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Selective exclusion and selective binding both contribute to ion selectivity in KcsA, a model potassium channel

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Abstract

The selectivity filter in potassium channels, a main component of the ion permeation pathway, configures a stack of binding sites (S1 to S4) to which K^+ and other cations may bind. Specific ion binding to such sites induces changes in the filter conformation, which play a key role in defining both selectivity and permeation. Here, using the potassium channel KcsA as a model, we contribute new evidences to reinforce this assertion. First, ion binding to KcsA blocked by tetrabutylammonium at the most cytoplasmic site in the selectivity filter (S4) suggests that such site, when in the nonconductive filter conformation, has a higher affinity for cation binding than the most extracellular S1 site. This filter asymmetry, along with differences in intracellular and extracellular concentrations of K^+ versus Na^+ under physiological conditions, should strengthen selection of the permeant K^+ by the channel. Second, we used different K^+ concentrations to shift the equilibrium between nonconductive and conductive states of the selectivity filter in which to test competitive binding of Na^+. These experiments disclosed a marked decrease in the affinity of Na^+ to bind the channel when the conformational equilibrium shifts toward the conductive state. This finding suggested that in addition to the selective binding of K^+ and other permeant species over Na^+, there is a selective exclusion of nonpermeant species from binding the channel filter, once it reaches a fully conductive conformation. We conclude that selective binding and selective exclusion of permeant and nonpermeant cations, respectively, are important determinants of ion channel selectivity.

Potassium channels are highly selective in allowing permeation of K^+ versus the nonpermeant Na^+ (1). Our current knowledge on such a selective process were laid down by MacKinnon's (2) and Perozo's (3) groups in their reports on the structure of KcsA, a potassium channel from *Streptomyces lividans*. This channel is a homotetramer in which each of its four subunits contains two transmembrane segments connected by a pore region formed by a tilted short helix (pore helix) and an ion selectivity filter with the sequence TVGYG, homologous to the eukaryotic K^+ channels (2). Early crystallography studies reported a stack of four K^+ binding sites (S1 to S4), formed primarily by the backbone carbonyls within the filter sequence (2,4). Moreover, crystal

structures under different ionic conditions indicate that the selectivity filter adopts distinct conformations depending on K^+ concentration (4-8). Such K^+ concentration-dependent conformational changes were also seen in NMR studies (9-14). Low K^+ concentrations leads to a nonconductive conformation of the selectivity filter in which K^+ binds only at the extreme S1 and S4 sites, with an average occupancy of just one K^+ distributed between those two sites. As the K^+ concentration increases, a second ion goes into the middle of the filter (sites S2 or S3) and a conformational transition to a conductive state occurs, which has an average occupancy of two K^+ ions per channel (4,7). Such transitions occur in a step-wise manner, through several structural intermediates showing X-ray structures with a different filter width and a different occupation of the available binding sites (7,15,16). X-ray crystallography also revealed that the use of Tl^+, instead of K^+, resulted in an identical occupation of filter sites by the former, while other permeant cations such as Rb^+ or Cs^+ could not occupy the S2 site, although their average occupation was also two ions per channel, similar to that found under high K^+ conditions (8). On the other hand, setting aside the differences in coordination geometries, Na^+ has been found at S1 and S4 sites in a nonconductive conformation (17) or more recently, at a "B" site near S4 (18), but never associated to the S2 and S3 sites, showing an average occupancy of just one ion per channel.

At the intracellular side of the channel, the C-terminal ends of the four subunits extend into the cytoplasm to form a bundle of helices, which constitute the most intracellular domain of the ion permeation pathway (3). In KcsA and in most K^+ channels, channel opening results from the concerted action of two gates along the ion path. First, the so called inner gate is formed by the cytoplasmic helical bundle referred above, whose change in conformation from a closed to an open state is driven by acidic pH (19-21). Secondly, the outer gate corresponds to the selectivity filter itself, which adopts distinct conductive or nonconductive conformations depending on the type and concentration of cations (4,8). Therefore, channel opening requires simultaneously an open inner gate and a conductive filter. Nonetheless, such a combination of conformational states is unstable and at acidic pH, rapidly evolves to an inactivated state, similar to C-type inactivation in eukaryotic K^+ channels (22-24).

We previously reported ion binding assays based on monitoring the intrinsic fluorescence of KcsA (25). These experiments were carried out at neutral pH i) to avoid inactivation, ii) to emphasize events at the selectivity filter and iii) to refer our results to the reported crystallographic structures, which are solved at neutral pH. Our first observation was that K^+ caused a reversible blue-shift and an increase in the intensity of the KcsA fluorescence greater than those observed in Na^+, consistent with the reported K^+-induced change in protein conformation (25). Second, studies using tryptophan to phenylalanine mutants of KcsA assigned the cation-induced fluorescence changes to the W67 and W68 residues (26), both located at the short helix, with their indole side chains practically in contact with the polypeptide backbone of the selectivity filter (2,6,27). These latter findings support the notion from X-ray studies that the selectivity filter itself and/or its immediate surroundings are the site(s) involved both in cation binding and in the cation-induced protein conformational changes. Third, it was found that fluorescence monitoring of thermal denaturation of KcsA, which includes the dissociation of the tetrameric protein into its subunits and their partial unfolding (26,28-30), provides an experimental observable, the apparent t_m of the native to denatured thermal transition, ideally suited to study cation binding (26,31). The nonpermeant species Na^+ or Li^+ bind with low affinity (millimolar K_D's) to a single set of binding sites contributed by the crystallographic S1 and S4 sites. On the contrary, permeant K^+, Rb^+, Tl^+ and even Cs^+ bind to two different sets of sites as their concentration increases, which seems consistent with the crystallographic evidence on the ability of these cations to induce concentration-dependent transitions between non-conductive and conductive conformations of the selectivity filter. The first set of such sites, assigned also to the crystal S1 and S4 sites, shows similarly high affinities for all permeant species (micromolar K_D's), thus, securing displacement of competing non-permeant cations. The second set of sites i) results from the contribution of all S1 through S4 crystallographic sites, ii) is available only to permeant cations when the filter is in the conductive conformation and iii) shows low

