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Mutations in the K^+-Channel KcsA Toward Kir Channels Alter Salt-Induced Clusterization and Blockade by Quaternary Alkylammonium Ions

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Abstract Protein aggregation is a result of malfunction in protein folding, assembly, and transport, caused by protein mutation and/or changes in the cell environment, thus triggering many human diseases. We have shown that bacterial K^+-channel KcsA, which acts as a representative model for ion channels, forms salt-induced large conductive complexes in a particular environment. In the present study, we investigated the effects of point mutations in the selectivity filter of KcsA on intrinsic stability, aggregation, and channel blocking behavior. First, we found that a low sodium chloride concentration in potassium-containing media induced fast transfer of single channels to a planar lipid bilayer. Second, increasing the sodium chloride concentration drastically increased the total channel current, indicating enhanced vesicle fusion and transfer of multiple channels to a planar lipid bilayer. However, such complexes exhibited high conductance as well as higher open probability compared to the unmodified KcsA behavior shown previously. Interestingly, the affinity of aggregated complexes for larger symmetric quaternary alkylammonium ions (QAs) was found to be much higher than that for tetraethylammonium, a classical blocker of the K^+ channel. Based on these findings, we propose that mutant channel complexes exhibit larger pore dimensions, thus resembling more the topological properties of voltage-gated and inwardly rectifying K^+ channels.

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The conversion of proteins into highly insoluble aggregates is associated with at least 25 well characterized human disorders, including Alzheimer's and Parkinson's diseases and various systemic amyloidoses (Kourie and Henry 2001). It has also been shown from in vitro experiments that mutations can favor amyloid formation when they facilitate the subsequent step in the aggregation process of globular proteins (or the primary step for natively unfolded proteins or for unstructured peptides), i.e., the conversion of unfolded or partially folded states into oligomeric species. Aggregation in such cases has been found to be facilitated by mutations that either increase the hydrophobicity of the polypeptide chain or its propensity to convert from α-helical to β-sheet structure or decrease the overall net charge on the protein molecule (Tjernberg et al. 2002; Chiti et al. 2003).

The oligomerization of protein macromolecules on cell surfaces is believed to play a fundamental role in the regulation of cellular function, including signal transduction and the immune response (Ullrich and Schlessinger 1990; Metzger 1992). A better understanding of the formation of well-defined homo- and hetero oligomers as well as larger aggregates of membrane proteins could enhance our insight into regulatory processes (Veenhoff et al. 2002). Single or multiple amino acid mutations can significantly change the stability of a protein structure. Thus, biologists and protein designers need accurate predictions of how amino acid mutations affect the stability and function of a protein structure (Splitt et al. 2000; Looger et al. 2003).

It is well known that K^+-channel KcsA forms an extremely stable tetrameric complex even in the presence of a detergent like SDS (Splitt et al. 2000). However, mutations within the pore region of KcsA significantly affect the tetrameric stability and channel activity (Splitt et al. 2000; Zhou and MacKinnon 2004). One useful tool to investigate the channel folding properties is to functionally probe the pore of a K^+ channel using a group of TEA analogues collectively referred to as quaternary ammonium ions (QAs). Recently, we have shown that KcsA forms large conductive complexes in the presence of salt and such complexes are efficiently blocked by TEA, suggesting that the inner pore properties of KcsA channels are not affected in aggregated complexes (Raja and Vales 2009a). In the present study, we investigate the effect of mutations on protein stability as well as on salt-induced protein aggregation of the mutant channel and compare it to wild-type (WT) KcsA channel behavior (Raja and Vales 2009b). We demonstrate that mutant channel complexes exhibit distinct properties with regard to their inner pore dimensions. We also compare channel inhibition properties of mutant protein by QAs with an inhibitor binding mode of eukaryotic K^+ channels, especially inwardly rectifying and voltage-gated K^+ channels, and propose that their topological properties are similar to those of the KcsA mutant channel in aggregated form.

