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Potassium Ion Channels of Chlorella Viruses Cause Rapid Depolarization of Host Cells during Infection

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Previous studies have established that chlorella viruses encode K⁺ channels with different structural and functional properties. In the current study, we exploit the different sensitivities of these channels to Cs⁺ to determine if the membrane depolarization observed during virus infection is caused by the activities of these channels. Infection of *Chlorella* NC64A with four viruses caused rapid membrane depolarization of similar amplitudes, but with different kinetics. Depolarization was fastest after infection with virus SC-1A (half time [*t*_{1/2}], about 9 min) and slowest with virus NY-2A (*t*_{1/2}, about 12 min). Cs⁺ inhibited membrane depolarization only in viruses that encode a Cs⁺-sensitive K⁺ channel. Collectively, the results indicate that membrane depolarization is an early event in chlorella virus-host interactions and that it is correlated with viral-channel activity. This suggestion was supported by investigations of thin sections of *Chlorella* cells, which show that channel blockers inhibit virus DNA release into the host cell. Together, the data indicate that the channel is probably packaged in the virion, presumably in its internal membrane. We hypothesize that fusion of the virus internal membrane with the host plasma membrane results in an increase in K⁺ conductance and membrane depolarization; this depolarization lowers the energy barrier for DNA release into the host.

Paramecium bursaria chlorella virus type 1 (PBCV-1) (family Phycodnaviridae, genus *Chlorovirus*) is the type member of a group of large, icosahedral, plaque-forming, double-stranded DNA viruses that replicate in certain unicellular, eukaryotic chlorella-like green algae (27). Chlorella viruses can be isolated from freshwater collected throughout the world, and they are among the largest and most complex viruses known. PBCV-1 contains an internal lipid bilayered membrane surrounded by an outer glycoprotein capsid (33). The 330-kb PBCV-1 genome has ~370 nonoverlapping, protein-encoding genes and 11 tRNA-encoding genes. One of these genes encodes a 94-amino-acid protein, called Kcv, that forms a functional K⁺ channel in heterologous systems, including *Xenopus* oocytes (21), human embryonic kidney (HEK) 293 cells (19), and Chinese hamster ovary (CHO) cells (10). Kcv conductance is blocked in *Xenopus* oocytes by the same molecules (Ba²⁺ and amantadine, but not Cs⁺) that inhibit PBCV-1 replication in its host, *Chlorella* NC64A (12, 21), suggesting that Kcv serves an important function in virus replication. Since Ba²⁺ is a charged molecule and hence membrane impermeable, it presumably acts from outside the cell (10). This observation led to the hypothesis that Kcv is present in the PBCV-1 particle (17), possibly as a component of its internal membrane. Fusion of the virus membrane with the host plasma membrane during infection could result in increased K⁺ conductance in the host. In freshwater algae, such as *Chlorella*, the prevailing membrane potential is always more negative than the K⁺ equilib-

rium voltage. Hence, an increase in K⁺ conductance will inevitably result in membrane depolarization (1).

This hypothesis is consistent with three observations. (i) PBCV-1 infection leads to immediate depolarization of the host membrane (17). This depolarization is host specific and sensitive to the same compounds that inhibit Kcv conductance and PBCV-1 replication. (ii) PBCV-1 infectivity (25) and virus-induced depolarization do not occur if the virus internal membrane is destroyed by organic solvents prior to infection (17). (iii) The *kcv* gene is transcribed as a late gene during PBCV-1 replication (11); typically, virion-associated proteins are expressed as late genes.

While these results are consistent with the hypothesis that Kcv alters the host plasma membrane during PBCV-1 infection, the evidence is circumstantial. For example, it is possible that the sensitivities of PBCV-1-induced membrane depolarization to Ba²⁺ and Cs⁺ could reflect different sensitivities of ion channels in the host plasma membrane.

Recently, genes encoding Kcv-like proteins were isolated from 40 additional chlorella viruses (12). Collectively, these viruses encode six Kcv homologs, with amino acid substitutions occurring in most of the functional domains of the protein. Furthermore, the electrophysiological properties of some of these channels differ from those of the PBCV-1 reference channel Kcv. For example, Kcv channels encoded by the NY-2A and IL-5-2s1 viruses are sensitive to Cs⁺, while Kcv channels from SC-1A virus and PBCV-1 are not (9, 12). Here, we use the differential sensitivities of Kcv channels to Cs⁺ to examine the role of the viral K⁺ channels in host membrane depolarization. We report that infections with PBCV-1 and three other viruses cause rapid depolarization of the host cell membrane. However, Cs⁺ inhibits virus-induced depolarization only in viruses that encode a Cs⁺-sensitive

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K⁺ channel. Consequently, the differences in responses to PBCV-1 infection in the presence of Ba²⁺ or Cs⁺ cannot be explained by host properties. Instead, the differences are related to the viruses and hence to the different sensitivities of their K⁺ channels to channel blockers.

