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ORIGINAL ARTICLES

The "Flipped" State in E71A-K^+-Channel KcsA Exclusively Alters the Channel Gating Properties by Tetraethylammonium and Phosphatidylglycerol

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Abstract Mutation E71A in the bacterial K^+-channel KcsA has been shown to abolish the activation-coupled inactivation of KcsA via significant alterations of the peptide backbone in the vicinity of the selectivity filter. In the present study, we examined channel-blocking behavior of KcsA-E71A by tetraethylammonium (TEA) from both the extra- and the intracellular sides. First, we found that E71A is inserted either in *cis* or *trans* orientation in a planar lipid bilayer; however, it exhibits only one orientation in proteoliposomes as determined by extravesicular partial chymotrypsin digestion. Second, E71A exhibits a lower extracellular TEA affinity and is more sensitive to intracellular TEA compared to wild-type KcsA, which apparently has >50-fold higher affinity for extracellular TEA and ~2.5-fold lower affinity for intracellular TEA compared to E71A. In additional experiments, we investigated the influence of negatively charged phosphatidylglycerol (PG) on channel-gating properties in phosphatidylcholine lipid bilayers. It was found that high PG content decreases the single-channel conductance and increases the channel open time and open probability. Taken together, our data suggest that the "flipped" conformation of the selectivity filter present in E71A allows weaker extracellular and stronger intracellular TEA binding, whereas higher PG content decreases channel conductivity and stabilizes the channel open "flipped" state via electrostatic interaction in the proximity of the channel pore.

Keywords Potassium channel KcsA · Mutation · E71A · Channel orientation · Tetraethylammonium · Phosphatidylglycerol · Selectivity filter · Electrostatic interaction

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Introduction

In 1995, a unique prokaryotic potassium channel (KcsA) was identified in the gram-positive soil bacterium *Streptomyces lividans*. The KcsA gene encodes a predicted 17.6-kDa protein with two transmembrane helices linked by a central domain (Schrempf et al. 1995). The amino acid sequence of this integral membrane protein shows high similarity to all known K^+ channels including those of vertebrates and invertebrates, particularly in the pore region (Doyle et al. 1998). X-ray analysis showed that KcsA is a tetramer with four identical subunits, creating a central pore. Each subunit has two α-helices connected by a 30-amino acid pore region consisting of the turret, pore helix and selectivity filter. A subunit is inserted into the tetramer such that one transmembrane (inner helix) faces the central pore while the other (outer helix) faces the lipid membrane (Doyle et al. 1998). When an ion enters the selectivity filter, it evidently dehydrates. To compensate for the energetic cost of dehydration, the carboxyl oxygen atoms must take the place of the water oxygen atoms and act like surrounding water (Hille 1973; Bezanilla and Armstrong 1972).

Tetraethylammonium (TEA) is a classical blocker of K^+ channels. In the absence of TEA, potassium ions can occupy up to seven sites in or near the selectivity filter of the KcsA potassium channel (Lenaeus et al. 2005). Major

binding sites for this quaternary ammonium ion are located at the external and internal entrances of the narrow selectivity filter. Binding of TEA to one site shifts the ion occupancy in the selectivity filter in a way that destroys the TEA-binding site on the other side. The unblocked selectivity filter exists predominantly and alternatively in two distinct states where K^+ ions occupy either 1/3 or 2/4 positions. TEA does not penetrate through K^+ channels but blocks them both from the external and internal sides of the cell membrane (Luzhkov and Aqvist 2001). Internal TEA binding occurs only if the selectivity filter is in the 2/4 state, whereas external TEA binding requires the selectivity filter in the 1/3 state. The ratio of the internal and external blocked states is proportional to the ratio of their binding affinities, where the external site of KcsA has a 10-fold higher binding affinity to TEA than the internal site (Lenaeus et al. 2005).

Furthermore, molecular dynamics (MD) simulations revealed that TEA effectively binds at the external side of the KcsA channel when the first binding site is vacant, i.e., not occupied by an ion or water molecule, so that the ethyl group can partially penetrate into the filter. From mutagenesis studies, it has been shown that external blockade is strongly dependent on the presence of an aromatic amino acid residue in KcsA (Tyr82) located near the extracellular entrance to the pore (Crouzy et al. 2001). Therefore, TEA interacts simultaneously with the aromatic tyrosine residues of all four monomers with partial insertion of one of the ethyl groups into the filter pore. Furthermore, external TEA prevents inactivation by stabilizing a permeant ion in the external pore, the exit of which is required for inactivation (Lenaeus et al. 2005).

Channel activation is dependent on proton binding somewhere in the intracellular side of the KcsA channel. At low pH, proton binding promotes the opening of the TM2 inner bundle, initiating ion flow through the channel, which is already primed to conduct. However, the conductive conformation of the selectivity filter is metastable and can undergo a spontaneous transition into a long-lived nonconducting state (Cordero-Morales et al. 2006a). This inactivation process can be suppressed by an E71A mutation in the pore helix (Cordero-Morales et al. 2006b). Although the intracellular gate is structurally open, the channel is functionally inactivated. In the wild-type protein, an interaction between Glu71, Asp80 and Trp67 leads to destabilization of the conductive conformation of the filter and promotes inactivation. Eliminating the carboxyl–carboxylate interaction between E71 and Asp80 increases the flexibility of the side chain of Asp80, weakening its hydrogen bonding to Trp67 and therefore preventing the channel from entering the inactivated state (Cordero-Morales et al. 2006b).

Two X-ray crystal structures of E71A-Fab have been determined. One structure is quite similar to wild-type (WT)-KcsA, whereas the other shows conformational changes in three regions of the pore (the selectivity filter backbone, Asp80 at the outer end of the pore and Tyr67 along the pore helix). These two structures are called "flipped" and "nonflipped" states. The nonflipped conformation is quite similar to wild type; a slight upward displacement of the carboxylate side chain of residue Asp80 of 0.5 Å toward the extracellular side is observed. In contrast, the carboxylate side chain moves 8 Å in the flipped conformation. The structural rearrangements in the flipped state have an impact on the position and the occupancy of the ions in the selectivity filter, whereas the ions in the nonflipped conformation match the positions and occupancy of those in the WT-KcsA structure (Cordero-Morales et al. 2006b).

It is not clear whether KcsA-channel inhibition by TEA either from the extracellular or the intracellular side of the channel pore is dependent on an inactivation state of the channel. In the present study, we used E71A-KcsA mutant channel to investigate how removal of the inactivated state affects the binding of TEA to the extra- and intracellular sides of the channel. Furthermore, we also demonstrate how negatively charged phosphatidylglycerol (PG) affects the channel-gating properties of E71A in a lipid bilayer.

