Molecular mechanism of pH sensing in KcsA potassium channels

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The bacterial potassium channel KcsA is gated by high concentrations of intracellular protons, allowing the channel to open at pH < 5.5. Despite prior attempts to determine the mechanism responsible for pH gating, the proton sensor has remained elusive. We have constructed a KcsA channel mutant that remains open up to pH 9.0 by replacing key ionizable residues from the N and C termini of KcsA with residues mimicking their protonated counterparts with respect to charge. A series of individual and combined mutations were investigated by using single-channel recordings in lipid bilayers. We propose that these residues are the proton-binding sites and at neutral pH they form a complex network of inter- and intrasubunit salt bridges and hydrogen bonds near the bundle crossing that greatly stabilize the closed state. In our model, these residues change their ionization state at acidic pH, thereby disrupting this network, modifying the electrostatic landscape near the channel gate, and favoring channel opening.

ion channel | proton sensor | salt bridge network | pH gating

Activity of ion channel proteins is modulated by signaling molecules that tightly control the opening and closing of the channel pores, allowing ions to cross the membrane in response to cellular signals. Protons are ubiquitous modulators of ion channel gating and permeation, likely because of the presence of titratable residues located near channel gates, pores, allosteric sites, and regulatory interfaces. Ion channels sensitive to either cytoplasmic or extracellular pH include: transient receptor potential (TRP) and acid-sensing (ASIC) channels, inward rectifier potassium channels (Kir), CLC chloride channels, NMDA receptors, and Ca-activated potassium channels (1–7). Strict modulation of channel gating near neutral pH is often crucial for the physiological roles of these channels. Despite the importance of pH modulation in these channels, the molecular mechanisms of proton gating are not completely understood, partly because of the absence of detailed structural information.

The prokaryotic potassium channel KcsA, the first K^+ channel characterized with x-ray crystallography (8), is modulated by pH in a very narrow acidic pH range (9, 10). The availability of an atomic structure combined with a sensitive functional assay (electrophysiological current recordings with purified channel protein) make KcsA an ideal system for locating and dissecting its pH sensor. KcsA senses pH at its intracellular side (10) and the pH sensor location has been further narrowed by truncation constructs that maintain the pH sensitivity of the full-length channel (11). Moreover, an NMR study recently implicated a histidine located near the bundle crossing of KcsA as the pH sensor (12). Despite major advances in our understanding of this archetypal ion channel, there is no detailed molecular picture of the pH sensor. Elucidating the mechanism underlying KcsA pH sensing may provide a foundation for understanding similar pH-gating dependencies in eukaryotic potassium channels.

Here, we describe a mechanism for pH sensing in KcsA based on the behavior of channel mutants rationally designed by inspecting the closed structure. We constructed a series of mutants that remain open at progressively higher pH values, as high as pH 9, for a highly pH-insensitive mutant. The pH sensor mechanism is not composed of a single amino acid but consists of a network of ionizable residues that form complex inter- and intrasubunit interactions at the cytoplasmic ends of the TM1 and TM2 transmembrane helices.

Results

Neutralizing Two Glutamates at the C Terminus of TM2 Dramatically Shifts pH Sensitivity. We initially investigated cytoplasmic glutamates and aspartates, because their expected pK_a values are similar to the acidic pH values that modulate KcsA activity. There are 10 glutamates and one aspartate at the cytoplasmic C terminus of KcsA. Of those, only two possibly play a role in pH sensing: E118 and E120, located on TM2 just below the junction between the membranous and cytoplasmic parts of the channel (Fig. 1*A*). The other nine are located beyond F125, the start of a previous C-terminal truncation that was shown to have an intact pH sensor (11). For this study we made mutations on the background of a noninactivating KcsA mutant (E71A KcsA), which was previously shown to be nearly 100% open at pH 4 (13). The high P_o of E71A KcsA insures confidence in our assessment of channel number and open probability, which makes the effect of pH sensor mutations easier to ascertain (Fig. 1*B*). This mutant's ability to sense pH ($pH_{0.5} = 5.3 \pm 0.007$, Fig. 2*A*) appears similar to wild-type KcsA, although a thorough comparison with the wild-type KcsA pH dependence is difficult because of inactivation (14, 15).

