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Concanamycin A blocks influenza virus entry into cells under acidic conditions

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Abstract

The selective inhibitor of the vacuolar proton-ATPase, concanamycin A, powerfully blocks influenza virus entry into cells, if present during the initial times of virus infection. Attachment of virus particles to cells is not prevented by concanamycin A, rather the exit of influenza virus from endosomes is the step blocked by this macrolide antibiotic. Inhibition of influenza virus entry into cells by concanamycin A or by nigericin takes place under acidic conditions. Moreover, if the pH gradient is abolished by pre-incubation of cells in acidic pH, influenza virus entry does not occur even in the absence of any inhibitors. These results indicate that acidic conditions per se are not sufficient to promote virus entry into cells; rather this step of virus infection requires a pH gradient.

Key words: Concanamycin; Proton ATPase; Enveloped virus; Virus entry

1. Introduction

Most animal viruses enter cells by receptor-mediated endocytosis. Viruses possessing a lipid membrane that surrounds the nucleocapsid contain glycoproteins involved in receptor recognition and virus entry into cells. After binding to cellular receptors virions are internalized and delivered to endosomes where the acid pH generated by the vacuolar proton-ATPase induces the glycoproteins to undergo a conformational change [1,2]. These conformational changes in the virus fusogenic proteins that are located on the virion membrane expose hydrophobic regions that permit their interaction with cellular membranes. This interaction triggers fusion between the virion and cellular membranes [3–5]. Experimental information favors this mechanism, particularly in the case of Semliki Forest virus and influenza virus [3–5]. However, it is still uncertain to what extent receptor binding alone or in combination with low pH contributes to these conformational changes in virion components [6,7]. The use of compounds that raise the pH in endosomes has been crucial to define this model of animal virus entry. However, results obtained with these lysosome-active agents should be interpreted with caution because some of them have side effects on several cellular and viral functions [8]. Therefore, the use of more selective and potent inhibitors is desirable to confront the current models of virus entry into cells.

The concanamycins are a family of macrolide antibiotics isolated from *Streptomyces diastatochromogenes* that are highly active and selective inhibitors of the vacuolar proton-ATPase (v-[H^+]ATPase) [9]. Here we show that the most selective and potent inhibitor of the v-[H^+]ATPase, concanamycin A, blocks the entry of enveloped animal viruses into cells, even under acidic conditions in the medium. Our present results lend support to the concept that a pH gradient, in addition to low pH, is necessary to drive animal virus entry into cells.

2. Materials and methods

2.1. Viruses and cell culture

Madin Darby canine kidney (MDCK) cells were grown on 100 mm plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (Gibco). Infection with influenza A virus (Victoria strain) was carried out in DMEM plus trypsin at 37°C in a CO_2 incubator.

2.2. Antibiotics and ionophores

Concanamycin A was obtained from Dr. K. Altendorf (University of Osnabrück, Germany). Nigericin was obtained from Sigma.

2.3. Electrophoretic analysis of protein synthesis

MDCK cells grown in 24-well plates were infected with influenza virus at a moi of 10–50 pfu/cell. After virus adsorption (time 0 of infection) the cells were washed and incubated in DMEM until 5.5 hpi. Then cells were labelled for 1 h with methionine-free medium containing 20 μCi [^{35}S]methionine per ml (1.45 Ci/mmol, Amersham). The radiolabelled cell monolayers were dissolved in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 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 1 M sodium salicylate. The gels were finally dried and exposed to X-ray films.

2.4. Electron microscopy

MDCK cells grown on 35 mm diameter dishes were infected with influenza virus at a moi of 100 pfu/cell and incubated at 4°C for 1 h. The samples were fixed after 12 min incubation at 37°C with 2% (v/v) glutaraldehyde and 2% (w/v) tannic acid in phosphate-buffered saline (PBS) at room temperature for 30 min and washed three times with PBS. Post-fixation was carried out with 1% (w/v) OsO_4 in PBS at 4°C

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Abbreviations: hpi, hours post-infection; moi, multiplicity of infection; pfu, plaque-forming units.

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for 1 h. Samples were then dehydrated through a 30–100% (v/v) ethanol series and embedded in Epon 812 (Fluka Chemie AG) after infiltration with ethanol:epon 812 (3:1, 1:1 and 1:3, v/v). En bloc staining was performed during the 70% ethanol step with 2% (w/v) 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 at 80 kV.