Materials and Methods

Reagents

Escherichia coli total lipid extract, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and 1,2-diphytanoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DPhPG) were purchased from Avanti Polar Lipids (Birmingham, AL). For clarity, the lipids DPhPC and DPhPG are abbreviated to PC and PG, respectively. *n*-Decyl-β-D-maltoside (DM) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) were from Fluka (Buchs, Switzerland). Nickel nitriloacetic acid (Ni^{2+}-NTA) agarose beads were obtained from Qiagen (Chatsworth, CA). The 100-nm membrane filters were obtained from Avestin (Ottawa, Canada). α-Chymotrypsin from bovine pancreas was purchased from Sigma (St. Louis, MO). The following chemical reagents were purchased from either Fluka, Merck (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany), available in the highest purity: Tris, KCl, imidazole, isopropyl-β-D-thiogalactopyranoside (IPTG) and TEA.

Mutagenesis, Protein Expression and Purification

Site-directed mutagenesis was performed using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA),

and mutations were confirmed by DNA sequencing of the entire gene as reported previously (Raja and Vales 2009a). Protein was expressed with a C-terminal His-tag from pQE60-KcsA in *E. coli* strain BL-21 (DE3). Purification was performed according to the procedure described previously (Raja and Vales 2009a). Briefly, the solubilized membranes in 40 mM DM were incubated with prewashed 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 mutant protein was purified with a yield of ~2 mg/l culture. Protein purity was assessed by SDS-PAGE.

Analysis of Channel Stability by SDS-PAGE

To determine channel stability, conventional gel electrophoresis experiments were performed using SDS detergent as described previously (Raja and Vales 2009b). Gels were run at 120 V until the blue dye front reached the edge of the gel. Proteins were detected by staining with Coomassie brilliant blue G-250.

Preparation of Liposomes and Protein Reconstitution

Small unilamellar vesicles (SUVs) were prepared either from *E. coli* total lipid extract or from a PC:PG mixture by extrusion with filters of 100 nm pore diameter (MacDonald et al. 1991), and protein reconstitution was carried out as described previously (Raja and Vales 2009b). Briefly, SUVs (10 mg/ml) prepared in vesicle buffer (150 mM KCl, 10 mM KH_2PO_4) at pH 7.0 were solubilized with 35 mM CHAPS and mixed with DM solubilized protein at a 1:100 protein:lipid molar ratio. The detergent was removed by dialysis. Reconstituted vesicles were collected by centrifugation (1 h, 130,000×*g*, 4°C). Proteoliposomes were finally resuspended in vesicle buffer (pH 4.0).

Analysis of Channel Activity and Channel Blocking

Functional reconstitution of E71A mutant into a bilayer made from *E. coli* lipids or PC:PG (at various mol% ratios) was confirmed by simultaneous measurements of membrane conductance (Schindler 1989). Briefly, planar lipid bilayers were formed from a solution of 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 (pH 4.0). After the bilayers were formed, proteoliposomes (with protein concentration of ~30 μg/ml) were added to one side of a preformed planar membrane with gentle mixing. As a control, similar experiments were performed with planar lipid bilayer without channel protein. All experiments were performed at room temperature. 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 (s) (Colquhoun and Sigworth 1995). All recordings and data analysis were carried out according to the procedure described previously (Raja and Vales 2009a).

Results

In the present study, we investigated the role of E71A mutation in determining the pore properties of a selective K^+ channel as a function of TEA binding either at the extracellular or the intracellular side of the KcsA channel. Figure 1a depicts the full-length KcsA amino acid sequence in which the positions of Arg residues at 52, 64 and 89 positions and Glu71 are highlighted. Figure 1b represents the schematic diagram of a single KcsA subunit where the locations of respective residues are also indicated with regard to the membrane.

Orientation of KcsA-E71A in Planar Lipid Bilayers and Membrane Vesicles

We first checked channel orientation in planar lipid bilayers formed from *E. coli* membranes. Usually, pH gradient is established for definite orientation of KcsA in the membrane (Heginbotham et al. 1999). We investigated whether mutant channels have any specific definite orientation, without establishing a pH gradient across the membrane, when they are transferred from proteoliposomes to a planar lipid bilayer. The channel current traces for −50, −100 and +75 mV in symmetrical 150 mM K^+ are illustrated in Fig. 2a and their respective amplitude histograms in Fig. 2b. The current–voltage (*I–V*) curves from −175 to +175 mV representing both orientations are shown in Fig. 2c. Figure 2d represents combined curves from Fig. 2c to show that two different current levels at positive or negative potentials are due to opposite orientation of the channel in a lipid bilayer. For uniformity, high current levels are plotted at positive potentials and low current levels are shown at negative potentials. It is, however, interesting to note that one orientation represents rectifying behavior at negative potential as shown in previous studies (Schrempf et al. 1995) and that the second orientation shows channel rectification at positive potentials. It is also worth noting that the planar bilayer contains channels oriented either in one or in the other direction and not both simultaneously.

Next, we determined if channels have specific orientation in proteoliposomes. Therefore, we performed partial proteolytic cleavage of KcsA using chymotrypsin, which

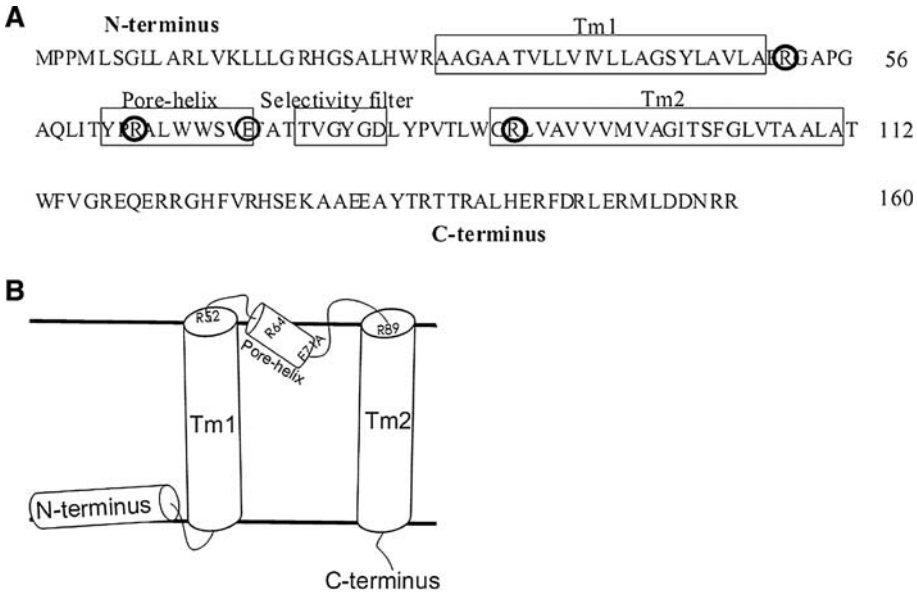

Fig. 1 **a** Amino acid sequence of the KcsA potassium channel from *S. lividans* (SWISS-PROT accession number Q54397). Transmembrane segments Tm1 and Tm2, pore helix and selectivity filter are highlighted by *open boxes*. Arg residues at positions 52, 64 and 89 and Glu-71-Ala (E71A) are also highlighted in *dark gray circles*. **b** A schematic representation of KcsA subunit pointing out the location of Arg residues at 52, 64 and 89 and E71A in the membrane

