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Characterization of the Ion Channels Formed by Poliovirus in Planar Lipid Membranes

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The steps in poliovirus infection leading to viral entry and uncoating are not well understood. Current evidence suggests that the virus first binds to a plasma membrane-bound receptor present in viable cells, leading to a conformational rearrangement of the viral proteins such that the virus crosses the membrane and releases the genomic RNA. The studies described in this report were undertaken to determine if poliovirus (160S) as well as one of the subviral particles (135S) could interact with membranes lacking poliovirus receptors in an effort to begin to understand the process of uncoating of the virus. We report that both forms of viral particles, 160S and 135S, interact with lipid membranes and induce the formation of ion-permeable channels in a manner that does not require acid pH. The channels induced by the viral particles 160S have a voltage-dependent conductance which depends on the ionic composition of the medium. Our findings raise the possibility that viral entry into cells may be mediated by direct interaction of viral surface proteins with membrane lipids.

Poliovirus is a prototypical member of the *Picornaviridae* family. The plus-stranded RNA genome of this nonenveloped virus is tightly packed within an icosahedrally symmetric spherical shell which is composed of 60 copies of each of four proteins (VP1 to VP4). The myristoylated VP4 and the amino-terminal sequences of the other three capsid proteins are located on the inner surface of the poliovirus virion (8).

Infection of cells by poliovirus starts as the virus particle attaches to the receptor at the cell membrane (2, 6). Receptor binding leads to a conformational transition that results in altered particles which lack VP4 and sediment with a coefficient of 135S (compared to 16 0S for the intact virion) (3a, 6, 9). This alteration in sedimentation behavior is further accompanied by the relocation and exposure of most of the amino-terminal regions of VP1 from the inner to the outer surface of the virion shell. Exposure of the amino-terminal regions of VP1 confers membrane binding properties to the 135S particles, which has been observed by Fricks and Hogle (5), using a liposome flotation method. However, the subsequent stages of penetration and uncoating remain poorly defined.

For viral replication to take place, the viral RNA must traverse the plasma membrane and be uncoated and released to the cytosol. Several hypotheses have been put forth to explain viral entry and uncoating, ranging from receptor-mediated endocytosis of the whole virion, to direct penetration of the plasma membrane by the 160S particle (hereafter referred to as simply 160S), to entry of the subviral particle 135S by either of those mechanisms (references 1 and 13 and references therein). Binding and anchoring of the viral particle to a membrane are required steps in any of these hypotheses.

In this report, we present evidence that domains of the native virion (160S) and the receptor-altered viral particle (135S) bind and interact with lipid bilayers to form ion-permeable channels. These channels could explain the changes in the ionic permeability of virus-infected cells observed by Carrasco and colleagues (4, 18). Moreover, the channels formed by 135S and 160S have distinctly different conductances and dependence on ionic environment, voltage, and temperature, suggesting that they might play a role at different stages of the virus life cycle.

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MATERIALS AND METHODS

Cells and media. HeLa cells in suspension culture were maintained in Joklik's modified minimal essential medium supplemented with 5% horse serum (GIBCO). Virus stocks were stored and diluted in phosphate-buffered saline (PBS) containing no calcium or magnesium.

Virus propagation and purification. Poliovirus (serotype 1, Mahoney strain) was grown by infecting suspension HeLa cells with a virus stock grown at low multiplicity from a plaque derived by transfection of the infectious cDNA (15). Virus was labeled continuously by the addition of [^{35}S]methionine from 3 h postinfection until the infected cells were harvested at 6 h postinfection (15). Virus was purified by banding on cesium chloride gradients, and virus concentration was estimated by optical density at 260 nm, using an extinction coefficient of 7.7 optical density units/mg/ml (19).

Preparation of 135S. Purified [^{35}S]methionine-labeled virus (approximately 100 virions per cell) was allowed to attach to HeLa cells in suspension (10^7 cells/ml) at 4°C for 60 min. Cells were washed with PBS to remove unattached virus, resuspended in minimal essential medium, and incubated at 37°C for 60 min. After pelleting of the cells, the eluted virions in the supernatant were purified as the 135S particles on 15 to 30% sucrose gradients (16). The fractions were pooled, desalted, and concentrated on Centricon-10 filters (Amicon) into PBS.

