 on these steps, radioactively labeled virus was prepared, and its binding or internalization into cells was studied in the absence and in the presence of BFLA1. Figure 2A shows that the attachment of virion particles to MDCK cells at 4°C is not affected by BFLA1, whereas a partial inhibition is observed in both the binding and the internalization of labeled particles at 37°C (Fig. 2B). This effect does not account for the strong blockade on virus replication observed for the drug. Since BFLA1 is an inhibitor of the v-[H+]ATPase, it is possible that the step affected by BFLA1 is the release of viruses from endosomes, as a result of the drug's inhibition of endosome acidification.

The binding and entry of influenza virus into MDCK cells were also analyzed by electron microscopy. When the virus was added to the cell monolayer and the mixture was kept on ice, most of the virus particles were adsorbed onto the cellular surface, i.e., the plasma membrane (Fig. 3A). In the presence of BFLA1, virus adsorption (Fig. 3B) still occurred, indicating that this step is not affected by the inhibitor. When the cells were warmed to 37°C, the virus was rapidly internalized, and after 15 min of incubation, virus particles were not detectable in the endosomes of cells that were not treated with drug (data

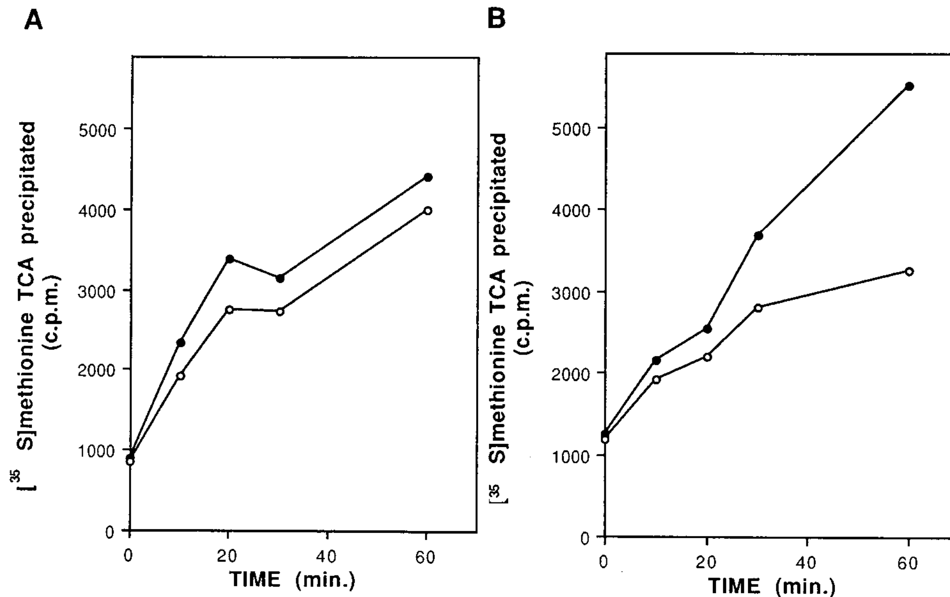

FIG. 2. Binding and internalization of influenza virus into MDCK cells in the presence of BFLA1. [^{35}S]methionine-labeled virus was obtained as described in Materials and Methods; 20 μl of radioactive virus (10,000 cpm/μl) was added to the cells, which were incubated at 4°C to estimate virus attachment (A) or at 37°C to measure viral internalization (B). BFLA1 (5 μM) was added at the same time as the virus. At the times indicated, cells were washed, scraped, and centrifuged, and TCA-precipitable radioactivity was measured. ●, Control without BFLA1; ○, with BFLA1.

not shown). However, in the presence of BFLA1, virus particles remained inside coated vesicles (Fig. 3C). These results are consistent with previous findings on other inhibitors of endosome function (58) and indicate that BFLA1 affects neither virus binding nor endocytosis but instead blocks virus release from endosomes.

