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Relevance of Lysine Snorkeling in the Outer Transmembrane Domain of Small Viral Potassium Ion Channels

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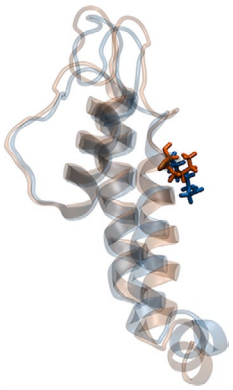
Abstract

Transmembrane domains (TMDs) are often flanked by Lys or Arg because they keep their aliphatic parts in the bilayer and their charged groups in the polar interface. Here we examine the relevance of this so-called "snorkeling" of a cationic amino acid, which is conserved in the outer TMD of small viral K^+ channels. Experimentally, snorkeling activity is not mandatory for Kcv_{PBCV-1} because K29 can be replaced by most of the natural amino acids without any corruption of function. Two similar channels, Kcv_{ATCV-1} and Kcv_{MT325}, lack a cytosolic N-terminus, and neutralization of their equivalent cationic amino acids inhibits their function. To understand the variable importance of the cationic amino acids, we reanalyzed molecular dynamics simulations of Kcv_{PBCV-1} and N-terminally truncated mutants; the truncated mutants mimic Kcv_{ATCV-1} and Kcv_{MT325}. Structures were analyzed with respect to membrane positioning in relation to the orientation of K29. The results indicate that the architecture of the protein (including the selectivity filter) is only weakly dependent on TMD length and protonation of K29. The penetration depth of Lys in a given protonation state is independent of the TMD architecture, which leads to a distortion of shorter proteins. The data imply that snorkeling can be important for K^+ channels; however, its significance depends on the architecture of the entire TMD. The observation that the most severe N-terminal truncation causes the outer TMD to move toward the cytosolic side suggests that snorkeling becomes more relevant if TMDs are not stabilized in the membrane by other domains.



The transmembrane domains (TMDs) of many proteins contain positively charged amino acids near the polar–/apolar membrane interface.(1-5) Because of their long and flexible side chains, these amino acids can keep their hydrocarbon part inside the membrane while the positive charge reaches into the interface region.(6, 7) This phenomenon has been termed "snorkeling" and is critical for the precise positioning and orientation of TMDs in the membrane and for the compensation of hydrophobic mismatches between proteins and membranes.(7, 8)

The small viral K^+ channel Kcv can serve as a model for studying the relevance of Lys or Arg snorkeling in the TMDs of K^+ channels. This Kir-like channel has the structural and functional hallmarks of more complex K^+ channels (9, 10) but with a monomer size of 94 amino acids consists of just the pore module component present in all K^+ channels. Examination of the Kcv structure indicates that the channel contains the basic amino acid Lys in the outer TMD at the predicted membrane–aqueous interface.(10, 11) Recent computational and experimental studies of the role of this Lys in Kcv from virus PBCV-1 (Kcv_{PBCV-1}) produced a surprising result. Molecular dynamics (MD) simulations revealed that a protonated Lys exhibits vivid snorkeling activity with the consequence that the protein structure becomes unstable and the channel does not transport ions.(11) In contrast, modeling the protein with a deprotonated Lys produced a stable channel, capable of spontaneous single-file ion transport. These results suggest that the Lys at position 29 (K29) in Kcv_{PBCV-1} could be uncharged and still form a functional channel. This hypothesis was supported by three site-directed mutations showing that K29 could be replaced with Ala, Ser, or Trp without impairing channel function in human HEK293 cells.(11) The apparent tolerance of the channel for different amino acids in position 29 is rather surprising because this equivalent position is very conserved among other viral K^+ channels. Recently, more than 40 Kcv-type viral K^+ channels were isolated (ref 12 and unpublished data of J. L. Van Etten), and some were successfully tested previously for function.(13) All of these channels have either a Lys or Arg in the equivalent position of TMD1.

