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Changing Val-76 towards Kir channels drastically influences the folding and gating properties of the bacterial potassium channel KcsA

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ABSTRACT

All K^+-channels are stabilized by K^+-ions in the selectivity filter. However, they differ from each other with regard to their selectivity filter. In this study, we changed specific residue Val-76 in the selectivity filter of KcsA to its counterpart Ile in inwardly rectifying K^+-channels (Kir). The tetramer was exclusively converted into monomers as determined by conventional gel electrophoresis. However, by perfluoro-octanoic acid (PFO) gel electrophoresis mutant channel was mostly detected as tetramer. Tryptophan fluorescence and acrylamide quenching experiments demonstrated significant alteration in channel folding properties via increase in hydrophilicity of local environment. Furthermore, in planar lipid bilayer experiments V76I exhibited drastically lower conductance and decreased channel open time as compared to the unmodified KcsA. These studies suggest that V76I might contribute to determine the stabilizing, folding and channel gating properties in a selective K^+-channel.

1. Introduction

Many membrane proteins are active as stable oligomers. The oligomerization of these complexes is crucial for their function as they, e.g., contribute to the control of ion flow, cell volume, release of hormones and neurotransmitters [1,2]. A K^+-channel from the soil bacteria Streptomyces lividans named KcsA [1], a homotetramer made up of identical 160-amino acid subunits, was the first of such complexes to be solved [3]. The X-ray structure showed the assembled tetramer with 4-fold symmetry around a central pore. Each subunit contributes its C-terminal transmembrane α-helix to the lining of the pore, while the other (N-terminal) helix faces the lipid membrane [2–4].

Like KcsA, inwardly rectifying K^+-channels (Kir's) are also 2-transmembrane (TM) proteins that have two segments, M1 and M2, which flank a P segment. The latter portion of P form a series of K^+ binding sites and thus determines the selectivity of the pore [5,6]. Kir's have numerous functions including modulation of electrical activity of cardiac and neuronal cells, insulin secretion, and epithelial K^+ transport [5–7]. Like KcsA, Kir channels also assemble as tetramers and may be either homo- or heteromeric channel proteins [7,8].

Previous mutational studies using various eukaryotic channel genes, as well as analysis of the channel activity led to the conclusion that the GYG motif is necessary to determine K^+ ion selectivity [9]. Furthermore, mutant proteins carrying single mutations within the pore region of KcsA did not at all or only to a very small degree assembled as tetramers and lacked channel activity [10,11]. Thus, the simplicity and the ease of expression in *E. coli* make KcsA a good model protein to study channel gating and oligomerization in a selective K^+-channel.

All K^+-channels are believed to be stabilized by K^+-ions in the selectivity filter [4–6]. KcsA is an extremely stable tetramer in a wide range of detergents, even in SDS [4]. However, Kir channels do not form stable tetrameric complexes and are exclusively detected in monomeric forms on SDS-gel [12,13]. This raises the possibility that KcsA and Kir channels differ in their intrinsic stability. However, the stabilizing differences among these channels are not known. In the present study, we checked the hypothesis that selectivity filter might contribute to such differences among KcsA and Kir channels. Site-specific mutation was performed to change a critical residue in the selectivity filter of KcsA towards Kir channels (Val-76-Ile). By combining biochemical and biophysical techniques we determine as how V76I mutation affect channel oligomerization, folding and gating properties of a K^+-channel KcsA.

2. Materials and methods

2.1. Mutagenesis, expression and purification

Site-directed mutagenesis was performed using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA) as described previously [14]. Proteins were expressed with a C-terminal hexahistidine (6X His) tag from pQE60-KcsA in Escherichia coli strain BL-21 (DE3). The solubilized membranes in 40 mM DM were incubated with pre-

Abbreviations: SDS, sodium dodecylsulphate; LDS, lithium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate; PFO, perfluoro-octanoic acid; Trp, tryptophan; WT, wild type; DM, decyl-β-D-maltoside; Ni^{2+}-NTA, nickelnitriloacetic acid; BSA, bovine serum albumin.

