Involvement of the vacuolar H+-ATPase in animal virus entry

Luis Pérez and Luis Carrasco*

Centro de Biología Molecular 'Severo Ochoa' (CSIC-AUM), Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain

Semliki Forest virus (SFV) enters cells by receptor-mediated endocytosis, followed by acidification of endosomes by the action of the vacuolar H+-ATPase. Fusion of the viral and the endosomal membrane delivers the viral genome to the cytoplasm. Direct blockade of the vacuolar H+-ATPase by the selective inhibitor bafilomycin A1 (BFLA1) prevented the infection of cells by SFV, if the compound was present during the first minutes of infection. Attachment and penetration of virus particles were not the targets of the antibiotic. BFLA1 and the ionophore monensin potently blocked SFV infection even at low pH, indicating that acidic pH is not sufficient for SFV to deliver its genome to the cytoplasm, but the proper functioning of the H+-ATPase pump is necessary. Other enveloped RNA-containing viruses, such as vesicular stomatitis virus or influenza virus were also blocked by BFLA1, whereas no effect was observed with Sendai virus, which enters into cells by direct fusion with the plasma membrane. Enveloped DNA-containing viruses, such as herpesviruses and vaccinia virus, infected the cells even when the vacuolar H+-ATPase was inhibited by BFLA1; similar behaviour was observed with poliovirus and adenovirus. Animal virus particles promote the internalization of proteins and other macromolecules during entry. BFLA1 blocked co-entry of the toxin α-sarcin when induced by SFV, but not when induced by Sendai virus. The inhibition of the enzyme responsible for acidification of endosomes by means of the potent inhibitor BFLA1 constitutes a selective and powerful tool to analyse the low-pH dependent mechanism(s) during virus entry and will aid in understanding the mechanisms and routes of entry of animal viruses into cells.

Introduction

The infection of mammalian cells by viruses begins with the recognition and attachment of viral particles to specific receptors located at the cell surface (Dales, 1973; Dillmock, 1982). This is followed by the delivery of the viral genome to the cell interior in a process that involves the crossing of a cellular lipid membrane by the viral nucleic acid (Hoekstra & Kok, 1989). Enveloped viruses accomplish the delivery of their genomes into the cell by fusion of their lipid envelope with a cellular membrane (White, 1990; Lamb, 1993), whereas viruses devoid of lipid envelopes traverse the cell membrane by mechanisms much less understood. Viruses that belong to the Paramyxoviridae family, such as Sendai virus, directly fuse their lipid membrane with the plasma membrane of the cell leaving their nucleocapsid free in the cytoplasm (Morrison & Portner, 1991). These viruses contain in their envelope fusogenic proteins that, upon binding of the virus to its receptor, induce fusion of the viral and the cellular membranes (Morrison & Portner, 1991; Lamb, 1993). A similar mechanism could also occur with more complex viral particles that contain DNA as their genetic material. Thus, members of the Poxviridae and Herpesviridae families are thought to fuse their external lipid envelopes directly with the plasma membrane, liberating the genome and other capsid envelopes into the cell interior (Wittels & Spear, 1991; Fuller & Lee, 1992; Doms et al., 1990).