Materials and Methods

Reagents

Escherichia coli total lipid extract was purchased from Avanti Polar Lipids Inc. DM (*n*-decyl-*β*-D-maltoside) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonatewere from Fluka. Nickel nitriloacetic acid (Ni^{2+}-NTA) agarose beads were obtained from Qiagen. The 100-nm membrane filters were obtained from Avestin Inc. The following chemical reagents were purchased from either Fluka (Switzerland), Merck (Germany), or Aldrich (Germany), available at the highest purity: Tris, KCl, NaCl, imidazole and isopropyl-*β*-D-thiogalactopyranoside. Triethylammonium (TEA), hexyl-TEA, and decyl-triethylammonium were obtained from Sigma.

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 2009b). 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 as described previously (Raja and Vales 2009b). 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. The purity of protein was assessed by lithium dodecyl sulphate (LDS)-polyacrylamide gel electrophoresis (PAGE).

Analysis of Channel Stability by LDS-PAGE

To facilitate detection of channel oligomerization gel electrophoresis experiments were performed in LDS detergent at low temperatures (4–8°C) 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 from *E. coli* total lipid extract (Avanti Polar Lipids) 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 2009a). Briefly, SUVs (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-solubilized KcsA mutant protein at a 1:200 protein:lipid molar ratio. The detergent was removed by dialysis. 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).

Analysis of Channel Activity and Channel Blocking

Functional reconstitution of KcsA mutants into a bilayer made from *E. coli* lipids (Avanti Polar Lipids) was confirmed by simultaneous measurements of membrane conductance (Schindler 1989). Briefly, planar lipid bilayer was 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 in diameter) in a homemade Teflon chamber between aqueous bathing solutions of vesicle buffer (pH 4.0). After bilayers were formed, proteoliposomes (with a protein concentration of ~5 μg/ml) were added to one (cis) side of a preformed planar membrane, with gentle mixing. As a control, similar experiments were also performed with planar lipid bilayer without channel protein. All experiments were performed at room temperature. All recordings and data analysis were carried out

according to the procedure described previously (Raja and Vales 2009a).

Results

The present study was primarily set out to explore the role of selectivity filter in determining the pore properties in a selective K^+ channel by mutating specific residues in KcsA toward eukaryotic inwardly rectifying K^+ (Kir) channels (Fig. 1), including ROMK1 and Kir6.1/6.2, and in addition, we wanted to investigate the effect of mutations on channel folding kinetics in aggregated complexes as a function of inhibitor binding.

Salt-Induced Vesicle Fusion and Transfer of Channels to a Planar Lipid Bilayer

We first checked the activity of mutant protein in a planar lipid bilayer formed from *E. coli* membranes. In principle, if channels fail to appear within 5 min, the bilayer is ruptured and the procedure is repeated. Typically, channels are observed in ~50% of such attempts (Heginbotham et al. 1999). From our experience, it seems that efficient channel transfer to planar lipid bilayer depends on protein stability and proper folding. However, we also observed that the solubility of mutant protein differed significantly in different protein expression trials, which might be related to improper folding or insertion of the mutant channel in the inner membrane during protein expression in the *E. coli* host.

After several attempts at rupturing and forming planar bilayers we did not observe channels. Interestingly, we found that the addition of 5–10 mM Na^+ in symmetrical 150 mM K^+ results in the appearance of channels in a short period of time, which could be due to the effect of NaCl on fast vesicle fusion and transfer of channels to a planar lipid bilayer as reported previously (Raja and Vales 2009a). We could not monitor single channels, suggesting that more than one or multiple channels, presumably in aggregated form, are transferred to a planar lipid bilayer. The channel current traces for ±100 and ±150 mV in symmetrical 150 mM K^+ and 10 mM Na^+ are illustrated in Fig. 2a. Figure 2b represents amplitude histograms of recordings shown in Fig. 2a. A chord conductance of 135 pS for ±100-mV and zero-voltage conductance of 140 pS (Fig. 2c) were recorded. In addition to the main current level (20–22 pA), several additional sublevels were also observed at +150 and −150 mV. The appearance of several current levels indicated that channels either are oriented bidirectionally or have several different conductance sizes, suggesting that oligomers/aggregates of varying number and size formed irregular channel structures. It is important to mention that the protein:lipid molar ratios used in our study to demonstrate salt-induced vesicle fusion and channel aggregation are quite similar to those used by other groups to show single-channel activity of KcsA channels (Cortes et al. 2001; Heginbotham et al. 1999). Similar results were obtained for different protein:lipid molar ratios, varying from 1:200 to 1:5,000 (not