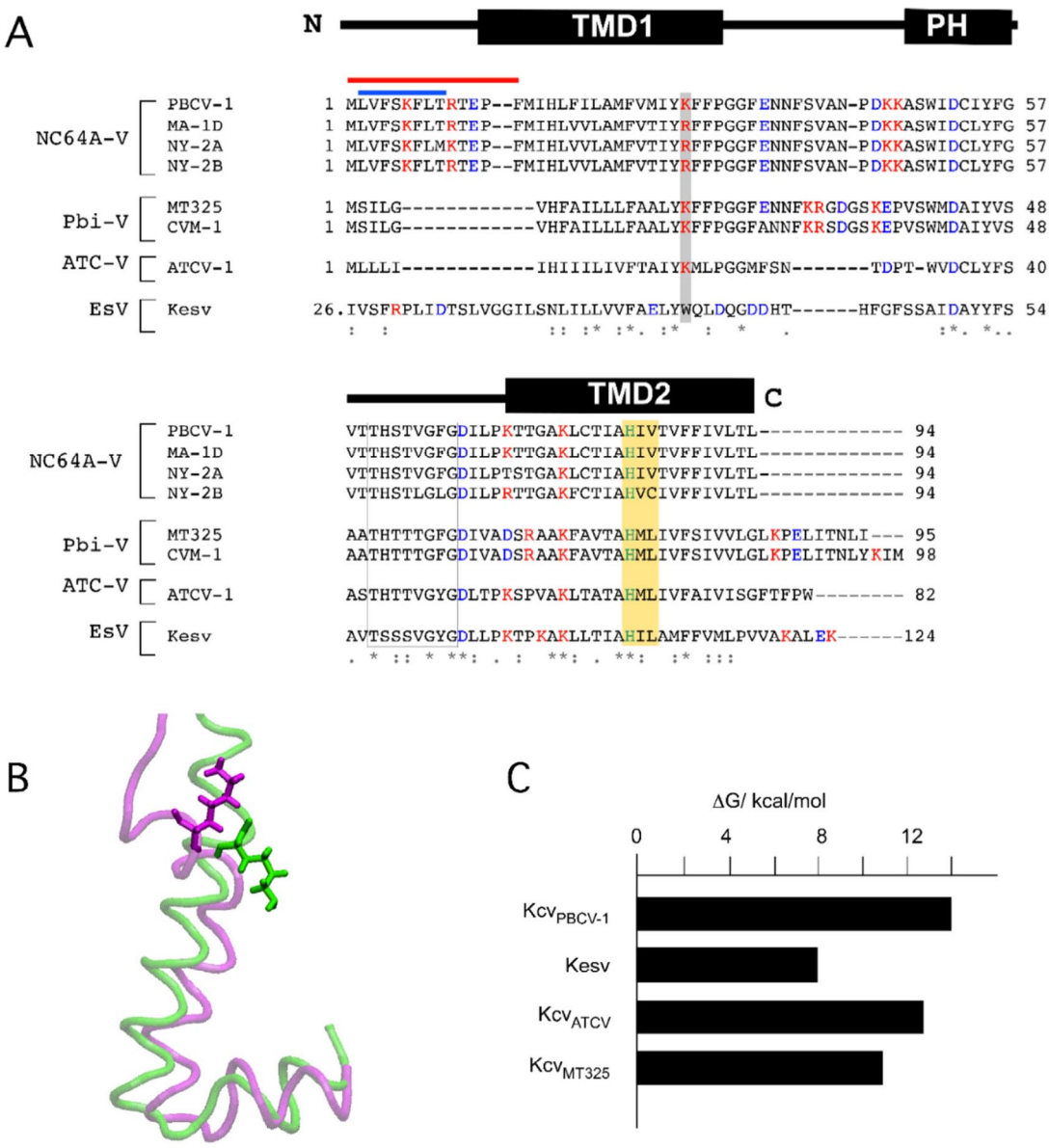


Figure 1. Position of a conserved basic amino acid in the outer transmembrane domain of viral K^+ channels. (A) Alignment of different viral K^+ channels. Several algal viruses (names given on the left), which infect different hosts, encode K^+ channels (second row). Viruses PBCV-1, MA-1D, NY-2A, and NY-2B infect *Chlorella variabilis* (formerly named *Chlorella* NC64A). Viruses MT325 and CVM-1 infect *Micractinium conductrix* (formerly named *Chlorella* Pbi). Virus ATCV-1 infects *Chlorella heliozoae* (formerly named *Chlorella* SAG.3.83). Virus EsV infects *Ectocarpus siliculosus*. The canonical K^+ channel selectivity filter is boxed, and the basic amino acid (K29 in Kcv from virus PBCV-1), which is conserved in all *Chlorella* viruses, is highlighted in gray. The amino acids, which presumably are in the vicinity of K29 in Kcv$_{PBCV-1}$ in TMD2, are highlighted (yellow). The acidic and basic amino acids in the protein are colored blue and red, respectively. Identical amino acids are denoted with asterisks, and similar and related amino acids are denoted with colons and periods, respectively. The structural organization of Kcv from PBCV-1 with the position of the two transmembrane domains (TMD1 and TMD2) as well as the pore helix (PH) is shown in the top panel. The amino acids at the N-terminus, which are truncated in the Kcv$_{PBCV-1}$-Δ8 and Kcv$_{PBCV-1}$-Δ14 mutants, are denoted with blue and red bars, respectively. (B) Average structures of TMD1 from Kcv$_{PBCV-1}$ taken from MD simulations. (11) The structures show TMD1 with the respective K29 simulated in its protonated (magenta) and deprotonated (green) forms. Snorkeling occurs only in the protonated state. (C) Hydropathy of different TMD1s calculated with MPEx. The calculated octanol scale is a measure of the total energetics of the helix stability of TMD1.(22, 23)

In this study, we continue to examine the functional role of this Lys in the structure–function context of viral K^+ channels by both experimental and computational analyses. Experimentally, we discovered that K29 in Kcv$_{PBCV-1}$ can be replaced with each natural amino acid with the exception of Pro without losing channel function. This result implies that channel function is insensitive to the nature of the amino acid at this position. Comparison of Kcv$_{PBCV-1}$ with some other viral K^+ channels indicates that this position is conserved, e.g., in Kcv$_{ATCV-1}$ and Kcv$_{MT325}$. Functional studies of these two viral channels indicate that Lys is more important in this position for function than in Kcv$_{PBCV-1}$. Computer simulation data shed some light on the structural significance of the protonation state of K29 in the context of the N-terminal length and the membrane positioning of TMD1. Collectively, the data indicate that the contribution of a single amino acid to the structure and function of a channel protein can only be understood in the context of the entire protein.

Materials and Methods

Constructs and Mutagenesis. Kcv genes from *Chlorella* viruses PBCV-1, ATCV-1, and MT325 were cloned either in the pEGFP-N2 vector (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) for electrophysiological measurements in HEK293 cells or in a modified pYES2 vector (Invitrogen GmbH, Karlsruhe, Germany) for yeast complementation experiments. (14) The genes were cloned into the *Bgl*II and *Eco*RI site in the pEGFP-N2 vector without their stop codons and in frame with the downstream green fluorescent protein (EGFP). For the yeast experiments, the genes were cloned with their stop codons into the *Eco*RI and *Xho*I site of the pYES2 vector. For insertion of the site-directed mutations, the QuikChange site-directed mutagenesis method (Stratagen) was used and the resulting constructs were verified by DNA sequencing.

Saccharomyces cerevisiae *Complementation Assays.* Yeast complementation assays were conducted as described previously.(14) Yeast strain SGY1528 lacks an endogenous K^+ uptake system and, therefore, does not grow on media with K^+ concentrations of <10 mM. Complementation assays were conducted on agar plates either under nonselective conditions (100 mM K^+ agar plates) or under selective conditions (1 or 0.5 mM K^+). The plates were incubated for 3 days at 30 °C. Experiments in liquid cultures were performed in selective medium with 0.5 mM K^+, and growth was monitored by measuring the optical density at 600 nm (OD_{600}). The selective medium was inoculated with a yeast suspension at a final OD_{600} of 0.1 and incubated at 30 °C and 230 rpm. After 0 and 24 h, the OD_{600} was measured in a spectrophotometer.

