Entry of Poliovirus into Cells Is Blocked by Valinomycin and Concanamycin A†

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ABSTRACT: Poliovirus contains a virus particle devoid of a lipid envelope that does not require an intact pH to enter into susceptible cells. Thus, the blockade of pH gradient generated in endosomes is not sufficient to impede the translocation of poliovirus particles to the cytoplasm, suggesting that translocation takes place at the plasma membrane. Measuring both viral protein synthesis and eIF4G-1 cleavage mediated by poliovirus protease 2A has been used to monitor productive entry of poliovirus into cells. Translation of the input poliovirus RNA produces enough $2A^{pro}$ to cleave eIF4G-1, providing a sensitive assay to estimate poliovirus RNA delivery to the cytoplasm followed by its translation. Combination of concanamycin A, a vacuolar proton-ATPase inhibitor, and valinomycin, an ionophore that promotes K^+ efflux from cells, powerfully prevented poliovirus infection. Moreover, modifying the ionic conditions of the culture medium (increasing the concentration of K^+ and decreasing the concentration of Na^+), together with concanamycin A, also significantly interfered with poliovirus entry. These findings suggest that poliovirus RNA requires an intact concentration of K^+ ions inside the cells to be uncoated and to gain access to the cytoplasm. In addition, the actual contribution of concanamycin A (as well as other inhibitors of endocytosis) to the total inhibition of productive poliovirus entry points to the idea that at least some percentage of polioviral subparticles translocates from the endosomes.

Entry of animal viruses into cells commences by attachment of virus particles to cell surface receptors (*1*). At least five different entry pathways are potentially exploited by viruses: direct fusion at plasma membrane, endocytosis via chlatrin-coated vesicles (110 nm), endocytosis via caveolae (60 nm) and entry by macropinocitosis (50−200 nm) and micropinocitosis (95 nm) (*2*). Most animal viruses follow the receptor-mediated endosomal route to obtain access to the cell interior (*3*, *4*). Viruses that enter through endosomes and contain a lipid envelope fuse their membrane with the endosomal membrane (*5*). Very little is known, however, about the mechanisms involved in passage across host membranes by virus particles devoid of a lipid envelope (*3*).

The infectious entry route of poliovirus remains still obscure. When poliovirus attaches to its receptor (*6*, *7*), the 160S native virion undergoes some irreversible conformational changes such as externalization of N-terminus of VP1 (*8*) and the loss of VP4, rendering a 135S altered and more hydrophobic A particle insensitive to RNAse (*9*, *10*). 135S particle seems to be an essential intermediate in the cellular entry of poliovirus (*10*−*14*). After this structural alteration, viral RNA has to be released and translocated to the cytosol to be expressed. However, the site of uncoating has not been clearly defined. Polioviruses could pass directly through the plasma membrane after receptor binding (*15*, *16*) or they can be internalized by receptor-mediated endocytosis and then cross the endosomal membrane (*17*, *18*). In both cases, the viral genome has to pass across a lipid membrane aided by virion proteins (*19*, *20*).

It has been pointed out that poliovirus particles have never been shown unequivocally inside the endosomes (*21*). However, early studies claimed that agents that alter pH inside these organelles showed a moderate inhibition of poliovirus infection (*17*, *22*, *23*). More recently, it was shown that infection of some animal viruses that do not contain a lipid envelope, such as poliovirus or adenovirus are not inhibited by compounds that block endosomal function, i.e., monensin or vacuolar proton-ATPase inhibitors (*19*, *24*, *25*) whereas other viruses, such as rhinoviruses or reoviruses do not infect cells if these compounds are present during the initial stages of infection (*24*, *26*−*30*). Since poliovirus entry was not inhibited by bafilomycin A1 (*24*), the requirement of low pH for this virus seems to be less stringent than for Semliki Forest Virus (SFV) or vesicular stomatitis virus (VSV) (*11*). Co-localization of native poliovirions with early endosomal markers has proved that at least a fraction of the virus population can enter through the endosomal system (*31*) although chlatrin-coated vesicles are not the route followed by these poliovirus particles (*32*). Other data suggest that endocytosis is dispensable for poliovirus infection, leading this route to an abortive infection (*11*, *33*).

Selective inhibition of poliovirus entry into cells has not been described yet. Thus, the finding of such an inhibitor would allow the possibility to characterize in more detail the cellular route and the molecular basis of poliovirus internalization. During a preliminary experiment using several drugs to alter energy production in the cell, we found that one ionophore, valinomycin, was able to partially inhibit poliovirus infection. Valinomycin is a K^+ carrier ionophore

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well-known to cause the collapse of the mitochondrial membrane potential by depolarization of the isolated organelle (*34*, *35*). In addition, valinomycin induces K^+ efflux from cells in normal medium (*36*−*38*). Bafilomycin and concanamycin A potently block the vacuolar proton-ATPase, preventing endosomal acidification (*39*, *40*). In the present work, we have found that valinomycin in combination with concanamycin A inhibit productive poliovirus entry. Both inhibitors needed to be present throughout entry to be actually effective, pointing to a reversible action. Therefore, the cellular route followed by poliovirus to enter cells implies both direct passage across plasma membrane and endosomal pathway. In addition, ion fluxes (K^+ and H^+) seem to be determinant to allow a productive poliovirus entry**.**

EXPERIMENTAL PROCEDURES

Cell Cultures, Media, and Virus. Dulbecco-modified Eagle's medium (DMEM)[1] supplemented with 10% newborn calf serum was used for growth and maintenance of HeLa cell cultures. Stably transformed tTA HeLa cells transformed with either wild-type dynamin (wt) or K44A dynamin (element I mutant) were also used, kindly provided by Sandra Schmidt (Department of Cell Biology, The Scripps Research Institute, La Jolla, CA) and cultured as described previously (*41*). Poliovirus type 1 (Mahoney strain) was propagated, grown, and titrated by plaque assay in HeLa cells. The viral infections were performed in DMEM with 2% newborn calf serum at the multiplicity of infection (MOI) indicated in each case. When indicated, DMEM without NaCl and KCl was used, supplemented with 100 mM KCl (high K^+ medium or DMEM*). This medium contains 45 mM Na^+, because of $NaHCO_3$ (40 mM) and NaH_2PO_4 (5 mM). In cytometry experiments, DMEM and DMEM* also lack $NaHCO_3$ and were supplemented with 20 mM Hepes (pH 7.0). In other experiments, the following treatments were used to block clathrin-coated pit formation: hypertonic medium (DMEM plus 0.43 M sucrose), cytosol acidification (DMEM plus AcONa plus 20 mM Hepes pH 7.0, to a final pH 6.05) and potassium depletion (DMEM without KCl) (*42*−*45*). To all type of media were also added 2 mM Gln and antibiotics (50 units of penicillin/mL and 50 μg of streptomycin/mL).

Chemicals. Valinomycin, monensin, nocodazol, chlorpromazine, and chloroquine were purchased from Sigma Chemical Co (St. Louis, MI). Concanamycin A was obtained from Dr. K. Altendorf (University of Osnabrück, Germany). α-sarcin was a generous gift from D. M. Shuurmans (Department of Public Health, Lansing, Mich.). Paraformaldehyde and ammonium chloride were purchased from Merck (Darmstad, Germany). Mowiol 40−88 was from Aldrich (Milwaukee, WI) and Hepes, sucrose, Nonidet P-40 and Triton X-100 were from Sigma.

Fluorescent and Radioactive Compounds. Carbocyanine dye $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) was purchased from Molecular Probes Inc. (Eugene, OR) and dissolved in DMSO. Fluorescein isothiocyanate-labeled dextran (FD or FITC-D) (average molecular weight 167 000) was from Sigma and was dissolved in distilled water. L-[^{35}S]-methionine/cysteine (>1000 Ci/mM) was purchased to Amersham (U.K.).