affinity (millimolar K_D's), thus, favoring cation dissociation and permeation. The differences in affinities between permeant and non-permeant cations and the similarities in binding behavior within each group correlate fully with their permeabilities relative to K^+ and therefore, it was concluded that cation binding may be an important determinant of ion selectivity (31). Here we report new evidences that greatly reinforce such hypothesis. First, we find asymmetry in the selectivity filter regarding non-equivalency in binding behavior between the S1 and S4 sites, which favors further the selective binding of permeant species. Second, we find a selective exclusion of non-permeant species from binding to the channel once the selectivity filter changes its conformation to a conductive state.

Results

The selectivity filter binds cations asymmetrically

Similar to the conditions used in crystallography, KcsA in our experimental system (purified channel protein in detergent solutions) has both, the extra- and intra-cellular ends of the selectivity filter, exposed to the same media and not subjected to any electrochemical gradient. This causes that, in principle, ions may access the channel pore through either end. Nonetheless, the pore could presumably be asymmetrical (non-equivalent S1 through S4 cation binding sites) and therefore, in an attempt to let pore asymmetry be manifested in our ion binding experiments, we have used tetrabutylammonium (TBA^+), a well-known blocker of potassium channels that binds to the intracellular end of the pore (32-34) in a practically irreversible manner. Figure 1A and B show that 0.5 mM TBA^+, in the absence of any other added ion, causes that the apparent t_m for thermal denaturation of wild-type KcsA, increases by approximately 13-14º C. Moreover, Figure 1A shows that titration of the TBA^+-blocked channel with increasing Na^+ concentrations increases only partly the thermal stability of the nonconductive channel protein and exhibits a lower slope than that seen in the absence of channel blockade (Figures 1A and C), indicative of a loss in Na^+ binding affinity (Table I). This suggests that the stabilizing effect of Na^+ seen in the absence of TBA^+ arises mainly from its binding to the S4 site at the intracellular end of the channel pore, whose access in these experiments is blocked by TBA^+. Likewise, K^+ titration of the TBA^+-blocked channel provides further evidence on the asymmetry of the selectivity filter (Figure 1B). Here, the binding curve in the lower K^+ concentration range shows also a much lower slope than in the absence of TBA^+, indicating a loss in K^+ binding affinity (inset to Figure 1B and Table I). In such low K^+ concentration range, the filter is still in a nonconductive state and K^+ is expected to bind with a micromolar K_D to the S1 and S4 sites (4). Under TBA^+ blockade, however, access to the S4 site is blocked both, by the bound TBA^+ at the cytoplasmic side and by the collapsed selectivity filter at the S2/S3 sites in the nonconductive conformation. Therefore, the decreased affinity under TBA^+ blockade should be attributed exclusively to the binding of K^+ to the S1 site in the nonconductive channel state. Nonetheless, as the K^+ concentration increases and the filter undergoes the K^+-induced transition to the conductive conformation, the observed extent of thermal stabilization becomes practically identical to that of potassium alone, added to that provided by the bound TBA^+ *per se* (Figures 1B and C). This suggests that in spite of TBA^+ blockade, once the filter is in the conductive conformation, K^+ eventually reaches all its binding sites within the pore to provide the characteristic thermal stability to the protein (26).

Cation binding to KcsA under competitive conditions

Competitive binding of cations to the KcsA channel has been carried out using two different approaches in which the starting points in the titration experiments were i) a nonconductive state of the selectivity filter caused by the continuous presence of 100 mM Na^+ (17) and ii) either nonconductive or conductive filter states resulting from the presence of varying concentrations of K^+ (4).

Figure 2 illustrates the first of such approaches in which the channel in a nonconductive state caused by 100 mM Na^+, was titrated with the permeant K^+, Rb^+ or Cs^+. Similarly to that observed under non-competitive conditions (35), i.e., where titrations started with a channel prepared in just 1.5 mM Na^+, the addition of increasing concentrations of any of the

permeant cations, resulted in a large thermal stabilization of the channel protein (Figure 2A, B and C). This indicates that all such permeant cations can efficiently displace Na^+ from binding to the channel pore; although K^+ and Rb^+ were always more efficient in this regard than Cs^+. Nonetheless, instead of the characteristic two binding events reported for permeant cations under non-competitive conditions (35), three different binding events are now clearly detected in the titration curves (Figure 2D, E and F). This was not unexpected because, as described in the previous paragraph, the S1 and S4 sites have different affinities for Na^+, which must be competitively displaced from both sites by the incoming permeant cation. Thus, the first binding event, seen at the lowest concentration range of permeant cations, should correspond to Na^+ displacement from the site having the lowest affinity for Na^+, i.e., the S1 site. Likewise, the second binding event seen at higher concentrations of permeant cations should correspond to Na^+ displacement from the S4 site still in the nonconductive conformation, which is the site with the higher affinity for Na^+. Finally, the third binding event reflects the occupancy of the most internal sites within the selectivity filter as the concentration of permeant cations is increased, that is, the S2 and S3 sites in the case of K^+, or just the S3 site for Rb^+ or Cs^+. Such latter sites become accessible to permeant cations only upon the concentration-dependent conformational transition to the conductive state. The K_D's of these three individual binding events for the permeant cations are summarized in Table II. The criteria used to define the range of concentrations in which each of the three binding events take place are explained in the Supplementary Information.