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washed Ni^{2+}-NTA agarose beads for 30 min at 4 °C. The bound His-tagged proteins were eluted with 500 mM imidazole pH 7.5 and 10 mM DM. The proteins were purified with a yield of 1–2 mg/l culture. The purity of proteins was assessed by SDS–PAGE. The presence of DM precluded the use of the Bradford assay and therefore the protein concentration was assessed by SDS gel after staining with Coomassie Blue, using a standard of BSA.

2.2. Stability assay by LDS/PFO–PAGE and immunoblotting

To facilitate detection of oligomeric forms of proteins, electrophoresis was performed at low temperature (4–8 °C) as described previously [15]. The His-tagged proteins were detected with a monoclonal anti-his_6-tag antibody. For LDS electrophoresis setups, gels, and buffers were chilled before use and cooled continuously throughout each run. Gels were run at 120 V until the blue dye-front reached the edge of the gel. Protein was detected by staining with Coomassie Brilliant Blue G-250. The His-tagged KcsA mutants were detected with a monoclonal anti-his_6-tag antibody (1:1000 dilution, Qiagen) in PBS containing Tween-20. After washing, the immunoblots were incubated with a secondary horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000, Sigma). Immunoreactive proteins were visualized by chemiluminescent ECL detection reagent (Amersham Biosciences). The method employed for PFO–PAGE was similar to that employed for LDS/SDS–PAGE as described previously [13].

2.3. Preparation of liposomes and protein reconstitution

Small unilamellar vesicles (SUVs) were prepared from *E. coli* total lipid extract (Avanti Polar Lipids) by extrusion with filters of 100 nm pore diameter [16,17]. Small unilamellar vesicles (SUV's) (10 mg/ml) prepared in vesicle buffer (150 mM KCl, 10 mM KH_2PO_4) at pH 7.0 were solubilised with 35 mM CHAPS and mixed with DM solubilised KcsA proteins at a 1:500 protein: lipid molar ratio. The detergent was removed by dialysis [18]. The reconstituted vesicles were collected by centrifugation (1 h, 40,000 rpm, 4 °C). The proteoliposomes were finally resuspended in vesicle buffer (pH 4.0) and centrifuged (5 min, 3000 rpm, room temperature) to remove aggregated fractions. The resulting supernatant was used for further analysis and the protein concentration in the vesicles was finally determined by SDS–PAGE using BSA as a standard.

2.4. Tryptophan fluorescence and acrylamide quenching

All experiments were performed in vesicle buffer at room temperature using a Hitachi F-4500 fluorescence spectrometer in a quartz cuvette. Trp fluorescence and acrylamide quenching experiments were performed as described previously [19,20].

2.5. Analysis of functional reconstitution and data analysis

Functional reconstitution of KcsA mutants into a bilayer made from *E. coli* lipids (Avanti Polar Lipids) was confirmed by simultaneous measurements of membrane conductance [18]. All measurements were made at room temperature. Briefly, planar lipid bilayer was formed from a solution of *E. coli* lipid (1–2 mg/ml), thus opposing the two monolayers within the aperture in the Teflon septum (150–200 μm diameter) in a homemade Teflon chamber between aqueous bathing solutions of vesicle buffer. As a control, no currents were detected for planar lipid bilayer without proteins. After the bilayers were formed, proteoliposome solution was added at ~1:1000 protein: lipid molar ratio to the *cis* compartment with gentle mixing.

The *cis* compartment (voltage command side) was connected to the head stage input, and *trans* compartment was held at virtual ground via a pair of matched Ag–AgCl reference electrodes which were immersed into the buffer solutions at both sides of the planar bilayers. Under voltage clamp conditions, the membrane current was measured by a patch clamp amplifier (model EPC9; HEKA Electronics). The recording filter was a 4-pole Bessel with 3-dB corner frequency of 0.1 kHz. The acquired raw data were analyzed with the help of the TAC soft ware package (Bruxton Corp., Seattle, WA). A Gaussian filter of 0.3 Hz was applied to reduce noise. Open time durations were displayed using the square root of the number of events per bin and fitted with a mixture of exponential densities by the method of maximum likelihood to define the respective time constants τ (ms) [21].