Actions of different inhibitors of proton-ATPases on influenza virus. The three types of proton-ATPases present in mammalian cells are inhibited to different extents by several compounds. *N,N′*-dicyclohexylcarbodiimide (DCCD) is a nonspecific inhibitor that blocks the three types of ATPases, whereas elaiophilin, a macrolide antibiotic produced by a *Streptomyces* sp., acts on the P-type (plasma membrane) [H^+]ATPases. BFLA1 is selective against the V-type (vacuolar) [H^+]ATPases but it also blocks the P-type [H^+]ATPases when added at high concentrations (10). The most selective and powerful inhibitor of the V-type proton-ATPases of mammalian cells so far described is concanamycin A, another macrolide antibiotic produced by a *Streptomyces* sp. (10, 36). All four drugs were used to unravel the functions of the different [H^+]ATPases during influenza virus entry (Fig. 4A). Both, BFLA1 and concanamycin A powerfully blocked influenza virus when present during the first hour of virus infection (Fig. 4A), but did not do so when added 1 h after the virus (data not shown). Concanamycin A was fully active at a concentration of 8×10^{-7} M, indicating that it resembles BFLA1 in being a potent inhibitor of an early step in influenza virus infection. In fact, concanamycin A totally prevented infection at a concen-

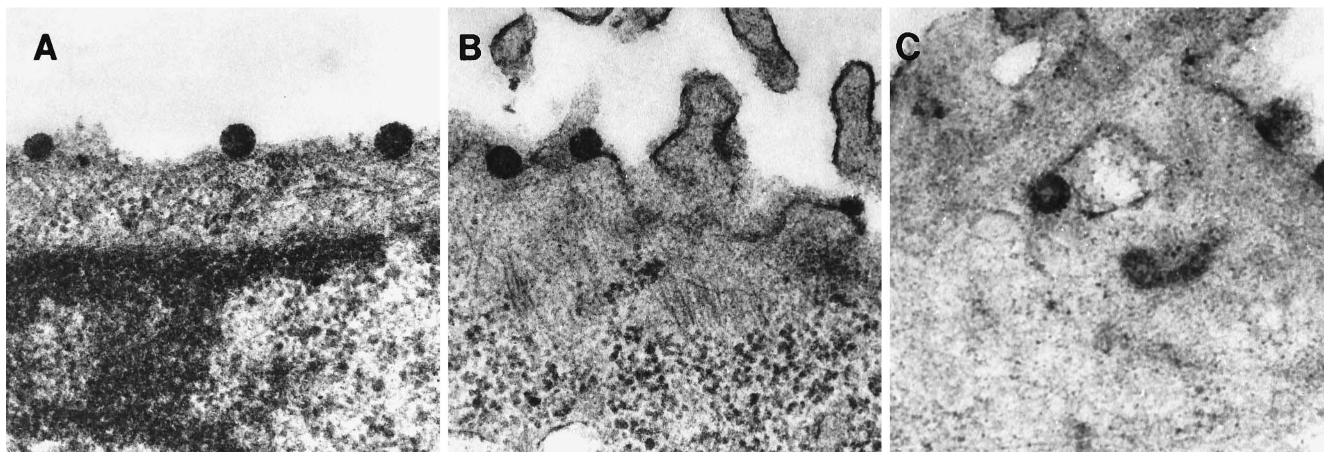

FIG. 3. Electron microscopy of influenza virus attachment and entry into MDCK cells: effect of BFLA1. (B and C) MDCK cells were grown in 35-mm-diameter dishes. Cell monolayers were washed with DMEM and incubated in the presence of 4 μM BFLA1 for 15 min. Incubation with influenza virus (100 PFU/cell) was then performed at 4°C for 1 h. After this time (A and B) or following incubation at 37°C for 15 min (C), the cells were washed and fixed for 30 min. Electron microscopy was performed as indicated under Materials and Methods.

