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Viruses infecting marine picoplancton encode functional potassium ion channels

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Abstract
Phycodnaviruses are dsDNA viruses, which infect algae. Their large genomes encode many gene products, like small K+ channels, with homologs in prokaryotes and eukaryotes. Screening for K+ channels revealed their abundance in viruses from fresh-water habitats. Recent sequencing of viruses from marine algae or from salt water in Antarctica revealed sequences with the predicted characteristics of K+ channels but with some unexpected features. Two genes encode either 78 or 79 amino acid proteins, which are the smallest known K+ channels. Also of interest is an unusual sequence in the canonical α-helixes in K+ channels. Structural prediction algorithms indicate that the new channels have the conserved α-helix folds but the algorithms failed to identify the expected transmembrane domains flanking the K+ channel pores. In spite of these unexpected properties electrophysiological studies confirmed that the new proteins are functional K+ channels.

Keywords: K+ channel evolution, Kcv, Chlorella viruses, Algal viruses, Virus evolution

Introduction
Several viruses have genes that encode proteins with ion channel activity (e.g., Fischer and Sansom, 2002; Wang et al., 2010; Nieva et al., 2012). Depending on the virus, these channels are either involved in viral entry, viral replication or viral exit from the host (Hsu et al., 2004; Thiel et al., 2009; Nieva et al., 2012). Bioinformatic analyses of these viral encoded channels have not revealed any obvious sequence similarities to channel proteins from cellular organisms (Fischer and Sansom, 2002). One exception is viruses in the family phycodnaviridae, which code for proteins with the structural and functional hallmarks of K+ channels (Plugge et al., 2000; Thiel et al., 2011). The prototype K+ channel Kcv is coded by chlorovirus PBCV-1 (KcvPBCV-1) (Plugge et al., 2000) and like its prokaryotic and eukaryotic homologs, it functions as a tetramer (Shim et al., 2007; Pagliuca et al., 2007). Each Kcv monomer has two transmembrane domains (TMDs), which are linked by a pore helix (Tayefeh et al., 2009). The pore helix contains a motif of 8 amino acids, which is the signature sequence for all K+ channels (Heginbotham et al., 1994). Assembly of four Kcv monomers creates a central pore with a selectivity filter that allows passage of K+ across the membrane (Tayefeh et al., 2009). The major difference between KcvPBCV-1 and K+ channels from other organisms is the small size of the monomers, which are only 94 amino acids (Plugge et al., 2000); that is, KcvPBCV-1 basically consists of the pore module present in all K+ channels (Thiel et al., 2011). However, in spite of its small size the KcvPBCV-1 channel has all the functional hallmarks of more complex K+ channels when expressed in heterologous systems, including selectivity for K+ and sensitivity to many of the known K+ channel blockers (Thiel et al., 2011).

After discovering KcvPBCV-1, we realized that K+ channel encoding genes are common in members of the Phycodnaviridae family. K+ channel sequences have been detected in more than 80 phycodnaviruses (Kang et al., 2004; Gazzarrini et al., 2006; Gazzarrini et al., 2009; Hamacher et al., 2012; Thiel and Van Etten, unpublished data). From an evolutionary point of view it is interesting that K+ channel coding sequences are found in members, representing four genera in the Phycodnaviridae; these viruses infect different algal hosts. Three of these viruses replicate in species of unicellular green algae from fresh water habitats, Chlorella variabilis, Chlorella heliozoae, and Micróctinum conductrix (Fitzgerald et al., 2007a, 2007b, 2007; Jeanniard et al., 2013); collectively these viruses are called chloroviruses. A fourth virus, EsV-1, also encodes a K+ channel protein named Kesv. EsV-1 infects the marine filamentous brown alga Eckocarpus siliculosus; EsV-1 is distantly related to the viruses that infect fresh water green algae (Van Etten et al., 2002). Several
studies have established that the Kcv channels are located in the internal membrane of the chloroviruses (Romani et al., 2013; Frohns et al., 2006) and that they serve an important role in the early steps of infection and DNA ejection into the host (Neupärtl et al., 2008; Greiner et al., 2009). The biological role of the K* channel in the marine EsV-1 is unknown but presumably it is different from that in the chloroviruses. The reason for this assumption is that chloroviruses have a lytic life cycle while EsV-1 is lysogenic (Delaroque et al., 1999; Van Etten et al., 2002). Also the energetic barrier for ejecting viral DNA into the host, which is lowered by Kcv activity in the fresh water algae (Neupärtl et al., 2008; Thiel et al., 2009), is not relevant in the marine habitat because virus EsV-1 infects the sporophytes of the host cells, which lack a cell wall and hence have no turgor pressure (Delaroque et al., 1999).