A different route of entry, receptor-mediated endocytosis, is followed by many other animal viruses (Hoekstra & Kok, 1989). These viruses capitalize on the continuous cellular process of endocytosis for internalization and follow the same route of entry as other macromolecules and physiological effectors, in order to obtain access to the cell interior (Wileman et al., 1985). Studies with members of the Togaviridae and Myxoviridae groups indicate that once the virus particles are bound to their receptors, they are internalized in coated vesicles that soon mature to endosomes (Hoekstra & Kok, 1989; Marsh & Helenius, 1989). Endosomes become acidified by the action of H+-ATPase pumps (Mellman et al., 1986; Schneider, 1987; Nelson & Taiz, 1989). This acidification, in turn, triggers conformational changes in the fusogenic viral glycoprotein(s) located in the virus envelope (White, 1990; Stegmann et al., 1989;
Lamb, 1993). These conformational changes lead to the exposure of a hidden hydrophobic region in the glycoprotein permitting its insertion in the endosomal membrane. In this way, fusion between the viral and cellular membranes ensues, liberating the free viral nucleocapsid into the cytoplasm (Stegmann et al., 1989; White, 1990). The extent to which receptor binding, alone or in combination with low pH, contributes to the conformational changes induced in the viral glycoproteins is still a matter of debate (Edwards et al., 1983; Kielian & Helenius, 1985; Edwards & Brown, 1991; Meyer et al., 1992; Haywood, 1994). In addition, it has not been demonstrated yet if an H + gradient, rather than a low pH, is the actual requirement for virus entry (Marsh & Helenius, 1989). Central to this model is the finding that compounds known to raise the pH in lysosomes and other vesicles of the vacuolar system block virus infection (Miller & Lenard, 1981; Cassell et al., 1984; Seglen, 1983; Helenius et al., 1982). However, caution should be taken in the interpretation of some of the data obtained with these lysosomotropic agents because of the side-effects that these compounds have on other cellular and viral functions (Seglen, 1983; Cassell et al., 1984).

Models that account for the entry of animal virus particles devoid of a lipid envelope are more scarce (Hoekstra & Kok, 1989; Marsh & Helenius, 1989). In principle, these viruses could pass directly through the plasma membrane after receptor binding. Some evidence suggests this mode of entry for certain viruses, such as adenoviruses (Brown & Burlingham, 1973), poliovirus (Dunnebacke et al., 1969), rotaviruses (Kaljot et al., 1988) and reovirus subviral particles (Borsa et al., 1979). Alternatively, the virus particles can be internalized by receptor-mediated endocytosis and then cross the endosomal membrane. In both cases, the viral genome has to pass across a lipid membrane, aided by the virion proteins. But it is still debated whether some of these viruses need a low-pH step that would trigger the crossing of the endosomal membrane, or if they enter cells by a pH-independent mechanism (Varga et al., 1991; Svensson, 1985; Brown & Burlingham, 1973; Sturzenbecker et al., 1987; Gromeier & Wetz, 1990; Madshus et al., 1984b).

One approach to understanding the mechanism by which animal viruses traverse the cellular membrane is to investigate the co-entry of protein toxins and other macromolecules together with viral particles in molecular terms (Carrasco et al., 1989, 1993; Fernández-Puentes & Carrasco, 1980). Cells do not possess receptors for these toxins and the viral particle replaces the function of these receptors. Thus, both enveloped or naked virus particles efficiently permeabilize mammalian cells to a number of proteins and compounds unable to penetrate cells under normal conditions (Fernández-Puentes & Carrasco, 1980; Carrasco & Esteban, 1982; Carrasco, 1981; Almela et al., 1991; Otero & Carrasco, 1987; Wagner et al., 1992; Cotten et al., 1992). This co-entry mechanism indicates that animal viruses permeabilize cellular membranes during the process of entry not only to their genomes, but also to the surrounding macromolecules (Fernández-Puentes & Carrasco, 1980; González & Carrasco, 1987; FitzGerald et al., 1983; Wagner et al., 1992; Cotten et al., 1992). Receptor binding and proper virion uncoating are necessary for the co-entry of proteins to take place (Almela et al., 1991), but the exact mechanism by which these toxins cross the endosomal membrane aided by virus particles remains unknown.

Methods

Cells, viruses and media. Baby hamster kidney (BHK-21) cells were used for growth and titration of Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV, Indiana strain). BHK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum. Human epithelial carcinoma cells (HeLa) and African green monkey kidney cells (Vero) were cultured in DMEM supplemented with 10% newborn calf serum.

Analysis of proteins by SDS–PAGE. Cells grown in 24-well plates were infected at a multiplicity of infection (m.o.i.) of 50 p.f.u./cell. After virus attachment (time zero of infection) the cells were incubated in DMEM plus 2% calf serum. Protein labelling was done with 20 μCi/ml [35S]methionine (145 Ci/mmol; Amersham) in methionine-free medium. The radiolabelled cell monolayers were dissolved in sample buffer (62.5 Tris-HCl pH 6.8), 2% SDS, 0.1 M dithiothreitol, 17% glycerol and 0.024% bromophenol blue as indicator). Samples were heated at 90 °C for 5 min, applied to a 15% polyacrylamide gel and run overnight at 80 V. Fluorography was carried out in I M-sodium salicylate. The gels were finally dried and exposed to an X-ray film (Agfa).

