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Minireview

Possible function for virus encoded K^+ channel Kcv in the replication of chlorella virus PBCV-1

Mario Mehmel[a], Maike Rothermel[a], Tobias Meckel[a], James L. Van Etten[b], Anna Moroni[c], Gerhard Thiel[a,*]

[a] *Membrane Biophysics, Botany Institute, Darmstadt University of Technology, Schnittspahnstrasse 3, 64287 Darmstadt, Germany*
[b] *Department of Plant Pathology and Nebraska Center of Virology, University of Nebraska, Lincoln, NE 68583-0722, USA*
[c] *Department of Biology, Istituto di Biofisica del CNR and I.N.F.M., Istituto Nazionale Fisica Materia, Unitá Milano-Universià, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy*

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Abstract The K^+ channel Kcv is encoded by the chlorella virus PBCV-1. There is evidence that this channel plays an essential role in the replication of the virus, because both PBCV-1 plaque formation and Kcv channel activity in *Xenopus* oocytes have similar sensitivities to inhibitors. Here we report circumstantial evidence that the Kcv channel is important during virus infection. Recordings of membrane voltage in the host cells *Chlorella NC64A* reveal a membrane depolarization within the first few minutes of infection. This depolarization displays the same sensitivity to cations as Kcv conductance; depolarization also requires the intact membrane of the virion. Together these data are consistent with the idea that the virus carries functional K^+ channels in the virion and inserts them into the host cell plasma membrane during infection.

Key words: Chlorella virus PBCV-1; Membrane depolarization; Viral K^+ channel

1. Introduction

Several viruses encode small membrane proteins, some of which can function as ion channels [1]. One member of this group of viral proteins is the K^+ channel protein Kcv encoded by *Paramecium bursaria* chlorella virus (PBCV-1) [2]. While potential ion channel forming proteins from other viruses lack homology to eukaryotic ion channel proteins [1], the 94 amino acid Kcv protein has the essential structural features of K^+ channels from prokaryotes and eukaryotes [2,3]. Expression of Kcv in *Xenopus* oocytes [2] and mammalian HEK or CHO cells [3,4] produced a K^+-selective and slightly voltage-sensitive conductance. This conductance is inhibited by the K^+ channel blocker Ba^{2+} [2,4].

Except for the M2 protein from influenza A virus, the physiological roles of viral channel proteins are largely unknown [1]. It is well established that the M2 channel allows H^+ to enter the virion, which results in a pH dependent fusion of endosomal and viral membranes [6,7]. For other viral channels current hypotheses suggest a role in infection, physiological modification of the host, or in release of progeny viruses (rev. in [5]).

Experiments to determine the function of the PBCV-1 Kcv channel in the virus replication cycle are described herein. We provide circumstantial evidence that Kcv is present in the PBCV-1 virion, probably as part of the virus internal membrane, and that Kcv becomes associated with the host plasma membrane during infection.

2. Evidence that Kcv is required for viral replication

The 330 kb genome of PBCV-1 encodes ~375 proteins [8]. Many of these proteins are essential in one or more stages of viral replication [8]. However, analysis of PBCV-1 deletion mutants revealed that PBCV-1 replication can proceed in the host *Chlorella NC64A*, at least in the laboratory, in the absence of some virus encoded proteins. Current estimates indicate ~12% of the genome encoding 31 ORFs are not required for PBCV-1 replication in the laboratory [9].

There is some evidence that the Kcv channel is essential for PBCV-1 replication. When expressed in oocytes the Kcv channel is blocked by inhibitors such as amantadine and Ba^{2+} but is somewhat insensitive to Cs^+ [2]. Essentially the same sensitivity to Ba^{2+} and amantadine and insensitivity to Cs^+ occurs in PBCV-1 plaque assays [2]. This similarity in sensitivity is not only qualitative but also quantitative. Plaque formation by PBCV-1 showed roughly the same half-maximal inhibition by amantadine as the Kcv current in oocytes [2].