Analysis of proteins by SDS−polyacrilamide gel electrophoresis and immunoblot against cellular translation factor eIF4G-1 were performed as described earlier (*46*, *47*).

Immunofluorescence Microscopy. Studies were performed on HeLa cells grown on glass coverslips. Cells were treated with different compounds or media. FITC-dextran (FD, molecular weight 167 000) stock was centrifuged and filtered through 0.8/0.2 μm filters Supor Acrodisc (Gelman Sciences, MI) before use. FD (3 mg/mL) was added at the same time as purified poliovirus (MOI 25) at 37 °C to cells and incubated 2 h in dark. After the incubation times, cells were washed 5 times RT with PBS, and 2% paraformaldehyde in PBS was added to fix them for 30 min. Paraformaldehyde was washed and neutralized with 0.1 M ammonium chloride for 30 min. Coverslips were washed again with PBS and mounted with Mowiol to be observed in a Zeiss fluorescence microscope at 100×. Fluorescent vesicles inside the cells are part of the endocytic compartment (*48*). The effects of compounds or high K^+ medium on the scattered patron of FD were quantified by choosing random fields of cells on the coverslips. Data are expressed as percentage of cells in the field with the punctuate patron due to FD scattering.

Flow Cytometric Determination of Membrane Potential. For analysis in the flow cytometer, HeLa cells monolayers were detached from 100-mm-diameter dishes with trypsin, centrifuged in DMEM without $NaHCO_3$ containing 10% calf and resuspended in the same medium containing 2% calf serum. Cells were distributed in eight eppendorf tubes to be preincubated at 37 °C in a termomixer in the presence or in the absence of 50 μM valinomycin, 80 nM concanamycin A or both. Pretreatments with the compounds took 15 min and after this time, the cells were infected or not by adding a purified poliovirus at MOI 50. At different times postaddition of poliovirus, a small volume was transferred to another tube containing the fluorescent cationic probe to be loaded at a final concentration of 5 nM $DiOC_6$ (3) for 10 min at 37 °C. Propidium iodide (5 μg/mL) was also added to the cell suspension, and dead and/or membrane-damaged cells were gated out of analysis. Loading with the probe was made 10 min prior to the fluorescence measurements. The cells were analyzed using an Epics Profile II cytofluorometer (Coulter Miami, FL). The probe was excited with an argon ion laser (488 nm, 200 mW) and its emission detected at 525 nm. Fluorescence intensity was displayed in logarithmic scale on the *x*-axis and the number of cells having a given fluorescence on the *y*-axis. The background light level of nonloaded cells was measured to subtract the cell autofluorescence from all measurements. Histograms were obtained at five different times from addition of poliovirus to 1 h later, from two independent experiments. The mean value of the fluorescence intensity distribution of the cell population was calculated at each time for each treatment. Data are presented as the percentage mean of fluorescence of their particular control cells at each time of measurement. Treatments with high K^+ medium (DMEM*) were made in the same way described but in the absence of serum.

Preparation of [^{35}S]Methionine-Labeled Poliovirus and Heated Virions. Monolayers of HeLa cells cultured in 100 mm plates were infected with poliovirus at MOI 5. At 3 h

[1] Abbreviations: PV, POLIOVIRUS; MOI, multiplicity of infection; VAL, valinomycin; CA, concanamycin A; DMEM*, DMEM with 45 mM Na^+ and 100 mM K^+; FD, FITC-dextran.

postinfection the medium was replaced by methionine-free medium containing 30 μCi/mL [^{35}S]methionine. After a 15 h incubation period, when cytophatic effect (CPE) was complete, the medium and the cells were collected and medium was clarified by low-speed centrifugation. A 5 mL cushion of 15% sucrose, 50 mM NaCl, 10 mM Tris-HCl (pH 7.4) was underlayered beneath clarified medium containing extracellular labeled-poliovirus. Viruses were pelleted by centrifugation at 47000*g* for 2 h at 4 °C in a T-865 rotor. The supernatant was removed and the pellet was suspended in DMEM to be centrifuged over a 5 mL sucrose cushion at 92000*g* in the same rotor for 2 h at 4 °C. Viral pellet was suspended in DMEM and small aliquots were taken to determine the radioactivity associated and the rest of the sample was stored frozen at −70 °C. Titration of viral suspension was performed by plaque assay in monolayers in 35-mm plates. To obtain a control of the 80S fraction of [^{35}S]methionine-labeled poliovirus for sucrose gradients centrifugation, purified radiolabeled virus was heated for 10 min at 56 °C in the presence of 0.05% Nonidet P-40. The solution was cooled on ice before being layered onto a 15 to 30% sucrose gradient (see below).

Virus Attachment and Virus Entry Assays. HeLa cells were pretreated with valinomycin, concanamycin A or DMEM* as described earlier. [^{35}S]Methionine-labeled poliovirus (170 000 cpm, MOI 100) was added in 2% calf serum medium to 4 °C precooled cell monolayers. One hour later, cells were shifted to 37 °C for the entry assay. After different incubation times during adsorption period (4 °C), and during entry period (37 °C), the medium was removed and the cells were washed three times with cool PBS. To measure virus binding, after precipitation with 5% trichloroacetic acid (TCA), the cells were washed twice with ethanol and allowed to dry before TCA-precipitable material was collected in 0.1 M NaOH, 1% SDS. To measure virus entry, cells were treated for 15 min at 4 °C with 50 μg/mL proteinase K (Boehringer Mannheim) in PBS to eliminate non internalized viral particles during incubation at 37 °C. Then, the TCA-precipitable material was collected in 0.1 M NaOH, 1% SDS as described above. In both cases, samples were redissolved in the liquid scintillation cocktail Optiphase "HiSafe" (Wallac Yo, England) and the radioactivity associated was quantified in a liquid scintillation counter (1219 Rackbeta, LKB).

Analysis of Virus Particle Alteration by Sucrose Gradient Centrifugation. HeLa cells were pretreated with valinomycin, and/or concanamycin A as described above. Purified native [^{35}S]methionine-labeled poliovirus (300 000 cpm, MOI 100) was attached to cells at 4 °C by addition onto the same media. One hour later, cells were washed to remove unattached virus, and new medium containing the compounds was added to cells to be incubated at 37 °C for 2 h. Cells were washed three times with PBS and dissolved in 0.5% Triton X-100 in PBS. The nuclei were removed by centrifugation and supernatants were adjusted to 0.2% SDS and layered on top of a 15−30% sucrose gradients in 10 mM phosphate buffer (pH 7.4) containing 0.14 M NaCl (*49*). Gradients were centrifuged for 2 h at 206000*g* and at 4 °C in a SW41 rotor. Fractions were collected from the top and the radioactivity in 250 μL of each fraction was measured in a liquid scintillation counter. Native and heated virions were used as gradient markers to identify 160S and 80S particles position.

RESULTS

Effects of Different Compounds on Early Steps of Poliovirus Infection. Our previous findings on the action of vacuolar proton-ATPase inhibitors revealed that none of these agents impeded poliovirus entry into cells (*24*). We then tested two ionophore antibiotics, alone or in combination with the inhibitor of the vacuolar proton-ATPase, concanamycin A (*50*): monensin (*51*) and valinomycin (*52*). Three different protocols were designed (Figure 1A): (1) the compounds were present 15 min before poliovirus addition and kept for half an hour of poliovirus entry, then poliovirus and the inhibitors were removed; (2) the compounds were added fifteen minutes before poliovirus and kept during the virus replication cycle until protein synthesis was measured; and (3) the compounds were added after poliovirus entry and kept during the virus replication cycle until protein synthesis was estimated. The inhibitor of the vacuolar proton-ATPase concanamycin A had no effect on poliovirus infection under the three experimental conditions employed (Figure 1A). However, monensin alone or in combination with concanamycin A had a clear inhibitory action on poliovirus when protocols 2 and 3 were used. These results indicate that monensin, a compound that interferes with the vesicular system, blocks poliovirus replication, even when added after the entry period (Figure 1A). These findings are not surprising given that the replication of poliovirus genomes requires an intact vesicular system (*53*−*55*). On the other hand, monensin is less effective if present only during the entry process than when protocol 2 is assayed. This means that the inhibitory action of monensin is reversible as previously described (*55*) since removal of the compound allows poliovirus replication.