Lipid bilayers. (i) Membrane formation. The membranes used in this study were formed from apposition of two monolayers of soybean lecithin (asolectin; Associated Concentrate, Woodside, N.Y.), using the method described by Montal and Mueller (14). Briefly, the two chambers with a volume of 1.5 ml each were separated by a thin (12-μm) Teflon partition (fluorofilm; Dielectrix Corp., Farmingdale, N.Y.) containing a small hole approximately 80 μm in diameter which was pretreated with a 3% solution of squalene in pentane. Approximately 0.5 ml of salt solution was added to each compartment, and the lipid was spread from a solution in pentane (15 mg/ml) onto the surface of the electrolyte solutions. The membrane was formed by raising the liquid level in each compartment until the hole was completely covered. Membrane formation was monitored continuously through measurement of the membrane capacitance.

(ii) Interaction of viral particles with lipid membranes. An aliquot of a suspension of viral particles (160S or 135S) was added to one of two compartments (*cis*) under continuous stirring to a final concentration of ~10^5 particles/ml (160S) or ~10^7 particles/ml (135S). The amount of particles in the chamber was chosen so that the interaction with the lipid bilayers would take place within 15 to 30 min after addition of viral particles. The electrolyte composition at the time of interaction is specified where appropriate. Changes in the

salt composition (or pH) were made through the addition of a small aliquot of a concentrated KCl (or HCl) solution to one or both compartments. After addition, the current response to an applied voltage (usually 60 mV) was tested every 5 min, and data collection started 5 min after channel activity was first detected. We determined that the single-channel conductance did not change with time within the first 2 h after detection but increased after this time. As a consequence, all experiments were performed within 2 h after the appearance of channels. The experiments were conducted at either 21 or 31°C, and in both cases, temperature was controlled by setting the chamber in the sample holder of a precision temperature controller (Microdevices model 500 KT; RPI, Wayland, Mass.). Changes in temperature could be completed within 4 min. All experiments involving temperature changes were started at 21°C.

(iii) Data acquisition analysis. Electrical contact was made through Ag-AgCl electrodes connected to the solution in the chambers via 3 M NaCl–5% agar bridges. The voltage-controlled side was the *cis* side. The electrical recordings and data processing were done as previously described (22). Briefly, the current in response to applied voltages (3-min duration) was recorded with a Dagan 3900 patch-clamp amplifier (Dagan Instruments, Minneapolis, Minn.) after it was filtered at 2 kHz and digitized at 0.1 or 0.5 ms per point for subsequent analysis. The single-channel conductance was calculated by using the mean value obtained from Gaussian fits (Peak Fit; Jandel Scientific Software, San Rafael, Calif.) to the distribution of the current amplitude. The permeability of the bilayer for cations relative to that of anions (p) was determined through measurements of the reversal potential (V_{rev}, the potential at which there is no current through the channel) in the presence of a salt concentration gradient and applying the Goldman-Hodgkin-Katz equation to calculate the relative permeability (7):

$$V_{rev} \frac{F}{RT} = \ln \frac{p[C]_0 + [A]_i}{p[C]_i + [A]_0}$$

RESULTS

General characteristics of the conductance induced by poliovirus particles. When poliovirus interacts with its receptor at the membrane of the host cell, a substantial fraction of the bound virus is released in an altered form: there is a loss of the internal capsid protein VP4, and the new particles sediment at 135S. To test whether the native virus as well as 135S could interact with lipid bilayers devoid of receptor, we exposed membranes to either particle under the same set of ionic conditions, at 31°C.

(i) General characteristics of the conductance produced by 160S. Upon addition of virus to a preformed lipid bilayer, measurements of the current across the membrane in response to a fixed potential showed abrupt changes characteristic of the presence of ionic channels (Fig. 1A, trace b). The current fluctuations are dependent on the presence of virus, because there are no current changes in the absence of virus (trace a). Thus, the native virion can bind and interact with the lipid bilayer at 31°C, producing a change in its permeability. Figure 1B depicts the results of the analysis of the current amplitude distribution, using data such as shown in Fig. 1A, trace b. The first peak (on the left) corresponds to the closed state of the channel (and is superimposable on the peak that we obtained when analyzing traces such as trace a in Fig. 1A). The second peak corresponds to the current through the open channel in response to a +75-mV applied potential. The mean value of the current distribution (8 pA) yields a single-channel conductance of 110 pS for the open state of the channel. Figure 1C shows the conductance-voltage relationship calculated from data obtained in the manner described, in response to other applied voltages to the same bilayer. The conductance-voltage curve indicates that the single-channel conductance has a strong voltage dependence, increasing 45 pS per 10-mV change in voltage (at voltages greater than 50 mV).

