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Entry of Poliovirus into Cells Does Not Require a Low-pH Step

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The requirement of a low-pH step during poliovirus entry was investigated by using the macrolide antibiotic bafilomycin A1, which is a powerful and selective inhibitor of the vacuolar proton-ATPases. Thus, viruses such as Semliki Forest virus and vesicular stomatitis virus that enter cells through endosomes and need their acidification, are potently inhibited by bafilomycin A1, whereas poliovirus infection is not affected by the antibiotic. The presence of lysosomotropic agents such as chloroquine, amantadine, dansylcadaverine, and monensin during poliovirus entry did not inhibit infection, further supporting the idea that poliovirus does not depend on a low-pH step to enter the cytoplasm. The effect of bafilomycin A1 on other members of the *Picornaviridae* family was also assayed. Encephalomyocarditis virus entry into HeLa cells was not affected by the macrolide antibiotic, whereas rhinovirus was sensitive. Coentry of toxins, such as α-sarcin, with viral particles was potently inhibited by bafilomycin A1, indicating that an active vacuolar proton-ATPase is necessary for the early membrane permeabilization (coentry of α-sarcin) induced by poliovirus to take place.

Poliovirus has a virion particle 30 nm in diameter with icosahedral symmetry that is composed of 60 copies each of four structural proteins, VP1, VP2, VP3, and VP4, that surround the 7.5-kb genome covalently linked to genome-bound protein VPg (15, 25). The poliovirus infective cycle commences by interaction of poliovirus particles with specific cell surface receptors (20). Then, virus internalization proceeds by receptor-mediated endocytosis (31). Entry of poliovirus particles directly through the plasma membrane, after binding to its receptor, has also been suggested as a portal of virus entry (8). Once the viral particles are in endosomes, they must exit this organelle to start translation of the genomic RNA in the cytoplasm. It is still a matter of debate to what extent poliovirus requires acidification of endosomes to allow passage of the genome through the endosomal membrane (16, 18, 31) or whether poliovirus accomplishes this task in a pH-independent fashion (12). Indirect experiments in which poliovirus particles were complexed with neutral red and incubated with weak amines or monensin for several minutes and then incubated for a very long time (18 to 40 h) suggested that poliovirus requires a low-pH step for infectivity (16–18). Virus entry was evaluated by measuring the cytopathic effect caused by poliovirus complexed with neutral red after several rounds of viral replication (16–18). High concentrations of amines, i.e., 0.3 mM chloroquine or 70 mM NH_4Cl (31), have been used in some of these studies, in which poliovirus polymerase production was strongly inhibited even when the drugs were added 1 or 2 h after virus entry (31). Certainly, the lysosomotropic agents employed have a number of side effects that influence a wealth of enzymes and functions apart from raising the endosomal pH (13, 27). Thus, it is not surprising that during the long incubation times, during which several rounds of viral replication take place, there was inhibition of poliovirus, particularly if the drugs used were irreversible (18). Shorter incubation times, testing the inhibition of poliovirus by chloroquine or monensin during the uncoating period, led to the idea that poliovirus entry is pH independent (12).

Studies on the requirement of a low-pH step for entry of other members of the *Picornaviridae* family indicate that foot-and-mouth disease virus replication is inhibited by lysosomotropic compounds (2, 6, 7). However, a direct action of these agents in the initial steps of virus infection is still uncertain, because chloroquine was still active in the inhibition of foot-and-mouth disease virus replication even when added 2.5 h after infection (2), whereas monensin blocked the internalization of labeled rhinovirus particles in HeLa cells (22). It has been claimed that this ionophore might even increase the infection of cells by encephalomyocarditis (EMC) virus when low-pH medium is used during virus penetration (18).