The second approach used in competitive binding experiments is based on X-ray evidence showing that conformational transitions from nonconductive to conductive states of the selectivity filter, can be induced by permeant cations such as K^+ or Rb^+ over a similar range of concentrations (5 to 30 mM) (7). Nonetheless, when extrapolating results from crystallography to dilute solutions, as we used in our experiments, it is possible that variables such as the high protein density or the presence of a bound antibody in the KcsA crystal cause the conformational transition to take place at cation concentrations different than those needed in solution. In order to test such possibility, we determined those critical concentrations in dilute solution conditions for the three permeant cations used here. K_D values of 2.5, 3.4 and 15 mM, for K^+, Rb^+ and Cs^+, respectively, were determined for the low affinity binding event inducing the conformational change in the selectivity filter (35), which are similar to those determined from crystallography (7). Therefore, in the experiments described below, we use K^+ at concentrations within such critical range to give raise to conductive or nonconductive filter states in which to test competitive Na^+ binding. Under non-competitive conditions, the nonconductive state was reported to bind K^+ with a micromolar K_D, while it increased to millimolar values in the final, conductive state (26). On the other hand, Na^+ binds the channel also with a millimolar K_D (26). Therefore, based on such reported K_D values, it would be expected that Na^+ competes with K^+ for binding to the S1 and S4 sites, only when the equilibrium between nonconductive and conductive states is sufficiently shifted towards the latter. In apparent agreement with such an expectation, Figure 3A shows that in the presence of 5 mM K^+, where the conformational equilibrium allows the coexistence in solution of nonconductive and conductive channel states along with the corresponding intermediates, fairly high concentrations of Na^+ cause an additional thermal stabilization of the protein, indicative of Na^+ binding in competition with K^+. The observed competitive binding, however, is not very potent (200 mM Na^+ *vs* 5 mM K^+). Moreover, in 25 mM K^+, where the equilibrium is shifted even further to the conductive state and should favor competitive Na^+ binding, the addition of Na^+ has no measurable effects on the thermal stability (Figure 3A). This indicates that binding of Na^+ does not occur under these circumstances and suggests that, when in competition with K^+, the conductive channel behaves differently regarding its ability to bind such cation. In order to test such possibility, we compared the apparent dissociation constants for the KcsA-Na^+ complex obtained from Na^+ titrations in the presence of different concentrations of K^+ (Figure 3B), with those predicted theoretically from a simple model in which two competing ligands bind to the same receptor maintaining constant their K_D values obtained under non-competitive conditions (26).

Figure 3C shows that there is a reasonable agreement between theoretical and experimental apparent K_D values for Na^+ when the K^+ concentration was below 5-10 mM. However, at higher K^+ concentrations there is a divergence between such values, as the apparent experimental K_D values for Na^+ binding keep increasing exponentially, at least up to a concentration of 20 mM K^+, suggesting that the affinity for Na^+ is greatly decreased when the channel selectivity filter completes its transition from the non-conductive to a fully conductive state. To further confirm such possibility, we turned to use the mutant channel E71A-KcsA. This channel does not inactivate because the lack of the E71 residue disrupts the W67-E71-D80 inactivation triad that confers such property (6,36) and although it deviates from the canonical conductive conformation of KcsA (5) it serves to model a "frozen", permanently conductive state of the selectivity filter. Figure 4 shows that, indeed, Na^+ concentrations much higher than those required by the wild-type channel are now needed to provide thermal stability to the protein under non-competitive conditions (panel A). The observed binding curve can still be fitted to a single Na^+ binding event (panel B), but the corresponding K_D is now over 250 mM, which is almost two orders of magnitude higher than that determined for Na^+ binding to the nonconductive state in the wild-type KcsA channel (26). These observations on Na^+ binding are in contrast with those reported for K^+ binding to the E71A-KcsA channel (37), in which the observed K_D is very similar to that determined from K^+ binding to the conductive state of the wild-type channel. Therefore, the above competitive binding experiments suggests that, while binding of K^+ remains unaltered regardless of Na^+ presence, the latter cation becomes selectively excluded from binding to the conductive state of the selectivity filter.

Discussion

This paper reports on two new contributing factors to ion selectivity in KcsA: i) the role of an asymmetric selectivity filter in the binding of cations to the S1 and S4 sites and ii) the selective exclusion of Na^+ from binding to the channel filter once it reaches a fully conductive conformation.

The evidence for the non-equivalency between filter sites S1 and S4 to bind either Na^+ or K^+ comes first from experiments using TBA^+-blocked KcsA. At the concentration of TBA^+ used in this work, there is no modification of the pore structure (32-34) and therefore, the response of the wild-type channel to cation binding in the absence of TBA^+ blockade, can still be used as a structural reference for our results. K^+ binding to TBA^+-blocked KcsA shows a decreased affinity in the lower K^+ concentration range compared to that observed in the absence of TBA^+. At such low K^+, the selectivity filter is in a nonconductive conformation and only the S1 and S4 sites are available for K^+ binding. When blocked by TBA^+, however, the S4 site becomes unavailable for cation binding and thus, the observed decreased affinity must be attributed to K^+ binding to the S1 site. This could be physiologically relevant as it points out to an S4 site with a markedly higher affinity to bind K^+, which is actually present in the intracellular media at a higher concentration than extracellularly, thus, facilitating even further the selection of the permeant cation by the channel. Regarding the nonpermeant Na^+, it is known that it binds to both the S1 and S4 sites (17) in a nonconductive pore conformation. Such binding is characterized by a K_D value of approximately 3 mM (26), which should represent the overall K_D coming from binding to both the S1 and S4 sites. Upon blockade of the S4 site by TBA^+, there is still Na^+ binding to the available S1 site, but the extend of Na^+-induced thermal stabilization decreases greatly and the K_D for the binding process increases by almost an order of magnitude, indicating that the S1 site has a lower affinity for binding Na^+ than the S4 site. This could also be relevant physiologically, as the S1 site is exposed to the extracellular media, which contains a higher concentration of Na^+ than the cytoplasm. Therefore, a decreased affinity for Na^+ at the S1 site should help prevent even further the association of the channel pore to this nonpermeant cation. The above observations on the binding of either Na^+ or K^+ to TBA^+-blocked KcsA, along with reported crystallographic evidence on the different occupancy of the S2 and S3 sites by permeant cations (8), provides a picture of the selectivity filter in which the stack of S1 to S4 sites seen in the KcsA crystal is clearly

symmetrical in terms of its ability to associate with either permeant or nonpermeant cations.

