SHORT COMMUNICATION

Entry of Semliki Forest Virus into Cells: Effects of Concanamycin A and Nigericin on Viral Membrane Fusion and Infection

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Semliki forest virus (SFV) was biosynthetically labeled with pyrene phospholipids and used to investigate two alternative routes of entry of SFV into BHK-21 cells: (1) receptor-mediated endocytosis followed by fusion of the viral envelope with the endosomal membrane and (2) direct fusion of SFV with the plasma membrane induced by low pH treatment. The selective inhibitor of the vacuolar proton-ATPase, concanamycin A, abolished fusion and subsequent infection only when the virus utilized the endocytic route to enter cells. The inhibitory effect of this macrolide antibiotic was bypassed by low pH treatment of cells. However, the ionophore nigericin was inhibitory irrespective of the route used by the virus to infect cells, suggesting the necessity of a transmembrane pH gradient for the entry process. According to our results, concanamycin A emerges as a suitable tool for selectively investigating the involvement of endosomal function in animal virus entry.

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range effectively block the entry of a number of enveloped animal viruses into cells (Guinea and Carrasco, 1994a,b; Carrasco, 1995). Nigericin and monensin, on the other hand, are carboxylic ionophores widely used in studies of virus entry that induce endosome neutralization (Ohkuma and Poole, 1978). Nigericin functions as a mobile cation carrier exchanging potassium for protons across membranes (Pressman, 1976), leading to inhibition of the low pH-dependent entry of animal viruses into cells (Guinea and Carrasco, 1994b, 1995). Given the differences in the mechanism of action between this ionophore and concanamycin A, we examined the effects displayed by these two compounds on SFV entry into cells. Viral entry was detected by two complementary assays: fusion of viral and cellular membranes and biosynthesis of viral proteins.

For viral fusion assays, the SFV membrane was biosynthetically labeled with pyrene as previously described (Pal et al., 1988; Wahlberg et al., 1992; Bron et al., 1993; Nieva et al., 1994). Briefly, BHK cells growing in 850-cm² glass spinning bottles were cultured in the presence of 15 μg/ml of the fluorescent probe 16-(1-pyrenyl)hexadecanoic acid (Molecular Probes, Eugene, OR) for 2 days to allow its incorporation to cellular membranes. When cells reached confluency, pyrene medium was replaced by fresh pyrene-free medium containing SFV at a m.o.i. of 10 PFU/cell, 1% fetal serum, and 30 mM HEPES (pH 7.4). After 90 min adsorption time, more medium was added to achieve a final concentration of 5% fetal calf serum. Cells were infected for 1 day at 37°C and new, fluorescent progeny virus was harvested and purified as described (Bron et al., 1993; Nieva et al., 1994). The pyrene-labeled SFV was titrated by plaque assay and diluted 1:5 in (pH 6.8) TXA medium [DMEM buffered with HEPES and MES (Sigma) and lacking phenol red and bicarbonate], supplemented with 1% BSA (Sigma) prior to use for infections. Pyrene labeling of SFV according to this procedure did not affect the infectivity of the virus.

Pyrene-labeled SFV was subsequently used to monitor fusion of the virus particles with the endosomal membrane after internalization. To this end, 7–8 × 10⁵ BHK cells growing on glass coverslips inside P₃₅ plates were washed twice with cold TXA medium (pH 6.8) supplemented with 1% BSA prior to incubation with pyrene-labeled SFV at m.o.i. 50 PFU/cell in the same medium for 1 hr at 4°C. Unbound viral particles were removed by washing with Hank’s medium (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.9 mM MgCl₂, 0.9 mM CaCl₂, 1.11 g/liter glucose, 0.88 g/liter citrate, and 98.6 mg/liter MgSO₄) plus 10 mM HEPES (pH 7.4) and supplemented with 1% BSA. BHK cells with the bound SFV were detached at 4°C with a rubber policeman. To initiate internalization, cells with the bound SFV were added to a stirring cuvette containing prewarmed Hank’s medium (pH 7.4; 37°C). Emission spectra were collected at several time points and corrected for background emission of cells in order to obtain the pyrene fluorescence signal arising from SFV lipids.

