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Chlorella viruses evoke a rapid release of K⁺ from host cells during the early phase of infection

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Abstract

Infection of *Chlorella NC64A* cells by PBCV-1 produces a rapid depolarization of the host probably by incorporation of a viral-encoded K⁺ channel (Kcv) into the host membrane. To examine the effect of an elevated conductance, we monitored the virus-stimulated efflux of K⁺ from the *Chlorella* cells. The results indicate that all 8 *Chlorella* viruses tested evoked a host specific K⁺ efflux with a concomitant decrease in the intracellular K⁺. This K⁺ efflux is partially reduced by blockers of the Kcv channel. Qualitatively these results support the hypothesis that depolarization and K⁺ efflux are at least partially mediated by Kcv. The virus-triggered K⁺ efflux occurs in the same time frame as host cell wall degradation and ejection of viral DNA. Therefore, it is reasonable to postulate that loss of K⁺ and associated water fluxes from the host lower the pressure barrier to aid ejection of DNA from the virus particles into the host.

Keywords: *Chlorella* viruses, Viral K⁺ channel Kcv, K⁺ efflux, Phycodnaviridae, PBCV-1

Introduction

The M2 protein from influenza virus A was the first protein encoded by a virus gene reported to form a functional ion channel in heterologous systems (Pinto et al., 1992). Since these initial studies, gene products from several other viruses have been described that either form functional ion channels (Schubert et al., 1996, Plugge et al., 2000, Wilson et al., 2004, Melton et al., 2002 and Lu et al., 2006) or alter the conductance properties of their host cell membrane during infection (Piller et al., 1998). Although these viral-encoded membrane proteins are not always absolutely required for virus replication, all of them enhance viral replication (Gonzalez and Carrasco, 2003). With the exception of the proton conducting M2 channel, not much is known about the specific function(s) of these membrane proteins in the viral life cycles although protein VPU from virus HIV1 is involved in the release of progeny viruses (Carrasco, 1995, Fischer and Sansom, 2002 and Hsu et al., 2004).

Paramecium bursaria Chlorella virus (PBCV-1) was the first virus discovered to encode a functional K⁺ channel protein. The 94-amino acid protein (called Kcv) produces a K⁺-selective and slightly voltage-sensitive conductance in *Xenopus* oocytes (Plugge et al., 2000) and mammalian HEK293 (Moroni et al., 2002) and CHO cells (Gazzarrini et al., 2003). Kcv is the smallest protein known to form a functional tetrameric K⁺ selective channel and its predicted transmembrane (TM1)-pore-TM2 structure resembles canonical K⁺ channels (Gazzarrini et al., 2004, Pagliuca et al., 2007, Shim et al., 2007 and Tayefeh et al., 2007).

PBCV-1 is a large, icosahedral (diameter of ~ 1900 Å), plaque-forming, dsDNA-containing virus that infects certain *Chlorella*-like green algae (Van Etten, 2003 and Yamada et al., 2006). It is the prototype of a rapidly expanding group of viruses in the family *Phycodnaviridae*. The 331-kb PBCV-1 genome has ~ 365 protein-encoding genes and 11 tRNA-encoding genes. The PBCV-1 virion is a multi-layered structure composed of the genome, a lipid bilayer membrane and an

outer icosahedral capsid shell (Yan et al., 2000). PBCV-1 infects its host by attaching to the external surface of the algal cell wall. Attachment occurs at a unique virus vertex (Onimatsu et al., 2006) and is followed by degradation of the host wall at the attachment point by virion-packaged, wall digesting enzymes (Meints et al., 1984). Following host cell wall degradation, the internal membrane of the virus probably fuses with the host membrane leading to entry of the viral DNA and virion-associated proteins. An empty capsid is left on the cell surface. Infection results in rapid depolarization of the host membrane (Frohns et al., 2006 and Mehmel et al., 2003), and physiological studies have led to the hypothesis that this rapid depolarization is caused by the Kcv channel that is predicted to be located in the virus internal membrane. That is, during infection the Kcv channel is inserted into the plasma membrane of the host causing a short circuit of the host membrane potential. Presumably this depolarization is essential for virus infection because certain K⁺ channel blockers partially prevent the virus-induced membrane depolarization and the ejection of viral DNA into the host cells; thus subsequent virus replication in the host is inhibited (Frohns et al., 2006). We predict that the PBCV-1-induced depolarization and the augmented K⁺ conductance effects K⁺ fluxes in the host cells. In this manuscript, we examine the effect of infection by 8 different *Chlorella* viruses, including PBCV-1, on K⁺ fluxes in

host *Chlorella* cells. The results indicate that the host cells release measurable amounts of K⁺ during the first few minutes after infection and that this loss of K⁺ can be mechanistically related to the properties of the Kcv channel.