In addition to the TBA^+ blockade approach, the competitive displacement of Na^+ from its binding sites by different permeant cations (Figure 2) also serves to detect the non-equivalency between S1 and S4 sites. Such feature was not detected in Na^+ binding experiments under non-competitive conditions (26), but the competitive displacement used here allows to see a first binding event at the lowest concentration of permeant cations, corresponding to the displacement of Na^+ from the site having the lower affinity for Na^+, i.e., the S1 site. This is followed at higher concentrations of permeant cations, by Na^+ displacement from the S4 site, which is the site with the higher affinity for Na^+.

Our present results also provide evidence to support a combined selective binding/selective exclusion mechanism to contribute to ion selectivity in the KcsA channel. Here we take advantage of crystallographic reports (4,7,8) showing that nonconductive or conductive conformations of the channel selectivity filter can be attained in the presence of different concentrations of permeant cations. In spite of the differences in experimental conditions, such concentrations are similar to those found in our dilute solutions for the three permeant cations; K^+ being slightly and much more efficient, respectively, than Rb^+ or Cs^+ in causing such an effect. Therefore, we used K^+ concentrations within such critical range to displace the equilibrium between nonconductive and conductive filter states and perform competitive Na^+ binding. The conclusion from these experiments is that the affinity of Na^+ to bind the channel is greatly decreased as the conformational transition of the selectivity filter progresses from the nonconductive state, throughout the structural intermediates, to the final fully conductive state. Such a conclusion received confirmation from experiments of Na^+ binding to the mutant E71A-KcsA channel, which models a "frozen" conductive state and indeed exhibits a two orders of magnitude lower affinity for Na^+ than previously reported for the nonconductive state of the wild-type channel (37). In conclusion, the decreased affinity for the binding of Na^+ to the conductive channel contributes an additional selective exclusion mechanism to reinforce the selective binding favoring K^+ over Na^+ reported previously (26) and in this paper. The combination of selective binding and selective exclusion mechanisms should therefore be an important determinant of ion channel selectivity. Indeed, such possibility was hypothesized by Armstrong and coworkers in the early seventies (38), although to the best of our knowledge, no experimental evidence such as that reported here had been provided until now.

Experimental Procedures

Expression and purification of channel proteins

Protein expression and purification were carried out as previously reported (29,39). Briefly, kanamycin-resistant *Escherichia coli* M15 (pRep4) cells were transformed with the wild-type *kcsA* or mutant *E71A-kcsA* gens cloned in frame into the pQE30 vector (Qiagen), which provided ampicillin resistance and an N-terminal hexahistidine tag. Cells were grown at 30° C in 2xYT media (MP Biomedicals) supplemented with antibiotics to an optical density of 0.6-0.8 (600 nm) and channel expression was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells were pelleted and resuspended in lisozyme-containing buffer (20 mM HEPES, pH 7.5, 0.45 M sucrose, 0.4 mg/ml lysozyme; supplemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail). The mixture was then sonicated on an ice bath using a Branson probe-type apparatus and centrifuged 1h at 100.000xg to yield a crude membrane fraction. Membrane proteins in this membrane pellet were solubilized in 20 mM HEPES buffer, pH 7.5, containing 100 mM KCl, 10 mM imidazole and 10 mM of the detergent n-dodecyl β-D-maltoside (DDM, Calbiochem). After centrifugation of insoluble remains, the supernatant was incubated overnight with Ni^{2+}-Sepharose gel beads (G.E. Healthcare), placed into a column and washed with 20 mM HEPES, pH 7.5, 100mM KCl, 10 mM imidazole and 5 mM DDM. The gel-bound protein was finally eluted using the previous buffer containing 0.5 M imidazole. Finally, a dialysis step was introduced to remove the imidazole and to prepare the stocks for the ion binding experiments. The wild-type protein stock at 70-80 μM (in terms of KcsA monomers) was dialyzed against 20 mM HEPES buffer (adjusted to pH 7.0 by addition of

approximately 5 mM N-methyl-D-glucamine ($NMDG^+$)), containing 5 mM DDM and either 100 mM NaCl (KcsA-Na^+ stock), 100 mM KCl (KcsA-K^+ stock) or 5 mM TBA^+ (KcsA-TBA^+ stock). For the E71A mutant channel stock the dialysis was performed similarly, but in presence of 2 M NaCl in order to retain tetramer integrity. Unless indicated otherwise, all salts and reagents were analytical grade from Sigma-Aldrich. Protein concentration was determined from the absorbance at 280 nm, using a molar extinction coefficient of 34950 M^{-1} cm^{-1} for the KcsA monomer (19)

Sample preparation

All sample aliquots for the thermal denaturation experiments were prepared individually, as described below, by dilution of the indicated protein stocks to a final protein concentration of 1 µM and in a total volume of 550 µl.

(a) *Binding of Na^+ and K^+ to the TBA^+-blocked channel:* The wild-type KcsA-TBA^+ stock referred above was diluted into 20 mM HEPES/$NMDG^+$ buffer, pH 7.0, containing 5 mM DDM and increasing concentrations of either NaCl or KCl.