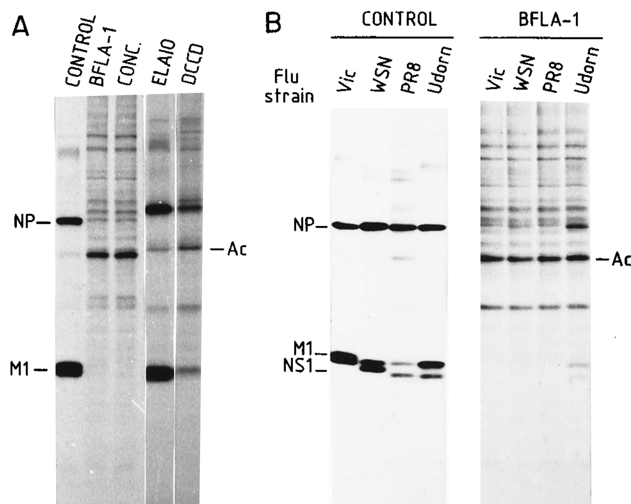

FIG. 4. Comparison of the action of BFLA1 with other proton-ATPase inhibitors: effects of BFLA1 on different influenza virus strains. (A) MDCK cells were infected with influenza virus at an MOI of 50 PFU/cell. At the time of virus addition, 5 μM BFLA1, 0.8 μM concanamycin A (CONC.), 5 μM elaiophylin (ELAIO), or 30 μM DCCD was added to the cells. Incubation in the presence of the inhibitor was carried out for 1 h. Afterwards, the cell monolayers were washed and incubated at 37°C in DMEM until protein labeling from 5 to 6 h postinfection. (B) MDCK cells were infected with the A/Victoria/3/75 (Vic), A/WSN/33 (WSN), A/PR/8/34 (PR8), or A/Udorn/72 (Udorn) influenza virus strain in the absence or presence of 5 μM BFLA1 and were then incubated for 1 h at 37°C. Then the cells were washed and incubated with DMEM at 37°C until protein labeling from 4.5 to 5.5 h postinfection.

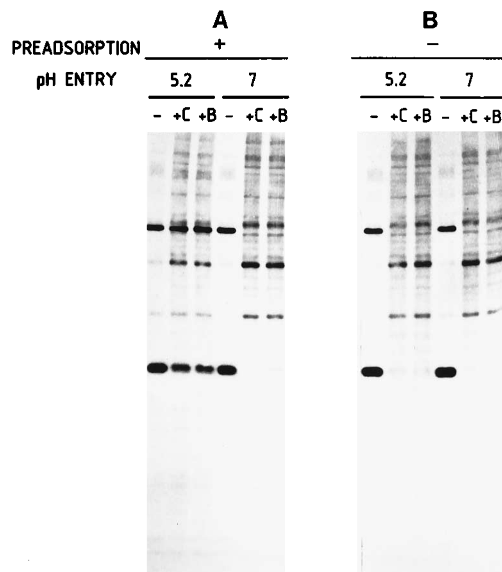

FIG. 5. Effect of acid pH on inhibition of influenza virus by BFLA1 or concanamycin. (A) Cells were grown in L-24 Linbro dishes and incubated with either BFLA1 (4 μM) or concanamycin A (50 nM) for 15 min at 37°C. Then cells were cooled at 4°C, and influenza virus was added to at 10 PFU/cell; the cells were then kept on ice for 1 h. After that time, the medium was removed and was replaced by DMEM without bicarbonate, buffered with MES (pH 5.2) or HEPES (pH 7). Cells were incubated at 37°C in an atmosphere free of CO_2 in the presence or absence of the antibiotics. After 15 min, the medium was replaced by DMEM, and the cells were incubated at 37°C until the labeling period from 4.5 to 5.5 h postinfection. (B) Cells were incubated with concanamycin A (50 nM) or BFLA1 (4 μM) for 15 min in DMEM without bicarbonate. Then concentrated influenza virus was added along with 20 mM MES (pH 5.2) or 20 mM HEPES (pH 7). Cells were incubated for a further 15 min in an atmosphere free of CO_2, and then they were washed and incubated in DMEM until the labeling period from 4.5 to 5.5 h postinfection.

tration as low as 5×10^{-9} M (16, 17). Influenza virus was partially inhibited by DCCD, whereas elaiophilin was devoid of activity. These results clearly implicate v-[H$^+$]ATPase in influenza virus entry and suggest that either BFLA1 or concanamycin A could be used in future studies aimed at testing the roles of v-[H$^+$]ATPases during animal virus entry into cells.

Finally, BFLA1 inhibits all the influenza virus strains tested (Fig. 4B), indicating that the drug blocks a step during infection that is common to all these strains.

Effects of BFLA1 on the entry of influenza virus at different pHs. In culture medium, direct entry of influenza virus through the plasma membrane occurs under acidic conditions (58). At pH 5.5 or below, influenza virus is able to fuse its lipid envelope directly with the plasma membrane, leading to virus infection (31). To assay the effects of BFLA1 and concanamycin A on the entry of influenza virus into cells at neutral or low pH, two different protocols were followed (Fig. 5). In protocol 1, the virus was first attached to cells at 4°C, and warmed medium at low or neutral pH was then added. In protocol 2, the virus was added directly (without preadsorption) to neutral or low-pH medium in the absence or presence of the antibiotics. It is well established that incubation of influenza virus alone, in the absence of cells, under acidic conditions inactivates its infectivity (41, 42, 49). However, addition of influenza virus directly to pH 5.2 medium does not block virus infection (Fig. 5). The v-[H$^+$]ATPase inhibitors powerfully prevent virus infection when the virus is added at the same time as the medium, even under low-pH conditions, whereas the drugs allow partial entry of influenza virus if the virus is bound before the addition of warm low-pH medium (Fig. 5). In addition, the simultaneous presence of two agents that interfere with endosome function, such as concanamycin A plus nigericin, powerfully prevented virus infection, even in the presence of low-pH medium (16).

