

12-2008

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## Chlorovirus-Mediated Membrane Depolarization of *Chlorella* Alters Secondary Active Transport of Solutes<sup>∇</sup>

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Received 7 August 2008/Accepted 30 September 2008

***Paramecium bursaria* chlorella virus 1 (PBCV-1) is the prototype of a family of large, double-stranded DNA, plaque-forming viruses that infect certain eukaryotic chlorella-like green algae from the genus *Chlorovirus*. PBCV-1 infection results in rapid host membrane depolarization and potassium ion release. One interesting feature of certain chloroviruses is that they code for functional potassium ion-selective channel proteins (Kcv) that are considered responsible for the host membrane depolarization and, as a consequence, the efflux of potassium ions. This report examines the relationship between cellular depolarization and solute uptake. Annotation of the virus host *Chlorella* strain NC64A genome revealed 482 putative transporter-encoding genes; 224 are secondary active transporters. Solute uptake experiments using seven radioactive compounds revealed that virus infection alters the transport of all the solutes. However, the degree of inhibition varied depending on the solute. Experiments with nystatin, a drug known to depolarize cell membranes, produced changes in solute uptake that are similar but not identical to those that occurred during virus infection. Therefore, these studies indicate that chlorovirus infection causes a rapid and sustained depolarization of the host plasma membrane and that this depolarization leads to the inhibition of secondary active transporters that changes solute uptake.**

Chloroviruses belong to the family *Phycodnaviridae*. They are large (190 nm in diameter), icosahedral, plaque-forming viruses with linear double-stranded DNA genomes (41, 45). The type member of the genus *Chlorovirus* is *Paramecium bursaria* chlorella virus 1 (PBCV-1); it has a genome of 331 kb that encodes ~366 proteins and 11 tRNAs. The PBCV-1 virion has a glycoprotein shell that surrounds a lipid bilayered membrane. Proteomic experiments established that the PBCV-1 virion contains more than 100 different virus-encoded proteins (D. Dunigan et al., unpublished data).

Chloroviruses infect certain freshwater, unicellular, eukaryotic chlorella-like green algae, which normally exist as endosymbionts in protists. The addition of PBCV-1 to its host, *Chlorella* strain NC64A, leads to the following program of events: (i) virus attachment to the cell wall is host specific and occurs at a unique virus vertex (45), followed by wall degradation at the point of attachment (25); (ii) rapid host membrane depolarization (11) and potassium ion release (28) occur within minutes of virus attachment; (iii) host nuclear DNA degradation begins at 3 to 5 min postinfection (p.i.) (1); (iv) early viral transcripts begin to appear at 5 to 10 min p.i. (17, 36); (v) virus DNA replication begins at 60 to 90 min p.i. (42); (vi) late virus transcription begins at 60 to 90 min p.i. (36); and (vii) viral-induced lysis and particle release occur at 6 to 8 h p.i.

*Chlorella* cells typically have a negative resting membrane potential of  $-120$  to  $-150$  mV (19) (Fig. 1). As in other plant cells, this negative voltage is achieved by a  $H^+$  ATPase, which

pumps  $H^+$  out of the cytoplasm, and a low conductance of all passive transporters. This resulting proton motive force across the plasma membrane provides the principal driving force for solute fluxes across the plasma membrane via secondary active transporters (also referred to as secondary transporters) (22).

Many chloroviruses encode a channel protein (called Kcv) that forms a functional  $K^+$ -selective channel. PBCV-1 Kcv was the first virus-encoded  $K^+$  channel protein to be discovered, and it is also the smallest protein (94 amino acids) known to form a functional  $K^+$  channel when expressed in *Xenopus* oocytes (33) and mammalian HEK293 (27) and CHO (12) cells. Chloroviruses expressing Kcv homologs have selective sensitivity to known  $K^+$  channel blockers, such as  $Ba^{2+}$  and  $Cs^+$ , resulting in diminished virus replication (24). Kcv is postulated to be located in the internal  $\sim 40$ -Å-thick membrane of the virus particles and to play the following role during virus infection: after PBCV-1 infects its host by degrading the cell wall, presumably there is contact between the virus and host membrane, resulting in a fusion of the viral and the host cell membranes. Because of the high conductance of the Kcv channel in the viral membrane, this fusion of membranes causes a rapid elevation of the  $K^+$  conductance and a consequent depolarization of the entire host plasma membrane/virus membrane continuum. The combination of membrane depolarization and elevated  $K^+$  conductance leads to  $K^+$  release from the host cell. This release reduces the host cell osmotic pressure, which can reach 0.7 MPa in plant cells (44), and facilitates viral DNA ejection into the host (24, 28).

The present report further examines the effect of chloroviruses on membrane transport features of the host cell. The data show that virus infection results in a drastic decrease in the secondary active transport of solutes across the plasma

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<sup>∇</sup> Published ahead of print on 8 October 2008.

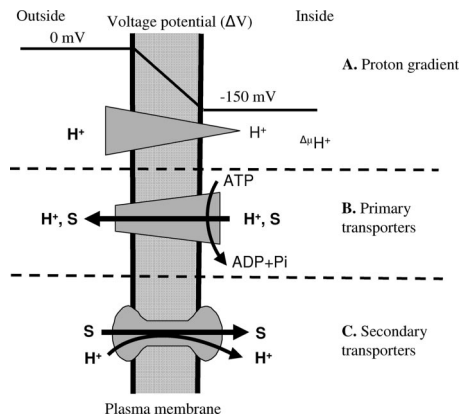


FIG. 1. Electrochemical potential and transport mechanisms of a plant cell (adapted from reference 22). (A) Plant cell electrochemical potential is usually based on a proton ( $H^+$ ) gradient. A cell establishes the  $H^+$  gradient across the cell plasma membrane by using ATP energy to pump protons out of the cell. (B and C) Active transporters can be classified as primary (ATP powered) or secondary (driven by the electrochemical gradient potential). Secondary transporters translocate a solute across the cell membrane into the cell using the energy of the proton moving down its concentration gradient. Some solutes have very complex transport pathways. A cell can have several transporters for a solute, it can be an active transport system (ATP powered; e.g., ABC transporters), and it can have secondary active transporters working at the same time. All of these transporters may be controlled differently; some of them are constitutively expressed, some need induction for expression, and many of them are regulated by a feedback, depending on solute concentration or other factors (22).

membrane into the host cell. This decrease provides additional support for the hypothesis that chlorovirus infection causes a depolarization of the host cell membrane; as a consequence, the uptake of solutes by secondary active transporters rapidly decreases. This means that all of the material which is required for the synthesis of virus progeny must be recruited from within the host cell. In addition, the data also imply that infected host cells are impaired in taking up radioactive adenine so that results from previous experiments on RNA synthesis during virus infection must be reinterpreted.

#### MATERIALS AND METHODS

**Strains and culture conditions.** The growth of the host *Chlorella* strain NC64A on bold basal medium (BBM) modified by the addition of 0.5% sucrose and 0.1% peptone (MBBM) and the production and purification of viruses PBCV-1 and NY-2A have been previously described elsewhere (1, 43).