cleaves the C terminus at position 125, thus generating Δ125-160-KcsA (Molina et al. 2004). Figure 3 shows the SDS-PAGE, illustrating partial chymotrypsin digestion of E71A in proteoliposomes. As control, untreated E71A protein is also shown, which exclusively forms a tetramer running at ~68 kDa. A monomer band of lower molecular weight (~18 kDa) can also be seen. Upon chymotrypsin digestion, the size of the tetramer (ΔC-T) and monomer (ΔM-T) was reduced upon cleavage of the extravesicular C terminus (Δ125-160), thus indicating that E71A has a distinct orientation in proteoliposomes despite the fact that it has either a *cis* or *trans* orientation in a planar lipid bilayer. It is also noteworthy that cleavage of the C terminus significantly destabilizes the tetrameric structure because the tetramer intensity is drastically decreased compared to the control sample, indicating the effect of E71A mutation on tetrameric stability compared to WT-KcsA (Molina et al. 2004).

Extracellular Block of E71A by TEA

To prove whether E71A has two different orientations in a planar lipid bilayer, we performed channel-blocking experiments by adding TEA to either the *cis* or the *trans* side of the membrane to selectively block channels either from the extra- or the intracellular side or vice versa. A zero potential was applied for 10 s after formation of a planar lipid bilayer, followed by +100 and −100 mV applied potential to record single-channel activity for 5 min. This procedure is called the "potential-switch" method. Based on orientation experiments, TEA was first added to the *trans* side (selectivity filter region, see schematic model in Fig. 4a) of the membrane to block channels from the extracellular side. The potential-switch method was therefore applied to measure single-channel current at +100 and −100 mV as a function of TEA concentration in 150 mM K^+, as shown in Fig. 4b and c, respectively. The respective amplitude histograms of representative recordings are shown in the right panel. In the absence of TEA (0 mM), the current levels of 9.6 pA at +100 mV and −13.0 pA at −100 mV were recorded. Upon addition of 5 mM TEA to the *trans* side, the channel current decreased by only ~0.3 and -0.5 pA at +100 and −100 mV, respectively. The channel current was not completely blocked even at higher TEA concentrations; e.g., the current was decreased to 3 and −7.1 pA from 9.6 (+100 mV) and −13 (−100 mV) pA at ~90 mM TEA, respectively. Figure 4d represents semi-logarithmic current concentration curves in which I/I_0, the remaining single-channel current, varies with TEA concentration ranging from 5 to 90 mM. The data are well fit assuming a bimolecular interaction between the channel and TEA with $K_{1/2}$(+100 mV) of 50.5 mM and $K_{1/2}$(−100 mV) of ~100 mM as described by

$$K_{1/2} = \frac{I/I_0}{1 - I/I_0}[\mathrm{TEA}] \quad (1)$$

If the channel is oriented with its selectivity filter pointing to the *cis* side (see schematic diagram in Fig. 5a), the channel rectification is observed at negative potentials and extracellular TEA blocks the channel current at positive potentials. Similarly, if the channel is oriented with its selectivity filter pointing to the *trans* side (see schematic diagram in Fig. 5b), the channel rectification is observed at positive potentials and extracellular TEA blocks the channel current at negative potentials. Furthermore, we determined if increasing TEA concentration to 150 mM might eventually block channel activity. The *I–V* curves representing both orientations in the absence and presence