Several interpretations of these results are possible. One is that virions need to modify their conformation by receptor binding before they encounter a low pH that would further influence virion conformation prior to fusion. This possibility is unlikely, because virions added directly to low-pH medium, thereby encountering a low pH before receptor binding, efficiently infect cells in the absence of inhibitors. Another possibility (4, 40) is that efficient virus entry into cells relies upon the existence of a pH gradient rather than low pH itself. Thus, the addition of warm low-pH medium instantly creates a pH gradient between the medium and the cytoplasm. When the virus is internalized under these conditions, the endosomes are already acidified and do not require further acidification by the v-[H$^+$]ATPase, since the pH gradient that exists between the endosome and the cytoplasm suffices to drive virus translocation. In contrast, incubation of cells at 37°C in acidic medium partially acidifies the cytoplasm and thus lowers the pH gradient. Direct addition of the virus to such an acidic medium implies that the virus has to bind to receptors during the time required for the drop in the cytoplasmic pH. The activity of the v-[H$^+$]ATPase is therefore necessary to create a pH gradient that is sufficient to promote virus genome delivery into the cytoplasm.

To test the effects of pH 5.2 medium on influenza virus infectivity, the virus was added directly to culture cells in pH 5.2 or 7.0 medium; after 15 min of incubation, the medium was removed, and plaque formation was estimated. The virus titer was equal under both conditions (results not shown). However, direct incubation of influenza virus in pH 5.2 medium drops

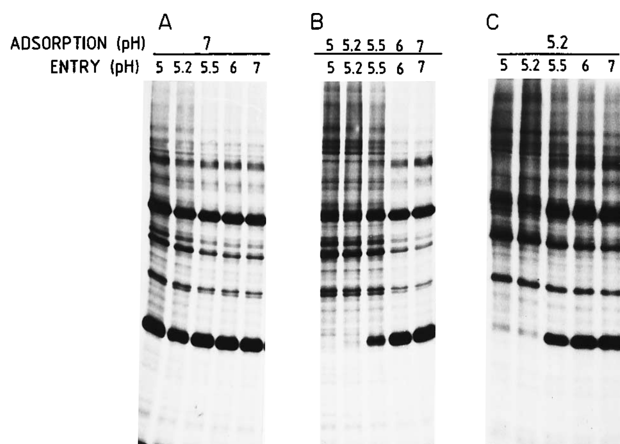

FIG. 6. Inhibition of influenza virus replication by disruption of the pH gradient. Cells were washed, and DMEM was replaced by DMEM without bicarbonate and buffered at the indicated pH. Influenza virus was added at the same time to the cells, and viral adsorption was allowed to occur for 1 h at 4°C. Excess virus was removed, and warm DMEM at different pHs was added for 15 min. The cells were washed again and incubated in DMEM until the labeling period from 4.5 to 5 h postinfection.

virus infectivity 1,000-fold after 5 min of incubation at 37°C. Finally, plaque formation was estimated by preincubation of the virus at 4°C in pH 5.2 medium or in pH 7.0 medium, followed by addition of warm pH 7 or 5.2 medium, respectively. No effect on influenza virus PFU by these treatments was observed (results not shown). Therefore, in agreement with previous findings (41, 42, 49), direct incubation of influenza virus with acidic medium diminishes virus infectivity, while this effect is not observed when the virus is incubated with cells.

The internal pH of cells rises upon incubation with low-pH medium (26). Hence, we reasoned that preincubation of cells with acidic medium would acidify the cytoplasm and block influenza virus entry, even in the absence of inhibitors. To test this possibility, three protocols were followed. (i) Influenza virus was prebound to cells at 4°C for 1 h in neutral medium; aliquots of warm medium at different pHs were then added, and the cells were further incubated for 15 min (Fig. 6A). (ii) The virus was prebound at 4°C at different pHs, and entry of the virus was studied at the same pHs but at 37°C (Fig. 6B). (iii) The virus was prebound at 4°C with acidic medium (pH 5.2), and then virus entry was studied at 37°C over a range of pHs (Fig. 6C). The results show that preincubation of cells with low-pH medium (5.0 and 5.2) followed by incubation at 37°C at the same pH renders the cells refractory to virus entry, whereas at pH 5.5 or above, virus infection is apparent (Fig. 6B). In contrast, preincubation of cells and virus at 4°C for 1 h in acidic medium does not inactivate the virus, since it readily infects such cells when they are subsequently incubated in medium at pH 5.5 or above (Fig. 6C). On the other hand, addition of warm pH 5.2 medium to influenza virus bound to cells does not destroy infectivity (Fig. 6A). The conclusion from these results is that influenza virus entry can be powerfully prevented by low-pH conditions, suggesting that low pH per se does not promote virus entry.

