Residues in a Jellyfish Shaker-Like Channel Involved in Modulation by External Potassium

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Grigoriev, Nikita G., J. David Spafford, and Andrew N. Spencer. Residues in a jellyfish Shaker-like channel involved in modulation by external potassium. J. Neurophysiol. 82: 1740–1747, 1999. The jellyfish gene, jShak2, coded for a potassium channel that showed increased conductance and a decreased inactivation rate as [K+]out was increased. The relative modulator effectiveness of K+, Rb+, Cs+, and Na+ indicated that a weak-field-strength site is present. Cysteine substituted mutants (L369C and F370C) of an N-terminal truncated construct, (jShak2Δ2–38) which only showed C-type inactivation, were used to establish the position and nature of this site(s). In comparison with jShak2Δ2–38 and F370C, L369C showed a greater relative increase in peak current when [K+]out was increased from 1 to 100 mM because the affinity of this site was reduced at low [K+]out. Increasing [K+]out had little effect on the rate of inactivation of L369C; however, the appearance of a second, hyperbolic component to the inactivation curve for F370C indicated that this mutation had increased the affinity of the low-affinity site by bringing the backbone oxygens closer together. Methanethiosulphonate reagents were used to form positively (MTSET), negatively (MTSES), and neutrally (MTSM) charged side groups on the cysteine-substituted residues at the purported K⁺ binding site(s) in the channel mouth and conductance and inactivation kinetic measurements made. The reduced affinity of the site produced by the mutation L369C was probably due to the increased hydrophobicity of cysteine, which changed the relative positions of carbonyl oxygens since MTSES modification did not form a high-field-strength site as might be expected if the cysteine residues project into the pore. Addition of the side chain -CH2-S-S-CH3, which is similar to the side chain of methionine, a conserved residue in many potassium channels, resulted in an increased peak current and reduced inactivation rate, hence a higher affinity binding site. Modification of cysteine substituted mutants occurred more readily from the inactivated state confirming that side chains probably rotate into the pore from a buried position when no K ions are in the pore. In conclusion we were able to show that, as for certain potassium channels in higher taxonomic groups, the site(s) responsible for modulation by [K+]out is situated just outside the selectivity filter and is represented by the residues L369 and F372 in the jellyfish Shaker channel, jShak2.

INTRODUCTION

Various voltage-gated potassium channels are modulated by the external potassium ion concentration, [K+]out (Baukrowitz and Yellen 1995; Lopez-Barneo et al. 1993; Pardo et al. 1992; Safronov and Vogel 1996; Tseng and Tseng-Crank 1992). Recently we were able to demonstrate that a Shaker-like, high-threshold potassium channel (α-subunit), encoded by a gene jShak2 cloned from the jellyfish Polyorchis penicillatus, when heterologously expressed in Xenopus oocytes, also demonstrates strong modulation of an A-like current by [K+]out (Grigoriev et al. 1999a,b). Increasing [K+]out increases the potassium conductance of jShak2 and simultaneously decreases the inactivation rate. Both these effects are associated with C-type inactivation (Grigoriev et al. 1999a,b). A possible physiological role for such modulation is to compensate for the effect of potassium ions accumulating in extracellular space during repetitive firing and leading to a change in the potassium equilibrium potential. We were interested in examining the mechanism of this modulation by determining the site(s) responsible in the channel vestibule. Because this is an evolutionarily early channel, it is more likely to have retained the ancestral mechanisms, without superimposed specializations, than K⁺ channels from more derived phyla.