Valinomycin inhibits poliovirus infection when present during the entry period (Figure 1A; protocols 1 and 2), while poliovirus protein synthesis was still detected when valinomycin was added after poliovirus entry (Figure 1A; protocol 3). The inhibition of poliovirus protein synthesis in the presence of valinomycin was observed either when the ionophore was assayed alone or in combination with concanamycin A. However, the effect is stronger when both compounds are continuously present (Figure 1A, protocol 2). The conclusion from these results is that an ionophore such as valinomycin, that promotes K^+ efflux, is a potent inhibitor of poliovirus entry (measured as viral protein synthesis), while compounds that block endosomal acidification had no effect (Figure 1A; protocols 1 and 2). To our knowledge, valinomycin represents the first compound that interferes with this early step of poliovirus infection (*56*).

Cleavage of eIF4G-1 as a Sensitive Test To Estimate Productive Poliovirus Entry. Our test to estimate poliovirus entry and infectivity relies upon the capacity of poliovirus to enter cells and to synthesize proteins at late times of infection. This test has the advantage of measuring productive entry of viruses i.e., those viruses able to give rise to a replication cycle. However, with some compounds such as monensin or nigericin that inhibit the replication of viral RNA (*55*), the use of this assay may not provide clear results when the compounds are maintained after virus entry. In addition, [^{35}S]methionine-labeled protein bands might be somewhat difficult to interpret, since polioviral proteins might

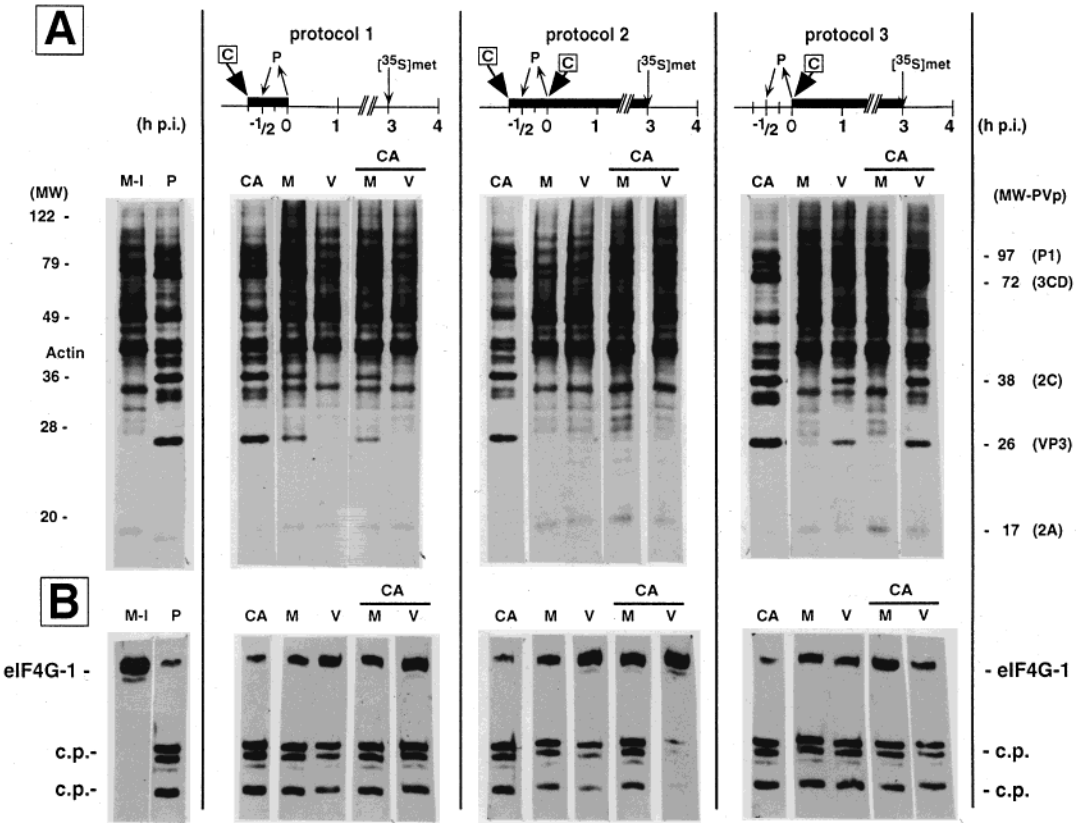

FIGURE 1: Effect of several compounds on poliovirus infection of HeLa cells. HeLa cells were mock-infected (M−I) or infected with poliovirus (P) at 25 PFU/cell. Treatment with 50 μM monensin (M), 50 μM valinomycin (V), and 80 nM concanamycin A (CA), was carried out according to three different protocols: (1) the compounds (C) were present 15 min before poliovirus addition and kept during half an hour of poliovirus entry until the virus inoculum was removed; (2) the compounds were added fifteen minutes before poliovirus and kept during virus replication until protein synthesis was estimated; and (3) the compounds were added after poliovirus entry and kept until protein synthesis was measured. Where indicated, treatments with the inhibitors in combination with concanamycin A were made. [^{35}S]-Methionine-labeling of HeLa cells was carried out from 3−4 h p.i. and processed for electrophoresis. (A) Measurements of protein synthesis. The samples were run in a 15% SDS−PAGE and processed as described. Molecular weight markers as well as some poliovirus proteins (PVp) are indicated. (B) Analysis of eIF4G-1 cleavage. The same samples were run in a 7.5% SDS−PAGE, transferred to nitrocellulose membrane and incubated with anti-eIF4G-1 polyclonal antibodies as described in the text. c.p. = eIF4G-1 cleavage products.

be hidden by cellular proteins due to delayed onset of host cell shut-off by these compounds. Therefore, we decided to investigate poliovirus entry by measuring an event prior to viral RNA synthesis, such as the translation of the input viral RNA, to ensure that this RNA is located in a functional form in the cytoplasm. Perhaps the most sensitive assay in this regard is provided by the analysis of eIF4G cleavage. The synthesis of small amounts of the poliovirus $2A^{pro}$ results in the proteolytic cleavage of the polypeptide eIF4G, a component of initiation factor eIF4F (*57*−*59*). Two forms of eIF4G have been described (*60*). Our antibodies recognize one of the forms of this factor known as eIF4G-1. Inhibitors of poliovirus RNA synthesis do not block this cleavage, revealing that the $2A^{pro}$ generated over the primary translation of the input poliovirus RNA accomplished eIF4G-1 cleavage (*54*, *55*, *61*, *62*).