DISCUSSION

Two different modes of entry have been described for enveloped animal viruses: (i) direct fusion of the viral lipid envelope with the plasma membrane, as occurs with Sendai virus (38), and (ii) internalization of virus particles in endosomes, followed by fusion of the viral and the endosomal membranes (29, 50, 56). The second pathway is followed by animal viruses such as Semliki Forest virus, vesicular stomatitis virus, and influenza virus (55, 56). It is believed that these viruses fuse with and enter directly from the plasma membrane, provided that an acidic culture medium is present during virus entry (50). Fundamental to this second model is the concept that viral glycoproteins present in the envelope change their conformation at acidic pH in such a way that exposure of a hydrophobic domain within the protein leads to its insertion into the cellular membrane, thereby promoting fusion between the viral and the cellular lipid bilayers (8, 50, 56). This model further suggests that fusion leads to virus entry and hence infection of the cell (29, 50, 56). Both morphological and biochemical evidence supports the idea that some enveloped viruses follow the receptor-mediated endosome route to enter cells, as indeed do many other macromolecules. Such viruses need a low-pH step for entry, a requirement that can be demonstrated by the use of compounds that raise the pH in endosomes (18, 19). Our present results lend support to this idea and provide direct evidence that the activity of the v-$[H^+]$ATPase is required for the generation of low pH in endosomes (33).

The high specificity of BFLA1 and concanamycin A in their inhibition of the v-$[H^+]$ATPase (1, 10) and the fact that very low concentrations of these antibiotics inhibit virus entry suggest that the compounds could be used as exquisite tools for investigating the requirements for cell infection by animal viruses. Indeed, we have recently demonstrated that the entry of poliovirus into cells does not require a low-pH step (4, 40); in fact, such a requirement for poliovirus entry has been disputed for almost a decade (15, 27, 28). BFLA1 and concanamycin A might also be employed to obtain a more precise understanding of the mechanisms that govern virus entry and the exact role that low pH plays during the early steps of virus infection. We stress that the use of lysosomotropic agents to assay the requirement for a low-pH step during virus entry would be an inappropriate approach for the experiments described in this article. Direct addition of lysosomotropic agents to low-pH media would raise the pH in such media. In addition, the protonated amines would not pass through membranes and therefore would not accumulate in endosomes under these conditions. Further studies on the exact mode of action of the macrolide antibiotics used in this work will shed more light on their effects on other viral functions, apart from virus entry, that are dependent on the v-$[H^+]$ATPase activity.

Several processes that are involved with virus entry are still poorly understood. For example, the precise role played by the interaction of virus ligands with cell receptors in the modification of the structural conformations of virions remains to be elucidated. As indicated above, low pH by itself changes the conformation of viral glycoproteins and of virion particles (8, 21, 34, 39, 56). In some instances, these modifications are so drastic that they eliminate virus infectivity (49). Recent evidence indicates that naked virion particles, or viral glycoproteins that are present in virus envelopes, undergo conformational changes upon interaction with their receptors (11, 35). Such interactions, in addition to a low pH, may be required for the correct insertion of the viral glycoprotein into the cellular membrane. For example, attachment of togavirus and influenza virus particles to their receptors at neutral pH does not trigger fusion, whereas fusion of virions with artificial liposomes takes place at acidic pH (49). However, it is not known if this fusion event would lead to virus infection, since the possibility exists that only viruses that enter cells via the endo-

cytic pathway are infectious, even under low-pH conditions in the medium (6).