Although, the K* channels from phycodnaviruses are similar, they do exhibit significant structural and functional diversity. An obvious structural difference is their monomer size, which ranges from 124 amino acids in the Kesv channel from virus EsV-1 (Bals et al., 2008) to 82 amino acids in viruses, which infect Chlorella heliozoae (Gazzarrini et al., 2009). These size differences are mostly due to the presence or absence of cytoplasmic domains and an extracellular turret domain in the channels (Thiel et al., 2009). Diversity also exists in the functional properties of the channels when they are expressed in heterologous systems. For example, Kcv_{PBCV-1} has a lower open probability than the corresponding channel from chlorovirus ATCV-1, Kcv_{ATCV-1}. Also, Kcv_{PBCV-1} conducts RB better than K*, whereas the situation is reversed in Kcv_{ATCV-1} (Gazzarrini et al., 2009). In addition to their functional differences the K* channels are sorted differently. The chlorovirus encoded Kcv channels are sorted into the secretory pathway and finally targeted to the plasma membrane in either HEK293 cells or in yeast, the Kcv channel from EsV-1 is targeted to the mitochondria (Bals et al., 2008).

Another interesting question is the origin and the evolution of the viral K* channel proteins. The fact that all K* channels from cellular organisms contain a pore, which resembles the viral K* channels, is consistent with the traditional assumption that viruses are ‘pick pockets’ (Moreira and Lopez-Garcia, 2009) and acquire their genes from their host via molecular piracy. However, this traditional view on the evolution of viral K* channels has been challenged recently. Comparative analysis of the Kcv channels from different chloroviruses and from virus EsV-1 with those coded by the two host cells found no evidence of co-evolution between the viruses and their hosts (Hamacher et al., 2012). Instead, a phylogenetic analysis indicated that the viral channels form, in spite of their structural and host diversities, a defined clade; i.e., the viral channels are clearly separated from their host K* channels and from K* channels from other cellular organisms (Thiel et al., 2013). This analysis clearly argues against the hypothesis that viruses have acquired their K* channels from their current hosts. This conclusion is further supported by a bioinformatics analysis of 41 chloroviruses with one of their hosts. The results of this study did not find any evidence to indicate a major transfer of genes from the host to the chloroviruses. For a few genes the results even indicated a flow of genes in the opposite direction, i.e., from virus to host (Jeaniard et al., 2013).

In the context of the question about the origin of viral K* channels, recent sequencing projects of viruses infecting marine unicellular algae (Moreau et al., 2010; Derelle et al., 2006, 2008) and metagenomic sequencing of an organic lake in Antarctica (Zhou et al., 2013; Yau et al., 2011) revealed open reading frames that were annotated as K* channels. Furthermore, in the context of the minimal size required for a functional K* channel, two of the newly detected putative K* channels have a monomer size of 78 and 79 amino acids, which is even smaller than the 82 amino acid Kcv_{ATCV-1} channel (Gazzarrini et al., 2009).

In this manuscript we report a detailed structural and functional examination of three of the new putative K* channel proteins, as well as their phylogenetic relationships. The results revealed considerable variability among the viral K* channels; a phylogenetic analysis indicated that the K* channels from the fresh water viruses clearly separated from those from the marine/salt water habitats. These results support the notion of a long evolutionary history for the viral K* channels.