Results

To define the early events associated with PBCV-1 infection, the time of virus attachment, cell wall degradation and virus DNA ejection was measured by electron microscopy. *Chlorella* NC64A cells were incubated with virus PBCV-1 at an m.o.i. of 100; infection was stopped at various times post infection (p.i.) by transferring cells into a mixture of cold glutaraldehyde/formaldehyde. After additional fixation steps (Frohns et al., 2006), the samples were examined by electron microscopy. Figure 1A shows representative images obtained 6 min p.i.: (i) the virus is attached to the host cell, (ii) the virus has digested a hole in the cell wall and (iii) the virus has ejected its DNA into the host. A statistical analysis of more than 1200 electron micrographs (see Frohns et al., 2006) provided the dynamics of the three stages of infection (Figure 1B). Attachment occurs in a biphasic manner with a rapid and a slow component; the half time ($t_{1/2A}$) of this process is about 20 s. After a short time, cell wall degradation ($t_{1/2L}$ = 2 min) and DNA ejection ($t_{1/2D}$ = 4.1 min) occur.
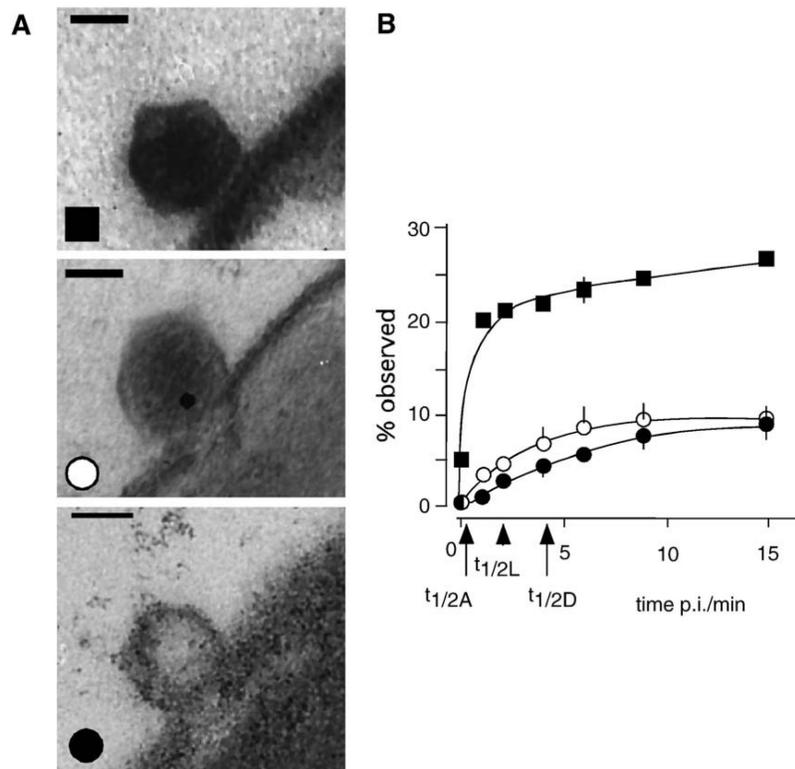
Figure 1. Time course of early stages of virus PBCV-1 infection of *Chlorella* NC64A cells. The electron micrographs on the left (A) were taken 6 min p.i. after infecting *Chlorella* cells with an m.o.i. of 100. The images present three defined and sequential stages of infection: attachment (■), degradation of the host cell wall (○) and empty virus capsids after ejection of DNA into the host cell (●). Electron micrographs of infections terminated at different times p.i. were analyzed with respect to the relative occurrences of these three stages. The relative frequency of the three stages is shown as a function of time p.i. in (B). From the graph the following half times can be calculated: attachment ($t_{1/2A}$) = 20 s; wall degradation ($t_{1/2L}$) = 2 min; DNA ejection ($t_{1/2D}$) = 4.1 min. At each time a total of 1200 cells from three independent experiments were analyzed. Data are given as mean ± standard deviation. Scale bar: 100 µm.

To determine if PBCV-1 infection of *Chlorella* NC64A is associated with K^+ release, cells were incubated in a K^+ free MBBM (MBBM$_{-K}$) medium with or without PBCV-1 (m.o.i. of 10). The K^+ concentration in the incubation medium ($[K^+]_o$) was determined during the first 30 min p.i. The results reported in Figure 2A indicate that cells release very little K^+ in the absence of viral infection. In contrast, PBCV-1 infection leads to a rapid increase in $[K^+]_o$; this increase has monotonic saturation kinetics and reaches its half maximal concentration by 3.3 min p.i. Note that this increase in $[K^+]_o$ occurs during the same time frame the cell walls are being degraded (Figure 1B).