Additional evidence that Kcv is important in PBCV-1 replication comes from our recent finding of a gene encoding a Kcv-like protein in another virus, MA-1D, that infects *Chlorella NC64A* [M. Kang, A. Moroni, unpublished results]. The MA-1D Kcv-like protein differs by five amino acids from PBCV-1 Kcv. Unlike PBCV-1, both MA-1D plaque formation and MA-1D Kcv channel activity in *Xenopus* oocytes is inhibited by Cs^+. The similar pharmacology of the K^+ channel and of viral infection support the conclusion that the K^+

*Corresponding author. Fax: (49)-6151-164630.
E-mail address: thiel@bio.tu-darmstadt.de (G. Thiel).

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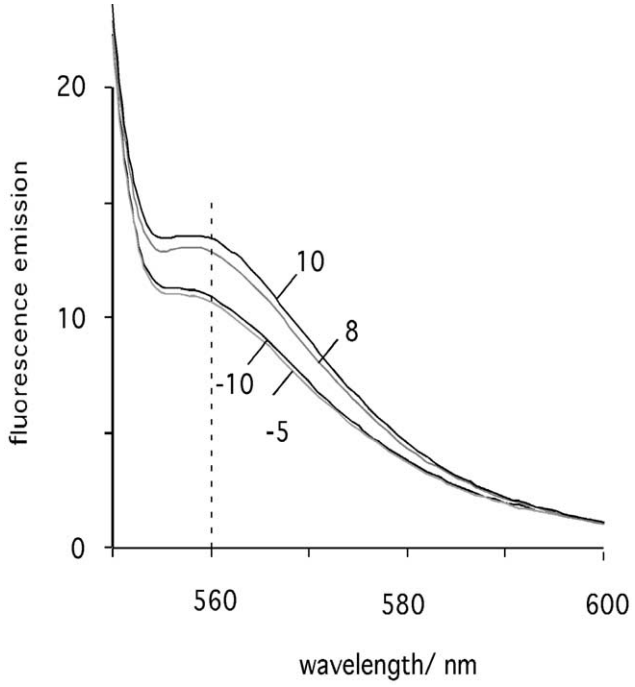

Fig. 1. Effect of ionophore nystatin on fluorescence of voltage dependent dye bisoxonol in a suspension of *Chorella NC64A* cells. *Chlorella NC64A* cells (ca. 3.5×10^6 cells ml^{-1}) in MBBM medium [20] with 1 μM bisoxonol (from Molecular Probes, Eugene, OR, USA) were kept in a 1 cm quartz cuvette with magnetic stirring. Fluorescence was excited at 540 ± 5 nm and monitored using a RF-5001PC fluorimeter (Shimadzu, Kyoto, Japan) with emission slits of 5 nm. The emission spectrum reveals fluorescence emission 10, and 5 min before and 8 and 10 min after addition of 50 μM nystatin to the suspension. In response to the ionophore the fluorescence at 560 nm increases over 10 min by ca. 30%.

channel is required at one or more stages in viral replication [M. Kang, A. Moroni, unpublished results].

3. PBCV-1 infection may induce membrane depolarization in host cells:

Accumulating evidence indicates that Kcv function is probably required for PBCV-1 replication. However, the stage(s) of the viral life cycle that requires Kcv activity as well as the membrane(s) that the Kcv protein is associated with are unknown. We would like to conduct electron microscopic studies using an antibody to Kcv to determine the location of Kcv. However, the Kcv protein is predicted to be mostly embedded in the membrane [2], consequently, there are only limited antigenic sites. Furthermore, our attempts to produce polyclonal antibody to either intact or peptide fragments of Kcv have been unsuccessful.

Therefore, studies on Kcv location and function must rely on indirect evidence and physiological experiments. One indication as to Kcv's function comes from the observation that Ba^{2+} inhibits PBCV-1 replication in *Chlorella NC64A* cells [2]. Ba^{2+} cannot cross the plasma membrane of the alga. Also the Ba^{2+} concentrations required to inhibit Kcv activity and plaque formation are in the range of about 1 mM and hence high enough that a small leakage of Ba^{2+} into the cytoplasm of the alga would not be sufficient to block the Kcv channel. Consequently, the inhibitory effect of Ba^{2+} on viral infection can only be explained if we assume that the Kcv channel is important in a stage of replication that is exposed to the external medium. One stage in which this occurs is during virus infection. This explanation requires the channel to be present in the virion.