(b) *Binding of permeant cations in competition with 100 mM Na^+:* The wild-type KcsA-Na^+ stock was diluted into 20 mM HEPES/$NMDG^+$ buffer, pH 7.0, containing 5 mM DDM, 100 mM NaCl and increasing concentrations of either K^+, Rb^+ or Cs^+ chloride salts.

(c) *Binding of Na^+ in competition with different concentrations of K^+:* The wild-type KcsA-K^+ protein stock was diluted into 20 mM HEPES/$NMDG^+$ buffer, pH 7.0, containing 5 mM DDM, KCl at concentrations of either 5, 10, 15, 20 or 25 mM, and increasing concentrations of NaCl.

(d) *Na^+ binding to E71A-KcsA*: The E71A-KcsA protein stock was diluted into 20 mM HEPES/$NMDG^+$ buffer, pH 7.0, containing 5 mM DDM and increasing concentrations of NaCl. In order to prevent the dissociation of the tetrameric mutant channel (37), 100 mM Na^+ was the lowest concentration tested in these assays.

Monitoring cation binding to channel proteins

Thermal denaturation of DDM-solubilized KcsA is an irreversible process that can be followed by monitoring the temperature dependence of the protein intrinsic fluorescence (30). Experiments were carried out in a Varian Cary Eclipse spectrofluorometer in a 1 cm-path quartz cuvette (500 µL final volume), using a temperature up-scan rate of 0.6 ºC/min. The excitation wavelength was 280 nm and the fluorescence emission at 340 nm was recorded at 1 ºC intervals.

The midpoint temperature of the thermally-induced protein denaturation process (T_m) at different cation concentrations was calculated from the thermal denaturation curves by fitting the data to a two-state unfolding model (40). The stabilization of the native state of KcsA by cation binding (i.e., the increase in T_m) depends both, on the concentration of the ligand cation, [L], and on the dissociation constant of the protein-ligand complex, K_D. The concentration-dependent increase in T_m was used to estimate the K_D of the KcsA-cation complex using the following equation (*Equation 1*):

$$\frac{\Delta Tm}{Tm} = \frac{Tm - (Tm)_0}{Tm} = \frac{R \cdot (Tm)_0}{\Delta H_0} \cdot \ln\left[1 + \frac{[L]}{K_D}\right]$$

where T_m and $(T_m)_0$ refer to the apparent denaturation temperature (in Kelvin) for the protein in the presence and in the absence of added ligand, respectively, R is the gas constant and ΔH_0 is the enthalpy change upon protein denaturation in the absence of added ligand (26,37). For the comparison of K_D values we used their confidence intervals (C.I.'s), using a percentage of confidence of 95%. The criterion of "non-overlapping 95% confidence intervals" was used to determine significant difference between K_D's.

Essentially the same model was used to address cation binding to more than one set of binding sites with sufficiently different K_D's (26), as detailed in the Supplementary Information. Also, binding of two competing cations to one or more than one set of binding sites was analyzed as detailed previously (25)

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Author contributions: JMGR conceived and coordinated the study and wrote the paper. MLR, along with EM, also conceived the study as it evolved and designed, performed and analyzed the experiments. JAP, AM and AFC supervised all experiments and help in the design of additional ones based on the observed results. AMG was mainly in charge of the expression and purification of wild-type and mutant proteins. All authors analyzed the results in joint sessions, provide a discussion and critical revision of the manuscript and approved its final version for submission.

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FOOTNOTES

The abbreviations used are: KcsA, potassium channel from *Streptomyces lividans*; DDM, dodecyl β-D-maltoside; t_m, midpoint denaturation temperature (in degrees Celsius); T_m, midpoint denaturation temperature (in degrees Kelvin); ΔH_o, enthalpy change upon protein unfolding in the absence of ligand; K_D, dissociation constant; TBA^+, tetrabutylammonium; $NMDG^+$, N-methyl-D glucamine.

TABLES

TABLE I: Binding of Na^+ and K^+ to wild-type KcsA under blockade by 0.5 mM TBA^+.

Target channel	Tested cation	Sets of binding sites detected	K_D (M) [(a)]	95% C.I.
KcsA[(b)]	Na^+	1	3.3×10^{-3}	$(2.54 – 4.28) \times 10^{-3}$
	K^+	2	1.9×10^{-6}	$(1.25 – 2.88) \times 10^{-6}$
			2.5×10^{-3}	$(1.80 – 3.48) \times 10^{-3}$
TBA^+-KcsA[(c)]	Na^+	1	2.7×10^{-2}	$(2.52 – 2.90) \times 10^{-2}$ (#)
	K^+	2	1.6×10^{-4}	$(1.07 – 2.38) \times 10^{-4}$ (#)
			1.6×10^{-3}	$(1.43 – 1.80) \times 10^{-3}$

(a) K_D values are the average from fitting to Equation 1 (see Methods) the results from three independent titrations covering a wide range of cation concentrations (Figure 1).
(b) As previously reported (26).
(c) It should be noticed that when blocked by TBA, the cations may access the filter only from the extracellular side.
(#) Significant differences respect to KcsA group ($p<0.05$)

TABLE II: Competitive binding of K^+, Rb^+ and Cs^+ to wild-type KcsA in the presence of 100 mM Na^+.