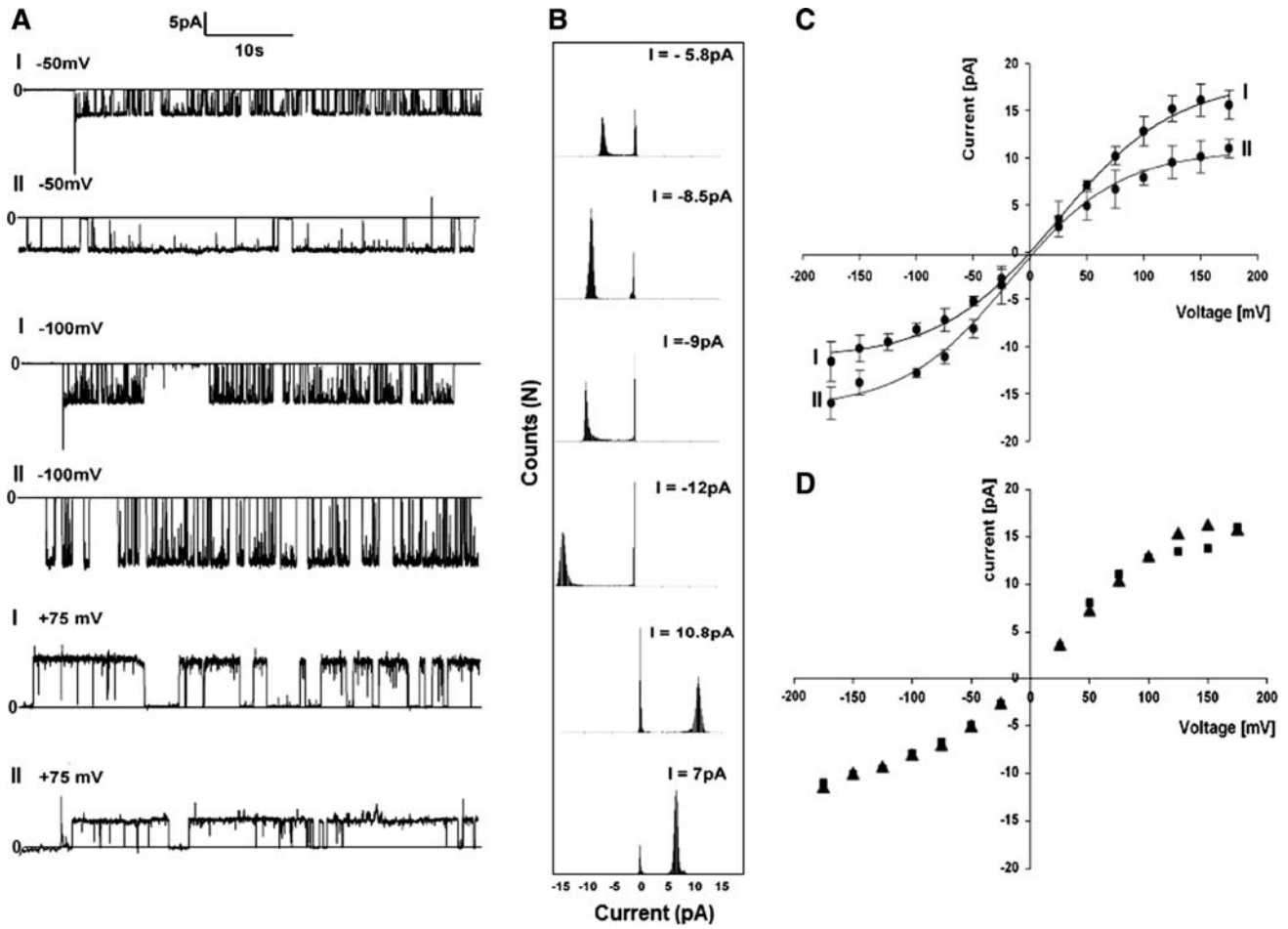

Fig. 2 **a** Representative single-channel current traces of KcsA-E71A in a planar lipid bilayer formed from *E. coli* lipid in symmetrical 150 mM K^+. *I* and *II* represent two distinct open channel conductances at similar applied potentials. The channel closed state is denoted by 0. **b** Detailed amplitude histograms of recordings shown in **a**. **c** Open-channel *I–V* relation representing single-channel characteristics, which is oriented in either the *cis* or the *trans* direction. **d** A combination of both *I–V* curves shown in **c**. For unification, all higher and lower currents are plotted at positive and negative potentials, respectively. All data points correspond to the average ± SD of at least three experiments

of 150 mM TEA are shown in Fig. 5a, b. No significant decrease in channel current compared to 90 mM TEA (as shown in Fig. 4) was further detected. These results indicated that the E71A channel is not completely blocked even at higher concentrations of extracellular TEA compared to WT-KcsA channels, which are effectively blocked by <10 mM extracellular TEA (Raja and Vales 2009b; Heginbotham et al. 1999).

Intracellular Block of E71A by TEA

As a next step, we performed channel-blocking experiments by adding TEA to the *cis* side (bundle cross region, see schematic model in Fig. 6a) of the membrane to block channels from the intracellular side. Again, the potential-switch method was applied to measure single-channel current at +100 and −100 mV as a function of TEA concentration in 150 mM K^+. Figure 6b, c shows representative

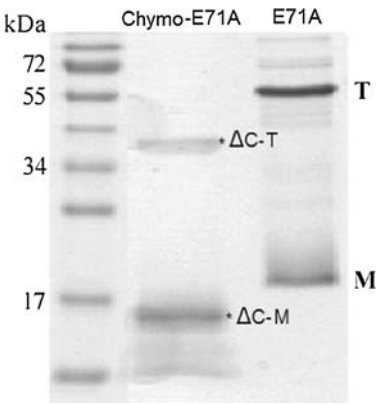

Fig. 3 Partial proteolytic cleavage of KcsA-E71A reconstituted in proteoliposomes from *E. coli* membrane. SDS gel (11%) stained with Coomassie brilliant blue shows chymotrypsin-treated E71A in proteoliposomes (*Chymo-E71A*) and a control sample of E71A revealing a stable tetramer (*T*) and unassembled monomeric populations (*M*). ΔC-T and ΔC-M represent tetramer and monomer lacking 125–160 residues, respectively

