Exploring the open pore of the potassium channel from *Streptomyces lividans*

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Received 5 November 1999

Edited by Ulf-Ingo Flügge

Abstract The tetrameric potassium channel from *Streptomyces lividans* (KcsA) embedded in planar bilayers exhibits the following electrophysiological characteristics: (i) K^+ ions can cross the pore in a highly hydrated state ($n_{H2O} \gtrsim 6$), (ii) the selectivity for K^+ exceeds that for Na^+ ions by 11 times, and both Ca^{2+} and Mg^{2+} are permeant, (iii) the internal side is blocked by Ba^{2+} ions in a voltage-dependent manner, (iv) intrinsic rectification is due to gating, depending on the direction of the electric field, (v) the internal side is pH-sensitive, and (vi) the open pore has a diameter of ~5.8 Å. In conclusion, our results show that ion conduction and selectivity of KcsA cannot easily be reconciled with the properties deduced from the rigid crystal structure [Doyle et al., Science 280 (1998) 69–77], which must be concluded to have the pore trapped in its closed state.

Key words: Block; Ion permeation; KcsA; Pore; Potassium channel; *Streptomyces lividans*

1. Introduction

Potassium (K^+) channels are abundant in cell membranes of eukaryotic organisms. The currents generated by the action of diverse channel types play key parts in electrical excitability, osmotic processes and metabolic regulation [2,3]. Malfunction of these channels may lead to diseases [4]. The two main families of K^+ channels comprise four subunits of a protein with six or two, respectively, transmembrane (TM) helices, as well as a small pore (P—) region (25 aa) determining the selectivity for K^+ ions. The fortuitous discovery of the two-TM K^+ channel gene (*kcsA*) within the bacterium *Streptomyces lividans* allowed the purification [5], functional reconstitution [5–7] and subsequent crystallization [1] of a tetrameric protein which meets the general electrophysiological and structural characteristics of K^+ channels. Being an in vitro reconstitutable channel, KcsA is currently the best-suited model system [8] to elucidate molecular details of the function–structure relationship of ion channels [9].

2. Materials and methods

2.1. Generation of a mutant protein and isolation of proteins

After cloning in pQE32 and transformation of *Escherichia coli* M15 pRep4, the wild type and the mutated Y82V protein (PCR mutagenesis) were obtained by growing to the mid-logarithmic phase at 30°C. After addition of 0.5 M ITPG, incubation continued for 2 h. The cells were harvested by low-speed centrifugation and washed with 1% NaCl solution. The cell pellet was suspended in 10 ml per g wet cells of 20 mM HEPES pH 7.5, 0.45 M sucrose, 8 mM EDTA, 0.75 mM Pefabloc (protease inhibitor), and 0.4 mg/ml lysozyme, and kept on ice for 1 h. The mixture was briefly sonicated (Branson sonifier), and the cell debris was removed by centrifugation (10 min, 8000×*g*). After removal of the supernatant by high-speed centrifugation (1 h, 100 000×*g*), the membrane proteins within the pellet were solubilized in the same volume as before of 20 mM HEPES pH 7.5, 100 mM KCl, 10 mM imidazole, 0.75 mM Pefabloc and 10 mM dodecylmaltoside (DDM) for 1 h at room temperature. Having removed insoluble remains (1 h, 100 000×*g*), the solubilized His-tag protein was purified using affinity chromatography, as described [5].

2.2. Electrophysiology

Using a dilution method the protein was reconstituted into liposomes prepared from L-α-asolectin (Sigma type IV-S). 400 µl L-α-asolectin (20 mg/ml) in 10 mM MOPS/KOH pH 7.0 and 10 mM DDM was mixed with 300 µl protein solution (about 1 mg/ml), weakly sonicated and rapidly diluted 100 times with 10 mM MOPS/KOH pH 7.0. The liposomes were collected after high-speed centrifugation (90 min, 100 000×*g*), resuspended in 200 µl 10 mM MOPS/KOH pH 7.0, rapidly frozen in aliquots in liquid nitrogen, and stored at −70°C.