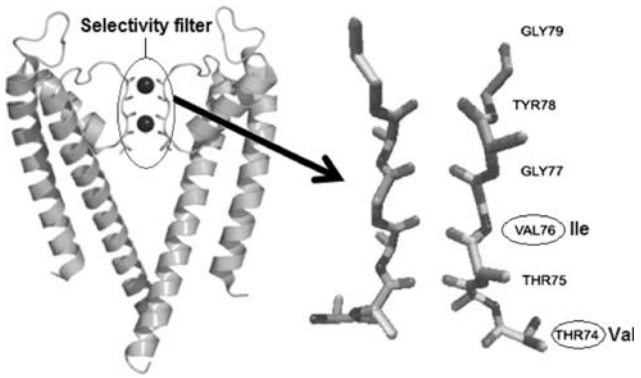

Fig. 1 The closed-state structure of the K^+-channel KcsA is shown on the *left-hand side* and a close view of selectivity filter on the *right-hand side*. In the filter, the residues are numbered according to their sequence in KcsA. The positions of Thr-74 and Val-76 are highlighted which were mutated to their counterparts Val and Ile, respectively, in Kir channels

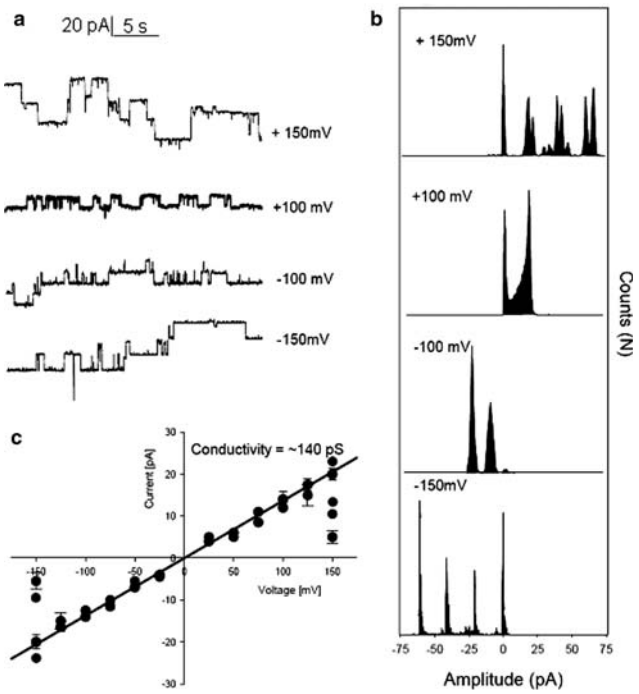

Fig. 2 **a** Single- and multiple-channel current recordings of mutant KcsA in symmetrical 150 mM K^+ and 10 mM Na^+ at the voltages indicated. **b** Detailed amplitude histograms of recordings shown in (**a**). **c** Open-channel *i–V* relation. All data points correspond to the average ± SD of at least three experiments. The *solid curve* has no theoretical meaning

shown), suggesting that aggregation phenomenon is not dependent on high number of protein molecules present in a vesicle.

Channel Activity of Aggregated Complexes

Although low salt induced fast transfer of channels of variable conductances, we expected that increasing salt concentrations might eventually block the channel activity since Na^+ is known to be a concentration-dependent and strong voltage-dependent blocker of KcsA (Heginbotham et al. 1999). This was not observed. Upon increasing the NaCl concentration (50 mM), the mutant channel exhibited increased channel conductivity and a high open probability (HOP). Figure 3a (upper panel) shows a representative recording from the mutant channel containing a bilayer in the presence of 50 mM NaCl at a +100-mV potential. Most strikingly, after ~30 s (patch 1), the total internal current was increased and multiple channels were recorded. For instance, the total internal current drastically increased, to ~450 pA (patch 1), and later several other current levels were recorded (patches 2–4). Upon analysis of patch 3 (Fig. 3b, left panel), a main current level of ~20 pA (~200 pS at this voltage) was observed. Furthermore, an open probability of ~0.85 was calculated as shown in the amplitude histogram (Fig. 3b, right panel). Approximately 10 s after application of a 100-mV potential (Fig. 3a, lower panel), the current level increased to ~500 pA. Patch 1 exhibited channels of a HOP pattern, which were followed by the opening of patches 2 and 3, consisting of variable channels. The ~22-pA current was the most frequently observed event throughout the whole recording as determined by analyzing patch 3 (level II; Fig 3c, left panel). In addition, another 'subconductance' or short-term closing event, of ~10 pA (level I), also occurred. The data indicate that NaCl induces fast vesicle fusion to the planar lipid bilayer, resulting in an increase in the mutant–channel current. These patterns exhibited well-defined channels with short closing and long opening that were stable for several hours at room temperature. It was also found that apparently NaCl-induced vesicle fusion with the planar lipid bilayer and channel aggregation do not depend on the ion/concentration gradient between the inside and the outside of the vesicle. Similar results were obtained when a proteoliposome mixture was premixed with NaCl in K^+ solution beforehand to form a planar lipid bilayer under symmetric or asymmetric concentration gradient conditions.