MATERIALS AND METHODS

Growth of viruses and cells. Growth of *Chlorella* NC64A cells on MBB medium, plaque assay, the production of the viruses, and the isolation of virus DNAs have been described (28, 29, 31). Plaque inhibition studies were conducted as follows. Actively growing chlorella cells in liquid culture were infected at a multiplicity of infection (MOI) of 5 at the same time the inhibitors were added. The mixtures were incubated at 25°C for 2 h with moderate shaking, briefly centrifuged to remove unattached virus, and then plated for infective centers on inhibitor-containing media as described previously (29). Effective inhibitors resulted in an inhibition of infective centers, as well as in a reduction of the plaque sizes. For quantification, we monitored infective centers because it is a more accurate way to measure the effects of blockers on virus infection.

For the fluorescent and electron microscopic measurements, cells from cultures in an exponential growth phase were incubated in a glass cuvette (1.7 ml⁻¹) with an average cell density of 3.5 × 10⁶ ml⁻¹ (fluorescent measurements) or 2 × 10⁷ ml⁻¹ (electron microscope) in MBBM. Sterile tetracycline (10 µg/ml) was added after autoclaving (18). The cells were continually stirred throughout the experiments.

Fluorescence measurements. Changes in membrane potential were monitored with the voltage-sensitive fluorescent dye bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (bisoxonol) (Molecular Probes, Eugene, OR) at a final concentration of 1 µM. Cells were incubated with the dye 15 min prior to the measurements. Fluorescence was monitored with a spectrofluorophotometer (RF 5001PC [Shimadzu, Kyoto, Japan] and FP-6200 [Jasco, Tokyo, Japan]) (see Fig. 5) with excitation at 540 ± 5 nm and emission at 560 ± 10 nm, respectively.

In control recordings with only *Chlorella* cells and bisoxonol, a continuous decrease in fluorescence occurs. This decrease is probably due to photobleaching of the dye during the short exposure to excitation light during the measurements. In a typical experiment, the continuous decrease could be fitted with a single exponential function, providing a mean time constant of 7.1 ± 0.5 min (*n* = 8). To correct for this change in background fluorescence, we first recorded the decrease in fluorescence over a period of 6 min before adding virus to the incubation medium. The initial decrease in fluorescence was extrapolated by fitting the data with an exponential function using the above time constant. We also tested the effect of adding viruses at the same concentration used in the experiments to a medium containing only bisoxonol. These measurements indicated that the viruses do not give a fluorescence signal by direct interaction with the dye.

The bisoxonol dye was not directly calibrated, because ionophores such as valinomycin have no effect on *Chlorella* cells. The dye was therefore calibrated by measuring the bisoxonol fluorescence of *Chlorella* NC64A cells at room temperature versus that at 4°C. Low temperature inhibits the H⁺-ATPase in plant cells with the result that the membrane depolarizes from a negative ATPase-dominated voltage to the diffusion potential. In control experiments, the effect of cooling resulted in a fluorescence increase of 19% ± 0.4% (*n* = 15).

All data are presented as the mean ± standard deviation of *n* independent replicates.

Electrophysiology. We used a two-electrode voltage clamp (Gene clamp 500; Axon Instruments) to record K⁺ currents from oocytes. Electrodes were filled with 3 M KCl and had a resistance of 0.2 to 0.8 MΩ (in 50 mM KCl). The oocytes were perfused at room temperature with a standard bath solution containing 20 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4, with KOH, at a rate of 2 ml min⁻¹. The osmolarity of the solution was adjusted to 215 mosmol with mannitol.

The standard clamp protocol consisted of steps from a holding voltage of -20 mV to voltages in the range +80 mV to -160 mV; tail currents were measured at -80 mV. Instantaneous and steady-state currents were sampled after 10 ms and at the end of the voltage step (usually 800 ms), respectively.

Electron microscopy. Cells were concentrated by centrifugation at various times postinfection (p.i.) and immediately fixed with a cacodylate-buffered (pH 6.8) 2% glutaraldehyde-2% formaldehyde (freshly prepared from paraformaldehyde) mixture. After being washed in buffer, samples were postfixed in OsO₄ (2% in the same buffer), dehydrated in a graded acetone series, and embedded in Spurr's medium (26). Ultrathin sections were obtained with diamond knives, poststained with uranyl acetate and lead citrate, and examined with a Zeiss EM

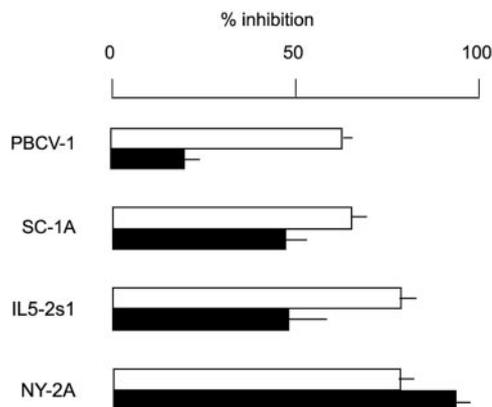


FIG. 1. Effects of two K⁺ channel blockers on plaque formation by four chlorella viruses. *Chlorella* NC64A was infected with PBCV-1, SC-1A, IL-5-2s1, or NY-2A in the absence or presence of 3 mM Ba²⁺ (open bars) or 3 mM Cs⁺ (solid bars). The results represent the inhibition of infective-center formation. The error bars indicate standard deviations.