Apart from the contribution that low pH makes to modification of viral glycoprotein conformation, it is conceivable that the pH gradient created by the v-$[H^+]$ATPase pumps is also required for virus entry, as we have recently indicated (2, 4, 40). The mechanistic model of virus entry can now be aligned with other models in which energy is necessary to push the virion nucleocapsid into the cytoplasm, and the use of the antibiotics described here may allow us to distinguish between the various possibilities. Animal viruses may simply fuse their membranes with cellular membranes for entry, or, alternatively, viruses may need an energized membrane for proper fusion, with the dissipation of the proton motive force propelling the virus into the cell. Indeed, the entry of both enveloped and naked animal viruses into cells disrupts the membrane potential (3, 22–24, 48). Poliovirus entry destroys the ionic gradients that exist in animal cells (3, 13). Paramyxoviruses also depolarize the cell membrane during entry, in such a way that there is a rapid efflux of potassium ions and a corresponding influx of sodium ions (12, 13, 60). Soon after Sendai virus entry, the membrane repolarizes again (12, 13, 60), and indeed, the entry of Semliki Forest virus does not occur in the absence of a membrane potential, even under low-pH conditions (18). Therefore, animal viruses dissipate the ionic gradients during entry, the physiological significance of this behavior perhaps being to couple the proton motive force with virus entry into the cytoplasm (4). The prediction of this model is that viruses will be unable to infect cells when the pH gradient has been destroyed, even in an acidic environment. Indeed, our present findings lend support to this concept and provide evidence that influenza virus infection can be prevented even under low-pH conditions (16). The addition of a low-pH medium to prebound virus instantly creates a pH gradient, avoiding the necessity for v-$[H^+]$ATPase to function. However, the dissipation of this gradient by preincubation with a low-pH medium compromises virus infection. Evidence from electron microscopy clearly shows that low-pH medium triggers the fusion of influenza virus directly with the plasma membrane (54, 55). However, it is not known if this fusion event requires exclusively low pH or if breakage of the pH gradient would hamper fusion. It needs to be ascertained whether fusion of influenza virus with the plasma membrane leads to infection or whether entry of the virus through endosomes even under low-pH conditions is a requisite for infectivity. Since monensin and concanamycin A are both required for preventing influenza virus entry at low pH, it seems that the v-$[H^+]$ATPase is involved in this process. Further clarification of the entry mechanisms for enveloped animal viruses at low pH could result from the use of the inhibitors described in this work.