Endosomes, like other organelles of the vesicular system, are acidified by the action of the vacuolar proton-ATPases (21, 26). These enzymes pump protons into the organelles at the expense of ATP hydrolysis in an electrogenic fashion (21, 26). The effect of specific inhibition of the vacuolar proton-ATPase on animal virus infectivity has not been examined. Only the action of the unspecific ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide on poliovirus has been assayed (17, 18). This compound was effective only when several rounds of viral replication occurred, but it had no effect on poliovirus entry when the amount of virus was increased (17, 18). The two major classes of inhibitors used raise the endosomal pH by accumulation of weak amines (NH_4Cl, chloroquine, and amantadine, etc.) or by dissipating the proton gradient of endosomes by ionophores (monensin and nigericin) (27). These two classes of compounds leave the endosomal proton-ATPase untouched, and in principle, this enzyme continues functioning even in their presence. Bafilomycin A1 (BFLA1) is a powerful and selective inhibitor of the vacuolar proton-ATPases that has recently been discovered (3, 21, 32). To elucidate the requirements of a low-pH step for poliovirus infectivity, we decided to test the effect this new antibiotic, and also those of the

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other two classes of inhibitors described above, on poliovirus entry. Our present results suggest that although poliovirus enters cells via an endosomal pathway, it does not require a low-pH step to infect cells.

MATERIALS AND METHODS

Cell lines, viruses, and media. Poliovirus type 1 (Mahoney strain) and EMC virus were propagated, grown, and titrated by plaque assay in human epithelioid carcinoma (HeLa) cells and mouse fibroblasts (L cells), respectively. Baby hamster kidney (BHK-21) cells were used for growth and titration of Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV; Indiana strain). Human rhinovirus (serotype 14) was kindly provided by M. G. Rossmann (Purdue University). BHK-21 and African green monkey kidney (CV2) cells were grown in Dulbecco modified Eagle medium supplemented with 8% fetal calf serum. HeLa and African green monkey kidney (Vero) cells were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum.

Inhibitors. BFLA1 was provided by K. Altendorf (University of Osnabrück, Osnabrück, Germany). α-Sarcin was a generous gift from D. M. Shuurmans (Department of Public Health; Lansing, Mich.). Monensin, chloroquine, and dansylcadaverine were purchased from Sigma Chemical Co., St. Louis, Mo.

Analysis of proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Cells grown in 24-well plates were infected at a multiplicity of infection (MOI) of 50 PFU per cell. After virus adsorption (min 30 postinfection), the cells were incubated in Dulbecco modified Eagle medium plus 2% calf serum. Protein labeling was performed with 20 μCi of [^{35}S]methionine per ml (1.45 Ci/mmol; Amersham International, Amersham, United Kingdom) in methionine-free medium. The radiolabeled cell monolayers were dissolved in sample buffer (62.5 mM Tris [pH 6.8], 2% SDS, 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue [as an indicator]). Samples were heated at 90°C for 5 min and electrophoresed on a 15% polyacrylamide gel overnight at 80 V. Fluorography was carried out in 1 M sodium salicylate. The gels were finally dried and exposed to Agfa X-ray films.

Measurement of radioactivity incorporated into trichloroacetic acid-precipitable material. The cells were incubated for 1 h with the virus and α-sarcin (50 μg/ml) in Dulbecco modified Eagle medium plus 2% calf serum, washed, and pulse-labeled for 1 h with 2 μCi of [^{35}S]methionine per ml in methionine- and serum-free medium. Radioactive medium was removed, and the cells were washed with phosphate-buffered saline, treated with 5% trichloroacetic acid, and washed twice with ethanol. The cell monolayer was allowed to dry before addition of 0.1 M NaOH–1% SDS. The samples were dissolved in liquid scintillation counting cocktail (Formula 989; Du Pont, Boston, Mass.), and radioactivity was quantitated in a liquid scintillation counter (1219 Rackbeta; LKB, Bromma, Sweden).

RESULTS

Effects of BFLA1 on entry of poliovirus. Comparison with enveloped RNA-containing viruses. To test the effect of BFLA1 on poliovirus entry, HeLa cells were treated with the compound in three different ways: (i) BFLA1 was present continuously, from virus addition until the cells were labeled with [^{35}S]methionine; (ii) the compound was present only during the first 20 min of infection, and then the excess virus and the inhibitor were removed; or (iii) BFLA1 was added 20 min after the virus and left throughout infection. Figure 1 shows that BFLA1 did not prevent infection of HeLa cells by poliovirus under any of the three conditions tested. In contrast, SFV and VSV were potently inhibited by BFLA1, but only when the compound was present during virus entry (i.e., conditions i and ii). Addition of BFLA1 20 min after the virus (condition iii) did not block SFV or VSV infection, indicating that this agent does not inhibit subsequent steps of viral infection that take place after the first 20 min of the replication cycle. We tested the inhibition of SFV attachment and internalization with radioactively labeled virions and found no inhibition by BFLA1 (results not shown).