**Results and discussion**

**New virus encoded K* channels**

Figure 1A shows an alignment of eight newly detected putative K* channel sequences from viruses infecting algae. Seven of the viruses with K* channel like sequences infect small unicellular algae, which are the main components of the so-called picoplankton community. Their hosts, Micromonas, Bathycoccus and Osterococcus species, belong to the class Prasinophyceae within the Chlorophyta; these algae are important ecologically because they are often the dominant photosynthetic species in marine habitats. Four of the viruses (MpV12T, MpVSP1, MpV1, MpPL1) infect Micromonas pusilla. Two viruses (BpV1, BpV2) infect Bathycoccus species; the sequence of the putative K* channel protein from these two viruses is identical. Two additional viruses (OlV4, ORT) infect Osterococcus species. The name of the gene products in Figure 1A is composed of K for K* channel, and the virus, which encodes the sequence, e.g., mpv is for Micromonas pusilla virus. The index specifies the virus isolate. Thus Kmpv_{12T} is the K* channel from M. pusilla virus isolate 12 T. Finally, a K* channel like sequence was detected in a metagenomic sequencing project of viral genomes in an organic lake in Antarctica (Yau et al., 2011). In this case, neither the host nor the virus encoding the K* channel from the organic lake phycodnavirus 2, Kolpv{2}, is known. Information on the gene accession numbers, on the source of the genes and on the protein nomenclature are summarized in Table 1.

<table>
<thead>
<tr>
<th>Gene accession number</th>
<th>From virus</th>
<th>Name of putative K* channel</th>
<th>Protein accession number</th>
<th>Number of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM004429</td>
<td>Micromonas sp. RCCI109 virus MpV1</td>
<td>Kmpv_{1}</td>
<td>YP_004062056</td>
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</tr>
<tr>
<td>HQ632826</td>
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<td>Kmpv_{12T}</td>
<td>YP_007676152</td>
<td>78</td>
</tr>
<tr>
<td>JF974320</td>
<td>Micromonas pusilla virus SP1</td>
<td>Kmpv_{SP1}</td>
<td>AET84893</td>
<td>86</td>
</tr>
<tr>
<td>HQ635037</td>
<td>Micromonas pusilla virus PL1</td>
<td>Kmpv_{PL1}</td>
<td>AET43568</td>
<td>85</td>
</tr>
<tr>
<td>HM004432</td>
<td>Bathycoccus sp. RCCI105 virus BpV1</td>
<td>Kbpv_{1}</td>
<td>YP_004061440</td>
<td>83</td>
</tr>
<tr>
<td>JF974316</td>
<td>Osterococcus lucimarinus virus OV4</td>
<td>Kolv_{4}</td>
<td>AET84496</td>
<td>102</td>
</tr>
<tr>
<td>JN225873</td>
<td>Osterococcus tauri virus RT-2011</td>
<td>Kolv_{RT}</td>
<td>AFTC34969</td>
<td>104</td>
</tr>
<tr>
<td>HQ704803</td>
<td>Organic Lake phycodnavirus 2</td>
<td>Kolpv_{2}</td>
<td>ADX06223</td>
<td>105</td>
</tr>
</tbody>
</table>
The sequences reveal some structural hallmarks of K⁺ channel proteins (Figure 1). All the predicted proteins contain a consensus or a consensus-like sequence of K⁺ channels (Heginbotham et al., 1994) including, either a GYG or GFG motif in the core of the selectivity filter. However, the second Thr in the consensus sequence is not conserved in all the channels. Worth noting is that four of the eight sequences have a Ser instead of the canonical Thr in the consensus sequence prior to the GY/FG motive. This same amino acid substitution is present in the selectivity filter of the viral Kesv channel (Balss et al., 2008). Even though this amino acid substitution is conservative, previously studies established that replacement of this canonical Thr with Ser resulted in a drastic reduction in the sensitivity of the channels to Ba²⁺ in KcvPBCV-1 and Kir channels (Chatelain et al., 2009). Even more unusual is the Kolpv₂ sequence, which has a Leu in this position. Structure function analyses of K⁺ channels have shown that they require a pair of aromatic amino acids upstream of the filter, which act as a cuff and keep the pore in the correct diameter for K⁺ passage (Doyle et al., 1998). The alignment in Figure 1A indicates that these obligatory aromatic amino acids are present in all 8 proteins.