To investigate the effects of valinomycin, concanamycin A and monensin on eIF4G-1 cleavage, 20 μL of the same [^{35}S]methionine-labeled samples obtained in Figure 1A were immunoreacted with a polyclonal antibody against human eIF4G-1 (*63*). The results obtained in the analysis of eIF4G-1 cleavage (Figure 1B) are consistent with the results obtained from the analysis of protein synthesis in the presence of the compounds (Figure 1A). However, measuring eIF4G-1 cleavage, the inhibitory action of valinomycin on poliovirus entry is partially reversed in the absence of the compound (Figure 1B, protocol 1), whereas in the presence of valinomycin either alone or in combination with concanamycin A, eIF4G-1 remains practically intact (Figure 1B, protocol 2). This result indicates that, upon removal of valinomycin (Figure 1B, protocol 1), poliovirus is able to enter productively in the cells. Notably, prevention of eIF4G-1 cleavage is much more powerful when both valinomycin and concanamycin A were present, indicating that eIF4G-1 cleavage does not occur when the amount of cytoplasmic K^+ and acidification of endosomes are altered (Figure 1B, protocol 2). Concanamycin A alone is not able to fully prevent eIF4G-1 cleavage under the three protocols used, in good agreement with the previously reported lack of effect of inhibitors of vacuolar proton-ATPase on poliovirus entry (*24*). The slight inhibitory effect of monensin alone or in combination with concanamycin A on eIF4G-1 cleavage under the two first protocols further suggests that only the blockade of the pH gradient is not sufficient to prevent poliovirus translocation. On the other hand, monensin added after poliovirus entry (Figure 1B, protocol 3), alone or in combination with concanamycin A, does not affect eIF4G-1 cleavage while still permitting cellular protein synthesis as it has been described (*55*). In addition, the use of monensin in protocol 3 represents a positive control of eIF4G-1

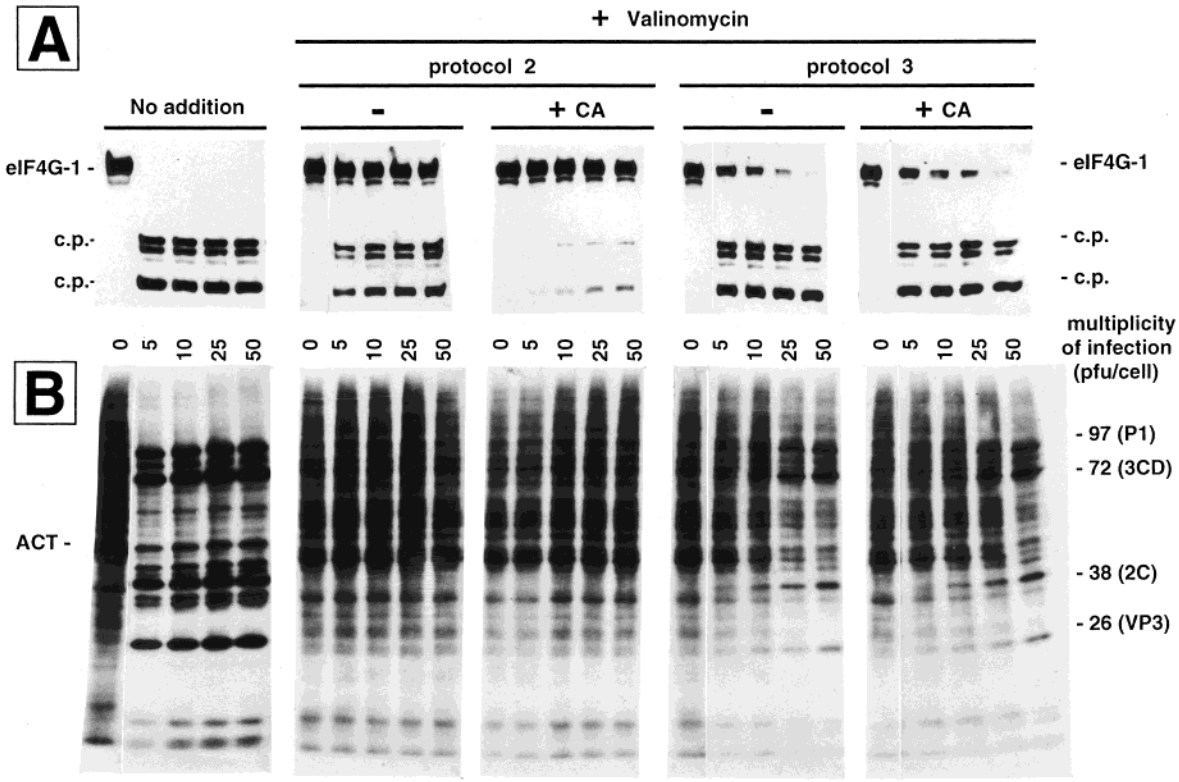

FIGURE 2: Influence of the multiplicity of infection on the inhibition of poliovirus entry by valinomycin alone or in combination with concanamycin A. HeLa cells were infected with poliovirus at the multiplicities of infection indicated. Addition of 50 μM valinomycin and/or 80 nM concanamycin A (CA) was made following protocols 2 and 3 described under Figure 1. Proteins were labeled from 3 to 4 h p.i. and cells were harvested and processed as indicated before. (A) Analysis of eIF4G-1 cleavage. The samples were applied to a 7.5% SDS−PAGE and processed as described. c.p. = eIF4G-1 cleavage products. (B) Measurement of protein synthesis. The same samples were run in a 15% SDS−PAGE. ACT: cellular actin. Molecular weights of some poliovirus proteins are indicated.

cleavage even when poliovirus gene expression is blocked; monensin allows RNA input translation, but not the further bulk of viral replication.

The action of both inhibitors was examined under different multiplicities of poliovirus infection of HeLa cells (Figure 2). Due to the fact that the inhibitory action of valinomycin on poliovirus entry was reversible (see Figure 1B, protocol 1), protocol 1 was omitted in this experiment. Cells were treated according to protocols 2 and 3 as indicated in Figure 1. Cell extracts were made after 4 h of infection and immunoreacted with polyclonal antibodies against eIF4G-1 (Figure 2A). In addition, protein synthesis was estimated by [^{35}S]methionine-labeling from 3 to 4 h postinfection (p.i.) (Figure 2B). Valinomycin alone partially inhibits cleavage of eIF4G-1 when protocol 2 is used, but not if added after poliovirus entry (protocol 3). Again, when valinomycin is combined with concanamycin A, inhibition of eIF4G-1 cleavage is even more apparent, indicating that eIF4G-1 cleavage does not occur when both cytoplasmic K^+ and endosomal H^+ are depleted. Analysis of protein synthesis indicates that valinomycin alone prevents poliovirus input translation under the different multiplicities tested (Figure 2; protocol 2). Nevertheless, under these circumstances partial cleavage of eIF4G-1 takes place, revealing that some poliovirus RNA can escape the valinomycin block and get translated. Notably, the simultaneous addition of concanamycin A totally prevents eIF4G-1 cleavage, suggesting a participation of the endosomal route in poliovirus entry. The inhibition of poliovirus genome translation by these compounds is lower when higher multiplicities are used (Figure 2B), perhaps reflecting partial entry through the plasma membrane at higher MOIs. In conclusion, analysis of eIF4G-1 cleavage provides a more sensitive test than the measurement of protein translation at late times of infection, although both tests complement each other to obtain information on the productive entry of poliovirus into cells. These findings point to the idea that poliovirus entry needs an intact K^+ concentration inside the cells, but the acidification of endosomes can also contribute to some extent to this translocation. Therefore, only when K^+ ions and endosomal pH are altered is poliovirus entry efficiently halted.