109 transmission electron microscope. After each treatment, several hundred cells were examined either for progeny viruses (5 h p.i.) or for the number of empty/filled capsids attached to host cells (0, 12, and 15 min p.i.). For each cell, only one cross section was inspected.

Multiplicity of infection. The virus particles are very efficient at infecting *Chlorella* NC64A cells. Experimentally, 20 to 50% of the virus particles form plaques on this host in the medium used in these experiments (29). Experiments were conducted with an MOI of 5 or 10 in order to ensure maximal infection, except in some electron microscopic studies, where a higher MOI was used to increase the likelihood of detecting particles.

RESULTS

Plaque assay of infective centers. Previous studies have established that Kcv channels encoded by some chlorella viruses differ structurally and functionally from the reference Kcv channel encoded by PBCV-1 and also SC-1A virus (12). All K⁺ channels are blocked by Ba²⁺. Expression of the reference Kcv channel from PBCV-1 and SC-1A in oocytes is only slightly inhibited by Cs⁺ (12). In contrast, Kcv channels from IL-5-2s1 and NY-2A viruses are inhibited by Cs⁺ (9, 12). The IL-5-2s1 and NY-2A Kcv homologs differ from the reference Kcv by 6 and 9 amino acid residues, respectively. To examine the role of the Kcv channels in viral replication, we monitored the numbers of infected cells (as infective centers) by plaque formation in the presence and absence of either Ba²⁺ or Cs⁺. Figure 1 summarizes the inhibitory effect of 3 mM Ba²⁺ or 3 mM Cs⁺ on the four viruses. Nonsaturating concentrations of the inhibitors were chosen in order to minimize any unwanted side effects of the cations on the growth and division of the host cells in these long-term experiments. The results show that all viruses exhibit about the same sensitivity to Ba²⁺; the number of infective centers is inhibited 60 to 70%. In contrast, the four viruses differ in their sensitivities to Cs⁺. NY-2A virus replication is nearly abolished by 3 mM Cs⁺; Cs⁺ reduces IL-5-2s1 and SC-1A replication by ~50% and PBCV-1 replication by ~20%. Notably, although PBCV-1 and SC-1A encode identical Kcv channels, their sensitivities to Cs⁺ differ slightly.

It should be noted that the SC-1A, IL-5-2s1, and NY-2A viruses form smaller plaques than PBCV-1. The difference

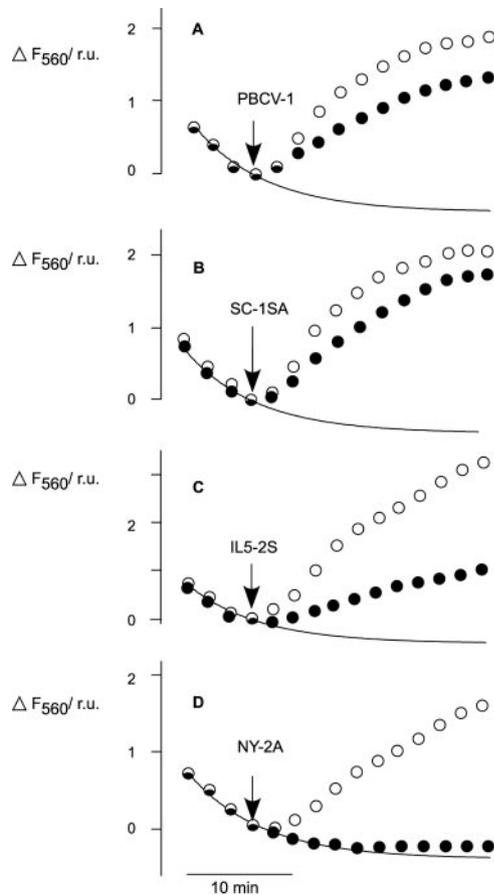


FIG. 2. Effects of four chlorella viruses on the fluorescence of bisoxinol at 560 nm (ΔF_{560}) in *Chlorella* NC64A cells in the absence and presence of the K^+ channel blocker Cs^+ . Approximately 3.5×10^6 chlorella cells ml^{-1} were maintained in MBB medium containing $1 \mu M$ bisoxinol without (open symbols) or with (closed symbols) 10 mM $CsCl$. After a 6-min preincubation, viruses were added at an MOI of 10 at the times indicated by the arrows. To extrapolate the fluorescence bleaching, the results obtained during the first 6 min of incubation were fitted by an exponential function with a time constant of 7.1 min^{-1} (see Materials and Methods). Fluorescence at the time of virus addition was adjusted to zero. Each data set is from the same batch of *Chlorella* NC64A cells. r.u., relative units.

in plaque size occurs because PBCV-1 replicates faster than the other three viruses (32). PBCV-1 progeny viruses first appear ~ 4 h p.i., and by 6 to 8 h p.i., virus release is complete. Small-plaque viruses require two to three times longer than PBCV-1 to replicate.

Depolarization assays. The abilities of the four viruses to depolarize *Chlorella* NC64A cells during infection were tested in the presence and absence of 10 mM Cs^+ . This should determine if the Cs^+ sensitivities of viral replication and Kcv activity correlate with virus-induced membrane depolarization. A representative experiment, in which the fluorescence of the voltage-sensitive dye bisoxinol was monitored before and after PBCV-1 (MOI = 10) was added to *Chlorella* NC64A cells, is shown in Fig. 2A.