Electrophysiological Measurements. The electrical properties of the viral channels in HEK293 cells were recorded as reported previously.(15) Different constructs of Kcv_{PBCV-1} and its homologues Kcv_{ATCV-1} and Kcv_{MT325} were transiently expressed as fusion proteins with GFP on the C-terminus using the liposomal transfection reagent TurboFect (Fermentas, St. Leon Rot, France). Measurements were performed at room temperature in a bath solution containing 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), and either 100 mM KCl or 100 mM NaCl; 10 mM $BaCl_2$ was added to the K^+-containing medium to block the channels. The osmolarity of all solutions was adjusted with mannitol to 330 milliosmolar. The pipet solution contained 130 mM potassium d-gluconic acid, 10 mM NaCl, 5 mM HEPES, 0.1 mM guanosine triphosphate (Na salt), 0.1 μM $CaCl_2$, 2 mM $MgCl_2$, 5 mM phosphocreatine, and 2 mM adenosine triphosphate (Na salt, pH 7.4).

Analyses of Molecular Dynamics Simulations. Microscopic insight was provided by atomistic MD simulations of wild-type (wt) Kcv_{PBCV-1} with both protonated and deprotonated K29 side chains as described previously (10, 11) and N-terminally truncated Kcv_{PBCV-1}-Δ8 and Kcv_{PBCV-1}-Δ14 with corresponding deprotonated Lys residues as described previously.(15) The truncated variants represent progressively shorter "slide" helices that align laterally with the cytosolic side of the membrane. The shortest mutant completely lacks the slide helix. Besides the average structures generated by mean-field annealing,(10, 11) we analyzed time series of various observables related to the position of the channel and its components in the membrane by calculating the distance of the center of mass (com) of fragments from the extracellular lipid phosphate headgroups. In particular, we analyzed (all numbers are for Kcv_{PBCV-1}-wt, truncated variants were shifted accordingly) the center of mass (com) of Lys29, the selectivity filter (residues 63–68), TMD1 (residues 15–29), and the entire tetrameric channel protein. All simulations include an initial ~30 ns rigid filter run, followed by a fully flexible model. In total, we obtained trajectory data for 90, 74, 103, and 100 ns for wt (deprotonated), wt (protonated), Δ8 (deprotonated), and Δ14 (deprotonated) variants, respectively.

Results

Status of Viral K^+ Channels and Snorkeling of Cationic Amino Acids. Like many membrane proteins,(1-8) viral K^+ channels typically have a cationic amino acid in the TMD near the lipid–aqueous interface. In the case of Kcv_{PBCV-1}, the outer transmembrane domain (TMD1) contains a Lys (K29) facing the extracellular solution (Figure 1A,B). Previous MD simulations of Kcv_{PBCV-1} indicated that this Lys behaves very differently in its protonated or deprotonated form.(11) The average structures in Figure 1B and the corresponding simulations by Tayefeh et al.(11) illustrate the dynamics and show a typical snorkeling activity for the protonated Lys.(11) The charged side chain of the amino acid is preferentially oriented toward the aqueous interface and is in contact with water. The vigorous movement of the Lys allows water molecules to penetrate the membrane, eventually leading to a breakdown of the TMD1 helical structure.(11) In contrast, the deprotonated form of Lys is fully desolvated and primarily directed toward the core of the membrane. In this configuration, the protein is stable and it is possible to observe spontaneous movement of K^+ ions through the channel. Consistent with the simulation results, separate experiments established that the channel does not require a protonated amino acid and hence does not require snorkeling in this position to function. For example, the channel still functions when the Lys is replaced with Ala.(11)

Lys29 in Kcv_{PBCV-1} Tolerates Mutations. We replaced Lys29 with each of the natural amino acids to determine if Kcv_{PBCV-1} function was altered; function was monitored by yeast complementation. The yeast mutant SGY1528 lacks endogenous K^+ uptake systems and consequently grows only in media with a high K^+ concentration. Yeast SGY1528 growth can be rescued in low-K^+ media (1 or 0.5 mM K^+) if the cells are supplied with a functional K^+ channel such as Kcv_{PBCV-1} (Figure 2A).

All of the mutants grew on a medium with 100 mM K^+ (Figure 2A); i.e., none of the mutations prevented SGY1528 growth. Surprisingly, all of the mutants also grew on the selective medium (1 or 0.5 mM K^+) with one exception, the substitution of Pro for Lys (Kcv_{PBCV-1}-K29P) (Figure 2A). These results indicate that Kcv_{PBCV-1} tolerates 19 amino acids at position 29 without losing function; only Pro is not tolerated. Because Pro is the amino acid with the strongest propensity for terminating α-helices(16, 17) and an amino acid that produces kinks in proteins,(18) we assume that the function of this position is to guarantee a proper α-helix and/or nonbent connection.

While the results in Figure 2A indicate that all amino acids except Pro can substitute for K29 in Kcv_{PBCV-1}, the amino acids differ in their ability to rescue growth. To quantify these differences in rescue efficiency, we performed liquid growth experiments with the different mutants. The rescue efficiency was estimated from the optical density of the cultures after 24 h. The results were similar to those obtained with agar plates (Figure 2B). All mutants, except for K29P, rescued yeast growth under selective conditions; however, some amino acids were