3. Results and discussion

Fig. 1A shows the sequence alignment of the selectivity filter of KcsA and Kir channels. The signature sequence of KcsA (TV GYG) differs from that of Kir (TI GYG). We therefore performed mutation to replace Val-76 by aliphatic Ile (highlighted) towards Kir channels. The mutant protein could be isolated from purified *E. coli* inner membrane vesicles with a yield of ~1 mg/L indicating that both membrane insertion and folding tolerate this mutation.

3.1. Analysis of tetrameric stability by LDS–PAGE

Because fully assembled wild type (WT) channel is relatively stable in detergent micelles, its tetrameric state is easily assessed by gel electrophoresis [4]. To improve the detection of oligomeric state of mutant protein, we performed LDS–PAGE at low temperature. Fig. 1B–I shows the LDS–gel of purified WT-KcsA and V76I. WT-KcsA yielded a stable tetramer running at ~68 kDa. However, V76I was found to be

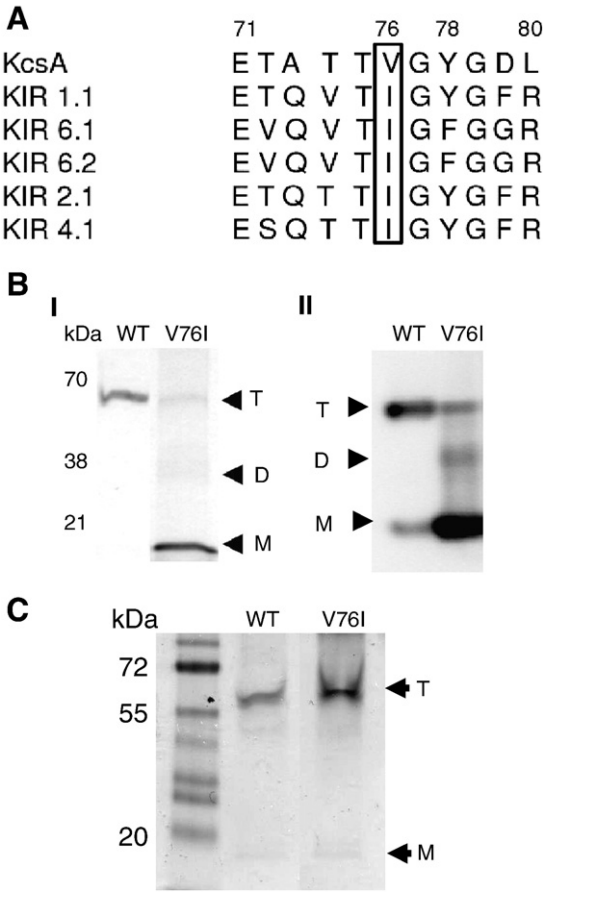

Fig. 1. (A), Differences and conservation of signature sequence among KcsA and Kir K^+ channels in the pore region. (B-*I*), LDS-PAGE (11% gel) and, (B-*II*), western blotting using Anti-His tag antibody showing WT and V76I. (C), Stability assay by PFO-PAGE (8% gel). The positions of molecular mass marker (in kDa), tetramer (T), dimer (D) and monomer (M) are indicated.

least stable and dissociated into its monomers (~18 kDa). Interestingly, the conversion of tetramer to monomer was followed by appearance of dimer (~35 kDa), which is more often observed for WT-KcsA during tetramer dissociation upon heat treatment [4,22]. Identification of tetrameric, dimeric and monomeric forms was further confirmed by western blotting (Fig. 1B-II). These experiments indicate that V76I mutation leads to destabilization of KcsA tetramer which might be related to altered packing of residues surrounding the filter region.