Typically, viral associated proteins are synthesized late in the viral replication cycle. To determine when *kcv* mRNA is transcribed, a Northern blot of total RNA from uninfected and PBCV-1 infected *Chlorella* was probed with a ^{32}P labeled ssDNA probe specific for *kcv*. This probe hybridizes early after infection to a putative bicistronic mRNA of 1.4 kb, which probably includes *kcv* and an upstream protein kinase gene. In addition, the probe hybridizes with an ~300 bp RNA late in infection. This RNA is large enough to encode the 94 aa Kcv. This smaller RNA first appears about 90 min post infection (p.i.), and remains at a high level until the cells lyse. PBCV-1 genes expressed 60–90 min p.i., when viral DNA replication begins, are considered to be late genes [8]. Hence the results indicate that the K^+ channel is transcribed during assembly of virus progeny and could be packaged in the mature virus.

Some viruses [10–13] cause depolarization of the host plasma membrane during an early phase of infection. These results led to the discovery that viral proteins can be incorporated into the host plasma membrane as a part of the infection cycle.

To examine electrical events in the early phases of PBCV-1 infection we monitored the membrane voltage of the host cells during the first 20 min of infection. *Chlorella NC64A* cells are small with a diameter of ~5 μm and they have a rigid cell wall. These two properties make recording the membrane voltage with microelectrodes difficult. As an alternative strategy we monitored the membrane voltage of the host cells with the voltage-sensitive fluorescent dye bisoxonol [14,15]. A cell suspension was incubated in a solution of 1 μM bisoxonol. This anionic dye partitions into the membrane after depolarization. Hence a positive shift in membrane voltage produces an increase in fluorescence [15].

To examine the responsiveness of the dye to voltage changes in *Chlorella*, we exposed the cells to the ionophore nystatin. In a related species *Chlorella vulgaris* this treatment produced a pronounced depolarization of the plasma membrane from a very negative, H^+-ATPase dominated membrane voltage of about −135 mV to one at about −20 mV [16]. Fig. 1 shows an emission spectrum of the bisoxonol dye with *Chlorella NC64A* cells before and after adding 50 μM nystatin to the suspension. At the reference wavelength of 560 nm nystatin causes a $30.7\pm3\%$ increase in fluorescence. In relation to the data reported previously [16] this corresponds to a depolarization of about 100 mV.

To investigate the effect of virus infection on the host cell voltage, we monitored bisoxonol fluorescence before and after infecting *Chlorella NC64A* cells with PBCV-1. Fig. 2A shows a typical time course of bisoxonol fluorescence in *Chlorella NC64A* cells in response to adding PBCV-1 at a multiplicity of infection (m.o.i.) of 10. Fluorescence begins to rise rapidly within the first few minutes after PBCV-1 addition and reaches a new steady state at about 15 min p.i. No such rise in fluorescence occurs without addition of virus (Fig. 2A). The mean steady state rise in fluorescence over the control 20 min p.i. is about 20%. This value is smaller than that obtained by nystatin depolarization of *Chlorella NC64A* cells (Fig. 1).