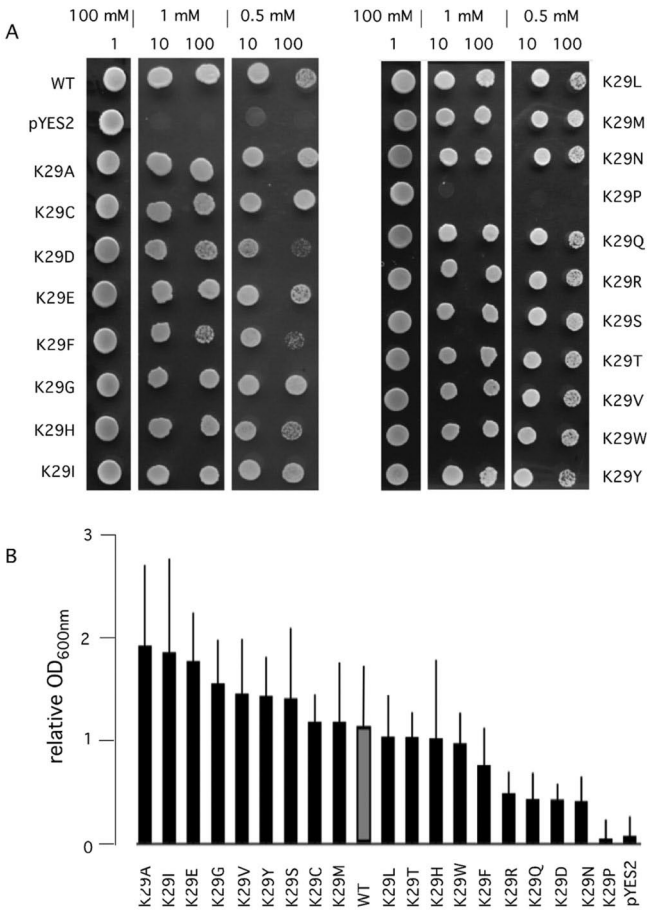


Figure 2. Most amino acid substitutions for K29 in Kcv_{PBCV-1} allow growth of K^+ uptake deficient yeast. (A) Growth of yeast mutants that express Kcv_{PBCV-1} wild-type channel or channel mutants in which K29 was replaced with each natural amino acid. The growth assay was performed on agar plates under nonselective (100 mM KCl) and selective (1 and 0.5 mM KCl) conditions. The yeast mutants expressing channels or empty vector (pYes2) were spotted in different dilutions: 1 (undiluted), 1:10 (10), and 1:100 (100). (B) Quantification of yeast growth with cells expressing empty vector or the aforementioned channels under selective conditions with 0.5 mM K^+ in liquid medium. Growth is shown as the increase in optical density at 600 nm (OD_{600}) 24 h after inoculation. Data are means ± the standard deviation of at least five independent experiments. With the exception of K29P, all mutants rescue yeast growth.

more effective in stimulating growth than others. Collectively, the data imply that the chemical properties of the amino acid at position of Kcv_{PBCV-1} have a structural impact on channel function; channel activity can be increased and decreased by amino acid substitutions with respect to the wt channel. In general, hydrophobic amino acids stimulate and polar amino acids inhibit channel function. However, similar amino acids such as Asp and Glu have very different effects on channel function; therefore, it is obvious that the yeast rescue data cannot simply be explained on the basis of a single property of the amino acid in this position. To explain the different results based on the physicochemical properties of the amino acids such as hydrophobicity, volume, etc., we tried to correlate yeast growth with several amino acid descriptors. However, none of the physicochemical parameters of the amino acids provided an obvious explanation for the yeast growth data (data not shown).

Chlorella *Virus-Encoded Kcvs Typically Have a Lys or Arg in the Same Position in TMD1.* The finding that Kcv_{PBCV-1} tolerates replacement of Lys with almost any amino acid contrasts with the high degree of conservation of basic amino acids in this position. An alignment of some viral Kcv-type channels (Figure 1A) shows that all but one of them has a Lys or an Arg in this position. (Note that K19 is equivalent to K29 of Kcv_{PBCV-1} in some of the *Chlorella* virus channel proteins.) Only channel Kesv, a K^+ channel from a distantly related algal virus, lacks a basic amino acid in or near this critical position. The conservation of a basic amino acid at this position suggests that a charge at this site is more important than expected from the experiments shown in Figure 2. In fact, channels from 40 additional *Chlorella* viruses homologous to Kcv_{ATCV-1} (n = 14), Kcv_{MT325}, (12) and Kcv_{PBCV-1}(14) have recently been sequenced, and all of them have a Lys or Arg in this position (ref 12 and unpublished results of J. L. Van Etten).

Neutralization of Lys in Two Additional Virus-Encoded K^+ Channel Proteins Inhibits Channel Function. Because of the conserved nature of basic amino acids in the Kcv channels, we conducted a similar series of amino acid replacements in the 82-amino acid Kcv_{ATCV-1} and the 95-amino acid Kcv_{MT325}. Previous experiments demonstrated that Kcv_{ATCV-1} and Kcv_{MT325} function when expressed in *Xenopus* oocytes. Kcv_{ATCV-1} is also functional in the yeast rescue assay.(20) Figure 3A shows yeast rescue experiments with K to A mutants in TMD1 of Kcv_{ATCV-1} and Kcv_{MT325}.

Yeast cells expressing Kcv_{ATCV-1}-K19A and Kcv_{MT325}-K19A grow on media with a high K^+ concentration, meaning that the channel is not deleterious for the cells. In contrast, growth tests on selective media indicate that neither of the mutants rescues the K^+ uptake deficient yeast mutants to the same extent as Kcv_{PBCV-1}-K29A (Figure 3A). However, a small amount of growth occurred under these conditions for both yeast mutants. To further examine this phenomenon, growth was tested with a higher yeast concentration and longer growth incubations. The results of these experiments indicate that the Kcv_{ATCV-1}-K19A and Kcv_{MT325}-K19A mutants exhibit some rescue capability and that neutralization of the charge does not render the channels completely inactive (Figure 3B). However, neutralization of this critical amino acid in these latter two channels has a more severe negative impact on channel function than it does for Kcv_{PBCV-1}.

The results described above suggest that Kcv_{PBCV-1} tolerates neutralization of the charged amino acid in TMD1 while the function of the other two Kcv channels is inhibited. To examine this observation, we measured the activity of a chimera of GFP with Kcv_{ATCV-1} or its mutants in HEK293 cells. The data in Figure 4 show a representative recording of mock-transfected HEK293 cells and a cell transfected with Kcv_{ATCV-1}::GFP. Like untransfected cells, the mock-transfected cells have the typical low conductance over a wide range of negative voltages. Cells transfected with Kcv_{ATCV-1}-wt respond differently to the standard voltage protocol. These cells have an elevated quasi-linear conductance at voltages between ~80 and +80 mV. At more hyperpolarized voltages, the current–voltage (I–V) relation shows a pronounced negative slope conductance. In this respect, the I–V relation of Kcv_{ATCV-1} is similar to that measured in *Xenopus* oocytes and the negative slope can be attributed to a fast gating of the channel at negative voltages.(20) The typical Kcv_{ATCV-1}-type I–V relation was seen in 6 of 13 cells, revealing positive expression of the channel. A similar fraction (~60%) of HEK293 cells transfected with Kcv_{PBCV-1}::GFP had characteristic Kcv channel activity.(15)