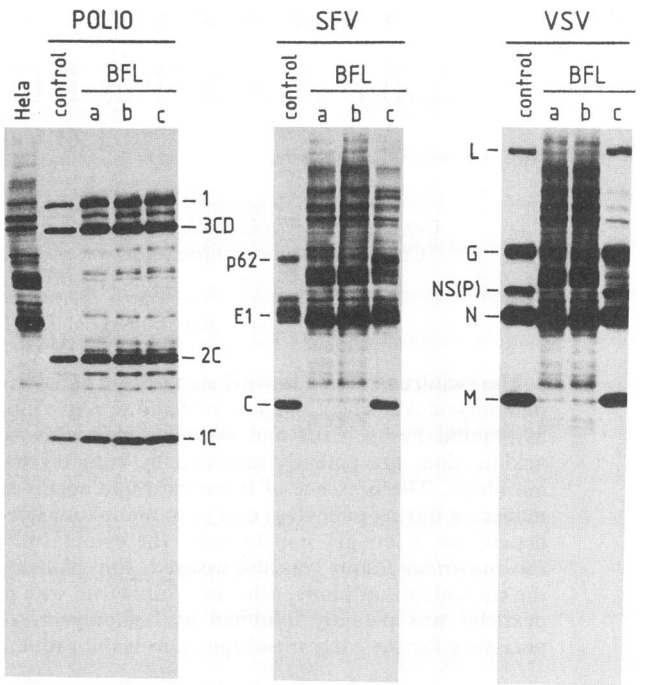

FIG. 1. Effects of BFLA1 added at different times on poliovirus (POLIO), SFV, and VSV. The MOI was 50 PFU per cell for the three viruses. Labeling of infected HeLa cells was carried out from 3.5 to 4.5 h postinfection, as described in Materials and Methods. Except in the control lanes, 1 μM BFLA1 was present from addition of the virus to the cells (0 h postinfection) until the labeling period (lanes a), during only the first 20 min of infection (lanes b), or from 20 min postinfection (lanes c) until the labeling period. Lanes: control, infected cells not treated with the compound; HeLa, protein synthesis in uninfected cells not treated with BFLA1.

There are two additional points in Fig. 1 that should be emphasized. One is that BFLA1 does not inhibit cellular translation, even after long incubation times (see lane a for both SFV and VSV). After 6 h of incubation at 1 μM BFLA1, cellular protein synthesis was 100% of the control value (data not shown). The second aspect is the very low concentrations of BFLA1 used, i.e., 1 μM, which is about 100-fold lower than the concentrations of chloroquine normally used to block SFV replication. The concentration dependence of the effect of BFLA1 on SFV and poliovirus replication is shown in Fig. 2. Concentrations of the antibiotic as low as 0.5 μM inhibited SFV replication, whereas an almost 10-fold higher concentration of BFLA1 (4 μM) had no effect on poliovirus.