**Solute uptake analysis.** *Chlorella* cells ( $5 \times 10^7$  to  $6 \times 10^7$  per ml) were infected with virus at a multiplicity of infection (MOI) of 10 PFU per cell. Mock-infected cells (using Tris buffer; 50 mM Tris-HCl, pH 7.8) served as a control. The radioactively labeled solutes (Table 1) were added for 10 min, starting at 10 min p.i.; radioactive solutes not taken up by the cells were removed by diluting the samples 20-fold with ice-cold growth medium, followed by centrifugation at  $3,000 \times g$  for 5 min at  $4^\circ C$ . The cell pellets were resuspended in fresh growth medium, and three samples (500  $\mu$ l) were taken from each treatment, mixed with 5 ml of Opti-Fluor (PerkinElmer, Boston, MA), and counted in a liquid scintillation counter.

**Nystatin treatment.** Prior to infection, *chlorella* cells ( $5 \times 10^7$  to  $6 \times 10^7$  per ml) were incubated for 10 min with nystatin (Sigma, St. Louis, MO) at a final concentration of 5  $\mu$ g/ml (nystatin was dissolved in methanol at 5 mg/ml). An equal amount of methanol (1  $\mu$ l/ml) was added to control cells. The radioactively labeled solutes were added for 10 min from 10 to 20 min p.i., and the samples were then processed as described above.

**Sodium azide treatment.** Sodium azide (100  $\mu$ M) was added to *chlorella* cells ( $1.2 \times 10^7$  to  $1.5 \times 10^7$  cells/ml) for 3 min before virus infection or the addition

of nystatin (5  $\mu$ g/ml). The radioactively labeled solutes were added for 10 min from 10 to 20 min p.i. Reactions were stopped by adding ice-cold growth medium, and the samples were processed for radiometric analysis as described above. Mock-infected cells (Tris buffer) and *chlorella* cells with methanol (1  $\mu$ l/ml) served as controls.

**Growth medium pH adjustments.** *Chlorella* cells grown in MBBM to  $1.2 \times 10^7$  to  $1.5 \times 10^7$  cells per ml were collected by centrifugation; the cells were resuspended in either MBBM at pH 5.0 (adjusted with  $H_3PO_4$ ), MBBM at pH 8.0 (adjusted with NaOH) or regular MBBM (pH 7.0) to  $5 \times 10^7$  to  $6 \times 10^7$  cells per ml. The cells were equilibrated in the modified media for 3 h prior to initiating experiments. Cells were infected with PBCV-1 at an MOI of 10. Mock-infected cells (Tris buffer) in MBBM with adjusted pH values served as the controls. Radioactively labeled solutes were added for 10 min, starting at 10 min p.i., and then the radioactive tracer was removed by dilution and the samples were processed as described above.

**Exogenous digestion of the *chlorella* cell wall.** The cell walls of *Chlorella* NC64A were disrupted using a crude mixture of cell wall-digesting enzymes (called "lysin") prepared from *Acanthocystis turfacea* chlorovirus 1 (ATCV-1) (5). ATCV-1 has a different host (*Chlorella* SAG 3.83), and the virus does not attach to *Chlorella* NC64A cells. *Chlorella* NC64A cells ( $4 \times 10^8$  to  $5 \times 10^8$  cells/ml) in BBM were treated for 1 h with the ATCV-1 lysin. Control cells had an equal amount of Tris buffer. Radioactive [ $^3H$ ]adenine was added for 10 min from 10 to 20 min p.i., and the samples were processed as described above.

**Lysin preparation and activity assay.** An ATCV-1 virus preparation (3 mg/ml) (5) in Tris buffer was sonicated (Tekmar ultrasonic processor; 100 W model) in an ice bath for 2 min (5-s pulses for 24 pulses) and centrifuged at  $4^\circ C$  for 15 min at  $18,000 \times g$ . The supernatant fraction (200  $\mu$ l) containing the soluble virion-associated proteins (ATCV-1 lysin) was mixed with 800  $\mu$ l *Chlorella* NC64A cells ( $4 \times 10^8$  to  $5 \times 10^8$  cells/ml) and incubated for 1 h at  $20^\circ C$ . *Chlorella* NC64A wall digestion by ATCV-1 lysin was assayed by measuring sodium dodecyl sulfate (SDS)-mediated chlorophyll release (18). After ATCV-1 lysin treatment, SDS was added to the samples to a final concentration of 2% and incubated for 30 min at  $20^\circ C$  before centrifuging for 10 min at  $18,000 \times g$ . The optical densities of the supernatant fractions were determined at a  $\lambda$  of 435 nm to measure chlorophyll release, indicating the extent of cell wall damage.

**Treatment with potassium ion channel blockers.** *Chlorella* cells in MBBM ( $1.2 \times 10^7$  to  $1.5 \times 10^7$  cells/ml) were infected with viruses at an MOI of 10. The  $K^+$  channel blockers at 10 mM  $Ba^{2+}$  (as  $BaCl_2$ ) and  $Cs^+$  (as  $CsCl$ ) were added to the cells 3 min before virus infection. Mock-infected cells (Tris buffer) with the corresponding  $K^+$  channel inhibitor served as controls. The radioactively labeled solute was added for 10 min, from 10 to 20 min p.i., and then the samples were processed for radiometric analysis as described above.

**RNA synthesis analysis.** Exponentially growing *chlorella* cells at  $1.2 \times 10^7$  to  $2.0 \times 10^7$  cells/ml were collected by centrifugation and then resuspended in fresh growth medium at a concentration of  $5 \times 10^7$  to  $6 \times 10^7$  cells/ml. Virus was added at an MOI of 10. Mock-infected control cells had an equal amount of Tris buffer.

**Pulse-labeling cells with adenine.** Infected cells were pulse-labeled for 5 min with [ $^3H$ ]adenine at 5, 15, 25, 40, 55, and 85 min p.i. Infections were stopped by adding an equal volume of 10% trichloroacetic acid (TCA) to the reaction mixtures. Cells were washed three times with 5% TCA, resuspended in 0.5 M NaOH, incubated overnight at  $37^\circ C$ , and centrifuged for 10 min at  $18,000 \times g$ , and three samples (250  $\mu$ l) of the supernatant fraction were neutralized with an

TABLE 1. Characteristics of the radioactive solutes tested<sup>a</sup>

Solute	Properties and type of transporter/class	Isotope	Specific activity (MBq/ml)	Solute concn (nM)
Adenine	Nucleobase	$^3H$	37	215
Glucose	Monosaccharide	$^3H$	37	80
Proline	Hydrophobic amino acid	$^3H$	37	50
Histidine	Basic amino acid	$^{14}C$	1.85	0.8
Methionine	Hydrophobic amino acid	$^3H$	37	40
Ornithine	Basic amino acid	$^{14}C$	1.85	4.8
Putrescine	Polyamine	$^{14}C$	1.85	2.34

<sup>a</sup> All tested solutes were obtained from Amersham/GE Healthcare, Piscataway, NJ.

equal volume of 10% TCA, mixed with 5 ml of Opti-Fluor, and counted with a liquid scintillation analyzer as an indicator of RNA synthesis.