Measurement of radioactivity incorporated into acid-precipitable material. Labelling of cells was done by incubation in methionine-free medium containing 2 μCi/ml [35S]methionine for 1 h. Radioactive medium was removed and the cells were washed with phosphate-buffered saline (PBS), treated with 5% trichloroacetic acid and washed twice with ethanol. The cell monolayer was allowed to dry before treatment with 0.1 M NaOH, 1% SDS. The samples were redissolved in LSC cocktail (Formula 989, Du Pont) and the radioactivity quantified in a liquid scintillation counter (1219 Rackbeta, LKB).

Preparation of [35S]methionine-labelled SFV. Monolayers of BHK cells cultured in 100 mm plates were infected with SFV at an m.o.i. of 5 p.f.u./cell. At 3 h post-infection the medium was replaced by methionine-free medium containing 30 μCi/ml [35S]methionine. After a 12 to 14 h incubation period, the medium and the cells were collected. The cells were lysed by freezing and thawing and mixed with the medium before distributing in 15 ml portions. A 5 ml cushion of 20% sucrose was underlayered beneath each sample. Viruses were pelleted by centrifugation at 38000 r.p.m. for 2 h at 4 °C in a T-865 rotor. The supernatant was removed and the pellet was redisolved in DMEM. Small aliquots were taken to determine the radioactivity in TCA-precipitable material and the rest of the sample was stored frozen at -70 °C.

Virus attachment assay. [35S]Methionine-labelled SFV (2 × 10⁵ c.p.m.) in 2% calf serum medium was added to 4 °C pre-cooled cell
monolayers. After different incubation periods (4 °C) the medium was removed and the cells were washed twice with PBS and harvested in 1 ml PBS. The radioactivity of each sample was determined by filtration of TCA-precipitable material through glassfibre filters (GF/C, Whatman).

**Virus entry assay.** The cells were incubated with [35S]methionine-labelled SFV at 37 °C for different periods of time. After removing the medium with the virus, the cells were placed on ice, washed three times with PBS at 4 °C and incubated for 15 min at 4 °C in PBS containing 50 mg/ml proteinase K (Boehringer). The cells were then collected, centrifuged (5 min at 2000 r.p.m.) and washed twice. Radioactivity in TCA-precipitable material was determined by filtration of the redissolved samples through GF/C filters.

### Results

**Bafilomycin A1 (BLFA1) inhibits entry of SFV into HeLa cells, but not attachment**

To assay the effectiveness of the vacuolar H⁺-ATPase inhibitor BLFA1 in the inhibition of attachment and/or entry of SFV we first tested this drug at different concentrations. The antibiotic was only present during the first hour of infection and was added 10 min before the virus. Virus entry and infectivity were assayed by measuring the proteins synthesized in the infected cells after 5 h of infection. At this time of infection host translation has been shut-down and the infected cells mostly synthesize SFV late viral proteins, i.e. p62, E1, E2 and C. Fig. 1 shows that BFLA1 is very effective in the blockade of SFV, since BLFA1 concentrations as low as 0.5 μM are sufficient to prevent infection by SFV, as determined by the lack of viral proteins synthesized in the cells. Early during infection of cells by animal viruses, the virus first attaches to receptors (Dales, 1973; Dimmock, 1982); this event is followed by internalization in the vacuolar system (Hoekstra & Kok, 1989). To assay virus attachment and internalization, radioactively labelled SFV was obtained and purified. BFLA1 (1 μM) did not interfere with virus attachment (Fig. 1b) indicating that an active H⁺-ATPase pump is not needed for this event to occur. A partial inhibition of virus internalization (30% of control) was observed after 1 h of incubation (Fig. 1c), perhaps as a consequence of the inhibitory effect that BLFA1 has on membrane and/or receptor recycling (Zeuzem et al., 1992). This partial inhibition of virus internalization does not account for the blockade of virus replication observed by measuring the synthesis of SFV proteins.
of proteins at late times of infection (Fig. 1a). BFLA1 effectively blocked SFV infection if added with the virus, but did not inhibit infection if added 30 min after the virus (results not shown).

