

Functional Role and Affinity of Inorganic Cations in Stabilizing the Tetrameric Structure of the KcsA K⁺ Channel

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Crystal structures of the tetrameric KcsA K⁺ channel reveal seven distinct binding sites for K⁺ ions within the central pore formed at the fourfold rotational symmetry axis. Coordination of an individual K⁺ ion by eight protein oxygen atoms within the selectivity filter suggests that ion-subunit bridging by cation–oxygen interactions contributes to structural stability of the tetramer. To test this hypothesis, we examined the effect of inorganic cations on the temperature dependence of the KcsA tetramer as monitored by SDS-PAGE. Inorganic cations known to permeate or strongly block K⁺ channels (K⁺, Rb⁺, Cs⁺, Tl⁺, NH₄⁺, Ba²⁺, and Sr²⁺) confer tetramer stability at higher temperatures ($T_{0.5}$ range = 87°C to >99°C) than impermeant cations and weak blockers (Li⁺, Na⁺, Tris⁺, choline⁺; $T_{0.5}$ range = 59°C to 77°C). Titration of K⁺, Ba²⁺, and other stabilizing cations protects against rapid loss of KcsA tetramer observed in 100 mM choline Cl at 90°C. Tetramer protection titrations of K⁺, Rb⁺, Cs⁺, Tl⁺, and NH₄⁺ at 85°C or 90°C exhibit apparent Hill coefficients (*N*) ranging from 1.7 to 3.3 and affinity constants ($K_{0.5}$) ranging from 1.1 to 9.6 mM. Ba²⁺ and Sr²⁺ titrations exhibit apparent one-site behavior ($N \cong 1$) with $K_{0.5}$ values of 210 nM and 11 μM, respectively. At 95°C in the presence of 5 mM K⁺, titration of Li⁺ or Na⁺ destabilizes the tetramer with $K_{0.5}$ values of 57 mM and 109 mM, respectively. We conclude that specific binding interactions of inorganic cations with the selectivity filter are an important determinant of tetramer stability of KcsA.

INTRODUCTION

All known K⁺-selective channel proteins share a pore domain exemplified by KcsA, a prokaryotic K⁺ channel whose structure has been studied at atomic resolution by X-ray crystallography (Doyle et al., 1998; Morais-Cabral et al., 2001; Zhou et al., 2001; Zhou and MacKinnon, 2003). A key feature of KcsA is the fourfold rotational symmetry axis along the transmembrane pore corresponding to the tetrameric quaternary structure formed by four KcsA monomers (Cortes and Perozo, 1997; Heginbotham et al., 1997; Doyle et al., 1998). It has been speculated that the fourfold coordination axis of a K⁺ ion hydrated by eight H₂O molecules in aqueous solution fundamentally dictates the tetrameric architecture of K⁺-selective channels (Zhou et al., 2001). High-resolution crystal structures of KcsA show that inorganic cations are located at up to seven distinct binding sites along the central pore axis (Zhou et al., 2001). The existence of multiple binding sites for K⁺ arranged in a single file is consistent with a large body of electrophysiological evidence interpreted within the context of multiple occupancy of K⁺ channel pores by permeant cations (Hodgkin and Keynes, 1955; Hille and Schwarz, 1978; Neyton and Miller, 1988; Stampe and Begenisich, 1996). A related question concerns the role of inorganic cations in stabilizing the tetramer. Given that K⁺ ions bind

along the symmetry axis and participate in chemical and electrostatic interactions with the protein, this paper addresses the following questions. (1) What is the functional significance of K⁺ binding for stability of the KcsA tetramer? (2) How do various cations behave with respect to tetramer stabilization?

The most intimate molecular contact between K⁺ ions and the KcsA protein occurs in the selectivity filter, a tunnel-like region 12 Å in length with a diameter of only ~2–3 Å (Doyle et al., 1998), originally hypothesized to be necessary for discriminating K⁺ and Na⁺, the major physiological inorganic cations with Pauling crystal radii of 1.33 and 0.95 Å, respectively (Bezanilla and Armstrong, 1972; Armstrong, 2003). Four sites for K⁺ are located directly within the filter. Counting from the outer vestibule, the first three filter sites, S₁, S₂, and S₃, are formed by four rings of backbone carbonyl oxygen atoms contributed by Y₇₈, G₇₇, V₇₆, and T₇₅ of the signature sequence (T₇₅VGYG₇₉), while the inner S₄ site is formed by both backbone carbonyl oxygens and side chain hydroxyl oxygens of T₇₅ (Morais-Cabral et al., 2001; Zhou and MacKinnon, 2004a). At each of these sites, K⁺ is bound by electrostatic interactions with eight oxygen atoms that form a square antiprism cage around the ion. Since each K⁺ ion in the filter is coordinated by both an “upper” and “lower” oxygen atom from each of the four subunits, for a total of eight oxygens per cation, it may be expected that K⁺

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contributes a certain stabilization energy to the structure of the filter and possibly, to the tetramer itself.

The role of K^+ in maintaining the structure of the filter is supported by crystallographic observations of two different conformations of the selectivity filter, a well-ordered “conducting” conformation observed at high K^+ concentration and a disordered “nonconducting” conformation observed at low K^+ or Tl^+ concentration (Zhou et al., 2001; Zhou and MacKinnon, 2003). The change in conformation of the filter from an open to an apparent closed state appears to be largely driven by repulsive electrostatic interactions of the 20 oxygen atoms of the filter that move away from each other in the absence of stabilizing counterions. Conformational changes of the selectivity filter that depend on the species and concentration of inorganic cations may underlie well-documented functional phenomena of K^+ channels such as ion-dependent gating, C-type inactivation, changes in selectivity, long-lived nonconducting states, and irreversible loss of channel function that occur upon exposure to low K^+ concentration (Almers and Armstrong, 1980; Swenson and Armstrong, 1981; Korn and Ikeda, 1995; Yellen, 1998; Vergara et al., 1999).

Although many aspects of ion–channel interactions have been deduced by electrophysiological assays of K^+ current interpreted by kinetic models and by more recent direct structural analyses, there is relatively little quantitative information on the binding of inorganic cations to K^+ channel proteins in the absence of competing ions. In principle, ion binding measurements on purified K^+ channel proteins can provide complementary information on the number, affinity, and thermodynamics of ion binding sites that may help to develop a more comprehensive picture of K^+ channel structure and function. With this goal in mind, we are interested in devising biochemical approaches that can be used to monitor ion binding to K^+ channels. In this paper, we introduce a novel method for studying interactions of inorganic cations with $C_{12}M$ -solubilized KcsA based on the ability of permeant cations to stabilize the tetrameric quaternary structure against denaturation at elevated temperatures. Using SDS-PAGE to monitor the oligomeric state of KcsA under various ionic conditions, we document the apparent affinity of various inorganic cations in stabilizing the KcsA tetramer at high temperature. The results are discussed in the context of known functional aspects of ion permeation, selectivity, and block of K^+ channels. This work was initially presented in abstract form (Krishnan et al., 2005).

MATERIALS AND METHODS

Purification of KcsA

An expression vector (pASK90 plasmid) containing a synthetic gene for the native KcsA protein modified with a hexahistidine sequence inserted following Met₁ was supplied by L. Heginbotham

(Yale University, New Haven, CT). Methods similar to those of Heginbotham et al. (1997) were used for expression of KcsA and purification of the protein at 4°C. Typically, a 7-liter culture of *Escherichia coli* strain BL21, transformed and selected for the above plasmid, was grown in LB medium to an absorbance of 0.5 at 550 nm followed by induction with 200 µg/ml anhydrotetracycline for 1.5 h. Cells were harvested by centrifugation (5,500 g, 10 min), washed with buffer A (100 mM NaCl, 5 mM KCl, 50 mM Mops-NaOH, pH 7.0), resuspended in buffer A (5 ml/liter of culture) containing EDTA-free protease inhibitor cocktail (Roche), and sonicated with a Branson Sonifier 450 (three pulses of 40 s). Membranes containing KcsA were harvested by centrifugation (110,000 g, 45 min). Protein was extracted by resuspension of membranes in buffer B (5 mM KCl, 100 mM NaP_i, pH 7.0) at 2 ml per liter of original culture plus 20 mM C₁₂M (n-dodecyl-β-maltoside from Anatrace) and 50 µl Triton X-100 per 2 ml resuspended membranes. Following gentle mixing for 2 h at 4°C, the suspension was centrifuged at 85,000 g for 30 min. The supernatant was gently shaken with Ni-NTA Agarose (QIAGEN, 50 µl of matrix/ml extract) for 1 h. Contaminating proteins were removed by extensive washing of a small column of the collected Ni-NTA agarose with 40 ml buffer B containing 1 mM C₁₂M. The column was then washed with 40 times the bed volume of buffer B containing 40 mM imidazole and 1 mM C₁₂M. KcsA was eluted with 20 times the bed volume of buffer B containing 400 mM imidazole and 1 mM C₁₂M.