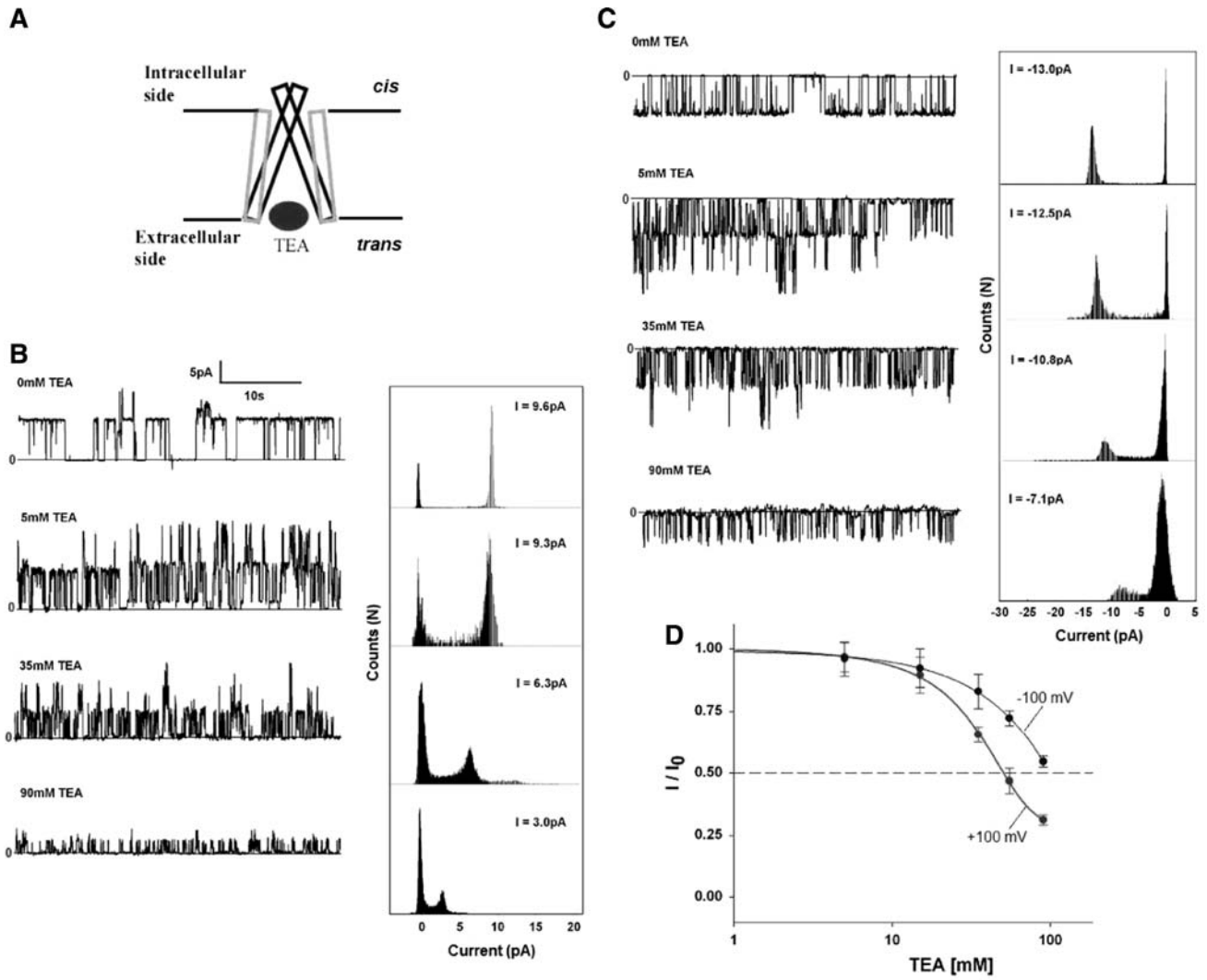

Fig. 4 **a** A schematic representation of KcsA-E71A channel depicting channel orientation in the membrane for extracellular TEA blocking experiments. Representative single-channel current traces of E71A in a planar lipid bilayer in the presence of symmetrical 150 mM K^+ and extracellular TEA (ranging 0–90 mM) at 100 mV (**b**) and −100 mV (**c**) applied potential. The channel closed state is denoted by 0. **b, c** (*left panel*) Detailed amplitude histograms of representative recordings shown in *right panels*. **d** Current remaining (I/I_0) was plotted against the concentration of TEA at +100 mV (*gray circle*) and −100 mV (*black circle*). $K_{1/2}$ was calculated as 50.5 and ~100 mM for +100 and −100 mV, respectively. All data points correspond to the average ± SD of at least three experiments

recordings from single-channel measurements and their respective amplitude histograms at +100 and −100 mV, respectively. Upon addition of 5–15 mM TEA to the *cis* side, the channel current significantly decreased at +100 mV and no significant current was detected in the range of 35–90 mM TEA. At −100 mV, addition of 5 mM TEA exhibited no significant channel blocking; however, the channel current was drastically decreased upon addition of 15 mM TEA and again no significant current could be detected at 90 mM TEA. Figure 6d represents a remaining current (I/I_0) as a function of TEA concentration. The $K_{1/2}$ values for TEA inhibition were 10.3 and 9.2 mM at +100 and −100 mV, respectively. These results are quite different from the WT-KcsA behavior which exhibited the $K_{1/2}$ value of ~25 mM for intracellular TEA channel inhibition (Heginbotham et al. 1999; Kutluay et al. 2005).

Effect of Anionic Lipid PG on Channel-Gating Properties of E71A

The influence of the lipid environment on ion channel properties (gating, conductance and inactivation) has become evident in the past few years (Lee 2005). These studies provide useful insight into structural rearrangements during KcsA-channel gating and into how K^+ ions are transported through the channel pore (Cordero-Morales et al. 2006a, b). From TEA binding data, we assumed that the altered pore conformation in E71A-KcsA might alter

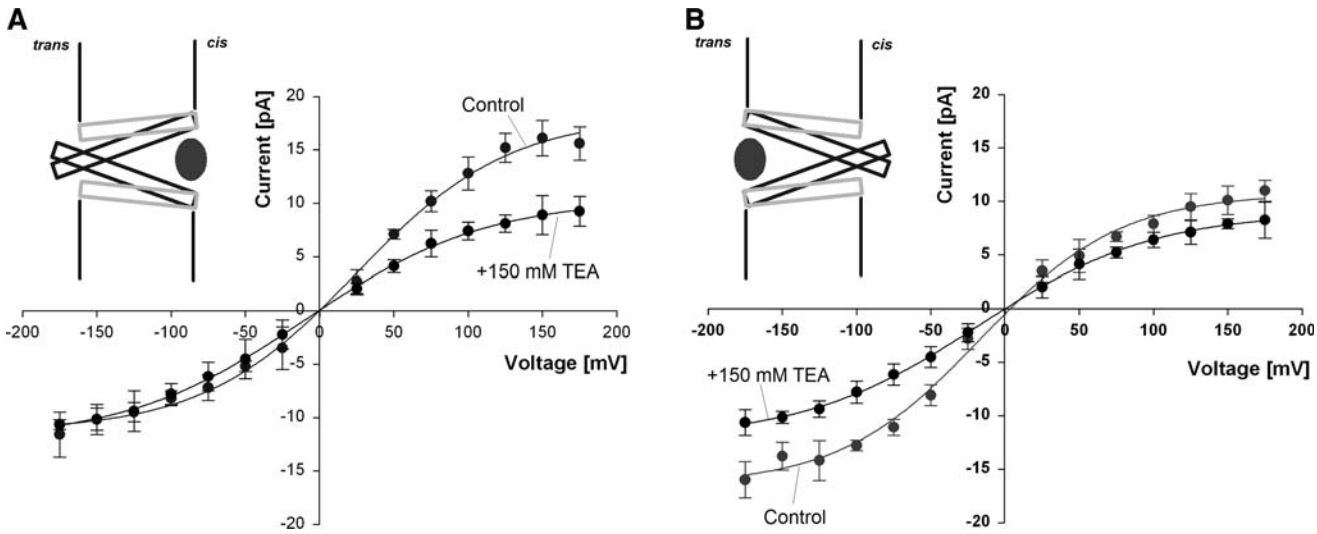

Fig. 5 Open-channel *I–V* relation from single-channel oriented in either the *cis* (**a**) or the *trans* (**b**) direction in symmetrical 150 mM K^+ in the presence and absence of 150 mM extracellular TEA. All data points correspond to the average ± SD of at least three experiments

the interaction mode of anionic lipid PG around the channel pore, hence affecting the channel-gating properties differently. Therefore, E71A was reconstituted in bilayers of PC with varying concentration of PG (20–40 mol%). Based on extra- and intracellular TEA blocking experiments, it is obvious that channels facing their intracellular side (bundle cross region) to the side where TEA was added were efficiently blocked at relatively lower concentrations of TEA. We also found that the single-channel activity differed in a planar lipid bilayer formed from PC and PG since two conductance levels were observed simultaneously, indicating that channels are oriented bidirectionally, which is different from the situation when E71A is inserted in a lipid bilayer formed from *E. coli* membranes. Therefore, 10 mM TEA was also added in order to block any channel which might be facing its intracellular side to the side where TEA was added. Thus, channels facing their extracellular side to the side of the membrane where TEA was added remained unaffected at this concentration of TEA. Figure 7a illustrates representative single-channel current traces at +100 mV and respective amplitude histograms of E71A reconstituted in PC lipid bilayers in the presence of varying concentration of PG (20–40 mol%). At 20 mol% PG the single-channel current was recorded as ~13.8 pA, which was drastically decreased to ~6.8 pA in the presence of 40 mol% PG in PC bilayers. In addition, we observed frequent "flickering" behavior, especially at 20 mol% PG, as if the channel goes through fast gating kinetics while switching between conductive and nonconductive states. However, this flickering characteristic seems to disappear upon increasing PG content to 35–40 mol%. It is also interesting to note that the single-channel characteristics of E71A in a planar lipid bilayer formed from *E. coli* lipid are quite similar to channel characteristics observed for PC:PG (70:30 mol%) lipid bilayer, which mimics biologically relevant PG contents (mol%) in natural membranes.

