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Changes in activation gating of I_{sK} potassium currents brought about by mutations in the transmembrane sequence

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Abstract Expression of the rat kidney I_{sK} protein in *Xenopus* oocytes produces slowly-activating potassium channel currents. We have investigated the relationship between structure and function of the single putative membrane-spanning domain using site-directed mutagenesis. Six mutants were constructed in which consecutive individual amino acids (53 to 58) of the transmembrane region were substituted by cysteine. Expression of four of these mutants in *Xenopus* oocytes resulted in currents which were similar to wild-type. However, for one mutant (position 55) activation curves were shifted in a hyperpolarising direction and for another mutant (position 58) activation curve were shifted in a depolarising direction. This suggests that the hydrophobic phenylalanine residues at positions 55 and 58 may play a critical role in I_{sK} activation gating. This spacing of functional amino acids at every third residue may indicate an α-helical conformation for the membrane-spanning domain of I_{sK}. Furthermore, these results also indicate that one face of the helix may represent a region of subunit association.

Key words: Potassium channel; Delayed rectifier

1. Introduction

Potassium channels play an essential role in the generation of electrical responses of all excitable cells [1]. Different classes of potassium channel protein have been categorised by their molecular structure. One type, exemplified by the delayed rectifier potassium channel, consist of proteins which have a relatively large molecular weight (up to around 100 kDa) and possess six putative transmembrane segments with a characteristic H5 pore-forming region [2]. Recently, several inwardly rectifying potassium channels have also been sequenced which appear to be rather similar to this type in structure except that they only seem to possess two putative transmembrane segments [3]. Much attention has been focused on the above channel types which has widened our insight into their structure–function relationships [4]. The subject of this study, however, is the further type of potassium channel known as I_{sK} or minK which has a smaller molecular weight (around 15 kDa), possessing only around 130 amino acids [5]. Much less has been reported concerning the relationship between structure and function for this channel type.

Hydropathy analysis of the amino acid sequence of I_{sK} from rat kidney suggests a single putative transmembrane sequence and current models predict an extracellular amino-terminal domain and an intracellular carboxyl-terminal domain [5]. Expression of I_{sK} in *Xenopus* oocytes [5] and in HEK293 cells [6] has been shown to induce a very slowly-activating voltage-dependent potassium current with no inactivation. It is unclear, however, how the protein is able to evoke potassium currents. Two mechanisms have been proposed. It has been suggested that several I_{sK} subunits may aggregate together to directly form a pore-containing structure [7]. An alternative proposal is that I_{sK} itself is not a channel forming protein but acts as a modulator of endogenous oocyte channels [8,9].

In this study we have investigated the functional role of a region of the putative transmembrane domain of I_{sK} by constructing several site-directed mutants, in which individual amino acid residues have been substituted by cysteine, followed by expression in *Xenopus* oocytes.

2. Materials and methods

2.1. Preparation of I_{sK} mutant RNA

To the C-terminus of the I_{sK} cDNA in pGEM2, a hexahis tail was added by recombinant PCR (to be described elsewhere). The I_{sK}-His_6 cDNA was subcloned into pKS Bluescript and from the resultant construct, pKS I_{sK}-His_6, cRNA was transcribed using T7 RNA polymerase (MEGAscript, Ambion). Site-directed mutagenesis was performed using the method of Kunkel [10].

2.2. Oocyte preparation and electrophysiology

Dumont stage V or VI *Xenopus* oocytes were prepared and injected with mutant or wild-type RNA (25–50 ng) as previously described [11] and maintained at 19°C in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris-HCl (pH 7.6), 10 mg/l penicillin, 10 mg/ml streptomycin).