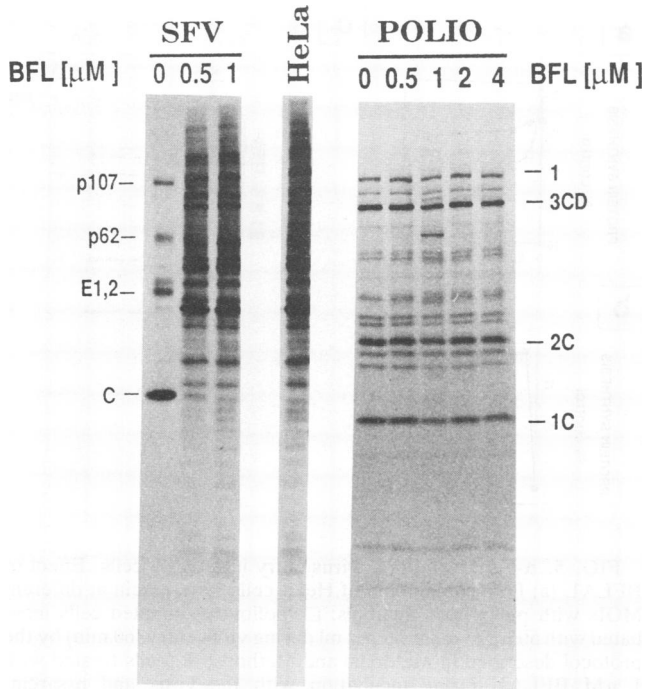

FIG. 2. Effects of different concentrations of BFLA1 on SFV and poliovirus (POLIO) infection of HeLa cells. Cell monolayers were pretreated with the indicated concentrations of BFLA1 15 min before infection with the virus (0 h postinfection; MOI, 50 PFU per cell). After 1 h of incubation with the virus and BFLA1, the monolayers were washed and incubated with fresh medium for 5 h postinfection. Labeling with [^{35}S]methionine was carried out as indicated in Materials and Methods. Proteins were labeled from 5 to 6 h postinfection with 15 μCi of [^{35}S]methionine per ml in methionine-free medium and analyzed by SDS-PAGE.

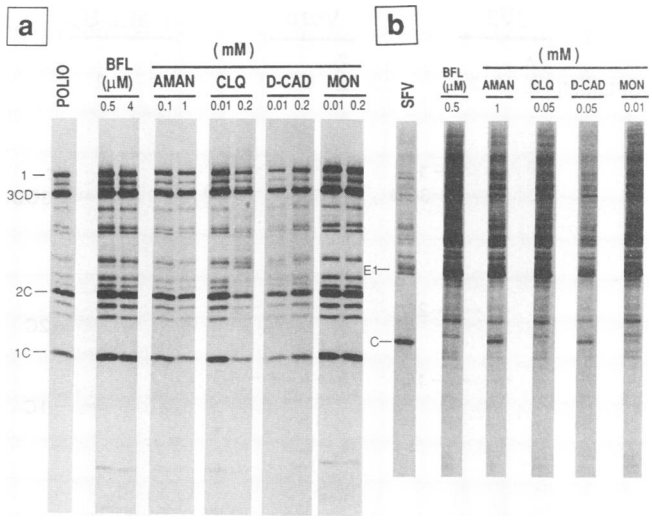

FIG. 3. Effects of several inhibitors on infection of HeLa cells by poliovirus (POLIO) (a) or SFV (b). Each compound was added to cells at the concentrations indicated at 15 min before virus infection and were treated as described in the legend to Fig. 2. Synthesis of late viral proteins was analyzed by SDS-PAGE as described in the legend to Fig. 2. Protein synthesis was measured in cells infected with poliovirus or SFV and treated with the following compounds: BFL, BFLA1; AMAN, amantadine; CLQ, chloroquine; D-CAD: dansylcadaverine; MON, monensin.