3.2. Analysis of channel stability by PFO–PAGE

Next, we examined the efficacy of PFO detergent to protect interactions within protein oligomers and to permit the molecular mass determination when it replaces SDS or LDS in gel electrophoresis [13]. Fig. 1C shows a typical PFO-gel (8%). For comparison, WT-KcsA was also investigated. For both, WT and V76I, soluble fractions of protein band (between 55–72 kDa) were detected which correspond to the molecular weight of tetramer. Another band of low intensity corresponding to the size of monomer (~18 kDa) was also detected. These results indicate that presence of LDS destabilizes the oligomeric forms of KcsA as compared to the least denaturing conditions in the presence of PFO detergent since it is a weak detergent [13].

3.3. Effect of V76I mutation on Trp fluorescence

A complicating factor in analyzing tetrameric stability from gel electrophoresis experiments is the presence of detergent in the media. As a complementary approach, we therefore used Trp fluorescence to analyze the folding properties of mutant and compared it to WT-KcsA in the membrane. KcsA contains five Trp residues located at the membrane water interfacial regions of the transmembrane (TM) helices and the pore helix [23]. To get insight into channel's active and inactivated states [24], experiments were performed both at basic and acidic pH, respectively. The Trp fluorescence emission spectra [$\lambda_{ex} = 295$ nm] at pH 7 and pH 4 are shown in Fig. 2A and B, respectively. The calculated parameters are compiled in Table 1. WT exhibits a typical emission maximum as shown previously [23]. In addition, a slight increase and non-significant decrease in fluorescence intensity was observed for V76I at pH 4 and 7, respectively, as compared to WT which might be related to distinct conformation of mutant protein in the membrane.

3.4. Effect of V76I on Trp acrylamide accessibility

Changes in fluorescence intensities and shifts in emission maxima suggested distinct conformational changes in V76I protein. This was assessed in a more direct manner by using the collisional quencher acrylamide to detect changes in the 'availability' of Trp to the aqueous environment [19,20]. The Stern–Volmer quenching plots in which F_0/F is plotted against the acrylamide concentration were linear and are shown in Fig. 2C and D. The Stern–Volmer constants representing acrylamide accessibility are compiled in Table 1.

An increase in acrylamide quenching was observed for V76I as compared to WT indicating that this mutation significantly affects the folding properties of tetramer. Such effect might be related to partially destabilized/unfolded tetramer. It has been previously shown that KcsA is not stable upon increase in accessibility of Trp residues and