Exchange of KcsA into Solutions of Defined Ionic Composition

Investigation of the ion dependence of the KcsA tetramer requires a convenient and reliable method to thoroughly change the ionic composition of the test protein solution. For this purpose, an ultrafiltration method based on repetitive concentration and dilution using a small disposable centrifugal filter device (Amicon Ultra-4, 10-kD nominal cutoff limit) proved to be adequate. This technique efficiently exchanges ions and small molecules; e.g., seven rounds of concentration of the KcsA preparation to ~50 µl by ultrafiltration followed by dilution with 4 ml of the desired final solution effectively dilutes cations in the original KcsA preparation by a factor of ~10⁻¹⁴. In the procedures described below, inorganic salts were obtained from vendors cited in parentheses: LiCl, KCl, RbCl, CsCl, Tl acetate, SrCl₂ (ultrapure grade from Alfa Aesar), BaCl₂ (Johnson Matthey), choline Cl (>99% grade from Fluka), and NaCl (Sigma-Aldrich).

Purified KcsA (1 ml) in buffer B with 1 mM C₁₂M was concentrated to 50 µl by centrifugation at 3,000 g in an Amicon Ultra-4 ultrafiltration device (Millipore). The 50-µl concentrate was diluted 80-fold by addition of 4 ml buffer C (10 mM Hepes-Tris, pH 7.4, 0.75 mM C₁₂M, and 100 mM of choline Cl, Tris-HCl, LiCl, NaCl, KCl, RbCl, CsCl, Tl acetate, or NH₄Cl; or 25 mM of BaCl₂ or SrCl₂). This sample was concentrated again to 50 µl in the same fashion. The preceding ultrafiltration step was repeated six more times using 4 ml of the corresponding buffer C without C₁₂M as the diluent. The final 50 µl of KcsA concentrate was diluted to 1 ml by addition of buffer C without C₁₂M and spun for 10 min at 15,000 g in a microcentrifuge to remove any insoluble material. C₁₂M was omitted from the dilution buffer in the final six ultrafiltration steps since the large average micellar molecular weight (70,000) and low critical micelle concentration (0.1–0.6 mM) of C₁₂M (Bhairi, 1997) results in incomplete removal of this detergent. An excessively high concentration of C₁₂M was found to interfere with protein mobility and staining in subsequent analysis of KcsA tetramer by SDS-PAGE.

Dependence of KcsA Tetramer on Temperature or Cation Concentration

Samples of KcsA (0.4–2 µg) in 10 µl final volume of defined solution were heated for 10 min at various temperatures in the range

of 50°C to 99°C using a thermal cycler machine. After 10-min incubation at high temperature, samples were rapidly cooled to 0°C, mixed with 2 μ l of 6 \times sample buffer (final concentrations: 10% glycerol, 62.5 mM Tris HCl, pH 6.8, 2% SDS, and 0.01% bromophenol blue), and subjected to SDS-PAGE (8 μ l sample per lane) using a precast 12% acrylamide Tris-HCl Ready Gel (Bio-Rad Laboratories) and a MiniProtean 3 Cell gel electrophoresis apparatus (Bio-Rad Laboratories). The gel running buffer was 25 mM Tris base, 192 mM glycine, pH 8.3, 0.1% SDS.

Cation titration experiments were performed by mixing 8 μ l of 550 ng KcsA (previously exchanged into 10 mM Hepes-Tris, pH 7.4, and 100 mM choline Cl as described above) with 2 μ l stock solution of an appropriate salt (KCl, NaCl, RbCl, etc.). The resulting KcsA sample was allowed to equilibrate for 1 h at 22°C. Samples were then heated for 10 min at 90°C (for K⁺, Rb⁺, Cs⁺, Tl⁺, and Ba²⁺) or 85°C (for Sr²⁺ and NH₄⁺), rapidly cooled to 0°C, and processed for SDS-PAGE. The dependence on Na⁺ or Li⁺ concentration in the presence of K⁺ was studied at constant ionic strength by replacement of an equal concentration of choline Cl (up to 200 mM) with LiCl or NaCl using a KcsA sample prepared in 10 mM Hepes-Tris, pH 7.4, 5 mM KCl, and 300 mM choline Cl. After equilibration for 1 h at 22°C, samples were heated for 10 min at 95°C, cooled to 0°C, and processed for SDS-PAGE.

SDS-PAGE gels were washed with deionized water for 5 min, stained for protein with GelCode Blue (Pierce Chemical Co.) for 1 h, and destained for 2 h using deionized water changed once after 1 h. Images of stained gels were analyzed by digital scanning. The density of the stained KcsA tetramer band was measured using ImageJ software available at <http://rsb.info.nih.gov/ij/>. KcsA tetramer bands were expressed as the fraction of total tetramer by normalizing the measured peak areas to the density of an unheated control sample or to the highest expected tetramer density extrapolated to the maximal peak area at high cation concentration. Data points for KcsA tetramer are generally plotted as the mean \pm SD of three separate gels except for the tetramer decay experiment (Fig. 6), which plots the mean \pm range of duplicate gels. Titration data were fit to Eq. 1 using the regression wizard (Marquardt-Levenberg algorithm) of SigmaPlot (Systat Software).

The effect of correcting densitometric measurements of tetramer bands for the weak nonlinearity of stain absorbance versus protein was examined by fitting the standard curve of Fig. 4 B to a power function (given in the figure legend) and using this function to calculate protein content from measured absorbance. Since this correction changed the values of the fitted N and $K_{0.5}$ parameters of Eq. 1 by <5% (not depicted), we ignored this correction in the presentation and analysis.

RESULTS

Thermal Stability of KcsA Tetramer Exhibits Cation Selectivity

Previous studies established that the KcsA tetramer is an extraordinarily stable oligomeric complex. KcsA migrates as a tetramer on SDS-PAGE when the pure detergent-solubilized protein is incubated at room temperature for long periods of time in C₁₂M micelles or even in the presence of SDS (Cortes and Perozo, 1997; Heginbotham et al., 1997). Dissociation of the tetramer into monomeric form is, however, greatly enhanced by exposure to elevated temperature, pH 12, and high concentrations of various alcohols (Cortes and Perozo,

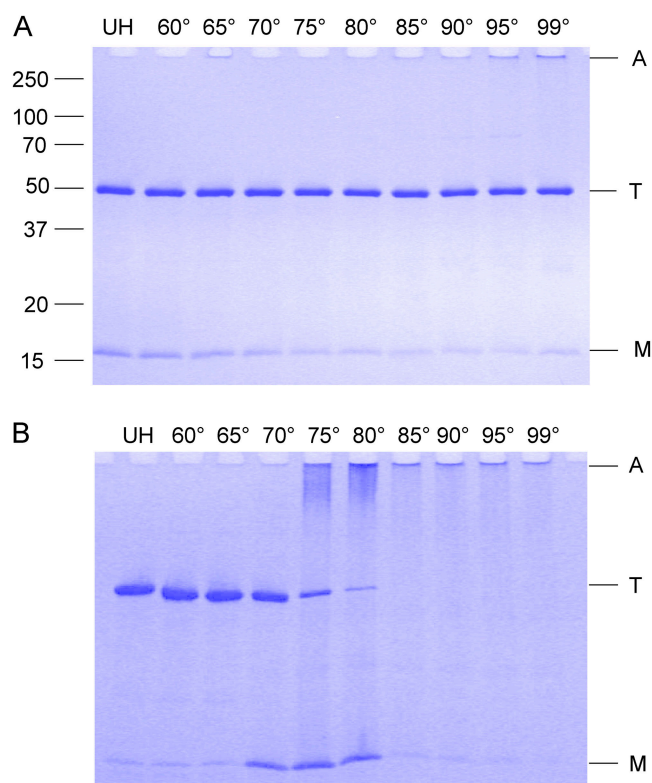


Figure 1. Thermal stability of KcsA tetramer in the presence of K⁺ vs. Na⁺. Purified KcsA was exchanged into buffer containing \sim 4 mM C₁₂M, 10 mM Hepes-Tris, pH 7.4, and either 100 mM KCl (A) or 100 mM NaCl (B). Identical protein samples were incubated at room temperature (UH, unheated) or heated for 10 min at indicated temperatures ranging from 60°C to 99°C and analyzed by SDS-PAGE. Positions of molecular mass markers (kD) are indicated on the left side of A. Positions of three observed forms of KcsA are noted on the right of each gel and labeled as A, aggregate; T, tetramer; M, monomer. The gel of B was purposely not completely destained in order to preserve the smeared bands of aggregated KcsA near the top of the gel in the two lanes labeled 75°C and 80°C.