**Preloading cells with adenine.** *Chlorella* cells were incubated with [<sup>3</sup>H]adenine for 5 min, and then the radioactive tracer was removed by centrifugation and the cells were washed with growth medium. A sample taken at the time of infection served as a baseline. Samples were collected at 10, 20, 30, 45, 60, and 90 min p.i., and infection was stopped by adding an equal volume of 10% TCA. Samples were processed for radiometric analysis as described above.

**Membrane potential recordings.** Changes in membrane voltage of *Chlorella* NC64A cells were recorded with the voltage-sensitive dye bisoxonol as described previously (11). To account for photobleaching during the long measurements, each recording was accompanied by monitoring the fluorescence under the same conditions but without viruses. The data were normalized to the value at the beginning of the recording, and the data that were obtained without virus infection were subtracted from those measured in the presence of viruses.

**Cytosolic pH.** The cytosolic pH of *Chlorella* cells was measured with the ratiometric pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Mobictec, Göttingen, Germany). *Chlorella* cells ( $3.5 \times 10^6$  cells/ml) were incubated for 90 min in the dark in BBM containing 20  $\mu$ M dye in its acetoxymethyl ester form (BCECF-AM). This molecule is membrane permeable; the ester is cleaved by esterases inside the cell, and the pH-sensitive dye is then trapped inside the cells and fluoresces. Ratiometric measurements were performed in a fluorescence spectrometer (Jasco FP-6200; Tokyo, Japan), switching between the two excitation wavelengths  $490 \pm 5$  nm and  $436 \pm 5$  nm. The emitted fluorescent light was collected at  $530 \pm 10$  nm. Cells with a density of  $3.5 \times 10^6$  cells/ml were incubated in a 3.5-ml quartz cuvette; after obtaining a stable baseline, the virus was added at an MOI of 10. In vivo calibrations were performed according to Dixon et al. (9) as follows: *Chlorella* cells were first loaded with dye as described above and were then transferred into MBBM with defined pH values of between pH 5.0 and pH 9.0. Buffering was achieved with either 10 mM MES (morpholineethanesulfonic acid) or HEPES buffers. The permeability of the plasma membrane for protons was increased by adding 20  $\mu$ g/ml of nigericin (Sigma, Deisenhofen, Germany) to the incubation buffer. The fluorescence ratio F<sub>490</sub>/F<sub>436</sub> was recorded after reaching a new steady state, assuming an equilibration of the cytoplasmic pH with that of the bath medium. The calibration curve was fitted as previously described by Plieth et al. (32). From the calibration, we estimated a resting pH of about pH 6.5 for the *Chlorella* NC64A cells. This value is low for a cytoplasmic pH and may indicate that the measurement is influenced by some dye uptake into the chloroplast.

**Statistical analysis.** All experiments were conducted at least twice with three subsamplings in each treatment. The effect of a particular treatment on the transport of different solutes was calculated as the percentage of control for each subsampling in the experiment. The results are expressed as the mean  $\pm$  the standard deviation (SD). Statistical analysis between different solutes and treatments was carried out using the least squares means method with SAS software (SAS Institute, Inc., Cary, NC). The differences between mean values were considered significant if the *P* value was less than 0.05.

## RESULTS

**Effect of virus infection on adenine uptake.** Adenine is commonly used as a precursor to monitor RNA and DNA synthesis in vivo. Adenine uptake typically occurs by a secondary transporter named purine permease (PUP) (8, 13). PUP is an integral membrane protein that transports adenine and cytosine with high affinity. The protein is a secondary cotransporter that uses the proton motive force across the plasma membrane to take up the nucleosides. Adenine uptake occurs against a concentration gradient and is sensitive to protonophores (13).

An experiment to monitor the effect of virus PBCV-1 infection on adenine uptake indicates that adenine transport was inhibited about 77% in virus-infected cells from 10 to 20 min p.i. (Fig. 2A). The cells were also monitored for adenine uptake from 170 min to 180 min p.i. to determine if the initial inhibition of uptake was transitory (Fig. 2A). Adenine transport did not return to the mock-infected level during the first 3 h p.i., which is well into the virus DNA synthesis phase of infection.

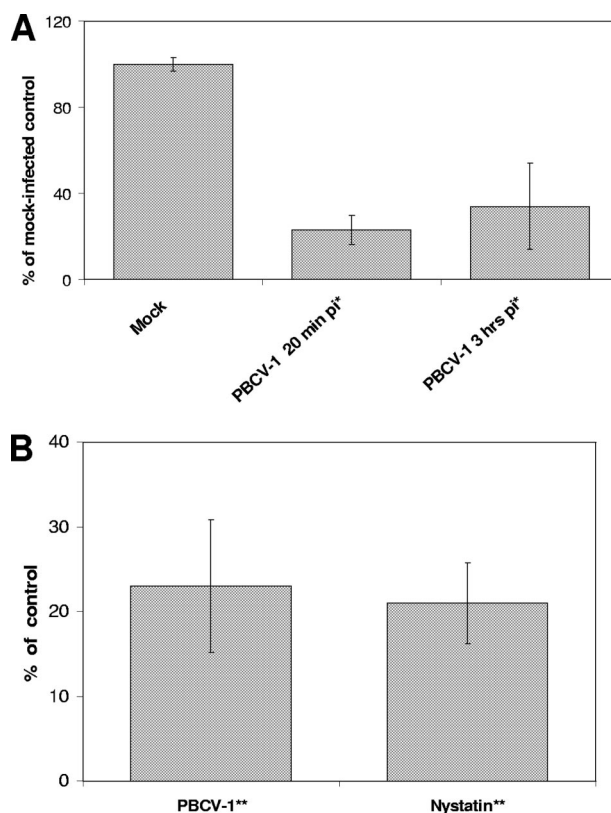


FIG. 2. Effect of virus PBCV-1 infection and nystatin treatment on *Chlorella* NC64A adenine uptake. (A) Effect of PBCV-1 infection on adenine uptake. *Chlorella* cells were infected with PBCV-1 at an MOI of 10. Cells were pulse labeled with [<sup>3</sup>H]adenine 10 min before the indicated time (a 10-min pulse). Mock-infected *Chlorella* cells served as a control. Results are plotted as the mean  $\pm$  SD ( $n = 6$ ). Asterisk,  $P \geq 0.05$ . (B) Comparison of PBCV-1 infection to nystatin treatment. *Chlorella* cells were treated with nystatin 5  $\mu$ g/ml for 10 min before labeling with [<sup>3</sup>H]adenine for 10 min. Untreated cells served as a control. Results are plotted as the mean  $\pm$  SD ( $n = 9$ ). Two asterisks,  $P \geq 0.05$ .

The reduced import of adenine could in principle be due to depolarization of the host membrane and/or acidification of the cytosol. Both processes would result in a decrease in the proton motive force, which facilitates adenine import. Therefore, we explored both possibilities. First, we monitored *Chlorella* membrane voltage over the entire 3 h p.i. The results indicate that infection of *Chlorella* NC64A rapidly depolarized the cell membrane (Fig. 3). After passing a peak depolarization of about 700 s p.i., the membrane only partially repolarized; during the entire period, the membrane remained more positive than the resting voltage prior to infection. Collectively, this means that virus infection is associated with a sustained lowering of the electrical driving force for secondary transport.