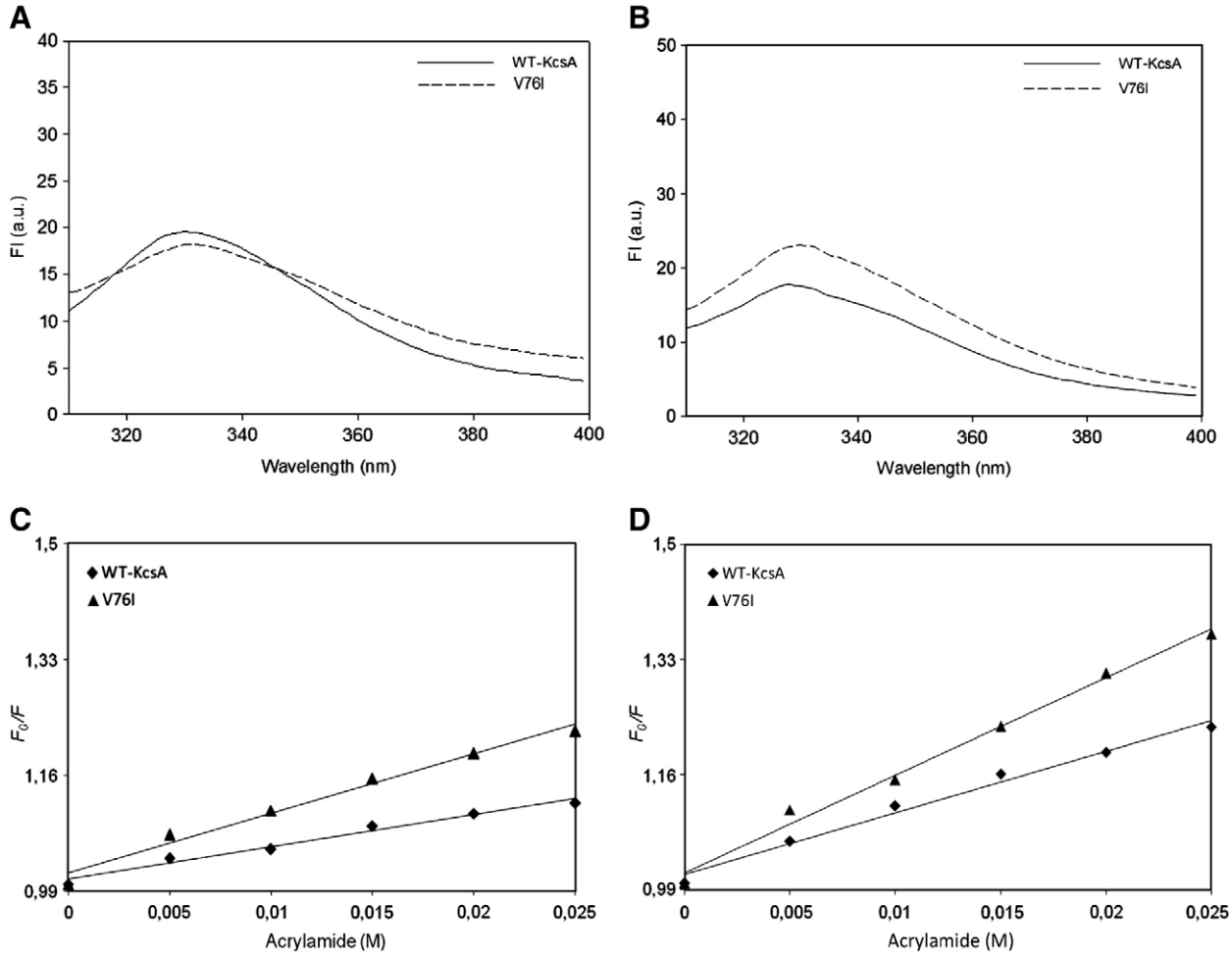

Fig. 2. Trp fluorescence spectra of WT and V76I at pH 7 (A) and pH 4 (B) in *E. coli* lipid bilayer. (C and D), Stern–Volmer plots of Trp fluorescence quenching by acrylamide at pH 7 and 4, respectively. The data points show the mean values of three independent experiments.

Table 1
Effect of V76I on KcsA Trp fluorescence and acrylamide accessibility.

Protein	pH	Wavelength (nm)[b]	K_{SV} (M^{-1})[c]
WT-KcsA	7	327	4.7 ± 0.8
	4	329	8.3 ± 0.9
V76I	7	328	8.7 ± 0.2
	4	330	14 ± 1.3

[a,b] The parameters were derived from Trp fluorescence emission maxima shown in Fig. 2A and B.
[c] The Stern–Volmer quenching constants were determined from the slopes of the lines of $F_0/F = 1 + K_{SV}$ [Q] as shown in Fig. 2B and C. Values are the means ± S.D. of three experiments.

more likely it is converted into monomers [19]. These results suggest that V76I has distinct folding properties either in its closed or open state. We therefore determined the channel activity of this mutant.

3.5. Single channel properties of WT-KcsA as a control

For comparison, we first checked the single channel properties of WT-KcsA in the membrane. All experiments were performed in symmetrical 150 mM K^+ solutions at pH 4.0 since KcsA is closed at pH 7 [24–26]. For sidedness of channels, pH gradient was used to eliminate activity of channels inserted backward into the bilayer [27]. Single-channel current traces for ±50 and 100 mV are illustrated in Fig. 3A. The detailed histograms of the single channel recordings of WT-KcsA are shown in Fig. 3B. The *i–V* curve shows a typical mild outward rectification (Fig. 3C) as previously reported [26–28] with chord conductances of 84 pS and 66 pS for +150 and −150 mV, respectively, and zero-voltage conductance of 90 pS in 150 mM K^+. However, open-channel current of KcsA is accompanied by excess noise, as if the channel undergoes rapid unresolved fluctuations, an effect particularly severe at positive potentials than at negative potentials. Such findings however might be related to different conditions like reconstitution of KcsA in a particular lipid system as described in several studies [28–30].