Generation of planar lipid bilayers and fusion of proteoliposomes with the bilayer was performed as described [5,10]. If not indicated otherwise, the measurements were done in buffers containing 245 mM KCl and 5 mM KOH/citric acid pH 4.0. The Ag/AgCl electrode of the *trans* compartment was directly connected to the headstage of a current amplifier (EPC 7, List Medical). Currents were recorded and analyzed as reported previously [5,10]. Modelling of the voltage- and concentration-dependent potassium currents was done by a fitting program (D. Meuser, unpublished) using Turbo Pascal 5.0 (Borland). Fetchan and Pstat from pClamp 7.0 (Axon Instruments) were used for dwell time analysis. All results are given as mean ± S.D., and the number of experiments ranged from $n = 3$ to 11.

3. Results and discussion

3.1. Gating of KcsA depends on the orientation of the transmembrane electric field

After fusion of proteoliposomes containing His-tag KcsA tetramers (in the following referred to as KcsA) into planar bilayers, voltage-dependent currents were observed with larger mean overall currents in one direction (Fig. 1A). Since gating depended on the orientation of the electric field (Fig. 1B), the

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Abbreviations: DDM, dodecylmaltoside; QA, quaternary ammonium ion; TBA, tetrabutylammonium; TEA, tetraethylammonium; THepA, tetraheptylammonium; THexA, tetrahexylammonium; TM, transmembrane; TMA, tetramethylammonium; TPrA, tetrapropylammonium; TPenA, tetrapentylammonium

0014-5793/99/$20.00
PII: S0014-5793(99)01579-3

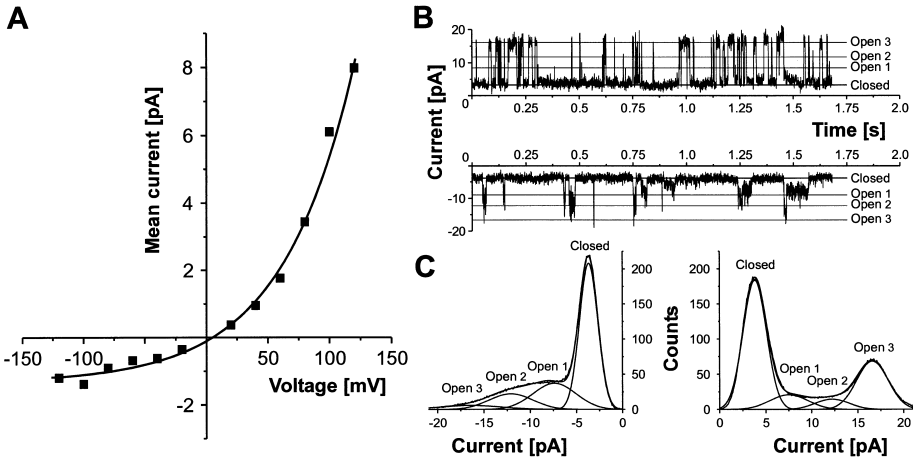

Fig. 1. Rectification of potassium currents. A: Voltage dependence of the mean current (250 mM KCl symmetric). B: Traces at +100 mV (upper lane) and −100 mV (lower lane) showing at least three conductance levels. C: Histograms deduced from the traces given in B. The occurrence of the closed and open levels is indicated.

relative orientation of KcsA within the bilayer could be determined during each experiment. In most cases, the orientations of the individual channels were unidirectional, however, their absolute orientation varied. This finding can probably be attributed to the sonication procedure prior to the addition to the *cis* chamber, which may have led, at least in part, to an inversion of the proteoliposomes.