Membrane currents were recorded from oocytes 2–4 days following RNA injection using two-electrode voltage-clamp (Axoclamp 2A) at room temperature (22–24°C). Electrodes were filled with 3 M KCl and resistances were 1–5 MΩ and 0.6–1 MΩ for the voltage and current electrodes respectively. Oocytes were held in a 200 μl chamber and were continually perfused (2 ml/min) with Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2). Membrane potential was held at − 80 mV and 10s duration depolarising steps were applied in 10 mV increments at an interval of 30 s in order to construct current–voltage relationships. A series of 10–20 hyperpolarising steps of 10 mV were also applied to allow leak subtraction (see [11]). Currents were sampled to hard disk at 50 Hz using a CED 1401plus, and analysed off-line using CED voltage clamp software. Normalised conductance curves were constructed for each oocyte and fitted by a least-squared method to a Boltzmann equation, $G/G_{max} = 1/(1 + \exp[ze(V_{0.5} - V_{test})/kT])$, where z is the effective gating valance, e is the elementary charge, $V_{0.5}$ is the potential for half maximal activation, V_{test} is the test potential, k is the Boltzmann constant and T is the absolute temperature. Values for the oocyte resting membrane potential (V_m) and cell input resistance (R_m) were also obtained in 'Bridge' mode. Data are expressed as means ± standard error of the mean (S.E.M.) and statistical comparisons between control and test data were made using a Student's t-test. Statistically significant results were taken as $P < 0.05$.

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     N_  45        50        55        60        65    _C
WT-IsK   A L Y I L M V L G F F G F F T L G I M L S Y I
G53C     - - - - - - - C - - - - - - - - - - - - - - -
F54C     - - - - - - - - C - - - - - - - - - - - - - -
F55C     - - - - - - - - - C - - - - - - - - - - - - -
G56C     - - - - - - - - - - C - - - - - - - - - - - -
F57C     - - - - - - - - - - - C - - - - - - - - - - -
F58C     - - - - - - - - - - - - C - - - - - - - - - -
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Fig. 1. Amino acid sequence of putative transmembrane region of rat kidney I_{sK} showing amino acids at positions 53–58 at which cysteine substitutions were made.

3. Results

As described above, site-directed mutagenesis was used to construct six mutant forms of rat kidney I_{sK} in which six consecutive amino acid residues in the centre of the putative transmembrane sequence (53 to 58) have been substituted by cysteine. The positions of these mutations and the nomenclature used are shown in Fig. 1. Injection of RNA encoding for each of these mutants or for wild-type I_{sK} into *Xenopus* oocytes resulted in very slowly-activating, voltage-dependent outward currents, characteristic of wild-type I_{sK} currents reported by others [5]. Typical current traces at test potentials of − 30 mV and + 30 mV are shown in Fig. 2 for wild-type I_{sK}, F55C and F58C along with current–voltage relationships averaged from several cells. The current–voltage relationships appeared to be shifted in a hyperpolarising direction for F55C and in a depolarising direction for F58C in comparison to the wild-type I_{sK} curve. The current–voltage relationships for the other four mutants, on the other hand, were similar to wild-type curves (data not shown). In order to investigate this apparent effect on the voltage-dependence of activation further, normalised conductance curves were constructed for each cell and fitted to a Boltzmann relationship (see section 2). Typical fits are shown in Fig. 3 for cells expressing wild-type I_{sK}, F55C and F58C. These fits allowed an estimation of $V_{0.5}$, the potential at which currents were half-maximally activated and z, the gating valence. The mean values found for all six mutants and for wild-type I_{sK} channel currents are shown in Table 1. As expected from the apparent shifts seen in the current–voltage relationships, $V_{0.5}$ was significantly shifted for mutants F55C and F58C in comparison to $V_{0.5}$ for wild-type conductance curves, indicating a shift in activation gating. For F55C, the shift was approximately 23 mV in the hyperpolarising direction, while for F58C the shift was approximately 18 mV in the depolarising direction. For the other four mutants (G53C, F54C, G56C and F57C), $V_{0.5}$ was unchanged. The gating valence, z, was not significantly altered for any of the mutants.

Expression of exogenous potassium channels in *Xenopus* oocytes has been shown to decrease cellular resting membrane potential [12]. We investigated whether I_{sK} was capable of producing this decrease and if the shift in activation gating observed above for mutants F55C and F58C influenced this. Table 2 shows the mean values of resting membrane potential (V_m) and cell input resistance (R_{in}) for oocytes injected with each mutant, for wild-type I_{sK} channels and for oocytes not injected with RNA. None of the various RNA injections used caused any significant change in R_{in} in comparison to non-injected oocytes. In our hands, V_m for non-injected oocytes was around −33 mV. Expression of wild-type I_{sK} caused a decrease in V_m of around 8 mV. A similar decrease was also seen in mutants G53C, F54C, G56C and F57C. V_m for mutants F55C and F58C, however, differed significantly from that of wild-type I_{sK}, being shifted in the same directions as seen for $V_{0.5}$ (Table 2). This therefore provides further support for a shift in activation gating in these two mutants.