We replaced both E118 and E120 (Fig. 1*A* and Fig. 2*A Inset*) with glutamines, in an effort to mimic protonated glutamates. If either of these residues is the pH sensor, then this double mutation should generate a channel open beyond pH 5.5 into the neutral pH range. Surprisingly, the E118Q/E120Q mutant ($pH_{0.5} = 5.5 \pm 0.005$) had a behavior similar to the background channel (Fig. 2*A*, black circles).

We examined further how pH sensing depends on the chemical properties at sites 118 and 120. Although E to Q is a charge-neutralizing mutation, this change may preserve the potential for hydrogen bonding and introduce new hydrogen-bonding capabilities (glutamines possess an amine where glutamates have oxygen). Hence, we replaced the glutamates with alanines because they are minimal in H-bonding capacity and less likely to contact the interaction partners of the glutamines. Indeed, the E118A/E120A KcsA mutant opens at pH < 7 (Figs. 1*B* and 2*A*) shifting the P_o vs. pH curve more than one pH unit beyond the background ($pH_{0.5} = 6.6 \pm 0.004$). Both the background channel and the two glutamate mutations show a very steep pH dependence because their activity falls from nearly 100% open to almost completely closed over half a pH unit (Fig. 2*A*).

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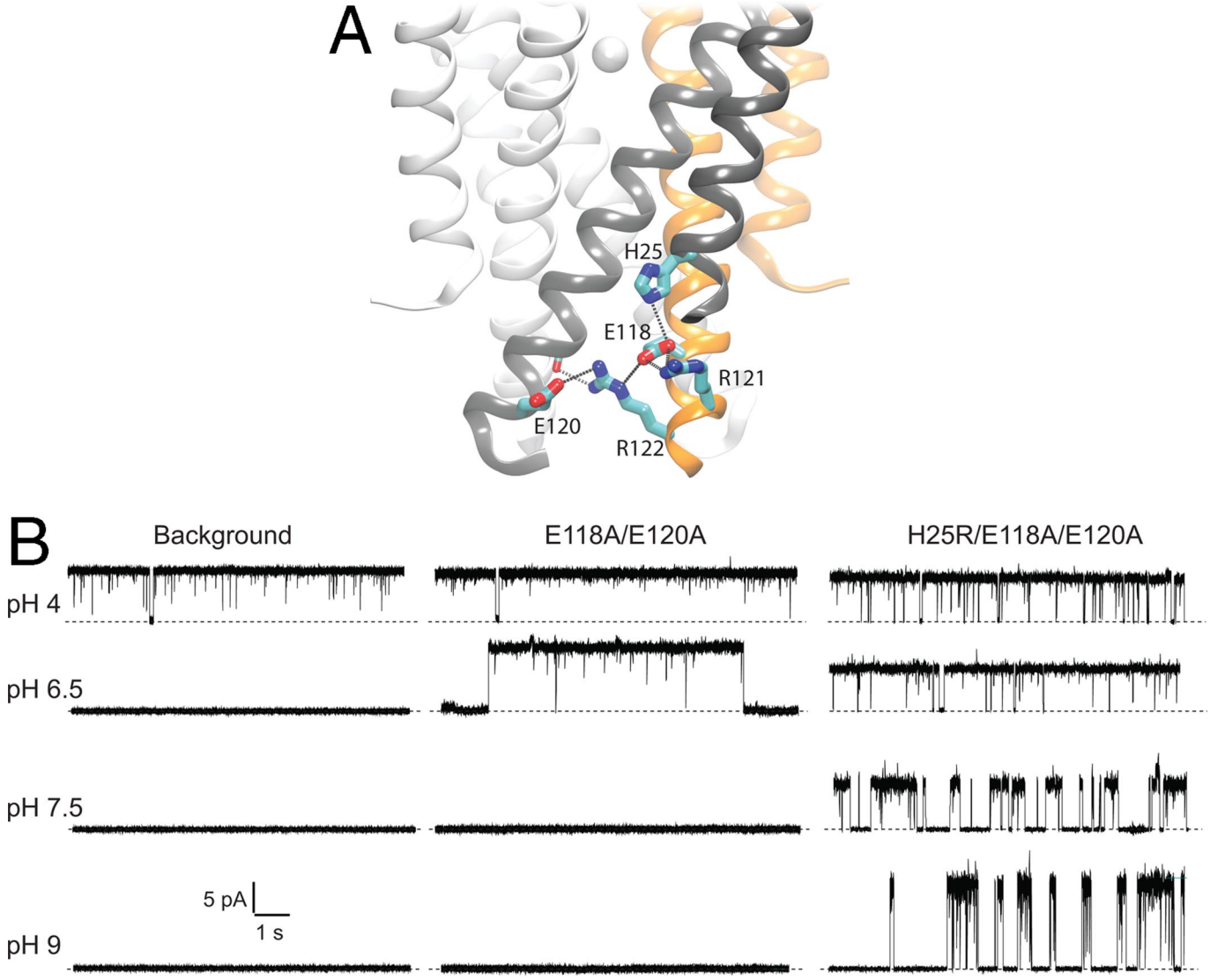