In order to determine the orientation of KcsA a point mutation at position 82 was introduced into the gene, leading to the formation of the mutant protein KcsA Y82V. Within the crystal structure of KcsA [1], Y82 (corresponding to position 449 in the *Shaker* channel, known to be the external binding site for tetraethylammonium (TEA) ions [11,12]) resides in the outer region of KcsA. The characteristics of the Y82V mutant protein as to tetrameric assembly, conductance and internal block by tetrabutylammonium (TBA) corresponded to those ascertained for the wild type KcsA (data not shown). At the side of the wild type and the mutant protein which was more highly conducting, the TEA IC_{50} values were almost identical. In contrast, at the opposite side of the mutant channel the blocking effect decreased drastically (24-fold) (Fig. 2A). These results allowed us to unequivocally allocate the two electrophysiologically determined distinct sides to the external or the internal face within KcsA. Moreover, gating was faster in the wild type than in the mutant KcsA (Fig. 2B,C). The wild type and the mutant channel had about identical mean open durations of $\tau_1 = 1$ ms and $\tau_2 = 8.8$ ms, but the amplitude ratios of the fast and the slow gating component for KcsA ($A_1 : A_2 = 0.79 : 0.21$) and for the mutant protein ($A_1 : A_2 = 0.39 : 0.61$) differed significantly.

Previous flux measurements [6] had shown that gating is sensitive to the pH at one side of the KcsA-carrying proteo-

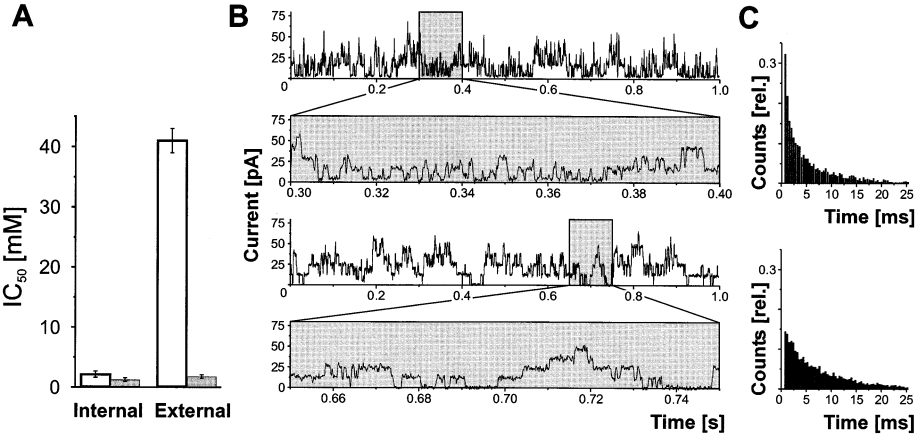

Fig. 2. Comparison of the wild type and of Y82V mutant channels. A: TEA inhibitory IC_{50} values for the wild type (▩) and the mutant protein (□) are given. B: Traces obtained for the wild type (lanes 1 and 2) and for the mutant protein (lanes 3 and 4). C: Dwell time histograms deduced for the open 3 level for the wild type (top) and the mutant protein (bottom). Dwell times revealed distributions which could be described as double exponential decay: $A = A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2)$, $A_1 + A_2 = 1$. IC_{50} values were either calculated from the mean of current traces of 2 min duration or from single channel currents (fast blocks) at +100 mV (internal block) or −100 mV (external block) by the equation:

$$\text{Block}_{\text{rel}} = 1 - \frac{I}{I_0} = \frac{[\text{blocking ion}]}{[\text{blocking ion}] + \text{IC}_{50}},$$

I_0: control current with [blocking ion] = 0.

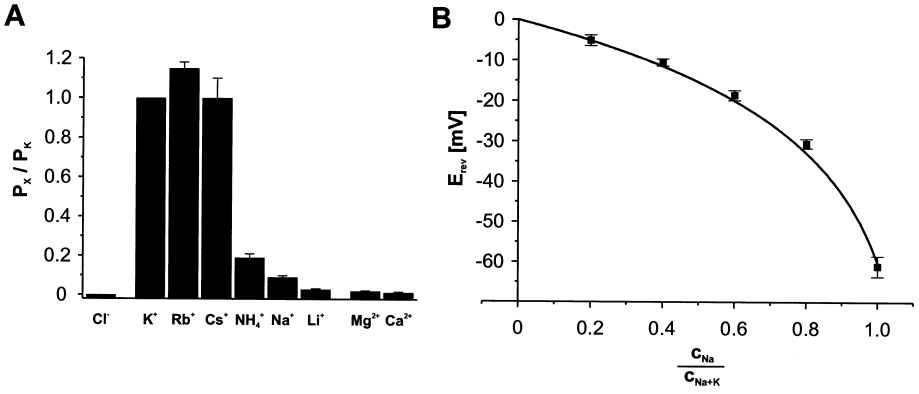

Fig. 3. Selectivity of KcsA. A: Permeability ratios were calculated from reversal potentials using equations derived from the Goldman–Hodgkin–Katz current equation [15]:

$$\frac{P_X}{P_K} = \frac{K_o \cdot \exp(-FE_{rev}/RT) - K_i}{X_i - X_o \cdot \exp(-FE_{rev}/RT)},$$

K_i, X_i, K_o, X_o: internal and external concentrations, for monovalent cations and

$$\frac{P_X}{P_K} = \frac{K_o \cdot \exp(-FE_{rev}/RT) - K_i}{X_i - X_o \cdot \exp(-2FE_{rev}/RT)} \cdot \frac{1 - \exp(-2FE_{rev}/RT)}{1 - \exp(-FE_{rev}/RT)}$$

for divalent counter cations. The potassium permeability was set as 1. B: Reversal potential in dependence on the mole fraction; (■) experimental data; (—) theoretical slope for a permeability ratio $P_K/P_{NA} = 10.8$.

liposomes. Within the KcsA-containing planar bilayer system it could be ascertained that the internal side of reconstituted KcsA is pH-sensitive (data not shown), as was recently confirmed [7]. Due to this pH sensitivity, the following experiments were performed at a pH of 4 at both sides.

The electrophysiological characteristics of the wild type and the Y82V mutant protein disclosed that KcsA is an intrinsically outward-rectifying channel. Thus, by convention the *trans* chamber was set as the internal compartment, with outward-directed potassium currents at positive voltages.

Analysis of single channel currents revealed at positive and negative potentials at least three conductance states (Fig. 1B), as was reported previously [5]. All point histograms of traces at ±100 mV (Fig. 1C) showed that rectification is a consequence of different occupancies of the individual open states at opposite voltages with almost the same overall open probabilities ($NP_O^{out} = 0.45$ and $NP_O^{in} = 0.43$). Outward K^+ currents were mainly carried by the open 3 state, inward currents primarily due to the open 1 state. As the conductance of open 3 is higher ($\Lambda_3 = 97$ pS at 100 mV) than that of open 1 ($\Lambda_1 = 41$ pS), the mean overall currents are rectified outwards. The observed subconductance states are likely the result of conformational changes [13]. The peaks from the three open states within the all-point histograms showed rather broad