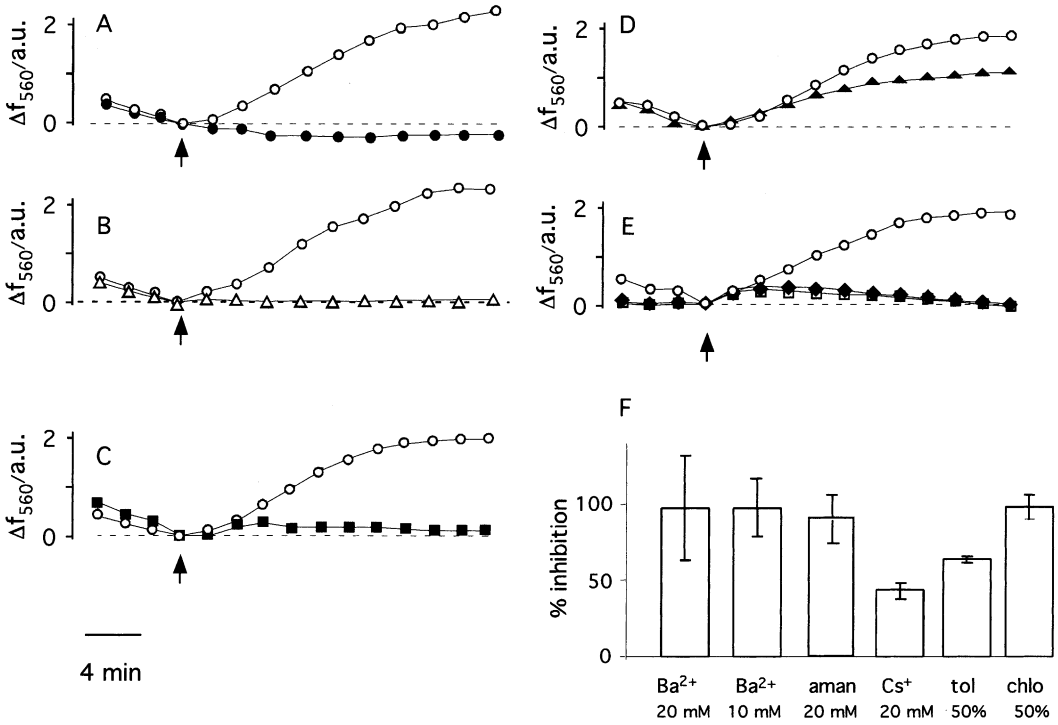

Fig. 2. Effect of PBCV-1 on the fluorescence of bisoxonol in *Chlorella* cells. For experiments ca. 3.5×10^6 cells ml^{-1} were kept in MBBM medium [20] containing 1 μM bisoxonol. After 6 min of pre-incubation ca. 3.5×10^7 PBCV-1 ml^{-1} were added at the time indicated by an arrow. Traces A–E show typical recordings of fluorescent changes (Δf_{560}) of experiments with respective controls measured with the same batch of algae and virus. The fluorescence at the time of virus addition was set to zero. A: *Chlorella NC64A* with (○) and without (●) addition of PBCV-1; B: PBCV-1 added to *Chlorella NC64A* (○) or *C. vulgaris* (△); C: PBCV-1 added to *Chlorella NC64A* in absence (○) or presence (■) of 20 mM $BaCl_2$; D: in absence (○) or presence of 20 mM CsCl (▲); E: *Chlorella NC64A* with PBCV-1 untreated (○) or pre-incubated with chloroform as described in [23] for 5 days (♦); also the effect of adding the same amount of chloroform containing buffer without PBCV-1 is shown (□). F summarizes mean inhibition of PBCV-1 induced rise in bisoxonol fluorescence 20 min p.i. by different cations or by pretreating virus for 5 days in organic solvents chloroform (chlo) or toluene (tol) (for details see [23]). Data are means ± standard deviation from ≥ 10 experiments per treatment.

Without a real calibration of the fluorescent signal it is premature to assign voltage amplitudes to the PBCV-1 induced depolarization. However, if we assume that nystatin is a non-selective ionophore, when added to one side of the membrane [17,18], and that the equilibrium voltages for all relevant cations and anions in plants are under the prevailing ionic conditions more positive than that of K^+ [19], the data are consistent with the hypothesis that the PBCV-1 induced depolarization levels off at the K^+ equilibrium voltage. Additional experiments will be required to test this hypothesis.

Fig. 2B also includes a representative experiment in which PBCV-1 was added to *C. vulgaris* cells at a m.o.i. of 10; PBCV-1 neither attaches to nor replicates in *C. vulgaris* [20]. Incubating *C. vulgaris* with PBCV-1 had no perceivable effect on bisoxonol fluorescence and hence membrane voltage. Similar results were obtained in five other experiments with *C. vulgaris*. We conclude that the increase in fluorescence observed by exposing *Chlorella NC64A* to PBCV-1 indicates a depolarization of the host membrane that is associated with virus infection.