Fig. 1. The KcsA pH sensor is a complex network of ionizable residues. (*A*) Proposed interactions between key residues are highlighted in the KcsA structure (PDB ID code 1k4c) with carbon (cyan), nitrogen (blue), and oxygen (red). Intersubunit connections are possible (dotted lines) between R122, E120, G116 (backbone carbonyl shown), H25, and E118; intrasubunit connections are possible (dotted lines) between E118 and R121 or R122. Two adjacent subunits are shown in the foreground in gray and orange. (*B*) Representative single KcsA current traces at 100 mV, in symmetric 100 mM [K^+] for background (E71A KcsA), E118A/E120A, and H25R/E118A/E120A channels oriented with the intracellular side facing the *trans*-side of a horizontal lipid bilayer chamber at various intracellular pH values (*Left*). The traces shown at different pH values for each mutant are from the same bilayer. The *cis*-side of the chamber was pH 7 for all experiments. Dotted lines represent the closed-channel level.

Histidine 25 at the N Terminus of TM1 Contributes to pH Sensing. The two glutamates at the C terminus of TM2 appear to be major contributors to the KcsA-gating mechanism. However, their mutation to alanines was not sufficient to open the channel at pH 7 and higher. This indicates the existence of additional components to the KcsA pH sensor. Histidine 25 is located at the N terminus of transmembrane segment-1 (TM1) within interaction distance of E118 (3.95 Å) on the transmembrane segment-2 (TM2) of an adjacent subunit (Figs. 1*A* and 2*B Inset*) and it was previously implicated as a sole pH sensor residue in an NMR study (12). The solution pK_a of histidine ($pK_a = 6.0$) is near the pH range modulating the E118A/E120A mutant, making it an excellent candidate for the remaining pH sensor component in this channel.

To test the importance of H25 for pH sensing near neutral pH, we made H25R to mimic a constitutively protonated histidine. The P_o vs. pH curve for H25R KcsA is not shifted compared with the background channel ($pH_{0.5} = 5.3 \pm 0.01$; Fig. 2*B*, blue circles). However, the P_o vs. pH relationship appears shallower indicating that the H25R mutant may have affected channel gating. When we combined the H25R mutation with the E118Q/E120Q, we saw a large right shift in the P_o vs. pH curve ($pH_{0.5} = 6.3 \pm 0.04$; Fig. 2*B*, black circles) relative to the background channel, suggesting that H25 and E118/E120 act in concert to open the channel.