We obtained essentially identical results when the pH of the solutions in both the *cis* and *trans* compartments was lowered to 5.4 in the presence of virus, as well as when virus was added to membranes formed at acid pH. Thus, the low pH is not a necessary condition for either channel formation or opening (Fig. 2). This result is consistent with that of Pérez and Carrasco (18), who have shown that poliovirus infection is not affected by increases of the endosomal pH.

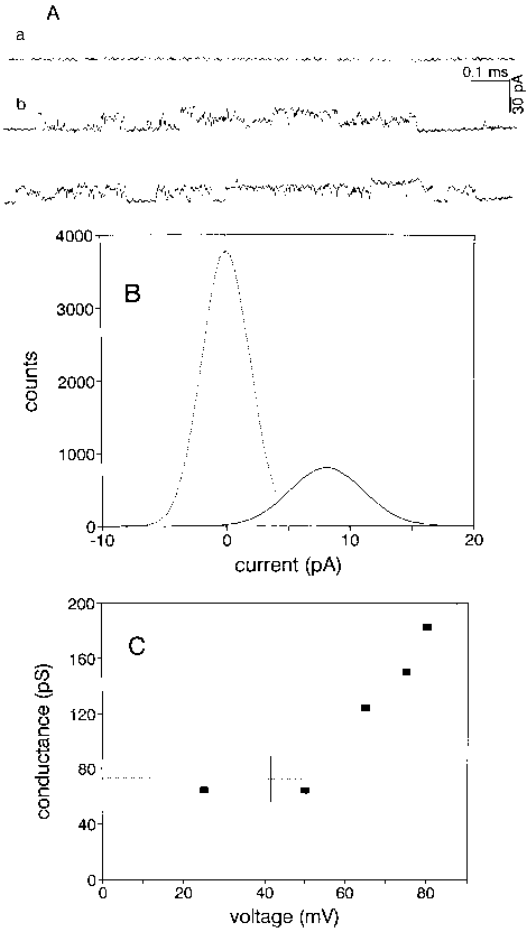

FIG. 1. Modification of the bilayer conductance induced by 160S. (A) Time course of the current in response to a +75-mV applied potential. Trace a corresponds to the current response in the absence of added virus to the *cis* compartment (V = +75 mV). Trace b was taken 60 min after addition of virus (2×10^5 particles), and the two traces shown are continuous. (B) Fitted amplitude histogram corresponding to the currents shown in panel A, trace b. The voltage was applied for 3 min, and the current response was digitized and stored. The digitized current traces were processed further after completion of the experiment, to obtain the amplitude histogram. The histograms were subsequently fitted with Gaussian curves, which are displayed as the number of times a particular value of the current was obtained (counts) in the ordinate as a function of the current (abscisa). The closed state was arbitrarily set to zero current to highlight the value of the open state. In general, the current through the closed state equaled that of the untreated bilayer at the same potential. (C) Conductance-voltage relationship for the state shown in panel B. The conductance was obtained by dividing the mean current (obtained from the Gaussian curves from the fit [see above]) by the value of the applied voltage. The points shown correspond to mean values obtained in 10 different bilayers. The horizontal line corresponds to the mean value of the conductance at potentials lower than 55 mV, and the vertical line corresponds to 1 standard deviation. Salt, 300 mM NaCl, pH 7.4; temperature, 31°C.