Weak bases and ionophores act on animal virus infection by mechanisms different from BFLA1 (Seglen, 1983). Therefore, we considered it interesting to compare the action of these agents with that of BFLA1. Fig. 2 shows that the three weak bases used, amantadine, chloroquine and dansylcadaverine, inhibited the infection of cells by SFV. The compound that showed an inhibitory pattern most similar to BFLA1 was the ionophore monensin. At a concentration of 10 μM, monensin blocked virus entry with no significant effect on cellular protein synthesis. The mode of action of BFLA1 and monensin on SFV may differ. Thus, treatment of cells with monensin will leave the H+-ATPase functioning, whereas BFLA1 blocks the action of this enzyme.

**Influence of pH on the inhibition of SFV by BFLA1**

At normal concentrations of monovalent cations, lowering the pH of the culture medium induces direct fusion of SFV with the plasma membrane (Kielland & Helenius, 1985). Lysosomotropic agents do not block SFV entry...
Fig. 3. Effect of the pH of the medium on SFV infection. Effect of BFLA1. Infection of HeLa or BHK cells with 2 μM-BFLA1 or 100 μM-monensin was done as described for Fig. 2. During virus entry (1 h period) the cells were incubated in NaHCO₃-free medium supplemented with 20 mM-MES (pH 5 and 6) or 20 mM-HEPES (pH 7.2, 8, and 8.9). After washing off excess virus, fresh medium (normal DMEM supplemented with 2% calf serum) was added. At 5 h post-infection proteins were labelled as described in Methods and then analysed by SDS-PAGE. Mock, cells not infected with SFV. BFLA1, cells infected with SFV and treated with BFLA1. The pH during the first hour of virus entry is indicated.

<table>
<thead>
<tr>
<th></th>
<th>HeLa Cells</th>
<th>BHK Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BFLA1</td>
<td>Monensin</td>
</tr>
<tr>
<td>pH . . .</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Table: HeLa and BHK Cells under different pH conditions

- **Mock** (no infection)
- **SFV** (infection with SFV)
- **BFLA1** (treatment with BFLA1)
- **Monensin** (treatment with Monensin)

- **BFLA1 (2 μM)**
- **pH** 7.2

- **SFV**
- **pH** 7.2
Fig. 4. Effect of BFLA1 on SFV, VSV, influenza virus and Sendai virus infection. The cell lines indicated were mock-infected (C) or infected with the virus indicated. Treatment with BFLA1 at the concentrations indicated or with other compounds (MDCK cells only) was as in Fig. 3. The m.o.i. for SFV and VSV was 50 p.f.u./cell. Vero cells were infected with Sendai virus at 50 p.f.u./cell. MDCK cells were infected with influenza virus at 10 p.f.u./cell. For MDCK cells treatments are indicated as follows. C, control uninfected cells; influenza, cells infected with influenza virus; -, infected cells not treated with any compound; B, 1 μM-BFLA1; C, 0.1 mM-chloroquine; M, 0.1 mM-monensin. Labelling of cells with [35S]methionine was carried out from 5 to 6 h post-infection in the cases of SFV and VSV infections, and from 7 to 8 h post-infection in the case of the influenza- or Sendai virus-infected cells. The migration of viral proteins is indicated as follows. SFV proteins: p107; p62; E2; E1 and C. VSV proteins: L; G; NS(P); N and M. Influenza virus proteins: NP; M1 and NS1. Sendai virus proteins: P; HN; F; NP and M.
under these acidic conditions (White et al., 1980). The effect of low pH in the medium during SFV entry is shown in Fig. 3. None of the media assayed blocked SFV infection, probably because the H^+-ATPase pump is functional in all conditions tested. Surprisingly, BFLA1 prevented SFV infection, even when the culture medium was acidic (5.0 or 6.0) (Fig. 3a). To further test if SFV entry can be blocked by inhibitors of endosome function other than BFLA1, we analysed the action of monensin on SFV entry at low pH. Fig. 3(b) indicates that this ionophore prevents infection of cells when present during the early steps of SFV infection. We interpret these results as an indication that simple acidification of the medium is not sufficient to promote the entry of an infectious SFV genome into the cell, but the proper functioning of endosomes is a key factor for virus entry.