2.5. Influenza virus entry at low pH

MDCK cells were grown in L-24 linbro dishes in DMEM supplemented with 5% fetal calf serum. Two different protocols were followed for influenza entry to the cells: (i) cells were cooled to 4°C and incubated with influenza virus (10 pfu/cell) on ice for 1 h. The inoculum was removed and replaced by DMEM without bicarbonate, buffered with 20 mM MES, pH 5.2, or 20 mM HEPES, pH 7. Then, cells were incubated at 37°C in an atmosphere free of CO_2. After 15 min the medium was replaced by DMEM and cells were incubated at 37°C until the labelling period. (ii) Cells were washed and concentrated influenza virus was added together with DMEM without bicarbonate buffered with 20 mM MES, pH 5.2, or HEPES, pH 7. Cells were incubated for a further 15 min in an atmosphere free of CO_2 and then washed and incubated in DMEM until the labelling period.

3. Results and discussion

To analyze the inhibitory effect of concanamycin A on virus infection, different concentrations of this compound were added during the initial stages of influenza virus infection. Concanamycin A was active at 5 nM and completely blocked influenza virus infection at 10 nM in MDCK cells (Fig. 1A). Treatment of uninfected cells with the same concentrations of the antibiotic showed no toxic effects, as measured by the capacity of these cells to synthesize proteins, even at 50 nM concanamycin A (Fig. 1A). The high specificity of concanamycin A in the inhibition of the v-[H$^+$]ATPase [10], and the fact that very low concentrations of this antibiotic inhibit virus entry, suggest that this compound could be a useful tool for investigating the requirements for acidic conditions during different steps of infection of animal cells by viruses. These findings suggest that concanamycin A blocks viral replication by inhibiting the v-[H$^+$]ATPase, thus preventing acidification of endosomes and release of virions into the cytoplasm. To test whether the action of this agent was restricted to the early events of infection, concanamycin A was added after virus entry (Fig. 1A). Addition of the macrolide antibiotic 60 min after the virus did not inhibit infectivity, even if the compound was continuously present. These results suggest that an early event in virus infection is the target of concanamycin A. Certainly this event is not related to virus binding to receptors or internalization, since radioactive influenza virus binds to cells at 4°C and is internalized after binding with the same kinetics and to the same extent in the absence or in the presence of the antibiotic (data not shown). Finally, binding of influenza virus to the cell surface takes place both in the absence and presence of the inhibitor, as shown by electron microscopy (Fig. 1B). These findings suggest that the inhibition of the v-[H$^+$]ATPase by concanamycin A prevents endosomal acidification, inhibiting virus release from endosomes.

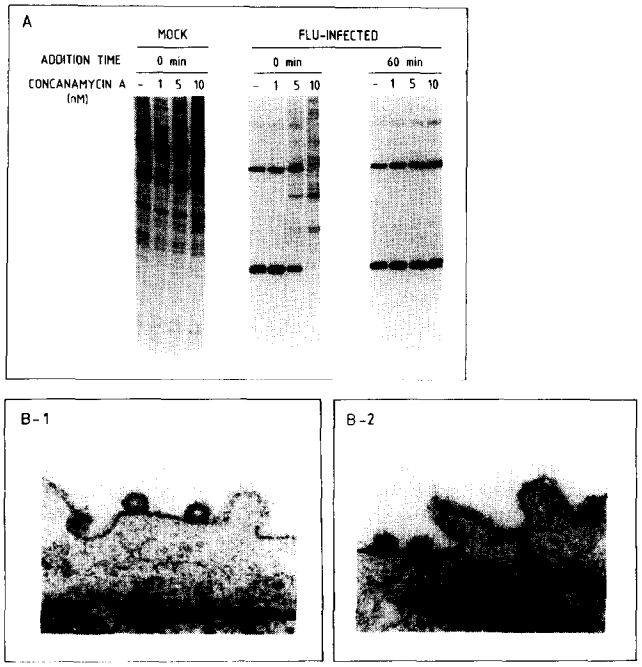

Fig. 1. Concanamycin A inhibits an early step of influenza virus infection. (A) MDCK cells were mock-infected or infected with influenza virus at 50 pfu/cell. Different concentrations of concanamycin A were added at the same time as the virus and cells were incubated for 1 h (0 min). Alternatively, concanamycin A was added after the cells were washed to remove the unadsorbed virus (60 min). Cells were incubated in DMEM until 5.5 hpi, then labelled for 1 h with methionine-free medium containing 20 μCi [^{35}S]methionine per ml. (B) MDCK cells grown on 35 mm diameter dishes were infected with influenza virus at a moi of 100 pfu/cell and incubated at 4°C for 1 h in the presence (B-2) or absence (B-1) of concanamycin A (50 nM). The samples were fixed after 12 min incubation at 37°C. Electron micrographs were taken with a JEOL 1010 microscope at 80 kV as described in section 2.