To determine if the increase in $[K^+]_o$ is specific for virus PBCV-1 or a general feature of the *Chlorella* viruses we performed the same experiment with 7 other viruses that infect *Chlorella* NC64A and encode a Kcv homolog. These experiments established that infection with all 7 viruses resulted in about the same increase in $[K^+]_o$ at 30 min p.i. (Figure 2B).

The increase in $[K^+]_o$ is related to virus infection

To determine if the increase in $[K^+]_o$ is correlated with virus infection, we repeated the same experiment described in Figure 2B with *Chlorella vulgaris*, a non-host for the viruses. A 30 min incubation of *C. vulgaris* with PBCV-1 had no effect on K^+ efflux (Figure 2C). This experiment establishes that the increase in external K^+ requires PBCV-1 infection of its host *Chlorella* NC64A and is not due to either a non-specific leakage from the virus particles or from the host cells.

K^+ is released from the host cells

Additional experiments addressed the question of the origin of the increase in $[K^+]_o$ in the host/virus system. One possibility is that K^+ is released from the viral particles in

an infection dependent manner. This seems unlikely, however, because of quantitative considerations. A viral particle has a diameter of about 190 nm; therefore all of the particles in an experiment have a total volume of 5.4×10^{-8} l, which is only 1/30,000 of the volume of the incubation medium. Hence the K^+ concentration in the viral particles would need to be in the molar range to explain the observed increase in $[K^+]_o$ in response to infection. This is unrealistically high and excludes the viral particles as the primary source of the increase in $[K^+]_o$.

Next we monitored the host cells for K^+ release in an infection specific manner. We repeated the experiments reported in Figure 2B except that the cells were also analyzed for their relative intracellular K^+ content ($[K^+]_{ri}$). The procedure used does not give an absolute value for the K^+ concentration in the *Chlorella* cells, but it provides a relative measure of the virus-induced changes in cellular K^+ content. The cellular K^+ concentration of fresh water algae is about 100 mM (Beilby and Blatt, 1986). If we assume that a single *Chlorella* cell has a diameter of 4 μm and a volume of 3.3×10^{-17} m^3 and that 70% of this volume is liquid, we calculate that 10^9 ml^{-1} cells will lead to a $[K^+]_{ri}$ of 2.3 mM if all its K^+ is released upon the freeze/thaw procedure into the medium. This is close to the concentration measured with control cell (Figure 3). Concomitant with K^+ efflux the $[K^+]_{ri}$ value drops to ~ 1.2–1.6 mM after infection with each of the 6 viruses tested (Figure 3). Consequently, the intracellular K^+ content in *Chlorella* NC64A is reduced 50–60% from its initial concentration by 30 min p.i. As expected, the content of the non-host *C. vulgaris* K^+ concentration (1.72 ± 0.04 mM) is not altered by 30 min incubation with PBCV-1.