The PBCV-1 induced membrane depolarization occurs very rapidly, within min after mixing the host and the virus. The fluorescence signal exceeded twice the standard deviation of the measurements taken prior to infection within 3 to 4 min p.i. (Fig. 2A–E). This speed in the infection process is not surprising because it is possible to detect the synthesis of early viral mRNAs within 10 min p.i. [8].

4. Similarities between inhibition of Kcv and PBCV-1 induced membrane depolarization

As mentioned above the viral Kcv channel is blocked by Ba^{2+} but is not very sensitive to Cs^+. Therefore, we tested the effect of these two cations on PBCV-1 evoked changes in bisoxonol.

Fig. 2C illustrates the effect of viral infection on bisoxonol fluorescence in *Chlorella NC64A* cells in the presence and absence of 20 mM BaCl. The PBCV-1 induced increase in bisoxonol fluorescence is strongly affected by Ba^{2+} in the incubation medium (Fig. 2C,F). The fluorescence first increased transiently before returning to the control level. On average 10 and 20 mM $BaCl_2$ almost completely inhibited the increase in fluorescence at 20 min p.i. as compared to the control (Fig. 2F).

A similar parallel inhibition of Kcv conductance, plaque formation and change in bisoxonol fluorescence is observed with the tricyclic drug amantadine. Fig. 2F shows that amantadine causes at 20 mM, i.e. at a concentration at which it abolishes Kcv conductance and PBCV-1 induced plaque formation [2], also the PBCV-1 evoked rise in bisoxonol fluorescence. The tricyclic drug amantadine is a high affinity inhibitor of the influenza M2 channel [21]. But at concentrations of two to three orders in magnitude higher than those required to block M2 it also inhibits K^+ channels (e.g. [22]). Hence the inhibitory effect of amantadine on the PBCV-1 evoked rise in

bisoxonol fluorescence is best understood in the context of a low affinity block of K^+ channels.

Cs^+ is a weak inhibitor of Kcv conductance in *Xenopus* oocytes and also PBCV-1 plaque formation [2]. Adding 20 mM CsCl to the incubation medium produced smaller inhibitory effects on the PBCV-1 induced increase in bisoxonol fluorescence. The change in fluorescence at 20 min p.i. was ~50% less in the presence of CsCl as compared to the control (Fig. 2D,F).

Collectively, the effect of inhibitors on membrane voltage during PBCV-1 infection of *Chlorella NC64A* cells exhibit the same pattern of membrane depolarization as observed for Kcv and the infectivity of the virus. Thus the results support the view that Kcv serves an essential role in the infection process, and that the virus inserts the Kcv channel into the plasma membrane of the host cell.

5. PBCV-1 induced membrane depolarization requires intact viral membrane

PBCV-1 contains an internal membrane that is located inside the virus capsid [23,24]. Previous experiments established that the PBCV-1 membrane is disrupted by organic solvents, making the viruses non-infectious. For example, 1 h exposure to chloroform reduces PBCV-1 infection by many orders of magnitude. Toluene also inhibits infectivity but requires a longer incubation time. One hour incubation reduces infectivity ~50% [23]. To test the hypothesis that the Kcv channel is associated with the viral membrane, we incubated PBCV-1 for 5 days in chloroform or toluene (for details see [21]). *Chorella NC64A* cells were then incubated with untreated or solvent treated viruses. As a control we added buffer with solvent but without virus. The data in Fig. 2E,F indicate that viruses pretreated with chloroform did not produce any change in bisoxonol fluorescence above the background. The pretreatment in toluene also was effective in reducing the PBCV-1 evoked increase in fluorescence. However the latter treatment only reduced the increase in fluorescence by ~65% (Fig. 2F).

These results indicate a good qualitative and quantitative correlation between an attenuation of infectivity [23] and the reduced ability of PBCV-1 to induce membrane depolarization in the host cells. The results provide additional evidence that the viral membrane is required for infection.