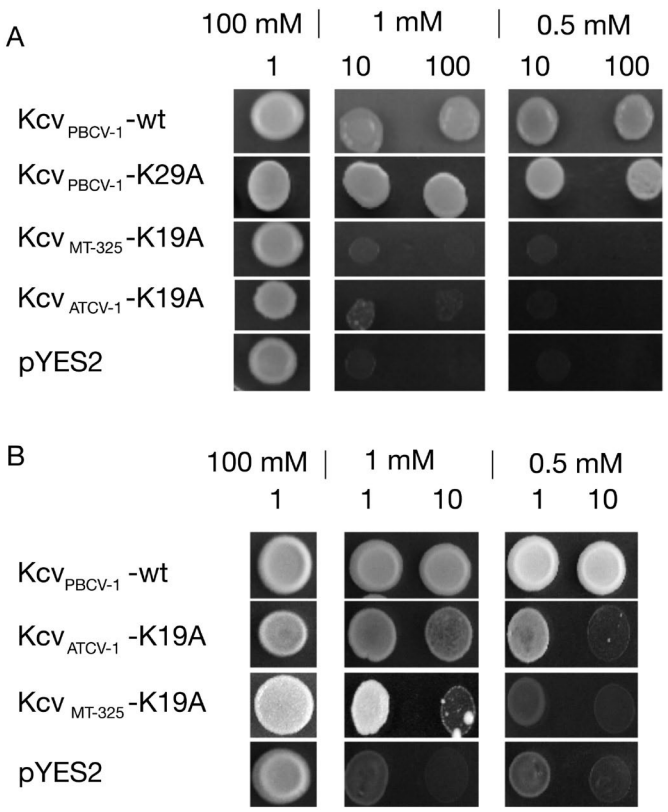


Figure 3. Replacement of Lys19 with Ala19 in Kcv_{MT325} and Kcv_{ATCV-1} does not rescue yeast growth. (A) Data show a complementation assay of a K^+ uptake deficient yeast expressing Kcv_{PBCV-1}-wt and mutants Kcv_{PBCV-1}-K29A, Kcv_{MT325}-K19A, and Kcv_{ATCV-1}-K19A with the empty vector as a control. (B) When the Kcv_{ATCV-1}-K19A and Kcv_{MT325} mutants are tested undiluted, some growth is detected under selected conditions. The yeast complementation assay was conducted as described in the legend of Figure 2.

To test the relevance of the charged amino acid at the lipid–aqueous interface, we expressed Kcv_{ATCV-1}-K19A in HEK293 cells. In the cells that expressed the mutant channel (n = 14), as judged by the GFP fluorescence, the current voltage relations were similar to those of nontransfected cells (Figure 4A–E). However, close scrutiny reveals that the Kcv_{ATCV-1}-K19A mutant generates a small characteristic current in addition to the background current of the mock-transfected cells. In the example of I–V relations shown in Figure 4E, a distinct difference occurs in the voltage window between 0 and −80 mV. This Kcv_{ATCV-1}-K19A specific current is also evident when we compare a large number of I–V relations from mock- and Kcv_{ATCV-1}-K19A-transfected cells (Figure 4G). Figure 4F shows the mean I–V curves of mock- and Kcv_{ATCV-1}-K19A-transfected cells. The ΔI–V relation (Figure 4F, inset) reveals features of the Kcv_{ATCV-1}-wt current with its negative slope conductance at negative voltages. The results of these experiments confirm the data from the yeast rescue experiments (Figure 3) in that the crucial Lys can be neutralized without completely abolishing channel activity. However, unlike the Kcv_{PBCV-1}-K29A situation, this neutralization has a strong negative effect on channel activity. This interpretation is consistent with the finding that transfection of HEK293 cells with the mutant Kcv_{ATCV-1}-K19R, which conserves the positive charge, produced larger currents. The currents recorded from HEK293 cells expressing the Kcv_{ATCV-1}-K19R mutant are similar to those measured with Kcv_{ATCV-1}-wt; only the mean current amplitudes of the mutant currents are smaller than those of the wild type (Figure 4E,G). In this case, the bulky, positively charged guanidino group of Arg may be less favorable than the terminal amino group on Lys.

Extracellular Snorkeling and Cytosolic Anchoring. Channels, which do or do not require a basic amino acid in TMD1, differ in their cytosolic N-termini (Figure 1). Those that require a basic amino acid in TMD1 (Kcv_{ATCV-1} and Kcv_{MT325}) lack a cytosolic N-terminus.(19, 20) Kcv_{PBCV-1} in contrast, which does not require a charged amino acid in this position, has a short cytoplasmic 12-amino acid N-terminus.(11) A reasonable assumption is that this cytosolic domain serves as an anchor for Kcv_{PBCV-1}, which supports the positioning of TMD1 in the membrane. Because of this additional support, a charged and snorkeling amino acid may not be required to maintain channel function.

To test this hypothesis, we analyzed the dynamics of Kcv_{PBCV-1} with Lys29 in a deprotonated and protonated form in its full-length form and in mutants in which the N-terminus was truncated. The truncated proteins resemble the structures of full-length Kcv_{ATCV-1} and Kcv_{MT325} channels. Relevant observables for characterizing the membrane position of various fragments are shown in Figure 5 and typical simulation snapshots in Figure 6. In all simulations, the initial setups of the mutants and the protonated wt channels were constructed by aligning the filter residues with the deprotonated wt model, which is perfectly centered in the 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) membrane. The shorter channels should therefore have a measurable drift toward the membrane center during the 100 ns simulation times unless specific sequence and structural features counteract this tendency. An important result of the simulations is that all the constructs, irrespective of the protonation state of Lys29 and irrespective of the length of the N-terminus, remain stable inside the membrane; the relative differences in their total center of mass position are very small (Figure 5D). Therefore, the truncation effect manifests itself predominantly in the form of internal protein distortions.