In principle, the release of K^+ could come from the protoplast or from the cell wall of *Chlorella* NC64A. For quantitative reasons, the cell wall is unlikely to be the source of K^+. If we assume that a single *Chlorella* cell has a diameter of 4 μm, the volume of 1.5×10^9 cells in 1.83 ml incubation
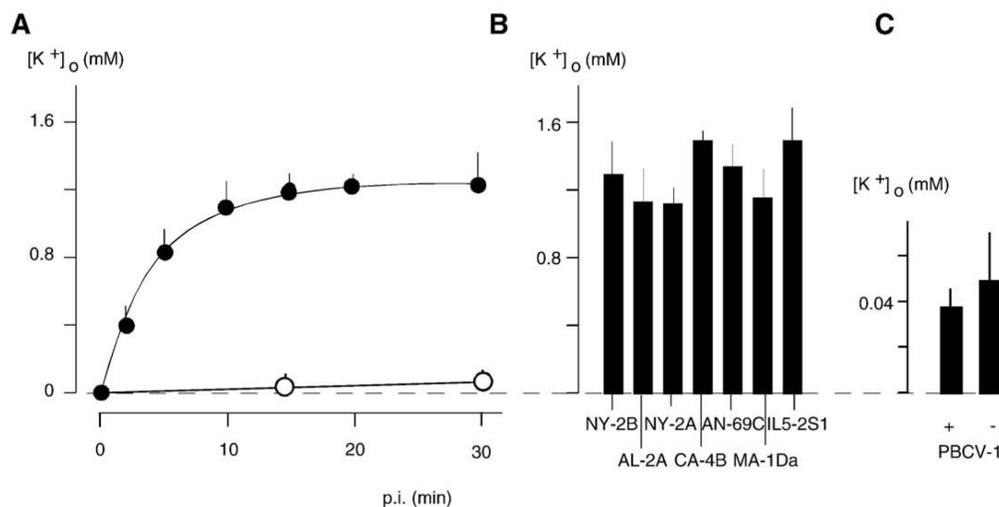
Figure 2. *Chlorella* viruses evoke an increase in $[K^+]_o$ early during infection. (A) Increase of $[K^+]_o$ as a function of time in the presence (●) or absence (○) of virus PBCV-1. Data fitted with first order saturation kinetics reveal a time of 3.3 min for a half maximal rise in $[K^+]_o$. (B) The mean value of $[K^+]_o$ measured 30 min p.i. of *Chlorella* NC64A with the 7 viruses indicated. All data were obtained using an m.o.i. of 10. Data are mean ± standard deviation (sd) of ≥ 4 experiments. (C) Virus PBCV-1 has no effect on $[K^+]_o$ when incubated with non-host *C. vulgaris.* Mean value of $[K^+]_o$ measured 30 min after incubation of *C. vulgaris* with (+) or without (−) PBCV-1 at an m.o.i. of 10. Data are mean ± standard deviation (sd) of 4 experiments.
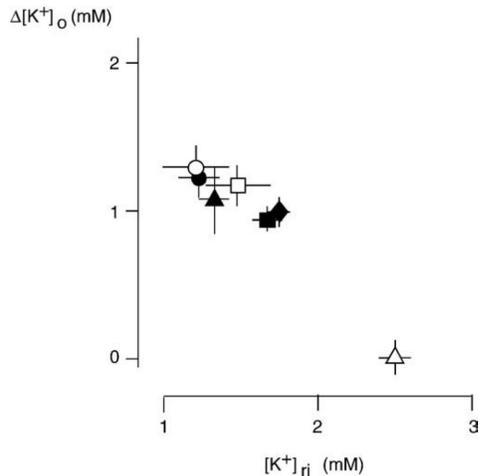
Figure 3. The virus induced increase in $[K^+]_o$ is correlated with a decrease in $[K^+]_i$ in *Chlorella* NC64A cells incubated without (△) or with viruses Al-2A (■), NY-2B (●), CA-4B (○), AN-69C (□), MA-1D (▲) or IL2-5s1 (♦). The $[K^+]_o$ and $[K^+]_i$ were measured 30 min p.i. The results are mean ± sd of ≥ 3 experiments.

Figure 4. Pre-treating *Chlorella* cells in order to lower K^{+} in cell walls has no effect on the virus-induced increase in $[K^+]_o$. Mean value of $[K^+]_o$ measured 30 min after infection of *Chlorella* NC64A with virus PBCV-1 at an m.o.i. of 10. Cells were pre-incubated in standard $MBBM_{-K}$ pH 6.7 or in $MBBM_{-K}$ pH 5.5 or in $CaSO_4$. Data are mean ± standard deviation (sd) of 4 experiments.

medium is 5×10^{-5} l. Assuming the cell walls are ~ 0.1 μm thick, the total volume of the walls contributes to < 10% of the total cell volume. This means that the K^{+} concentration in the cell wall would need to be several hundred millimolars in order to explain the measured increase of $[K^+]_o$ of about 1 mM. This value is high for plant cell walls and suggests that K^{+} is not released from the cell wall.

Nonetheless, we conducted two experiments to measure the contribution of K^{+} release from cell walls to the overall virus induced increase in $[K^+]_o$. *Chlorella* NC64A cells in $MBBM_{-K}$ were incubated in either an acidic pH or in a 5 mM $CaSO_4$ solution. Both treatments reduce the amount of exchangeable K^{+} ions in plant cell walls (Marre et al., 1992). The pretreated cells were infected with virus PBCV-1 as in Figure 2 and the increase in $[K^+]_o$ measured 30 min p.i. Neither of the pre-treatments affected the ability of virus PBCV-1 to release K^{+} from its host (Figure 4). This means that most, if not all, of the released K^{+} must be from the protoplasm of the cell.