Comparison of BFLA1 and other lysosomotropically active compounds. Although BFLA1 potently blocked replication of the enveloped RNA-containing viruses tested, it was possible a priori that, for unknown reasons, this compound was active only on these viruses but not on poliovirus. It could be speculated that poliovirus enters cells in an endosomal subpopulation where the vacuolar proton-ATPase is not susceptible to BFLA1. To assay the effects of other agents known to raise the pH in endosomes, we tested the amines amantadine, chloroquine, and dansylcadaverine and the ionophore monensin against poliovirus. Amines are thought to accumulate in endosomes and other organelles of the vesicular system, where they become protonated and are thus less permeable, leading to an increase in the endosomal pH (27). On the other hand, monensin is an ionophore that exchanges Na^+/H^+ in favor of an H^+ gradient, thus dissipating the ΔpH created by the proton-ATPase pump (27). Since there is no net movement of charges by monensin, $\Delta\Psi$ is not affected by the ionophore. These compounds were assayed over a wide range of concentrations. Figure 3 shows the results obtained with the lowest and highest concentrations tested on poliovirus. None of these compounds blocked poliovirus replication. The highest concentrations of amantadine and chloroquine analyzed, and also dansylcadaverine, decrease the synthesis of poliovirus proteins, most likely because of the toxic effects that these agents have under those conditions (27). In no case was there even a slight increase of cellular protein synthesis that would be an indication that the entry of poliovirus was compromised by these compounds. As a control, the effects of these agents on SFV infection were also tested (Fig. 3). All of them had an inhibitory effect on SFV infection, although amantadine and dansylcadaverine were less effective than the other inhibitors at the concentrations tested (compare levels of viral peptide C). Therefore, entry of poliovirus is not affected by agents which increase the endosomal pH by three different mechanisms, i.e., (i) blockade of the vacuolar proton-ATPase with BFLA1, (ii) buffering of H^+ by weak amines, and (iii) dissipation of the ΔpH by monensin.

In previous studies, HeLa S3 cells were used to analyze the requirement for a low-pH step for poliovirus entry into cells (16–18). Therefore, we decided to test the effects of BFLA1 on poliovirus entry in different cell lines. Figure 4 shows that BFLA1 did not reduce poliovirus infection of HeLa S3, CV2, or Vero cells, suggesting that the uptake mechanisms are likely to be similar in all of these cell types.

Coentry of α-sarcin with poliovirus. Effect of BFLA1. Poliovirus is able to cointernalize and efficiently deliver protein toxins during entry into cells (1, 4). The exact mechanism by which this occurs is not fully understood, but virion uncoating is necessary for this process to occur (1). It has been speculated that delivery of protein toxins by animal viruses involves disruption of endosomes (5, 10), but other more selective mechanisms are possible. α-Sarcin had no effect on protein synthesis when added to uninfected HeLa cells, with or without BFLA1 (Fig. 5c), whereas in agreement with previous findings, the toxin efficiently blocked translation when added together with virion particles (Fig. 5a). This cointernalization of the protein toxin by poliovirus was potently blocked by BFLA1. These results suggest that an active vacuolar proton-ATPase is necessary for efficient delivery of α-sarcin to the cytoplasm. In addition, they constitute indirect evidence that poliovirus entry occurs through the endosomal pathway, because the coentry of

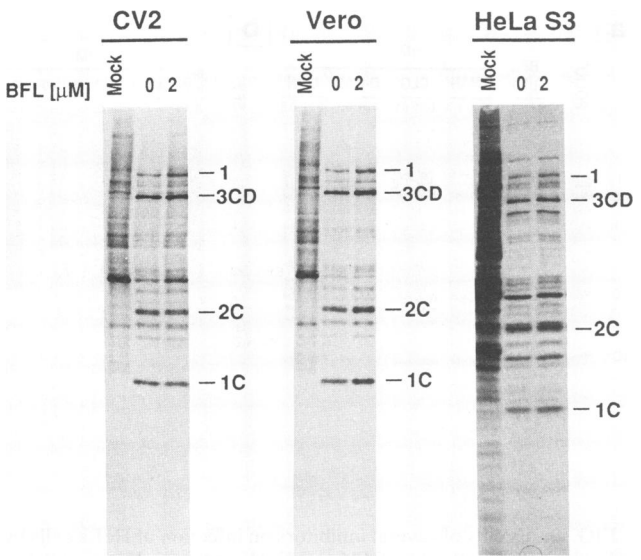

FIG. 4. Poliovirus infection of different cell lines. Effect of BFLA1. CV2, Vero, and HeLa S3 cells were infected with poliovirus at an MOI of 50 PFU per cell. The cells were pretreated (lanes 2) or not (lanes 0) at 15 min before infection with 2 μM BFLA1 as indicated and incubated in the presence of the compound and the virus for 1 h. At that time (1 h postinfection), the monolayer was washed and then incubated with fresh medium for 4 h. At 5 h postinfection, protein synthesis in the infected cells was determined as described in the legend to Fig. 2.