PBCV-1 causes an immediate increase in fluorescence; this increase indicates depolarization of the cell membrane (17). Similar results were obtained in 14 independent experiments

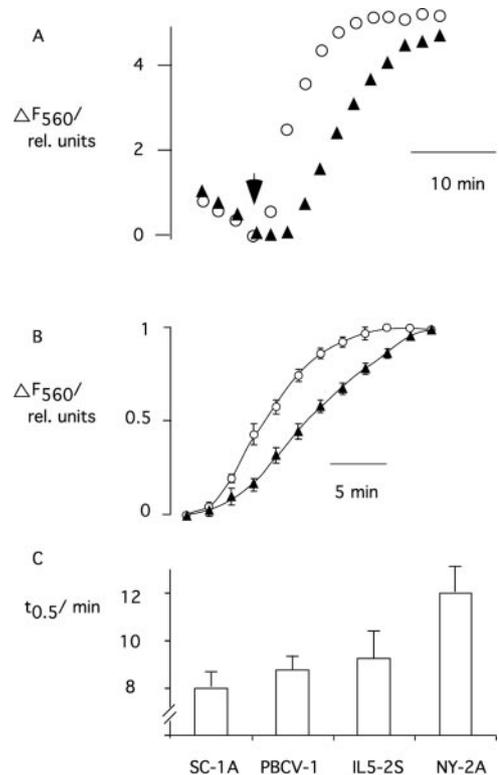


FIG. 3. Viruses induce depolarization of chlorella cells with similar amplitudes but different kinetics. (A) Comparison of membrane depolarization induced by PBCV-1 (open circles) and NY-2A virus (triangles) using the same batch of *Chlorella* NC64A cells. rel., relative. (B) Mean time course (mean \pm standard deviation; $n \geq 4$) of membrane depolarization induced by PBCV-1 (open circles) and NY-2A virus (solid triangles). The data were normalized to fluorescence values at 560 nm (ΔF_{560}) at 0 min and 22 min after virus addition. (C) Half-times ($t_{0.5}$) of virus-induced membrane depolarization by four different viruses. The error bars indicate standard deviations.

with PBCV-1. On average, the fluorescence at 560 nm (ΔF_{560}) increased in response to the same treatment by $22\% \pm 6\%$ ($n = 14$). This increase in fluorescence corresponds to depolarization from the negative H^+ -ATPase-dominated membrane voltage to the diffusion voltage (see Materials and Methods).

Comparison of individual data sets reveals that the kinetics of the increase in fluorescence were similar for all of the experiments. However, the amplitudes of the increases varied between experiments, resulting in a relatively large standard deviation. The reason for this variability is unknown, but it probably results from different batches of *Chlorella* cells.

To determine if membrane depolarization is a general feature of chlorella virus infection, we monitored fluorescence during infection with SC-1A, IL-5-2s1, and NY-2A viruses. Like PBCV-1, all three viruses depolarized the host cell membrane (Fig. 2B to D). However, as mentioned above, the amplitudes of the virus-induced depolarizations varied between experiments. Therefore, comparative experiments were conducted with PBCV-1 and each of the other viruses using cells from the same culture. As shown in Fig. 3A, infections with PBCV-1 and NY-2A evoked depolarizations with similar amplitudes. The amplitudes produced by the other two viruses were also similar to those produced by PBCV-1 (results not

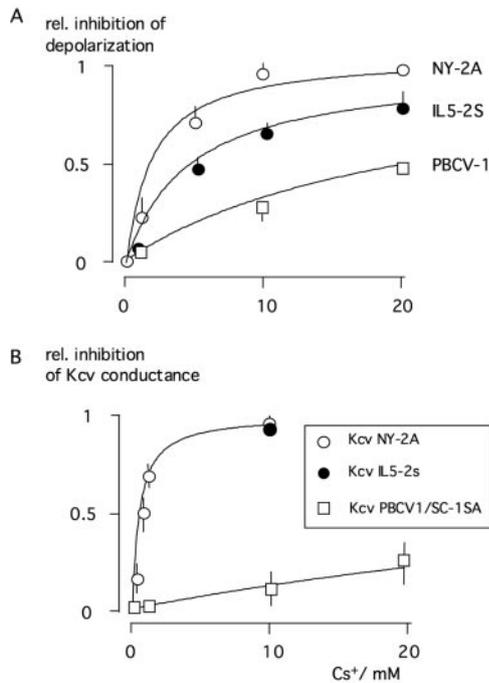


FIG. 4. Dose-response curves for the effects of Cs⁺ on virus-induced membrane depolarization (A) and on the conductances of three different Kcv channels expressed in *Xenopus* oocytes at a test voltage of -100 mV. The different Kcv channels are encoded by the viruses as indicated in panel B. The data in panel A show the amplitudes of virus-induced depolarization (22 min p.i.) in the presence of Cs⁺ in relation to that measured in the absence of Cs⁺. To improve comparison of measurements with and without Cs⁺, data were obtained with the same batch of algae. The data are means \pm standard deviations from ≥ 4 measurements. rel., relative.

shown). Altogether, these results indicate that infections with all four viruses induce similar amplitudes of membrane depolarization in *Chlorella* NC64A cells.