When we introduced H25R on the background of the previously described E118A/E120A channel, the resultant mutant remained open from pH 4 to 9 (Figs. 1*B* and 2*B*), suggesting that we had touched on all key components of the pH sensor. Most H25R/E118A/E120A channels display intermittent subconductance gating more frequently than the other mutants, a property that has yet to be explained [Fig. 1*B* and supporting information (SI) Fig. S1]. A majority of the bilayers (6) showed little decrease in P_o from pH 4 to pH 9 (Figs. 1*B* and 2*B*). Two other bilayers

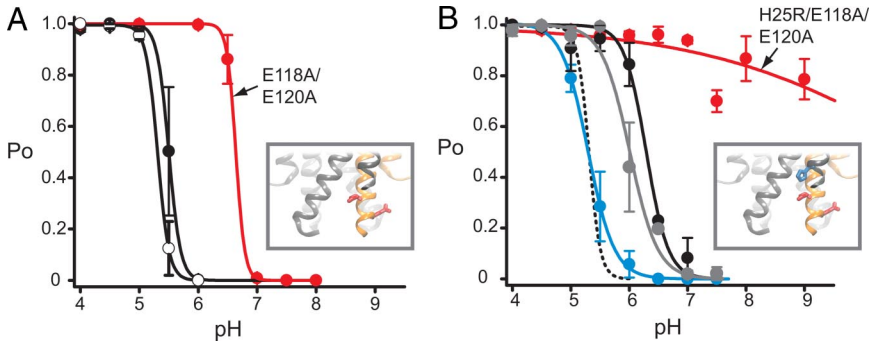

Fig. 2. Mutations of select residues just below the bundle-crossing shift KcsA pH sensitivity. (*A*) P_o vs. pH plots with fits to the Hill equation (lines). E71A, background channel (open black circles): $n_H = 4.4 \pm 0.1$, $pH_{0.5} = 5.3 \pm 0.007$; E118Q/E120Q (filled black circles), $n_H = 4.4 \pm 1.2$, $pH_{0.5} = 5.5 \pm 0.005$; E118A/E120A (filled red circles), $n_H = 5.8 \pm 0.2$, $pH_{0.5} = 6.6 \pm 0.004$. The locations of E118 and E120 in the KcsA structure are highlighted in red (*Inset*). (*B*) P_o vs. pH curves with Hill fits: background (dashed black line); H25R (filled blue circles), $n_H = 1.9 \pm 0.1$, $pH_{0.5} = 5.3 \pm 0.01$; H25R/E118Q/E120Q (filled black circles), $n_H = 2.5 \pm 0.5$, $pH_{0.5} = 6.3 \pm 0.04$; H25R/R117Q/R121Q/R122Q (filled gray circles), $n_H = 1.8 \pm 0.4$, $pH_{0.5} = 6.0 \pm 0.05$; H25R/E118A/E120A (filled red circles). The red line through the H25R/E118A/E120A red symbols has no theoretical meaning. The location of H25 is highlighted in blue with E118 and E120 in red (*Inset*). Error bars in *A* and *B* are the standard error of the mean for three to seven experiments. Error values for n_H and $pH_{0.5}$ are standard error estimates from the fit.

(not included in Fig. 2, shown in Fig. S1) have a P_o of ≈1 for pH < 7 and a P_o that drops <20% at pH > 7. These outliers illustrate that our mutant KcsA channel may retain a gating component independent of the protonation state of these key residues. In addition, H25R/E118A/E120A channels displayed significant gating modes, a property of KcsA channels that has been described (14) but not understood. It is possible that in these two bilayers the incidence of lower P_o modes is more prevalent. A majority of our channels, however, are constitutively open (Fig. S1).