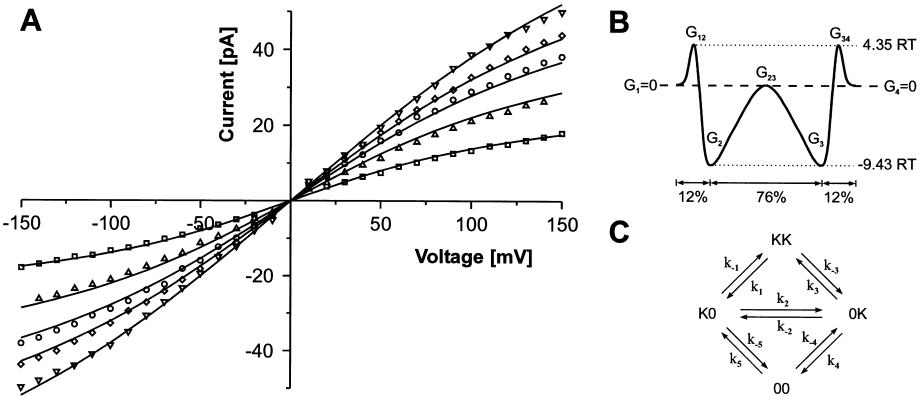

Fig. 4. Ion conduction in KcsA. A: Current–voltage relationship for conductance level 3. Measurements were done in the presence of 250 mM (□), 500 mM (△), 750 mM (○), 1 M (◇) and 1.5 M (▽) KCl. The slopes were deduced from a single-file–two-binding site model. (B) Energy profile and (C) occupancy states and rate constants of the applied model. The energy profile resulted from a fitting process permitting ion–ion interaction ($F_{out} = 102$). In modelling the voltage and concentration dependence of K^+ currents through reconstituted KcsA the probabilities of occupancy (P_{XX}) of the different states of the pore were calculated from their steady-state kinetic equations, in which the rates of creation and loss of a state are equal. The steady-state kinetic equation for the occupancy state KK, where both binding sites are occupied by potassium ions, for example, is: $k_{-1} \cdot P_{K0} + k_3 \cdot P_{0K} = (k_1 + k_{-3}) \cdot P_{KK}$. Together with the conservation equation $\Sigma P_{XX} = 1$, this represents a system of four independent equations, from which the current can be calculated for each combination of voltage and potassium concentration. The rate constants and the ion–ion interaction factor F_{out} were as defined [30].

current distributions indicating that these open states correspond to rather flexible conformations [14]. Moreover, key elements of the gating machinery in KcsA are sensitive to the direction of the electric field.

3.2. Cation selectivity of KcsA

Further basic electrophysiological properties of the channel such as ion permeation and selectivity were studied. The relative permeabilities (P_X/P_K) of several mono- and divalent cations to those of potassium ions were calculated from the bi-ionic reversal potentials in 250 mM K^+/250 mM X^+ (or 125 mM X^{2+}) using the constant field approach (Goldman–Hodgkin–Katz voltage equation [15]). The ratios of anion and cation selectivity were determined from the reversal potentials in asymmetric potassium solution (245 mM KCl/20 mM KCl). KcsA has no detectable permeability for chloride ions. It is strongly selective for cations (Fig. 3A); among those tested, it showed the highest preference for Rb^+ (1.15 ± 0.03), K^+ (set as reference) and Cs^+ (1.0 ± 0.2), but was less permeable for NH_4^+ (0.19 ± 0.02), Na^+ (0.09 ± 0.01) and Li^+ (0.03 ± 0.003). Thus selectivity follows the Eisenman III sequence, implying a weaker field interaction at the selectivity filter and a larger radius of the cation binding site. The reversal potentials and conductances determined in solutions with different concentration ratios of potassium and sodium ions (internal 250 mM K^+/external X mM K^+, Y mM Na^+; $X+Y=250$) displayed independence of ionic movement through the channel (Fig. 3B). Notably, the bivalent cations Mg^{2+} and Ca^{2+} were found to pass the selectivity filter, although their relative permeabilities were comparatively low (0.022 ± 0.002 and 0.015 ± 0.003, respectively). In contrast, Ba^{2+} ions efficiently blocked the K^+ current from the internal side (details see below).

3.3. K^+ currents show saturation

The K^+ currents carried by KcsA in the open 3 state were measured in symmetrical solutions ranging from 250 mM to 1.5 M KCl. In contrast to a linear current–voltage relationship, which would be expected for ionic movement governed solely by electrodiffusion, currents saturated in dependence on the applied voltage as well as on the potassium concentration (Fig. 4A). The data can be explained by a three-barrier–two-site model with multiple occupancies and ion–ion interaction (Fig. 4B,C).

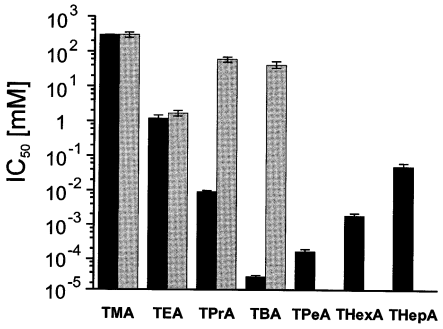

Fig. 5. Internal (■) and external (▨) block of KcsA by quaternary ammonium ions. IC_{50} values were determined at +100 mV (internal block) and −100 mV (external block).