It is well established that the mouth of the aqueous pore of potassium channels as well as the selectivity filter are formed by the P-region (Doyle et al. 1998; Heginbotham et al. 1994; Lipkind et al. 1995), which is structurally conserved among most of the voltage-gated K⁺ channels (Chandy and Gutman 1994). C-type inactivation of Shaker channels appears to be associated with specific residues in the P-region (Baukrowitz and Yellen 1995; Lopez-Barneo et al. 1993). Mutation of threonine449 dramatically alters the rate of C-type inactivation of Shaker mutants and the channel’s sensitivity to changes in external potassium. The presence of charged residues such as lysine or glutamate at this site increases the channel’s sensitivity to [K+]out and the rate of C-type inactivation. The lipophilic residues, tyrosine or valine at position 449 render the channel insensitive to changes in [K+]out and cause slow inactivation (Lopez-Barneo et al. 1993). Despite the homologous position being occupied by phenylalanine, which is aromatic and lipophilic, jShak2 demonstrated fast C-type inactivation and a high sensitivity to [K+]out. jShak2 also carries leucine369 in a position occupied by methionine in most known voltage-sensitive channels. To elucidate the role of these residues in sensing [K+]out, we tested two jShak2 mutants: L369C and F370C.

METHODS

Molecular biology

All jShak2 mutants were constructed using cassette, PCR-based, site-directed mutagenesis as described previously (Grigoriev et al. 1999).
1997). Mutants were verified by sequencing in both directions using a Perkin-Elmer ABI 373A sequencer and an ABI Prism Dye-Terminator Cycle Sequencing Kit. Construction of the mutant jShak1Δ2–24 (jShak1T) was described by Jegla et al. (1995). Capped mRNAs were prepared by run-off transcription using a T7 mMessage mMachine kit (Ambion).

Whole cell, two-electrode recording from Xenopus oocytes

Xenopus oocytes were prepared and injected with mRNA as previously described (Grigoriev et al. 1997). From 1 to 5 ng of mRNA were injected in each oocyte using a volume of 50 nl. The amount of injected RNA was adjusted for each expressed channel type to minimize the experimental artifacts introduced by a high level of channel expression (Grigoriev et al. 1999a). Whole cell currents were recorded between 2 and 3 days after injection using a two-microelectrode voltage clamp (CA-1, Dagan Corporation). Cells were constantly microperfused either with a gravity-fed system or computer-controlled pneumatic syringe pumps when a stable perfusion volume (3 ml min⁻¹) was required, such as with the experiments using MTS reagents.

The potassium-free extracellular solution, \([K^+]_{\text{out}} = 0\), contained 100 mM NMG-Cl, 3 mM MgCl₂, 10 mM HEPES-acid adjusted to pH 7.5 with NMG-Cl; while the solution, \([K^+]_{\text{out}} = 100\) mM contained KCl 95 mM, MgCl₂ 3 mM, 10 mM HEPES¹⁻,K, pH 7.5. Intermediate concentrations of K⁺ were made by mixing these two solutions in the required proportion. The MTS reagents, MTSES, MTSET, and TMTM were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada) and were dissolved in extracellular solution at a concentration of 500 μM and kept on ice. Reservoirs were loaded with the appropriate MTS reagent solution immediately before each modification experiment. Tetraethylammonium (TEA), tetramethylammonium (TMA), tetrapropylammonium (TPA), and tetrabutylammonium (TBA) chloride salts were obtained from Sigma-Aldrich. Experiments were carried out at 20°C using a temperature controller TC-10 (Dagan Corporation, Minneapolis, MN).

Data acquisition and experimental control

All data acquisition and experimental control was through a Digidata 1200 (Axon Instruments, Foster City, CA) acquisition system running pClamp 6.1 software. For most of the recordings currents were elicited by depolarizing pulses from a holding potential of −80 to +65 mV, with a duration of 100 ms. Analysis and fitting of experimental data were done using the Clampfit program in pClamp 6.1 (Axon Instruments) and SigmaPlot 4.00 (SPSS, Chicago, IL). Fitting of \([K^+]_{\text{out}}\)-effect curves were done by using a combination of an equation similar to a Michaelis-Menten relationship, and a linear portion of the curve. Recovery from inactivation for L369C was slightly less (\(K_{\text{app}}\) around 1 mM, and the second component had \(K_{\text{app}}\) of 30 mM (Fig. 3D)). It is likely that the appearance of a second hyperbolic component and the disappearance of the linear component represents an increased K⁺ affinity of the low-affinity site, which, before mutation, is represented by the linear portion of the curve. Recovery from inactivation for F370C was slowed dramatically (>27-fold) relative to jShak2Δ2–38 (Fig. 3E).

