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Review

Minimal art: Or why small viral K^+ channels are good tools for understanding basic structure and function relations

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ABSTRACT

Some algal viruses contain genes that encode proteins with the hallmarks of K^+ channels. One feature of these proteins is that they are less than 100 amino acids in size, which make them truly minimal for a K^+ channel protein. That is, they consist of only the pore module present in more complex K^+ channels. The combination of miniature size and the functional robustness of the viral K^+ channels make them ideal model systems for studying how K^+ channels work. Here we summarize recent structure/function correlates from these channels, which provide insight into functional properties such as gating, pharmacology and sorting in cells.

Contents

1. Introduction

Minimalism is a movement in various forms of contemporary art and design where the work is reduced to its most fundamental features. The goal of the artists is to achieve a maximal esthetic or functional effect with a minimal amount of material. In the spirit of this movement we can also view viruses as minimal artists. Throughout evolution they have designed proteins for their replication, which often are very small in size but still robust in function. The analysis of structure/function relationships in these proteins can teach us valuable lessons about the minimal structural requirements for achieving an enzymatic function.

Interesting examples of such a minimal design of functional proteins are ion channels, which are now found in an increasing number of viruses including the influenza viruses A, B and C, HIV and many others. Structure and function of these viral proteins are reviewed elsewhere [1,2] and more information can be found in this issue. Here we concentrate on the design of very peculiar viral channels, which are closely related to eukaryotic K^+ channels. Sequencing the 330 kb genome of chlorella virus PBCV-1, a large dsDNA virus in the family *Phycodnaviridae* [3], revealed an open

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reading frame encoding a protein with the structural hallmarks of prokaryotic and eukaryotic K^+ channels; the protein was named Kcv for K^+ channel from *c*hlorella *v*irus. While K^+ channels from prokaryotes and eukaryotes typically consist of several hundred amino acids, Kcv only has 94 amino acids [4]. More recently, a related K^+ channel was discovered in chlorella virus ATCV-1 that is only 82 amino acids long [5]. In spite of their small sizes, both viral proteins form functional channels that have many of the properties associated with larger K^+ channels such as selectivity, gating and sensitivity to inhibitors. The small viral proteins readily assemble into tetramers and they sort in cells to distinct target membranes [6–10]. This means that all of the structural requirements for correct assembly of the channel as well as for the basic functional properties of a K^+ channel exist in the minimal design of the viral K^+ channels. This combination of small size and robust function makes viral K^+ channels an interesting model system for discovering basic structure/function correlates of K^+ channels. Here we review recent progress in understanding basic principles of K^+ channel function and sorting in cells using the viral K^+ channels.

2. Miniature viral channels provide information on how K^+ channels work

2.1. Viral K^+ channels represent the pore module of all K^+ channels

K^+ channels are found in most membranes of eukaryotic cells and in many bacteria [11,12]. They function as a selective and regulated pathway for the selective diffusion of K^+ ions across membranes. In this context they play prominent roles in many physiological reactions ranging from membrane excitation to osmoregulation in animals and plants [13,14]. In spite of their many functions, the general structure of K^+ channels is conserved throughout prokaryotes and eukaryotes. K^+ channels are generally homo- or heterotetramers. Four pore forming domains are symmetrically arranged around an ion conducting water filled pore. Each subunit contains transmembrane domains (TMD); the number of TMDs varies between two and eight depending on the type of channel [13–15].

Fig. 1 summarizes the architecture of the monomers which compose main types of K^+ channels. The simplest channels consist of 4 proteins, each with 2 transmembrane domains (TMD) channels, which are linked by a pore helix. The eukaryotic Kir channels (K^+ inward rectifiers) [16] and the bacterial K^+ channels KcsA and KirBac [17,18] are well known examples of this type of channel. Another type of K^+ channel consists of 4 proteins, each with 6 TMDs. The eukaryotic Kv channels (for voltage gated K^+ channels) are members of this group [19]. In the so-called tandem channels two TMD channels are linked in a protein. When two of the tandem units come together they form the canonical pore. Finally in the 8 TMD type channel the functional channels are made of a tandem of two subunits [20].

The structural element, which is common to all K^+ channels is the pore module. This element consists of two TMDs linked by the pore helix. The latter contains a highly conserved sequence of amino acids, the K^+ channel consensus motive TXXTXGY/FG [21]. These amino acids align the narrow part of the water filled pore and its distinct architecture is the basis for channel selectivity [17].

The prototype channel Kcv from virus PBCV1 contains all of the structural elements present in the pore module of all K^+ channels [22], e.g., the two TMDs, a pore helix with the consensus motif in the selectivity filter. Also a loop at the extracellular side, the so-called turret, which exists in all K^+ channels, is present. The main difference between Kcv and other K^+ channels is the small size of the viral channel. While the 333 amino acid bacterial K^+ channel KirBac1.1 has extensive cytoplasmic domains [18] the viral channel is quasi fully embedded in the membrane [22,23]. The C-terminus of the inner TMD is virtually at the end of the membrane. The extension and crossing of the inner TMDs, which occurs in larger K^+ channels and is important for gating of K^+ channels [25,26], is absent. The size of the inner TMDs in Kcv is too short for the canonical bundle crossing observed in larger K^+ channels. Also the N-terminus of Kcv is much shorter than that of other K^+ channels; the viral channel only has 12 amino acids upstream of the first TMD. This cytosolic domain is probably helical and positioned at the inter-phase between the membrane and aqueous environment [22]. Viral protein Kcv not only looks like a K^+ channel, it also functions as a K^+ channel. The protein has been expressed in several heterologous systems including *Xenopus* oocytes [4], mammalian cells [27] and yeast [5], and in all cases the protein generated a K^+ selective conductance. The protein can also be purified after expression in the yeast *Pichia pastoris* [6]) or after in vitro translation with bacterial lysate [8]. When purified Kcv is reconstituted in planar lipid bilayers it produces K^+ conductances with the same properties that occur in heterologous expression systems [6,8]. Collectively, these data clearly establish that Kcv generates an active K^+ channel. The basic functional details of the channel have been reviewed elsewhere [28].