Second, we measured the cytoplasmic pH of *Chlorella* NC64A cells during virus infection. *Chlorella* NC64A cells were loaded with the fluorescent pH-sensitive dye BCECF. The ratio of fluorescence obtained at an excitation of F<sub>490</sub> and F<sub>436</sub> is pH sensitive (32); an increase in the ratio indicates alkalinization of the cell, while a decrease indicates acidification. As expected, loading of the cells with the weak acid by adding 10 mM acetate to the extracellular medium resulted in

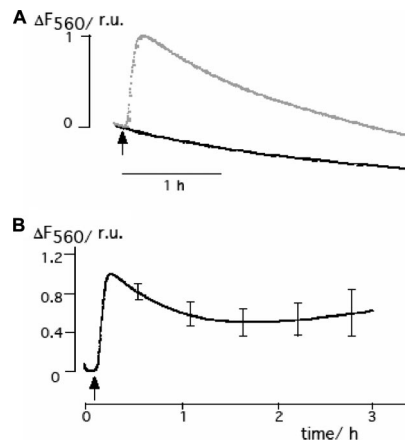


FIG. 3. Long-term effect of PBCV-1 on the membrane potential of *Chlorella* NC64A cells as reported from the fluorescence of bisoxonol at 560 nm (F560). Approximately  $3.5 \times 10^6$  chlorella cells/ml were maintained in MBBM containing  $1 \mu\text{M}$  bisoxonol. Viruses were added at an MOI of 10 at the time indicated by the arrow. (A) Change in bisoxonol fluorescence in solution with *Chlorella* NC64A cells with (gray line) and without (black line) the addition of viruses. The black curve reports the photobleaching of the fluorescent dye; the virus-induced increase in bisoxonol fluorescence, which results from a depolarization of the cell, is given by the difference between the two curves. (B) Mean difference ( $\pm$  SD;  $n = 4$ ) in PBCV-1-induced bisoxonol fluorescence. To compare different measurements, the data were normalized to the peak of the fluorescence increase.

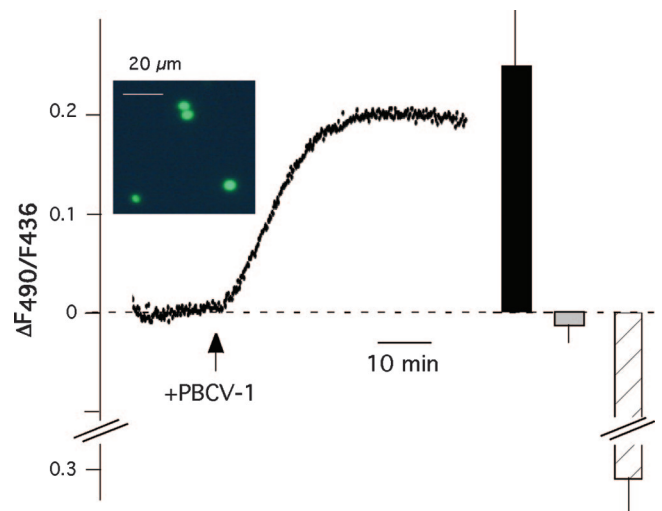


FIG. 4. Virus PBCV-1 infection evokes an alkalization of the internal pH of *Chlorella* NC64A cells. Fluorescence of *Chlorella* NC64A cells was monitored with the preloaded BCECF dye (inset). The continuous recording indicates the trajectory of the ratiometric fluorescence recording of cells before and after (at arrow) infection with PBCV-1 at an MOI of 10. The bar graph shows the mean difference ( $\pm$  SD;  $n > 6$ ) in fluorescence ratio  $\Delta F_{490}/F_{436}$  over a period of 3,000 s in response to virus infection of *Chlorella* NC64A (solid bar) or *Chlorella vulgaris* (gray bar). The hatched bar reports the decrease in fluorescence ratio  $\Delta F_{490}/F_{436}$  upon the addition of 10 mM acetate to the incubation buffer.

a decrease in the fluorescence ratio (Fig. 4). The trajectory of the fluorescence ratio in Fig. 4 shows that virus infection of *Chlorella* NC64A cells (an MOI of 10) resulted in an increase in the ratio, indicating alkalization of the cytoplasm. Similar results were obtained in eight independent experiments, giving a mean increase in the fluorescence ratio of  $0.25 \pm 0.06$ . From in vivo calibrations, this increase in ratio translates into a pH difference of 0.4 to 0.5 pH units. The effect of PBCV-1 infection on the cytosolic pH was specific for the host. Similar experiments with *Chlorella vulgaris*, a nonhost for the virus, produced no appreciable change in pH (Fig. 4). Collectively, these data indicate that infection of *Chlorella* NC64A cells is associated with an increase in the cytosolic pH of the host cells. However, this alkalization does not explain the inhibition of adenine import; in contrast, for thermodynamic reasons, alkalization should favor adenine import via secondary transport. Hence, the primary reason for the reduced uptake of adenine after virus infection must be due to the rapid and sustained depolarization of the plasma membrane.

#### Effect of nystatin on *Chlorella* NC64A adenine transport.

Cells were treated with different concentrations of nystatin (0.5 to 200  $\mu\text{g}/\text{ml}$ ), a drug known to reduce chlorella cell membrane potential (24, 30), in order to evaluate the secondary active nature of adenine transport in *Chlorella* NC64A cells. Nystatin reduced adenine uptake about 80% at 5  $\mu\text{g}/\text{ml}$  ( $\sim 5 \mu\text{M}$ ) (Fig. 2B); higher concentrations did not increase the inhibition of adenine uptake significantly. Apparently, 5  $\mu\text{g}/\text{ml}$  nystatin is a saturating dose for *Chlorella* NC64A cells. Separate experiments established that 5  $\mu\text{g}/\text{ml}$  nystatin inhibits *Chlorella* NC64A growth and prevents PBCV-1 replication (results not shown).

The inhibition of adenine uptake by nystatin mimicked the inhibition by PBCV-1 infection, indicating that plasma membrane potential is required for adenine transport into *Chlorella* NC64A cells. This similarity between virus infection and nystatin treatment also supports the concept that the change in adenine uptake in virus-infected cells is due to virus-mediated membrane depolarization, probably leading to an inhibition of PUP.

**Transporter proteins encoded by *Chlorella* NC64A.** The *Chlorella* NC64A genome was recently sequenced by the Joint Genome Institute (U.S. Department of Energy), and ninefold coverage data are available for annotation (<http://www.jgi.doe.gov/NC64A/>). The *Chlorella* NC64A genome was analyzed for genes encoding cytoplasmic membrane transport proteins. This analysis also led to the identification of the types of solutes that might be altered by depolarization of the plasma membrane.