Notably, a slight drift of the filter region toward the membrane center occurs with the truncated models (Figure 5B), but not with the full-length, protonated form even though the crucial Lys residues snorkel, as expected, only in the latter case (Figure 1 and Figure 5A, gray line). In the wt and truncated mutants, all deprotonated Lys residues penetrate the membrane down to roughly the same depth (Figure 5A, red, green, and blue lines), indicating a strong localization force. A visible difference between full-length and truncated mutants that shows up early in the simulations concerns the center of mass of TMD1 (Figure 5C). Here, the shortest mutant (blue line), which lacks the entire cytosolic slide helix, shows a stretch toward the membrane center, while the Lys residues apparently stabilize the outer protein region relative to the lipid headgroups, as seen in Figure 5A. This process occurs irrespective of their protonation states because the Lys29 (or equivalent) residues maintain their relative membrane penetration depth. Both the deprotonated Lys and the protonated Lys take very distinct positions and are therefore both relevant for understanding the overall protein location. Apparently, the hinge that connects the Lys29 residue with the protein backbone represents a stable, protonation- and truncation-independent position, which controls the penetration depth of the protein region encompassing the filter. Hence, in response

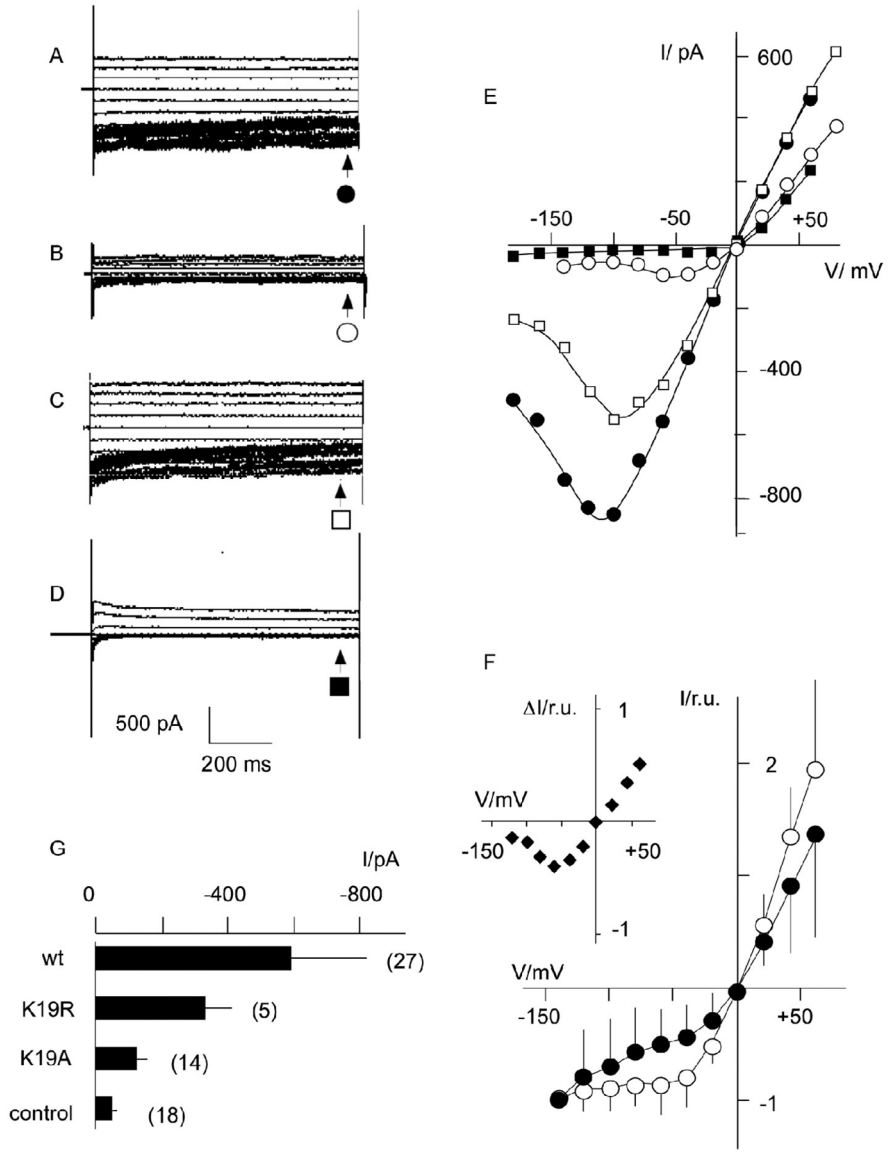


Figure 4. Electrophysiological measurements of HEK293 cells transfected with wt and mutant Kcv_{ATCV-1}::GFP. (A–D) Current responses of HEK293 cells expressing Kcv_{ATCV-1}-wt::GFP (A), Kcv_{ATCV-1}-K19A::GFP (B), Kcv_{ATCV-1}-K19R::GFP (C), or only GFP (D) to standard pulse protocol. (E) Corresponding current–voltage relationships. The symbols of the *I–V* relationships cross-reference with the symbols in the current traces. (F) Mean current–voltage relations of mock-transfected (●) and Kcv_{ATCV-1}-K19A::GFP-transfected cells (○) normalized to voltage at −140 mV. Data are means ± the standard deviation of at least 14 measurements. The inset shows the Δ*I–V* curve of mean data. (G) Mean currents at a reference voltage of −140 mV from at least five measurements. Currents were measured in a whole cell configuration with 100 mM K^+ in the bath by stepping cells from a holding voltage (0 mV) to test voltages between 60 and −160 mV.

to the strong localization forces acting on the extracellular region exhibited by Lys residues, the lack of a cytosolic anchor (provided by the laterally aligned slide helix in Kcv_{PBCV-1}-wt) leads to a significant distortion of the overall protein architecture. Distant protein regions apparently react and correspondingly rearrange. In the case of a drastic cytosolic truncation, a substantial stretch of TMD1 and a slight inward shift of the filter region are the obvious consequences.

On the other hand, the Lys-protonated full-length channel shows the most stable (i.e., only marginally drifting) set of all observables examined here, even in comparison with those of the deprotonated wt. Despite the stable Lys backbone positioning in the membrane for both the protonated and the deprotonated variants, the deprotonated form shows greater structural variability for Kcv_{PBCV-1}, although it conducts ions in contrast to the protonated structure because of a disintegrating TMD1(11) (note that we cannot rule out the possibility that even the protonated variant would conduct ions over longer simulation time scales). One can therefore expect that a snorkeling, protonated Lys is required in a situation where the lack of a cytosolic anchor would otherwise lead to potentially inactivating protein distortions, for example, Kcv_{ATCV-1}.