**Predicted TMDs in the new viral K⁺ channels**

A surprise arose from in silico analyses of possible TMDs in these new putative K⁺ channel proteins. The general architecture of a K⁺ channel requires one TMD downstream and one TMD upstream of the selectivity filter (Doyle et al., 1998; Tayefeh et al., 2009). However, one of the most established prediction programs for TMDs, the TMHMM2.0 algorithm (Amico et al., 2006), did not predict the expected second TMD in the Kmpv₁₂T and Kolpv₂ sequences (Supplement Figure SI). In the Kmpv₁₂T, Kbpv₁, and Kotvᵣ sequences two TMDs were predicted but the second TMD was in the wrong position, i.e., in the center of the selectivity filter (Supplement Appendix A); this hydrophobic domain probably represents the pore helix of the K⁺ channel proteins (Doyle et al., 1998; Tayefeh et al., 2009). In the two remaining viral channels the algorithm predicts a protein architecture that is compatible with a K⁺ channel. That is, they
have two peripheral TMDs, which are connected by a stretch of hydrophobic amino acids; this stretch is in the correct position of the pore helix of K⁺ channel proteins.

Because of the ambiguous predictions of the TMDs in six viruses we selected the Kmpv₁ channel, e.g., a channel in which the aforementioned algorithm did not predict any TMD. A consensus prediction for Kmpv₁ from all the algorithms is shown in Supplement Figure S1A; the data illustrate that a TMD downstream of the selectivity filter seem rather unlikely on the basis of structural predictions. In the 10 prediction platforms tested only one (MINNOUN, Cao et al., 2006) suggested for the Kmpv₁ channel TMDs in positions, which are in agreement with a canonical K⁺ channel structure (Supplement Figure S1A). This analysis, which is based on the predicted solvent accessibility and secondary structure of each amino acid in a sequence (Cao et al., 2006) appears to be the most suitable tool for the analysis of the apparent unusual structures of the viral proteins. The same tool was used for a scrutiny of all putative channel proteins. The data presented in Supplement Figure S1A indicate that this prediction suggests for all sequences two TMDs one upstream and one downstream of the selectivity filter.

Because of the ambiguous predictions for TMDs, we also examined the proteins with respect to their predicted folds. The structure of a K⁺ channel requires two α-helices, which form the two TMDs and an α-helix upstream of the filter, which forms the pore helix (Doyle et al., 1998; Tayefeh et al., 2009). Analysis of known viral channels such as Kcv₁ of PBCV-1, with a structure prediction algorithm PRED 3 revealed α-helices in the expected positions (Figure 1B). Analyses of the new sequences indicated that all 8 proteins have an α-helix in the position of the Kcv₁ channel protein and a third helix in front of the pore helix. The results from this analysis indicate that all the new putative K⁺ channel proteins have the potential to fold according to the architecture of a functional K⁺ channel; the putative channels apparently have a high conservation for folding but achieve this with different amino acid sequences.