3.6. Effect of V76I on single channel activity

Mutations that alter pore properties of potassium channels often drastically affect the channel gating [9]. Single-channel current traces of V76I for ±50 and 100 are illustrated in Fig. 3A and open channel histograms are shown in Fig. 3B. The *i–V* curve shows a mild outward rectification, with significantly reduced chord conductances of 9 pS and 5 pS for +150 and −150 mV, respectively, and zero-voltage conductance of ~15 pS in 150 mM K^+ (Fig. 3C). It had previously been shown that mutations V76A or V76E lead to an unstable and inactive tetramer [10]. Similarly, an exchange of the corresponding Val residue (V443) by

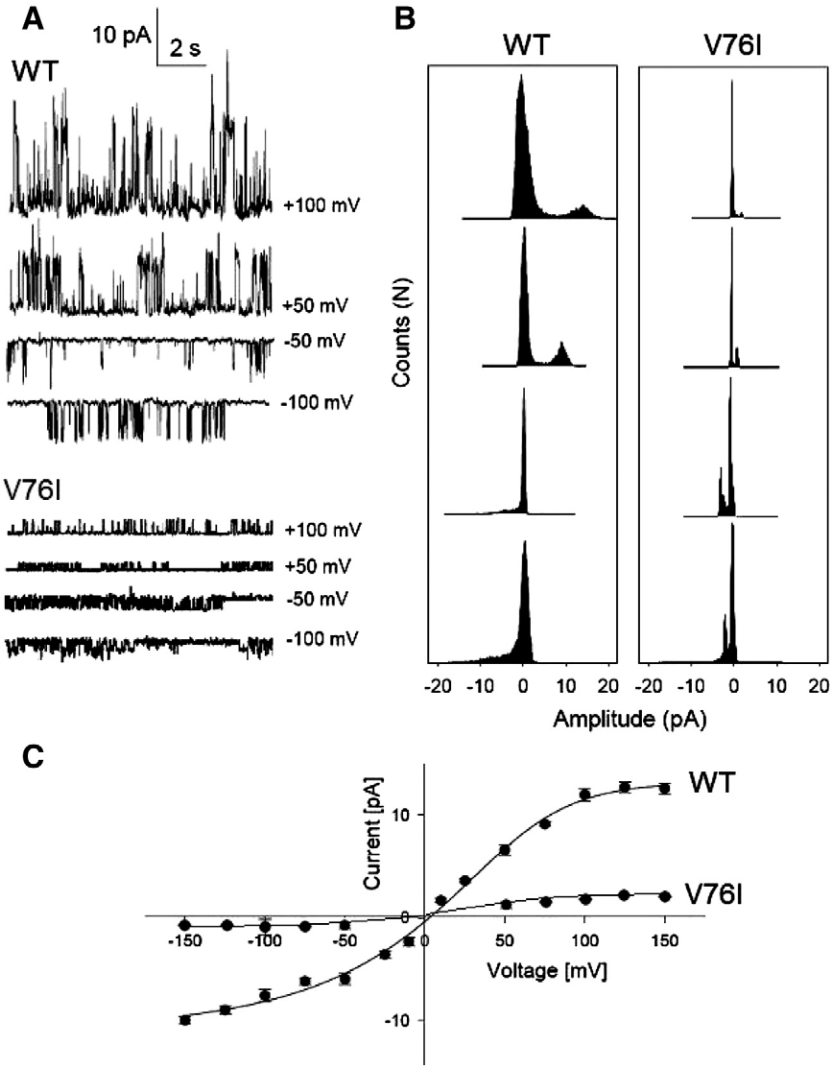

Fig. 3. (A) Representative single-channel currents through WT-KcsA and V76I in planar lipid bilayers. Recordings are shown of single channels in symmetrical 150 mM K^+ at voltages indicated. (B), Detailed histogram of the recording shown in panel A. (C), Open-channel current–voltage (*i–V*) relation of WT and V76I. Mean standard deviations are shown. Solid curve has no theoretical meaning.