Discussion

Kcv-type channel proteins have a conserved Lys or Arg at the membrane–aqueous interface in the outer TMD. A cationic amino acid in this position is common in many TMDs, and it is

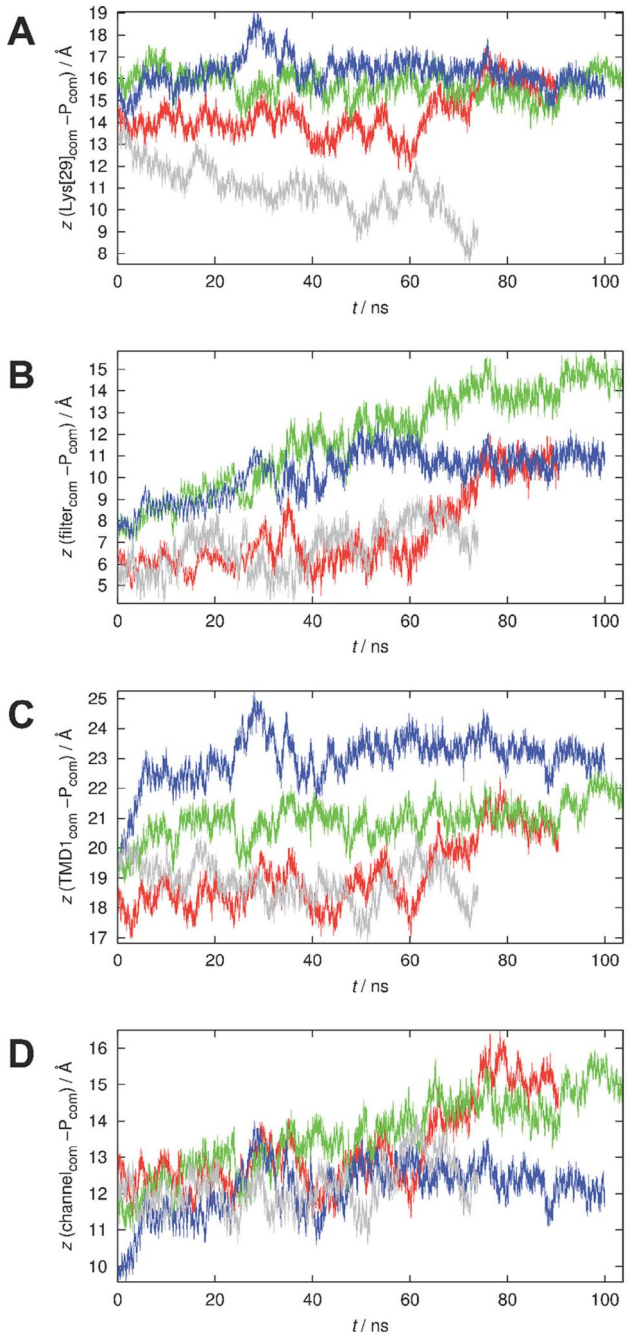


Figure 5. Time series of several dynamical quantities obtained from MD simulations. From top to bottom: *z* components, averaged over all monomers, of (A) the center of mass (com) of K29 (or corresponding sequence index in truncated channels), (B) the selectivity filter, (C) the N-terminal transmembrane domain, and (D) the entire channel protein, all relative to the average com of the lipid phosphate headgroups on the external solution-exposed side. Red denotes Kcv_{PBCV-1}-wt (deprotonated K29), gray Kcv_{PBCV-1}-wt (protonated K29), green Kcv_{PBCV-1}-Δ8 (deprotonated K29), and blue Kcv_{PBCV-1}-Δ14 (deprotonated K29). Note that increasing numbers correspond to an inward movement of the respective entities. The negative trend of the gray trajectory in panel A for example shows the outward movement of the protonated Lys29 for snorkeling. This is not the case for the deprotonated Lys (red), for which the trajectory remains stable. The seemingly drifting filter region of Kcv_{PBCV-1}-Δ8 (green line in the second plot) is the result of a structural breakdown of three of the four subunits; one stays at the positions of the other mutants.

thought that their long positively charged side chains snorkel. Experimental and computational experiments on model peptides indicate that as a consequence membrane proteins can adjust the orientation and positioning of their transmembrane helices in the bilayer.(4-7) These results provide information about the structure–function correlates of such basic amino acids in the context of small K^+ channel proteins. The conclusion is that the interfacial position of a basic amino acid in a TMD helix by itself is not sufficient for understanding their impact on structure and function. While the activity of two viral K^+ channels (Kcv_{ATCV-1} and Kcv_{MT325}) was severely inhibited by neutralization of the critical cationic amino acid, the function of a third channel protein, Kcv_{PBCV-1}, was not. Hence, snorkeling activity of the basic amino acid is required neither for Kcv_{PBCV-1} channel function nor for K^+ channel function in general. This view is supported by the fact that the structurally related algal virus-encoded Kesv channel lacks a basic amino acid in an equivalent position (Figure 1A).(14) Also, Kir channels, which have the same overall architecture as the viral channels, have no obligate requirement for basic amino acids in their TMD1. Only Kir2.1 from *Xenopus laevis* (GenBank entry ABQ44516.1) has an Arg in TMD1 at the membrane–water interface, which could snorkel.

The high degree of positional conservation of Arg/Lys in TMD1 of viral K^+ channels and the explanation for the different dependencies of these channels on a charged amino acid in the membrane–aqueous interface must be related to the small size of the viral channels and systematic differences in their architecture. A comparison of Kcv_{PBCV-1} with Kcv_{ATCV-1} and Kcv_{MT325} shows no appreciable differences in their amino acid sequences in the vicinity of their lysines or arginines, which could explain this difference. The amino acids that are separated from the Lys/Arg site by an α-helical turn have similar properties in all three Kcv channels, and there is no evidence of a specific interaction with the Lys. We recently reported that TMD1 in Kcv_{PBCV-1} is connected with the outer TMD via a π–π interaction between Phe30 and His83.(21) Inspection of the amino acids in TMD1, which are presumably close to the Lys in TMD1, also reveals no obvious differences among the three Kcv channels (Figure 1). Collectively, these findings exclude any local interactions as an explanation for the differential significance of the Lys in TMD1. On a more macroscopic scale, the different TMDs are similar; but still there are some differences in the composition of the hydrophobic amino acids. To test if these sequence differences have an impact on the hydrophobicity of the outer TMD, we calculated their hydrophobicity using the Wimley and White hydrophobic scale.(22) The hydrophobicity of the three K^+ channels for the outer TMD is reported in Figure 1C. There is no systematic difference between the channels, which explains their differential requirement for a charged amino acid in the TMD.