The sequence analysis revealed that *Chlorella* NC64A encodes at least 482 putative transporters and ion channels (Table 2). This number of transport-associated proteins is similar to that of other algae whose genomes have been sequenced (35), e.g., *Chlamydomonas reinhardtii* (486 transporter proteins), *Thalassiosira pseudonana* (429 transporters), and *Ostreococcus tauri* (323 transporters). The density of genes encoding transporters for *Chlorella* NC64A is about 11.3 transporter proteins per 1 Mb of genome. Other algae range between 4.9 transporters per 1 Mb of genome for *C. reinhardtii* and 28 transporters per 1 Mb for *O. tauri*.

Sixty-four of the *Chlorella* NC64A genes are predicted to encode ion channel proteins; such channel proteins transport solutes in a passive fashion. The transporter group driven by

TABLE 2. Putative solute transporters encoded by *Chlorella* NC64A

Characteristic	Value <sup>a</sup>
Genome size .....	46.2
Transporters per Mb genome .....	11.3
ATP dependent .....	194
Ion channels .....	64
Secondary active transporter .....	224
Amino acid, polyamine, and oligopeptide transporter .....	73
Nucleobases and nucleoside transporter .....	17
Hexose and carbohydrate transporter .....	37
Inorganic ion transporter .....	60
Other .....	37
Total .....	482

<sup>a</sup> All values are number of transporters except for genome size (Mb).

ATP hydrolysis consists of 194 proteins; transporters in this class are often referred to as primary active transporters. These proteins couple energy released during ATP hydrolysis to translocate a substance against its electrochemical potential. Transmembrane transporters of the other major class are referred to as secondary active transporters. In plants, this type of transport utilizes energy stored in the electrochemical gradient of protons across the cell membrane (22). *Chlorella* NC64A encodes 224 such genes, as follows: 73 amino acid, polyamine, and oligopeptide transporters; 17 nucleobase and nucleoside transporters, including PUP; 37 hexose and sugar transporters; 60 inorganic ion transporters; and 37 other secondary transporters.

*Chlorella* NC64A annotation is preliminary, and the final number of transporters encoded by the alga may differ slightly from these estimates. Nevertheless, these results indicate that *Chlorella* NC64A shares many aspects of solute transport with other eukaryotic organisms.

**Effect of virus infection on solute uptake.** Members of three of the five families of secondary transporters were evaluated for their response to virus infection as follows: (i) the amino acid, polyamine, and oligopeptide family; (ii) the nucleobase and nucleoside family; and (iii) the hexose and carbohydrate family. The amino acid, polyamine, and oligopeptide family was evaluated using four amino acids (proline, methionine, histidine, and ornithine) and one polyamine precursor (putrescine). Adenine was used to evaluate the nucleobase and nucleoside family, and glucose was used to evaluate the hexose and carbohydrate family. These compounds are commercially available as radioactive tracers (their properties are listed in Table 1).

All experiments on the effect of virus infection on solute uptake were accompanied by experiments with nystatin to evaluate the secondary nature of the particular solute transport system. Experiments with different solutes revealed that virus infection caused changes in the uptake of all the solutes tested, but the degree of effect depended on the solute (Table 3). The uptake changes caused by virus infection and nystatin treatment were similar but not identical for most of the solutes. Both virus infection and nystatin had the highest inhibitory effect on proline uptake, which was nearly abolished, about 3% to 6% of the control in both cases.

TABLE 3. Effect of PBCV-1 infection on solute transport in *Chlorella* NC64A cells

Solute	Transporter class	Solute uptake as a % of that of the mock-infected control <sup>a</sup>		
		Virus	Nystatin	P value
Adenine	Nucleobase	23 ± 7.9 (37)	21 ± 4.9 (9)	0.476
Glucose	Monosaccharide	66 ± 15.1 (9)	30 ± 9.2 (6)	0.014
Proline	Hydrophobic amino acid	3 ± 3.4 (28)	6 ± 4.7 (9)	0.8521
Methionine	Hydrophobic amino acid	19 ± 8.3 (9)	25 ± 7.2 (9)	0.657
Histidine	Basic amino acid	66 ± 6.8 (9)	82 ± 8.2 (6)	0.273
Ornithine	Basic amino acid	63 ± 9.3 (18)	100 ± 13.0 (9)	0.016
Putrescine	Polyamine	35 ± 13.0 (12)	10 ± 2.7 (9)	0.096

<sup>a</sup> Values are means ± SD (number of experimental replicates).

Glucose and putrescine uptake was suppressed by both PBCV-1 infection and nystatin, but it was significantly less for both solutes in virus-infected cells than in nystatin-treated cells. Glucose transport in nystatin-treated cells was about 30% of that of the control, whereas in PBCV-1-infected cells it was 66% of that of mock-infected cells (Table 3). Putrescine uptake was 10% compared to that of the control in nystatin-treated cells versus 35% of that in virus-infected cells.

Histidine and ornithine (both basic amino acids) transport were more affected by virus infection than by nystatin treatment (Table 3). In PBCV-1-infected cells, ornithine uptake was 63% of that of control cells. Nystatin treatment did not alter ornithine uptake, indicating that this solute is likely not transported by secondary transporters. Histidine transport was 66% in PBCV-1-infected cells compared to that in mock-infected cells, and in nystatin-treated cells it was 82% of that of the control cells.

**Effect of sodium azide on *Chlorella* NC64A glucose and putrescine transport.** The inhibition of both glucose and putrescine uptake was less in virus-infected cells than in nystatin-treated cells. To determine the impact of ATP on these transport processes, cells were treated with sodium azide (NaN<sub>3</sub>), an uncoupler of oxidative phosphorylation. Studies of putrescine and glucose uptake were conducted in the presence of 100 μM NaN<sub>3</sub> added 3 min before virus infection or nystatin treatment. NaN<sub>3</sub> diminished the differences in glucose and putrescine uptake between virus infection and nystatin treatment and reduced the uptake of both solutes to ~10% of that of the control (results not shown). These results are consistent with the view that glucose and putrescine uptake into *Chlorella* NC64A depends on energy from ATP hydrolysis. The results of these experiments further imply that the effect of virus-induced depolarization on the proton motive force for solute uptake is less severe than that obtained by the ionophore nystatin or by depletion of the cellular ATP concentration in response to azide.