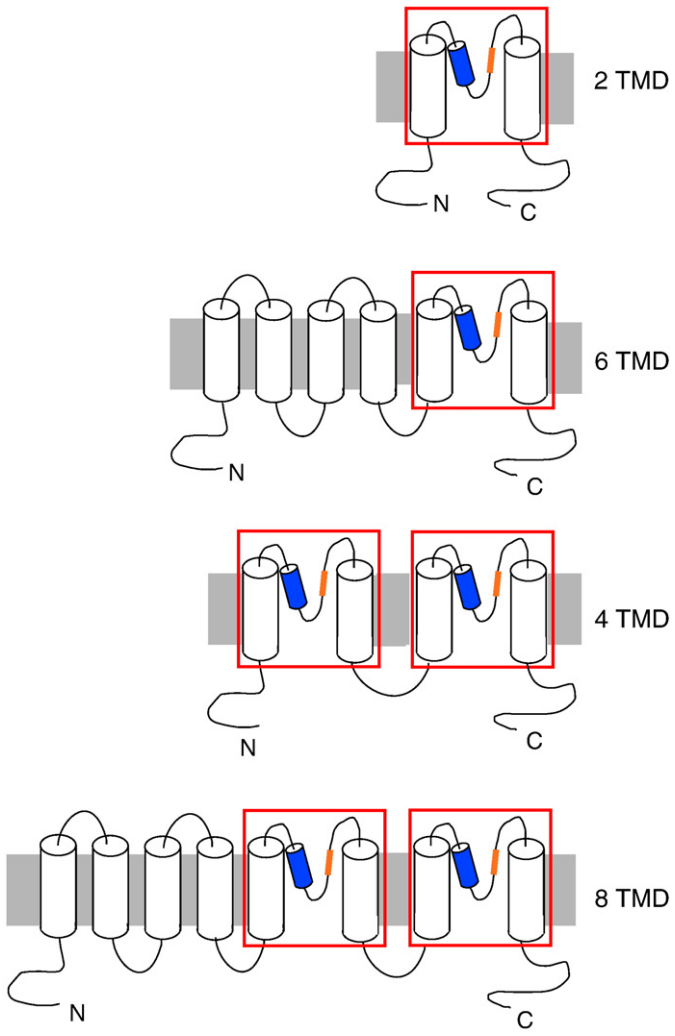

Fig. 1. Membrane topology of the main K^+ channel subunits. The subunits are composed of between 2 and 8 transmembrane domains (TMDs). A functional channel is a tetramer of 2-TMD or 6-TMD subunits or a dimmer of 4-TMD or 8-TMD subunits. The pore module of the channels including the pore helix (in blue) and the selectivity filter (in orange) is framed by red box.

2.2. Algal viruses are a source of K^+ channels with different structural and functional features

The understanding of structure function correlates in Kcv type channels is favored by an exploitation of the known evolutionary

pressure on viruses. The channel can be seen as undergoing a quasi-unlimited complexity of naturally evolving proteins. Each spontaneous mutation is selected through millions of years in the context of the surrounding sequence to generate a modified but still functional variant of the original sequence. In an attempt to use this unbiased pool of genetic information for structure/function analysis, a natural collection of functional Kcv variants from other virus isolates was examined [29,30]. Genes encoding the K^+ channel were cloned from 40 additional chlorella viruses, which infect the same host as virus PBCV-1, *Chlorella* NC64A. Because a functional Kcv channel is probably required for the virus to replicate [31–33], it is not surprising that all the Kcv variants, generated by the long evolutionary history of these viruses [3], are functional. They constitute a pool of prescreened functionalized genetic diversity in which selection had removed non-advantageous diversity from the pool of functionality. From this gene pool six natural Kcv variants were identified containing 4–12 amino acid substitutions when compared to the reference channel PBCV-1 Kcv. Functional tests revealed that these six variants have different kinetic and permeability properties relative to PBCV-1 Kcv [29,30]. The comparison of amino acid substitutions with channel properties identified three sets of substitutions associated with three specific channel properties:

- K^+ current inactivation,
- high Rb^+ permeability, and
- blocking by Cs^+.

The power of this unbiased method for structure/function research is underscored by the fact that the relevant amino acid exchanges were localized in domains of the protein not previously considered to be important. For example, the relative conserved substitution of amino acid 19 in TMD1 resulted in a drastic difference in Cs^+ sensitivity of the channel [29,30]. Neither the type of amino acid exchange nor the position would have been assumed to be important using a rational approach as functional relevant. Another surprise was that a channel variant had an amino acid substitution from a Phe to a Leu in the selectivity filter. Other than this unexpected amino acid substitution, this natural mutant exhibited typical K^+ channel behavior including K^+ selectivity [30].

Mutational analysis was also used to determine whether amino acid substitutions in each set of Kcv variants contributed singly or in combination to the channel property. The results of these experiments established that none of the property changes resulted from a single amino acid substitution. Indeed, mutations that do not alter the channel behavior alone nevertheless exert a strong influence reversing the effects of other mutations [30]. Each property is controlled by a synergistic interaction between amino acid pairs and networks located in different pore regions with no obvious physical interactions. Properties such as Rb^+ permeability and Cs^+ blocking expected to localize in the selectivity filter, are the result of a synergistic interaction at two positions, amino acid Phe19 in the outer helix, TM1, and amino acid Ile54 in the pore helix [30]. Model predictions for Kcv suggest that these two positions are distant from each other and do not seem to contact the selectivity filter [22,29,30]. Thus, these natural Kcv variants reveal interactions between distant domains and, most importantly, they could not be predicted from the current static understanding of channel pore structure.

Kcv type K^+ channel are not only found in viruses with the same host specificity but also in 3 other members of the *Phycodnaviridae*, which infect different hosts (Fig. 1, Table 1; [34–36]). Particularly interesting among these viruses is virus EsV-1, because the host of this virus is a filamentous brown alga while the hosts for the other viruses are unicellular green algae. In terms of evolution, the brown and the green algae are far apart [37]. Worth noting is also the difference in life cycle among the listed viruses: EsV-1 is a lysogenic virus, which integrates its entire genome into that of the hosts and coexists with the host [34]. The other viruses are lytic meaning that they kill their host in the process of replication [3]. Genomic sequencing of the viruses listed in Table 1 indicates that they also code for full size K^+ channel-like proteins. Comparison of the derived amino acid sequences with the PBCV-1 reference channel Kcv shows that the new virus proteins resemble one another; however, some of their details differ significantly (Fig. 2). A few obvious differences include:

Table 1
Phycodnaviruses, which code for K^+ channels. The table lists some details about the virus and their hosts.

Virus	Genome size	Host	Habitat	Replication cycle	Viral K^+ channel	Size/amino acids
PBCV-1	331 kb	*Chlorella* NC64A	Fresh water	Lytic	Kcv_{PBCV1}	94
ATCV	288 kb	*Chlorella* SAG 3.83	Fresh water	Lytic	Kcv_{ATCV}	82
MT325	314 kb	*Chlorella* Pbi	Fresh water	Lytic	Kcv_{M235A}	95
EsV-1	336 kb	*Ectocarpus siliculosus*	Sea water	Lysogenic	Kesv	124

- The 82 amino acid Kcv-ATCV is missing the turret and is smaller than all the other proteins.
- Kcv-ATCV and the 95 amino acid Kcv-MT325 lack cytoplasmic N-termini. However, unlike Kcv-PBCV-1, they have an extended C-terminus.
- The 124 amino acid Kesv has a longer C- and N-terminus than the other three virus channels.

MT325-Kcv and ATCV-Kcv have been successfully expressed in *Xenopus* oocytes indicating that these channels are also functional [5,38]. Indirect evidence also suggests that Kesv generates a functional channel [39]. Further analysis uncovered the structural basis for the apparent functional differences between the different channels. For example, whereas Kcv-PBCV-1 has a rather low channel open probability [40], Kcv-ATCV has a very high open probability [5]. In terms of understanding structure/function correlates it will be interesting to find which of the structural differences between the channels accounts for this functional difference.

2.3. The selectivity filter is responsible for assembly of the channel into functional tetramers

After providing this general overview of the structure of the small viral K^+ channels we now go into more detail and present some insight into structure and function relationships.