Furthermore, the increase in channel current clearly indicated that NaCl induces efficient vesicle fusion and eventually transfer of a large number of channels to a planar lipid bilayer. This observation is quite similar to behavior reported previously for the WT-KcsA channel

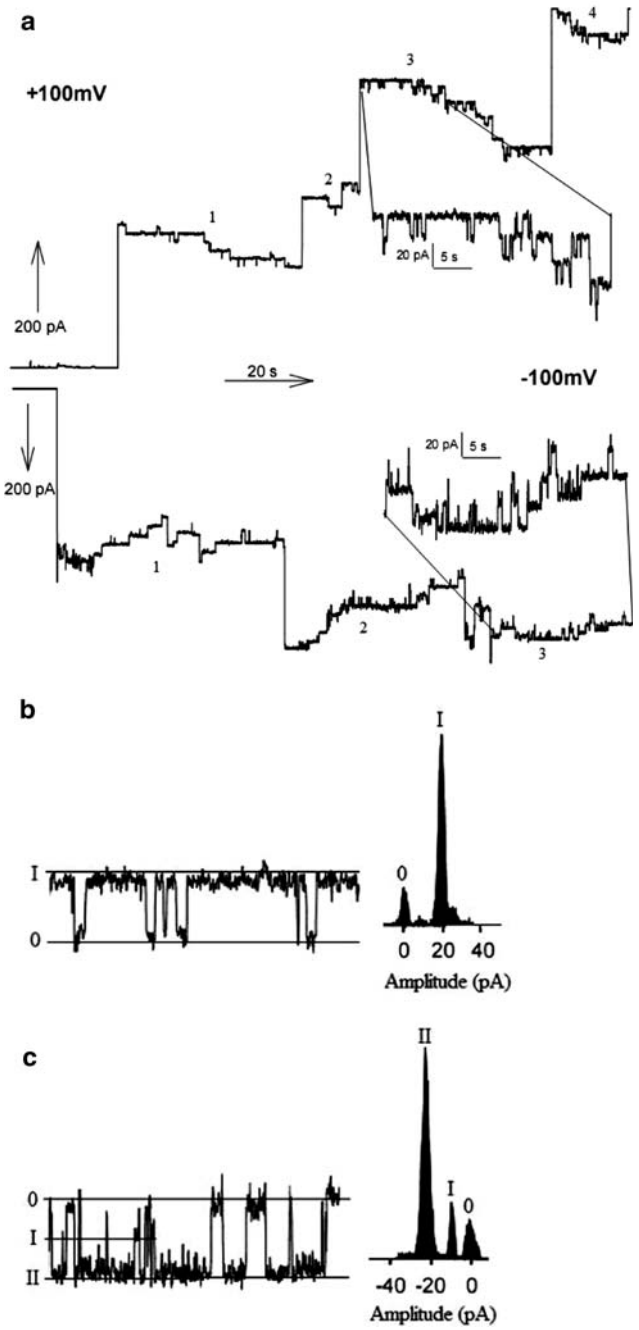

Fig. 3 **a** Induction of mutant channel aggregation upon addition of 50 mM salt in *E. coli* membranes. Representative whole records after applying +100- and −100-mV voltages are shown. Several patterns are indicated by *numbers*. **b** Closeup view of patch 3 from multiple channel patterns with a detailed histogram of the recording showing the open probability of patch 3 at +100 mV. **c** Closeup view of patch 3 from multiple channel patterns with a detailed histogram of the recording showing the open probability of patch 3 at −100 mV. Traces were analyzed to determine the single-channel current (pA) and open probability (P_o). The closed state is indicated by '0'