While the increases in depolarization are similar for all four viruses, the kinetics differ among the viruses. The normalized rise in fluorescence induced by PBCV-1 and NY-2A are reported in Fig. 3B. On average, depolarization induced by PBCV-1 occurs about 1.3 times faster than that elicited by NY-2A. Similar analyses were performed for all four viruses, and the times for half-maximal depolarization are reported in Fig. 3C. Virus SC-1A produces the fastest depolarization, ~ 8 min.

Cs⁺ effect on depolarization. To investigate the effect of Cs⁺ on the virus-induced increase in bisoxonol fluorescence, cells were infected with each of the four viruses in the presence of 10 mM Cs⁺ (Fig. 2). The high Cs⁺ concentration was chosen to ensure maximal inhibition of the Cs⁺-sensitive process. Figure 2 shows that 10 mM Cs⁺ slightly inhibits membrane depolarization by PBCV-1 and SC-1A. In contrast, Cs⁺ reduces the depolarization caused by IL-5-2s1 about 50 to 60% and completely abolishes membrane depolarization by NY-2A.

To quantify the inhibitory effect of Cs⁺, PBCV-1, IL-5-2s1, and NY-2A were incubated with several concentrations of Cs⁺. The Cs⁺ effect is concentration dependent (Fig. 4A). The strongest inhibition occurs with NY-2A and the weakest with PBCV-1. The dose-response curves were fitted by a Michaelis-

Menten-type kinetic model, yielding K_i values for NY-2A, IL-5-2s1, and PBCV-1 of 1.8 mM, 4.6 mM, and 21 mM, respectively.

In principle, the results can be explained in two ways. The short exposure to Cs⁺ may alter the capsids of the NY-2A and IL-5-2s1 viruses and make them noninfective, or at least less infective. However, pretreatment of the viruses with 10 mM Cs⁺ followed by dialysis does not change virus infectivity, eliminating this possibility. The alternative explanation is that the virus causes membrane depolarization by integrating the viral channel Kcv into the membrane of the host. Viruses with a Cs⁺-sensitive channel would in this case fail to depolarize the host because of a block in the channel. This hypothesis predicts that the membrane conductance of the host cell in the depolarized state has a higher sensitivity to the external K⁺ concentration than in the hyperpolarized state prior to the virus-induced depolarization. To test this hypothesis, we first elevated the K⁺ concentration ($[K^+]_o$) of the bath medium of the *Chlorella* NC64A cells in the absence of virus. Figure 5A and C show that this has only a small enhancing effect on bisoxonol fluorescence. However, when the $[K^+]_o$ was elevated at a time when the *Chlorella* cells were already depolarized by PBCV-1, the fluorescence increased steeply (Fig. 5B and C). The results of these experiments indicate that the depolarization is, at least to a large extent, the consequence of an elevated K⁺ conductance of the host cell membrane. To further examine whether the rise in K⁺ conductance is associated with the function of Kcv, the response of the bisoxonol fluorescence to a 10-fold elevation of Na⁺ was monitored. The data in Fig. 5C show that the elevation of $[Na^+]_o$, when added after virus-induced depolarization, also results in a rise in fluorescence. However, this relative increase is on average three times smaller than that obtained with K⁺. This observation is qualitatively consistent with the properties of Kcv, which preferentially transports K⁺ over Na⁺ (21).

Electrophysiology. For quantification of the sensitivities of the PBCV-1 and NY-2A Kcv channels to Cs⁺, both channels were expressed in *Xenopus* oocytes to compare their electrophysiological properties in the absence and presence of 10 mM CsCl in the bath medium (12). Inward currents reflecting the activities of Kcv channels were elicited by stepping the membrane voltage from a holding voltage of -20 mV to a test voltage of -100 mV. The results indicate that 10 mM Cs⁺ has a small inhibitory effect on the PBCV-1/SC-1A-type Kcv; in contrast, NY-2A Kcv conductance is abolished by Cs⁺ (Fig. 4B). The dose-response curve shows that Cs⁺ inhibits NY-2A Kcv conductance in a concentration-dependent manner with a half-maximal concentration of about 0.9 mM, whereas PBCV-1 Kcv conductance is inhibited by Cs⁺ with a half-maximal concentration of 68 mM (Fig. 4B).

It is important to note that Cs⁺ sensitivity of the depolarization and of the K⁺ channels in Fig. 4A and B can only be compared in a qualitative manner. For a real quantitative comparison, the effects of Cs⁺ on the K⁺ channels would have to be estimated at the free-running potential of the *Chlorella* cells; this value could not be accurately determined in our experiments.