1997; Heginbotham et al., 1997; van den Brink-van der Laan et al., 2004b). Investigations thus far reveal that the tetramer is also destabilized by certain mutations of the selectivity filter, pore helix, and extracellular portion of the outer (M1) and inner (M2) transmembrane helices (Splitt et al., 2000; Irizarry et al., 2002; Choi and Heginbotham, 2004; Molina et al., 2004). In addition, tetramer stability of reconstituted KcsA is influenced by lipid composition of the membrane bilayer (Valiyaveetil et al., 2002; van Dalen et al., 2002; van den Brink-van der Laan et al., 2004a). These results suggest that intersubunit interactions in the external half of the protein, protein-lipid interactions, and/or membrane lateral pressure are important determinants of tetramer stability. Heretofore, the possibility that specific ion-protein interactions contribute to the tetramer stabilization energy has not been systematically explored.

The effect of physiologically relevant inorganic cations on the stability of KcsA tetramer at elevated temperature is shown in Fig. 1. In this experiment, KcsA was exchanged into a buffer solution containing 10 mM HEPES-Tris, pH 7.4, ~ 4 mM $C_{12}M$, and either 100 mM KCl or 100 mM NaCl. Samples were heated for 10 min at various temperatures in the range of 60–99°C, rapidly cooled at 0°C, mixed with SDS-PAGE sample buffer at room temperature, and analyzed by SDS-PAGE. The protein bands labeled M and T, respectively correspond to monomer (18.5 kD calculated mass) and tetramer (74 kD calculated mass), which run anomalously fast in this gel system. KcsA tetramer prepared in $C_{12}M$ and 100 mM KCl is fully stable under these conditions at temperatures up to 99°C (Fig. 1 A). In contrast, tetramer prepared in 100 mM NaCl is progressively lost at temperatures $>65^\circ\text{C}$ (Fig. 1 B). This experiment reveals a large shift in temperature stability of the tetramer that depends solely on the inorganic cation present in solution, K^+ versus Na^+ .

As reported by other workers (Heginbotham et al., 1997; Irizarry et al., 2002; van den Brink-van der Laan et al., 2004a), purified preparations of His₆-tagged KcsA sometimes contain variable amount of monomer (M) band that is observed even before heating. This fraction of “intrinsic” KcsA monomer has been reported to be due to an incorrectly folded fraction of the protein present in the *E. coli* host cell (Irizarry et al., 2002). Irrespective of its origin and variability, the minor monomer band present in the KCl sample of Fig. 1 A gradually disappears with heating $>70^\circ\text{C}$. The monomer band in the NaCl experiment (Fig. 1 B) first increases in samples heated $>65^\circ\text{C}$, corresponding to the loss of tetramer, then it disappears altogether at higher temperatures. Disappearance of the monomer at high temperature seems to be associated with the appearance of high molecular weight aggregate (labeled A in Fig. 1) that can be seen as a dark smear at the top of the gel in Fig. 1 B for samples heated at 75°C and 80°C , or at higher temperatures, as a faint thin band at the top of the gel that does not enter from the sample well (Fig. 1, A and B). Other studies of tetramer stability also report high molecular weight bands of KcsA under various conditions, particularly upon exposure to high temperature (Cortes and Perozo, 1997; Molina et al., 2004). Since the aggregate (A) band is observed under conditions of tetramer loss that coincide with the appearance and loss of the monomer band (Fig. 1 B), it is tempting to suggest that the process of KcsA denaturation at high temperature first involves dissociation of tetramer into monomers followed by unfolding and aggregation of the monomeric protein. Because this phenomenon is poorly defined and difficult to analyze, we henceforth focus solely on the disappearance of the tetramer band as a quantitative measure of tetramer stability.

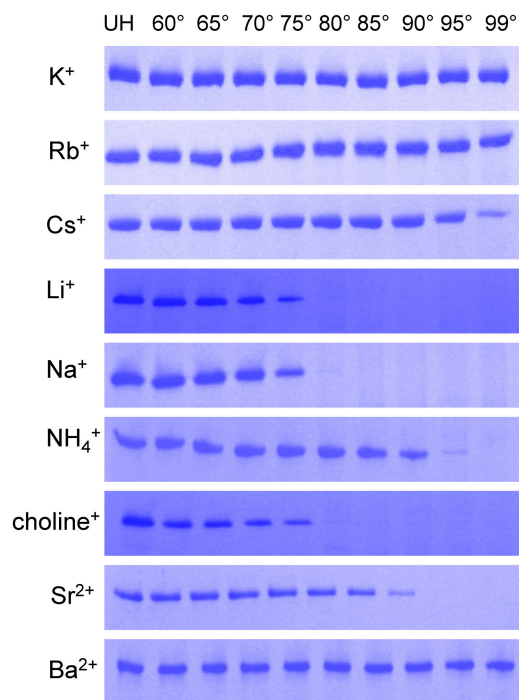


Figure 2. Dependence of the thermal stability of KcsA tetramer on various monovalent and divalent cations. Purified KcsA was exchanged into buffer containing ~ 4 mM $C_{12}M$, 10 mM HEPES-Tris, pH 7.4, and one of the following salts: 100 mM KCl, 100 mM RbCl, 100 mM CsCl, 100 mM LiCl, 100 mM NaCl, 100 mM NH_4Cl , 100 mM choline Cl, 25 mM SrCl_2 , 25 mM BaCl_2 . Identical protein samples in each solution were incubated at room temperature (UH, unheated) or heated for 10 min at indicated temperatures ranging from 60°C to 99°C and analyzed by SDS-PAGE. Only the horizontal portion of the stained gel corresponding to the tetramer band is shown for each cation.

If the shift in temperature stability of the tetramer observed in the presence of 100 mM K^+ versus 100 mM Na^+ (Fig. 1) reflects a specific functional difference in the interaction of alkali cations with the K^+ channel selectivity filter, one might generally expect that cations that permeate or strongly block K^+ channels by binding within the filter would stabilize the tetramer more effectively than impermeant cations or weak blockers. A test of this hypothesis is presented in Figs. 2 and 3, where the temperature dependence of the tetramer band is compared for KcsA preequilibrated with a variety of different inorganic and organic cations. The gel images of Fig. 2 show that loss of the tetramer band is dependent on the major cation species present in the test solution. Fig. 3 (A and B) summarizes data from experiments illustrated in Fig. 2, where the fractional loss of the tetramer band relative to an unheated sample is plotted as a function of temperature for 13 different cations.