Modifying Intracellular K^+ by Altering External Cations. To further assess the role of intact cellular K^+ content for poliovirus entry, we sought means other than valinomycin to alter it. Increasing the concentration of K^+ and decreasing the concentration of Na^+ in the culture medium would prevent K^+ efflux from cells (*64*). Poliovirus entry was tested following the three protocols depicted in Figure 1, in medium containing 100 mM K^+/45 mM Na^+ (High K^+ medium or DMEM*) plus or minus concanamycin A (Figure 3). Testing both protein synthesis (Figure 3A) and eIF4G-1 cleavage (Figure 3B) monitored productive poliovirus entry. Treatment of control uninfected HeLa cells with high K^+ medium neither affects translation nor the integrity of eIF4G-1. This treatment has by itself no effect on poliovirus protein synthesis or eIF4G-1 cleavage when using protocol 1 and 3 (Figure 3). However, if high K^+ medium is present throughout poliovirus infection (Figure 3; protocol 2) there is a reduction in cellular and viral translation, but no effect on eIF4G-1 cleavage is found. This result agrees with the idea

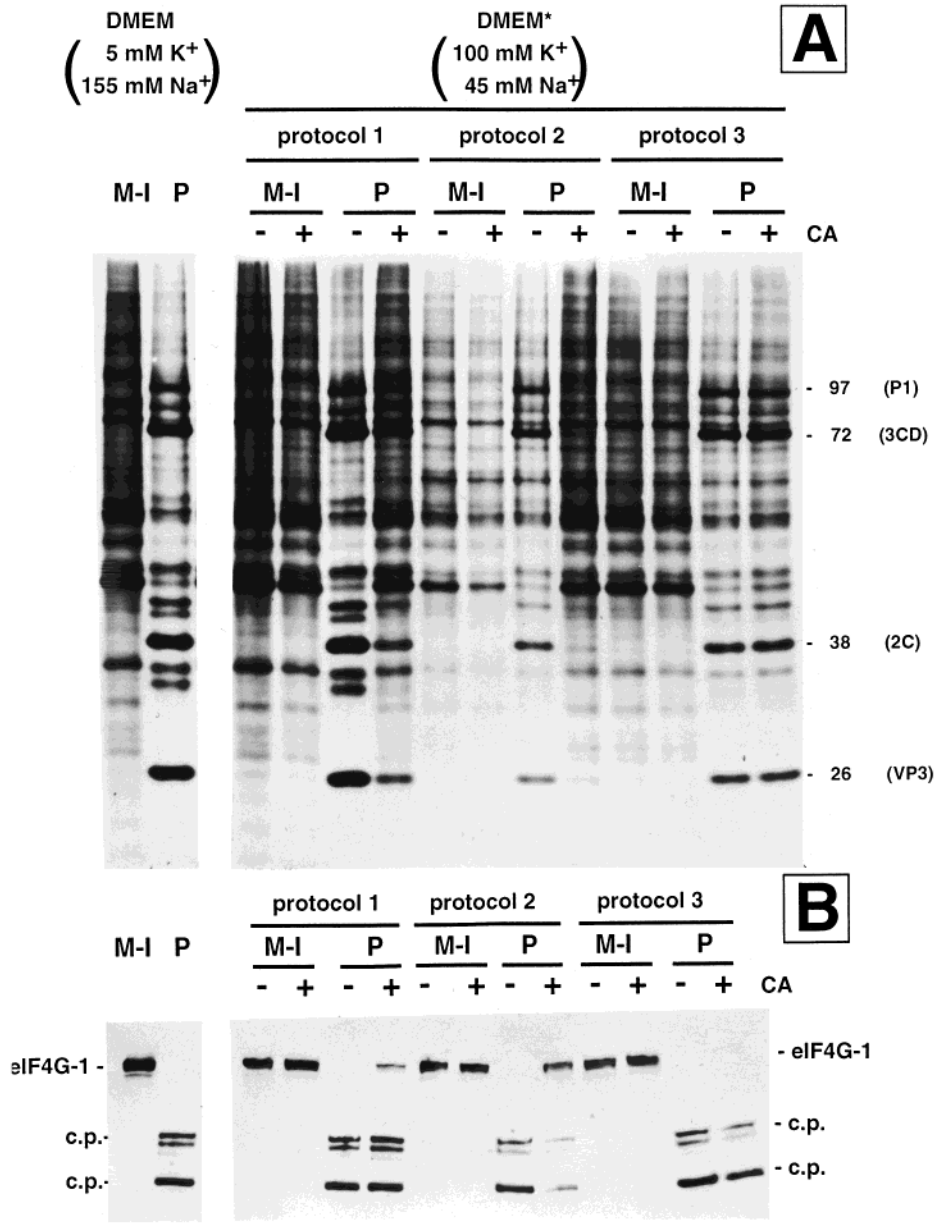

FIGURE 3: Effects of high K^+ medium plus or minus concanamycin A on poliovirus infection of HeLa cells. HeLa cells were mock-infected (M-I) or infected with poliovirus (P) at MOI 25. Cells were washed five times prior to the incubation with DMEM containing 100 mM K^+ and 45 mM Na^+ (high K^+ medium or DMEM*). Protocols 1, 2, and 3 (see Figure 1) were followed for the addition and incubation with the high K^+ medium, in the absence or in the presence of 80 nM concanamycin A (CA). Proteins were labeled at 3 h p.i. and harvested 1 h later to be processed. (A) Protein synthesis. The samples were applied to a 15% SDS−PAGE and processed as described. Molecular weights of some poliovirus proteins are indicated. (B) Analysis of eIF4G-1 cleavage. The same samples were run in a 7.5% SDS−PAGE. c.p. = eIF4G-1 cleavage products.

that small amounts of poliovirus $2A^{pro}$ efficiently cleave eIF4G-1. These findings also suggest an effect of high K^+ medium on poliovirus replication when this medium is continuously present during infection. Notably, simultaneous treatment with high K^+ medium and concanamycin A powerfully blocks the appearance of poliovirus proteins (Figure 3A), when protocol 2 is used but not with protocol 3. In addition, the presence of high K^+ medium, plus concanamycin A is the treatment that interferes with eIF4G-1 cleavage, suggesting that poliovirus entry is prevented to some extent under these conditions. In conclusion, combination of high K^+ medium with a vacuolar proton-ATPase inhibitor affects poliovirus entry. The effect seen on eIF4G-1 cleavage is due to the combined effects of concanamycin A and high extracellular K^+, since concanamycin A alone does not significantly interfere with the poliovirus-induced eIF4G-1 hydrolysis (Figure 1B).

Effects of Poliovirus Entry on HeLa Cells Membrane Potential. The K^+ ions inside the cells are the major factor that contributes to membrane potential (*65*−*68*). The generation of plasma membrane potential (PMP) results from the unequal distribution of charges between the cytosol and the external medium (*69*). The net loss of positive charges makes the cells and its membrane electronegative, rendering a membrane potential between −60 and −90 mV (*37*). The effect of both valinomycin/high K^+ medium and concanamycin A on membrane potential on HeLa cells infected or not with poliovirus have been monitored by means of flow cytometry using the carbocyanine dye $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) as a probe (*37*, *70*) (Figure 4A).