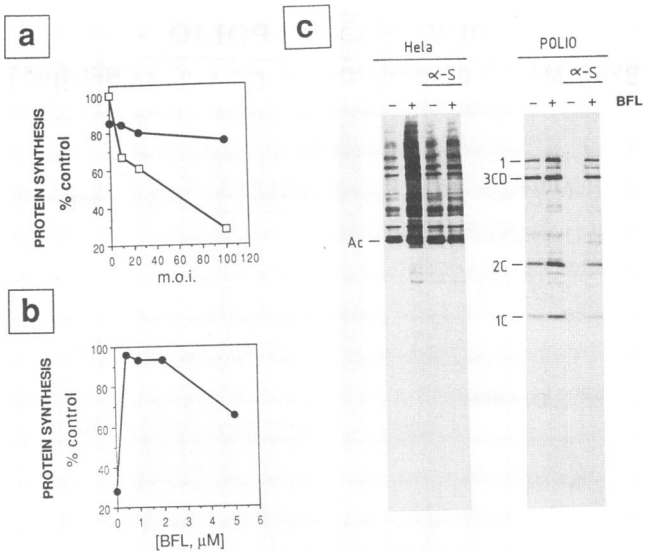

FIG. 5. α-Sarcin and poliovirus entry into HeLa cells. Effect of BFLA1. (a) Permeabilization of HeLa cells for α-sarcin at different MOIs with poliovirus. Symbols: □, poliovirus-infected cells incubated with 50 μg of α-sarcin per ml during virus entry (60 min) by the protocol described in Materials and Methods; ●, cells treated with 1 μM BFLA1 during incubation with the virus and α-sarcin. [^{35}S]methionine incorporation into mock-infected cells (6.0×10^5 cpm) was 100% of the control value. Infection with the virus or treatment with BFLA1 or α-sarcin in uninfected cells yielded values of over 85% of the control. (b) Effects of different concentrations of BFLA1 on the permeabilization of poliovirus-infected HeLa cells (MOI, 100 PFU per cell) for α-sarcin. BFLA1 was added to the cells 15 min before α-sarcin and virus infection. α-Sarcin entry was determined as inhibition of protein synthesis measured as described for panel a. (c) Electrophoretic analysis of [^{35}S]methionine-labeled proteins in cells infected with poliovirus (POLIO) in the presence of α-sarcin and BFLA1. Mock-infected or poliovirus-infected HeLa cells were incubated with or without 50 μg of α-sarcin (α-s) per ml with (+) or without (−) and 1 μM BFLA1 for 1 h. The cells were washed and incubated with fresh medium until labeling of proteins from 5 to 6 h postinfection as described in the legend to Fig. 2. Ac, cellular protein actin.

α-sarcin induced by poliovirus does not occur in the presence of an inhibitor of an endosomal enzyme. In fact, the coentry of alpha-sarcin induced by Sendai virus is not blocked at all by BFLA1, because this virus enters by direct fusion with the plasma membrane (14, 19) (results not shown). These results lead to the conclusion that most, if not all, the toxin delivered in the cytoplasm by poliovirus entry comes from endosomes, because poisoning of HeLa cells by α-sarcin was totally prevented by inhibition of the vacuolar proton-ATPase with BFLA1. For a model for poliovirus entry illustrating the coentry of α-sarcin and the site of action of the inhibitors, see Fig. 7.

Effect of BFLA1 on replication of other members of the *Picornaviridae* family. The *Picornaviridae* family is divided into four genera: the enteroviruses, (poliovirus), rhinoviruses, cardioviruses (EMC virus), and aftoviruses (foot-and-mouth disease virus). The enteroviruses and rhinoviruses are closely related by a number of characteristics, whereas the other two genera are more distant in evolutionary terms (24). We tested the action of BFLA1 against EMC virus and rhinovirus and compared its effect against the enveloped RNA-containing virus VSV (Fig. 6). Infection of HeLa cells by EMC virus did not seem to be blocked by the macrolide antibiotic (e.g., compare viral peptide alpha with and without BFLA1), as occurs with poliovirus, whereas rhinovirus or VSV infection was inhibited by BFLA1. Remarkably, treatment of EMC virus-infected cells with BFLA1 interfered with the inhibition of host translation by the virus. We did not investigate this effect. Figure 6 also shows that α-sarcin efficiently entered the cytoplasm in the presence of the three virus particles. BFLA1 efficiently blocked this coentry mediated by any of the three viruses. Although more detailed analyses with each of these viruses are needed for a more firm conclusion regarding their mode of entry, our results certainly suggest that only rhinovirus requires a low-pH step for efficient entry into cells.