The permeant cations, K^+ and Rb^+ , and the well-known K^+ channel blocker, Ba^{2+} , exhibit the greatest degree of tetramer stabilization with an undetermined midpoint temperature for 50% loss of tetramer ($T_{0.5}$)

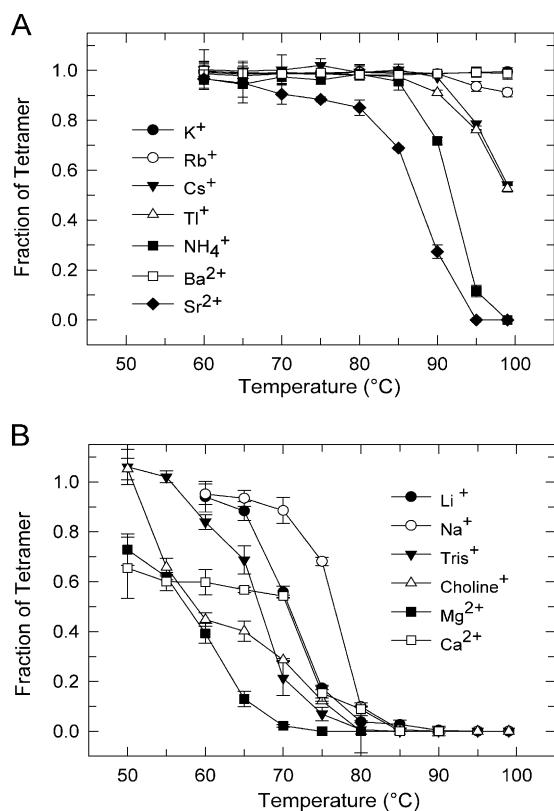


Figure 3. Temperature dependence of KcsA tetramer in the presence of various cations. Results of experiments described in Fig. 2 were analyzed by measuring the relative protein content of KcsA tetramer bands by densitometry. Integrated areas of tetramer bands were normalized to that of the unheated sample from the same gel. Data points correspond to the mean \pm SD of three experiments. (A) Incubation buffer for KcsA contained \sim 4 mM C₁₂M, 10 mM Hepes-Tris, pH 7.4, and either 100 mM KCl, 100 mM RbCl, 100 mM CsCl, 100 mM Tl acetate, 100 mM NH₄Cl, 25 mM BaCl₂, or 25 mM SrCl₂, as indicated by the symbol legend. Note that data points for KCl and BaCl₂ are completely superimposed. (B) Incubation buffer for KcsA contained \sim 4 mM C₁₂M, 10 mM Hepes-Tris, pH 7.4, and either 100 mM LiCl, 100 mM NaCl, 100 mM TrisCl pH 7.4, or 100 mM choline Cl as indicated. In the case of Mg²⁺ and Ca²⁺, the buffer contained \sim 4 mM C₁₂M, 10 mM Hepes-Tris, pH 7.4, 100 mM choline Cl, and either 5 mM MgCl₂ or 5 mM CaCl₂.

that is $>99^\circ\text{C}$ (Fig. 3 A). Other highly effective tetramer-stabilizing cations include Tl⁺ ($T_{0.5} \cong 98^\circ\text{C}$), Cs⁺ ($T_{0.5} \cong 98^\circ\text{C}$), NH₄⁺ ($T_{0.5} = 92^\circ\text{C}$), and Sr²⁺ ($T_{0.5} = 87^\circ\text{C}$). Of these latter cations, Tl⁺ and NH₄⁺ are well known to permeate through K⁺ channels (Eisenman et al., 1986; Hille, 2001; LeMasurier et al., 2001). Cs⁺ typically blocks many K⁺ channels, including KcsA (Gay and Stanfield, 1977; Cecchi et al., 1987; Heginbotham et al., 1998; LeMasurier et al., 2001), but also carries current for certain members of the K⁺ channel family (Shapiro and DeCoursey, 1991; Heginbotham and MacKinnon, 1993). Sr²⁺, like Ba²⁺ and Cs⁺, exhibits strongly voltage-dependent block of various K⁺ channels, which is generally taken as evidence of interaction

with the selectivity filter region (Armstrong and Palti, 1991; Jow and Numann, 1998; Sugihara, 1998; Zeng et al., 2005). The high-affinity blocking interaction of Ba²⁺ has often been attributed to the close similarity of the ionic radii (r) of Ba²⁺ ($r_{\text{Ba}} = 1.35 \text{ \AA}$) and K⁺ ($r_{\text{K}} = 1.33 \text{ \AA}$). The smaller Group IIA divalent cations, Mg²⁺, Ca²⁺, and Sr²⁺, are typically less effective and lower-affinity blockers of K⁺ channels than Ba²⁺ (Armstrong and Palti, 1991; Ferguson, 1991; Jow and Numann, 1998). The results compiled in Fig. 3 A thus show that highly permeant monovalent cations (K⁺, Rb⁺, and Tl⁺) stabilize the KcsA tetramer at a higher temperature range than less permeant monovalent cations (Cs⁺ and NH₄⁺). Similarly, the high-affinity divalent blocker, Ba²⁺, stabilizes KcsA tetramer at a higher temperature range than the lower affinity divalent blocker, Sr²⁺. These correlations suggest that ion-dependent temperature stabilization is due to specific ion interactions with the selectivity filter.

As further evidence for this conclusion, the impermeant Group IA cations, Li⁺ ($T_{0.5} = 71^\circ\text{C}$) and Na⁺ ($T_{0.5} = 77^\circ\text{C}$), promote dissociation of the tetramer at a lower temperature range, slightly higher than that observed for organic cations such as Tris⁺ ($T_{0.5} = 67^\circ\text{C}$) and choline⁺ ($T_{0.5} = 59^\circ\text{C}$) (Fig. 3 B). The latter organic cations are likely to be excluded from the narrow selectivity filter region of K⁺ channels on the basis of molecular size. The effects of Mg²⁺ and Ca²⁺ on tetramer stability are difficult to assess since we find that exchange of KcsA into solutions containing >10 mM concentration of these particular divalent cations results in severe loss of protein due to precipitation. However, we were able to measure the temperature dependence of KcsA tetramer in solutions containing 100 mM choline Cl plus 5 mM CaCl₂ or 5 mM MgCl₂. The results indicate that Ca²⁺ or Mg²⁺, which are also relatively weak blockers of K⁺ channels, have modest effects on the temperature dependence observed in the presence of 100 mM choline Cl alone. Addition of 5 mM Mg²⁺ to 100 mM choline Cl appears to destabilize KcsA at lower temperatures, whereas 5 mM Ca²⁺ appears to exhibit a small protective effect at temperatures $>60^\circ\text{C}$ (Fig. 3 B). Based on results of Fig. 3, the effectiveness of Group IA monovalent (Li⁺, Na⁺, K⁺, Rb⁺, and Cs⁺) and Group IIA divalent (Mg²⁺, Ca²⁺, Sr²⁺, and Ba²⁺) cations in stabilizing KcsA tetramer at high temperature appears to be well correlated to known functional interactions of these cations with respect to ion permeation and block of K⁺ channels.

Tetramer Protection as a Function of Cation Concentration

The dependence of tetramer stability on different cations suggests that the relative binding affinity of inorganic cations may be investigated by titration of a destabilized form of KcsA with stabilizing cations. We took

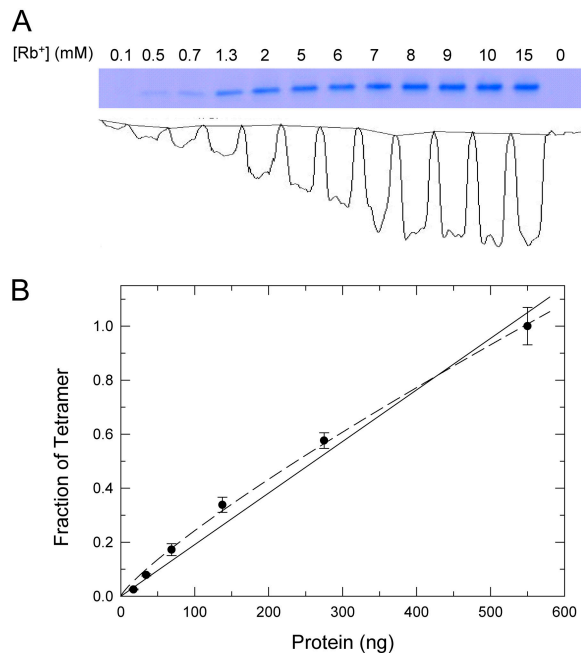


Figure 4. Method for analyzing the dependence of tetramer stability on cation concentration. (A) Rb⁺ titration. Identical samples of KcsA (550 ng) were incubated for 1 h at room temperature in ~4 mM C₁₂M, 10 mM Hepes-Tris, pH 7.4, 100 mM choline Cl, and various concentrations of RbCl as indicated. The samples were then heated at 90°C for 10 min and subjected to SDS-PAGE. The horizontal section of the gel corresponding to the tetramer band is shown above the tracing of a densitometric scan of each lane using ImageJ software. Stain absorbance increases in the downward direction. The baseline for area integration of stained protein peaks is shown as a linear interpolation between adjacent lanes. (B) Dependence of measured density of the tetramer band on KcsA concentration. Various samples of KcsA in the range of 0 to 550 ng were subjected to SDS-PAGE under conditions similar to those used in cation titration experiments. The area of each tetramer band was measured by densitometry and normalized by dividing by the area of the largest protein sample (550 ng) in each gel. Data points are the mean ± SEM of five separate experiments. The solid line is a fit to $Y = (0.00191)X$ and the dashed line is a fit to $Y = (0.0052)X^{0.835}$, where Y is the normalized area (fraction of tetramer) and X is the amount of protein.