Furthermore, we determined mean open time (τ_{open}) as a function of PG concentration in a lipid bilayer. Figure 7b represents open channel histograms in which τ_{open} significantly increases to ~3.3 s in the presence of 40 mol% PG compared to the τ_{open} value of ~0.2 s in the presence of 20 mol% PG. Increasing PG content also led to a marked increase in the open probability (N*P*o), which is quite similar to what has been observed in WT-KcsA (Marius et al. 2008). As shown in Fig. 7c, the open probabilities in a bilayer of 40% and 15% PG were ~0.98 and ~0.75, respectively. Despite any uncertainties in the measurements, it is clear that the relationship between N*P*o and PG content shows cooperativity. These results clearly indicate that increasing PG concentration in the membrane decreases the channel conductivity and rather increases the channel mean open time and open probability, suggesting that PG stabilizes the open channel conformation of E71A-KcsA differently from WT-KcsA channel (see "Discussion").

Discussion

Quaternary alkyl ammonium ions like TEA provide an indispensable tool for understanding conduction properties in a selective potassium channel. KcsA has been extensively studied to investigate TEA blocking effects on ion permeation behavior (Lenaeus et al. 2005; Kutluay et al. 2005; Crouzy et al. 2001). In the present study, we

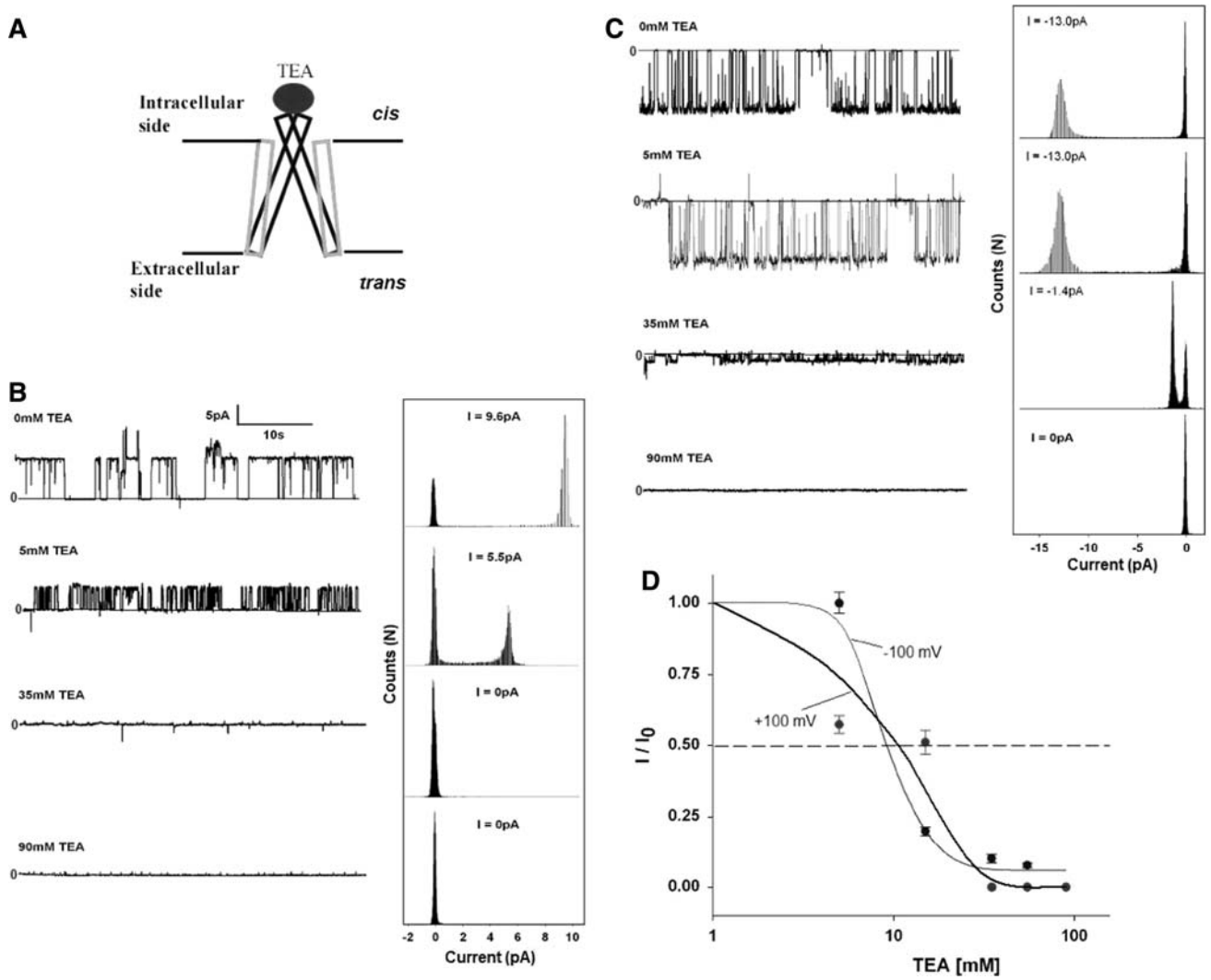

Fig. 6 **a** A schematic representation of KcsA-E71A channel depicting channel orientation in the membrane for intracellular TEA blocking experiments. Representative single-channel current traces of E71A in a planar lipid bilayer in the presence of symmetrical 150 mM K^+ and intracellular TEA (ranging 0–90 mM) at 100 mV (**b**) and −100 mV (**c**) applied potential. The channel closed state is denoted by 0. **b, c** (*left panel*) Detailed amplitude histograms of representative recordings shown in *right panels*. **d** Current remaining (I/I_0) was plotted against intracellular TEA concentration at +100 mV (*gray circle*) and −100 mV (*black circle*) applied potentials. $K_{1/2}$ was calculated as 10.3 and 9.2 mM for +100 and −100 mV, respectively. All data points correspond to the average ± SD of at least three experiments

investigated whether KcsA-E71A can be blocked more efficiently from its intracellular side, which is not the case for WT-KcsA as it is efficiently blocked by extracellular TEA (Heginbotham et al. 1999). Thus, inefficient extracellular blocking behavior of E71A can be well explained on the basis of distinct conformational changes in E71A due to loss of the stabilizing hydrogen bond network and the existence of two distinguishable conformations (see schematic models in Fig. 8a, b). It has been proposed that H-bonds are likely to influence the conformation of residues Asp80–Pro83, which in turn can trigger deformation of the selectivity filter (Cordero-Morales et al. 2006b). However, Tyr82, which is a key residue in determining extracellular TEA binding (Crouzy et al. 2001), also lies in the region of Asp80–Pro83. We suggest that a change in the conformation of these residues might directly influence extracellular TEA binding. In KcsA, TEA binding prevents the formation of an inactivated state (Lenaeus et al. 2005), which is not present in E71A, suggesting that extracellular blocking behavior is related to an inactivation process.