(Raja and Vales 2009a). However, it should be noted that the mutant channel exhibited significantly higher total current, higher channel conductance, and higher open

Table 1 Comparison of total current, channel gating kinetics, and channel blocking by QAs in WT and mutant KcsA

Protein	Total current[a]	Channel current[a]	NPo[a]	TEA blocking[b]	Hexyl-TEA blocking[b]	Decyl-TEA blocking[b]
WT-KcsA[c]	~155 pA	~8 pA	~0.5	~5 mM		
Mutant	~1000 pA	~20 pA	~0.85	–[d]	~8 mM	~2.5 mM

[a] Parameters were determined from representative recordings at +100 mV in the presence of 150 mM KCl and 50 mM NaCl

[b] Refers to the concentration of QAs determined from Figs. 5b, 6b, and 7b at which the channel current was blocked by 50%

[c] Parameters for WT KcsA were derived from data described in a study (Raja and Vales 2009a)

[d] Refers to an unknown concentration of TEA which could not be achieved to block the channels at +100 mV

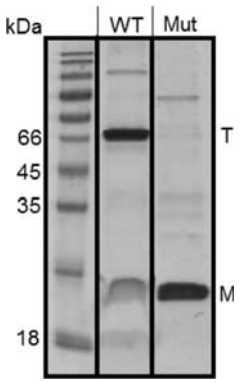

Fig. 4 Determination of channel aggregation/clusterization in the presence of 150 mM K^+ and 50 mM Na^+ by LDS-PAGE (11% gel). The positions of the molecular mass marker (kDa), tetramer (T), and monomer (M) are indicated

channel probability in oligomeric complexes compared to WT KcsA (see Table 1), which is indicative of efficient aggregation or oligomerization of channel as a function of mutations.

Analysis of Channel Aggregation by Gel Electrophoresis

We next determined if mutant aggregation can be monitored by gel electrophoresis. Figure 4 shows the LDS gel of the purified mutant channel in the presence of 150 mM K^+ and 50 mM Na^+. As a control, WT KcsA is also shown, which yields a stable tetramer running at ~68 kDa. It is obvious that the mutant tetramer is unstable and dissociates into its monomers (~18 kDa), indicating that the energy barrier for conversion of monomers to tetramer is drastically decreased. Furthermore, some other species of higher molecular weight were also observed which indicate oligomeric/aggregated complexes. However, the sizes of such complexes differ in the two proteins, which could be due to altered stability of such complexes or instability of the tetrameric structure of the mutant compared to WT KcsA. This observation is well supported by the fact that in additional experiments NaCl-containing proteoliposomes were centrifuged and the resultant pellet was detected as aggregated complexes running at the top of the gel (not shown).

Effect of TEA on Aggregated Complexes of the KcsA Mutant Channel

TEA has long been known to be a potent blocker of K^+ currents flowing across the cell membrane. TEA has been widely used to separate different components of the membrane currents and to probe the dynamics of ion permeation across K^+ channels (French and Shoukimas 1981). We assumed that channel aggregation might alter the function of mutant KcsA. Because of TEA's unique tendency to block KcsA (Guidoni and Carloni 2002), we next performed TEA blocking experiments. After channel aggregation TEA was added to the cis side of the chamber. Figure 5a shows representative records of the effect of 55 and 120 mM TEA at +100 and −100 mV. Figure 5b represents the current-concentration curve, in which i/i_0 denotes the remaining current as a function of TEA concentration. It is clear that channels are not efficiently blocked even at higher concentrations of TEA, compared to WT-KcsA clusters, which are efficiently blocked by 10 mM TEA (see Table 1). A decrease in single-channel current by ~4 pA was calculated at 120 mM TEA compared to the control reaction (~20 pA in the absence of TEA). However, at a negative potential (−100 mV) the single-channel current decreased to 8–10 pA, suggesting that most of the channels are blocked and therefore pass less current at a negative potential, possibly due to the altered TEA binding mode of mutant channel complexes (see Table 1).