(ii) General characteristics of the conductance induced by 135S. Figure 3A shows the current-time plots of a bilayer before (trace a) and after (trace b) addition of the receptor-modified poliovirus particle, 135S. As was the case when native poliovirus was added to the aqueous solutions, trace b clearly shows that the exposure of bilayers to 135S changes the permeability of lipid bilayers and that a domain of the particle binds and interacts with the membrane to form ion-permeable channels. Comparison of Fig. 1C and 3B indicates that the

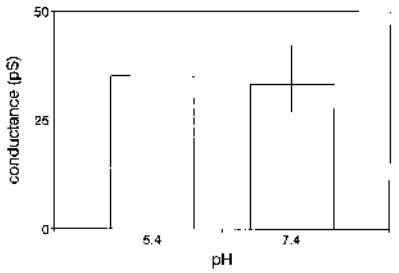

FIG. 2. Conductance induced by 160S when the pH of the medium was changed from 7.4 to 5.4. Bilayers were formed at pH 7.4 and virus was added. After data such as those in Fig. 1 were obtained, the pH was shifted to 5.4 through addition of an aliquot of HCl and the currents at various potentials were determined again. The pH of the solutions was measured at the end of the experiment. In one instance the bilayer was formed at pH 5.4 and virus added at this pH. The conductance obtained under these circumstances was indistinguishable from that found by shifting the pH. Data at pH 7.4 are shown with the standard deviations. The data shown at pH 5.4 represent the mean from two different experiments, not including the one in which the bilayer was formed at pH 5.4. Applied potential, +50 mV; salt, 150 mM NaCl–50 mM KCl.

values of the single-channel conductance formed by the two viral particles are of the same order of magnitude, with those formed by 135S being about twice as large as those formed by 160S at voltages lower than +50 mV. The main differences in the channels induced by the two particles are evident by comparing Fig. 1C and 3B. The channels induced by 160S open only when the *cis* side is positive, while those produced by 135S open at positive as well as at negative potentials. The conductance of the channels induced by 160S is voltage dependent, whereas those induced by 135S are voltage independent.

To determine the ion selectivity of the channels induced by either 160S or 135S, we measured the reversal potential by creating a gradient in the concentration of NaCl and the cat-

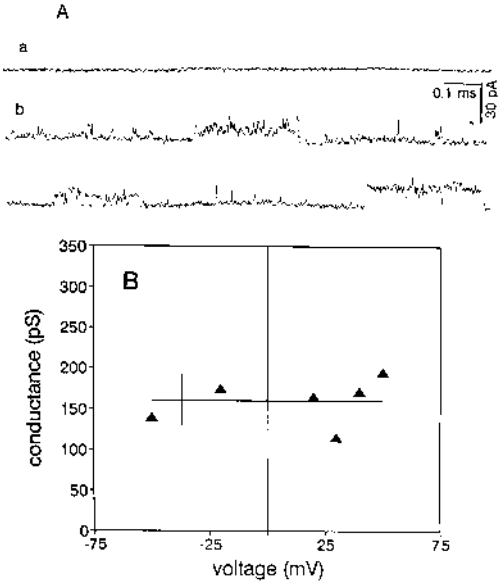

FIG. 3. Conductance induced by 135S in a lipid bilayer. (A) Time course of the current in response to a +50-mV applied potential. Trace a corresponds to the current response in the absence of added viral particles. Trace b was taken 35 min after addition of 135S (9×10^6 particles), and the traces are continuous. (B) Conductance-voltage relationship obtained as detailed in legend to Fig. 1. The points shown correspond to mean values obtained in 5 different experiments. The line corresponds to the mean value of the current and the bar corresponds to 1 standard deviation. Salt, 300 mM NaCl, pH 7.4; temperature, 31°C.

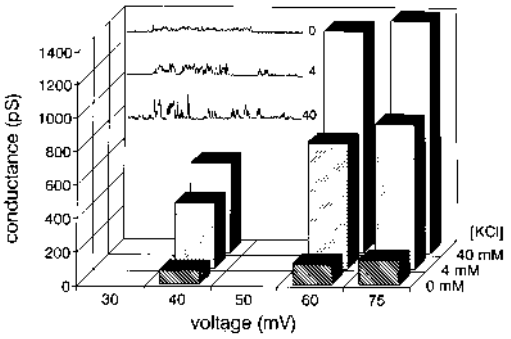

FIG. 4. Conductance of bilayers exposed to 160S as a function of voltage, as the concentration of KCl in the medium is varied. Bilayers were formed in the presence of 300 mM NaCl (pH 7.4), and 160S was added. After characterization of the conductance at the potentials indicated, an aliquot of concentrated KCl was added under continuous stirring, to have 4 mM in each compartment and the conductance determined again. After this sequence, another aliquot of KCl was added to have total concentrations of 40 mM in both compartments and the conductance was determined again. The sequence was repeated in three different experiments, and care was taken to avoid data gathering 2 h after addition of viral particles. We also established that the conductance, at each [KCl] reported, attained a steady value within 5 min after addition. The data shown represent the mean value of the three experiments. The inset shows the current fluctuations at +60 mV obtained at the [KCl] indicated. Temperature, 31°C.