We were able to use the substituted cysteine accessibility method (SCAM) (Akabas et al. 1994) to evaluate the accessibility of residues in positions 369 and 370. In addition, this method allowed us to examine their state-dependent reactivity using thiol-specific methanethiosulphonate reagents (MTS). Treatment of cysteine with these reagents enabled us to attach functional side groups through disulphide bonds. The reagent MTSET attached a group carrying positive charge, whereas the jellyfish Shaker channel jShak2 does not conduct in the absence of external potassium (Fig. 1A, inset) (Grigoriev et al. 1999a,b). Peak amplitude of jShak2 current is increased (termed the “amplitude effect”) and the inactivation rate decreased as \([K^+]_{\text{out}}\) is increased (Fig. 1). This modulatory effect was shown to be associated with a C-type inactivation mechanism (Grigoriev et al. 1999b). Other monovalent cations produced qualitatively similar effects on jShak2 current, but with substantially lower efficiency than potassium ions (Fig. 1A).

Their effectiveness at modulating both current amplitude and inactivation kinetics (Fig. 1B) could be ranked as follows: K⁺ > Rb⁺ > Cs⁺ > Na⁺. This order corresponds to the IVth Eisenman sequence for equilibrium ion exchange and may indicate the presence of a comparatively weak-field-strength binding site for monovalent cations (Eisenman 1961; Hille 1992). As for many K⁺ channels, Cs⁺ blocked jShak2 channels when applied to the cytoplasmic side of the membrane (data not shown), which ruled out the possibility that the sensor for external [K⁺] is associated with the selectivity filter in the pore of jShak2.

Inactivation of jShak2 channels is predominantly of the C-type, whereas N-type inactivation is very weak and can be completely eliminated by N-terminal sequence truncation (Grigoriev et al. 1999b). To observe C-type inactivation un-contaminated by N-type inactivation, P-region mutants (Fig. 2) were constructed in truncated jShak2Δ2–38. Both mutants, L369C and F370C, retained external potassium sensitivity (Fig. 3, A and B).

For L369C the peak current increased dramatically in response to elevation of the extracellular potassium concentration from 1 to 100 mM (Fig. 3A), which could be accounted for by the lower affinity of this mutant to potassium ions at low concentrations. The conductance versus \([K^+]_{\text{out}}\) curve for L369C was less steep (\(K_{\text{app}}\) increased 8-fold, from 2 to 16 mM) than for jShak2Δ2–38 (Fig. 3C). There was only a moderate decrease in the inactivation rate for L369C (Fig. 3, A and D) without any change in \(K_{\text{app}}\). The rate of recovery from C-type inactivation for L369C was slightly less (\(\tau = 150\) ms) than for Wild jShak2Δ2–38 (\(\tau = 415\) ms; Fig. 3E).

The potassium sensitivity of the amplitude effect in F370C remained virtually unchanged relative to jShak2Δ2–38 (Fig. 3C). Unlike L369C, F370C showed a very different sensitivity of inactivation to \([K^+]_{\text{out}}\) with two components appearing, the first had a \(K_{\text{app}}\) around 1 mM, and the second component had a \(K_{\text{app}}\) of 30 mM (Fig. 3D). It is likely that the appearance of a second hyperbolic component and the disappearance of the linear component represents an increased \(K^+\) affinity of the low-affinity site, which, before mutation, is represented by the linear portion of the curve. Recovery from inactivation for F370C was slowed dramatically (>27-fold) relative to jShak2Δ2–38 (Fig. 3E).
MTSES introduced a negative charge, and MTSM added an uncharged group. Two of the reagents, MTSET and MTSES, did not affect the peak current of $j_{Shak2}^{\Delta2-38}$, whereas MTSM treatment reduced current by 23% (Figs. 3F and 4A). We assume that the six endogenous cysteine residues located on membrane-spanning and cytoplasmic regions of $j_{Shak2}^{\Delta2-38}$ are inaccessible from the outside because the hydrophilic reagents MTSES and MTSET had no effect. However, the small inhibitory effect of MTSM could result from its lipophilicity (Fig. 3F).