advantage of the large shift of the temperature dependence of KcsA tetramer equilibrated in choline⁺ (Fig. 3 B) versus that of the stabilizing cations of Fig. 3 A to investigate the cation concentration dependence of protection against high temperature. Fig. 4 A shows results of an experiment where the same amount of KcsA (550 ng) prepared in 100 mM choline Cl was equilibrated with various concentrations of RbCl in separate tubes at ambient temperature (~22°C) for 1 h, then heated for 10 min at 90°C, cooled, and analyzed for tetramer content by SDS-PAGE. The intensity of the stained tetramer band increases in a saturable fashion over the range of 0.1 mM to 15 mM RbCl. A standard calibration curve of densitometric area as a function of protein content applied to different lanes of similar gels

TABLE I
Best-fit Parameters for Cation Titrations of Tetramer Stability

Cation	Radius (Å)	Parameters Fit to Eq. 1	
		K _{0.5} (mM)	N
K ⁺	1.33	1.5 ± 2.5%	3.2 ± 8.1%
Rb ⁺	1.48	1.7 ± 3.3%	2.9 ± 9.2%
Cs ⁺	1.69	4.1 ± 3.5%	3.3 ± 10%
Tl ⁺	1.40	1.1 ± 5.5%	1.7 ± 9.1%
NH ₄ ⁺	1.8	9.6 ± 6.8%	2.8 ± 18%
Ba ²⁺	1.35	0.00021 ± 5.9%	0.93 ± 5.1%
Sr ²⁺	1.13	0.011 ± 5.0%	1.0 ± 4.2%

Cation titrations of tetramer stability shown in Fig. 5 were fit to Eq. 1 to obtain K_{0.5} and N. The standard error derived from the fit is expressed as a percentage of the fitted parameter (coefficient of variation). Listed radii are based on values determined by Pauling as cited in Hille (2001) for all cations except for NH₄⁺, which is estimated by a molecular graphics approach described in Sun et al. (1997).

(Fig. 4 B) indicates that this relationship is nearly linear under the conditions of our assay. Thus, densitometric scanning of protein gels may be used to reliably quantify the dependence of the fraction of KcsA tetramer on cation concentration.

Fig. 5 summarizes the results of tetramer protection titrations for seven different stabilizing cations plotted as the fraction of maximum observed tetramer (f_T) versus total cation concentration in the assay mixture. As shown by comparison of the data to the shallow dashed theoretical curve in Fig. 5 A, the observed dependence on K⁺ concentration is distinctly steeper than expected for a one-site binding reaction. To analyze this dependence, titration data for the various cations were fit to the following form of the Hill equation:

$$f_T = 1 - \left(1 + \frac{[X]^N}{K_{0.5}^N} \right)^{-1}, \quad (1)$$

where $[X]$ is the cation concentration in the assay, N is an apparent Hill coefficient, and $K_{0.5}$ is the cation concentration at half-maximal stabilization. As summarized in Table I, best-fit Hill coefficients for the monovalent cations (K⁺, Rb⁺, Cs⁺, Tl⁺, and NH₄⁺) are >1.0, ranging from 1.7 for Tl⁺ to 3.3 for Cs⁺. In contrast, Hill coefficients for Sr²⁺ and Ba²⁺ are not significantly different from 1.0. K_{0.5} values for the monovalent cations range from 1.1 mM for Tl⁺ to 9.6 mM for NH₄⁺, whereas K_{0.5} for Ba²⁺ and Sr²⁺ are 210 nM and 11 μM, respectively (Table I).

Effect of K⁺ Concentration on the Time Course of Tetramer Decay

The titration experiments of Fig. 5 compare the effect of various inorganic cations in protecting the tetrameric form of KcsA from denaturation upon exposure to high temperature for a fixed duration of time

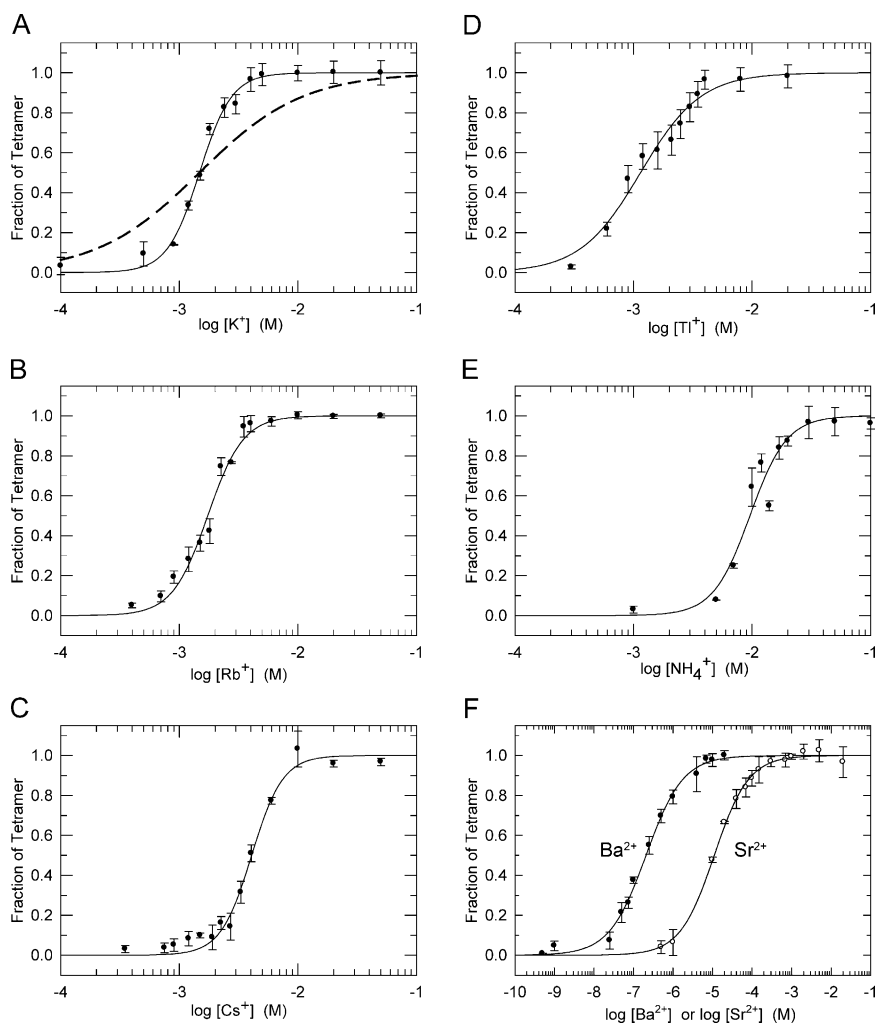


Figure 5. Comparison of the concentration dependence of various inorganic cations for stabilization of KcsA tetramer. Samples of KcsA (550 ng) in ~ 4 mM $C_{12}M$, 10 mM Hepes-Tris, pH 7.4, and 100 mM choline Cl were incubated with increasing concentrations of various inorganic cations for 1 h at room temperature. Samples were then heated at 90°C (A, KCl; B, RbCl; C, CsCl; D, Tl acetate; E, NH_4Cl ; F, $SrCl_2$) or at 85°C (E, NH_4Cl ; F, $SrCl_2$) for 10 min before analysis by SDS-PAGE. Tetramer bands were normalized to the maximal densitometric peak area of scanned gels as described in MATERIALS AND METHODS. Each data point is the mean \pm SD of three experiments. Solid lines are fits to Eq. 1 with best-fit parameters summarized in Table I. For purpose of illustration, the dashed line in Fig. 5 A shows the behavior of a one-site model for K^+ binding ($N = 1$, $K = 1.48$ mM using Eq. 1).

(10 min). To investigate the actual kinetics of the underlying tetramer denaturation process, we examined the effect of K^+ concentration on time course of loss of the tetramer band by stopping the reaction by rapid cooling of samples to 0°C after various times of exposure to 90°C. The cooled samples were subsequently analyzed by SDS-PAGE to quantitate the fractional loss of tetramer. Fig. 6 shows that tetrameric KcsA equilibrated in 100 mM choline Cl rapidly decays upon exposure to high temperature. The time course of tetramer loss in 100 mM choline⁺ and the absence of K^+ is well described by a single exponential decay with a time constant of 16.5 s (fit not shown). The loss of KcsA tetramer observed upon exposure to high temperature in the absence of K^+ is essentially irreversible since incubation of previously heated samples for up to 24 h at room temperature fails to recover the tetramer band (unpublished data).