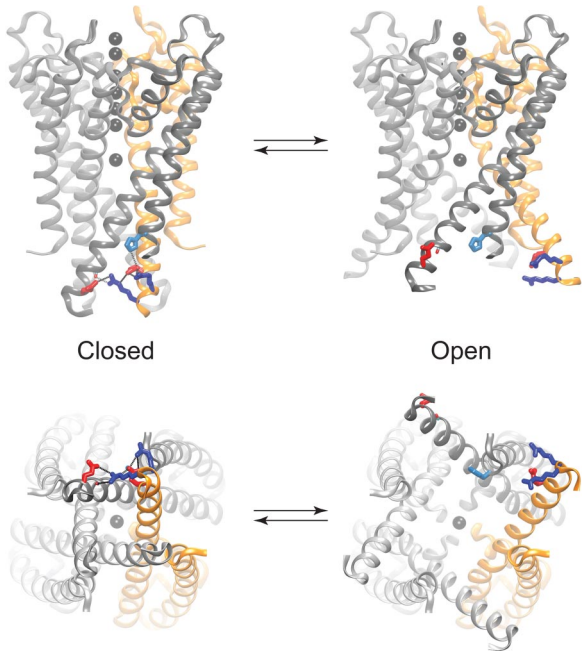

Fig. 3. Model of how KcsA transitions between closed and open states. A simple model of the KcsA open state was generated by bending the TM2 helices at the ''glycine hinge'' in a direction similar to that of the MthK channel structure (see *Methods*). The closed-to-open transition illustrates that the intersubunit interactions that stabilize the closed state (*Left*) are inhibited by protons, allowing key residues to move far from each other in the open state (*Right*). This proton-induced structural change is shown to widen the entrance to the K^+ pore (intracellular view, *Lower*). Two adjacent subunits (dark gray and orange) are shown in the foreground with key residues highlighted: glutamates 118 and 120 (red), histidine 25 (cyan), arginines 121 and 122 (blue).

Model for KcsA pH-Sensing Mechanism. We propose a model for pH gating in KcsA based on residues mutated in the constitutively open channel. In this model, both glutamates, E118 and E120, interact with arginine 122 in the closed state, making an inter- and intrasubunit network of salt bridges and hydrogen bonds (Fig. 1*A*). Histidine 25 can also make an intersubunit interaction with glutamate 118 (Fig. 1*A*). We suggest that at neutral pH, these intersubunit interactions constrain the ends of the TM2 helices at the bundle crossing, greatly stabilizing the closed conformation. At acidic pH, the glutamates protonate and become neutral, breaking all inter- and intrasubunit salt bridges and increasing the net positive charge on the TM2 helices; the histidine becomes positively charged, destabilizing the bundle crossing in two possible ways: (*i*) electrostatic repulsion with TM2 arginines 117, 121–122, and/or (*ii*) increased hydrophilicity in the mainly hydrophobic environment of the bundle crossing. We propose that these changes induce the TM2 helices to separate either through electrostatic repulsion or by strain-dependent rearrangement, destabilizing the closed state and opening the channel (Fig. 3).

The model above for KcsA pH gating predicts that neutralization of the glutamates resulted in the disruption of a key salt bridge with the TM2 arginines. Neutralization of the partner arginines is therefore predicted to yield a similar result. We neutralized all three TM2 arginines (R117Q/R121Q/R122Q) on the background of the H25R channel and measured a P_o vs. pH curve similar to the H25R/E118Q/E120Q channel, suggesting arginine–glutamate interactions (Fig. 2*B*, gray symbols). The H25R/R117Q/R121Q/R122Q mutant is still pH-sensitive, likely because of the preservation of hydrogen-bonding capability when glutamines are substituted for arginines (keeping in mind that glutamates 118 and 120 are still present in this mutant). A similar mechanism is likely responsible for the pH-dependent gating observed in the E118Q/E120Q mutant, discussed earlier.

In addition, because protonation of H25 increases the destabilization of the closed state, neutralization of this histidine should result in the stabilization of the closed-channel conformation. To test this, we mutated H25 to an alanine on the background of a channel with a more stable open conformation (E118A/E120A). Indeed, the H25A/E118A/E120A KcsA mutant is not open at pH values as high as the E118A/E120A mutant (Fig. S2), further supporting the proposed role of H25 protonation in opening the channel.

Discussion

Since the appearance of the first KcsA structure in 1998, a plethora of structure-related techniques have been directed at

determining the open conformation of the KcsA channel, including EPR, solution NMR, mass tagging and spectrometry, or theoretical calculations and simulations (12, 16–20). Two articles have proposed molecular mechanisms for pH gating although neither study contains functional ion channel data to support their conclusions. In a theoretical study, Miloshevsky and Jordan (20) performed Monte Carlo simulations that suggested that protonation of E118 and E120 is sufficient to open KcsA channels. Although our results agree with the simulation in that protonation of the two glutamates is a major determinant within the gating mechanism, we found that mutation of both glutamates to glutamines or to alanines produced channels that were closed above pH 5.5 and 6.5, respectively. Our data indicate that other residues also contribute to a network of inter- and intrasubunit interactions that underlie proton sensing.