**Effect of K<sup>+</sup> channel blockers on solute transport in virus-infected *Chlorella* NC64A.** As mentioned in the introduction, the virus-encoded K<sup>+</sup> channel protein (Kcv) is predicted to be responsible for cell membrane depolarization during virus infection. Chlorovirus-encoded Kcv channels have selective sensitivity to known K<sup>+</sup> channel blockers (24, 28). PBCV-1 Kcv is blocked by Ba<sup>2+</sup> and is less sensitive to Cs<sup>+</sup>. The virus NY-2A

TABLE 4. Effect of K<sup>+</sup> channel inhibitors on adenine and proline transport in virus-infected *Chlorella* NC64A cells<sup>a</sup>

Solute	Treatment	Solute uptake as a % of that of the mock-infected control			
		PBCV-1		NY-2A	
		Mean ± SD	P value	Mean ± SD	P value
Adenine	None	23 ± 7.9 (37)		12 ± 6.8 (6)	
	Ba <sup>2+</sup>	84 ± 6.8 (21)	0.0001	74 ± 12.0 (6)	0.025
	Cs <sup>+</sup>	38 ± 12.7 (6)	0.177	72 ± 13.7 (6)	0.034
Proline	None	3 ± 3.4 (28)		2 ± 0.2 (6)	
	Ba <sup>2+</sup>	42 ± 10.4 (15)	<0.0001	4 ± 1.6 (6)	0.021
	Cs <sup>+</sup>	9 ± 1.5 (6)	0.300	7 ± 4.7 (6)	0.049

<sup>a</sup> *Chlorella* cells were treated with 10 mM Ba<sup>2+</sup> or Cs<sup>+</sup> for 3 min and infected with PBCV-1 at an MOI of 10. Radioactively labeled solutes were added for 10 min starting at 10 min p.i. Control cells had an equal amount of inhibitor. Results are presented as means ± SD (number of experimental replicates). The differences between mean values were considered significant if the *P* value was less than 0.05. PBCV-1 Kcv is blocked by Ba<sup>2+</sup> and is less sensitive to Cs<sup>+</sup>. The NY-2A Kcv ion channel is blocked by both Ba<sup>2+</sup> and Cs<sup>+</sup>.

Kcv ion channel is blocked by both Ba<sup>2+</sup> and Cs<sup>+</sup>. Adenine and proline uptake were measured in PBCV-1- and NY-2A-infected cells in the presence of either Ba<sup>2+</sup> or Cs<sup>+</sup> to test the hypothesis that virus infection induced changes in the membrane potential and, consequently, that the inhibition of secondary transporter proteins is dependent on the Kcv channels.

Adding Ba<sup>2+</sup> decreased the inhibition of adenine transport by PBCV-1 infection. Adenine uptake went from 23% of that of the control to 84% of that of the control in Ba<sup>2+</sup>-treated cells. Ba<sup>2+</sup> had a similar effect on proline uptake, reducing the inhibitory affect of virus infection from 3% of the mock-infected control to 42% (Table 4). Cs<sup>+</sup> did not have a significant effect on the uptake of either adenine or proline by PBCV-1-infected cells, consistent with the linkage to Kcv and membrane depolarization sensitivity.

In NY-2A infection, the inhibition of adenine uptake was significantly altered by Ba<sup>2+</sup> treatment (from 12% of that of the mock-infected control to 74% for adenine) and Cs<sup>+</sup> treatment (from 2% to 72% for adenine). However, the effect of Ba<sup>2+</sup> and Cs<sup>+</sup> on proline uptake is difficult to explain. The addition of both of these cations led to increased proline uptake, which was statistically significant (Table 4); however, the increase from 2% to 4% for Ba<sup>2+</sup> and 2% to 7% for Cs<sup>+</sup> in absolute values does not represent a major reversal of virus-mediated inhibition.

One possible explanation for these observations is that different transporters have different dependencies on membrane potential. Such differences are expected, depending on the mode of operation of the respective secondary transporters. An electrogenic symporter, for example, exhibits a much stronger dependency on the membrane potential than an electro-neutral transporter.

**Effect of medium pH on adenine and proline transport in *Chlorella* NC64A.** Most secondary transporters in plants utilize protons to cotransport solutes against their gradient. For thermodynamic reasons, it can be expected that external acidification increases while alkalization decreases secondary active transporters, because the cell has to invest less or more energy, respectively, to maintain the proton gradient across the plasma

membrane. The full impact of extracellular pH on transport, however, is more complex because the activity of transport enzymes usually shows an inherent pH dependency, and the protonation of the transporter could be sensitive to the pre-voltage membrane voltage (37). As a result of these concerted actions, the activity of extracellular H<sup>+</sup> on secondary transporters usually shows a sigmoidal decrease in activity, with progressive alkaline pH values (37).

To examine pH dependency, adenine and proline uptake were measured in PBCV-1-infected *Chlorella* NC64A cells at different medium pHs (Fig. 5A and B). Transport of adenine into *Chlorella* NC64A cells did not differ significantly between pH 5.0 and pH 7.0. Consistent with the hypothesis of H<sup>+</sup>-coupled transport, adenine uptake was lower at pH 8.0 than at both pH 5.0 and 7.0 (Fig. 5A). The amount of adenine taken up in virus-infected cells was about the same at all three pH values, suggesting that H<sup>+</sup>-coupled import is no longer relevant in infected *Chlorella* cells.

The proline results support the view that uptake of this amino acid into cells is coupled to proton transport (Fig. 5B). In mock-infected cells, proline uptake is about 37% higher when the pH of the medium is 5.0 than when it is pH 7.0 (the normal pH of MBBM), and uptake is about 70% lower when the pH of the medium is 8.0 than when it is pH 7.0. At all three pH values, PBCV-1 infection resulted in about 95% inhibition

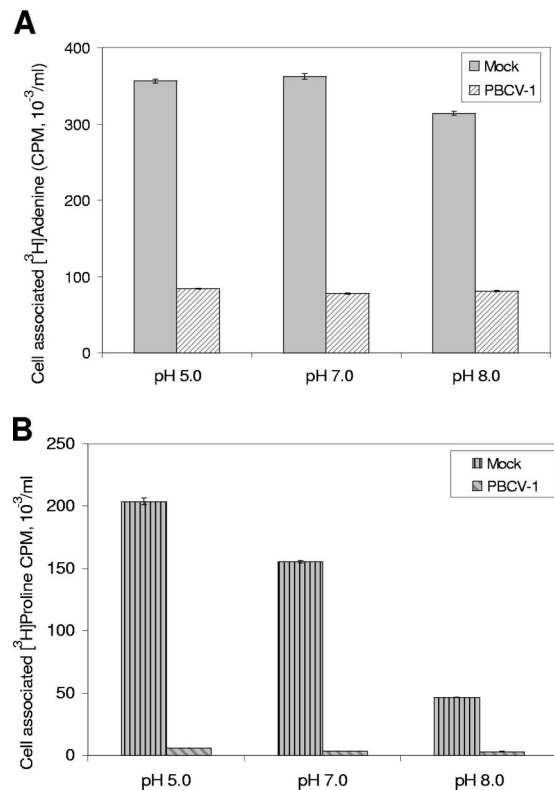


FIG. 5. Effect of pH on solute uptake in PBCV-1-infected *Chlorella* NC64A cells. Cells in media with different pHs were infected with PBCV-1 at an MOI of 10. Radioactively labeled solutes were added for 10 min, starting at 10 min p.i. Mock-infected cells in medium with a corresponding pH served as a control. Results are plotted as the mean ± SD (*n* = 6). (A) Effect of pH on adenine uptake. (B) Effect of pH on proline uptake.