ion/anion permeability ratio calculated by using the Goldman-Hodgkin-Katz equation (see Materials and Methods). The value obtained for the ratio of cation to anion permeability is 98 ± 10 (standard deviation) ($n = 3$), indicating that both types of channels are more permeable to cations than to anions.

Effect of KCl on the conductance induced by poliovirus particles. Since the viral particle-induced channels are selective for cations, we explored the possibility that ions other than Na could have an effect on the conductance of the channels.

Figure 4 illustrates the effect of KCl on the voltage dependence of the single-channel conductance at various potentials and KCl concentrations, showing that as the concentration of KCl is increased, the single-channel conductance increases. In the absence of KCl, the conductance-voltage curves of the single channel are voltage dependent for voltages up to 100 mV. In contrast, in the presence of KCl, the conductance-voltage curves approach a maximum value, independent of the voltage but dependent on the concentration of KCl.

In contrast to the conductance response shown in Fig. 4 for the conductance of the channels induced by 160S, the conductance of the single channel induced by 135S is practically insensitive to the concentration of KCl at all voltages and temperatures tested (not shown).

Effect of temperature on the conductance induced by poliovirus particles. Taking advantage of the fact that specific domains within the interior of the poliovirus capsid are reversibly accessible at the surface of the virion with increasing temperatures, we determined the characteristics of the channels as the temperature was varied. When bilayers were formed at 21°C, 135S was capable of inducing channels (Fig. 5A, trace a), with a lower conductance than that at 31°C. In contrast, no channel openings were observed upon exposure of lipid bilayers to 160S at 21°C (trace b). When the aqueous solutions surrounding the membrane to which 160S had been added were heated, channels opened with the same characteristics as those detected at 31°C in Fig. 1 (Fig. 5A trace c). Since opening of channels induced by 160S was detected immediately after temperature equilibration at 31°C, this result suggests that the 160S particles were previously adsorbed to the membrane at 31°C and that either channel openings at 21°C were too small

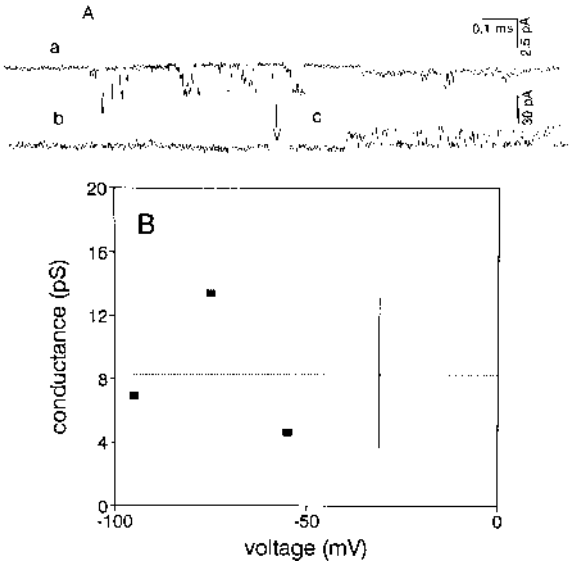

FIG. 5. Effect of temperature on the conductance induced by poliovirus particles. (A) Time course of the current in response to an applied potential. Trace a was obtained after addition of 135S to the *cis* compartment at 21°C and an applied potential of 95 mV. Trace b shows the current response to an applied potential of V +90 mV 60 min after addition of 160S to the *cis* compartment. Note that there are no current fluctuations. At the arrow, the temperature in the chamber was changed to 31°C in approximately 4 min (no voltage applied). Trace c was obtained as soon as the temperature was equilibrated. at an applied potential of +90 mV. (B) Conductance-voltage relationship obtained as detailed in legend to Fig. 1 for bilayers in the presence of 135S at 21°C. Of note is that no openings were observed when the polarity of the applied pulse was reversed. The horizontal line corresponds to the mean value of the conductance obtained in the three different bilayers, and the vertical line represents 1 standard deviation. Salt, 300 mM NaCl, pH 7.4. The time marker (100 ms) applies for all traces.

or the channel kinetics were too fast for detection, or both. However, we cannot rule out the possibility that protein domains important for channel formation were not accessible at the lower temperature.