**New virus encoded K⁺ channels are functional**

To determine if the new viral proteins form functional K⁺ channels, we selected three proteins, namely Kmpv₁, Kmpv₁₂T and Kbpv₁, to test for channel activity in HEK293 cells; Kmpv₁ and Kmpv₁₂T represent the smallest proteins and Kbpv₁ represents the proteins with a longer sequence. The HEK293 cell expression system was successfully used previously to record currents from several viral K⁺ channels (Moroni et al., 2002; Braun et al., 2013). Figure 2A reports the typical currents of a non-transfected HEK293 cell, which serves as a control. Voltage steps from −160 mV to +80 mV elicit only small currents in these cells; the I/V relation is typically linear at voltages negative of ca. 0 mV. At more positive voltages a K⁺ current develops (Figure 3A and C). The experiments with Kmpv₁ were performed in a buffer where extracellular K⁺ was replaced with Na⁺. These cells had a lower inward current and a concomitant left shift of the reversal voltage (Figure 2D and E). These results are consistent with a functional K⁺ selective channel. Using a mean negative shift of the reversal voltage by −53 mV in between experiments with either K⁺ (n=13) or Na⁺ (n=8) in the bath medium, we estimated that the channel was, according to the constant field model (Hille, 2001), >10 times more selective for K⁺ than Na⁺. This value is similar to other viral K⁺ channels in the same expression system (Moroni et al., 2002). As a further test of typical K⁺ channel function, currents were recorded in a solution containing 50 mM K⁺, with/without 10 mM Ba²⁺. A typical result is shown in Figure 2F–H. The HEK293 cell expressing Kmpv₁ had a large and quasi-linear conductance before Ba²⁺ addition. The channel blocker inhibited the current. As expected for a voltage-dependent block by Ba²⁺, the inward current decreased the most. It is interesting that this high concentration of Ba²⁺ did not completely inhibit the channel; under the same conditions the reference channel Kcv₁ of PBCV-1 is completely inhibited by Ba²⁺ (Chatelain et al., 2009). As noted above, Kmpv₁ has Ser at residue 44 in the selectivity filter sequence. Since substitution of Thr for Ser in this position lowers the sensitivity of a channel to Ba²⁺ block in other channels (Chatelain et al., 2009), we speculate that the natural occurrence of Ser in this critical position renders the channel less sensitive to Ba²⁺.

Similar experiments were repeated with Kbpv₁ fused to GFP. This protein also generates a significant and quasi-linear increase in membrane conductance in HEK293 cells (Figure 3A and C). Worth noting is a negative slope conductance of the I/V relation at negative voltages (Figure 3C). This decrease in conductance at extreme voltages is a typical feature of Kcv type channels (Abenavoli et al., 2009; Arrigoni et al., 2013) and is probably due to a flickering block of the filter (Abenavoli et al., 2009). At a reference voltage of −100 mV, Kbpv₁ expressing cells had a mean current of 3.75 nA±2.5 nA (n=8). Also the Kbpv₁ generated conductance is K⁺ selective. Exchange of K⁺ for Na⁺ caused a −90 mV left shift of the reversal voltage (Figure 3B and C). On average the reversal voltage was 63.5 mV more negative in recordings with Na⁺ (n=6) versus K⁺ (n=8) in the bath medium. This result implies an even higher selectivity for K⁺ over Na⁺. Collectively, these data indicate that Kbpv₁, which contains a K⁺ consensus sequence, generates K⁺ conductance in HEK293 cells.

Further experiments established that Ba²⁺ (10 mM) blocked Kbpv₁ activity (Figure 3D and E). Like Kmpv₁, the block was voltage dependent but not complete (Figure 3E and F). This result is surprising because Kbpv₁ has a canonical Thr and not a Ser in the critical position in the selectivity filter (Figure 1A). The results of these experiments indicate that Ba²⁺ sensitivity is not due to a single amino acid but determined by additional structural elements in the filter domain.

We also tested the potential channel function of the smallest protein, Kmpv₁₂T, by expressing it in HEK293 cells. Unlike the experiments with Kmpv₁ and Kbpv₁, cells transfected with Kmpv₁₂T rarely exhibited a conductance that was significantly different from un-transfected control cells (data not shown). Since these results were not convincing enough to establish channel function, we synthesized the protein in vitro and reconstituted it in planar lipid bilayers. This procedure was used previously to measure channel function of small viral proteins (Braun et al., 2013). When the recombinant Kmpv₁₂T protein was tested in a synthetic DPhPC membrane, channel fluctuations were routinely detected (Figure 4A and B). These channel fluctuations only occurred after adding the protein; hence the results were not due to contamination or unspecific membrane pores. Channel fluctuations and the corresponding I/V relation for a recording of channel activity in a solution with symmetric 100 mM KCl are reported in Figure 4A and B. From the linear slope of the I/V relation we estimate a conductance of 42 pS (n=5); this value is smaller than the viral K⁺ channels studied previously (Pagliuca et al., 2007; Braun et al., 2013).