Electron microscopy. The preceding experiments are consistent with the virus-encoded K⁺ channel being involved in virus infection. However, we tested this hypothesis further by mon-

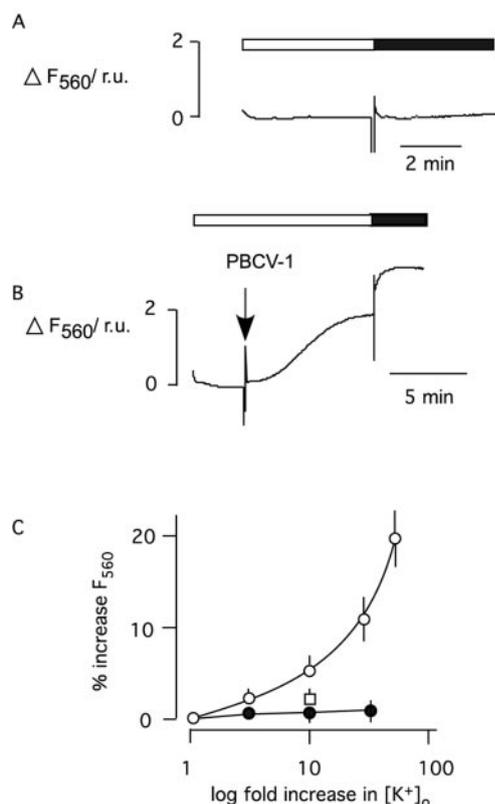


FIG. 5. Virus-induced membrane depolarization is associated with a rise in K^+ conductance of the *Chlorella* plasma membrane. (A and B) Representative recordings of bisoxonol fluorescences at 560 nm (ΔF_{560} of chlorella cells (3.5×10^6 cells ml^{-1}) before (open bar) and after (black bar) the concentration of K^+ in the incubation buffer ($[K^+]_o$) was increased 50-fold with K^+ -glutamate. In panel A, $[K^+]_o$ was increased in the absence of virus, and in panel B, after virus-induced depolarization. PBCV-1 was added at an MOI of 10 to *Chlorella* NC64A cells at the time indicated by the arrow. r.u., relative units. (C) The relative cation-induced increase of bisoxonol fluorescence as a function of the elevation of $[K^+]_o$ in the absence of virus (closed circles) or after a virus-induced depolarization (open circles). The response of the bisoxonol fluorescence to a 10-fold elevation of $[Na^+]_o$ in the incubation buffer (as Na^+ -glutamate) after a PBCV-1-induced depolarization is also shown (square). The error bars indicate standard deviations.

itoring infection/replication of the chlorella viruses in their host by electron microscopy. *Chlorella* cells were infected with either PBCV-1 or NY-2A at an MOI of 10 in the absence and presence of channel inhibitors. Figure 6A (top) shows a representative electron micrograph of *Chlorella* NC64A 5 h after PBCV-1 infection in the absence of Ba^{2+} . In these control cells, progeny virions can easily be seen as either empty or filled capsids. Examination of 250 micrographs with cell cross sections revealed virus progeny in about 67% of the micrographs (Fig. 6B). The percentage of cells with viral progeny is lower than the 90 to 95% expected under the prevailing conditions (29). This discrepancy is probably due to the fact that the ultrathin sections represent only a fraction of the entire *Chlorella* cell.

The same experiment as in Fig. 6B was repeated with either 10 mM $BaCl_2$ or 10 mM $CsCl$ in the incubation medium.

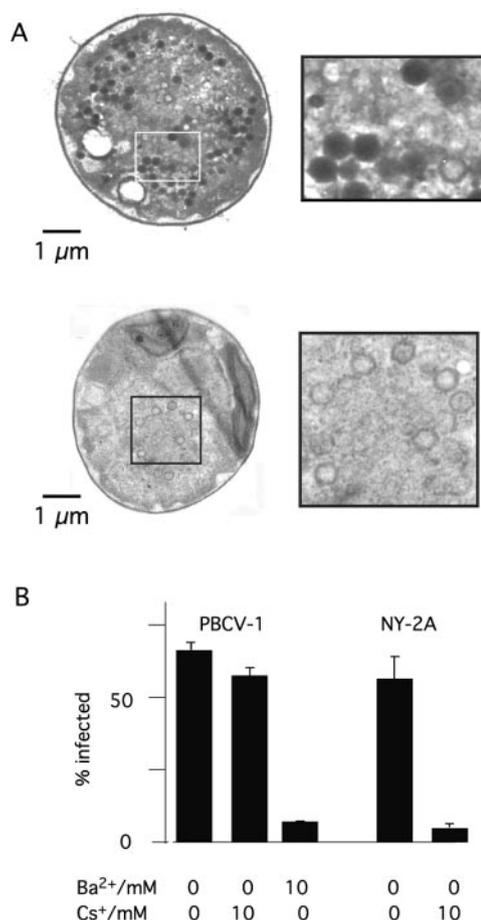


FIG. 6. Appearance of progeny viruses in *Chlorella* NC64A cells. (A) Thin section of chlorella cells at 5 h p.i. with PBCV-1 (top) or NY-2A virus (bottom) at an MOI of 10. Empty and filled progeny virus particles at 5 h p.i. are detectable within the cell. The bars indicate the magnifications of the particles in the expanded views of the the marked sections. Note that in the case of the slowly replicating virus NY-2A, only empty capsids were detected at this time point. (B) Percentages of cross sections of *Chlorella* NC64A with detectable progeny PBCV-1 (5 h p.i.) or NY-2A (5 h p.i.) particles in the absence or presence of 10 mM Ba^{2+} or 10 mM Cs^+ in the incubation medium. Micrographs from >250 cell cross sections were inspected for each time point.