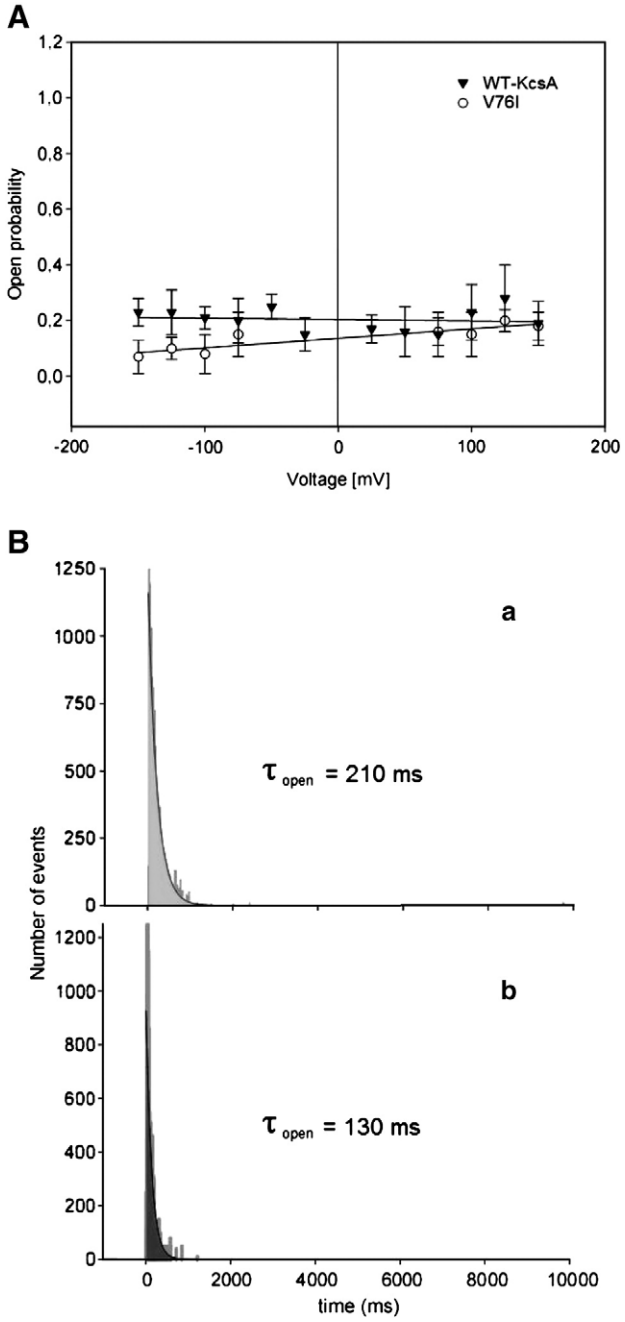

Fig. 4. (A) A plot of open probability (NPo) vs. voltage of WT-KcsA and V76I in symmetrical 150 mM K^+. The voltage independence of the open channel probability calculated as the fraction of time during which the channels were opened in recordings such as those shown in Fig. 3A. The values of NPo were calculated from open channel histogram of the recording shown in Fig. 3B. Data points correspond to the average and ±S.D. of two or three experiments. (B) Representative open-time histograms of WT-KcsA (a) V76I (b) in symmetrical 150 mM K^+. Time constants were determined from single-exponential fits to the histograms from two or three experiments.

Ala in Shaker H4 leads to a non-selective channel, whereas a substitution by Glu did not entail functional expression [9] most probably due to lack of assembly or low stability of the mutant tetramer. Our data suggest that although substitution of V76I drastically affects the rate of K^+ permeation it still retains channel activity.

3.7. Effect of V76I on open channel probability and gating kinetics

The detailed histogram of the single channel recordings representing open channel probability of WT-KcsA and V76I are shown in Fig. 3B and the calculated open probabilities (NPo) are combined in Fig. 4A. The NPo values were determined under similar gradient conditions at voltages from −150 mV to +150 mV. As shown, V76I exhibited relatively low open probability of ~0.15 which is similar to WT-KcsA (~0.2) as also shown in other studies [25–28]. Surprisingly and similar to mutant channel, WT also exhibited voltage independent open channel probability which is in contrast to the KcsA behavior suggesting that this difference might be related to reconstitution of KcsA in different lipid environment as described previously [25–28].