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Fig. 2. Inhibition of influenza virus entry by concanamycin A and nigericin under acidic conditions. (A) Cells were pre-incubated with concanamycin A (50 nM) for 15 min at 37°C. Then two different protocols were followed. Preadsorption +: cells were cooled to 4°C and incubated with influenza virus (10 pfu/cell) on ice for 1 h. Then, the inoculum was removed and replaced by DMEM at pH 5.2 and the cells were incubated at 37°C for 15 min in the presence or absence of the antibiotic. After that time the medium was replaced by neutral DMEM and cells were incubated at 37°C until the labelling period from 4.5 to 5.5 hpi. Preadsorption −: concentrated influenza virus was added to the cells in DMEM at pH 5.2. Cells were incubated for 15 min and then washed and incubated in neutral medium until the labelling period from 4.5 to 5.5 hpi. (B) Cells were treated with concanamycin A (50 nM) or nigericin (10 μM) or both for 15 min at 37°C. Then virus was added and allowed to absorb for 1 h at 4°C. After adsorption, the medium was replaced by DMEM buffered at pH 5.2 or 7. Cells were incubated for 15 min at 37°C in the presence or absence of the inhibitors and then washed and incubated in neutral DMEM before analysis of protein synthesis from 4.5 to 5 hpi.

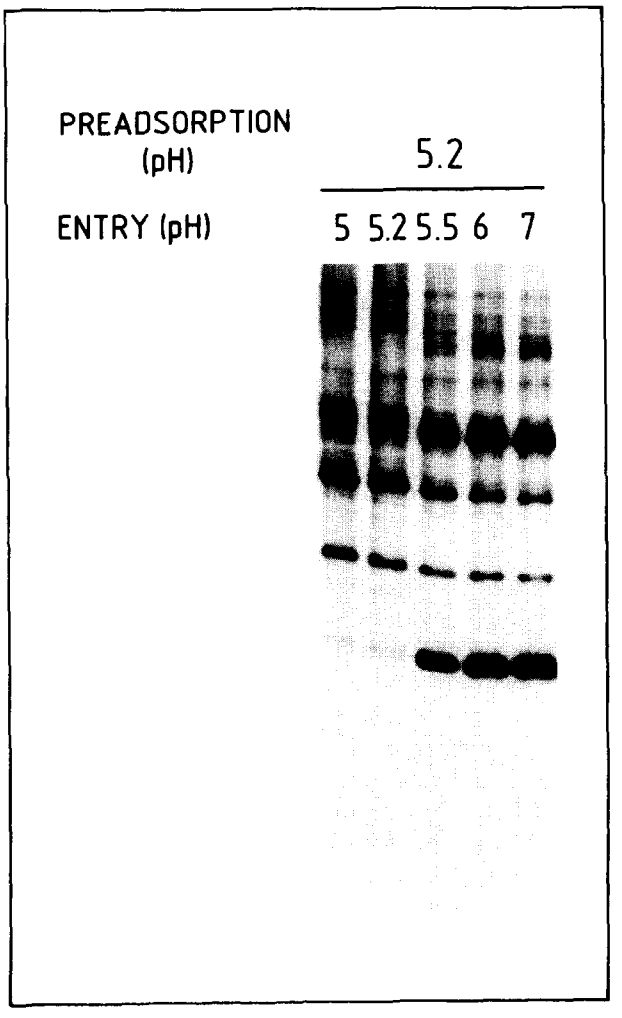
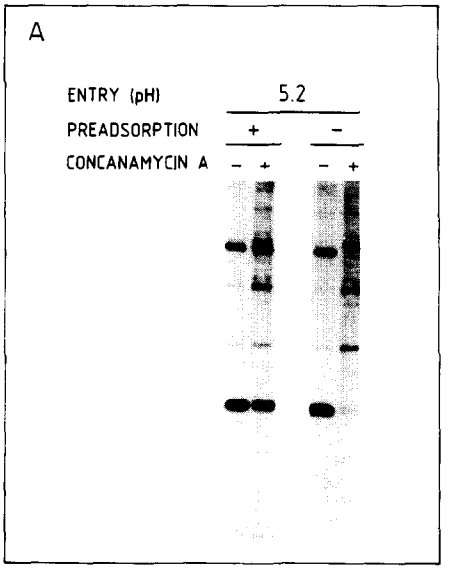
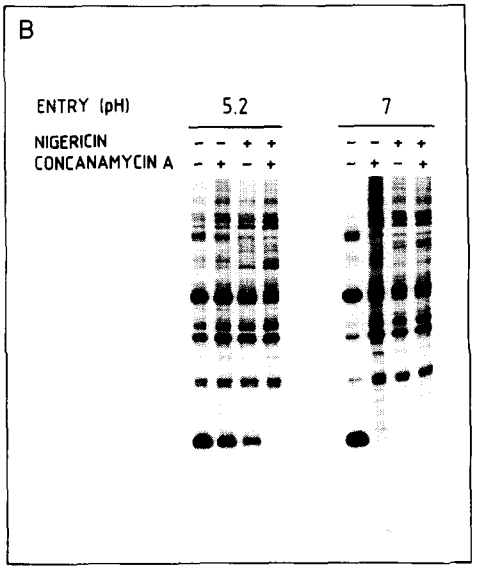