In a separate study, Takeuchi and colleagues (12), found that mutation of H25 to alanine abolished the conformational changes associated with varying pH in a solution NMR experiment. Thus, H25 was proposed to be the KcsA pH sensor. Again, our experiments indicate that this is not the full story. We found that H25 plays a significant role in pH sensing because the additional mutation of H25 to arginine was necessary to keep the E118A/E120A KcsA channel open as high as pH 9. Furthermore, changing H25 to alanine in the E118A/E120A mutant produced a small leftward shift in the P_o vs. pH curve, indicating that neutralization of the positive charge at position 25 favored channel closure. Interestingly, the H25R mutation did not shift the P_o vs. pH curve by itself, because the intersubunit salt bridges formed between the glutamates and arginines favor the closed conformation. However, H25R in addition to E118Q/E120Q produced a rightward shift in P_o vs. pH, suggesting a cooperative opening effect between these mutations. All of these results together suggest a complex interaction between ionizable and charged residues within the proposed pH sensor.

Although we cannot exclude the possibility that our mutations may have biased the open–closed equilibrium of KcsA channels through a mechanism other than altering the pH sensor, we believe mutating the minimal number of amino acids to their protonated counterparts minimized this potential effect. In addition, we introduced multiple mutations at key residues that together implicate these positions as proton-binding sites that modulate channel gating directly.

Model of an Open KcsA Channel. Our proposed mechanism for pH sensing in the KcsA channel is based on a proton-dependent disruption of intersubunit salt bridges and hydrogen bonding mediated by glutamates combined with bundle-crossing destabilization mediated by histidine 25. In Fig. 3 we show a simple diagram of the KcsA closed-to-open structural transition. The open channel is generated from the closed KcsA structure by bending the TM2 helices in a manner similar to the open-state MthK potassium channel structure (21, 22). Although we cannot speculate on the degree of opening at the KcsA inner mouth, we can illustrate how the proton sensor residues change their relative locations. In this model, the proposed interactions keeping the channel closed are disrupted in the open state because of the protonation of key residues. Intersubunit salt bridges between TM2 residues R122 and E120 are no longer possible. H25, now positively charged, also disrupts the packing of the TM2 helices near the bundle crossing. Protonation of these residues provides the force that destabilizes the tight TM2 packing in the closed conformation and can make such an open state the preferred conformation. Future high-resolution crystal structures of KcsA, perhaps of the open mutants described here, will elucidate the extent of TM2 opening and how it compares with other known K^+ channel structures.

Is it plausible for the disruption of intersubunit salt bridges and hydrogen bonds combined with the protonation-induced destabilization of the TM2 helices to underlie the significant conformational changes observed in KcsA gating? Based on data gathered from other proteins, the answer appears to be yes. In the bacterial colicin ion channel, the breaking of a salt bridge between transmembrane domains on acidification leads to activation of the channel (23). In AMPA receptors, mutation of an intersubunit salt bridge decreases dimer stability and speeds deactivation (24). In Kir 1.1 channels, an intrasubunit H bond stabilizes the closed state (25) whereas intersubunit salt bridges stabilize the open state (26).

Interestingly, in the KcsA structure, R122 is positioned such that three stabilizing interactions are possible: (*i*) the critical intersubunit salt bridge with E120, (*ii*) potential salt bridge or hydrogen bond with E118, and (*iii*) an intersubunit hydrogen bond with the backbone carbonyl oxygen of G116 (Fig. 1*A*). Such a network of intersubunit interactions probably enhances the stability of the closed structure significantly. A previous analysis of the distribution and geometry of salt bridges in a collection of proteins of known structure revealed that complex salt bridges are frequently used to connect protein subunits and domains important for allosteric regulations (27–29). Musafia and colleagues (28) found that the most conserved salt bridges appear to contain arginines and are either in catalytic sites or at interfaces between subunits, as in our model.