We next determined the channel gating properties of WT-KcsA and V76I. It is also apparent from the figure that not only does the channel conductivity decrease, but open time also greatly decreases. Fig. 4B, a and b show the representative open time histograms of WT-KcsA and V76I, respectively, in symmetrical 150 mM K^+. V76I drastically decreases the channel open time duration ($\tau_{open} = 130 \pm 25$ ms) as compared to the WT-KcsA which shows mean open time of 210 ± 40 ms. The data indicate that V76I mutation affects the pore conformation that reduces the open state of the channel.

It is clear that mutations in the filter region drastically affect the stability of KcsA tetramer although not as drastically as other mutations [10]. Furthermore, it was also reported that changing Val-76 to Ala or Glu leads to complete disappearance of channel currents [10] which is in contrast to our observation. Thus, the effects of V76I mutation on channel stability, folding and activity are unique which need to be taken into account when alterations in pore conformation are considered (see Conclusion).

4. Conclusions

Mutation V76I drastically affected the tetrameric stability in the presence of detergent like LDS. Most likely, the bulkier Ile side chain affects the packing of residues surrounding the filter thus affecting the tetramer stability in detergent and altering the channel folding properties in the membrane as observed by significant increase in acrylamide quenching either in channel open or closed conformation.

Within the crystal structure of KcsA [2,3], Val-76 is orientated toward the pore helix. Molecular dynamics (MD) simulations on Kir6.2 revealed the flipping of backbone carbonyl of Ile-131 (Val at the corresponding position in KcsA) so as to be no longer directed toward the pore thus narrowing the pore in this region [31]. We also propose that Ile substitution at position 76 results in narrowing of the pore center (Fig. 5) thus making the similar arrangement possible as observed for Kir6.2 [31,32].

The structural and functional characterizations regarding inactivation and conformational changes at the selectivity filter of KcsA as a function of mutation can be well supported by previous findings using solid-state NMR spectroscopy [33] where it was shown that transition of the KcsA-Kv1.3 mutant K^+ channel from a closed state to an inactivated state involves distinct structural changes within the pore. Later, it was

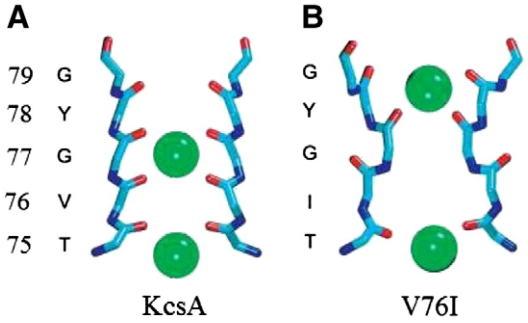

Fig. 5. Comparison of X-ray structure of the KcsA filter region with proposed conformation of V76I. The positions of residues are indicated at the left corner. In each case two subunits (and two K^+ ions) are shown. These models were adopted from Kir 6.2 MD simulation studies [32].

reported that channel opening is associated with a structural rearrangement thus affecting the water accessibility of protein surface of chimeric KcsA-Kv1.3 channel in a lipid environment [34].

The change in tryptophan fluorescence are quite informative, implying that long-range conformational changes (from the site of the perturbation to the periphery/membrane interface) can be detected as it has been previously shown by Mackinnon's group by lowering K^+ concentration or by using the mutant M96V [3,35]. Additionally, these changes in fluorescence are supported by our denaturation experiments which indicate that perturbation at position 76 does affect the structure of the protein and therefore its oligomeric stability. This seems to be a common motif in K^+ channels and it has been previously pointed out in a number of mutations and/or ionic conditions which seem to affect the tetrameric nature of these channels [4,36,37]. Additionally, our studies indicate that mutating critical residues in KcsA towards Kir channels might provide a step towards better understanding of mechanisms of structural or functional differences among KcsA and Kir channels.

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