**Unusual architecture of transmembrane domains**

Collectively the experimental results established that K⁺ channel like gene products from viruses Mpv1, Mpv12T and BpV1 function as K⁺ channels. A positive K⁺ channel function
occurred in spite of the fact that prediction algorithms for TMDs failed to identify a proper structure for a $K^+$ channel. It is reasonable to speculate that these proteins follow the general tendency of viruses to miniaturize the size of their genes and evolve efficient TMDs, which are structurally different from those in cellular organisms. Consequently, the inability of the TMHHM algorithm to predict the second TMD in some of the viral channels might be because the algorithm was developed to identify canonical TMDs in prokaryotic and eukaryotic membrane proteins; the low hydrophobicity of many of the viral channels is very unusual for TMDs. Interestingly, 3 of the $K^+$ channel proteins with very short sequences (Figure 1A) have a cationic amino acid at the C-terminus. These amino acids are well known for a phenomenon termed “snorkeling” (Killian and von Heijne, 2000). Because of their long and flexible side chains cationic amino acids can keep their hydrocarbon part inside the membrane while the positive charge reaches into the water-lipid interface (Segrest et al., 1990; Strandberg and Killian, 2003). This property can keep proteins in a transmembrane orientation even when the structure of the protein is not suitable as a TMD per se (Henkel et al., 2010).

**Phylogenetic analyses**

A phylogenetic analysis was conducted between the $K^+$ channels coded by viruses that infect algae from fresh water or marine water (Figure 5 and Figure 6, Supplement Figure S2). The chloroviruses PBCV-1, MT325 and ATCV-1, which have different fresh water hosts, were chosen to represent viruses infecting 3 fresh water algae (Thiel et al., 2011). Virus EsV-1 represents a virus, which infects a marine alga (Van Etten et al., 2002). All of these viral encoded $K^+$ channels were previously shown to function as $K^+$ channels (Gazzarrini et al., 2006, 2009; Balss et al., 2008).

Previous studies on structure function relations established that some amino acids are very important for Kcv channel activity (Gebhardt et al., 2011, 2012); these significant amino acids are labeled with small letters (a–f) in Figure 5. The aromatic amino acids a–c are required to anchor the first TMD in the lipid
The cationic amino acid d is a “snorkeling” amino acid and required for placing the TMD in the bilayer (Gebhardt et al., 2011). Finally a pair of Phe or Met (e) form together with a His (f) in the second TMD, a \( \pi: \pi \) -stacking or C–H:\( \pi \) interaction; this interaction attaches the inner TMD to the outer TMD (Gebhardt et al., 2011). The listed amino acids are conserved among more than 80 Kcv type channels from viruses, which infect fresh water algae (Thiel and Van Etten, unpublished data). This high degree of conservation indicates that they are important for the global architecture of the channel. Scrutiny of the new sequences reveals that the aromatic amino acids b and c are highly conserved throughout most of the viral encoded K\(^+\) channels; only the Kolpv\(_2\) protein differs from the rest. The result of this analysis indicates that the channels from fresh water viruses are more closely related to each other than to the other viral channels. The absence of amino acids in the new channel sequences, which are structurally important in the latter channels, implies that the newly discovered K\(^+\) channel proteins use a different architecture for generating a functional K\(^+\) channel.

Further scrutiny of the alignment indicates that, collectively, the channel sequences are quite diverse. Apart from the K\(^+\) channel consensus sequence, which is moderately conserved among all the viral channels, only a few amino acids are highly conserved. The general picture is that the N-terminal part of the channels is more diverse than the C-terminal part downstream of the filter region (Figure 5, supplement Figure S2). It will be interesting to examine the functional significance of the few remaining conserved amino acids among the population of viral channels.

The channel sequences in Figure 5 were used to construct a phylogenetic tree (Figure 6) with the maximum likelihood algorithm. The data show that the channel proteins can be separated with moderate statistical support between channels from fresh water viruses and marine or salt-water viruses. This result is consistent with previous suggestions that the viral K\(^+\) channels have a long evolutionary history (Hamacher et al., 2012; Thiel et al., 2013). However, the analysis does not indicate a strict separation of the channels on the basis of their viral hosts. The channels from virus BpV1/2 are on the same branch as those from virus MpV1. Also the Kesv channel, which infects a brown alga, is not clearly separated from those infecting green algae. The latter finding is in good agreement with previous analyses that indicate the viral channels are not the product of molecular piracy (Hamacher et al., 2012).