3.4. High-affinity block by quaternary ammonium ions

To explore structural features of the open channel the block of KcsA by quaternary ammonium compounds (QAs) with chain lengths of one (tetramethylammonium, TMA) to four C atoms (TBA) was systematically studied at both sides of the KcsA-containing bilayer. Moreover, the effects of QAs with alkyl chains up to C7 at the internal side were investigated (Fig. 5). Significant differences could be observed at the internal side of KcsA, where the block increased (up to 10^7-fold) in correlation with the chain length (up to C4) of the QAs, with TBA being the strongest inhibitor ($IC_{50} = 31 \pm 2$ nM). Compared to TBA, the block efficiency decreased for QAs with chain lengths of 5–7 atoms.

At the external side, TEA showed the strongest inhibition ($IC_{50} = 1.7 \pm 0.1$ mM). The block at the internal side by TMA and at the external side by TMA, TEA and tetrapropylam-

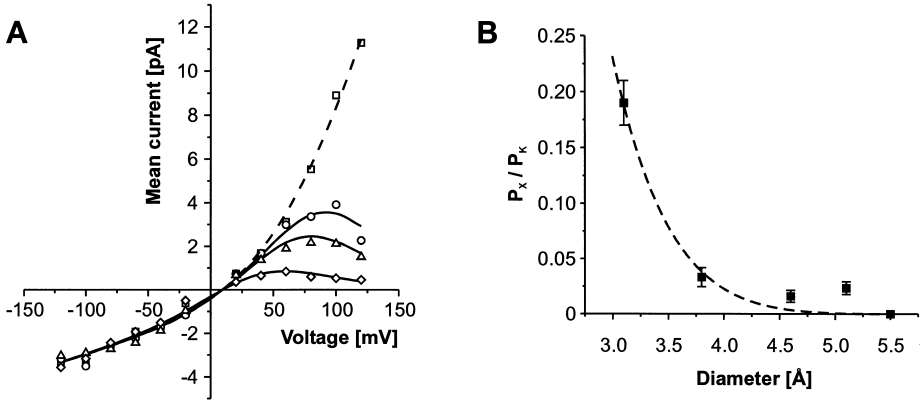

Fig. 6. Characteristics of the pore. A: Voltage-dependent block by Ba^{2+} ions at the internal side. Measurements were performed at 0 mM (□), 0.5 mM (○), 1 mM (△) and 5 mM (◇) $BaCl_2$; the outlined slopes represent the prediction for a single binding site model with the blocking ion traversing 94% of the electrical distance. B: Determination of the smallest diameter of the pore. A comparison of the experimental data (■) and the theoretical fit (—) is presented. The diameter of the open pore of several ion channels has been deduced from the permeability ratios of alkaline ions to larger monovalent inorganic and organic cations [21–23]. The pore diameter was determined by fitting the function

$$\frac{P_X}{P_K} = k \cdot (1-\alpha)^2 \cdot \frac{1 - 2.105\,\alpha + 2.0865\,\alpha^3 - 1.7068\,\alpha^5 + 0.72603\,\alpha^6}{1 - 0.75857\,\alpha^5}, \alpha = \frac{d_{ion}}{d_{pore}},$$

k: proportionality factor, to the experimental data.

monium (TPrA) was fast ($\tau_{on} < 100$ μs). As a consequence, fast current fluctuations could not be resolved and resulted in apparently reduced current amplitudes. All other blockers led to unchanged conductances with decreased mean currents, due to missing or shortened open events (data not shown). Several types of potassium channels show similar sensitivity sequences for QAs blocking from the internal side [16–18]. Previous investigations in Shaker channels [19] led to the model that QAs can be trapped in a cavity-like compartment. Thus it can be presumed that in KcsA the interaction site for the QAs is the hydrophobic surface of the cavity.