We mentioned above that the Kcv protein can be produced heterologously in *P. pastoris* or by in vitro translation. When the purified protein is electrophoresed on an SDS PAGE gel, the protein appears primarily as a tetramer [6–9]. This means that the protein has an inherently high propensity for tetramerization. This fact is supported by experiments, which show that the tetramer withstands high temperatures before it disassembles into monomers [6,9]. The unexpected high stability of the tetramer is influenced by the nature of the cations in the buffer solution: only cations, which can enter the pore of the channel for either transport or to block the channel, stabilize the oligomer [6,9]. These findings imply that the functional Kcv tetramer is due to electrostatic interplay between the protein selectivity filter with the ions in the filter.

The hypothesis that the tetramer is stabilized by an electrostatic interplay between the selectivity filter and ions in the filter is supported by recent experiments. A mutant of Kcv has a conservative amino acid exchange in the selectivity filter (S63T) in a site, which is associated with Ba^{2+} and K^+ binding [41]. The ability of Ba^{2+} to block this channel is drastically reduced in the mutant, which can be

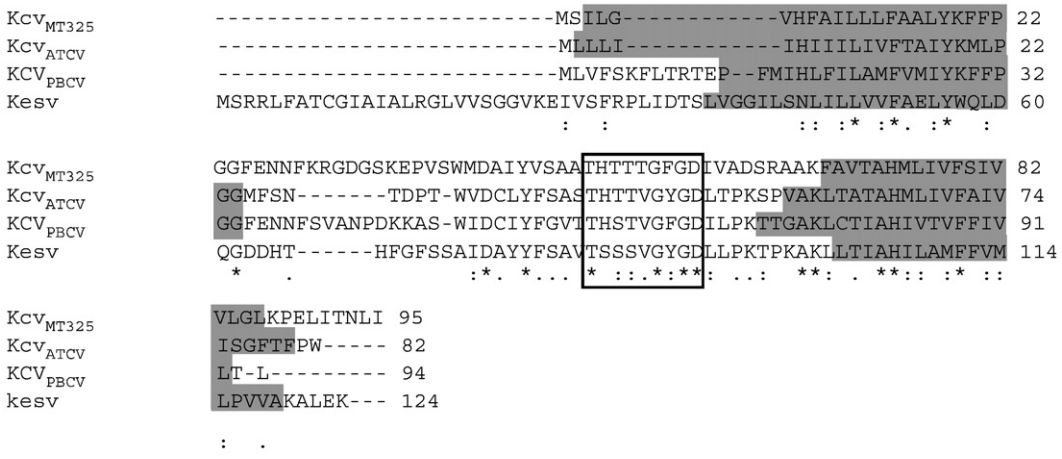

Fig. 2. Multiple alignment of derived amino acid sequences of K^+ channel proteins from four Phycodnaviruses. The transmembrane helices were determined with the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/alogirith) and they are shown in gray. The filter region of the channels is highlighted in a box.

explained by a reduced Ba^{2+} binding affinity to the channel [9]. In support of the hypothesis that binding of ions in the selectivity filter is important for tetramer stability, this mutant is much less stable in the presence of Ba^{2+} than the wt channel. This intimate connection between protein structure and ions in the pore also suggests that the channel architecture is affected by the transported ion and vice versa. We will return to this issue below in the context of channel gating.

2.4. Gating

In spite of the small size and even without a canonical bundle crossing, the Kcv channel is gated. On the functional level, two types of gates can be identified, a fast gate and a slow gate. When Kcv is expressed in *Xenopus* oocytes the channel current can be dissected into two kinetic components [40,42,43]. At negative test voltages the channel exhibits two kinetic components, namely an instantaneous activating conductance and a slow time dependent component. Both kinetic components are voltage dependent. The instantaneous current decreases with extreme positive and negative voltages; the result is a negative slope conductance at the voltage extremes. The slow component has the typical features of a slow inward rectifier in that the channel increases in a time dependent manner. Like typical voltage gated K^+ channels the speed of activation increases with increasingly negative voltages.

The two kinetic components of the macroscopic current can be assigned to different modes of single channel gating. Single channel recordings of Kcv in planar lipid bilayers [6] and in the membrane of *Xenopus* oocytes [40] reveal that the unitary channel current decreases at both voltage extremes. Very much like the macroscopic current, the single channel current I/V relation generates a negative slope at extreme voltages meaning that the decrease in the single channel amplitude is the underlying mechanism for the negative slope of the macroscopic I/V relation. Close scrutiny of the single channel data shows that the decrease in channel amplitude is correlated with an increase in open channel noise. A detailed analysis of the channel openings reveals that this noise is the product of very fast closures, which interrupt the open channel. These fluctuations are faster than the recording system so that the measured current appears smaller than the real open channel current [40].

The most straight-forward explanation for this fast gating is to assume that increasing voltages draw blocking ions into the pore of the channel. Such a mechanism is the basis for the rectification of the Kir channels in animals [44]. However at this point we conclude that the mechanism in Kcv is different, which is independent of blocking ions. The experimental data reveal that the fast gating process is more or less independent of ions other than K^+ in the buffer solution [40]. The results of these experiments imply that fast gating is unlike Kir channels not depending on any block; it must be a property, which is inherent in the passage of the K^+ ion through the channel.

Indeed, at increasing K^+ concentrations, the onset of the negative slope is shifted to higher voltages. This implies that fast gating is possibly caused by potassium depletion of the filter [43]. Schroeder and Hansen [45] observed a very similar effect in human BK channels, explaining it with a quantitative model for depletion-induced destabilization of the selectivity filter, as already documented in crystal structures [46] and MD simulations [47]. In Kcv, however, the current reduction is much more severe than in BK; this fits well with the aforementioned critical role of the permeating ion for tetramer stability in the viral channel.

Further analysis of the single channel data also shows that single channel activity is voltage dependent on a slower ms time scale. Single channel recordings on *Xenopus* oocytes expressing Kcv show that the open probability of the channel increases in a voltage dependent manner with negative voltages. Averaging current responses to multiple cycles of clamp voltage steps from +80 mV to −100 mV produces a slow activating mean current response. The time course of this mean single channel current is the same as that measured on the macroscopic level [40]. This means that voltage dependent single channel activity is responsible for the slow voltage dependent component of Kcv.

2.5. Gating of the channel is sensitive to the membrane environment

The two gating modes described above are not only a property of the channel protein but a combination of protein and transported ions. In addition, the surrounding membrane seems to be involved in gating.

When Kcv is expressed in *Xenopus* oocytes the channel has the aforementioned two kinetic components. When the protein is expressed in mammalian cells such as HEK293 cells or CHO cells it also generates a K^+ selective and Ba^{2+} sensitive conductance very much like in oocytes [27,42]. However, the channel has different kinetic properties in mammalian cells. The most obvious difference is that the time dependent component is not present in mammalian cells. Also the channel has much stronger inward rectification in mammalian cells; the channel barely generates an outward current in HEK293 cells. What is similar in both expression systems is the prominent negative slope conductance at negative voltages [43,48]. It cannot be completely ruled out that GFP, which was C-terminally attached to the channel for the expression and detection in the mammalian cells, influences gating. However, control experiments, in which a chimera of Kcv and GFP was expressed in oocytes still

exhibited time dependent inward conductance (B. Hertel private communication). Therefore, the presence or absence of GFP alone cannot explain the differences in channel kinetics. At first glance, it seems as if the channel in HEK cells differs from that in *Xenopus* oocytes. This impression, however, is wrong because it is possible to recover some of the kinetic features observed in oocytes by small modifications in the HEK293 system. The following paragraphs will discuss how the slow inward rectifying activation of Kcv can be rescued in HEK cells by using NH_4^+ as transported ion or by extending the length of the outer transmembrane domain.