$BaCl_2$, but not $CsCl$, drastically decreased the percentage of micrographs with detectable progeny virions; only 9% of a total of 250 inspected micrographs from Ba^{2+} -treated cells contained progeny virions (Fig. 6B).

A similar set of experiments was conducted with NY-2A virus, i.e., a virus with a Cs^+ -sensitive channel. Visible capsids were seen at 5 h p.i. Since NY-2A replicates more slowly than PBCV-1, the capsids were not yet packed with DNA. In 250 cross sections from control cells, 58% were found to contain newly forming virus capsids at 5 h p.i. (Fig. 6A, bottom). In contrast, only 7.5% of the cells contained detectable progeny virions in the presence of Cs^+ (Fig. 6B). The results obtained from the electron microscope experiments resemble those obtained by monitoring infective centers by the plaque assay (Fig. 1). However, the data in Fig. 1 indicate that the channel blockers inhibit some early event during infection prior to capsid formation.

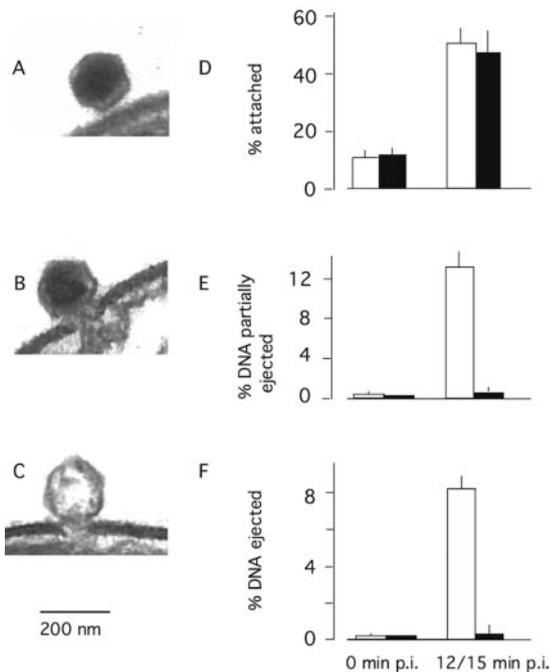


FIG. 7. Ba^{2+} has no effect on virus attachment but does inhibit DNA release from PBCV-1 particles into the host cells. The images on the left were taken at 15 min p.i. at an MOI of 200; three different stages of infection are discernible. (A) Particles attached to host. (B) Attached particles which have digested the cell wall and partially ejected their DNA. (C) Attached viruses (ghosts) which have completely ejected their DNA. (D to F) Ultrathin cross sections of *Chlorella* NC64A cells in the absence (open columns) and presence (filled columns) of 10 mM Ba^{2+} were prepared at 0, 12, and 15 min p.i.; the data from 12- and 15-min samples were pooled (12/15 min p.i.). In four independent experiments, a total of >1,800 cross sections were inspected for each time point and treatment. The percentages in panel D give the probabilities of detecting a cross section with ≥ 1 particle attached. In a fraction of these attached particles, viruses were detected which had already partially (E) or fully (F) ejected their DNA into the host. The percentages in panels E and F represent the probabilities of finding a particle with fully or partially ejected DNA with respect to the total number of attached particles. The data are means plus standard deviations from four experiments.

To explore the possibility that the function of the channel is related to ejection of DNA during virus infection, we monitored virus attachment and release of virus DNA into the host by electron microscopy. *Chlorella* NC64A cells were infected with PBCV-1 at an MOI of 200 in the absence or presence of 10 mM Ba^{2+} . The high MOI was used to increase the probability of observing virus activity within the first few minutes of infection. Figure 7 contains three representative micrographs from a sample at 15 min p.i. The micrographs demonstrate three events that occur during virus infection: viral attachment to the host (Fig. 7A), cell wall degradation and partial DNA release into the host (Fig. 7B), and virus ghosts after DNA release into the host (Fig. 7C).

To examine the effect of Ba^{2+} on virus attachment, we inspected micrographs from cells infected by PBCV-1 in the absence or presence of Ba^{2+} . For each data point, we inspected a total of ca. 1,800 micrographs in four independent experiments in which each micrograph represented a cross section of one cell. We found that immediately after infection (0 min p.i.), about 10%

of the micrographs exhibited as a background signal one or more attached viruses (Fig. 7D). When micrographs of cells 12 or 15 min p.i. were inspected, ca. 50% of the cross sections had one or more attached viruses. A comparison of the two treatments indicates that 10 mM Ba^{2+} has no appreciable effect on virus attachment (Fig. 7D). Examination of ca. 900 viruses attached to control cells at 12 to 15 min p.i. revealed that more than 200 had already partially or fully ejected their DNA (Fig. 7B and C). In contrast, few virions ejected their DNA in the presence of 10 mM Ba^{2+} (Fig. 7B and C). Comparison of the data obtained in the presence or absence of Ba^{2+} shows that this cation significantly reduced the frequency of observation of viruses that had partially or fully released their DNA into the host (Fig. 7E and F).