After differences in hydrophobicity had been excluded as an explanation, the most apparent systematic deviation in the channel proteins is the size and nature of the cytosolic N-termini. Only channel proteins that lack charged cytoplasmic N-terminal domains have a strong dependence on a cationic amino acid at the lipid–aqueous interface for snorkeling. One explanation for a stronger requirement for a charged Lys/Arg in this position is that a short/absent cytosolic N-terminus provides no anchoring for the TMD on the cytosolic side. This hypothesis is corroborated by the MD simulation data. Because Lys is in a uniform DMPC bilayer strongly localized in both the protonated and the deprotonated form (the former near the interface, the latter near the membrane center), the lack of an

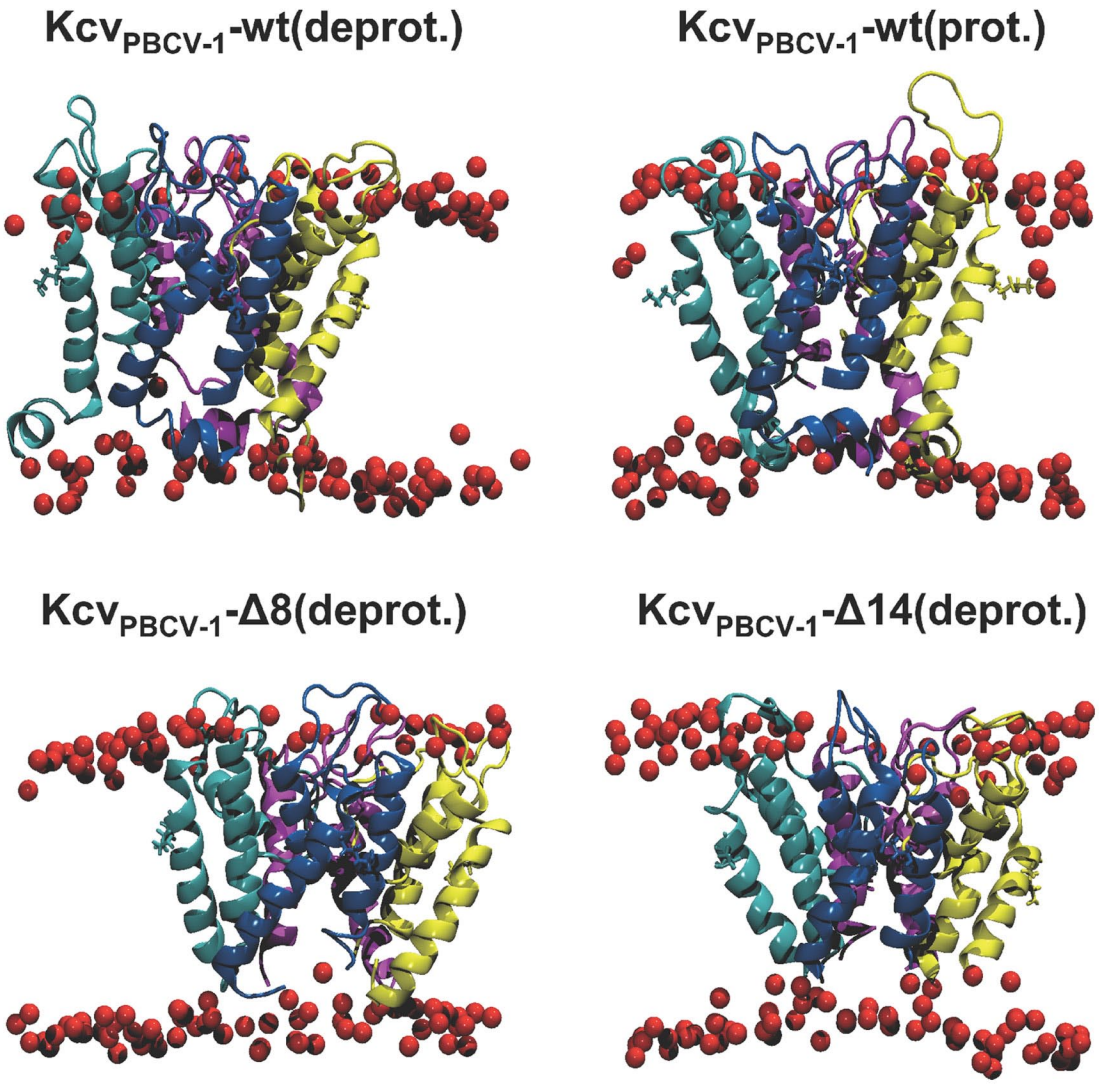


Figure 6. Cartoon representations of snapshots from MD simulations, each taken after 14.7 ns rigid filter runs for various wt and truncated mutants. Raw data described in refs 10 and 11 were used for the images shown in the top and bottom rows, respectively. Phosphate headgroups of the lipid molecules are represented as red balls, and the remaining species (lipid tails, water, and ions) were omitted for the sake of clarity. The individual channel monomers are shown in different colors. The residues of K29 (and corresponding Lys residues for truncated mutants) are shown explicitly.

N-terminal, anchoring slide helix leads to protein distortion. The membrane position of Kcv$_{PBCV-1}$-wt is apparently independent of the Lys protonation state, whereas that is not the case for the truncated, shorter forms. Therefore, it is reasonable to conclude that shorter channels such as Kcv$_{ATCV-1}$ and Kcv$_{MT325}$ need a stabilizing force at the exterior membrane side to penetrate more deeply without distortions that could potentially lead to inactivity. One such stabilizer could be provided by a protonated Lys equivalent in the interface regions that could shift the channel protein inward.

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