Fig. 3 shows a typical recording of a HEK293 cell expressing Kcv. When K^+ is in the buffer the channel activates in an instantaneous manner generating an inward rectifying conductance with a prominent negative slope conductance at voltages more negative than about −120 mV. Exchanging K^+ in the solution with NH_4^+ results in different current properties. First, the reversal voltage of the I/V relation shifts to negative voltages meaning that the channel passes NH_4^+ with a lower preference than K^+. The fact that Kcv is permeable to NH_4^+, albeit to a lower degree than to K^+, occurs in other more complex K^+ channels from eukaryotes [49]. This fact is further reflected in an overall decrease in the NH_4^+ generated inward current compared to that of K^+. The most important point, however, is the observation that at negative voltages the channel has a slow time dependent component with NH_4^+ as the carrying ion. The latter is similar to the K^+ current via Kcv in oocytes. These experimental results imply that the slow activating kinetics of the channel depends on an interaction of the transported ion with the protein. This hypothesis is further supported by the behavior of the channel in oocytes when Rb^+ is the permeable ion. Rb^+ is transported as well or even slightly better than K^+ [30]. However, with increasing negative voltages the current no longer exhibits a time dependent activation but a slow inactivation of the conductance [30]. This result underscores the hypothesis that the slow gating process of the Kcv channel depends on the interplay of the protein with the transported ion. The basis of the cation sensitive gating on the single channel level is not yet understood. However, in experiments in which Kcv was reconstituted in planar lipid bilayers, Rb^+ altered channel gating in a way that the typical unitary channel open/closed transitions were no longer resolvable with Rb^+; the channel generated a conductance which was higher than in K^+ but the channel no longer formed the typical step like fluctuations between a closed and open state [6]. Collectively, these data indicate that the protein conducts different cations with different selectivities. The transported ions however, reflect in some way back on the gating characteristics of the protein. The permeation of an ion through the channel occurs in a few ns. The Rb^+ generated inhibition of the Kcv current on the other hand, happens with a half time in the ms range. The same is true for the slow activation of the channel in the presence of NH_4^+. This means that Kcv slow gating cannot be explained by a direct interplay of the cation on its way through the channel. There must be other as yet unknown slow effects of the cation on the fold of the Kcv protein.

The Kcv time dependent component in HEK293 cells can also be rescued by a small modification in the protein fold. When the length of the outer TMD of the channel is extended by insertion of one Ala at the beginning of the transmembrane helix, the channel also transports K^+ ion in a time and voltage dependent activation in HEK293 cells [50]. The structural basis for this impact on gating is still not completely understood. However, it is tempting to speculate that it is

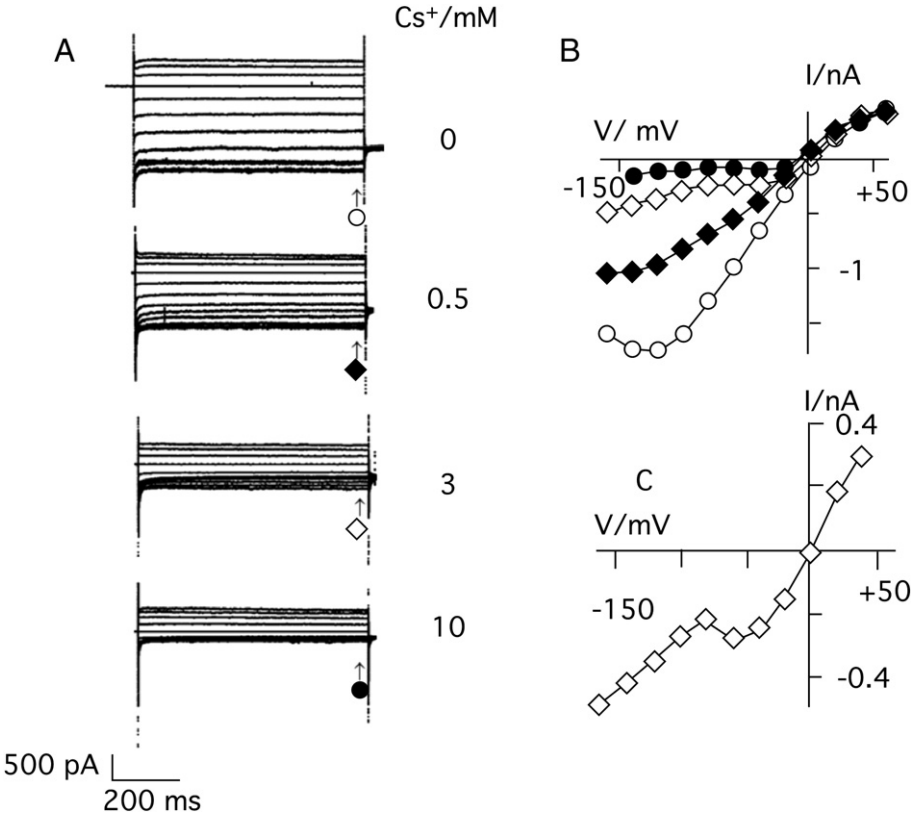

Fig. 3. Kcv generates different currents with either K^+ or NH_4^+ as transport substrate. Whole cell recordings of a HEK293 cell expressing a chimera of Kcv and GFP. Currents were elicited by voltage steps from a holding voltage of 0 mV to test voltages between +60 and −160 mV and a post pulse at −80 mV. Currents were measured either in a bath solution with 100 mM K^+ or 100 mM NH_4^+. The current response to a step to −140 mV in a buffer with 100 mM K^+ and 100 mM NH_4^+ is magnified in B. Note that the current decreases in K^+ but increases slowly in NH_4^+. The steady state currents indicated by an arrow in A are plotted as an I/V relation in C. Symbols in C correspond to those in A. Further experimental details are provided in [48].

due to a modified interaction between the outer and inner TMD. As noted below an interaction between the two TMDs is important for the access/exit of K^+ ions from or into the cavity of the channel.

Not only is the gating of the channel influenced by the environment but also its sensitivity to inhibitors. When Kcv is expressed in *Xenopus* oocytes, the channel is not very sensitive to the typical K^+ channel blocker Cs^+; this cation is only weakly transported by the channel [30]. Ten mM Cs^+ blocks the channel at −140 mV by less than 20%. Again a small modification in the structure, namely the exchange of I20V or K29R in the first TMD [30] makes the channel much more sensitive to Cs^+. These mutants are inhibited more than 50% by 10 mM Cs^+ at −140 mV. The experiments show that small changes in protein fold have a pronounced impact on basic functions of the channel.