Tested cation	Sets of binding sites detected	K_D (M) [(a)]	95% C.I.
K^+	3	3.1×10^{-5}	$(2.25 – 4.28) \times 10^{-5}$
		2.4×10^{-3}	$(1.81 – 3.18) \times 10^{-3}$
		1.4×10^{-2}	$(1.09 – 1.79) \times 10^{-2}$
Rb^+	3	1.4×10^{-4}	$(0.71 – 2.77) \times 10^{-5}$ (#)
		1.6×10^{-3}	$(1.07 – 2.38) \times 10^{-3}$
		1.6×10^{-2}	$(1.17 – 2.19) \times 10^{-2}$
Cs^+	3	3.0×10^{-4}	$(1.81 – 4.96) \times 10^{-4}$ (#)
		7.0×10^{-3}	$(5.85 – 8.37) \times 10^{-3}$ (#,*)
		2.4×10^{-2}	$(2.06 – 2.79) \times 10^{-2}$ (#)

(a) K_D values are the average from fitting to Equation 1 the results from three independent titrations covering a wide range of cation concentrations (Figure 2).
(#) Significant differences with respect to the K^+ group ($p<0.05$)
(*) Significant differences with respect to the Rb^+ group ($p<0.05$)

FIGURE LEGENDS

FIGURE 1. Effects of TBA^+ blockade on the binding of Na^+ and K^+ to wild-type KcsA. Briefly, in this and in the following figures, thermal denaturation of DDM-solubilized KcsA was recorded at the indicated cation concentrations by monitoring the temperature dependence of the protein intrinsic fluorescence. The midpoint temperature of the denaturation process (T_m) at the different cation concentrations was calculated from the thermal denaturation curves and plotted versus cation concentration to illustrate the stabilization of the native state of KcsA by cation binding (i.e., the increase in T_m). Panels A and B show such plots to describe the effects of Na^+ and K^+ binding on the thermal stability of KcsA in the presence (grey and open triangles for Na^+ and K^+, respectively) and in the absence (pink and dark blue spheres for Na^+ and K^+, respectively) of 0.5 mM TBA^+ as the starting condition in the cation titration experiments. Inset in panel B zooms on the lower concentration range used in the titrations, which cannot be clearly seen in the overall binding curves. Results shown here and in the following Figures are presented as the average t_m (in Celsius) ± SD from three to four independent titrations. The term "independent" used in this context means that the same samples were prepared from different protein stocks and the titration of such samples were carried out in different days. Experimental conditions are detailed under Methods. Panel C facilitates the comparison of the effects of TBA^+ on Na^+ and K^+ binding, by subtracting from the TBA^+-bound curves the thermal stabilization of the protein caused by the presence of the TBA^+ blocker *per se*, in the absence of added Na^+ or K^+.

FIGURE 2. Binding of K^+ (dark blue), Rb^+ (red) and Cs^+ (green symbols) to wild-type KcsA in competition with a fixed concentration of 100 mM Na^+. The changes in the apparent t_m with the concentration of each of the permeant cations (panels A, B and C) allows the detection of three sets of thermodynamically different binding sites (see the Supplementary Information). Insets in panels A, B and C zoom on the lower concentration range used in the titrations, which cannot be clearly seen in the overall binding curves. Panels D, E and F show the fitting of the experimental data (n = 3) to Equation 1 for the first (D), second (E) and third (F) sets of binding sites. The apparent K_D`s for the above competitive binding events are given in Table II.

FIGURE 3. Competitive binding of Na^+ to wild-type KcsA in the presence of different initial amounts of K^+. Panel A shows representative thermal denaturation curves of KcsA to illustrate that addition of 200 mM Na^+ to a sample containing 5 mM K^+ result in further protein thermal stabilization, indicative of competitive Na^+ binding (blue and pink spheres, respectively). On the contrary, addition of 200 mM Na^+ to a sample containing 25 mM K^+, at which the transition to a fully conductive state of the selectivity filter has been completed, results in no significant modification of the channel's t_m (blue and pink squares). Panel B shows the analysis of such Na^+ binding data in the absence of K^+ (circles; n = 3) and in the presence of several K^+ concentrations (triangles, inverted triangles and squares for 5, 10 and 25 mM K^+, respectively; n = 3 in all cases) used to determine the apparent K_D's for Na^+ binding (K_D app) under competitive conditions (25). Panel C shows a semi-log plot of the changes in the experimental (open circles) and theoretical (open squares) apparent K_D's for Na^+ when in competition with increasing concentrations of K^+ (25). The lines connecting experimental and theoretical data points are just a guide to the eye. The black symbols to the left of the panel (at zero K^+) correspond to the experimental K_D's for Na^+ binding under non-competitive conditions to the wild-type (black circle) (26) and to the mutant E71A KcsA (black triangle; see Figure 5) channels, as representative of non-conductive and conductive states of the channel selectivity filter, respectively.

FIGURE 4. Binding of Na^+ to the mutant E71A KcsA channel under non-competitive conditions. Panel A shows representative thermal denaturation curves of the mutant channel at several of the Na^+ concentrations used in the titration (0.1 M (black circle); 0.2 M (square); 0.5 M (grey circle); 1.25 M (inverted triangle); 2 M (triangle)). Panel B shows the fitting to Equation 1 (see Methods) of the experimental data (n = 3) from the whole range of Na^+ concentrations used in the titrations, which yields a K_D value of 256 mM (C.I. 195-336 mM) for the single Na^+ binding event detected.