In contrast, virtually no loss of tetramer was observed for KcsA equilibrated in 100 mM KCl during 30 min of incubation at 90°C, again indicating that the K^+ -bound conformation of KcsA tetramer is much more resistant

to thermal denaturation than the conformation in choline Cl. Addition of as little as 0.1 mM K^+ to the reaction mixture changes the time course of denaturation from a fast exponential decay to a complex slower biphasic decay process (Fig. 6). Definitive characterization of the kinetics of cation protection against thermal denaturation of the KcsA tetramer requires high-precision kinetic data that accurately capture the fast phase of decay as a function of cation concentration. Given the current kinetic sampling method of manual processing of separately heated reaction tubes and the fast initial time course, such an analysis is beyond our present technical capability. However, these experiments nevertheless demonstrate the strongly protective effect of K^+ on the kinetics of tetramer denaturation.

Evidence for Displacement of K^+ by Na^+ and Li^+

Since loss of the tetramer band for KcsA equilibrated with 100 mM NaCl or LiCl occurs over a temperature range only slightly higher than that observed for KcsA in 100 mM choline Cl or TrisCl (Fig. 3 B), cation protection titrations cannot be readily used to investigate

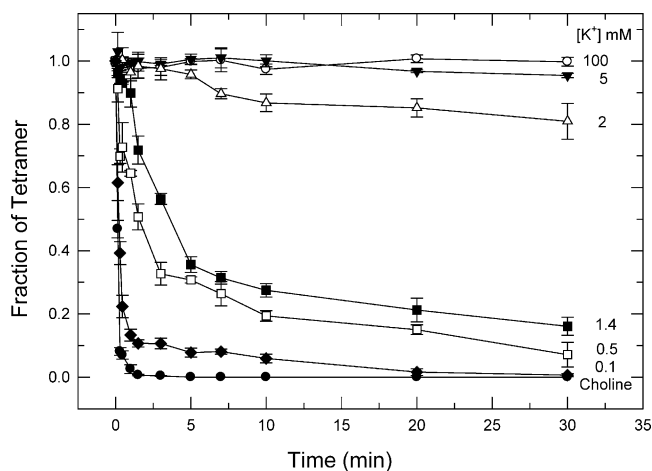


Figure 6. Time course of decay of KcsA tetramer measured as a function of K^+ concentration. Samples of KcsA were incubated for 1 h at room temperature in ~ 4 mM $C_{12}M$, 10 mM Hepes-Tris, pH 7.4, 100 mM choline Cl, and increasing concentrations of KCl as indicated. The samples were then heated to $90^\circ C$. Aliquots taken at various times after heating were placed on ice to halt tetramer decay. After analysis by SDS-PAGE, areas of tetramer bands were measured by densitometric analysis and normalized to an unheated sample (zero time point). Each data point corresponds to the mean of two separate experiments.

the intrinsic affinity of KcsA for Na^+ and Li^+ . However, based on the fact that Na^+ is a pore blocker of many K^+ channels, including KcsA (Nimigean and Miller, 2002), and that electron density corresponding to a Na^+ ion has been mapped to the central cavity of KcsA where K^+ , Rb^+ , Cs^+ , and Tl^+ also bind (Zhou and MacKinnon, 2004b), we hypothesized that Na^+ and Li^+ might bind competitively or noncompetitively to displace K^+ from the stabilized form of the tetramer. To test this idea, we analyzed samples of KcsA incubated with increasing concentrations of LiCl and NaCl up to 200 mM in the presence of 5 mM KCl. This experiment was also performed at constant ionic strength by molar replacement of 300 mM choline Cl with LiCl or NaCl to avoid complexities due to concentration-dependent changes in electrostatic potential. After equilibration for 1 h, the samples were heated for 10 min at $95^\circ C$, a temperature that preserves the tetramer in 5 mM K^+ (Fig. 3 A) but not in 100 mM Li^+ or 100 mM Na^+ (Fig. 3 B). Analysis by SDS-PAGE shows that KcsA tetramer is lost as a function of increasing concentration of both Li^+ and Na^+ under these conditions (Fig. 7). The results of Fig. 7 were analyzed by fitting the data to a descending Hill function (f_T equal to 1 minus the expression on the right side of Eq. 1). Best-fit values of N are $1.3 \pm 6.7\%$ (Li^+) and $1.1 \pm 4.5\%$ (Na^+); best-fit values of $K_{0.5}$ are $57 \text{ mM} \pm 4.7\%$ (Li^+) and $109 \text{ mM} \pm 2.6\%$ (Na^+). The observation that Li^+ and Na^+ inhibit the tetramer-protecting effect of 5 mM K^+ in a concentration-dependent fashion suggests that both of these

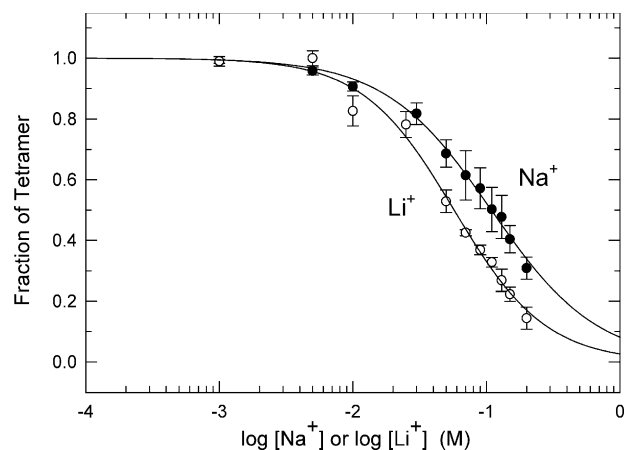


Figure 7. Destabilization of KcsA tetramer by Li^+ or Na^+ in the presence of 5 mM K^+ . Samples of KcsA (550 ng) in ~ 4 mM $C_{12}M$, 10 mM Hepes-Tris, pH 7.4, 300 mM choline Cl, and 5 mM KCl were titrated with an increasing concentration of LiCl or NaCl by replacement of an equal concentration of choline Cl and incubated at room temperature for 1 h. The samples were then heated at $90^\circ C$ for 10 min followed by analysis by SDS-PAGE. Areas of tetramer bands were measured by densitometric analysis and normalized to the peak area of identical samples not exposed to LiCl or NaCl. Data points are the mean \pm SD of three experiments. Solid lines are fits to a descending Hill function as described in the text.

smaller Group IA cations bind with low affinity to displace K^+ from sites in the pore, resulting in a temperature-destabilized form of the tetramer.

DISCUSSION

Molecular and Ionic Interactions Involved in Tetramer Stability

The major conclusion of this paper is that K^+ and certain other inorganic cations function in stabilizing the tetrameric structure of the KcsA K^+ channel. This role of inorganic cations is demonstrated by the remarkable ability of K^+ , Rb^+ , and Ba^{2+} to protect KcsA tetramer from thermal denaturation at $99^\circ C$, a temperature at which most proteins rapidly denature and unfold. Since our experiments were performed in the absence of a native phospholipid membrane using KcsA solubilized in a nonionic detergent ($C_{12}M$), contributions to the free energy of tetramer stabilization primarily consist of protein-protein, protein-water, and protein-ion interactions. This work establishes the significance of binding interactions between permeant cations, in particular K^+ , and the four KcsA protein monomers in maintaining the quaternary structure of the channel complex.

A functional role of phospholipids in stabilizing KcsA tetramer against thermal denaturation in detergent micelles has previously been described (van Dalen et al.,

2002; Valiyaveetil et al., 2002; Williamson et al., 2002; Molina et al., 2004; van den Brink-van der Laan et al., 2004a). The fact that certain phospholipids such as phosphatidylethanolamine or phosphatidylglycerol preferentially stabilize KcsA tetramer relative to phosphatidylcholine indicates that specific protein–lipid interactions and/or bulk mechanical properties of the membrane bilayer also contribute to tetramer stability (van Dalen et al., 2002). We have not yet examined whether cation-protection effects described here are influenced by interactions of KcsA with phospholipids. Thus it cannot be excluded that lipids or the membrane bilayer may modulate inorganic cation affinity or selectivity through allosteric or mechanical effects on protein conformation. However, our results can be interpreted in the context of crystallographic data, which were generated under conditions similar to the present work using KcsA protein solubilized in C₁₂M and crystallized in the absence of exogenous phospholipids.