It has been shown by van der Waals energy profile that TEA easily fits in the cavity of KcsA. However, the closed KcsA structure was used for this calculation, which could not address the question of where along the cavity TEA binds when inducing intracellular block. Interestingly, E71A exhibited efficient intracellular blocking by TEA

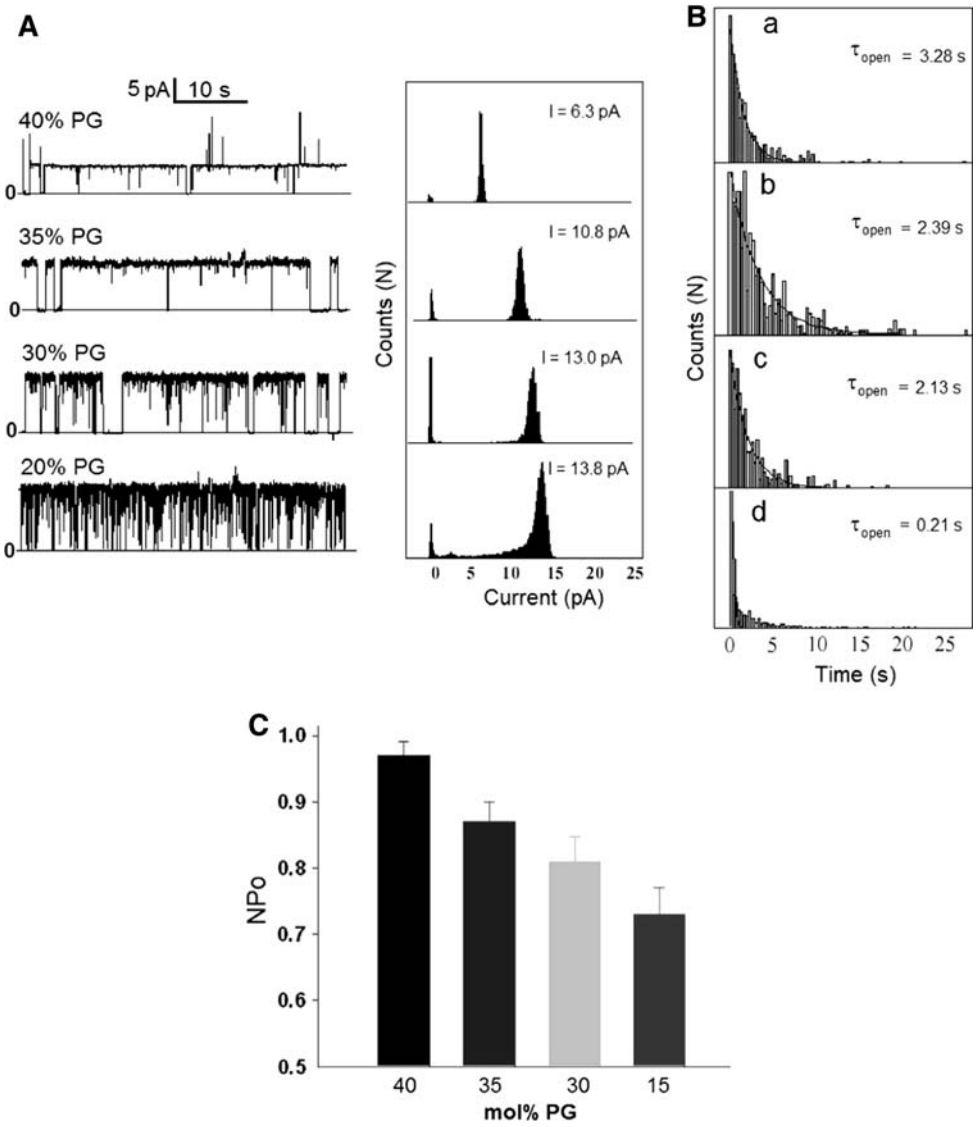

Fig. 7 **a** Representative single-channel recordings (*left panel*) and amplitude histograms of respective recordings (*right panel*) of KcsA-E71A reconstituted in PC bilayers with varying amounts of PG (mol%) in symmetrical 150 mM K^+ and 10 mM extracellular TEA. Traces were recorded at +100 mV potential. **b** Representative open-time histograms of channels shown in **a**. Samples include E71A reconstituted in PC:PG (mol%) of (*a*) 60:40, (*b*) 65:35, (*c*) 70:30 and (*d*) 80:20. Time constants were determined from single-exponential fits to the histograms. **c** Open probability (N*P*o) vs. PG concentration determined at +100 mV. All data points correspond to the average ± SD of at least three experiments

compared to WT-KcsA (Kutluay et al. 2005), suggesting that this blocking behavior is most likely related to the constitutively open state of E71A.

E71A is affected not only by the TEA blocking behavior at both sides of the channel but also by PG-dependent channel-gating properties. The interaction of charged lipids significantly alters the channel-gating behavior of ion channels (Lee 2005). This interaction might be quite specific: One way of achieving selective binding of both hydrophobic and hydrophilic ends of the lipid molecule is via deep cavities in the protein, especially between alpha-helical transmembrane domains. In KcsA, Arg64 and Arg89 close to the selectivity filter region are known as "binding sites" of anionic PG (Marius et al. 2008). Furthermore, it was shown that the presence of PG increases the open channel conductance as well as the open probability for the channel (Marius et al. 2008). Such a phenomenon was explained on the basis of charge effects on the concentrations of K^+ close to the membrane surface and pore of the channel, which might result in an increased conductance at varying concentrations of PG in the membrane. However, the possibility of direct effects of anionic lipids on channel properties could not be ruled out in their studies.