Sensitivity of K^{+} release to channel blockers

Previous experiments established that virus infection of *Chlorella* NC64A results in a rapid virus-induced depolarization of the host membrane; depending on the virus, this depolarization and subsequent virus replication are partially inhibited by K^{+} channel blockers Ba^{2+} and Cs^+ (Frohns et al., 2006 and Mehmel et al., 2003). The sensitivity of virus-induced depolarization and subsequent virus replication to the channel blockers correlates with the sensitivity of the channel blockers on Kcv conductance in *Xenopus* oocytes. That is, conductance of all virus Kcv channels expressed in *Xenopus* oocytes is partially inhibited by Ba^{2+}; however, Cs^+ only inhibits conductance of Kcv channels from some viruses (Gazzarrini et al., 2003, Gazzarrini et al., 2004 and Frohns et al., 2006). To test the hypothesis

that the viral induced K^{+} efflux is related to the activity of the Kcv channels, we monitored K^{+} efflux in the presence or absence of 10 mM Ba^{2+} or 10 mM Cs^+; this is a saturating concentration for the blockers in terms of inhibition of the Kcv channels including K^{+} outward current and virus-induced depolarizations (Plugge et al., 2000 and Frohns et al., 2006). The two blockers were added to the medium 10 min before infecting with the viruses.

The results of the experiments in which virus induced K^{+} efflux was monitored in the presence and absence of 10 mM $BaCl_2$ are reported in Figures 5A & B. Ba^{2+} reduced K^{+} efflux after infection with all 8 viruses. Depending on the virus, K^{+} efflux inhibition varied between 75% for PBCV-1 and 38% for virus IL5-2s; however, Ba^{2+} did not completely inhibit K^{+} efflux for any of the viruses (Figure 5A). The Ba^{2+} inhibition of virus-triggered K^{+} efflux is also reflected in a reduced release of K^{+} from the cells (Figure 5B). In the absence of Ba^{2+}, virus infection resulted in a 50–60% reduction in intracellular K^{+} content (Figure 3), whereas in the presence of Ba^{2+}, it is only reduced 20–30% (Figure 5B).

We also examined the Cs^+ sensitivity of virus-induced K^{+} efflux. The two viruses chosen for this experiment, PBCV-1 and NY-2A, respond differently to Cs^+ (Frohns et al., 2006). PBCV-1 replication and PBCV-1-induced depolarization are only slightly inhibited by Cs^+. In contrast, Cs^+ almost completely inhibits both NY-2A replication and NY-2A-induced depolarization. This difference in Cs^+ sensitivity correlates with the fact that NY-2A Kcv conductance is inhibited by Cs^+ while PBCV-1 Kcv is much less sensitive to Cs^+ (Frohns et al., 2006, Kang et al., 2004b and Mehmel et al., 2003). *Chlorella* NC64A cells were infected with either PBCV-1 or NY-2A and K^{+} efflux was monitored in the presence or absence of 10 mM CsCl (Figure 5C). K^{+} efflux induced by PBCV-1 was unaffected by Cs^+ at 30 min p.i. However, contrary to what we expected, Cs^+ only reduced NY-2A-induced efflux about 20% (Figure 5C).

Figure 6. Estimation of virus-induced changes in $[K^+]_o$ and $[K^+]_i$. (A) A simulation of K^+ fluxes (for details see Materials and Methods) in *Chlorella* cells predicts that a depolarization to -70 mV results in a rise of $[K^+]_o$ (black line) to about 1 mM and a roughly two-fold decrease of $[K^+]_i$ (gray line). (B) Model calculation of steady state values of $[K^+]_o$ and $[K^+]_i$ for depolarized membrane voltage (Vm) between -100 mV and -40 mV.

Calculation of K^+ efflux

To understand the relationship between membrane depolarization, K^+ conductance and the observed increase in extra-cellular $[K^+]_o$, we monitored the dynamics of the $\Delta[K^+]_i$ and $\Delta[K^+]_o$ based on the hypothesis that K^+ efflux is mediated by the viral Kcv channels. Assuming *Chlorella* cells are spherical and the typical cytoplasmic K^+ concentration for fresh water algae is 100 mM (Beilby and Blatt, 1986), we estimate the progressive decrease in $[K^+]_i$ and the concomitant increase in $[K^+]_o$. Figure 6A shows the estimated changes in $\Delta[K^+]_i$ and $\Delta[K^+]_o$ for an instantaneous depolarization from -120 mV to -70 mV. This depolarized voltage roughly resembles the experimental results, namely a rise of $[K^+]_o$ to about 1 mM and a two-fold decrease in $[K^+]_i$. Other depolarization values produce either higher or lower $\Delta[K^+]_o$ and $[K^+]_i$ values (Figure 6B). Comparison of Figures 6A and 2A shows that the calculated K^+ efflux is much faster than the measured K^+ efflux. This is consistent with the fact that virus particles have to first attach and digest the cell wall at the point of attachment before they can trigger K^+ efflux (Figure 1).