Mutational investigations have previously shown that amino acid residues affecting the tetramer stability of KcsA map primarily to the extracellular portion of the protein with respect to membrane topology, including sites in the selectivity filter, pore helix, and the M1 and M2 transmembrane helices (Splitt et al., 2000; Irizarry et al., 2002). Since this region of the protein essentially surrounds the selectivity filter where K⁺ binds by coordination to protein oxygen atoms, it is possible that the tetramer-destabilizing effects of some of these mutations involve perturbation of K⁺ binding in the filter region either separately or in addition to alteration of intersubunit contacts at the site of the mutation. The present findings suggest that perturbation of K⁺ binding may be a significant factor underlying the effects of KcsA mutations on tetramer stability. Further work is clearly required to determine the relative contributions of intersubunit protein contacts, cation–protein interactions, and the phospholipid bilayer to the overall energetics of the tetramer stability of K⁺ channels.

Location of Cation Binding Sites Involved in Tetramer Stability

Several lines of evidence indicate that cation binding sites mediating the tetramer-stabilizing effects of inorganic cations described in this paper are located along the pore axis and selectivity filter of KcsA. First, from a qualitative standpoint, there is a striking correspondence between permeation and/or blocking behavior of inorganic cations and the temperature dependence of tetramer stability (Fig. 3). Cations that readily permeate (K⁺, Rb⁺, Tl⁺, and NH₄⁺) or strongly block (Cs⁺, Ba²⁺, and Sr²⁺) K⁺ channels stabilize KcsA tetramer at a markedly higher temperature than cations that are typically impermeant and/or weak blockers (Na⁺, Li⁺, choline⁺, Tris⁺, Mg²⁺, and Ca²⁺). In particu-

lar, the fact that Ba²⁺ is the most effective stabilizing cation among those tested suggests that tetramer stabilization is at least partly related to the affinity of cation interactions with the selectivity filter. Ba²⁺ exhibits the strongest known blocking interaction (highest affinity and slowest dissociation rate) with K⁺ channels among the tested inorganic cations (Standen and Stanfield, 1978; Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983; Oberhauser et al., 1988). Ba²⁺ has also been found to occupy a single site equivalent to the internal S₄ site for K⁺ within the selectivity filter as determined by a crystallographic study of the KcsA complex with Ba²⁺ (Jiang and MacKinnon, 2000).

A second aspect of the results that implicates pore/filter cation binding sites in tetramer stability is the distinctly different concentration dependence of monovalent versus divalent cations in the cation protection titrations of Fig. 5. In particular, the concentration dependence of the monovalent cations (K⁺, Rb⁺, Cs⁺, Tl⁺, and NH₄⁺) is markedly steeper than that of Sr²⁺ and Ba²⁺ as indicated by apparent Hill coefficients (N) ranging from 1.7 to 3.3 for the monovalent cations versus N values of 0.93 for Ba²⁺ and 1.0 for Sr²⁺ (Table I).

To properly interpret values of N extracted from the data of Fig. 5 we must be careful to acknowledge that these experiments are performed under nonequilibrium conditions. To observe destabilization of the tetramer, we perturb the preexisting cation binding equilibrium by increasing sample temperature from 22°C to 85°C or 90°C for 10 min. The heat treatment promotes denaturation by irreversibly removing more labile (e.g., K⁺-free) forms of the protein from the preexisting binding equilibrium, and thus shifting the distribution of multiple KcsA forms present in the solution away from more stabilized (e.g., K⁺-bound) forms. Similar to kinetic assays of the affinity of substrates or ligands that protect enzymes from inactivation by irreversible chemical inhibitors (e.g., active site inhibitors), it can be shown that the K⁺ concentration dependence of the initial rate of tetramer loss reflects the true K⁺ binding equilibrium constant(s) before addition of the inhibitor. However, as illustrated by the results of Fig. 6, the 10-min time point used here to sample KcsA tetramer lies far from the initial rate, especially at lower K⁺ concentrations. Thus, the N and K_{0.5} values listed in Table I clearly do not represent actual Hill coefficients or dissociation constants of the cation–KcsA equilibrium binding reaction. Based on principles of mass action, we may expect that K_{0.5} values of Table I overestimate the underlying macroscopic cation binding dissociation constant; i.e., the actual cation affinities for KcsA are likely to be stronger than indicated by these values. Likewise, under the present conditions, N values >1.0 can arise from a true one-site binding system when irre-

versible decay kinetics are sampled at long times compared with the initial rate (Andersen, O., personal communication).

Despite these important caveats for quantitative interpretation of the fits to Eq. 1, we would argue that the qualitatively different behavior of N values for the monovalent versus divalent cations of Fig. 5 reflects fundamental aspects of K^+ channel behavior. Specifically, the higher N values of K^+ , Rb^+ , Cs^+ , and NH_4^+ in the range of 2.8–3.3 are evocative of multi-ion behavior of K^+ channel permeation. Such behavior first discovered by electrophysiologists provided the original biophysical evidence for the hypothesis that K^+ channels contain a narrow selectivity filter where multiple ions bind in a single file of discrete sites (Hodgkin and Keynes, 1955; Hille and Schwarz, 1978; Stampe and Begegnisich, 1996; Hille, 2001). Multi-ion binding to the K^+ channel pore has been more recently verified by the observation of four (K^+ and Tl^+) or three (Rb^+ and Cs^+) cations bound directly within the selectivity filter as observed for KcsA crystals formed in the presence of various inorganic cations (Zhou et al., 2001; Morais-Cabral et al., 2001; Zhou and MacKinnon, 2003). Evidence for the interpretation that two monovalent cations bind simultaneously to the filter region was also obtained from quantitative analysis of anomalous scattering of electron density of various KcsA crystal complexes that yielded the following values for the total absolute occupancy number of the S_1 – S_4 filter sites by various ions: K^+ , 2.1; Rb^+ , 2.2; Cs^+ , 1.9; Tl^+ , 2.5 (Zhou and MacKinnon, 2003). In contrast to simultaneous binding of multiple monovalent cations, the observation of a single crystallographic binding site for Ba^{2+} observed at the location of the S_4 site for K^+ in the KcsA filter (Jiang and MacKinnon, 2000) is consistent with our observation of N values near 1.0 for the Ba^{2+} and Sr^{2+} titrations (Fig. 5; Table I). An answer to the interesting question of whether features of our data concerning differences in apparent Hill coefficients for monovalent versus divalent cations actually reflect differences in the maximum number of cations that simultaneously bind to the KcsA selectivity filter will require more precise measurements that reflect true equilibrium conditions.

Another piece of evidence in favor of a pore location for inorganic cations that stabilize the KcsA tetramer comes from the observation that Na^+ or Li^+ ions appear to displace K^+ in stabilizing the tetramer at 95°C (Fig. 7). Neither Na^+ nor Li^+ have yet been directly observed within the selectivity filter by crystallographic studies. Na^+ and Li^+ appear to be too small to effectively stabilize the native structure of the filter relative to larger conductive cations such as K^+ , Rb^+ , Cs^+ , and Tl^+ . Electrophysiological studies of the BK channel have shown that Na^+ binds to an internal lock-in site

with respect to the Ba^{2+} -blocking site that displays approximately fivefold selectivity for K^+ over Na^+ (Neyton and Miller, 1988). Similarly, internal Na^+ blocks outward K^+ current through the KcsA channel at an internal site that is selective for K^+ with a 5–7-fold preference over Na^+ (Nimigeon and Miller, 2002). The weakly K^+ -selective blocking site for Na^+ in these latter blocking experiments is probably equivalent to the internal cavity site (S_{cavity}) where Na^+ has been observed to reside in crystallographic studies of KcsA (Zhou and MacKinnon, 2004b). The low apparent affinity of Na^+ ($K_{0.5} = 109$ mM) measured in the titration experiment of Fig. 7, is strikingly similar to the equilibrium dissociation constant, $K_d = 110$ mM, determined for Na^+ binding to the cavity site of KcsA (Zhou and MacKinnon, 2004b), albeit the latter value is an intrinsic K_d corrected for the effect of cation competition. The qualitative similarity between the behavior of Na^+ and Li^+ in tetramer stabilization and the known blocking interactions of Na^+ in various K^+ channels suggests that the destabilizing effect of Na^+ and Li^+ is mediated by interactions of these ions within the KcsA pore. The results suggest that simultaneous occupancy of the cavity by Na^+ or Li^+ and the filter by K^+ is a repulsive ligand–ligand interaction capable of driving K^+ from its sites in the filter. Once K^+ dissociates from the filter, the KcsA structure with a Na^+ or Li^+ ion bound primarily in the cavity is apparently not sufficiently stable to resist tetramer dissociation at the high assay temperature (95°C) of the experiment of Fig. 7.