4. Discussion

We have demonstrated that substitution by cysteine of the phenylalanine residues at position 55 or 58 in the putative transmembrane domain of the rat kidney I_{sK} potassium channel results in a shift in channel activation gating. The activation

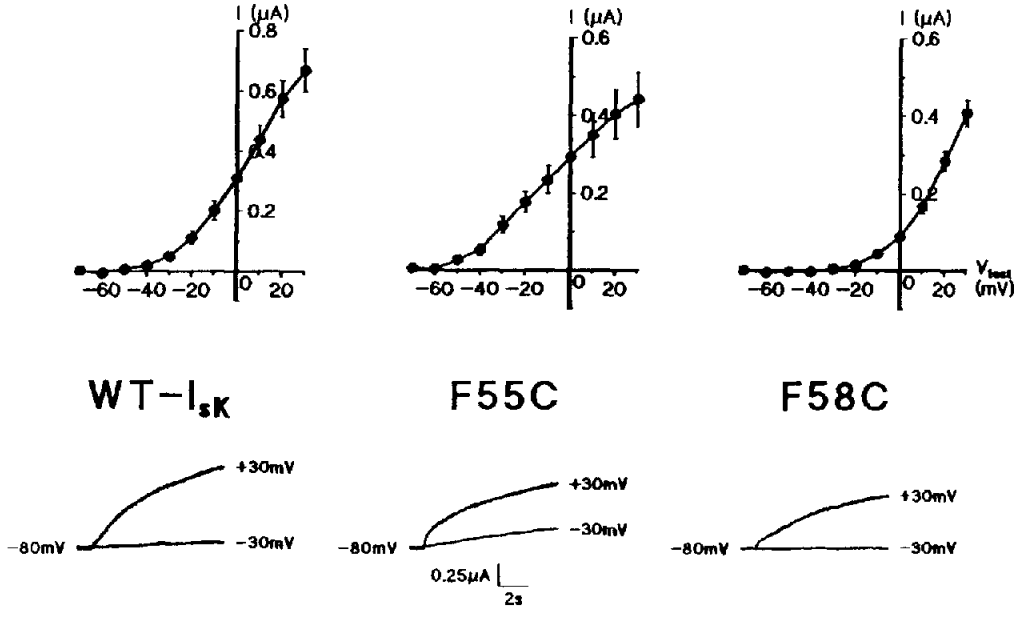

Fig. 2. Mean current–voltage relationships measured for oocytes injected with RNA encoding wild-type I_{sK} (upper left, n = 16 oocytes), mutant F55C (upper middle, n = 14 oocytes) and mutant F58C (upper right, n = 14 oocytes). Typical current traces are also shown for single oocytes injected with RNA encoding wild-type I_{sK}, F55C and F58C at test potentials of −30 mV and +30 mV.

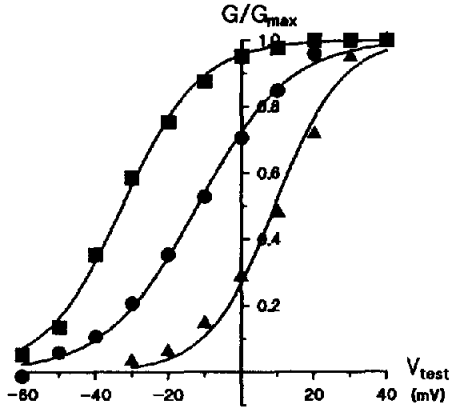

Fig. 3. Typical normalised conductance curves for single oocytes injected with RNA encoding wild-type I_{sK} (●), F55C (■) and F58C (▲) fitted using a Boltzmann relationship (see section 2).

curve was markedly shifted by around 23 mV in a hyperpolarising direction for F55C and by around 18 mV in a depolarising direction for F58C when compared to wild-type I_{sK}. Shifts in the same directions were also seen in the measured resting membrane potential for the oocytes.

I_{sK} currents have very slow activation kinetics which do not reach a steady-state even when during depolarising pulses of very long duration are applied [13]. We found that we could not accurately fit extrapolated steady-state currents and slow time constants to the kinetics of our current traces with any consistency. Hence, the current amplitude values used in this study were measured at the end of a ten second pulse rather than estimating extrapolated steady-state amplitude. Because of this we cannot completely rule out the possibility that mutations F55C and F58C may produce only an apparent shift in the activation curves by simply altering the kinetics of activation. This, however, is unlikely because the resting membrane potential was also shifted in a parallel manner.