Upon excitation at 343 nm, the pyrene probe forms excimers (excited dimers, E in Fig. 1A) which fluoresce at 475 nm, approximately 100 nm higher than the emission...
formation in the labeled virus is dependent on the surface density of labeled phospholipids. Upon fusion of the internalized pyrene-labeled virus particle with the unlabeled endosomal membrane, the pyrene phospholipids are diluted in the target membrane (mixing of viral and endosomal membrane lipids), resulting in a decrease of the pyrene monomer fluorescence at 396 nm. The excimer-to-monomer ratio (fluorescence intensity at 475 nm/fluorescence intensity at 396 nm, E/M ratio) is proportional to the surface density of pyrene-labeled lipids in the membrane and decreases after fusion (Galla and Hartmann, 1980). Figure 1A shows a decrease from 0.44 at time 0 to 0.28 at 30 min after SFV internalization.

To analyze the effects of concanamycin A (K. Altendorf, University of Osnabrück, Germany) and nigericin (Sigma) on SFV fusion during virus entry, the E/M ratios were measured after different times in the presence and absence of these compounds (Fig. 1B). A decrease in the E/M ratio was observed in control cells lacking inhibitor from the initial addition of the virus inoculum, which plateaued about 30 min after warming to 37°C. This indicates that the viral envelope of SFV bound to cells and internalized in endosomes fused with the endosomal membrane. The lipid mixing kinetics that we obtained (T_1/2 = 15 min) are comparable to the RNA uncoating and E1 conversion kinetics measured by Kielian et al. (1982). This fact suggests that the lipid mixing assay scores a kinetically coupled event during virus entry. The presence of concanamycin A blocked the E/M ratio decrease (Fig. 1B), in agreement with the concept that the pH in endosomes must diminish to bring about the fusion process (Marsh and Helenius, 1989). This result further corroborates the implication of a role for the v-H⁺ATPase in endosome acidification (Gluck, 1993). The concentrations of concanamycin A used were not toxic for control uninfected BHK cells as estimated by their capacity to support protein synthesis after incubation (Fig. 1C). Moreover, the antibiotic potently blocks virus infection when present at these concentrations (Fig. 1C). These results show that the inhibitor of the vacuolar proton-ATPase concanamycin A, previously shown to be the most potent inhibitor of animal virus entry (Guinea and Carrasco, 1994b), does so by interfering with the fusion step. The ionophore nigericin also blocked fusion and infection (Figs. 1B and 1C) in accordance with the ability of this compound to neutralize endosomes (Ohkuma and Poole, 1978).

Incubation of cell surface-bound virus in mildly acidic medium (pH 5.5–6.0) triggers fusion of the viral and the cellular membranes (Helenius et al., 1980; White et al., 1980). Thus, under these conditions, direct infection may occur through the plasma membrane without a need for endosomal function to drive the entry process. Since concanamycin A and nigericin are both specific endosomal inhibitors, it was also of interest to assay their effects on SFV membrane fusion and virus infection induced by low pH. After 1 hr adsorption at 4°C, BHK cells with fluorescent SFV bound to their surface were added to a prewarmed and stirring cuvette containing H_2NE buffer [5 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA (pH 7.4)] supplemented with 1% BSA, and after 10 sec, fusion was initiated by injection of a pretitrated volume of 0.3 M MES (pH 5.2) to achieve a final pH of 5.55 in the cuvette. Online monitoring of fluorescence at 475 nm was performed for several minutes. The extent of fusion was expressed as the relative decrease of excimer fluorescence (Fig. 2A). The fusion process under these conditions was very fast (T_1/2 = 2 sec) and was completed in about 5–10 sec, suggesting that the virus did not need to be transported into endosomes to obtain fusion. Again the lipid mixing kinetics showed a good correlation with the kinetics of viral RNA penetration measured by White et al. (1980), indicating that both methods monitored coupled events during entry. Moreover, the pH dependence of lipid mixing (inset in Fig. 2A) also paralleled the pH dependence of viral RNA penetration.