The calculations of K^+ efflux also agree with the measured K^+ fluxes in the presence of Ba^{2+}. If we assume that

Figure 5. Effect of Ba^{2+} or Cs^+ in the incubation medium on virus-induced increase in K^+ fluxes. (A) Virus induced increase in $[K^+]_o$ ($1 - \Delta[K^+]_{o, +Ba2+} / \Delta[K^+]_{o, -Ba2+}$) in the presence ($+ Ba^{2+}$) and absence ($- Ba^{2+}$) of 10 mM $BaCl_2$ in the incubation medium. Data are the mean ± standard deviation (sd) of ≥ 3 independent experiments. (B) $[K^+]_o$ and $[K^+]_i$ were determined from cells at 30 min p.i. in the presence of 10 mM Ba^{2+} and with or without viruses. Data are mean ± sd of ≥ 3 experiments. The $[K^+]_o$ was monitored as in Figure 2 with an m.o.i of 10 without (△) or with viruses Al-2A (■), NY-2B (●), CA-4B (○), AN69C (□), MA-1D (▲) or IL2-5s1 (◆). (C) The mean value of $[K^+]_o$ measured 30 min after infection of *Chlorella* NC64A cells with viruses PBCV-1 or NY-2A at an m.o.i. of 10 in the absence (−) or presence (+) of 10 mM CsCl. Data are mean ± standard deviation (sd) of 4 experiments.

the membrane depolarizes to only −90 mV instead of −70 mV, the calculated $[K^+]_i$ is reduced in the presence of Ba^{2+} by 70% of the resting value (Figure 6B). This value corresponds to a measured reduction of $[K^+]_i$ in the range of 40% to 80% (Figure 5). The same reduced depolarization predicts a $\Delta[K^+]_o$ of 0.5 mM; the measured value is 0.4 to 0.7 mM (Figure 5). The 20% inhibition of K^+ efflux evoked by virus NY-2A in the presence of Cs^+ (Figure 5C) can be explained if the membrane depolarization level is only −80 mV compared to the −70 mV in the absence of the inhibitor (Figure 6B).

Discussion

Experiments described in this report establish that viral infection of *Chlorella* NC64A is associated with a rapid and substantial loss of K^+ from the host cell protoplast. K^+ efflux was observed during infection by all 8 viruses tested and so it is reasonable to assume that K^+ efflux is an important step in the infection process. Furthermore, because K^+ efflux occurs during the first few minutes of infection, the efflux is functionally related to other very early events associated with infection such as ejection of DNA and probably some viral proteins into the host cells.

Fresh water algae have a high turgor pressure of about 1 MPa (Wendler and Zimmermann, 1982). It must be assumed that this pressure energetically opposes, like in bacteria, DNA ejection into the host (Evilevitch et al., 2003 and Evilevitch et al., 2005). Therefore, the present results can be interpreted in the context that the efflux of K^+ from the chlorella cells leads to a substantial drop in turgor pressure, which then favors ejection of the large viral DNA genome and proteins into the chlorella cells. The alternative interpretation that K^+ efflux is accidental seems unlikely because inhibition of K^+ efflux is correlated with an inhibition of DNA ejection and virus replication (Frohns et al., 2006). Also the possibility that an efflux of K^+ allows an influx of Mg^{2+} for neutralization of the DNA (Altaossava et al., 1987) is not compatible with the experimental data because the virus induced membrane depolarization abolishes the driving force for passive influx of Mg^{2+} into the host cells.

A comparison of the K^+ content of infected versus noninfected chlorella cells shows that approximately half of the cellular K^+ content is lost during this early period. K^+ is the major cation in the cell and it comprises the bulk of the osmotic potential in plant cells and fresh water algae, such as *Chlorella*; the resting concentration of K^+ in plants and fresh water algae is about 100 mM (Beilby and Blatt, 1986). If we assume that, for reasons of electro-neutrality, K^+ is lost together with anions, this loss reduces the cell's osmotic potential by about 100 mosM. This drop in the osmotic potential will cause an efflux of water (*J*) according to equation

$$J = Lp(\Delta P - \Delta \pi) \qquad (1)$$

where *Lp* is the water conductance, ΔP the turgor pressure and $\Delta \pi$ the difference in osmotic potential. Worth noting in this context is that recently a gene encoding an aquaglyceroporin channel was detected in another chlorella virus

(Gazzarrini et al., 2006). This water channel, if located in the viral membrane, could speed up the infection related water flux when inserted into the membrane of the host.