Both residues at positions 55 and 58 are phenylalanines, but it does not appear that substitution of phenylalanine residues per se by cysteine produces shifts in activation because expression of both F54C and F57C produced no such effect. It is therefore likely that the precise topological positions that the residues occupy influence their degree of involvement in the gating process. It has been previously demonstrated that substitution of a leucine residue at position 52 by isoleucine causes a negative shift in activation whilst substitution of the same residue by alanine causes a positive shift in activation [14,15]. A critical role for the leucine at position 52 has also been demonstrated in experiments measuring currents from a synthetic peptide form of I_{sK} reconstituted in lipid bilayers. In the latter experiments, substitution of this residue by cysteine resulted in an inactive peptide [16]. Combined with our data, this suggests that the amino acids at every third position (at least over this region of the transmembrane domain) play an important role in activation gating.

At present it is not certain whether the I_{sK} transmembrane domain is α-helical or β-strand in conformation, although there is some evidence for an α-helical conformation [8,16]. Our data may support this because, unlike a β-strand conformation, an α-helical conformation would align residues 52, 55 and 58 along one face of the helix. We may speculate that the phenylalanines at positions 55 and 58 (and the leucine at position 52) of I_{sK} may form a region of interaction either between individual homomultimeric subunits forming a pore or between an I_{sK} subunit and an endogenous channel protein. Mutation of the residues at such a region would be expected to perturb activation of I_{sK} channels as seen here. As the valence of gating was not significantly altered it can be concluded that these mutations stabilise certain structural conformations of the channel [1,17]; mutation F55C stabilising an open state of the channel and mutation F58C stabilising a closed state.

It has been proposed that I_{sK} activates endogenous potassium channels via a critical C-terminal region and that the transmembrane region may be responsible for only modest changes is pore property [8]. In the present experiments, perturbations of the transmembrane structure have produced quite marked changes in channel function. It is unlikely that our mutations would significantly affect the structural conforma-

Table 1
Mean values for the half-activation voltage ($V_{0.5}$) and the gating valence (z), measured for wild-type I_{sK} (control) and each of the six cysteine mutants (see section 2)

	WT-I_{sK}	G53C	F54C	F55C	G56C	F57C	F58C
$V_{0.5}$ (mV)	−7.5 ± 0.7	−9.0 ± 0.6	−1.8 ± 0.6	−31.6 ± 0.9*	−4.9 ± 0.5	−4.4 ± 0.5	+10.9 ± 1.4*
z	2.58 ± 0.07	2.11 ± 0.04	2.46 ± 0.04	3.24 ± 0.23	2.24 ± 0.04	2.38 ± 0.05	2.84 ± 0.22
n	16	16	17	14	17	17	15

n represents the number of oocytes and $^*P < 0.05$ as compared with the wild-type.

Table 2
Mean values for the measured resting membrane potential (V_m) and the cell input resistance, measured in oocytes injected with RNA encoding wild-type I_{sK} (control), each of the six mutants and for oocytes not injected with RNA

	No RNA	WT-I_{sK}	G53C	F54C	F55C	G56C	F57C	F58C
V_m (mV)	−32.9 ± 1.5*	−39.7 ± 1.1	−42.4 ± 1.2	−38.7 ± 1.0	−54.9 ± 1.8*	−40.8 ± 1.5	−38.8 ± 1.2	−33.0 ± 1.1*
R_{in} (Mohm)	1.10 ± 0.06	1.15 ± 0.05	1.13 ± 0.06	1.11 ± 0.05	1.09 ± 0.07	1.13 ± 0.06	1.11 ± 0.08	1.05 ± 0.04
n	36	46	36	39	42	37	38	43

n represents the number of oocytes and $^*P < 0.05$ as compared with the wild-type.

tion of the C-terminus, suggesting that, rather than playing a modest role, the transmembrane region may play a critical role in I_{sK} function.

In summary, we have shown that certain residues of the transmembrane region of I_{sK} may play a role in activation gating of the potassium channel currents expressed in *Xenopus* oocytes. These functionally important amino acids appear to be positioned at every third residue, supporting a structural model for I_{sK} in which the transmembrane domain is α-helical.

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