Modification of F370C with MTSET and MTSM suppressed the current almost completely, whereas MTSES did not produce a strong effect on current amplitude (Fig. 3F) or inactivation rate (data not shown). MTSES treatment of F370C did not produce significant changes in potassium sensitivity while the residual currents that remained after F370C modification with MTSM and MTSET reagents were too small for reliable quantification of their potassium sensitivity (data not shown).

In contrast, both MTSM and MTSES treatment of L396C increased conductance (Fig. 4B) and slowed inactivation (Fig. 4C), whereas MTSET had the opposite effect. All three reagents modified the cysteine-substituted mutants within several seconds after initiating perfusion with MTS reagents. However, rates of return to the unmodified state were very slow, which allowed us to test the potassium sensitivity of channels that remained modified after removal of MTS reagents from the external solution.

The potassium sensitivity of L369C mutants was dramatically altered as a result of cysteine modification (Fig. 4, B and C). Positively charged MTSET reduced the potassium sensitivity of channel conductance, making $K_{app}$ five times greater (17 to 90 mM) when compared with unmodified L369C (Fig. 4B). This was accompanied by a slight overall decrease in the steepness of the $\tau[K^+]_{out}$ curve, which is most noticeable in the linear component of these curves (Fig. 4C). Modification of L369C by negatively charged MTSES and neutral MTSM increased the sensitivity of whole cell conductance to $[K^+]_{out}$ making the $K_{app}$ value similar to that for $j_{Shak2}^{\Delta2-38}$. The $K_{app}$ after modification by MTSES was 1.45 ± 0.3 mM (mean ± SE, $n = 5$), and by MTSM it was 1.0 ± 0.3 mM ($n = 5$); although this value for $j_{Shak2}^{\Delta2-38}$ was 2.1 ± 0.2 mM.

**FIG. 1.** Relative effectiveness of modulation of peak current and inactivation rates of $j_{Shak2}$ by $K^+$ and other monovalent cations. A: currents recorded in the presence of 5 mM of the indicated monovalent cation applied externally. *Inset:* current traces recorded at $[K^+]_{out} = 0, 1$, and 100 mM. For this and all other figures the stimulation protocol used was a holding potential of $-80$ mV and a stimulus of $+65$ mV for 100 ms. B: top graph shows a plot of the peak currents obtained in the presence of the indicated monovalent cations after normalizing to the peak current obtained with $[K^+]_{out} = 1$ mM, and the bottom graph is a plot of the time constant of inactivation of these currents versus the extracellular concentration of the corresponding cations. Data for B obtained from a minimum of 5 experiments for each tested monovalent cation.

**FIG. 2.** Aligned amino acid sequences of the presumed pore or P-region of fruit fly Shaker, $jShak1$, Wild $jShak2$, and the mutants $jShak2^{\Delta2-38}$ L369C, $jShak2^{\Delta2-38}$ F370C. The consensus sequence of the P-region in the Kv family of potassium channels was adapted from Chandy and Gutman (1994). Absolute conservation of amino acids is shown by uppercase letters, whereas lowercase indicates conservative substitutions. Amino acid residues that are not conserved are shown in bold.
MTSES and MTSM treatment also slowed inactivation (Fig. 4, A and C) and made inactivation more sensitive to potassium (Fig. 4C). None of these modification agents appreciably affected the rate of recovery from inactivation (data not shown).