In contrast, when the wt channel is expressed in HEK293 cells it is blocked by Cs^+ (Fig. 4). In this case, 10 mM Cs^+ in the bath medium inhibits the channel at −140 mV by up to 100%. Interesting to note is the inhibition kinetics at lower Cs^+ concentrations. With 3 mM Cs^+ in the medium and at moderate voltages an inhibition occurs, which increases with negative voltage. At more negative voltages, e.g., at voltages more negative than −100 mV, the channel again increases in conductance (Fig. 4). A similar voltage dependent increase in conductance, as a consequence of a release of a block, is also observed in more complex animal and plant K^+ channels [50,51]. The best explanation for this phenomenon is that Cs^+ is drawn by voltage into a binding site within the selectivity filter with the result that it blocks the K^+ current. With higher clamp voltages the Cs^+ ion is pulled from the binding site by the electrical field so that a higher K^+ current is again possible [50]. Collectively the data imply that Cs^+ can pass through the Kcv channel. The environment in the HEK293 cells however, produces a small conformational change in the channel with the result that the channel imposes a higher energetic barrier for Cs^+ transport. Since the passage of an ion or the voltage dependent block of an ion is most likely decided in the selectivity filter we speculate that the environment sensitive fold is manifested in the fine structure of the selectivity filter.

To summarize, we conclude that the functional properties of Kcv depend on the expression system. The differences are most likely due to small changes in the fold of the protein in different environments. Interesting to note is that this high sensitivity of the channel to the environment is a particular feature of the miniature viral channel. Other more complex K^+ channels from eukaryotes are less affected by the expression system. The plant K^+ channel KAT1, which has been studied extensively in our laboratory, has the same kinetic properties whether expressed in *Xenopus* or HEK293 cells [52,53]. Also the sensitivity of channels to inhibitors is usually independent of the expression system. It is tempting to speculate that the minimal size of the Kcv protein and the fact that it is almost completely embedded in the membrane makes the viral channel more sensitive to the membrane environment.

2.6. Function of Kcv in relation to its structure

The next step in understanding structure function correlates in the mini viral K^+ channels will be to assign distinct gating modes, i.e., instantaneous and time dependent gating described above, to the dynamic behavior of structural elements. A step forward was the development of a computational model for Kcv that performed extensive molecular dynamic (MD) simulations. Kcv has only moderate similarity to channels with known crystal structures.

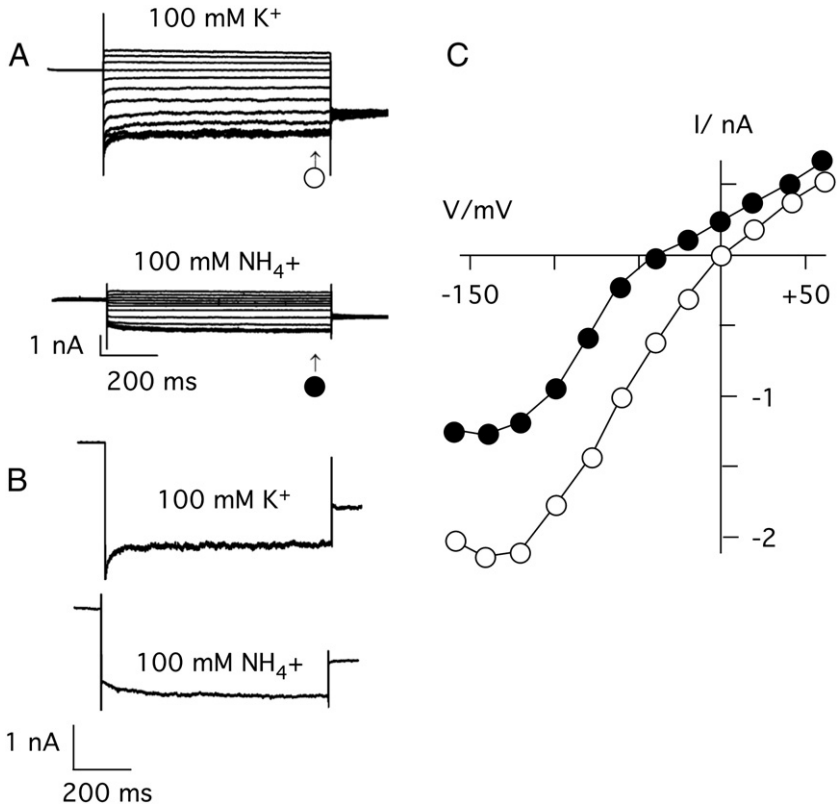

Fig. 4. Kcv is blocked by Cs^+ when expressed in HEK293 cells. Whole cell recordings of a HEK293 cell expressing a chimera of Kcv and GFP. A: Currents were elicited by voltage steps from a holding voltage of 0 mV to test voltages between +60 and −160 mV and a post pulse at −80 mV. Currents were measured in a bath solution with 100 mM K^+ without or with increasing concentrations of Cs^+. The steady state currents indicated by an arrow in A are plotted as an I/V relation in B. Symbols in B correspond to those in A. The plot in C magnifies the I/V relation in the presence of 3 mM Cs^+. Note the onset of voltage dependent inhibition at voltages negative of −50 mV and the release of block at voltages more negative than −100 mV. Further experimental details are provided in [48].

Therefore, a homology model for Kcv was developed in two steps. In the first step, the bacterial channel KirBac1.1 was chosen as a starting model because its structure is known [18] and it has some similarities to Kcv in various pivotal regions, such as the presence of a proline-linked short helix, the so-called "slide helix", at the N-terminus [23]. The KirBac1.1 channel has extended cytosolic termini that, for model building, was reduced to the size of Kcv, thus creating a functional analog to the viral channel, termed KirBac-Kcv. The resulting model was embedded in a neutral lipid bilayer and solvated by an aqueous KCl solution. The average structure of KirBac-Kcv, which was obtained from MD simulation is shown in Fig. 5. This system was found to be stable in the MD simulation and revealed features, which are expected for a K^+ channel. Most notably, K^+ ions entered the channel cavity spontaneously from the intracellular side through the mouth region. On the other hand, although the selectivity filter was stabilized by the characteristic cation configurations, no spontaneous translocation through the filter occurred over several tens of nanoseconds [23]. Thus, the artificial KirBac-Kcv system was useful for studying the impact of mutations near the intracellular mouth, but it did not represent an appropriate model for the functional Kcv channel.

In the next step, the KirBac1.1 structure was used as a template to generate a homology model for Kcv that was refined in extensive MD simulations in a solvated lipid bilayer [22]. Various models were initially tested that differ in the protonation state of titratable residues near the membrane interface (see below), as well as in the fold of the N-terminus. The most reliable and robust model was found to be extraordinarily stable for a homology approach and was selected for further studies of mutation effects. The average structure of Kcv from extensive MD simulation is shown in Fig. 5. A comparison with the KirBac-Kcv structure shows that both structures are very similar. As found earlier for KirBac-Kcv, K^+ ions entered spontaneously through the mouth into the cavity of the Kcv model. There was no need to artificially place ions in critical positions to elicit transport. Furthermore, the cavity ions rapidly exchanged positions with the filter ions ultimately leading to single-file motion with a concerted and directed movement of ions in the binding sites of the selectivity filter, in an agreement with the classical view [22]. The Kcv model is therefore the first simulation system for which spontaneous ion translocation through the entire pore has been observed. Altogether the data from the simulation stress that the structural model of Kcv is functional and therefore represents a plausible model for understanding basic structure/function correlates in this channel.