3.5. Barium ions block K^+ currents from the internal side of KcsA

A further property of the channel pore can be deduced from the observation that Ba^{2+} ions blocked K^+ currents from the internal side by entering but not passing the selectivity filter. With an IC_{50} of 0.52 ± 0.03 mM, at +100 mV, this side was about 120 times more sensitive than the opposite one ($IC_{50} = 60 \pm 20$ mM, at −100 mV). The internal block was highly voltage-dependent (Fig. 6A); it was nearly absent at negative values and considerably increased at positive potentials, when the Ba^{2+} ions were forced into the channel pore. The voltage dependence of the channel block can be described by the classical Woodhull model [20] with a single binding site at 94% of the electrical distance. Analysis of the K^+ conduction using the constant rate approach revealed the necessity of at least two binding sites. Possibly Ba^{2+} ions crossing most of the electrical distance from the internal side interact with the external binding site.

3.6. The opened channel pore has a diameter of about 5.8 Å

In order to gain further insight into the topology of the ion-conducting KcsA channel, we determined the smallest diameter of the pore by established electrophysiological techniques [21–23]. Ammonium, methylammonium, dimethylammonium and trimethylammonium ions were found to pass the pore of the reconstituted KcsA channel, but not tetramethylammonium ions ($d_{TMA} = 5.5$ Å, Fig. 6B). Using the equation first applied to channel proteins by Dwyer et al. [21], the smallest diameter of the open pore of the reconstituted KcsA was calculated from our permeability measurements to be 5.8 ± 0.7 Å. This finding together with the observed selectivity suggested that K^+ ions cross the selectivity filter surrounded by a considerable number of H_2O molecules. Using a modified electrodiffusion approach [24] and taking into account the diameter determined for the selectivity filter, a hydration number of $n_w \cong 6.4$ was calculated for permeating K^+ ions. The resulting activation barrier of 2.7 kcal/mol is in good accordance with the activation barrier of 2.6 kcal/mol predicted for K^+ permeation by the above used constant rate approach. From our data it is extremely unlikely that the selectivity of KcsA is achieved via rings of carbonyl oxygens acting as substitutes for H_2O molecules of largely dehydrated K^+ ions.

3.7. Summary

The conclusions are as follows. (i) The relatively broad current distributions of the three open states picture the dynamic changes during the opening process. (ii) The occupancy of the subconductance levels depends on the direction of the transmembrane electric field resulting in intrinsic rectification of the currents. The characteristic gating properties in accordance with previous ESR studies [25] point to a high plasticity of the pore. (iii) Like other in vivo investigated K^+ channels (BK [26,27], IRK [28] and SR [29]), KcsA exhibits moderate selectivity for K^+ ions ($P_K/P_{Na} = 11$). The KcsA pore with a smallest diameter of 5.8 ± 0.7 Å allows the passage of mono- and divalent ions ($Rb^+ > K^+ > Cs^+ \gg NH_4^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$). This implies that during permeation K^+ ions do not have to shed the major part of their hydration shell. From our data it can be deduced that each K^+ ion permeates surrounded by $n \cong 6$ H_2O molecules. In view of these findings one has to suggest that the crystal structure of KcsA pictures the pore ($d \approx 3$ Å) [1] in a closed state and consequently it cannot be the basis for explaining the mechanism of ion permeation and selectivity. (iv) The dependence of K^+ currents on voltage and concentration can be described by a rate theory model with two binding sites flanked by activation energy barriers consistent with the passage of highly hydrated ions. KcsA co-crystallized with K^+, Rb^+ or Cs^+ contains two ions within the selectivity filter [1] and their locations might correlate with the two binding sites derived by modeling. (v) The internal side is blocked specifically by quaternary ammonium ions probably due to interaction with the hydrophobic region in the channel cavity visualized by the crystal structure. To account for the movement of QAs into the cavity the internal entrance of the pore has to widen to a diameter larger than that found in the crystal structure (~ 6 Å). This could be achieved prior to or during opening by rotations of TM1 and TM2 identified by ESR studies [25].

Acknowledgements: H. Splitt and D. Meuser were supported by grants awarded to H. Schrempf through the Sonderforschungsbereich (SFB) 431/D5 and the Graduiertenkolleg, respectively. Costs for the experiments were financed by grants of the SFB 431 awarded to H. Schrempf (D5) and R. Wagner (D7). M. Lemme supported the writing of the manuscript.

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