Collectively, the data indicate that a block in the activity of the viral channel by Ba^{2+} inhibits the release of the virus genome into the host cell. Additional support for this suggestion was obtained by monitoring the number of cells producing progeny viruses when Ba^{2+} was added to cultures 15 min p.i. There were no differences between the numbers of cells producing progeny viruses in the control and Ba^{2+} -treated cells (results not shown).

DISCUSSION

The discovery that PBCV-1 has a gene encoding a functional K^+ channel, called Kcv, when expressed in *Xenopus* oocytes (21) or mammalian HEK293 (19) and CHO (10) cells is significant for several reasons. Kcv was the first virus-encoded K^+ channel protein to be discovered, and the 94-amino-acid protein is the smallest protein known to form a functional K^+ channel. Despite its small size, Kcv is predicted to have all the essential structural elements of larger K^+ channel proteins (19). Therefore, Kcv is an excellent model for studying basic channel functions (9, 10).

While progress is being made in characterizing the biophysical properties of the Kcv channel in heterologous systems (9), less is known about the function of Kcv in the PBCV-1 life cycle. Presumably, the channel serves an important role in PBCV-1 replication, because Kcv conductance in frog oocytes is blocked by the same molecules (Ba^{2+} and amantadine, but not Cs^+) that inhibit PBCV-1 replication in its host (12, 21). These results, and other observations mentioned in the introduction, led to the hypothesis that the Kcv channel is located in the PBCV-1 internal membrane and that after virus attachment and digestion of the cell wall, the virus membrane fuses with the host membrane (17). This fusion results in elevated K^+ conductance and subsequent depolarization of the host cell membrane.

The data indicate good qualitative agreement between the Cs^+ sensitivities of the different K^+ channels and the effect of Cs^+ on depolarization. The values for half-maximal inhibition of both processes by Cs^+ are on the same order of magnitude. The correlation, however, is not as strong between viral-channel activity and plaque formation under the influence of Cs^+ . Even though PBCV-1 and SC-1A encode the same K^+ channel (12), the Cs^+ sensitivities of plaque formation differ. This result suggests that the viral channel is not the only rate-limiting step in the infection mechanism that is Cs^+ sensitive. The contributions of other proteins, such as two putative ligand-gated ion channels coded by PBCV-1 (13), might play a role.

The rapid depolarization of the host cells to PBCV-1 infection with a half time of ~9 min is consistent with the finding that early PBCV-1 transcripts can be detected within 5 to 10 min p.i (14, 24). Even though PBCV-1 carries ~370 genes, the virus lacks a recognizable RNA polymerase gene, suggesting that the host supplies at least some of the enzymes required for virus transcription. Thus, the following events must occur in the first few minutes of infection. PBCV-1 attaches to the host cell wall and digests a hole in the wall at the point of attachment, and its internal membrane fuses with the host membrane, resulting in host membrane depolarization. Membrane depolarization probably plays a role in easing the ejection of viral DNA and associated proteins into the host cell; these components then probably move to the nucleus and commandeer at least some of the host transcriptional machinery to begin transcribing viral DNA within 5 to 10 min.

Like infection with the chlorella viruses, changes in membrane conductance in combination with changes in the host cell membrane voltage also occur during infection by other viruses (3), such as poliovirus (22, 23), human immunodeficiency virus type 1 (20), and Sendai virus (8), and by bacteriophages T4 (2), PRD1 (6, 34), and PM2 (15). At present, the advantage to the virus of depolarizing the host membrane is unknown. There is evidence that depolarization by PRD1 is related to the lysis of the host and release of virus progeny (34). Depolarization of the cell membrane by poliovirus has been suggested to trigger shutoff of the host metabolism and to direct it to virus replication (22, 23). Another possibility is that changes in membrane potential prevent infection by a second virus. In fact, previous infection studies have established that the chlorella viruses mutually exclude one another (4). Furthermore, this exclusion phenomenon occurs early in the infection cycle. As in the case of bacteriophages, the exclusion process could occur at the plasma membrane of the host cell (5).

An important observation in the present study is that the chlorella viruses are unable to release their DNAs in the presence of the channel inhibitors. This means that the channel has a key function very early in the infection cycle; its activity could be related to the energetics of DNA release from the virus into the host cell. This explanation seems reasonable, considering some parallels between the infection process of bacteriophages and chloroviruses (30). In the case of bacteriophage lambda, it was shown that the efficiency of DNA ejection from the phage is affected by the strength of the osmotic medium into which the DNA is injected; the higher the osmolyte concentration in the target medium, the more DNA ejection was suppressed (7, 16). This information may be crucial for understanding DNA injection from the chloroviruses into their host cells. Freshwater algae, such as *Chlorella*, are expected to have very high turgor pressure; the turgor values measured in other green algae, such as *Chara* and *Nitella*, are on the order of 0.5 to 0.6 MPa (35). Hence, the virus-induced depolarization of *Chlorella* cells might lower the energy barrier for DNA ejection by causing an efflux of salts and water from the *Chlorella* cells.

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