The MD simulations with both, the Kcv homology model and the KirBac-Kcv functional analog model, predicted some interesting structure/function correlates in Kcv. One observation was that the channel is unstable and non-conducting when amino acid Lys29 in the first transmembrane domain (which is present in Kcv type channels only) is protonated. In contrast, only when the channel is simulated with a deprotonated lysine in this position, the model is stable, capable of facilitating single-file ion movement, and allows K^+ to enter the cavity [22]. The results of these simulations imply that Lys29 may not be protonated in a functional channel. The hypothesis derived from the simulation is supported by experimental data. A mutant of Kcv in which lysine is replaced by alanine, for example, has the same conductance properties as the wt channel meaning that Lys29 is not necessarily protonated in a functional channel [22]. This is of interest because there is considerable discussion among channel researchers as to whether and to what extent lipid-embedded charged amino acids can be protonated [54–56].

The MD simulation studies also focused attention on the cytosolic entrance into the channel. This domain is of great importance in large K^+ channels because the inner transmembrane domain extends in these channels into the cytosol. By crossing of this helical bundle at the entrance into the cavity a gate is formed [17]. Diffusion of ions into or out of this cavity only occurs when a drastic conformational change results in the removal of the bundle crossing as an obstacle [25,26]. The MD simulations of both KirBac-Kcv and Kcv showed that the inner transmembrane domain of Kcv is too short to form a bundle crossing. This eliminates the possibility that Kcv is using the same mechanism of gating used by other K^+ channels with a cytosolic barrier as a gate. However, close scrutiny of the MD simulations indicates that entry into the Kcv cavity also depends on structure. The channel protein ends at the last amino acid of the inner TMD. As a consequence, all four subunits at the C-terminus have a negative charge at the entrance into the cavity. This ring of negative charges can catch an individual K^+ ion and bind it in a coordinated position. Further transport of the ion into or out of the cavity is only possible if the strong electrostatic interaction between the K^+ ion and the protein domains is weakened and ultimately disrupted by the fluctuating environment. Such a disruption is possible because of the transient formation of salt bridges, which can spontaneously occur between charged amino acids in the outer TMD and the inner TMD [22,23]. The competition between positive salt bridge partners for the negatively charged C-terminal group as a substitute for a cation is vital for facilitating rapid K^+ exchange between the cavity and the cytosol. The relevance of these salt bridges for channel function can again be demonstrated experimentally. Mutants of Kcv in which either the N-terminus is truncated or in which critical amino acids, which are required as partners for salt bridges, are neutralized reveal no or reduced channel activity [10]. This experimental result can be correlated with the

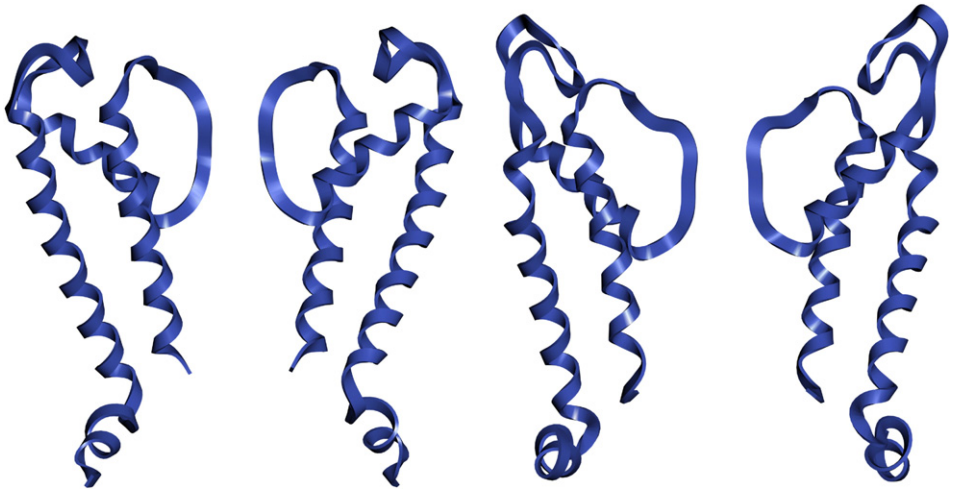

Fig. 5. The structures of KirBac-Kcv and Kcv are similar. The images show two opposing subunits of the average structures obtained from extensive MD refinements for KirBac-Kcv (on the left) and Kcv (on the right). Data from Ref. [22,23]. Images were created by MOLCAD [24].

observations in the simulations of both KirBac-Kcv and Kcv, which show that the truncation of the N-terminus corrupts the formation of salt bridges in this part of the protein [10,22,23]. As a result a K^+ ion, which is bound by the C-terminus of the inner helix, is no longer released and blocks exit and entry to the cavity.

In summary, the convergence of experimental and computational results allowed us to highlight the important role played by dynamic salt bridge patterns for ion transport through the mouth region. An important next step will be to clarify the connection between salt bridge formation and the molecular mechanism of the slow gate.

2.7. Two similar viral channels, Kcv and Kesv, are sorted to the secretory pathway and to the mitochondria, respectively

There are several examples in which investigations on the sorting of viral proteins have led to an understanding of the pathways used by cellular proteins [57–59]. In the spirit of this approach, the sorting of two viral K^+ channels was investigated. As mentioned above, small K^+ channel proteins were identified in several algal viruses. One of these channels, Kcv from virus PBCV-1 can be expressed heterologously in several systems including mammalian cells, *Xenopus* oocytes and yeast, where it produces a K^+ conductance in the plasma membrane. Kcv is therefore sorted to the plasma membrane *via* the canonical secretory pathway i.e., it is present in the endoplasmic reticulum (ER) and from there it is directed to the plasma membrane via vesicle trafficking [10]. The second channel, Kesv from virus EsV-1, is structurally similar to Kcv (Fig. 1). Nonetheless its sorting is completely different; the protein is imported into the mitochondria of HEK293 cells and yeast. This import, which occurs in a voltage dependent manner, requires the canonical pathway for protein entry into the mitochondria namely the TOM complex. This view is supported by in vitro import experiments into isolated yeast mitochondria. These experiments show that the Kesv protein is no longer imported when the multi protein TOM complex is disordered [39].

Kesv has an N-terminal amphiphatic helix with the hallmarks of a mitochondrial import sequence; this domain directs GFP into the mitochondria [39]. Experiments in which this putative signaling domain was mutated or deleted show that this domain is – other than in the GFP experiment – neither sufficient nor essential for sorting Kesv to the mitochondria. Structural comparisons and mutagenesis experiments instead revealed that the sorting of Kesv into the mitochondria depends on structural information in the second TMD of the Kesv channel: Kesv can be redirected from the mitochondria into the secretory pathway with a final destination in the plasma membrane after extension of the downstream part of the second TMD by $\geq$2 amino acids [39]. The degree of sorting Kesv into the ER increases with the length of the extension. With this modification in Kesv the channel protein sorts to the plasma membrane, where its activity can be measured. The result of this experiment is not only important in the context of protein sorting; it also demonstrates that the new viral channel Kesv from virus Esv1 is functional. In the context of protein sorting these data reveal a new type of discrimination between targeting of membrane proteins between the secretory pathway and the mitochondria.