Does the Identification of the KcsA pH Sensor Have Implications for Other K^+ Channels? KcsA is presently the only known K^+ channel that opens at extreme acidic pH values and closes steeply at pH ≈ 5 and higher, in contrast to the pH dependencies in the physiological range for eukaryotic channels. However, that does not necessarily imply that the mechanism KcsA employs to sense pH is different from other ion channels. We examined regions of inward rectifier potassium channels (Kir) equivalent to the pH sensor in KcsA (alignment in ref. 30) and found groups of charged residues just below the putative bundle-crossing region similar to the proposed KcsA pH sensor. Some Kir channels have histidines at equivalent positions to H25 in KcsA whereas others have arginines, also a good candidate for inter- and intrasubunit interactions. A semiconserved lysine, shown to be involved in pH sensing in Kir channels, is also located nearby (31). Glutamates and arginine residues are also encountered at the C terminus of TM2 in most inward rectifier channels. These residues may form part of the pH sensor in Kir channels by a similar mechanism as KcsA.

Recent studies also indicate the involvement of arginine-glutamate (R-E) intersubunit salt bridges in the pH gating of inward rectifier potassium channels [Kir1.1 (26) and Kir 6.2 (32)]. Although its location is on the cytoplasmic domain of these channels and not near the bundle crossing as proposed for KcsA, this R-E pair, conserved in almost all inward rectifier potassium channels, is an example of a critical intersubunit salt bridge. This interaction has been suggested to stabilize the open state of Kir, as opposed to KcsA, which has an R-E salt bridge likely stabilizing the closed state. Therefore, although the pH modulation of KcsA and various eukaryotic channels are qualitatively different, the basic underlying mechanism may be similar.

Conclusion

We report a set of KcsA mutations near the bundle crossing that mimic the protonated state of the wild-type channel with respect to charge. The most pH-insensitive mutant remains open from pH 4 to pH 9. We proposed that at neutral and basic pH a complex salt bridge and hydrogen-bonding network connects TM1 and TM2 helices, stabilizing the closed conformation. At acidic pH, glutamate and histidine residues protonate, disrupting these connections, and allow the channel to open. Similar pH-dependent interactions may modulate the gating properties of a wide variety of ion channels.

Methods

Protein Expression, Purification, and Reconstitution in Lipid Bilayers. KcsA channel protein expression and purification protocol is in refs. 10 and 33. In brief, N-terminal hexahistidine-tagged KcsA variants constructed in a pASK90 vector (34) were transformed into JM83 *Escherichia coli* (American Type Culture Collection) and grown at 37°C in Terrific Broth (TB) to $OD_{600} = 1$. Protein expression was induced for 90 min with 0.2 mg/ml anhydrotetracycline (ATC; Acros Organics). Induction at 20°C overnight was carried out for a subset of the mutants (H25R/E118A/E120A and H25R/R117Q/R121Q/R122Q) because of their low initial protein expression/aggregation/easy denaturation at 37°C as observed on gel filtration and SDS/Coomassie protein gels. Cells were harvested with centrifugation at 5,000 × *g* for 15 min at 4°C, resuspended in 100 mM KCl, 50 mM Tris, pH 7.5, and broken with probe sonication (Fisher Scientific). Membranes were extracted by shaking for 2 h at room temperature in 50 mM *n*-decyl maltoside (DM; Anatrace) and applied over a Ni-affinity column (Qiagen) in buffer B (100 mM KCl, 20 mM Tris, 5 mM DM, pH 7.5). KcsA was eluted with 300 mM imidazole and then purified with gel filtration (Superdex 200, GE) in buffer B. Tetramer integrity was verified on a Coomassie-stained SDS gel by examining the stability of the tetrameric band at ≈60 kDa compared with the monomeric band at ≈17 kDa. Immediately after purification, KcsA was reconstituted into liposomes at protein-to-lipid ratios of 0.1–10 μg of protein per mg of lipid (3:1 POPE:POPG; Avanti Polar Lipids) by removing the detergent over a hand-packed G50 fine (GE) gel filtration column in 400 mM KCl, 20 mM Tris, pH 7.5, as described (10, 33, 35), and 100-μl liposome aliquots were flash-frozen in liquid nitrogen and stored at −80°C. Before being used for lipid bilayer recordings, liposomes were thawed and sonicated for 5 sec.