DISCUSSION

The mechanisms used by animal viruses to cross cellular membranes and deliver their genomes into the cytoplasm to start infection is a matter of intensive research (14, 19). Animal viruses containing lipid membranes accomplish this task by fusion of the external envelope, either with the plasma membrane, as occurs with Sendai virus, herpesvirus or vaccinia virus, or with the endosomal membrane, as with SFV, VSV, or influenza virus (14, 19). In the latter case, fusion is promoted by conformational modifications of the viral glycoproteins after receptor binding and acidification of endosomes (28, 29). Prevention of this acidification by lysosomotropic agents leads to inhibition of infection by SFV, VSV, or influenza virus (14). Models to account for the entry of nonenveloped viruses, such as poliovirus or adenovirus, are less well developed. We support the idea that attachment of poliovirus and internalization in endosomes leads to conformational changes in the virion particle, particularly in VP1 (11), which can interact with the membrane

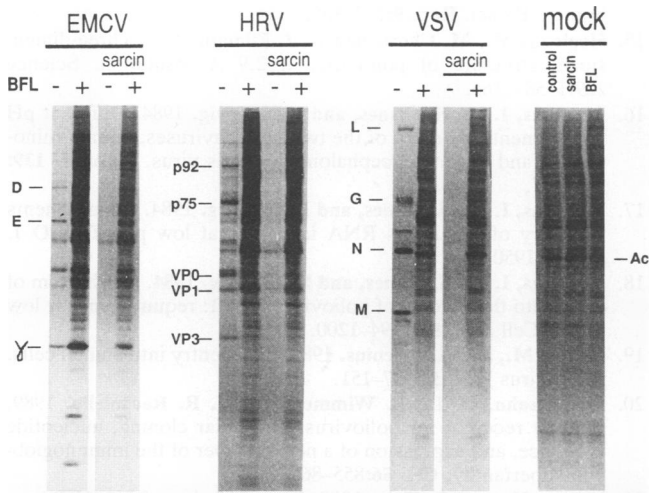

FIG. 6. Action of BFLA1 on the entry of several picornaviruses into HeLa cells. Effect on α-sarcin coentry. Monolayers of HeLa cells (Ohio strain) were mock infected (mock) or infected with human rhinovirus serotype 14 (HRV) at an MOI of 100 PFU per cell or with EMC virus or VSV at an MOI of 25 PFU per cell. Lanes: +, treatment of cells with 2 μM BFLA1 (BFL) as described in the legend to Fig. 2; −, cells not treated with BFLA1. As indicated (sarcin), 50 μg of α-sarcin per ml was added to the medium at the same time as the virus. After incubation for 1 h, the cells were washed and incubated with fresh medium. [^{35}S]methionine-labeled viral proteins were analyzed by PAGE. Samples containing equal amounts of total protein were loaded in all of the lanes. The cells were labeled at 5.5 h postinfection for VSV and EMC virus and at 7.5 h postinfection for rhinovirus. Ac, cellular protein actin.