We examined the sensitivity of L369C to various monovalent cations to determine if modification by MTSES altered the relative effectiveness of these ions in modulating K$^+$ current amplitude (Fig. 4E). Ions with atomic radii closer to that of potassium showed the greatest effect on peak current amplitude. Addition of a negatively charged group to L369C did not alter the order of the permeability sequence, but modification did alter the relative effect of these ions so that the sequence K$^+$ \geq$ \text{Rb}^+$ \geq$ \text{Cs}^+$ \geq$ Na$^+$ \geq$ Li$^+$ became K$^+$ \geq$ \text{Rb}^+$ \geq$ \text{Cs}^+$ \geq$ Na$^+$ \geq$ Li$^+$. Thus, this sequence shows a trend toward an Eisenman III sequence, which implies a weaker field-strength site than in the unmodified channel. This conclusion appears to contradict an obvious explanation that the increase in the apparent affinity of this mutant is a result of the stronger electrostatic interaction of K$^+$ with the negatively charged group acquired by cysteine$^{448}$ after modification with MTSES.

Liu et al. (1996) demonstrated that for Shaker channels, residues in homologous positions to methionine$^{448}$ and threonine$^{449}$ became preferentially available for modification in their inactivated state. Modification of both L369C and F370C was slow (from 3 to 5 M$^{-1}$s$^{-1}$) in the closed state (data not shown). However, the rate of modification was increased when the probability of channels being in an inactivated state was increased. This is seen in Fig. 4D, which shows that the rate of modification of L396C by MTSES using 10-ms depolarizing test pulses (5.5 ± 1.5 M$^{-1}$s$^{-1}$, n = 5) was indistinguishable from the rate in the closed state. However, long 100-ms pulses caused far more rapid (119.6 ± 6.5 M$^{-1}$s$^{-1}$, n = 6) modification. If MTSES interacted preferentially with channels in their open state, then one would expect a 5-fold increase (estimated by integration of current during the pulse) in the modification rate seen with 100-ms pulses relative to 10-ms pulses. Instead, we recorded a 20-fold increase in the rate. We also observed a somewhat slower rate of modification at high concentrations of [K$^+$] out (data not shown). The slow recovery from inactivation of F370C (Fig. 3C) did not allow us to use the same stimulus protocol as for L369C (>0.05 Hz), and when we used a lower frequency of stimulation we could not discriminate between the inactivated and open states. Nevertheless, we obtained qualitatively similar results for modification of cysteine$^{370}$, indicating that this...
residue became preferentially available in the open and/or inactivated states but not the closed state.

Introduction of the aromatic residue, phenylanine, in the P-region of Shaker channels (mutant T449F) makes it highly sensitive to TEA due to the formation of a bracelet of pore-lining aromatic residues (contributed by each subunit) in the channel mouth, which provides a structure that favors interaction with flat, symmetrical molecules such as TEA (Heginbotham and MacKinnon 1992). Administration of TEA to jShak2–38 channels caused partial channel block and a decrease in the inactivation rate (Fig. 5A), which can be explained by assuming that TEA in the channel mouth prevents potassium ions from passing through the pore while simultaneously preventing transition of the channel to an inactivated state (Grissmer and Cahalan 1989). As expected, the mutant (F370C), where phenylalanine had been replaced by cysteine at a homologous position, showed reduced sensitivity to TEA (Fig. 5B) when compared with the Wild, N-terminal deleted mutant (jShak2Δ2–38). Among all quaternary ammonium (QA) ions, TEA had the most noticeable effect on peak current and inactivation rate (Fig. 5C). The smallest ion, TMA, had no effect, whereas the larger ions, TPA and TBA, showed some blocking effect, but inactivation was affected minimally (Fig. 5C). TPA and TBA had no effect on current amplitude or inactivation rate at concentrations of 10 mM or less (data not shown). The sensitivity of jShak2 to TEA blockade ($K_d = 0.35$ mM) (Jegla et al. 1995) is lower than that reported for Shaker T449F ($K_d = 1.2$ mM) (Jegla et al. 1995) is lower than that reported for Shaker T449F ($K_d = 0.35$ mM), indicating that the relative positions of the aromatic rings in jShak2 are less favorable for the formation of a high-affinity receptor. Comparison of the blocking effects of other quaternary ammonium ions (TMA, TPA, and TBA) with TEA showed that this site is most specific for TEA (Fig. 5C). The smaller ion, TMA, had no effect, whereas the larger molecules, TPA and TBA, blocked at a lower efficiency. A similar profile of blocking by QA ions was reported for Shaker