3. Resume

When the first viral K^+ channel Kcv was detected in the chlorella virus PBCV-1 it was considered a curiosity of nature. But ten years of work on this and related proteins have shown that they are more than a footnote in the area of ion channel studies. Because of the simplicity of their structure and their robustness in function they became a versatile tool for uncovering some of the most basic features of structure/function correlates in K^+ channels. The last chapter in this review has shown that they are also providing valuable information on the mechanisms of protein sorting [39]. Last but not least the robustness of the protein together with its small size has made Kcv an interesting component in testing and constructing new devices for biosensors and drug screening [7,60].

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References

[1] W.B. Fischer, M.S.P. Sansom, Viral ion channels: structure and function, Biochim. Biophys. Acta 1561 (2002) 27–45.
[2] M.E. Gonzalez, L. Carrasco, Viroporins, FEBS Lett. 552 (2003) 28–34.
[3] J.L. Van Etten, Unusual life style of giant chlorella viruses, Annu. Rev. Genet. 37 (2003) 153–195.
[4] B. Plugge, S. Gazzarini, R. Cerana, J.L. Van Etten, M. Nelson, D. DiFrancesco, A. Moroni, G. Thiel, A potassium ion channel protein encoded by chlorella virus PBCV-1, Science 287 (2000) 1641–1644.
[5] S. Gazzarrini, M. Kang, A. Abenavoli, G. Romani, C. Olivari, D. Gaslini, G. Ferrara, J.L. Van Etten, M. Kreim, S.M. Kast, G. Thiel, A. Moroni, Eighty-two amino acids are sufficient for making a potassium selective channel, Biochem. J. 420 (2009) 295–303.
[6] C. Pagliuca, T.A. Goetze, R. Wagner, G. Thiel, A. Moroni, D. Parcej, Molecular properties of Kcv, a viral-encoded K^+ channel, Biochemistry 46 (2007) 1079–1090.
[7] J.W. Shim, L.Q. Gu, Stochastic sensing on a molecular chip containing a single-ion channel, Anal. Chem. 6 (2007) A–G.
[8] J.W. Shim, M. Yang, L.Q. Gu, In vitro synthesis, tetramerization and single channel characterization of virus-encoded potassium channel Kcv, FEBS Lett. 581 (2007) 1027–1034.
[9] F.C. Chatelain, S. Gazzarrini, Y. Fujiwara, C. Arrigoni, C. Domigan, G. Ferrara, C. Pantoja, G. Thiel, A. Moroni, D.L. Minor, Selection of inhibitor-resistant viral potassium channels identifies a selectivity filter site that affects barium and amantadine block, PLosOne 4 (2009) e7496.
[10] B. Hertel, S. Tayefeh, T. Kloss, J. Hewing, M. Gebhardt, D. Baumeister, A. Moroni, G. Thiel, S.M. Kast, Salt bridges in the miniature viral channel Kcv are important for function, Eur. Biophys. J. 39 (2010) 1057–1068.
[11] P.A. Anderson, R.M. Greenberg, Phylogeny of ion channels: clues to structure and function, Com. Biochem. Physiol. B Biochem. Mol. Biol. 129 (2001) 17–28.
[12] M.M. Kuo, W.J. Haynes, S.H. Loukin, C. Kung, Y. Saimi, Prokaryotic K^+ channels: from crystal structures to diversity, FEMS Microbiol. Rev. 29 (2005) 961–985.
[13] B. Hille, Ion Channels of Excitable Membranes, Sinauer Associates, Sunderland, MA, 2002.
[14] A. Leabudy, A.A. Very, H. Sentenac, K^+ channel activity in plants: genes, regulations and functions, FEBS Lett. 581 (2007) 2357–2366.
[15] C. Miller, An overview of the potassium channel family, Genomebiol. 4 (2000) 1–5.
[16] X. Tao, J.L. Avalos, J. Chen, R. MacKinnon, Crystal structure of the eukaryotic strong inward rectifier K^+ channel Kir2.2 at 3.1 A resolution, Science 326 (2009) 1668–1674.
[17] D.A. Doyle, J. Marales Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, R. MacKinnon, The structure of the potassium channel: molecular basis of K^+ conduction and selectivity, Science 280 (1998) 69–77.
[18] A. Kuo, J.M. Gulbis, J.F. Antcliff, T. Rahman, E.D. Lowe, J. Zimmer, J. Cuthbertson, F.M. Ashcroft, T. Ezaki, D.A. Doyle, Crystal structure of the potassium channel KirBac1.1 in the closed state, Science 300 (2003) 1922–1926.
[19] T.M. Shih, A.L. Golden, Topology of the Shaker potassium channel probed with hydrophilic epitome insertions, J. Cell Biol. 136 (1997) 1037–1045.
[20] K.A. Ketchum, W.J. Joiner, A.J. Sellers, L.K. Kaczmarek, S.A. Goldstein, A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem, Nature 376 (1995) 690–695.
[21] L. Heginbotham, Z. Lu, T. Abramson, R. MacKinnon, Mutations in the K^+ channel signature sequence, Biophys. J. 66 (1994) 1061–1067.
[22] S. Tayefeh, T. Kloss, M. Kreim, M. Gebhardt, D. Baumeister, B. Hertel, C. Richter, H. Schwalbe, A. Moroni, G. Thiel, S.M. Kast, Model development for the viral Kcv potassium channel, Biophys. J. 96 (2009) 485–498.
[23] S. Tayefeh, T. Kloss, G. Thiel, B. Hertel, A. Moroni, S.M. Kast, Molecular dynamics simulation of the cytosolic mouth in Kcv-type potassium channels, Biochemistry 46 (2007) 4826–4839.
[24] J. Brickmann, T. Goetze, W. Heiden, G. Moeckel, S. Reiling, H. Vollhardt, C.-D. Zachmann, Interactive visualization of molecular scenarios with MOLCAD/SYBYL, in: J.E. Bowie (Ed.), Data Visualisation in Molecular Science: Tools for Insight and Innovation, Addison-Wesley, Reading, 1995, pp. 83–97.