These results clearly agree with the findings that the simple presence of low pH is not sufficient to promote the delivery of SFV into the cytoplasm, but that other factors including membrane potential may be necessary (Helenius et al., 1985). Note that SFV glycoprotein synthesis is more prominent in BHK cells than in HeLa cells.

**Effect of BFLA1 on different animal viruses**

To compare the effect of BFLA1 on other enveloped animal viruses, we tested the activity of BFLA1 on BHK and Vero cells infected with SFV or VSV as well as the effect of this antibiotic on MDCK cells infected with influenza virus. The results shown in Fig. 4 indicate that the three enveloped RNA-containing animal viruses

---

**Fig. 5. Action of BFLA1, chloroquine and monensin on the entry of several animal viruses.** HeLa cells not treated (−) or treated with 1 μM-BFLA1 (B), 0.1 mM chloroquine (C) or 50 mM-monensin (M) were mock-infected (mock) or infected with the virus indicated. Treatment with the compounds was as in Fig. 3. The m.o.i. with poliovirus (PV) was 50 p.f.u./cell; with herpes simplex virus type 1 (HSV-1) and adenovirus (AdV) 25 p.f.u./cell; and 5 p.f.u./cell in the case of vaccinia virus (VV). Medium containing [35S]methionine (15 μCi/ml) was added to the cells at 5 h post-infection with PV and VV; at 16 h post-infection with HSV-1; or at 22 h post-infection (AdV). Protein synthesis was analysed as described in Methods.
Members of the *Paramyxoviridae* family, particularly Sendai virus, enter cells by direct fusion of the viral envelope with the plasma membrane (Hoekstra & Kok, 1989). However, this mechanism is not true for all members of the *Paramyxoviridae* family (Compans et al., 1964; Lamb, 1993). Sensitivity of Sendai virus to weak amines has been reported (Skehel *et al.*, 1978). The inhibition of Sendai virus by lysosomotropic agents may be due to the side-effects that these compounds have on cellular metabolism (Seglen, 1983). Thus, it was of interest to test the action of BFLA1 on Sendai virus. Fig. 4 shows that Sendai virus infection is not blocked by BFLA1. These results reinforce the idea that direct fusion of Sendai virus with the plasma membrane leads to an infectious cycle. In addition, the results indicate that BFLA1 is a powerful tool with which to distinguish between viruses that enter directly from the plasma membrane and those that use the endosomal route, followed by acidification. It must be pointed out that the lack of effect of BFLA1 indicates that the virus does not need a low pH to enter the cell, either directly by fusion with the plasma membrane or after internalization in endosomes.

Perhaps the most important evidence to indicate that a virus requires a low pH for entry and delivery of its genome into the cell comes from the inhibition of virus entry by weak bases (Marsh & Helenius, 1989). Therefore, we tested the effect of BFLA1 on other animal viruses and compared the action of this antibiotic with chloroquinone, one of the most commonly used amines that blocks virus infection, and with the ionophore monensin (Miller & Lenard, 1981; Coombs *et al.*, 1981; Helenius *et al.*, 1982; Cassell *et al.*, 1984). We tested the...
effect of BFLA1, chloroquine and monensin on two DNA-containing viruses that possess lipid envelopes, i.e. vaccinia virus and herpes simplex virus type 1. The results in Fig. 5 indicate that BFLA1 had no effect on either of these viruses, thus reinforcing the idea that they do not require a low-pH step for entry and can gain access to the cytoplasm by direct fusion with the plasma membrane (Doms et al., 1990; Wittels & Spear, 1991). Unlike BFLA1, however, chloroquine and monensin clearly affect the expression of proteins in vaccinia and herpesvirus-infected cells; perhaps these drugs exert side-effects on the replication cycle of the viruses. In conclusion, BFLA1 did not prevent the infection of vaccinia virus or HSV1, and the effect of this antibiotic is clear-cut as compared to other classical inhibitors of virus entry.