It seems that aggregated complexes are, apparently, deformed since they exhibit a lower affinity to TEA. How these mutations affect TEA binding is unclear. Perhaps mutations change the filter conformation, thus making arrangement of the filter in aggregated complexes incompatible for TEA binding.

Effect of Larger Quaternary Ammonium Ions on Aggregated Complexes

TEA insensitivity indicated that mutant complexes presumably exhibit larger pore dimensions, which do not allow the trapping of smaller QAs like TEA. We checked

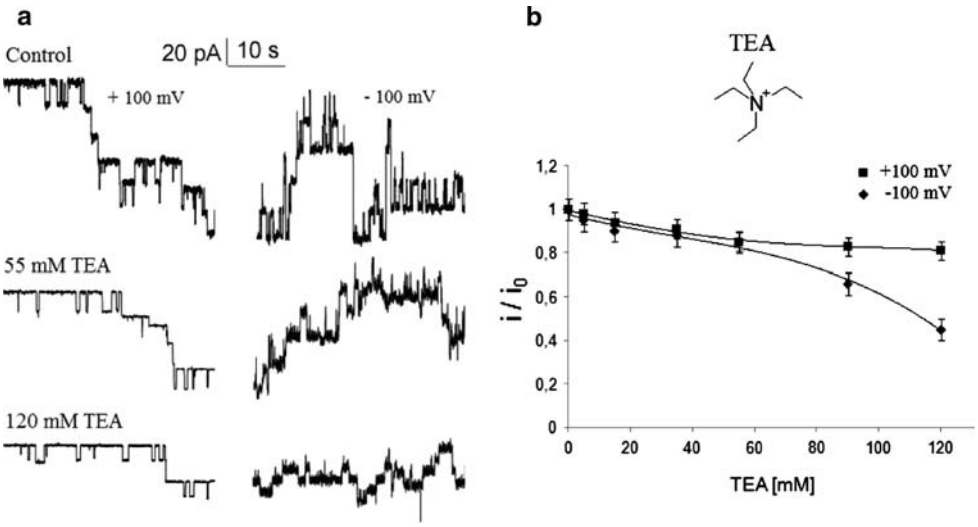

Fig. 5 **a** Effect of different concentrations of TEA on multiple-channel s in oligomeric complexes. TEA inhibition was monitored at potentials of +100 and −100 mV in the presence of 150 mM K^+ and 50 mM Na^+. **b** Current remaining (i/i_0) was plotted against different concentrations of TEA, where i or i_0 denote the channel current in the presence or absence of TEA, respectively. The *curves* through points are sigmoidal fits with no theoretical significance

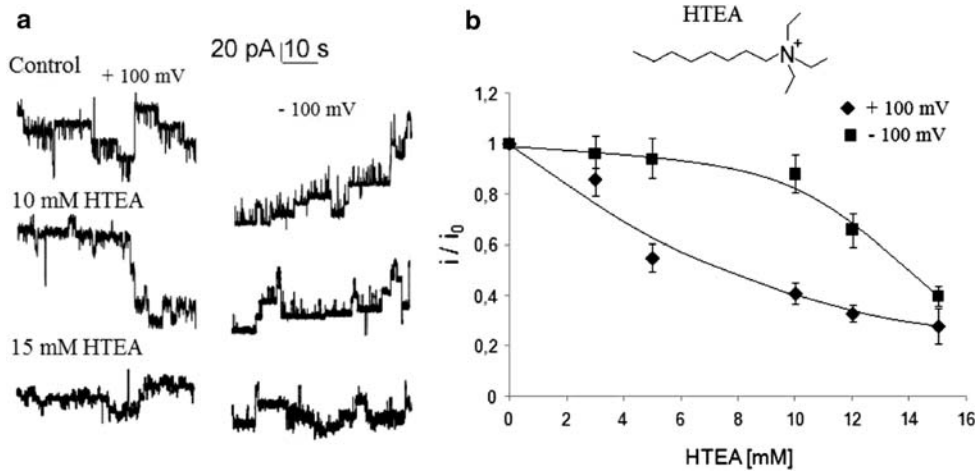

Fig. 6 **a** Effect of increasing concentrations of hexyl-TEA (HTEA) on multiple-channel currents in oligomeric complexes. HTEA inhibition was monitored at potentials of +100 and −100 mV in the presence of 150 mM K^+ and 50 mM Na^+. **b** Current remaining (i/i_0) was plotted against different concentrations of HTEA. The *curves* through points are sigmoidal fits with no theoretical significance