The efflux of water, which follows the discharge of K-salts from the host cell, will eventually result in a decrease in cell volume and a concomitant drop in turgor pressure. As in the case of bacteria, this decrease in pressure will reduce the physical hurdle for the virus to eject its DNA into the alga cell. Accordingly, when this process is inhibited or reduced by Ba^{2+} or Cs^+, respectively, infection and DNA ejection are also inhibited. At first glance it appears as if a 20% reduction of K^+ efflux by Cs^+ in experiments with virus NY-2A is not sufficient to explain an 80% reduction in virus infection and inhibition of DNA ejection into the host (Frohns et al., 2006). However experimental measurements of volume/pressure relationships in plant and algal cells (e.g. Steudle et al., 1977) have revealed that these two parameters are related in a highly non-linear fashion such that small changes in volume produce large changes in turgor pressure. Thus an apparent small inhibition of volume changes can also have dramatic effects on the decrease in turgor pressure.

Viral channels and K+ efflux

Plant cells including fresh water algae have a very negative membrane voltage (Beilby and Blatt, 1986), which produces a driving force for passive K^+ influx. In order to switch from passive influx to K^+ efflux the cells have to be depolarized to a voltage that is positive of the K^+ reversal voltage (Thiel et al., 1992). Previous experiments indicate that virus infection of chlorella cells is indeed intimately related to depolarization of their plasma membranes (Frohns et al., 2006 and Mehmel et al., 2003). The temporal coincidence of virus-induced depolarization and K^+ efflux strongly suggests that the depolarization creates the driving force for K^+ efflux. This hypothesis is supported by the model calculations which show that the observed quantitative changes in $[K^+]_o$ and $[K^+]_i$ can be reproduced by depolarization which substantially exceeds the K^+ reversal voltage. The fact that chlorella plasma membrane voltage has to be depolarized by about 30 mV in excess of the K^+ equilibrium voltage has consequences in interpreting the infection process. Such a strong depolarization in excess of the K^+ equilibrium voltage cannot be achieved solely by insertion of Kcv channels in the host cell membrane even if we assume that this channel has only a moderate selectivity for K^+ over Na^+ (Plugge et al., 2000). Hence, the data suggest that other channels with a more positive reversal voltage must also be activated in the process. These other channel proteins could be encoded by either the viruses or be the result of activation of endogenous host channels.

Several observations support the hypothesis that membrane depolarization of chlorella cells is triggered by the virus-encoded Kcv channel (Frohns et al., 2006, Mehmel et al., 2003 and Kang et al., 2004a). The current data are largely consistent with this model. The loss of K^+ from the cells is extremely rapid which means that K^+ conductance must be very high. Fresh water algae, including *Chlorella*

NC64A, typically have a very low resting K+ conductance. Consequently, endogenous K+ channels are unlikely to be sufficient to allow the rapid loss of K+ observed during infection; the high unitary channel conductance of Kcv channels (Pagliuca et al., 2007) on the other hand provides the required conductance.

The results also show that Ba^{2+}, which blocks Kcv channels, reduces the viral induced K+ efflux. The experimental results are qualitatively compatible with the assumption that this reduction in efflux is associated with a smaller virus induced membrane depolarization (Mehmel et al., 2003). The model however is not fully compatible with the results obtained with virus NY-2A infection in the presence of Cs+. We expected the NY-2A-induced K+ efflux to be more severely affected by Cs+ than was observed because the same treatment causes a severe inhibition of virus-triggered membrane depolarization (Frohns et al., 2006). The reason for this discrepancy is unknown, but a possible explanation for this discrepancy is that the Cs+ induced inhibition of membrane depolarization in the virus NY-2A was overestimated. This explanation is reasonable considering the fluorescence properties of the voltage sensitive dye bisoxonol, which was used to monitor the virus induced depolarization in the *Chlorella* NC64A cells (Mehmel et al., 2003 and Frohns et al., 2006). Calibration curves of bisoxonol show that the fluorescence of the dye becomes very small and decreases exponentially at negative voltages (e.g. Moreno et al., 1998); therefore, the small depolarization required for the measured K+-efflux in the presence of Cs+ may not have been fully resolved.

Materials and Methods

Growth of viruses and cells

Growth of *Chlorella* NC64A and *C. vulgaris* and the production of the viruses have been described (Van Etten et al., 1981 and Van Etten et al., 1983).