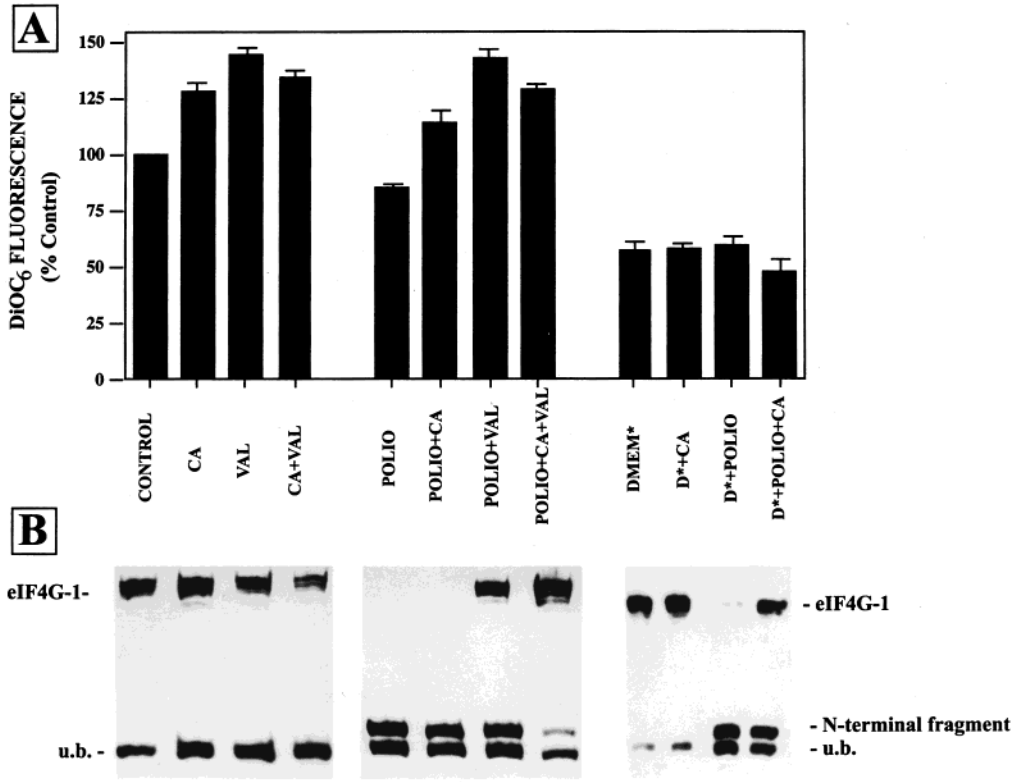

FIGURE 4: Effects of valinomycin, concanamycin A and high K^+ medium on (A) membrane potential and (B) eIF4G-1 cleavage in poliovirus-infected HeLa cells. (A) The fluorescence associated with cell membranes of HeLa cells infected or not with poliovirus at 50 PFU/cell, and pretreated with 50 μM valinomycin (VAL), 80 nM concanamycin A (CA), or both compounds, were measured with 5 nM $DiOC_6$ (3). Treatments with the compounds were made 10 min prior to the infection. Treatment with high K^+ medium (DMEM*) was as described earlier. During fluorescence measurements 50 000 events were recorded at different times post-addition (5, 10, 15, 20, 45, and 60 min) of purified poliovirus. The mean value of fluorescence in mock-infected cells at each time of measurement was taken to calculate the percentage of increase/decrease of fluorescence in poliovirus-infected cells. At each time of measurement the mean value of the fluorescence intensity distribution of mock-infected cell population (or the poliovirus-infected cell population) was used to calculate the percentage of increase/decrease of fluorescence in the corresponding mock-infected treated cells (or in the poliovirus-infected treated cell population). Data represent the mean of percentages and SEM from the six measurements in two independent experiments. (B) Entry of poliovirus was measured in parallel under the same culture conditions. Samples were collected at 3 h p.i. and run in a 7.5% SDS−PAGE, transferred to nitrocellulose membrane and incubated with a polyclonal antibody raised against amino-terminal peptide of eIF4G-1 (N-terminal fragment). u.b. = unspecific band.

Once cells have been equilibrated with the cationic dye, a subsequent electrical depolarization of the cells (i.e., a decrease in membrane potential) will cause release of dye from cells, while a hyperpolarization (i.e., an increase in membrane potential) will lead to additional dye entry into cells (*37*). Therefore, the fluorescence intensity correlates with membrane potential. Notably, fluorescence was reduced a 15% in poliovirus infected cells during the hour of adsorption. This result is consistent with the conclusion that viral adsorption and/or translocation depolarize biological membranes (*71*−*74*). Addition of 50 μM valinomycin causes a consistent hyperpolarization of HeLa cells, in agreement with the results described for other mammalian cells (*37*, *75*). Valinomycin increases the mean value of the fluorescence intensity distribution of the cell population by 44% in mock-infected cells and by 58% in poliovirus-infected cells. Concanamycin A alone also increases membrane potential in HeLa cells, whereas when combined with valinomycin it exerts a lesser effect on hyperpolarization as compared with valinomycin alone both in mock- and in poliovirus-infected cells (Figure 4A). On the other hand, increasing the concentration of K^+ and decreasing the concentration of Na^+ in the culture medium (DMEM*) drastically decreases PMP both in mock-infected and in poliovirus-infected HeLa cells, in agreement with previous works (*76*, *77*). However, PMP is not totally abolished, because the Na^+/K^+ ATPase and other ion pumps present at the plasma membrane continue to function (*37*, *65*, *66*, *76*, *78*, *79*). A parallel measurement of eIF4G-1 cleavage (Figure 4B) showed that modification of PMP (Figure 4A) either by hyper (valinomycin and concanamycin A treatments)- or depolarization (high K^+ medium incubation) clearly affects productive entry of poliovirus.

Effects of the Treatments on Uptake of Fluid-Phase Marker or Poliovirus. Chlatrin-dependent endocytosis could be selectively inhibited by cellular K^+ depletion (*43*, *44*, *80*). Since valinomycin promotes K^+ efflux from cells, we wonder if this compound would be exerting its inhibitory action by impeding endosome formation. We have assessed that valinomycin did not affect endosome and lysosome formation in HeLa cells by means of FITC-dextran, as a fluid-phase marker for these compartments (Table 1A). Control HeLa cells exhibited a punctuate fluorescence in their cytoplasm when incubated with FITC-dextran (data not shown). Fluorescence analysis of FITC-dextran endocytosis was significantly identical in nontreated and valinomycin-treated HeLa cells. However, the presence of concanamycin A quenched the fluorescence of FITC-dextran (Table 1A). This means

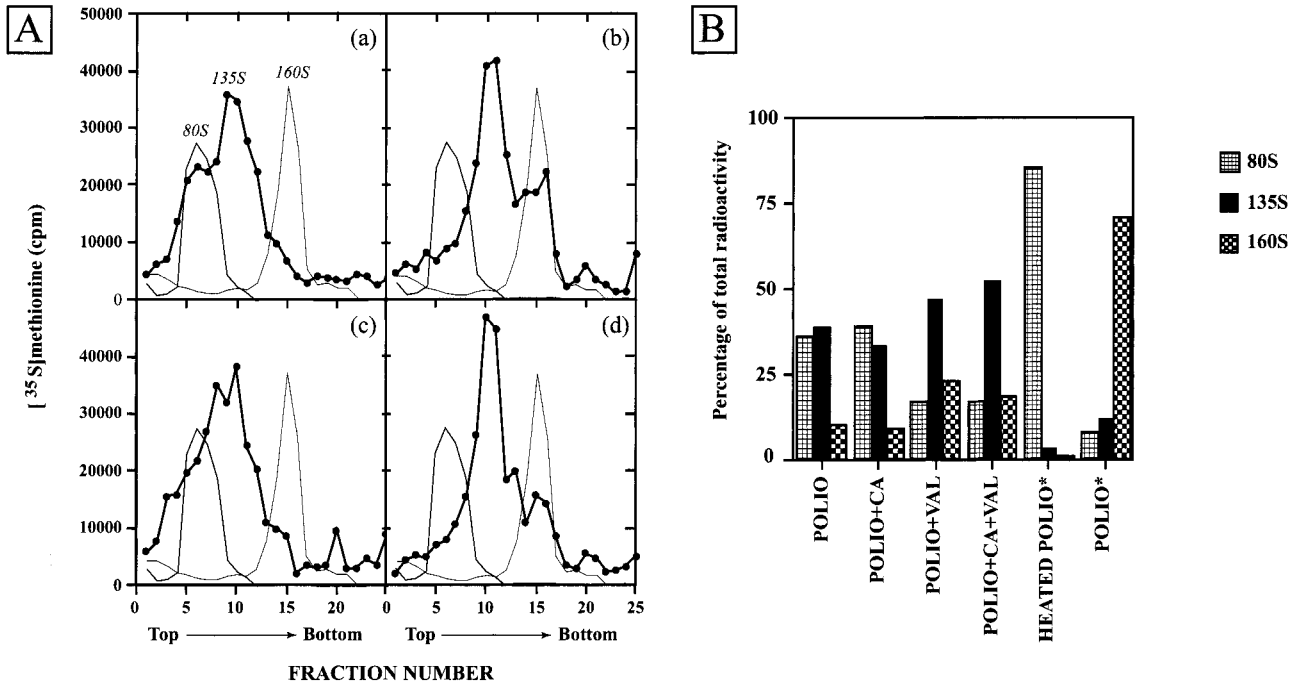

FIGURE 5: Effects of concanamycin A and valinomycin treatments in the generation of [^{35}S]methionine-labeled poliovirus subviral particles. (A) Sucrose gradient centrifugation of extracts of [^{35}S]methionine-labeled poliovirus infected cells were performed after incubation with cells in the absence (a) or in the presence of 50 μM valinomycin (b), 80 nM concanamycin A (c), or both compounds (d), following protocol 2 as described under Experimental Procedures. Gradients were fractionated from the top. Representative gradients for heat-inactivated 80S particles and 160S purified native virions are shown in all panels. (B) Quantification of radioactivity in the fractions corresponding to 80S (fractions 4−9), 135S (fractions 10−14), and 160S (fractions 15−20) particles of the gradients shown in (A). Data represent the percentage of total radioactivity in the gradients.