[25] S.M. Irizarry, E. Kutluay, G. Drews, S.J. Hart, L. Heginbotham, Opening the KcsA K^+ channel: tryptophan scanning and complementation analysis lead to mutants with altered gating, Biochemistry 41 (2002) 13653–13662.
[26] M. Rapedius, P.W. Fowler, L. Shang, M.S. Sansom, S.J. Tucker, T. Baukrowitz, H bonding at the helix-bundle crossing controls gating in Kir potassium channels, Neuron 55 (2007) 537–538.
[27] A. Moroni, C. Viscomi, V. Sangiorgio, C. Pagliuca, T. Meckel, F. Horvath, S. Gazzarrini, P. Valbuzzi, J.L. Van Etten, D. DiFrancesco, G. Thiel, The short N-terminus is required for functional expression of the virus encoded miniature K^+-channel Kcv, FEBS Lett. 530 (2002) 65–69.
[28] S. Gazzarrini, M. Severino, M. Lombardi, M. Moranti, D. DiFrancesco, J.L. Van Etten, G. Thiel, A. Moroni, The viral potassium channel Kcv: structural and functional features, FEBS Lett 552 (2003) 12–16.
[29] M. Kang, A. Moroni, S. Gazzarrini, D. DiFrancesco, G. Thiel, M. Severino, J.L. Van Etten, Small potassium ion channel protein encoded by chlorella viruses, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 5318–5324.
[30] S. Gazzarrini, M. Kang, J.L. Van Etten, S. Tayefeh, S.M. Kast, D. DiFrancesco, G. Thiel, A. Moroni, Long-distance interactions within the potassium channel pore are revealed by molecular diversity of viral proteins, J. Biol. Chem. 279 (2004) 28443–28449.
[31] T. Greiner, F. Frohns, M. Kang, J.L. Van Etten, A. Käsmann, A. Moroni, B. Hertel, G. Thiel, Chlorella viruses prevent multiple infections by membrane depolarization of the host, J. Gen. Virol. 90 (2009) 2033–2039.
[32] M. Neupärtl, C. Meyer, I. Woll, F. Frohns, M. Kang, J.L. Van Etten, D. Kramer, B. Hertel, A. Moroni, G. Thiel, Chlorella viruses evoke a rapid release of K^+ from host cells during early phase of infection, Virology 372 (2007) 340–348.
[33] G. Thiel, A. Moroni, D. Dunigan, J.L. Van Etten, Initial events associated with virus PBCV-1 infection of Chlorella NC64A, Prog. Bot. 71 (2010) 169–183.
[34] N. Delarouque, D.G. Müller, G. Bothe, T. Pohl, R. Knippers, W. Boland, The complete DNA sequence of the Ectocarpus siliculosus Virus EsV-1 genome, Virology 287 (2001) 112–132.
[35] L.A. Fitzgerald, M.V. Graves, X. Li, J. Hartigan, A.J. Pfitzner, E. Hoffart, J.L. Van Etten, Sequence and annotation of the 288-kb ATCV-1 virus that infects an endosymbiontic chlorella strain of the heliozoon *Acanthocystis turfacea*, Virology 362 (2007) 350–361.
[36] L.A. Fitzgerald, M.V. Graves, X. Li, T. Feldblyum, J. Hartigan, J.L. Van Etten, Sequence and annotation of the 314-kb MT325 and the 321-kbFR483 viruses, Virology 358 (2007) 459–471.
[37] P.G. Falkowski, M.E. Katz, A.H. Knoll, A. Quigg, J.A. Raven, O. Schofield, F.J.R. Taylor, The evolution of modern eukaryotic phytoplankton, Science 305 (2004) 354–360.
[38] S. Gazzarrini, M. Kang, S. Epimashko, J.L. Van Etten, J. Dainty, G. Thiel, A. Moroni, Chlorella virus MT325 encodes water and potassium channels that interact synergistically, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 5355–5360.
[39] J. Balss, M. Mehmel, D. Baumeister, B. Hertel, N. Delaroque, F.C. Chatelain, D.J. Minor, J.L. VanEtten, A. Moroni, G. Thiel, Transmembrane domain length of viral K^+ channels is a signal for mitochondria targeting, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 12313–12318.
[40] A. Abenavoli, M.L. DiFrancesco, I. Schroeder, S. Epimashko, S. Gazzarrini, U.P. Hansen, G. Thiel, A. Moroni, Fast and slow gating are inherent properties of the K^+ channel pore module, J. Gen. Physiol. 134 (2009) 219–229.
[41] Y. Jiang, R. MacKinnon, The barium site in a potassium channel by X-ray crystallography, J. Gen. Physiol. 115 (2000) 269–272.
[42] S. Gazzarrini, J.L. Van Etten, D. DiFrancesco, G. Thiel, A. Moroni, Voltage-dependence of virus encoded miniature K^+-channel Kcv, J. Membr. Biol. 187 (2002) 15–25.
[43] S. Gazzarrini, A. Abenavoli, D. Gradmann, G. Thiel, A. Moroni, Electrokinetics of miniature K^+ channel: open state V-sensitivity and inhibition by K^+ driving force, J. Membr. Biol. 214 (2007) 9–17.
[44] C.G. Nichols, A.N. Lopatin, Inward rectifier potassium channels, Annu. Rev. Physiol. 59 (1997) 171–191.
[45] I. Schroeder, U.P. Hansen, Saturation and microsecond gating of current indicate depletion-induced instability of the MaxK selectivity filter, J. Gen. Physiol. 130 (2007) 89–97.
[46] Y. Zhou, J.H. Morais Cabral, A. Kaufman, R. MacKinnon, Chemistry of ion hydration and coordination revealed by a K^+ channel-Fab complex at 2.0 A resolution, Nature 414 (2001) 43–48.
[47] G.V. Miloshevsky, P.C. Jordan, Conformational changes in the selectivity filter of the open-state KcsA channel: an energy minimization study, Biophys. J. 95 (2008) 3239–3251.
[48] B. Hertel, S. Tayefeh, M. Mehmel, S.M. Kast, J.L. Van Etten, A. Moroni, G. Thiel, Elongation of outer transmembrane domain alters function of miniature K^+ channel Kcv, J. Membr. Biol. 210 (2006) 21–29.
[49] A. Moroni, L. Bardella, G. Thiel, The impermeant ion methylammonium differently blocks K^+ and NH_4^+ currents through KAT1 channel: evidence for ion interaction in channel permeation, J. Membr. Biol. 163 (1998) 25–35.
[50] R.J. French, J.J. Shoukimas, An ion's view of the potassium channel, J. Gen. Physiol. 85 (1985) 669–698.
[51] G. Thiel, M.R. Blatt, The mechanism of ion permeation through K^+ channels of stomatal guard cells: voltage-dependent block by Na^+, J. Plant Physiol. 138 (1991) 257–363.
[52] B. Sottocornola, C. Visconti, S. Orsi, S. Gazzarrini, S. Giacometti, C. Olivari, L. Camoni, P. Aducci, M. Marra, G. Thiel, A. Moroni, The potassium channel KAT1 is activated by plant and animal 14-3-3 proteins, J. Biol. Chem. 281 (2006) 35735–35741.
[53] B. Hertel, F. Horvath, B. Wodala, A. Hurst, A. Moroni, G. Thiel, KAT1 inactivates at sub-millimolar concentrations of external potassium, J. Exp. Bot. 422 (2005) 3103–3110.
[54] U. Schulte, B. Fakler, Gating of inward-rectifier Kþ channels by intracellular pH, Eur. J. Biochem. 267 (2000) 5837–5841.
[55] J.L. MacCallum, W.F.D. Bennett, D.P. Tieleman, Distribution of amino acids in a lipid bilayer from computer simulations, Biophys. J. 94 (2008) 3393–3404.
[56] J.A. Freites, J.T. Douglas, G. von Heijne, S.H. White, Inter-face connections of a transmembrane voltage sensor, Proc. Natl. Acad. Sci. USA 102 (2005) 15059–15064.
[57] L. Pelkmans, A. Helenius, Insider information: what viruses tell us about endocytosis, Curr. Opin. Cell Biol. 15 (2003) 414–422.
[58] W.J. Lucas, S. Wolf, Connections between virus movement, macromolecular signalling and assimilate allocation, Curr. Opin. Plant. Biol. 2 (1999) 192–197.
[59] N. Nishimura, W.E. Balch, A di-acidic signal required for selective export from the endoplasmic reticulum, Science 277 (1997) 556–558.
[60] R. Syeda, M.A. Holden, W.L. Hwang, H. Bayley, Screening blockers against a potassium channel with a droplet interface bilayer array, JACS 133 (2008) 15543–15548.