During the course of these experiments, we also observed that the channels induced by 135S at 21°C opened only if the applied potential was negative (Fig. 5B). We do not have an explanation for this observation.

DISCUSSION

The experiments described in this report were designed to determine if poliovirus particles interact with lipid membranes and induce a change in their permeability properties, as has been shown to occur in the case of the subvirion particles of reovirus (21) and has been suggested to occur in the case of poliovirus (1). Our data show that not only the subvirion particle (135S) but also the intact virion (160S) of the Mahoney strain of poliovirus interacts with lipid bilayers in the absence of the receptor. This interaction results in the formation of channels which are selective for cations and are pH independent. This pH independence is consistent with the results of Pérez and Carrasco (18) indicating that a low-pH step is not required for poliovirus infection. However, the characteristics of the channels formed by the two types of virus particles are distinct and different. Channels formed by the 135S particle are temperature independent and are not voltage gated (except at 21°C). In contrast, appearance of the channels formed by 160S depends on the potassium concentration, temperature, and polarity of the membrane potential. Our findings are consistent with the studies of Fricks and Hogle (5), who showed that the poliovirus particle 135S binds to lipid vesicles and with those of López-Rivas et al. (12), showing that infection by poliovirus modifies the permeability of cells.

The biological function of either of the channels is not known at present. However, because both of the viral particles tested in these studies are present during the entry process, it is possible that these channels are important for poliovirus entry into cells. This hypothesis then raises the possibility of infection of cells believed heretofore to be nonpermissive and are consistent with the recent findings that 135S can infect nonsusceptible cells in a receptor-independent manner (3).

Several functions could be proposed for ion channels during viral entry. One model would be that the channels provide a pathway for transport of the RNA genome across the membrane into the cytosol, as hypothesized by Silva et al. (20) for the southern bean mosaic virus and by Rueckert (19) for poliovirus. In this context, we have shown that the channels formed by 160S open when the voltage is positive. If these channels are also formed during the initial stages of infection, then they could open because the potential across the plasma membrane is positive (extracellular phase with respect to cytosol). Because the approximate diameter for a channel with a conductance of 500 pS is 20 Å (7), the channels that we have described could be large enough to allow passage of a fully extended single-stranded RNA molecule (approximate diameter of 10 Å).

A second, alternative model would be that viral infection requires the endosomal route of entry (19). The channels could then play a role in the release of infectious particles to the cytosol via lysis of the endosomal membrane. This could occur as a consequence of the changes in the ionic permeability of the membrane brought about by the opening of the channels, since a conductance of 100 pS (a flux of approximately 10^7 ions/s) could lead to a substantial alteration of the ionic composition of the cellular compartment.

The specific domains that form these channels remain to be defined. A pore structure has been proposed to exist at the center of pentameric subunits (at fivefold axes) in the protein shell of icosahedral viruses (20). However, we do not think that this pore structure forms the channels described here. Rather, we favor the hypothesis that these channels are formed by the amino termini of VP1 alone or with the myristoylated VP4 protein. This is due to several lines of evidence. The amino termini of VP1 mediate membrane binding of the 135S particle (5). Exposure of VP4 and the amino termini of VP1 at the surface of the 160S particle and the channel openings are temperature dependent (11). In addition, the conversion of 160S to 135S results in the selective loss of VP4; the channels formed by these particles show different characteristics (Fig. 1 and 3). Finally, a mutation in VP4 leads to a nonviable mutant that is defective in the penetration/uncoating process (17).

Interactions of subviral particles with planar bilayers have been reported for another nonenveloped mammalian virus (21). In this work, the authors reported that the infectious subviral particles of reovirus form anion-selective channels in planar lipid bilayers. Even though the poliovirus channels have different ionic selectivities and properties, the finding that two structurally and biologically different nonenveloped viruses form channels suggests that this may be an important general feature of the replication cycle of nonenveloped viruses.

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