our hypothesis by using larger TEA analogues with longer alkyl side chains, hexyl-TEA and decyl-TEA. For channel blocking, each QA was added to the cis side of the membrane. Figure 6a shows the current traces of mutant complexes in the absence or presence of hexyl-TEA at 10 and 15 mM concentrations. Figure 6b represents the remaining current (i/i_0) as a function of hexyl-TEA concentration. It is obvious that these complexes are efficiently blocked at fairly low concentrations compared to TEA. Interestingly, the extent of channel inhibition increases with increasing QA size. Figure 7a shows the current traces of channel complexes in the absence or presence of a 5 mM DTEA concentration. Figure 7b represents the corresponding remaining current (i/i_0) at different concentrations of DTEA. These data indicate that mutant complexes exhibit a larger pore size, which might be explained by their variable affinities to different QAs (also see Table 1).

Discussion

It is quite evident that sodium chloride plays a crucial role in inducing fast vesicle fusion and, ultimately, aggregation of either the channel itself or a channel-lipid complex. However, the exact mechanism of this phenomenon is not understood. Also, hydration repulsion seems to be the limiting barrier for the fast fusion process in the presence of monovalent ions (Carmona-Ribeiro and Chaimovich 1986). Our data provide preliminary evidence that decreasing channel intrinsic stability upon mutations enhances the rate of protein aggregation as a consequence of distinct conformation of the pore region.

It is interesting to note that single-channel conductance also increases with an increase in total current upon increasing the salt concentration (compare Figs. 2 and 3), suggesting that mutant channels aggregate into

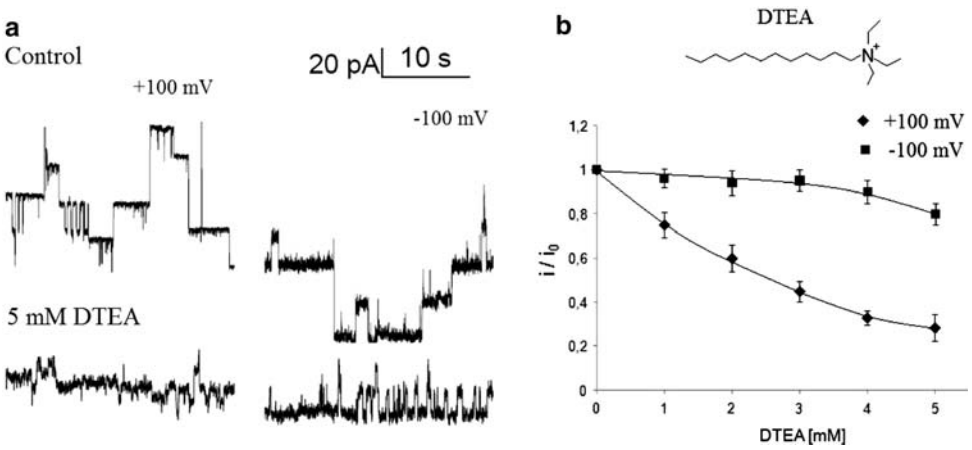

Fig. 7 **a** Effect of increasing decyl-TEA (DTEA) concentrations on multiple-channel currents in oligomeric complexes. DTEA inhibition was monitored at potentials of +100 and −100 mV in the presence of 150 mM K^+ and 50 mM Na^+. **b** Current remaining (i/i_0) was plotted against different concentrations of DTEA. The *curves* through points are sigmoidal fits with no theoretical significance

progressively larger oligomers with increasingly larger sizes and higher channel conductances. Similar phenomenon has been shown for matrix protein clusterization in the planar lipid bilayer (Schindler and Rosenbusch 1981). This seems to be a common motif in channel protein clusterization/aggregation and it has been pointed out in several other studies (Hirakura et al. 1999; Janson et al. 1999). It is also evident that many channels including K^+ channels form multiple complexes of tetrameric unit and function as large clusters in the membrane under physiological conditions (Marx et al. 2001; Jugloff et al. 2000; Laver et al. 2004; Molina et al. 2006).