Table 1: Effects of Treatments on (A) FITC-Dextran endocytosis and (B) Poliovirus Attachment and Poliovirus Internalization[a]

	A		B	
treatments	mock-infected	PV-infected	[^{35}S]met-PV attachment	[^{35}S]met-PV internalization
without treatment	95 ± 2	95 ± 3	100	100
50 μM VAL	95 ± 3	96 ± 2	96 ± 4	98 ± 4
80 nM CA	2 ± 1	3 ± 1	95 ± 7	105 ± 6
50 μM VAL + 80 nM CA	3 ± 1	2 ± 1	103 ± 3	100 ± 8
DMEM*	79 ± 2	94 ± 3	97 ± 6	94 ± 4
DMEM* + 80 nM CA	4 ± 2	10 ± 2	95 ± 7	99 ± 3

[a] (A) HeLa cells growing on coverslips were treated with 50 μM valinomycin (VAL), 80 nM concanamycin A (CA), or high K^+-medium (DMEM*) for 15 min prior to poliovirus addition (MOI 25). FD (MW: 167 000; 3 mg/mL) were then also added to cells. Two hours later, cells were processed as described in methods to be observed in a fluorescence microscope. Data are expressed as the mean of the percentage of cells showing FD endocytosed in different fields randomly chosen in each coverslip. SEM is also calculated. (B) Attachment of poliovirus and internalization assays were as described in methods. HeLa cells were pretreated with different media and compounds, and then incubated with [^{35}S]methionine-labeled poliovirus particles at 4 °C for one hour (attachment). Then, poliovirus was washed and cells shifted to 37 °C to measure internalization of adsorbed particles for one hour (internalization). At different times during the two incubation periods, cells were washed and viral-associated radioactivity measured. Data represent the mean of percentages of five measurement times in two independent experiments and SEM. PV: poliovirus.

that an inhibitor of v-H^+-ATPase might be affecting the progression of fluid-phase markers to lysosomes by preventing the acidification of early compartments. To study the possibility of an inhibitory effect of treatments on attachment and/or internalization of poliovirus particles into cells, [^{35}S]-methionine-labeled poliovirus was obtained and purified. Table 1B shows that attachment of labeled poliovirus particles at 4 °C was affected neither by valinomycin nor by high K^+ medium (DMEM*), alone or in combination with concanamycin A. In addition, the amount of [^{35}S]methionine-labeled poliovirus inside the cells (37 °C) did not vary despite the treatment performed, suggesting that viral particles are internalized. Therefore, valinomycin treatment does not affect FITC dextran uptake or poliovirus attachment or internalization. Since the entry assay of radiolabeled particles measures total entry of poliovirus, the step affected by the different treatments seems to be the release of productive genomes from the early endosomes to the cytoplasm. To further assess the action of the different compounds on poliovirus entry, analysis of the formation of the subviral particles by sucrose density gradients were carried out. Figure 5 reveals the formation of both 135S and 80S subviral particles in control poliovirus-infected cells. The presence of valinomycin partially blocks the generation of RNA-free 80S particles, while the presence of both valinomycin and concanamycin A fully inhibits the release of viral RNAs from 135S particles. These findings are further support to the idea that both the H^+ and K^+ gradients in endosomes should be intact to allow poliovirus entry to the cytoplasm.

Effects of Treatments Known To Affect Endocytosis on Productive Poliovirus Entry. We wonder if other ways described to inhibit endocytosis were able to impede poliovirus productive entry. For this purpose different media, compounds or cell lines were employed (Table 2). Endocytosis through clathrin-coated vesicles can be blocked by K^+ depletion (*44*, *80*), acidification of cytosol (*42*, *81*) or

Table 2: Effect of Known Inhibitors of Endocytosis on Poliovirus Infection of HeLa Cells[a]

treatments	proteins detected[b]		eIF4G-1 cleavage[c]		α-sarcin co-entry[d]
	−	+VAL	−	+VAL	
without treatment (DMEM)	viral	cellular	0.04	70	0
acidic medium (pH 6.05)	cellular	cellular	3.45	45	NA[e]
K^+-depleted medium	viral	viral	2.30	9.3	NA
hypertonic medium	ND[e]	ND	20[e]	8.8[e]	NA
10 μM chlorpromazine	viral	cellular	4.10	90	10
0.05 mM chloroquine	viral	cellular	11.20	60	100
20 mM ammonium chloride	viral	cellular	2.80	15	26
66 μM nocodazol	viral	cellular	4.40	82	26
80 nM concanamycin A	viral	cellular	5	88	100
WT-dynamin cell line	viral	NA	0.05	NA	0
K44A-dynamin cell line	viral	NA	0.05	NA	0

[a] HeLa Cells or HeLa Tet-off/on cells were infected with poliovirus at 25 PFU/cell. Fifteen minutes prior to infection, indicated HeLa cells were incubated with different media or compounds according to protocol 2. Wild-type dynamin HeLa Tet-off line and K44A mutant dynamin HeLa Tet-off line were induced as described (*92*) 48 h prior to infection. Where indicated, treatments with the inhibitors/media in combination with valinomycin (+VAL) were made. [^{35}S]Methionine labeling of HeLa cells was carried out from 4 to 5 h p.i in the same media/compounds but without methionine and cells were processed for 15% PAGE-SDS. The same samples were analyzed for eIF4G-1 cleavage. Entry of α-sarcin was estimated as described (*91*). [b] At hour 5 postinfection. [c] Percentage of eIF4G-1 at 5 h p.i., referred to intact eIF4G-1 in mock-infected and nontreated cells. [d] Percentage of protein synthesis at 1 h p.i. referred to mock-infected and nontreated cells (*91*). [e] No eIF4G-1 fragments detected. ND: no protein detected. NA: not assayed.

hypertonic medium (*45*). With exception of cytoplasmic acidification, neither of these treatments per se affect poliovirus entry, as assessed by protein synthesis assay (Table 2). The use of nocodazole as a microtubule depolimerizing agent (*82*−*86*) revealed that poliovirus entry is accomplished even when microtubules are disrupted (Table 2). These results agree well with previous findings showing that the transit of poliovirus particles to lysosomes was not an essential step for infection (*11*). Treatment with chlorpromazine, a cationic amphiphilic drug that induces a redistribution of the clathrin-coated pit component, AP-2, to endosomes (*87*−*89*) did not prevent the appearance of poliovirus proteins. Chloroquine, a prototype weak base (*11*, *90*), and ammonium chloride, a classical compound that raises endosomal pH (*90*) also failed in inhibiting poliovirus entry, measured as translation of viral proteins (Table 2). In a parallel experiment, Semliki Forest virus entry was only inhibited by acidic medium, chloroquine, and ammonium chloride, whereas K^+-depleted medium only partially prevented this togavirus entry (results not shown). Since co-entry of α-sarcin mediated by poliovirus (*91*) was totally inhibited only by chloroquine and concanamycin A (Table 2), these compounds are the ones that efficiently prevent endosome acidification.