FIGURE 1

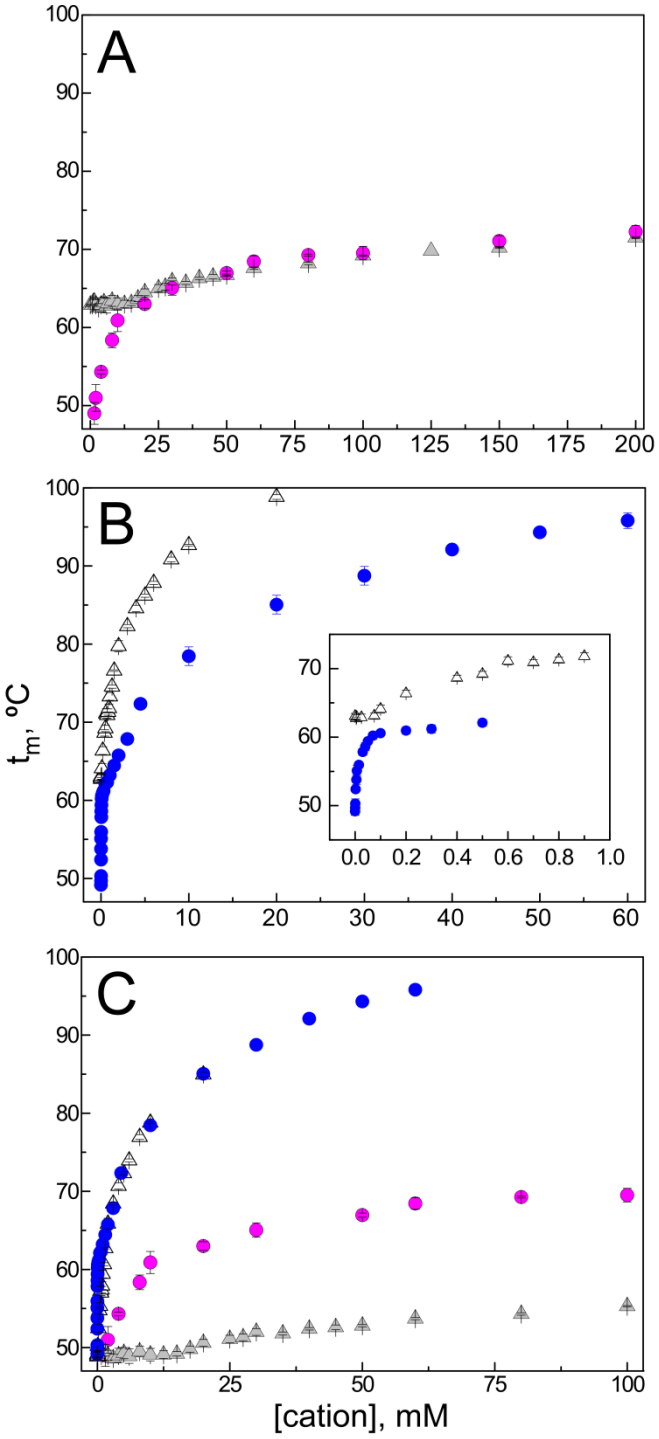


FIGURE 2

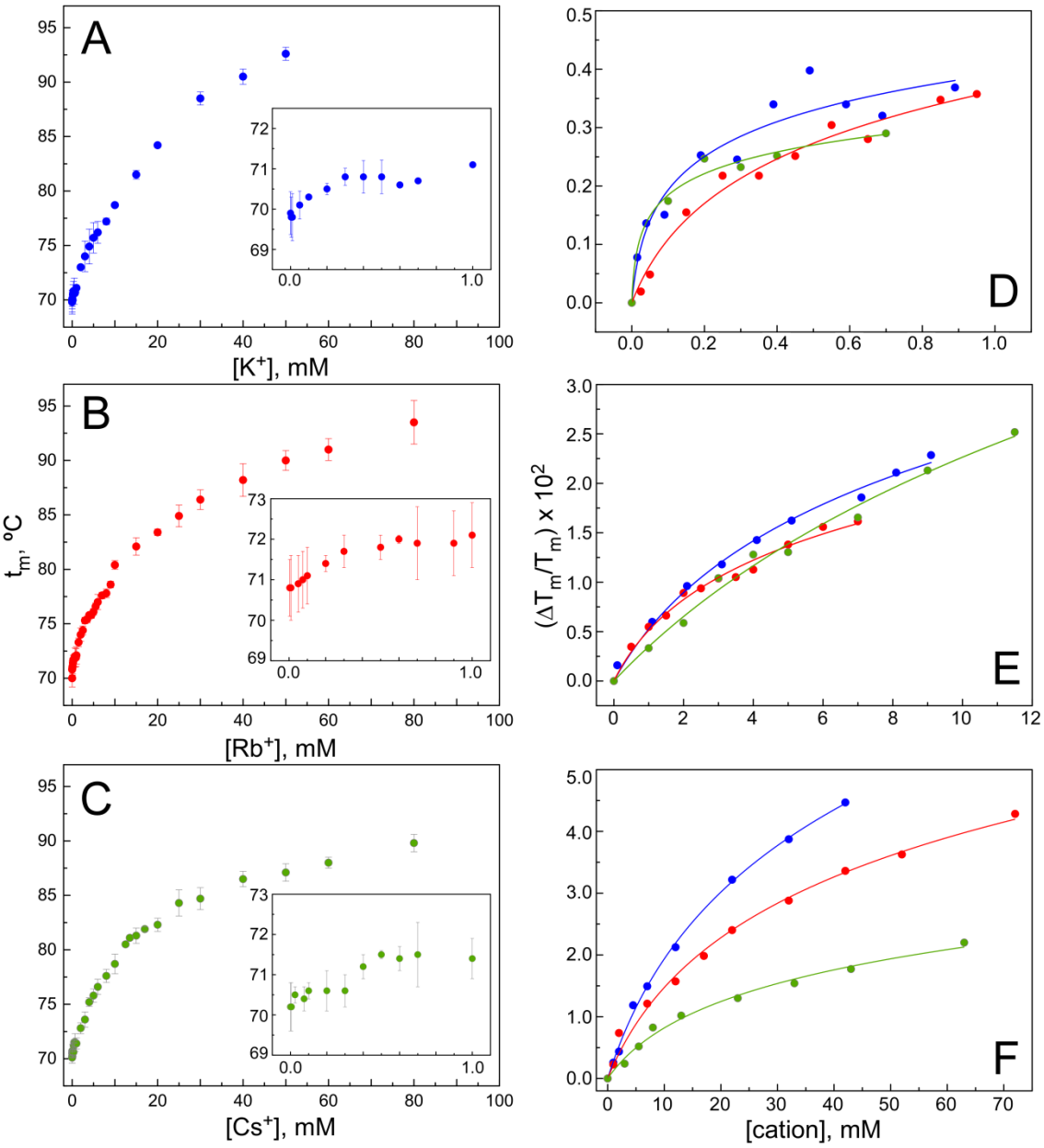


FIGURE 3

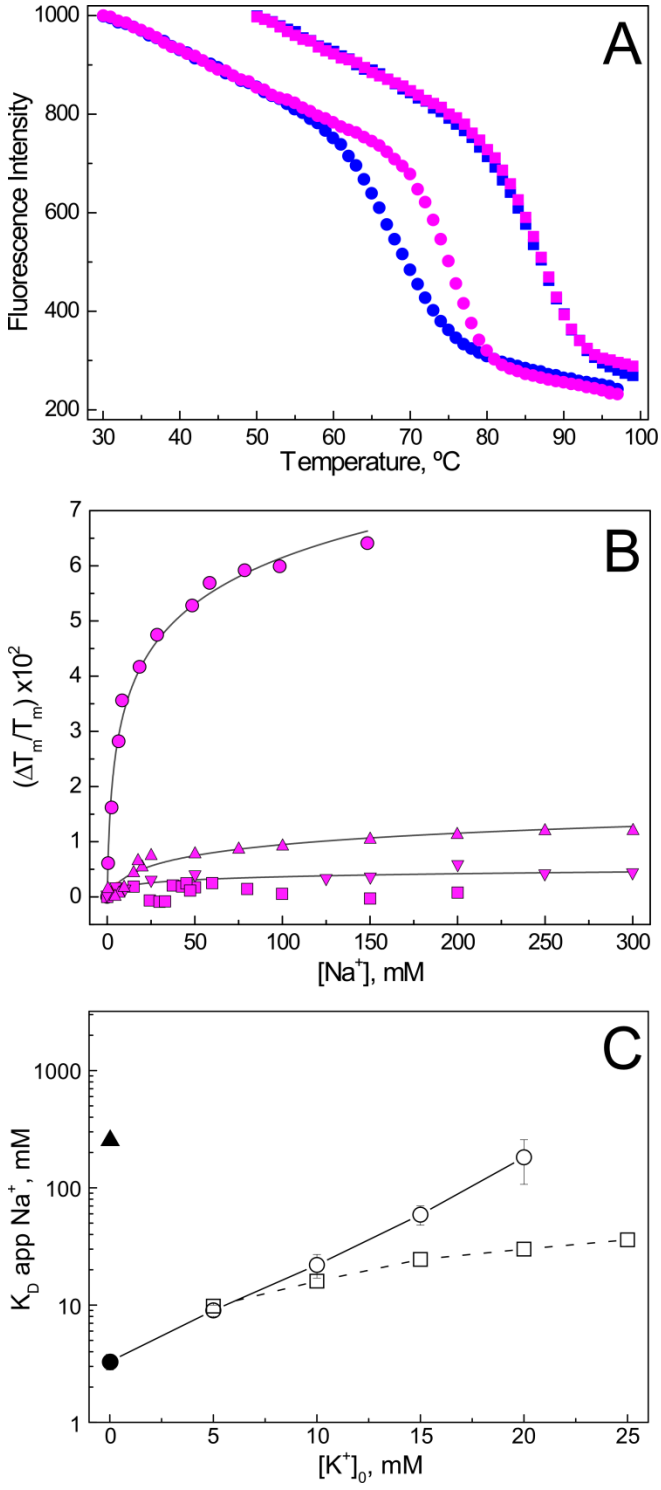
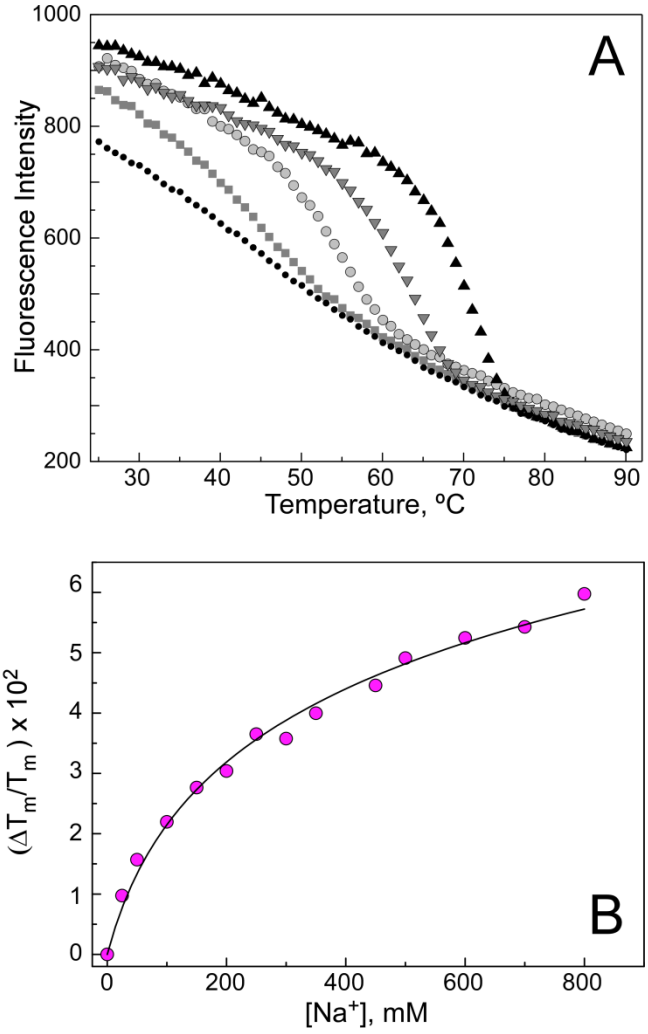


FIGURE 4



Selective exclusion and selective binding both contribute to ion selectivity in KcsA, a model potassium channel

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