6. Conclusions

All the results presented in this report are consistent with the hypothesis that the virus PBCV-1 encoded K^+ channel protein Kcv is present in the virion and that the Kcv channel serves an important role during infection of the algal host. A proposed model for the role of Kcv during infection is presented in Fig. 3; this model assumes that Kcv is located in the internal ~40 Å membrane of the virus [24]. PBCV-1 infects the chlorella by attaching rapidly to the external surface of the cell wall [24]. Attachment always occurs at a virus vertex and is followed by degradation of the wall at the attachment point. Presumably attachment alters the virus structure, at least at the attaching vertex, allowing the virus membrane to come in contact with and fuse with the host membrane. This fusion event, presumably aided by a virus packaged fusion protein, releases virus DNA and probably some DNA associated proteins into the interior of the host cell. Assuming Kcv channels exist in the virus membrane, fusion with the host membrane, produces rapid changes in the K^+ conductance and a depolarization of the entire host plasma membrane. Such a change in the plasma membrane could also explain the exclusion phenomenon associated with the chlorella viruses [25]. That is, infection by one chlorella virus, like many bacteriophage, results in poor infection by a second chlorella virus.

The combination of membrane depolarization, an elevated K^+ conductance together with depolarization activated anion

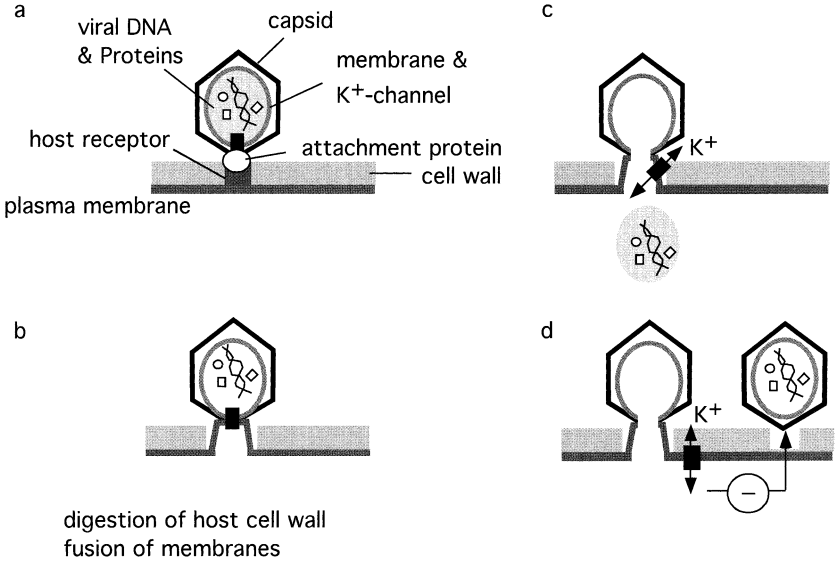

Fig. 3. Model for early events and the role of the K^+ channel Kcv in PBCV-1 infection of *Chlorella NC64A* cells. a: Sketch of PBCV-1 architecture including capsid, internal membrane and Kcv and schematic view of virus attachment to Chlorella cell. b: Digestion of cell wall and fusion of membranes may lead to incorporation of Kcv into host cell membrane. c: Elevated K^+ conductance in host cell would result in depolarization, loss of K^+ and consequent decrease in host cell turgor pressure. Altogether this may make it easier for the virus to insert large genome and proteins into the host cell. d: Membrane depolarization may be a signal, which avoids multiple infections.

channels, which are ubiquitous in plant cells [26], might lead to release of K^+ from the host cell. This release could reduce the high turgor pressure of the host cells (which can be as high as 1 MPa in plant cells [27]), and allow viral DNA to be released into the cell against a reduced internal pressure of the host cell.

Simple calculations indicate that only a few channel molecules are required in order to short circuit the *Chlorella* plasma membrane. With millimolar concentrations of ions in the external medium plant cells generally have a conductance of about 0.3 S m^{-2} [28]. Considering an average surface area of a *Chlorella* cell of 100 μm^2 this gives a conductance of 30 pS for the whole cell. Hence addition of a single channel of 30 pS will more than double the whole plant conductance for K^+.

Finally, assuming the suggested role for Kcv during infection is true, this does not exclude other functions for Kcv during PBCV-1 replication.

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