TABLE 5. Effect of cell wall damage on adenine uptake in *Chlorella* NC64A cells

Treatment	Chlorophyll released ( $A_{435}$ ) ( $n = 3$ )	Relative chlorophyll released ( $A_{435}$ )	Adenine uptake (CPM, $10^3$ ) ( $n = 3$ )
NC64A cells, untreated (control)	0.043 $\pm$ 0.002	1.0	254 $\pm$ 4.13
NC64A cells treated with ATCV-1 solubilized lysin	0.365 $\pm$ 0.062	9.3	242 $\pm$ 12.05

of proline uptake. Thus, the degree of inhibition of proline uptake by PBCV-1 infection was not altered by the pH of the medium. This result can be explained by the fact that virus infection inhibits proline transport to nearly background levels, but the uninfected cell is hypersensitive to changes in the membrane potential.

These results, along with those shown in Table 4, indicate that nucleobases and amino acid transporters in *Chlorella* NC64A may have very different mechanisms. While proline transport seems to use the  $H^+$  concentration gradient, the transport of adenine makes more use of the electrical gradient. But in spite of the different mechanisms of secondary transport, both uptake systems are strongly inhibited by virus infection.

**Effect of cell wall degradation on adenine uptake in *Chlorella* NC64A.** The cell wall is the first barrier that chloroviruses have to overcome when infecting their hosts. Chloroviruses encode and package several enzymes in the virion that are probably involved in degrading the host cell wall during infection (6, 38–40, 45). One possibility is that this enzymatic “tampering” with the host cell wall triggers a defense process in the host, which indirectly causes membrane depolarization and hence a drop in the proton motive force of the host. Such phenomena are known in higher plant cells (4). To evaluate this possibility, a crude cell wall-degrading enzyme preparation, called lysin, was isolated from virus ATCV-1 virions. Although virus ATCV-1 does not attach to *Chlorella* NC64A, the ATCV-1 lysin degrades *Chlorella* NC64A walls sufficiently so that the cells become sensitive to SDS lysis, resulting in the release of measurable chlorophyll. That is, a 1-h treatment of *Chlorella* NC64A cells with ATCV-1 lysin caused a ninefold increase in released chlorophyll from the treated cells (Table 5). However, the lysin treatment did not alter adenine uptake by *Chlorella* NC64A (Table 5). We conclude that the change in solute transport is not the result of cell wall digestion. The change probably results from the fusion of the virus membrane with the host membrane rather than a secondary effect due to a perturbed cell wall.

**Effect of PBCV-1 infection on adenine incorporation into RNA.** Several experiments have been conducted in the past 25 years on the relationship between PBCV-1 infection and the apparent rates of macromolecular syntheses (36, 42). These experiments have produced incongruent results, depending on the labeling technique. In the context of the current findings, we reevaluated the role of transport in the radiolabeling of nucleic acids during virus infection.

Nucleic acid synthetic rates (RNA and DNA) are commonly measured by pulse-labeling techniques, where radioactive tracers are used to track the incorporation of substrate molecules, including nucleobases and the corresponding nucleosides, into a polymer. Nucleotides are used less frequently as a synthesis tracer because these anionic molecules are usually not transported through cell membranes. The rates of RNA synthesis in certain *Chlorella* species can be estimated by incubating cells with an exogenous source of [ $^3H$ ]adenine for short time periods (e.g., 5 to 10 min); the alkaline soluble fraction of total cellular nucleic acid is monitored by scintillation counting. In light of the data presented here, we now believe that chlorovirus infection induces changes in *chlorella* membrane potential (i.e., depolarization) that alters the intracellular pool of the nucleotides by disrupting the proton motive force and consequently inhibiting PUP. This inhibition disrupts adenine uptake and thus alters the intracellular pool of radiolabeled ATP relative to that of the mock-infected control. Consequently, the net effect is an apparent suppression of transcription rate, when in fact the suppression occurs because of reduced substrate transport, resulting in an altered intracellular nucleotide pool size.

This phenomenon is illustrated with a 5-min pulse-labeling experiment with [ $^3H$ ]adenine during PBCV-1 infection; 94% inhibition of adenine incorporation into RNA occurred at 20 min p.i. compared to that of mock-infected cells (Fig. 6). In a companion experiment, [ $^3H$ ]adenine was preloaded into *Chlorella* NC64A cells for 5 min, and the exogenous radioactive adenine was removed from the medium before virus infection. In this experiment, the results indicated only a 60% decrease in RNA synthesis at 20 min p.i. (Fig. 6). A key feature of both labeling methods is the uptake of adenine from the exogenous environment, which is subsequently converted to ATP via a series of steps known as the salvage pathway (26). This discrepancy in the data produced by different labeling techniques can be explained by the fact that adenine uptake changes after virus infection.

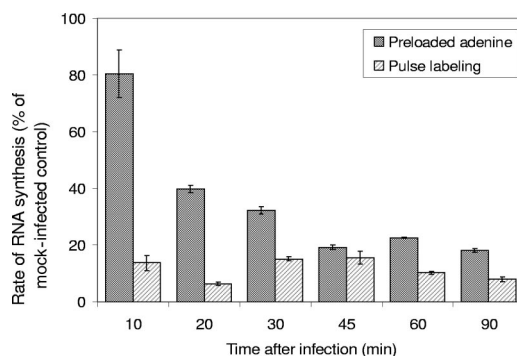


FIG. 6. Effect of PBCV-1 infection on *Chlorella* NC64A RNA synthesis. *Chlorella* NC64A cells were infected with PBCV-1 at an MOI of 10. Mock-infected *chlorella* cells served as a control. For “Pulse labeling,” *chlorella* cells were pulse labeled with [ $^3H$ ]adenine (a 5-min pulse) after virus infection, starting at 5 min p.i.; for “Preloaded adenine,” *chlorella* cells were labeled before virus infection with [ $^3H$ ]adenine for 5 min, and then the unincorporated radioactive tracer was removed by centrifugation. Results are plotted as the mean  $\pm$  SD.

## DISCUSSION

All viruses must pass through the host cell plasma membrane to establish an infection *de novo*. Many plant and animal viruses enter a cell intact through penetration or endocytic pathways. In contrast, most bacteriophages and certain animal and algal viruses deposit the genome and associated proteins in the cell, leaving structural or envelope elements of the virion on the exterior of the cell (14). This transgression of the plasma membrane has physiological consequences.

The immediate-early events of chlorovirus infection are pre-programmed into a complex virion. PBCV-1 contains over 100 unique virus-encoded proteins (Dunigan, unpublished data). In addition to structural functions, these proteins direct several important events leading up to the initial viral gene expression. As noted earlier, virus PBCV-1 has a gene encoding a protein that forms a functional K<sup>+</sup> channel (named Kcv) in heterologous cells (12, 27, 33). We hypothesize that Kcv is located in the inner membrane of the virus particle and plays the following role during infection (24). PBCV-1 infects its host by attaching rapidly, specifically, and irreversibly to the external surface of the algal cell wall (25). Attachment occurs at a unique virus vertex (29; C. Xiao, V. Kostyucherko, J. L. Van Etten, and M. Rossmann, unpublished data), followed by digestion of the cell wall at the point of attachment by virion-packaged, cell wall-degrading enzymes. Presumably, this leads to fusion of the virion membrane with the host plasma membrane and activation of Kcv, which leads to depolarization of the host membrane, followed by ejection of the virus DNA plus accompanying virus-packaged proteins into the cell.