The effects of concanamycin A and nigericin on SFV fusion with the plasma membrane were subsequently studied by monitoring the low pH induced decrease of excimer fluorescence (Fig. 2A). The presence of concanamycin A slightly affected this fusion step (19% inhibition), while the presence of nigericin had a relatively strong inhibitory effect (60% inhibition). This inhibitory effect is unlikely to be due to a direct action of nigericin on the membrane fusion activity of the virus as shown by experiments with liposomes. When SFV fusion activity was assayed using liposomes as target membranes (White and Helenius, 1980; Nieva et al., 1994), comparable activity was observed in the presence or absence of the inhibitors (Fig. 2B). Therefore, nigericin-induced inhibition of SFV fusion with BHK cells at low pH is confined to the cellular fusion process. In fact, as shown below, the partial inhibition of fusion induced by nigericin was followed by a total absence of productive infection.

The effects of concanamycin A and nigericin on SFV virus infection of BHK cells under acidic conditions are illustrated in Fig. 2C. The inhibitory effect of concanamycin A at neutral pH was prevented by the low pH treatment of SFV bound to the cell surface. It must be noted that to overcome the inhibition of SFV entry by concanamycin A under acidic conditions the virus needs to be prebound to cells (Guinea and Carrasco, 1994b). This result is in agreement with the idea that entry is independent of the endosomal acidification mediated by the v-H⁺ATPase under these conditions. On the contrary, a strong inhibition of virus entry was detected when nigericin was present in the medium. It is highly improbable that this short treatment with nigericin does interfere with viral translation. Infection inhibition at the level of RNA synthesis has been previously reported to occur...
in this case (Fig. 2C) the low pH treatment did not overcome the inhibitory effect of this carboxylic ionophore, further suggesting that low pH alone is not sufficient to promote virus entry through the plasma membrane (Carrasco, 1994; Guinea and Carrasco, 1995). We explain these findings in terms of a requirement of a pH gradient to drive the entry of SFV into BHK cells (Perez and Carrasco, 1994; Perez et al., 1994). Nigericin, as an acidic ionophore, readily incorporates into membranes and exchanges potassium for protons, dissipating the artificially generated transmembrane pH gradient under acidic conditions. Therefore, two competing processes occur: one is membrane fusion promoted both by low pH and the pH gradient generated when acidic medium is added; the other process that would block fusion is the accelerated dissipation of the proton gradient induced by nigericin. Therefore, fusion with the plasma membrane at low pH will occur as long as the pH gradient has not been destroyed by nigericin. In addition to the pH gradient, the maintenance of the electrochemical gradient has also been pointed out as necessary for the fusion of SFV with the endosomal membrane (Helenius et al., 1985). Surprisingly, however, neither transmembrane pH nor ion gradients are required for SFV–liposome fusion (data not shown; Helenius et al., 1985). Therefore, we conclude that these gradients appear to be a key exclusively for the case of SFV fusing with cells.

The need for pH gradients as an additional requirement for virus entry into cells has been widely documented (reviewed in Carrasco, 1994, 1995). This notion is extended in the present work by demonstrating that the fusion step may be severely impaired when the gradient is abolished, a phenomenon that, in the case of the SFV and BHK cells, is followed by the absence of productive infection as detected by the lack of viral protein synthesis. Nevertheless, nigericin did not completely block the membrane fusion step in acidic medium. If the remaining fusion activity was accompanied by release of nucleocapsids into the cytoplasm, other cellular factors must be involved in blocking the subsequent progression of the infection. In this respect, it is important to note that delivery of the nucleocapsid directly into the cytoplasm is not always sufficient to give rise to infection (White et al., 1980).

The results presented here show that concanamycin A is the most potent inhibitor of animal virus entry known to date. Clearly, this antibiotic at concentrations as low as 50 nM prevents the release of the nucleocapsid into the cytoplasm by inhibiting fusion between the viral and endosomal membranes. Since this inhibitory effect is now well characterized, we propose the use of concanamycin A as a selective tool to investigate the routes of animal viral entry into cells.

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