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 modulatory 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, (Shak2Δ2–38) 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, resulted in most potassium channels, resulting 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 F³⁷⁰ 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 threonine³⁴⁹ 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.

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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 MgCl\(_2\), 10 mM HEPES, pH 7.5 with NMG-Cl; while the solution, [K\(^+\)]\(_{\text{out}}\) = 100 mM contained KCl 95 mM, MgCl\(_2\) 3 mM, 10 mM HEPES\(\cdot\)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 MTSM were obtained from Toronto Research Chemicals (Toronto, Ontario, Canada) and were dissolved in extracellular solution at a concentration of 500 \(\mu\)M and kept on ice. Reservoirs were loaded with the appropriate MTS reagent solution immediately before each modification experiment. Tetraethylammonium (TEA), tetramethylammonium (TEA), 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 \(-80\) 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 (\(\tau = 150\) 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).

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 MTS binds through a group carrying positive charge, whereas...
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 \(jShak2\) 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 \(jShak2\) 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 \(jShak2\Delta2–38\). The \(K_{app}\) value for \(jShak2\Delta2–38\) 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 \(jShak2\Delta2–38\) was 2.1 ± 0.2 mM.
FIG. 3. Sensitivity of the cysteine-substituted mutants L369C and F390C to external potassium. A and B: typical current traces obtained using mutants L369C and F390C, respectively, at $[\text{K}^+]_{\text{out}} = 1$ and 100 mM. C: plot of normalized conductances of L369C (●) and F370C (●) vs. the extracellular potassium concentration. There is a change in the scale of the horizontal axis at 20 mM. D: plot of the inactivation time constants determined for L369C (●) and F370C (●) vs. the extracellular potassium concentration. Experimental data for L369C and F370C on C and D were fitted with Eq. 1, except the $t[\text{K}^+]_{\text{out}}$ curve for F370C (D) was obtained by fitting experimental data with an equation containing 2 hyperbolic functions (Eq. 2). Dashed lines result from fitting experimental data with Eq. 1 obtained for jShak2Δ2–38 in the previous study (Grigoriev et al. 1999b). The number of experiments used to generate the graphs in C and D was from 6 to 8. E: plot of the time course for recovery from inactivation using L369C (●) and F370C (●) compared with Wild jShak2Δ2–38 (---). The number of experiments used to generate the graphs in E was from 6 or 7. Recovery of channels from inactivation was determined by using a 2 pulse protocol (P1 and P2 were 300 ms pulses to ±65 mV applied from a holding potential of ±80 mV). Experimental data for L369C and F370C were fitted by a single exponent (—). The dashed line results from fitting experimental data with a single exponent obtained for jShak2Δ2–38 in a previous study (Grigoriev et al. 1999b). F: effect of MTSX treatment on the current amplitude of L369C and F370C. Bars represent the normalized peak current amplitudes as a result of modification of Wild jShak2, L369C, and F370C mutants with MTSES (black), MTSET (light gray), and MTSM (dark gray) reagents. The number of experiments used to generate the bar graph in F was from 5 or 8.

(n = 7). 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 $K^+ \approx \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ \approx \text{Li}^+$ became $K^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ > \text{Li}^+$ (data not shown). The slow recovering 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

these...
residue became preferentially available in the open and/or inactivated states but not the closed state.

Introduction of the aromatic residue, phenylalanine, 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 jShak2D2–38 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 on either peak current or inactivation, 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 ($K_d = 1.2$ 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...
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 \textit{jShak2}.

**DISCUSSION**

This study indicates that residues L369 and F370 participate in the formation of site(s) responsible for the modulatory effect of \([K^+]_{\text{out}}\) on 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 \textit{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 \textit{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 L369C 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
**REFERENCES**


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**Fig. 6.** Diagram of the jShak2 potassium channel and sequences participating in formation of the selectivity filter and the vestibule of the pore. Letters correspond to the amino acid residues from 2 opposing α-subunits forming the channel pore. Two hydrated potassium ions are depicted at the external mouth of the pore. Solid gray circles represent backbone oxygens forming several rings. The narrowest ring is the selective filter for K⁺, whereas the other rings form potential potassium binding sites with their affinity for potassium ions being determined by the distance between opposite oxygen atoms. The positions of the letters represent the position of the side chain in respect to the water-filled channel pore. Gray hexagons symbolize rings of aromatic residues. Negatively charged aspartic acid residues (D) are depicted with minus signs. Water-filled channel pore. Gray hexagons symbolize rings of aromatic residues. Negatively charged aspartic acid residues (D) are depicted with minus signs. Water-filled channel pore. Gray hexagons symbolize rings of aromatic residues. Negatively charged aspartic acid residues (D) are depicted with minus signs. Water-filled channel pore. Gray hexagons symbolize rings of aromatic residues. Negatively charged aspartic acid residues (D) are depicted with minus signs. Water-filled channel pore. Gray hexagons symbolize rings of aromatic residues. Negatively charged aspartic acid residues (D) are depicted with minus signs.