Several experiments support the following hypothesis: (i) host membrane depolarization occurs almost immediately after mixing PBCV-1 with its host *Chlorella* (11); (ii) PBCV-1 infection results in a rapid efflux of K<sup>+</sup> from the host cells (28); (iii) K<sup>+</sup> channel blockers that inhibit Kcv activity in heterologous cells also block host membrane depolarization, K<sup>+</sup> release from the host cells, and virus infection (11, 28); and (iv) the *kcv* gene is expressed late in infection (16) and typically virus-packaged proteins are expressed late. However, direct evidence that the Kcv protein is packaged in the virus particle is lacking. To test this Kcv hypothesis, one would like to delete the *kcv* gene from PBCV-1 and determine if the mutated virion is infectious, and if it is, if virus infection results in host membrane depolarization. Unfortunately, these experiments are not possible at the present time because procedures for manipulating the *Chlorella* virus genomes are lacking.

In the present manuscript, we demonstrate that one of the consequences of host cell membrane depolarization is a rapid and sustained effect on the secondary transport of seven solutes across the plasma membrane into the host cell. Agents that depolarize the plasma membrane inhibit the uptake of solutes dependent on secondary transporters. Virus infection results in the altered function of all tested transporters in the same time period as depolarization. The fact that PBCV-1 infection does not affect the uptake of all solutes equally argues against the possibility that the agent simply creates holes in the cell, such as what occurs with certain animal viruses and results in transient increased cell permeability (14).

Nystatin, a compound known to depolarize *Chlorella* cells (24, 30), caused the inhibition of secondary solute uptake in a

manner that was similar, but not identical, to virus infection. The inhibition of virus-mediated depolarization by Ba<sup>2+</sup> for PBCV-1 and virus NY-2A, or Cs<sup>+</sup> for NY-2A, also reversed the inhibition of the associated transport dysfunction. Thus, we conclude that *Chlorella* virus infection results in cell depolarization, which leads to the dysfunction of secondary solute uptake. Additionally, these experiments reveal the differential nature of certain solute transporters when the cells were treated with both virus and K<sup>+</sup> channel blockers.

In light of the fact that virus infection results in cell depolarization (11, 24), we evaluated the uptake of adenine solute into *Chlorella* NC64A cells, particularly in the context of RNA synthesis. The uptake of adenine, a nucleobase precursor of ATP, was inhibited ~75% within minutes of infection compared to what occurred with a mock-infected control. Uptake remained inhibited for at least 3 h p.i. (Fig. 2A), well past the transition point from early to late infection. Similar results were obtained with nystatin (Fig. 2B); thus, the transport of adenine is due primarily to a secondary transporter, and virus infection inhibits this transport function, consistent with the depolarization-sensitive transport model. This depolarization causes the dysfunction of other secondary transporters, which alters the uptake of many solutes into the cell, including adenine, glucose, proline, methionine, histidine, ornithine, and putrescine. Consequently, one has to use caution when studying changes in metabolic synthetic rates during virus infection of *Chlorella* NC64A using pulse-labeling procedures.

Virus-mediated cell permeabilization is a commonly observed event, especially with animal viruses. The virus agents responsible for permeabilization are called viroporins, and several viruses encode these small hydrophobic proteins that interact with the host membranes (14). The permeabilization observed is transient and often biphasic, where there is a period shortly after infection when the cells become permeable to both large and small molecules that are otherwise prevented from entering the cells. Later in infection, cells often become leaky to a wide range of molecules as the virus is about to be released. In conjunction with these events, ionic homeostasis is disrupted, and this can be a precursor to induced cell death (23). Mitochondrial membranes are known to depolarize with virus infection, leading to apoptosis (2). Certain mitochondrial-targeted regulators of apoptosis of viral origin are known to modify mitochondrial membrane permeabilization (MMP) (10). For example, human immunodeficiency virus type 1 Vpr protein, influenza virus p13<sup>H</sup> protein, and the hepatitis B virus HVB-X protein are proapoptotic factors that appear to be linked to MMP (3), whereas Kaposi's sarcoma-associated herpesvirus K7 protein, cytomegalovirus vMIA protein, and myxoma virus M11L protein act as antiapoptotic factors that tend to suppress MMP. However, in none of these cases have cell membrane dysfunctions been linked to inhibition of secondary transporters.

Bacteriophage infections are also known to cause membrane depolarization and in certain cases are known to be transient, MOI dependent, and biphasic (21). Indeed, certain types of virus-encoded holins depolarize bacterial cells, typically as a late event (31). In the cases of the double-stranded RNA phages  $\phi 6$  and  $\phi 13$ , virus entry is associated with a limited depolarization of the plasma membrane along with a transient increase in permeability of the gram-negative *Pseudomonas*

*syringae* outer membrane (7). Indeed, nucleocapsid binding of  $\phi 6$  to the plasma membrane is independent of the membrane potential ( $\Delta\Psi$ ), yet internalization requires a high  $\Delta\Psi$  (34). The subsequent transient depolarization is thought to preclude secondary infections. Likewise, bacteriophage T4 and T5 infections of *Escherichia coli* cause a reversible depolarization of the cell membrane, and the infection requires a high  $\Delta\Psi$  (less than  $-100$  mV) (15, 20). But again, the membrane permeabilization is nonspecific, unlike the alteration of the algal cell secondary transporters observed with chlorovirus infection.

In summary, PBCV-1 infection of *Chlorella* NC64A leads to rapid alterations in the physiology of the host cell. These alterations include a depolarization of the host plasma membrane and an alkalization of the cytoplasm. In particular, the depolarization causes the dysfunction of secondary transporters, which alters the uptake of many solutes into the cell, including adenine, glucose, proline, methionine, histidine, ornithine, and putrescine. The observations presented here indicate that virus infections may alter cell physiology significantly; however, little is known about how widespread virus-mediated depolarization is in the virosphere, the consequences of these events to productive infections, or how they relate to host-virus interactions in general. Nevertheless, in the case of *Chlorovirus* infection of *Chlorella* NC64A, it appears that these physiological manifestations lead to a productive and robust infection with a large burst size (approximately 1,000 particles per cell [41]), indicating that this altered cellular environment is conducive to acute lytic infections.

#### ACKNOWLEDGMENTS

We thank Martin Ehle (Darmstadt) for the in vivo calibration of BCECF. We thank the U.S. Department of Energy Joint Genome Institute for sequencing and assembling the *Chlorella* NC64A genome ([http://genome.jgi-psf.org/ChlNC64A\\_1/ChlNC64A\\_1.info.html](http://genome.jgi-psf.org/ChlNC64A_1/ChlNC64A_1.info.html)). We also thank Michael Graves and Xiao Li, Department of Biological Sciences at the University of Massachusetts-Lowell for the tools and *Chlorella* NC64A genome sequence, available at the GreenGene website (<http://greengene.uml.edu>).

This research was supported in part by Public Health Service grant GM32441 (J.L.V.E.) and NIH grant P21RR15635 from the COBRE program of the National Center for Research Resources (J.L.V.E. and D.D.) and by a grant from the Deutsche Forschungsgemeinschaft (G.T.).

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