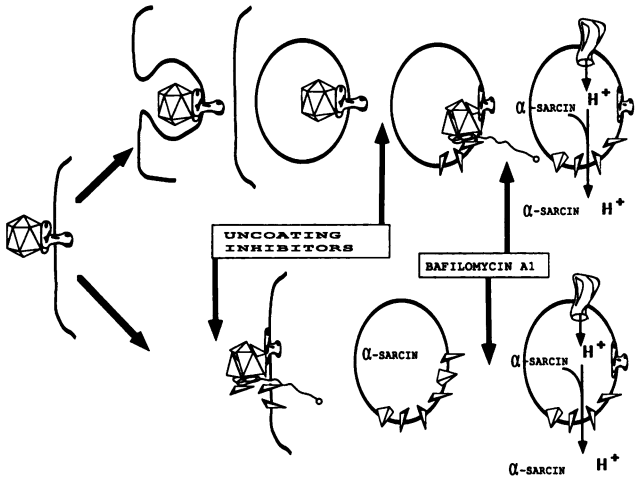

FIG. 7. Schematic model of two potential mechanisms of poliovirus entry into cells. Poliovirus internalization through endosomes or direct crossing of the plasma membrane would lead to insertion of structural proteins into the membrane. These proteins, together with the proton gradient between endosomes and the cytoplasm generated by the H^+-ATPase, would translocate the toxin α-sarcin to the cell interior.

and aid the insertion of myristoylated protein VP4 into the membrane (11). This insertion not only destabilizes the particle but may also allow interaction of some moieties of other viral proteins with the membrane (11). Such an insertion could open a pore in the membrane that allows passage of the genome to the cytoplasm. In this model, proteins of the virus particle act similarly to the B chains of some protein toxins (30). In fact, we have reported that animal virions, including poliovirus particles, are able to replace the B chains of protein toxins and promote the entry of the A chain or effector moiety of the molecule (5, 9, 23). We recently showed that for this co-entry of toxins with poliovirus, uncoating of the particle is required, in agreement with the model described above (1). Our present findings on the involvement of the vacuolar proton ATPase in this phenomenon are of interest in understanding not only the entry of nonenveloped viruses but also the coentry of protein toxins inside cells. Thus, we cannot dissociate poliovirus uncoating from the coentry mechanisms, arguing against previous models that suggested the rupture of endosomes during virus entry (1, 4, 5, 10). Therefore, poliovirus enters from endosomes into the cytoplasm and maintains the endosomal membrane intact, because this rupture would lead to leakage of the endosomal content, including α-sarcin, into the cytoplasm. Thus, after or during poliovirus entry, the modification introduced in the cellular membrane is used by α-sarcin to traverse the membrane. Our present results favor the view that the force that pushes α-sarcin through the membrane may be the proton gradient generated by the vacuolar proton-ATPase. Therefore, instead of a simple mechanistic model of creation of a pore through which molecules pass, other forces (proton motive, electrical) may be involved in protein translocation. In conclusion, the virus capsid protein would be able to couple these forces to the translocation of the proteins present in endosomes.

Two major questions must be answered to explain the mechanism of poliovirus entry into cells. One is whether the virus is internalized through endosomes, and the second is whether endosomes need to be acidified for the virus to enter cells. We believe that the second question has been clarified by the present work. Since none of the agents known to increase the endosomal pH prevent poliovirus infection when they are present during entry, we conclude that a low-pH step is not required for poliovirus to infect cells. We emphasize the unique mode of action of one of the inhibitors used in our work, i.e., BFLA1. Since this agent selectively blocks the vacuolar proton-ATPases (3, 21, 32) and shows activity at micromolar concentrations, we suggest that it should replace other compounds widely employed to analyze the low-pH requirements for animal virus entry (14, 19).

However, our results do not provide a definitive answer to the first question raised above, i.e., whether poliovirus gains access to the cytoplasm via endosomes. We believe that the two possible mechanisms of poliovirus entry, i.e., direct entry through the plasma membrane (8) and internalization in endosomes (31), are still open. It is even possible that poliovirus uses both of them. Although poliovirus permeabilizes cells for α-sarcin and this event occurs in endosomes, it is possible a priori that the virus has already released its genome into the cytoplasm after binding to the cell surface receptor and the virion proteins present in the membrane and internalized in endosomes are the ones that permeabilize the membrane for α-sarcin once the ΔpH has been created (model in Fig. 7). Alternatively, it is possible that the poliovirus particle can traverse the cell membrane only during the internalization process. Recent electron microscopic evidence illustrating entry of poliovirus into cells favors the second mechanism (31). Further experiments are needed to clarify this issue and give us a better idea of how the genome of a nonenveloped animal virus is able to cross a lipid membrane to start infection.

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