Finally, we analysed the effects of BFLA1 on two animal viruses, poliovirus and adenovirus, that do not possess lipid envelopes. There has been some controversy about the requirement of low pH for poliovirus entry (Gromeier & Wetz, 1990; Madshus et al., 1984a). Indirect studies based on the u.v.-sensitivity of dye-treated virions and the action of weak bases and monensin after prolonged incubation times (18 to 40 h) were taken as evidence that poliovirus required a low pH during entry for infectivity (Madshus et al., 1984a, b). These results have not been confirmed by others (Gromeier & Wetz, 1990; Pérez & Carrasco, 1993). BFLA1 had no effect on either poliovirus or adenovirus infection under conditions that prevented SFV entry (Fig. 5).

There are reports that adenoviruses enter cells directly through the plasma membrane (Brown & Burlingham, 1973) or by receptor-mediated endocytosis (Svensson & Persson, 1984; Svensson, 1985; Varga et al., 1991). Only high concentrations of NH$_4$Cl (30 mm) partially reduced (40%) infection by adenovirus as measured by hexon formation (Varga et al., 1991). The inhibition was due to a blockade of endocytosis itself because most virions remained attached to the cell surface (Varga et al., 1991). Our present results with BFLA1 suggest that the acidification of endosomes by the H$^+$/ATPase is not required for the efficient infection of HeLa cells by adenovirus.

Requirement of the vacuolar H$^+$/ATPase pumps for the co-entry of α-sarcin

During the entry of animal viruses into cells, concomitant co-entry of protein toxins and other macromolecules into the cytoplasm can occur (Carrasco et al., 1989, 1993). Thus, protein toxins not only enter in endosomes, but also traverse the endosomal membrane mediated by animal virus particles (Fernández-Puentes & Carrasco, 1980). Although the molecular basis of the so-called early membrane permeabilization by animal viruses is not yet fully understood, virus uncoating is required for the viral particle to permeabilize the endosome (Almela et al., 1991). To test the extent to which functional H$^+$/ATPase is required for the early permeabilization to occur we measured the uptake of α-sarcin mediated by SFV (Fig. 6). Uptake was assayed by the ability of α-sarcin to inhibit protein synthesis only after internalization into the cytosol (Fernández-Puentes & Carrasco, 1980). Fig. 6 shows the dependence of α-sarcin entry on the m.o.i. At a m.o.i. of 100 the entry of the toxin during the initial minutes of infection blocks protein synthesis more than 80%. The co-entry of α-sarcin is strongly inhibited by BFLA1 (Fig. 6), indicating that acidification of the endosome is indeed required for its permeabilization by animal virus particles. Fig. 6c indicates that incubation of α-sarcin with HeLa cells in the absence of virus particles does not affect translation, as analysed by separation of labelled proteins by PAGE, whereas α-sarcin readily enters cells and powerfully blocks protein synthesis in the presence of SFV. The entry of α-sarcin as measured by this assay is hampered by BFLA1.

Remarkably, the fusion of Sendai virus with the cellular membrane efficiently permeabilizes the cell to α-sarcin (Fig. 7) perhaps suggesting that passage through endosomes is not required for the early membrane permeabilization to occur. Clearly, BFLA1 had no effect at all on the permeabilization of the cell to α-sarcin induced by Sendai virus unlike SFV. These results suggest that the release of viral genomes to the cytosol
by enveloped RNA viruses and the co-entry of toxins into the cytoplasm are coupled phenomena.