**Fig. 4.** Modification of L369C with MTS reagents affects channel sensitivity to extracellular potassium and other monovalent cations. **A**: typical current traces in the presence of 1 mM $[K^+]_{out}$ when channels are modified by the addition of negative (MTSES), positive (MTSET), or neutral (MTSM) groups to cysteine $^{369}$ in the L369C mutant. Control traces are obtained from the unmodified L369C mutant. **B**: changes in potassium-dependent conductance. **C**: potassium dependence of inactivation that are associated with the current profiles shown in A. Data were obtained from 5 to 7 experiments. Solid lines result from fitting the experimental data by Eq. 1 except for the $g/[K^+]_{out}$ curve for MTSET modification, which was fitted by a single hyperbolic function (Eq. 3). **D**: modification of L369C with MTSES altered the relative sensitivity of the channel to monovalent cations. This plot shows the relative sensitivity of peak current to different monovalent cations normalized to the peak current at $[K^+]_{out} = 1$ mM vs. the atomic radius of each cation. The extracellular concentration of all monovalent cations was 1 mM ($n = 4–6$). **E**: plot of the normalized peak current recorded for the mutant L369C using stimulus pulses to +65 mV from a holding potential of 100 mV using durations of 100 ms (●) and 10 ms (●). Perfusion of oocytes with a solution containing 500 μM MTSES began at time 0 ($n = 8$).
T449F (Heginbotham and MacKinnon 1992), indicating that the relative positions of the four phenylalanine residues in the channel mouth of this mutant is similar to that for $jShak2$.

**DISCUSSION**

This study indicates that residues L$^{369}$ and F$^{370}$ participate in the formation of site(s) responsible for the modulatory effect of $[K^+]_{out}$ in an evolutionarily early voltage-gated potassium channel. Occupancy of these sites by potassium decreases the probability of C-type inactivation occurring by a “foot in the door mechanism” (Grigoriev et al. 1999b; Labarca and MacKinnon 1992; Lopez-Barneo et al. 1993). According to Eisenman’s theory (Eisenman 1961), the observed selectivity sequence for monovalent cations in $jShak2$ indicates that a low-field-strength binding site for $K^+$ is present at or close to the selectivity filter. A model of the pore suggested by Lipkind et al. (1995) shows that potassium ions interact with four carbonyl oxygens of the peptide backbone of the hairpin structures formed by four P-region sequences lining the pore. A recent study using X-ray analysis of the structure of the potassium channel pore from *Streptomyces lividans* clearly demonstrated that main chain carbonyl oxygen atoms line the selectivity filter (Doyle et al. 1998). The weak nature of the binding site is created by low energy interactions between permeable ions and oxygen atoms. The narrowest pore section formed by carbonyl oxygens occurs within the conserved GYG sequence (Heginbotham et al. 1994) and is the presumed selectivity filter (Fig. 6). Exterior to the selectivity filter, the vestibule becomes wider and the interaction of $K^+$ with other potential binding sites becomes weaker. The oxygen rings of the GDL sequence form a less narrow opening that presumably reduces affinity for $K^+$, but this site is able to hold the larger impermeable ion, Cs$^+$. The section of the pore formed by L and F has even less probability of interacting with potassium, or other monovalent cations and therefore may be a possible candidate for the sites with lowest affinity. The mutation L$^{369}$C produced a lower affinity site than in the Wild protein. If we assume that side chains project into the channel lumen, then this lowered affinity could be due to reduced steric hindrance of $K^+$ by the shorter side groups of cysteine. Alternatively, the lower hydrophobicity of cysteine alters interactions between residues in the hairpin structure ($\psi$ and $\phi$ angles of rotation of peptide backbone), which, in turn, changes the position of wall-forming carbonyl oxygens and decreases the strength of the potassium-oxygen interaction.