**Conclusions**

The present data show that K\(^+\) channel like genes are abundant in viruses that infect either fresh water or sea/salt water algae. All viral channels have maintained the overall architecture of K\(^+\) channel proteins with three \( \alpha \) -helices; two form the required TMDs and the central one is adjacent to the canonical pore helix signature sequence. Apart from these common features the viral K\(^+\) channels are quite diverse. A peculiarity of many of the channels coded by sea water viruses is the unusual TMDs, which are not detected by algorithms that predict protein structures. A further peculiarity of two of the newly discovered channel proteins is their small size. The present experimental verification of their function as K\(^+\) selective channels makes them the smallest proteins known to form a functional K\(^+\) channel. Sequences alignments and analysis of specific amino acids, which were identified in previous studies as functionally important (Gebhardt et al., 2011, 2012), support a clear
**Materials and methods**

**Sequences and algorithms**

Sequences of putative K⁺ channels were obtained from The National Center for Biotechnology database (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed with T-Coffee algorithm at http://www.phylogeny.fr. The phylogenetic tree was calculated with the maximal likelihood algorithm implemented on the same platform. The location of transmembrane domains was predicted by the following algorithms (1) TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), (2) TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), (3) DAS (http://www.sbc.su.se/~miklos/DAS/), (4) SPLIT (http://split.protserv.ncl.ac.uk/split/4/), (5) HMMTOP (http://hmm-top.biocomputing.ohio-state.edu/hmmtop/), (6) SOSUI (http://sosui.nig.ac.jp/sosui), (7) MFEx (http://blanco.biomol.uc.edu/mpes), (8) MELSAT (http://www.sacsc.ucsf.edu/cgi-bin/melsat.py), (9) PSIPREDV2.3 (http://bioinf.cs.ucl.ac.uk/psipred), and (10) MINNOU (http://www.enzim.hu/hmmtop). The α-helical folds were predicted with the Jpred algorithm (http://www.compbio.dundee.ac.uk/www-jpred) (Cole et al., 2008).

**Electrophysiological measurements**

The electrical properties of the putative viral channels in HEK293 cells were recorded as reported previously (Moroni et al., 2002). Currents were recorded with an EPC-9 Patch Clamp amplifier (HEKA, Lambrecht, Germany) and stored for further analysis (HEKA, Lambrecht, Germany). The currents were measured at room temperature in a standard medium containing: 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4) and either 50 mM KCl or 50 mM NaCl. 10 mM BaCl₂ was added to the K⁺ containing media to block channel activity. The osmolarity of all solutions was adjusted with mannitol to 330 mOsm. The pipette solution contained 130 mM potassium-d-gluconic acid, 10 mM NaCl, 5 mM HEPES, 0.1 mM guanosine triphosphate (Na salt), 0.1 μM CaCl₂, 2 mM MgCl₂, 5 mM phosphocreatine, and 2 mM adenosine triphosphate (Na salt, pH 7.4). Kmpv₁ and Kbpv₁ constructs were transiently expressed as fusion proteins with GFP on the C-terminus using the liposomal transfection reagent TurboFect™ (Fermentas, St. Leon Rot).