Discussion

Three major classes of H+-ATPases exist in mammalian cells: (a) the mitochondrial F-class ATPases; (b) the plasma membrane H+-ATPases of the P-class; and (c) the V-class of vacuolar H+-ATPases (Schneider, 1987; Nelson & Taiz, 1989). Each class is distinguished by several parameters including their sensitivity to inhibitors (Nelson & Taiz, 1989). BFLA1 is a highly selective inhibitor of the V-class H+-ATPases, blocking the acidification of endosomes and disrupting the H+ gradients that exist in vesicles of the vacuolar system (Yoshimori et al., 1991; Bowman et al., 1988). BFLA1 is a uniquely useful compound for assaying the roles that acidification of endosomes and the action of the vacuolar H+-ATPase play in the entry of animal viruses. Although agents such as weak amines or ionophores disrupt the H+ gradient of endosomes (Maxfield, 1982), these agents should leave the H+ pump functioning. On the other hand, the presence of an acidic pH in the culture medium does not inhibit the internalization of viruses through the endosomal pathway and the vacuolar H+-ATPase can function in vesicles even when the culture medium is acid. Therefore, even though fusion of SFV with the plasma membrane takes place under acidic conditions (White et al., 1980), it is not known whether direct uptake through the plasma membrane constitutes the infectious route for these viruses under such conditions (Hoekstra & Kok, 1989). Alternatively, only those virions that enter through endosomes even in acidic media may give rise to an infectious cycle. Addition of lysosomotropic bases does not block the infection of SFV at low pH (White et al., 1980), in agreement with the idea that these agents become protonated at low pH in the external medium and are thus unable to cross the cell membrane and accumulate in endosomes (Seglen, 1983). The fact that BFLA1 blocks SFV infection even during exposure to low pH demonstrates that low pH is not the only requirement for virus-induced membrane fusion and efficient delivery of the nucleocapsid to the cytosol. These results agree with other findings indicating that, although SFV binds to cell receptors at low pH, it cannot infect cells when the membrane potential is modified (Helenius et al., 1985). Recent experiments have revealed the conformational changes of Sindbis virus glycoproteins E1 and E2 that take places when the virus binds to the cell surface at neutral pH (Flynn et al., 1990; Meyer et al., 1992). These conformational changes in virion structure are similar, but not exactly the same, as the changes observed after incubation of SFV at 51 °C, low pH, or treatment with dithiothreitol (Meyer et al., 1992). It is possible that low pH is required for SFV to induce membrane fusion, but other factors, such as receptor attachment together with the functioning of the vacuolar H+-ATPase to create a pH gradient are also necessary.

The rapid internalization of protein toxins from the culture medium into the cytoplasm is a property of most animal virions tested (Carrasco et al., 1989, 1993; Carrasco, 1981; Fernández-Puentes & Carrasco, 1980). The exact mechanism used by the virus particle to facilitate transport of toxins is not yet fully understood (Carrasco et al., 1989, 1993). We know that the binding of poliovirus particles to their receptors is a necessary step. In addition, even though macromolecules are internalized with endosomes during virus entry, they are unable to access the cytosol if proper virion uncoating is blocked (Almela et al., 1991). SFV-mediated delivery of toxin to the cytoplasm does not occur when the vacuolar H+-ATPase is selectively inhibited, perhaps because the release of the virus nucleocapsid and the toxin is coupled phenomena. This idea is supported by the fact that co-entry of z-sarcin takes place in the presence of BFLA1 when Sendai virions are used, because uptake of this virus is not affected by interfering with the vacuolar H+-ATPase.

Our present results on the action of BFLA1 on other viruses indicate that Sendai virus does not need a low-pH step and may enter by direct fusion with the plasma membrane, with no requirement for an active vacuolar H+-ATPase. In agreement with current views that herpesvirus and vaccinia virus infection takes place by direct fusion with the plasma membrane (Wittels & Spear, 1991; Doms et al., 1990), the lack of inhibition of these two viruses by BFLA1 can be interpreted as favouring that mechanism. Finally, in the case of poliovirus and adenovirus our results support the view that they do not require a low-pH step during entry.

The expert technical assistance of M. A. Sanz is acknowledged. Luis Pérez was the holder of a C.A.M. fellowship. Plan Nacional project number BIO 92-0715; DGICYT project number PB90-0177 and Fundación Ramón Areces are acknowledged for financial support.

References


(Received 7 March 1994; Accepted 27 May 1994)