Only the combined use of all treatments or compounds with valinomycin showed no appearance of viral proteins (Table 2). Analysis of eIF4G-1 integrity revealed that despite the treatment performed all infected cells have a poliovirus infection since all exhibited a cleaved factor. However, all of them prevented the entry of a percentage of poliovirus particles (around 10%), whereas when combined with valinomycin, they practically inhibited productive poliovirus entry. Ammonium chloride had less effect and addition of valinomycin could not restore control levels.

We wanted to take advantage of the HeLa Tet-off line system that inducibly expresses a negative dominant mutant of the protein dynamin (K44A). Dynamin is required for pinching-off of chlatrin coated vesicles at the plasma membrane level (*41*). Therefore, the inducible expression of this mutant of dynamin might result in a blockade of the chlatrin-mediated endocytosis. Unfortunately, in our hands neither SFV nor α-sarcin entry were inhibited in this cell line even at a low MOI of SFV (results not shown). However, it has been reported that SFV also binds to galactosyl ceramide, a molecule expected to cluster in, and enter cells via caveolae (*2*) or via other noncoated pits (*32*). When it comes to poliovirus, we did not get any inhibition with this cell line, as expected (Table 2). Co-entry of α-sarcin was either not inhibited, suggesting that at least endosomes with this cargo are correctly working. We conclude that perhaps an alternative nonchlatrin-dependent pathway of endocytosis can be also activated in response to have inhibited the chlatrin-dependent endocytosis (*92*). Thus, the high multiplicity of infection used could account for the alternative ways of entry (*32*).

DISCUSSION

A still unresolved issue concerning the entry of naked virus particles into animal cells is the actual mechanism by which viral genomes cross the lipidic bilayer (*3*, *93*). Recently, a cryo-electron microscopy study has revealed movements of about 9 Å inside 135S poliovirus particles, that create gaps between adjacent subunits of VP1, VP2, and VP3. In this model, emergence from these gaps of VP4 and VP1 could form a transmembrane pore acting as a channel for RNA to enter the cells (*20*). The viral genome can reach the cytoplasm directly by penetrating across the plasma membrane and/or after endocytosis by trespassing the endosomal membrane. The use of inhibitors of virus entry provides useful tools to study the requirements of these models at the molecular level. Our present findings show that poliovirus entry is not abolished by concanamycin A or chloroquine treatments whereas the simultaneous addition of valinomycin (or high K^+ medium) produces nearly complete inhibition. Valinomycin induces an increased exit of ions, thus rendering a depletion of K^+ ions in the cells. In the opposite direction, an increased concentration of K^+ ions inside the cells (due to the incubation in high K^+ medium) also impaired poliovirus entry. In both instances, the K^+ gradient has been destroyed. The acidification of endosomes seems not to be strictly necessary although it might be contributing to some extent to productive poliovirus entry, since its impairment

by concanamycin A or chloroquine enhances the inhibitory effect of modifying the K^+ gradient. Both combined treatments are acting during an early stage of poliovirus entry since their late additions are no longer effective. Moreover, at least the inhibitory effect of valinomycin on poliovirus entry is reversible. All these data suggest that viral decapsidation and translocation of viral genomes may be dependent on several factors regulated by the ionic conditions within the cells.

Our results strongly suggest that endocytosis is somehow involved in productive poliovirus infectivity. First, concanamycin A, a well-known inhibitor of vacuolar H^+-ATPase, interferes with poliovirus RNA translocation when a factor that affects the K^+ gradient is present. Also the weak base chloroquine inhibits productive poliovirus entry combined with valinomycin. Second, the amount of radiolabeled poliovirus particles inside the cells is identical during both K^+-disrupting treatments plus concanamycin A, despite the fact that these combinations are able to fully prevent the formation of RNA-free 80S particles. This might possibly mean that, under these circumstances, some percentage of poliovirus particles (around 10%) is unable to decapsidate and gain access to the cytoplasm from the altered endosomes. The concanamycin A contribution to the inhibition of poliovirus productive entry is clearly due to its effect on vacuolar H^+-ATPase pump, since FITC-dextran does not progress to late endosomes when this compound is present.

The contribution of valinomycin (or high K^+ medium) to the inhibition of poliovirus entry is higher than concanamycin A (an inhibitor of endosome acidification) alone. We think that valinomycin (or high K^+ medium) is probably acting on the vast majority of poliovirus population, the one that enters through plasma membrane. There is a balanced equilibrium of ions throughout the plasma membrane, and both treatments (valinomycin and high K^+ medium) are clearly altering this equilibrium, thus modifying PMP. This could mean that massive entry of poliovirus through the plasma membrane would be inhibited, while concanamycin A or chloroquine could be altering only the endosomal pathway during virus entry. It is probable that the vesicles involved in poliovirus entry are micropinosomes (95 nm) sensitive to concanamycin A. In this regard, studies using free flow electrophoresis have recovered 30−40% poliovirus type 1 and 2 from compartments accessible by fluid-phase markers, whereas the rest of population is plasma-membrane bound (*31*, *33*). This partition of poliovirions population in two entry pathways could explain previous conflicting results (*17*, *19*, *22*−*25*). Our present work shows that the small fraction of poliovirus entering by endosomes (about 10%) is productive too. Moreover, this fraction could account for the co-entry of α-sarcin. Therefore, bafilomycin A1, concanamycin A or chloroquine inhibit α-sarcin co-entry by endosome pathway (*24*), whereas they are unable to impede the simultaneous entry of poliovirus through plasma membrane.

Bearing in mind the hypothesis that a small percentage of poliovirions enter by endocytosis, it is tempting to speculate that membrane potential alteration is also taking place in endosomes with poliovirus cargo. Endosomes would exhibit a high content in Na^+ and H^+ in HeLa cells: in the presence of valinomycin, K^+ from cytosol would be expected to increase these positive charges. In the opposite direction, influx of K^+ from high K^+ medium treatment leads to a decrease of positive charges inside endosomes. Some authors have found that valinomycin consistently increases the initial rate of acidification in isolated brain chlatrin-coated vesicles, rendering an interior positive membrane potential (*94*). As observed by several laboratories and from our present results, the initial stages of viral infection lead to depolarization of plasma membrane (*71*−*74*) and subsequently, of endosomes. Thus, any external change modifying the optimum ionic concentration inside the endosomes would block viral decapsidation as evidenced by the formation of 80S subviral particles. Valinomycin alone (or high K^+ medium) also might be altering the direct entry of poliovirus across the plasma membrane.

The effects of valinomycin on poliovirus entry may possibly be not restricted to its effects on membranes. This ionophore has been described to be an inhibitor of ATP production in mitochondria; therefore, it could not be ruled out a lateral effect on poliovirus entry due to the lack of energy. Nevertheless, it has been reported that concentrations of valinomycin of 50 μM did not impair cell viability: lactate dehidrogenase release was less than 2% of control (*95*) and cytofluorimetric analysis has also revealed that valinomycin had no gross effects on cell viability (*79*). A similar conclusion has been assessed in our present experiments, where 50 μM valinomycin incubations up to 4 h did not significantly affect cell viability and protein synthesis either in infected cells (Figures 1−3) or uninfected cells (data not shown). Therefore, a direct inhibition of RNA input translation by valinomycin is not likely.

In conclusion, productive entry of poliovirus would depend not only on K^+ gradient but also on the pH gradient. This points out to the idea that the chemiosmotic gradient, composed by membrane potential ($\Delta\varphi$) and pH gradient (ΔpH), provides the energy able to translocate the particles of animal viruses across membranes (*91*). However, caution should be taken in the interpretation of the data because of side effects of the performed treatments on other cellular compartments. To the best of our knowledge, these are the only treatments described that block productive poliovirus entry into cells. Further studies about naked virus entry may possibly benefit from the use of these two combined treatments. Finally, the use of a sensitive, fast, nonradioactive and reliable test to estimate productive poliovirus entry, such as the cleavage of eIF4G-1 described here, will be useful for poliovirus entry studies.

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