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REFERENCES

1. **Bowman, E. J., A. Siebers, and K. Altendorf.** 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. USA **85:**7972–7976.
2. **Carrasco, L.** 1994. Entry of animal viruses and macromolecules into cells. FEBS Lett. **350:**152–154.
3. **Carrasco, L., M. J. Otero, and J. L. Castrillo.** 1989. Modification of membrane permeability by animal viruses. Pharmacol. Ther. **40:**171–212.
4. **Carrasco, L., L. Perez, A. Irurzun, J. Lama, F. Martinez-Abarca, P. Rodrigez, R. Guinea, J. L. Castrillo, M. A. Sanz, and M. J. Ayala.** 1993. Modification of membrane permeability by animal viruses, p. 283–305. *In* L. Carrasco, N. Sonenberg, and E. Wimmer (ed.), Regulation of gene expression in animal viruses. Plenum Press, New York.
5. **Cassell, S., J. Edwards, and D. T. Brown.** 1984. Effect of lysosomotropic weak bases on infection of BHK-21 cells by Sindbis virus. J. Virol. **52:**857–864.
6. **Coombs, K., and D. T. Brown.** 1987. The penetration of animal cells by viruses, p. 5–20. *In* L. Carrasco (ed.), Mechanisms of viral toxicity in animal cells. CRC Press Inc., Boca Raton, Fla.
7. **Dean, R. T., W. Jessup, and C. R. Roberts.** 1984. Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. Biochem. J. **217:**27–40.
8. **Doms, R. W.** 1993. Protein conformation changes in virus-cell fusion. Methods Enzymol. **221:**61–72.
9. **Doms, R. W., A. Helenius, and J. White.** 1985. Membrane fusion activity of the influenza virus hemagglutinin: the low pH-induced conformational change. J. Biol. Chem. **260:**2973–2981.
10. **Dröse, S., K. U. Bindseil, E. J. Bowman, A. Siebers, A. Zeeck, and K. Altendorf.** 1993. Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphatases. Biochemistry **32:**3902–3906.
11. **Fricks, C. E., and J. M. Hogle.** 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J. Virol. **64:**1934–1945.
12. **Fuchs, P., E. Gruber, J. Gitelman, and A. Kohn.** 1980. Nature of permeability changes in membrane of HeLa cells adsorbing Sendai virus. J. Cell Physiol. **103:**271–278.
13. **Fuchs, P., and A. Kohn.** 1983. Changes induced in cell membranes adsorbing animal viruses, bacteriophages, and colicins. Curr. Top. Microbiol. Immunol. **102:**57–99.
14. **Gluck, S. L.** 1993. The vacuolar H^+-ATPases: versatile proton pumps participating in constitutive and specialized functions of eukaryotic cells. Int. Rev. Cytol. **137:**105–137.
15. **Gromeier, M., and K. Wetz.** 1990. Kinetics of poliovirus uncoating in HeLa cells in a nonacidic environment. J. Virol. **64:**3590–3597.
16. **Guinea, R., and L. Carrasco.** 1994. Concanamycin A blocks influenza virus entry into cells under acidic conditions. FEBS Lett. **149:**327–330.
17. **Guinea, R., and L. Carrasco.** 1994. Concanamycin A: a powerful inhibitor of enveloped animal-virus entry into cells. Biochem. Biophys. Res. Commun. **201:**1270–1278.
18. **Helenius, A., M. Kielian, J. Wellsteed, I. Mellman, and G. Rudnick.** 1985. Effects of monovalent cations on Semliki Forest virus entry into BHK-21 cells. J. Biol. Chem. **260:**5691–5697.
19. **Helenius, A., M. Marsh, and J. White.** 1982. Inhibition of Semliki Forest virus penetration by lysosomotropic weak bases. J. Gen. Virol. **58:**47–61.
20. **Hoekstra, D., and J. W. Kok.** 1989. Entry mechanisms of enveloped viruses: implications for fusion of intracellular membranes. Biosci. Rep. **9:**273–305.
21. **Hoover-Litty, H., and J. M. Greve.** 1993. Formation of rhinovirus-soluble ICAM-1 complexes and conformational changes in the virion. J. Virol. **67:**390–397.
22. **Jakeman, K. J., H. Smith, and C. Sweet.** 1991. Influenza virus enhancement of membrane leakiness induced by staphylococcal alpha toxin, diphtheria toxin and streptolysin S. J. Gen. Virol. **72:**111–115.
23. **Karadaghi, S. E., J. A. Zakomirdin, C. Shimane, D. J. Bucher, V. A. Tverdislov, and I. G. Kharitonenkov.** 1984. Interaction of influenza virus protein with planar bilayer lipid membranes. Biochim. Biophys. Acta **778:**269–275.
24. **Lanzrein, M., N. Käsermann, and C. Kempf.** 1992. Changes in membrane permeability during Semliki Forest virus induced cell fusion. Biosci. Rep. **12:**221–236.
25. **Lentz, T. L.** 1990. The recognition event between virus and host cell receptor: a target for antiviral agents. J. Gen. Virol. **71:**751–766.
26. **Madshus, I. H.** 1988. Regulation of intracellular pH in eukaryotic cells. Biochem. J. **250:**1–8.
27. **Madshus, I. H., S. Olsnes, and K. Sandvig.** 1984. Requirements for entry of poliovirus RNA into cells at low pH. EMBO J. **3:**1945–1950.
28. **Madshus, I. H., S. Olsnes, and K. Sandvig.** 1984. Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. J. Cell Biol. **98:**1194–1200.
29. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
30. **Marsh, M., J. Wellsteed, H. Kern, E. Harms, and A. Helenius.** 1982. Monensin inhibits Semliki Forest virus penetration into culture cells. Proc. Natl. Acad. Sci. USA **79:**5297–5301.
31. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. **91:**601–613.
32. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1982. Pathway of vesicular stomatitis virus entry leading to infection. J. Mol. Biol. **156:**609–631.
33. **Mellman, I., R. Fuchs, and A. Helenius.** 1986. Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. **55:**663–700.