All mutations were introduced with QuikChange (Stratagene) site-directed mutagenesis as per the manufacturer's instructions, and the coding region was sequenced. We experimented only with channel mutants where the tetrameric stability of KcsA channels was preserved as shown by the presence of a band at ≈60 kDa on Coomassie-stained SDS gels.

Single-Channel Recording and Analysis. Lipid bilayers are formed from a 10 mg/ml POPE:POPG (3:1) mix resuspended in *n*-decane (Sigma) on a 50-μm partition (transparency slide, IKON) stuck with vacuum grease on a larger hole separating two horizontal chambers containing the recording solutions as described in ref. 36. The TRANS chamber (lower) is grounded and the CIS chamber (upper) holds the recording electrode and is where the lipids are applied. Proteoliposomes were then applied and the insertion of channels into the bilayer was monitored electrically. Currents were recorded in Clampex 10 under continuous mode with an Axopatch 200 A and B, digitized with a Digidata 1440A and 1320 (Molecular Devices), sampled at 20 kHz and filtered at 2 kHz. Recording solutions were: 70 mM KCl, 30 mM KOH, 10 mM Mops, 10 mM succinate, and 10 mM Tris to achieve buffering for pH values from 4 to 9. We attempted insertion of channels with their cytoplasmic side facing the *trans* chamber at pH 4 whereas the *cis* chamber has pH 7. There seemed to be a preferential insertion of channels in this orientation because only ≈10% of the KcsA mutant channels open at neutral pH inserted the other way. Channel orientation was confirmed by typical conductance and open-channel noise properties of KcsA (10), a feature maintained in the E71A mutants (data not shown). Currents were analyzed in Clampfit 10 (Molecular Devices). Single-channel current amplitudes were measured by hand from at least 100 events, and open probabilities (NP_o) were determined from traces with at least 20 events with the Single-Channel Search module from Clampfit. Single-channel open probabilities (P_o) were then determined by dividing the NP_o to the total number of channels. The constancy of the number of channels during perfusions was verified by returning during the experiment periodically to pH 4–4.5 where channels are open all of the time. Bilayers where this was not achievable because of bilayer rupture or gain/loss of channels were not included in the final analysis. Consequently, all bilayers included in the analysis have at least two perfusions: from pH 4 to a neutral pH and back to pH 4 to verify the constancy of channel numbers. For each mutant presented, at least one bilayer was successfully perfused through the whole pH range to verify the phenotype of the mutants. P_o vs. pH curves were generated in Origin (Microcal) and fit with the Hill equation after conversion of the *x* axis from pH to proton concentration:

$$P_o = \frac{P_{o,\max}}{\left(1 + \frac{K_d}{[H^+]}\right)^{n_H}},$$

where $K_d = -\log(pH_{0.5})$ and n_H is the Hill coefficient.

All mutants investigated have permeation properties typical of KcsA channels, suggesting that the mutations do not deleteriously affect the integrity of the channels. Inward currents have much greater open-channel noise than outward currents (10, 36). The outward current amplitudes of all mutants increase with intracellular pH similarly to wild-type KcsA channels (37) (data not shown, with the exception of the H25R/E118A/E120A, where this is more difficult to ascertain because of the high frequency of subconductance gating). All channel mutants maintained tetrameric stability, observed as a 60-kDa band on Coomassie-stained SDS gels (38) (Fig. S3). Additionally, we found that the mutant channel chord conductances (at ±100 mV) are similar to the background channel (E71A KcsA) and Na^+ blocks the H25R/E118A/E120A KcsA in a voltage-dependent manner similar to the background channel (Fig. S3). We note that both the E71A and the mutants made on the background of E71A differ from wild-type KcsA in Na block and magnitude of outward current (33) (Fig. S3).

Open KcsA Model Generation. The open KcsA model was generated with least-squares structural alignment of specific KcsA residues with MthK by using the program O (39). Kcsa (PDB ID code 1K4C) residues 22–98 were placed according to the alignment of TM2 above the gating hinge between residues 87–98 (KcsA) and residues 71–82 (MthK), and the TM2 helices were individually placed by using alignment below the gating hinge between residues 99–102 (KcsA) and 83–86 (MthK). Structure figures were generated in VMD (40).

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