Fig. 3. Inhibition of influenza virus entry by pre-incubation in acidic medium. Cells were washed and DMEM was replaced by DMEM without bicarbonate and buffered at pH 5.2. Influenza virus was added at the same time to the cells, and viral adsorption was allowed for 1 h at 4°C. Then, excess virus was removed and pre-warmed DMEM at the pH indicated was added for 15 min. Cells were washed again and incubated in DMEM for a labelling period from 4.5 to 5 hpi. →

Influenza virus particles can be induced to fuse directly with the plasma membrane by direct acidification of the culture medium [3,11]. Two different protocols were used to analyze the effect of concanamycin A on virus entry under low pH conditions: either the virus was added directly to cells in low pH medium, or the virus was pre-bound at 4°C under normal pH conditions and the cells were then incubated at 37°C in warmed low pH medium. Significantly, concanamycin A totally blocked infectivity of influenza virus added directly to low pH media (Fig. 2A). However, some virus infection is detected in the presence of the inhibitor if the virus is pre-bound to cells followed by uptake at pH 5.2. Efficient blockade of pre-bound viruses is achieved by combining concanamycin A and nigericin treatment (Fig. 2B). Nigericin is an ionophore that facilitates exchange of H^+ for K^+ and thus dissipates any existing H^+ gradient across the endosomal membrane. Therefore, our results indicate that virus entry can be prevented by inhibitors of endosomal functions, even under low pH conditions. We interpret these results to mean that viruses pre-bound to cells at 4°C and incubated in pre-warmed acidic medium are soon internalized into endosomes that have a pre-existing low pH, even if the v-[H^+]ATPase is blocked. Hence, the viruses pre-bound to cell surface receptors can quickly leave the endosome before the pH gradient is dissipated due to the absence of a functioning v-[H^+]ATPase and acidification of the cytoplasm. If the viruses are added directly to low pH medium, however, virions must first bind to the cell surface; during this time protons from the external medium have acidified the





cytoplasm, with a consequent decrease in the pH gradient across the endosomal membrane. Under these circumstances, the activity of the v-[H^+]ATPase is required to create a pH gradient sufficient to promote virus genome delivery to the cytoplasm. In addition, nigericin, an ionophore that exchanges K^+ for H^+, dissipates the proton gradient. Consequently, a clear inhibition of virus entry is achieved by adding nigericin that rapidly dissipates this proton gradient in the absence of an active v-[H^+]ATPase, even in acidic media.

Since the presence of the inhibitors used could influence other unknown functions required for virus entry, we wanted to test whether entry of some animal viruses promoted by low pH required the existence of a pH gradient in the absence of any inhibitors. Cells were preincubated in acidic medium during virus binding. Under these conditions the intracellular pH rises, diminishing the pH gradient between the external medium (or endosomes) and the cytoplasm [12]. Under these circumstances influenza virus was unable to infect cells when incubated at 37°C and low pH, whereas infection did occur if cells were placed in neutral medium, indicating that virus infectivity was not affected by preincubation in acidic medium (Fig. 3). These results lend support to the concept that the entry of some enveloped animal viruses requires a pH gradient in addition to low pH to promote virus infection. The biological implications are that viruses may need an energized membrane for proper fusion and virus entry into the cell. Indeed, the entry of both enveloped or naked animal viruses into cells disrupts the membrane potential [13–18]. Therefore, animal viruses dissipate the ionic gradients during entry. The physiological significance of this behaviour may be to couple the proton motive force with virus entry into the cytoplasm [19].

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