The Kmpv₁₂T protein was synthesized cell-free with the MembraneMaxTM HN Protein Expression Kit (Invitrogen) following the manufacturer’s instructions. The gene of Kmpv₁₂T was cloned into a pEXP5-CT/TOPO®-vector. For protein expression in its native form, a stop-codon was inserted right before the gene of a 6xHis-tag. The DNA template was incubated with the synthesis reaction mix (MembraneMaxTM HN reagent carrying a polyHis-tag, ribosomes, T7 RNA polymerase and energy renewal system) for 35 min at 37 °C (1000 rpm). The feeding buffer was added and the reaction was incubated for 1 h 45 min at 37 °C (1000 rpm). After expression, the protein was loaded on a Ni–NTA column, which was equilibrated with an equilibration buffer [500 mM NaCl, 30 mM HEPES, 10% glycerin (all from AppliChem GmbH, Darmstadt, Germany), pH 7.5]. Unspecific binding was removed by washing the column with 20 mM imidazole (Sigma Chemical, Deisendorf, Germany) twice. The protein was then eluted with 250 mM imidazole in 7 fractions at 100 µl. After elution the protein was used directly in the planar bilayer (Braun et al., 2013). Planar lipid bilayer experiments were done with a vertical bilayer set up (IonoVation, Osnabrück Germany) following previously (Braun et al., 2013). A 1% hexadecane solution (MERCK KGaA, Darmstadt, Germany) in n-hexadecane (Carl ROTH, Karlsruhe, Germany) was used for pretreating the Teflon foil (Dielectric Corporation). The hexadecane solution (ca. 0.5 µl) was pipetted onto the hole (100 µm in diameter) in the Teflon foil with a bent Hamilton syringe (Hamilton Company, Reno, Nevada, USA) until the solvent evaporated. The experimental solution contained 100 mM KCl and was buffered to pH 7.0 with 10 mM HEPES/KOH. As a lipid we used 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) (from Avanti Polar Lipids, Alabaster, AL, USA) at a concentration of 15 mg/ml in n-pentane (MERCK KGaA, Darmstadt, Germany).
Figure 5. Multiple alignments of K+ channels from different viruses with a focus on the first TMD and the selectivity filter domain. The alignment includes established K+ channels from chloroviruses (KcvChy, KcvCy, KcvMCX2) and from Ectocarpus siliculosus virus (Ksv). The remaining sequences are K+ channels from viruses described in this manuscript. The dotted box highlights the area of the consensus sequence of all K+ channels; the arrows with letters a–f indicate amino acids that are highly conserved in K+ channels from fresh water viruses and which were previously found essential for channel function. The channels from fresh water viruses are in light gray and those from sea or salt water in dark grey. Alignment was constructed with T-COFFEE Version 6.85 software available at Phylogeny.fr (Dereeper et al., 2008). The colors depict the degree of similarity from conserved (red) to not conserved (blue). Fully conserved or similar amino acids are marked by: or , respectively.

Figure 6. Phylogenies of viral encoded K+ channel proteins. The tree was constructed using PhyML (Guindon and Gascuel, 2003) available at Phylogeny.fr (Dereeper et al., 2008) with WAG matrix and gamma distribution from gap free alignment (Supplement Figure S3). Branch labels indicate bootstrap percentages (>0.7) after 100 replicates. The tree is unrooted.

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Supplementary materials
Supplementary figures S1 and S2 are included following the References; supplementary data link to a zipped folder can be found in the online repository html cover page.

References


Fig. S1: Prediction algorithms fail to detect an expected K⁺ channel TMD in some of the viral sequences. (A) Amino acid sequences of putative K⁺ channels from marine and salt water viruses. The position of the TMDs was predicted with either the TMHMM or MINNOU algorithms. Amino acids, which were positively identified with TMHMM or MINNOU algorithms, are shown in red or are underlined, respectively. For orientation purposes the GYG or GFG motif of the selectivity filter is also highlighted by black background. Canonical amino acids at the C-terminal end of the proteins are marked by *.

(B) Consensus for prediction of TMDs in channel Kmpv₁. The plot was calculated as mean value from predictions with 10 different algorithms (See materials and methods). An amino acid was assigned the value 1 when it was predicted as part of a transmembrane domain and 0 if it was not. The y-axis shows the mean value from the predictions. The red bar indicates the location of the GYG motive in the filter.
Fig. S2: Full multiple alignment of K⁺ channels from viruses with different origins. The alignment includes established K⁺ channels from chloroviruses (KcvPBCV1, KcvATCV, KcvMT325) and from the *Ectocarpus siliculosus* virus (Kesv). The remaining sequences are K⁺ channels from viruses described in this manuscript. Alignment was constructed with T-coffee software. The colors depict the degree of similarity from conserved (red) to not conserved (blue). Fully conserved or similar amino acids are marked by either : or * respectively.