All voltage-gated and inwardly rectifying K^+ channels were found to be sensitive to QAs applied to the intracellular side; and QA molecules have been well established as open channel blockers, which have access only to channels that are open (Armstrong 1971). For example, the affinity of squid voltage-activated K^+ channels for larger QAs, such as tetrabutylammonium (~10 Å) and tetrapentylammonium (~11 Å), is much higher than that for TEA (~7 Å) (French and Shoukimas 1981). Similar phenomena were observed with ROMK1 inward-rectifier K^+ channels (Oliver et al. 1998), indicating that the inner pore is so wide that it may not sustain single-ion filing. The term 'inner pore' is used either literally or to refer to its narrowest part if the width is nonuniform.

Furthermore, it was shown that the TEA derivative nonyltriethylammonium (C9) can be trapped in the inner pore by the activation gate (Armstrong 1971). Although the Shaker channel itself traps neither TEA nor DTEA (C10) in the closed state, the I470C Shaker mutant channel is capable of trapping them both, as if the smaller cysteine side chain allows more room for TEA or C10 binding (Holmgren et al. 1997). Interestingly, Shaker residue I470 corresponds to a residue in the bacterial KcsA channel that lines the 'cavity' internal to the narrow K^+-selective pore formed by the signature sequence (Doyle et al. 1998). In

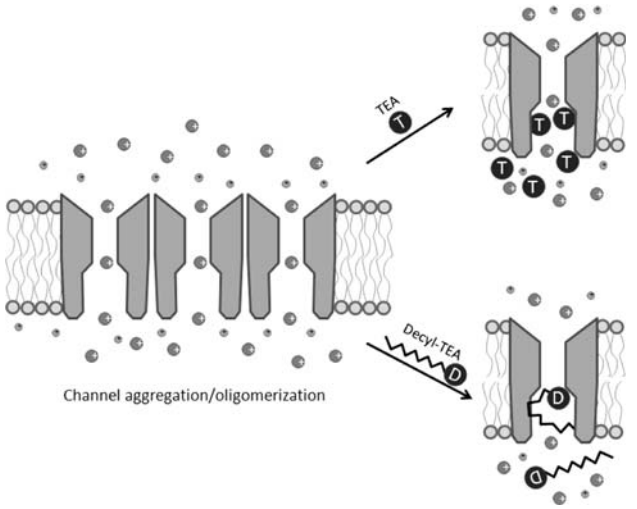

Fig. 8 Scheme depicting channel aggregation in the membrane and interaction of TEA or decyl-TEA with the channel complex. The inner pore diameter allows larger QAs to interact with the channel more strongly than TEA, thus hindering the larger QA's mobility and blocking of ion flow. For convenience, two subunits of each tetrameric structure forming a single channel are shown. The large and small positively charged spheres represent K^+ and Na^+ ions, respectively

light of these findings, we propose that the modified channel pore structure we studied here is probably similar to the one possessed by voltage-gated or inwardly rectifying K^+ channels. We also suggest that the diameter of the inner pore is wide enough to allow QAs larger than TEA to block the pore.

However, there exists uncertainty regarding absolute QA dimension; larger QAs may widen the pore as they elbow their way into it. Furthermore, as a larger QA travels toward its binding site, part of the longer alkyl chain may interact with and penetrate the netlike protein lining of the inner pore (see model in Fig. 8), significantly hindering the mobility of the larger QA and resulting in channel blocking. The larger dimension of the pore depicted in the model

(Fig. 8) is evident from the high conductances as well as the higher open probability. The relationship between the larger dimension of the pore and the high conductance is well supported by the observation that Ca^{2+}-activated K^+ (BK) channels, which exhibit the highest single-channel conductance among all K^+ channels, also exhibit a larger inner pore and the strongest sensitivity to the large QA molecule C10 (Li and Aldrich 2004). Through a combination of techniques applied we present a mutated K^+ channel KcsA with a view of a eukaryotic K^+ channel, as it has been shown that the KcsA channel is structurally very similar to eukaryotic K^+ channels (Capener et al. 2003). It seems that the inner pores of these channels likely share some common architectural features, although critical residues probably account for differences in pore properties among simple-structured KcsA and more complex eukaryotic K^+ channels.

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