In fact, we observed a different phenomenon. In E71A-KcsA, an increase in PG content significantly decreased the single-channel conductivity, which is in contrast to WT-KcsA behavior. However, similar to WT-KcsA, increasing PG also increased the mean open time and channel open probability, possibly due to the fact that charged PG head groups affect the charge repulsion between positively charged Arg residues. It seems that structural arrangements have a drastic effect on ion occupancy in the selectivity filter. Mutation of Arg64 in

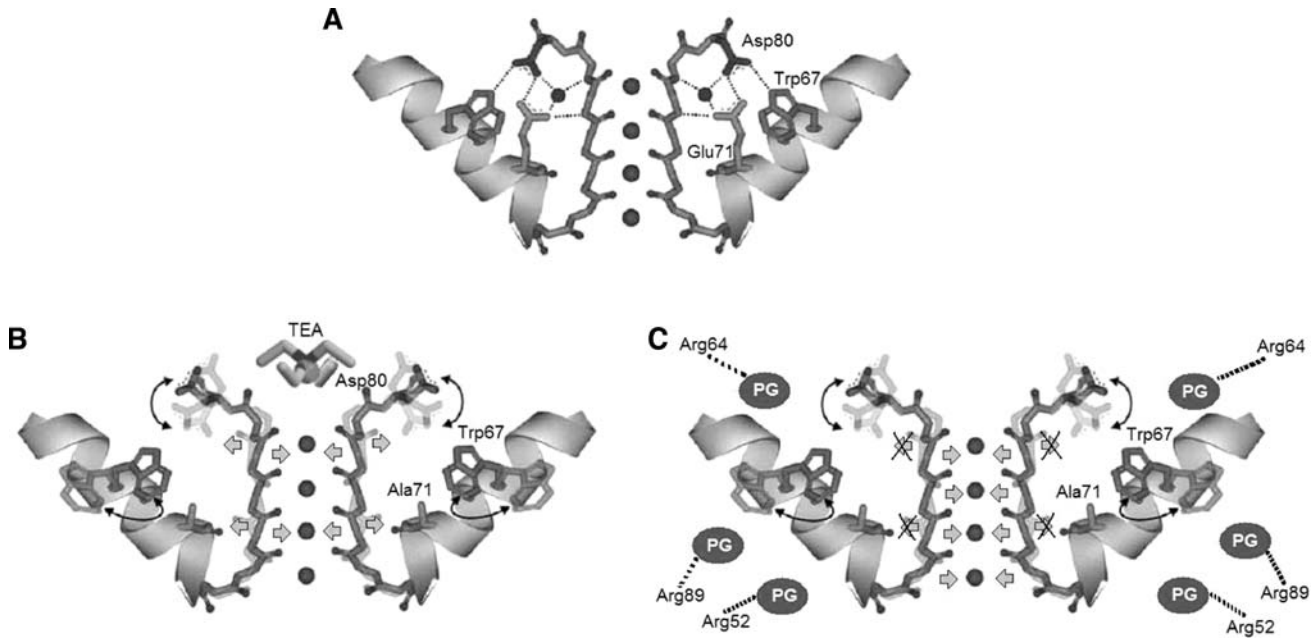

Fig. 8 A mechanistic interpretation of KcsA-E71A gating as a function of TEA and PG interaction. **a** In WT-KcsA the interaction between Asp80 and Trp67 destabilizes the conductive state of the filter and promotes inactivation. **b** Eliminating the Asp80-Trp67 carboxyl–carboxylate by E71A disrupts the hydrogen bonding network between the signature sequence and the pore helix (Cordero-Morales et al. 2006b). Such changes allow significant movement, leading to either the "flipped" or the "unflipped" conformation of the pore region, which hinders extracellular TEA from interacting with the channel pore. **c** Increase in PG content presumably stabilizes the "unflipped" conformation of the filter via electrostatic interaction with the positively charged Arg residues, which presumably pushes the filter region toward the pore center and reduces ion movement across the channel

KcsA, close to the protein–protein interface in the homotetrameric structure, also resulted in channels with an increased open probability, which is similar to the effect of E71A mutation (Cordero-Morales et al. 2006b). These observations indicated that packing of KcsA subunits at the interface influences channel opening. Furthermore, it was also suggested that binding of anionic lipids at the protein–protein interface affects packing at the interface and might therefore also be expected to affect channel opening. We also propose that the flipped state in E71A favors the decreased channel conductivity at higher PG content via increased accessibility at the protein–protein interface or via establishing stronger electrostatic interactions with other positively charged residues close to the transmembrane surface (see schematic model in Fig. 8c). The presence of a histidine tag at the C-terminal end does not, however, interfere with the channel activity in the presence of anionic PG. The reason is that the C-terminal bundle points away from the membrane surface and remains whole during gating (Uysal et al. 2009), which eliminates any potential effects due to electrostatic interaction between the histidine tag and anionic PG on channel gating.

However, a word of caution is necessary. The amount of 10 mM TEA was used in PG-dependent channel-gating kinetics to select channels in one orientation. There is a possibility that the decreased channel conductivity we observed in our experiments is an effect of TEA accumulated close to the membrane surface because of higher PG content. Therefore, the Gouy-Chapman approach (Aveyard and Haydon 1973) was applied to calculate the approximate concentration of TEA close to the membrane surface, which is presented as

$$C = C_0 \, e^{-\varphi/26\text{mV}} \quad (2)$$

where C describes the concentration of TEA (mM) close to the membrane, C_0 is the concentration in the bulk solution and φ is the surface potential of the membrane (mV). From the equation RT/zF, 26 mV is derived, where R describes the gas constant, T is the absolute temperature, z is the valence of the ion and F denotes the Faraday constant. If the membrane contains 20% negatively charged lipids and 10 mM TEA is present in the bulk solution, the TEA concentration close the membrane surface would be 22 mM. Similarly, if 40% negatively charged lipids are present in the membrane and 10 mM TEA is present in the bulk solution, the TEA concentration close to the membrane would be 67 mM. If 35 mM TEA is present in the bulk solution and the membrane contains 20% charged lipids, the accumulation of TEA close to the membrane would be 70 mM, which is quite similar to the concentration of TEA close to the membrane surface, which

contains 40% charged lipids and there is 10 mM TEA present in the bulk solution. Thus, it can be ruled out that the decreased channel conductivity at high PG is just an effect of accumulation of TEA close to the membrane surface. This estimation is also supported by the fact that in additional experiments 60% PG resulted in a drastic decrease in channel conductivity in the absence of TEA (not shown).

Another interesting effect of E71A-KcsA is that we observed a "flickering" behavior—a fast switch between "permeable" and "nonpermeable" states—between 15% and 30% anionic lipid content. Therefore, a stochastic model of the selectivity filter can be assumed in which the channel alternates between a cation-bound conformation (to hold ions) and a cation-free conducting conformation (to release ions). In the presence of high PG content in the membrane, such a fast change in conformational dynamics seems to disappear, leading to a stable permeable state. This seems to be a common motif in KcsA, signifying the role of two conformational states which exist both in the selectivity filter and near the C-terminal ends of the TM2 helices (Baker et al. 2007).

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