K+ efflux assay

For K+ efflux assays, cells from cultures in exponential growth were transferred to modified Bold's basal medium (MBBM$_{-K}$) (Van Etten et al., 1983) in which all of the K+ was replaced with Rb+. Measurements of the medium revealed that this nominally K+ free solution always contained a K+ contamination of some 100 μm. Separate experiments established that this medium does not alter virus replication or virus-induced membrane depolarization of *Chlorella* NC64A cells in the time frame of the experiments. The optimal cell density for measuring K+ efflux was determined by monitoring depolarization of *Chlorella* NC64A cells in 50 μM Nystatin, an antibiotic known to depolarize membranes (Komor and Tanner, 1967 and Mehmel et al., 2003). A measurable millimolar increase in the K+ concentration in the incubation medium occurred when the cell density was ≥ 1 × 10^9 ml^{-1}. Therefore, experiments on virus-induced K+ fluxes were conducted at a cell density of 1 × 10^9 ml^{-1}.

To monitor virus-induced K+ efflux, cells were incubated in 1.5 ml MBBM$_{-K}$ plus 0.33 ml of either a virus suspension or virus buffer. After incubating for up to 30 min, cells were pelleted by centrifugation (10 min at 4000 rpm, Biofuge, Kenro, Hanau, Germany). The supernatant was collected and the K+ content determined by flame photometry (Eppendorf FCM6341, Hamburg, Germany). The spectrometric read-out was calibrated separately over a range of K+ concentrations in the presence or absence of 10 mM BaCl$_2$ or 10 mM CsCl. Virus-induced K+ efflux was calculated as the difference in K+ concentration of samples with viruses at a multiplicity of infection (m.o.i.) of 10 minus parallel samples lacking virus.

To determine the contribution of K+ efflux from the cell walls, chlorella cells were either pre-incubated for 1 h in 5 mM CaSO$_4$ or in acidic (pH 5.5) MBBM$_{-K}$. We also measured the relative changes in K+ content inside the *Chlorella* cells by suspending pelleted cells in 1.5 ml MBBM$_{-K}$ and subjecting them to four freeze/thaw cycles with liquid nitrogen. Cells were subsequently pelleted by centrifugation and the K+ released into the supernatant was measured by flame photometry.

Calculation of K+ efflux

The efflux of K+, i.e. the infection stimulated outward K+ current (IK) is given by Equation (2):

$$I_K = (V_m - E_K) * \Delta G_K, \qquad (2)$$

where V_m is the membrane voltage after virus induced depolarization, E_K is the K+ equilibrium voltage and ΔG_K is the increase in K+ conductance. E_K is calculated from the Nernst equation assuming an internal resting K+ concentration of 100 mM. E_K was calculated by treating Rb+ in the extracellular medium like K+ because both cations are transported with about the same efficiency by Kcv channels (Gazzarrini et al., 2003). We also assume that the K+ efflux is caused by a rise in ΔG_K of the chlorella membrane. If a virus inserts one Kcv channel into the virus/chlorella membrane continuum, ΔG_K is approximately 100 pS. This value is a conservative estimate for G_K considering the high single channel conductance of Kcv (Pagliuca et al., 2007) and the possibility that more than one Kcv channel per virus might contribute to ΔG_K. Because Ba^{2+} and Cs+ cannot enter the *Chlorella* cells and because both cations block Kcv inward currents in a voltage dependent manner (Gazzarrini et al., 2003), we can, to a first approximation, use the same ΔG_K value for calculating K+ efflux in the presence of inhibitors. Furthermore, we assume that *Chlorella* NC64A cells have a mean diameter of 4 μm and that 70% of this volume is aqueous. Because cells are spherical the total volume of all cells (10^9 ml^{-1}) can be calculated and related to the volume (1.83 ml) of the incubation medium. These values allow us to estimate the progressive decrease in [K+]$_i$ and the concomitant increase in [K+]$_o$. It is important to note that the substantial efflux of K+ also alters E_K. To account for this dynamic behavior of E_K, this value is recalculated in Equation (2) for Δt increments of 1 s.

Electron microscopy

Cells were concentrated by centrifugation at various times post infection (p.i.) and treated as described previ-

ously (Frohns et al., 2006). The resulting ultrathin cross-sections of *Chlorella* cells collected at each time point were inspected for different states of viral infection namely for particles which (i) were attached, (ii) had digested the cell wall and (iii) had ejected their DNA. In those instances where more than one particle was seen in the same cross-section the most advanced state (e.g. DNA ejection over cell wall degradation) was considered in the analysis. With 1200 cross-sections inspected for each time point, a 10% observation for DNA ejection for example in Figure 1 translates into 120 *Chlorella* cells in which single virus particles had ejected their DNA.

Acknowledgments

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