MTSES modification of side chains that project into the pore should provide a structure that forms a high-field-strength binding site because of the introduction of strong negative charge. This appears not to be the case because adding a strong binding site should alter both the order of the modulatory effect produced by different monovalent cations and produce a shift to a higher Eisenman sequence. However, the Eisenman sequence remained at IV, and even showed a trend toward the
IIIrd sequence, indicating a weakening of the purported site. These results enable us to rule out the possibility that the cysteine side chains of L369C project into the pore during ion passage. Therefore changes in the relative positions of carbonyl oxygens as a result of conformational changes in the hairpin remains the most plausible explanation for changes in sensitivity to $[\text{K}^+]_{\text{out}}$ produced by mutation L369C, as well as changes evoked by its modification using MTS reagents. It is interesting that modification of L396C with MTSM produces side chains with the structure, $\text{-CH}_2\text{-S-S-CH}_3$, which enhances the apparent affinity of the modulatory sites to potassium. This side-chain construct resembles the side chain of methionine, $\text{-CH}_2\text{-CH}_3\text{-S-CH}_3$, which is conserved in $\delta\text{Shak1}$ and many other potassium channels. It is possible that the presence of such side-chain structure is optimal for formation of high affinity potassium sites by placing backbone oxygens in positions that favor high-affinity binding. If it can be assumed that the side chains of C$^{369}$ and C$^{370}$ are able to interact with neighboring side chains, then the positioning of backbone oxygens also explains the differences noted in the modification profiles of L369C and F370C (Fig. 3F). For example, it is likely that the absence or presence of negatively charged D$^{368}$ in proximity to side chains of C$^{369}$ and C$^{370}$ will affect $\psi$ and $\phi$ angles of rotation of the peptide backbone, which, in turn, will change the relative positions of carbonyl oxygens. Pascual et al. (1995) also observed different profiles after modifying cysteines in positions homologous to L369C and F370C in Kv2.1 channels with MTS reagents carrying different functional groups. This hypothesis presents a paradox because the side chains of C$^{369}$ and C$^{370}$ should be buried to prevent interaction between them and potassium ions, yet they are accessible for modification by hydrophobic MTS reagents. This paradox can be resolved if we assume that side chains can rotate in the pore when potassium is absent. Guy and Durell (1994) have suggested stabilization of the pore lining by potassium ions. The buried position is stabilized when K$^+$ is in the pore, thereby preventing cysteines being modified by MTS reagents. Disruption of potassium flux allows side chains to rotate and exposes them to the water-filled pore, where they are available for MTS modification. This scenario also explains the preferential modification of L369C in the inactivated state. A similar explanation for state-dependent modification of residues in homologous positions was suggested by Liu et al. (1996) for fruit-fly $\delta\text{Shaker}$ channels.

The appearance of a hyperbolic component in place of the linear component of $\tau$ versus $[\text{K}^+]_{\text{out}}$ curves when cysteine replaces phenylalanine at position 370 can be explained by assuming that loss of the rigid phenylalanine side chain brings the backbone oxygens closer together in this section of the pore producing a higher affinity site for potassium.

Such a change in the architecture of the external channel vestibule probably allows for a more severe conformational change during C-type inactivation, which can explain the drastic decrease in the rate of recovery from inactivation when cysteine replaces phenylalanine at position 369.

In conclusion we have been able to show that the modulatory effect of external K$^+$ is likely to be evolutionarily ancient and that the mechanism of K$^+$ binding to a specific site(s) just exterior to the selectivity filter so as to interfere with C-type inactivation has been conserved. Selection for different amino acids at this homologous site, leucine$^{369}$ and phenylalanine$^{370}$ in the case of $\delta\text{Shak2}$, presumably depended on the requirement for operating in different external K$^+$ regimes and on the variability of these regimes.

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