34. **Meyer, W. J., S. Gidwitz, V. K. Ayers, R. J. Schoepp, and R. E. Johnston.** 1992. Conformational alteration of Sindbis virion glycoproteins induced by heat, reducing agents, or low pH. J. Virol. **66:**3504–3513.
35. **Meyer, W. J., and R. E. Johnston.** 1993. Structural rearrangement of infecting Sindbis virions at the cell surface: mapping of newly accessible epitopes. J. Virol. **67:**5117–5125.
36. **Muroi, M., A. Takasu, M. Yamasaki, and A. Takatsuki.** 1993. Folimycin (concanamycin A), an inhibitor of V-type H^+-ATPase, blocks cell-surface expression of virus-envelope glycoproteins. Biochem. Biophys. Res. Commun. **193:**999–1005.
37. **Nelson, N., and L. Taiz.** 1989. The evolution of H^+-ATPases. Trends Biochem. Sci. **14:**113–116.
38. **Okada, Y.** 1993. Sendai virus-induced cell fusion. Methods Enzymol. **221:** 18–41.
39. **Olson, N. H., P. R. Kolatkar, M. A. Oliveira, R. H. Cheng, J. M. Greve, A. McClelland, T. S. Baker, and M. G. Rossmann.** 1993. Structure of a human rhinovirus complexed with its receptor molecule, p. 1–12. *In* L. Carrasco, N. Sonenberg, and E. Wimmer (ed.), Regulation of gene expression in animal viruses. Plenum Press, New York.
40. **Perez, L., and L. Carrasco.** 1993. Entry of poliovirus into cells does not require a low-pH step. J. Virol. **67:**4543–4548.
41. **Ramalho-Santos, J., S. Nir, N. Düzgünes, A. Pato de Carvalho, and M. C. Pedroso de Lima.** 1993. A common mechanism for influenza virus activity and inactivation. Biochemistry **32:**2771–2779.
42. **Ruigrok, R. W. H., E. A. Hewat, and R. H. Wade.** 1992. Low pH deforms the influenza virus envelope. J. Gen. Virol. **73:**995–998.
43. **Sato, S. B., K. Kawasaki, and S. Ohnishi.** 1983. Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments. Proc. Natl. Acad. Sci. USA **80:**3153–3157.
44. **Schlegel, R., R. B. Dickson, M. C. Willingham, and I. H. Pastan.** 1982. Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of alpha 2-macroglobulin. Proc. Natl. Acad. Sci. USA **79:**2291–2295.
45. **Schlegel, R., M. Willingham, and I. Pastan.** 1981. Monensin blocks endocytosis of vesicular stomatitis virus. Biochem. Biophys. Res. Commun. **102:** 992–998.
46. **Schneider, D. L.** 1987. The proton pump ATPase of lysosomes and related organelles of the vacuolar apparatus. Biochim. Biophys. Acta **895:**1–10.
47. **Seglen, P. O.** 1983. Inhibitors of lysosomal function. Methods Enzymol. **96:**737–764.
48. **Spruce, A. E., A. Iwata, and W. Almers.** 1991. The first milliseconds of the pore formed by a fusogenic viral envelope protein during membrane fusion. Proc. Natl. Acad. Sci. USA **88:**3623–3627.
49. **Stegmann, T., F. P. Booy, and J. Wilschut.** 1987. Effects of low pH on influenza virus: activation and inactivation of the membrane fusion capacity of the hemagglutinin. J. Biol. Chem. **262:**17744–17749.
50. **Stegmann, T., R. W. Doms, and A. Helenius.** 1989. Protein-mediated membrane fusion. Annu. Rev. Biophys. Biophys. Chem. **18:**187–211.
51. **Stegmann, T., J. M. White, and A. Helenius.** 1990. Intermediates in influenza induced membrane fusion. EMBO J. **9:**4231–4241.
52. **Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley.** 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature (London) **333:**426–431.
53. **White, J., A. Helenius, and M. J. Gething.** 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature (London) **300:**658–659.
54. **White, J., J. Kartenbeck, and A. Helenius.** 1982. Membrane fusion activity of influenza virus. EMBO J. **1:**217–222.
55. **White, J., K. Matlin, and A. Helenius.** 1981. Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. J. Cell Biol. **89:**674–679.
56. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52:**675–697.
57. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56:**365–394.
58. **Yoshimura, A., K. Kuroda, K. Kawasaki, S. Yamashina, T. Maeda, and S. Ohnishi.** 1982. Infectious cell entry mechanism of influenza virus. J. Virol. **43:**284–293.
59. **Yoshimura, A., and S. Ohnishi.** 1984. Uncoating of influenza virus in endosomes. J. Virol. **51:**497–504.
60. **Young, J. D., G. P. Young, Z. A. Cohn, and J. Lenard.** 1983. Interaction of enveloped viruses with planar bilayer membranes: observations on Sendai, influenza, vesicular stomatitis, and Semliki Forest viruses. Virology **128:**186–194.
61. **Yu, Y. G., D. S. King, and Y.-K. Shin.** 1994. Insertion of a coiled-coil peptide from influenza virus hemagglutinin into membranes. Science **266:**274–276.