Dual Role of K^+ as a Structural Cofactor and Substrate for Permeation through K^+ Channels

A functional role for K^+ in stabilizing the structure of K^+ channels has long been anticipated. Electrophysiological experiments on squid giant axon showed that complete removal of K^+ from the axon bathing solution results in an irreversible loss of K^+ conductance underlying the action potential (Chandler and Meves, 1970; Almers and Armstrong, 1980). Similarly, removal of K^+ from the external medium results in the loss of Ca^{2+} -induced K^+ efflux activity of human erythrocytes (Heinz and Passow, 1980), a physiological function known to be mediated by a Ca^{2+} -activated K^+ channel sensitive to charybdotoxin.

More recently, this phenomenon has been investigated using the *Drosophila* Shaker K^+ channel, where loss of function in the absence of K^+ is observed after several episodes of voltage-dependent activation, as if structural demise of the channel first requires channel opening for complete dissociation of K^+ trapped within the selectivity filter in the closed state (Gomez-Lagunas, 1997; Melishchuk et al., 1998). In the transition between the native state and the “defunct” state generated by the absence of K^+ , the Shaker channel becomes

transiently nonselective; i.e., strict ionic selectivity for K^+ is lost and current carried by Na^+ and large organic cations such as tetramethylammonium and N-methylglucamine is observed (Loboda et al., 2001). Certain gene isoforms of voltage-gated K^+ channels such as $K_v2.1$ readily exhibit Na^+ conductance in the absence of K^+ that is inhibited by low K^+ concentrations in the range of 0–3 mM (Callahan and Korn, 1994; Korn and Ikeda, 1995). This latter behavior has been interpreted as competitive binding of K^+ and Na^+ within the selectivity filter (Kiss et al., 1998), a ligand interaction that is also evident here with inhibition of KcsA tetramer stability by increasing Na^+ concentration in the presence of 5 mM K^+ (Fig. 7).

A debilitating effect of K^+ removal has also been described for single BK Ca^{2+} -activated K^+ channels studied in planar lipid bilayers (Vergara et al., 1999). In the presence of internal K^+ and the virtual absence of external K^+ , the BK channel enters a long-lived nonconducting state. Restoration of single BK activity from this silent K^+ -deprived state may be achieved by subsequent addition of external K^+ , Rb^+ , Cs^+ , and NH_4^+ . However, such recovery is not supported by external Na^+ or Li^+ . The fact that low concentrations of external K^+ that prevent collapse of channel conductance also block the exit of internal Ba^{2+} to the extracellular side of the channel implies that the K^+ site mediating the lock-in of internal Ba^{2+} and the preservation of channel function are located within the selectivity filter (Vergara et al., 1999).

The extensive evidence cited above indicates that K^+ channels in native cell membranes and reconstituted bilayers intrinsically rely upon the presence of K^+ to maintain the functional integrity of normal conductance and gating activity. K^+ is clearly also required to maintain strict ionic selectivity for itself over other inorganic cations. This behavior predicts that significant structural changes of K^+ channels must occur in the absence of K^+ . Indeed, crystallographic studies of KcsA have directly shown that the selectivity filter changes conformation from a well-ordered state in the presence of high K^+ or Tl^+ concentration to a disordered and presumably nonconducting conformation when the concentration of these latter ions is lowered to 3 mM and <65 mM, respectively, and ionic strength is maintained by NaCl (Zhou et al., 2001; Zhou and MacKinnon, 2003). The conformation of the KcsA selectivity filter in the presence of 100 mM K^+ is optimally organized for K^+ binding and conduction, with 16 carbonyl oxygens and four Thr hydroxyl groups precisely aligned to form the four S_1 – S_4 coordination sites for K^+ . At low K^+ or Tl^+ concentration, the Val₇₆ carbonyl group is positioned away from the pore axis, the α -carbon of Gly₇₇ occludes the pore, and a disruptive network of hydrogen bonds involving invasive water mole-

cules surrounds the perimeter of the selectivity filter, thereby defeating the function of the filter as a conducting channel (Zhou et al., 2001). Since a crystal structure of KcsA in the presence of Na^+ alone or in the complete absence of K^+ has not yet been obtained, one can only speculate that the disordered conformation of the filter described by Zhou et al. (2001) represents an intermediate state enroute to an even more drastic change in conformation that ultimately destabilizes the tetrameric subunit interface of the protein.

Zhou and MacKinnon (2003) have proposed that the change in conformation of the selectivity filter that occurs in the transition from low to high K^+ concentration involves a mandatory coupling energy that lowers the binding affinity of the second K^+ ion, thus permitting a high conduction rate. Other workers have uncovered substantial evidence supporting the idea that a change in conformation of the selectivity filter is the basis of a slow form of inactivation gating termed C-type inactivation (Starkus et al., 1997; Kiss and Korn, 1998; Yellen, 1998; Kiss et al., 1999). C-type inactivation is highly dependent upon external K^+ concentration. It appears to involve the transition of the channel from a state of high K^+ selectivity to a less-selective conducting state permeable to Na^+ before the final transition to the nonconducting inactivated state (Lopez-Barneo et al., 1993; Baukrowitz and Yellen, 1995; Kiss et al., 1999). Thus, the functional role of K^+ in preserving its own high conductance, selectivity, and gating kinetics appears to be a direct consequence of its structural role in organizing and maintaining the narrow open-tunnel configuration of the selectivity filter. The functional significance of conformational dynamics of the K^+ channel filter is further highlighted by the major contribution of thermal atomic fluctuations of protein atoms to selective ion permeation as revealed by molecular dynamics simulations (Noskov et al., 2004; Allen et al., 2004).

The preceding evidence supports the idea that the conformation of the selectivity filter depends on the species and concentration of inorganic cations present in solution phase on both sides of K^+ channels. The new findings described here extend the structural role of K^+ to maintenance of the quaternary structure of KcsA as probed at high temperature. Our results show that the same conditions of zero or low K^+ concentration that modulate gating and simultaneously disrupt K^+ selectivity and conductance of K^+ channels also disrupt the stability of the KcsA tetramer. Taken together, electrophysiological, biochemical, and structural evidence leads us to consider K^+ channel proteins in the context of metalloproteins and metalloenzymes (Lip-pard and Berg, 1994). In this sense, multiple K^+ ions serve as structural cofactors that support the open conformation of the selectivity filter and stabilize a tet-

rameric quaternary structure. Multiple K^+ ions can also be described as catalytic cofactors that enhance the rate of transitions among the ion occupancy states that give rise to fast and selective permeation, a hallmark feature of K^+ channels.

Finally, the role of K^+ in stabilizing KcsA against thermal denaturation may also be considered in light of the observation that an organic molecule such as tetrodotoxin protects the voltage-sensitive Na^+ channel protein from denaturation in the detergent-solubilized state (Agnew et al., 1978). The ability of ions and pore-binding ligands to stabilize channel structure and function may thus have broader implications for related families of ion channel proteins.

Summary

This work provides new evidence supporting the notion that K^+ ions serve an important structural role in KcsA and presumably other K^+ channels. The molecular conformation of the delicate cage-like selectivity filter of K^+ channels as revealed by crystallographic analysis of KcsA is intrinsically determined by the number, species, and location of inorganic cations bound within the pore. By virtue of the central location of the selectivity filter at the interface of the K^+ channel tetramer, the quaternary structure of KcsA as monitored at high temperature by SDS-PAGE is also dependent on the number and species of inorganic cations bound within the selectivity filter. Particular cations bound inside the central cavity and within the external vestibule of KcsA may also promote or inhibit tetramer stability. Biochemical analysis of tetramer stability provides information on specific binding interactions of inorganic cations to KcsA. Further application of this approach may provide molecular insight into other K^+ channel mechanisms coupled to ion binding interactions.

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