Molecular mechanism of pH sensing in KcsA potassium channels

Ameer N. Thompson, David J. Posson, Pirooz V. Parsa, and Crina M. Nimigean*

Department of Anesthesiology, and Department of Physiology and Biophysics, Weill Cornell Medical College, New York City, NY 10065

Edited by Christopher Miller, Brandeis University, Waltham, MA, and approved February 20, 2008 (received for review January 28, 2008)

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.

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. 1A). 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 nonactivating 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. 1B). This mutant’s ability to sense pH (pH_{0.5} = 5.3 ± 0.007, Fig. 2A) 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. 1A and Fig. 2A 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 ± 0.005) had a behavior similar to the background channel (Fig. 2A, 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 glutamates. Indeed, the E118A/E120A KcsA mutant opens at pH < 7 (Figs. 1B and 2A) shifting the P_o vs. pH curve more than one pH unit beyond the background (pH_{0.5} = 6.6 ± 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. 2A).

Author contributions: A.N.T. and D.J.P. contributed equally to this work; A.N.T., D.J.P., and C.M.N. designed research; A.N.T., D.J.P., and P.V.P. performed research; A.N.T., D.J.P., and C.M.N. analyzed data; and A.N.T., D.J.P., and C.M.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

*To whom correspondence should be addressed. E-mail: cm2002@med.cornell.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0800873105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA
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. 1A and 2B Inset) and it was previously implicated as a sole pH sensor residue in an NMR study (12). The solution pKa of histidine (pKa = 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 Popen vs. pH curve for H25R KcsA is not shifted compared with the background channel (pH0.5 = 5.3 ± 0.01; Fig. 2B, blue circles). However, the Popen 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 Popen vs. pH curve (pH0.5 = 6.3 ± 0.04; Fig. 2B, 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. 1B and 2B), 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. 1B and supporting information (SI) Fig. S1]. A majority of the bilayers (6) showed little decrease in Popen from pH 4 to pH 9 (Figs. 1B and 2B). Two other bilayers...
These two bilayers the incidence of lower been described (14) but not understood. It is possible that in significant gating modes, a property of KcsA channels that has residues. In addition, H25R/E118A/E120A channels displayed component independent of the protonation state of these key residues. In Fig. 1, (Inset). (B) Po vs. pH curves with Hill fits: background (dashed black line); H25R (filled blue circles), nH = 1.9 ± 0.1, pH0.5 = 5.3 ± 0.01; H25R/E1180/E120Q (filled black circles), nH = 2.5 ± 0.5, pH0.5 = 6.3 ± 0.04; H25R/R117Q/R121Q/R122Q (filled gray circles), nH = 1.8 ± 0.4, pH0.5 = 6.0 ± 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.

A plethora of structure-related techniques have been directed at new structure-related techniques have been directed 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).

Fig. 2. Mutations of select residues just below the bundle-crossing shift KcsA pH sensitivity. (A) Po vs. pH plots with fits to the Hill equation (lines). E71A, background channel (open black circles): nH = 4.4 ± 0.1, pH0.5 = 5.3 ± 0.007; E118Q/E120Q (filled black circles), nH = 4.4 ± 1.2, pH0.5 = 5.5 ± 0.005; E118A/E120A (filled red circles), nH = 5.8 ± 0.2, pH0.5 = 6.6 ± 0.004. The locations of E118 and E120 in the KcsA structure are highlighted in red (Inset). (B) Po vs. pH curves with Hill fits: background (dashed black line); H25R (filled blue circles), nH = 1.9 ± 0.1, pH0.5 = 5.3 ± 0.01; H25R/E1180/E120Q (filled black circles), nH = 2.5 ± 0.5, pH0.5 = 6.3 ± 0.04; H25R/R117Q/R121Q/R122Q (filled gray circles), nH = 1.8 ± 0.4, pH0.5 = 6.0 ± 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 nH and pH0.5 are standard error estimates from the fit.

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. 1A). Histidine 25 can also make an intersubunit interaction with glutamate 118 (Fig. 1A). 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 Po vs. pH curve similar to the H25R/E118Q/E120Q channel, suggesting arginine–glutamate interactions (Fig. 2B, 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 Po vs. pH curve, indicating that neutralization of the positive charge at position 25 favored channel closure. Interestingly, the H25R mutation did not shift the Po 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 E118O/E120Q produced a rightward shift in Po 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 mediate 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 intersubunit 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. 1A). 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.
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 (P_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_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:

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

where $K_d = -\log(pH_{50})$ 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 increased 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 Mode 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 channels. Inward currents have much greater open-channel noise than outward currents (10, 36). The outward current amplitudes of all mutants increased 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).

**ACKNOWLEDGMENTS.** We thank T. Y. Chen, J. Zheng, and M. Rosenblatt for critically reading the manuscript. This work was supported in part by grants and fellowships from the National Institutes of Health (to C.M.N.), the National Science Foundation (to A.N.T.), and the American Heart Association (to C.M.N.).
Fig. S1. Gating of H25R/E118A/E120A KcsA mutant. (A) Plot of each H25R/E118A/E120A bilayer individually. Each symbol is a different bilayer serially perfused to different pH values. The two pH-gating outliers mentioned in the text are the magenta triangles and brown hexagons. (B) Illustration of subconductance gating in one bilayer with H25R/E118A/E120A single-mutant channel at the indicated pH values. Dashed line indicates the closed-current level. Currents are recorded at +100 mV in 100 mM symmetric K⁺ and are filtered at 500 Hz for display.
Fig. S2. Plot of $P_o$ vs. pH for H25A/E118A/E120A (filled red with red Hill fit, $n_H = 2.5 \pm 0.15$, $pH_{0.5} = 6.2 \pm 0.01$). For comparison, the fits to background (black line) and E118A/E120A (dashed line) from Fig. 2A are shown. Error bars 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. Surprisingly, the H25A/E118A/E120A mutant is still able to sense pH. It is possible that other pH-sensitive residues are contributing to the modulation of this channel.
Fig. S3. KcsA mutants have biochemical and permeation properties similar to wild-type KcsA. (A) Coomassie-stained SDS gel analysis of gel-filtration protein fractions used for reconstitution and bilayer recording. For each mutant, a sample is heated to >95°C for 10 min to produce the monomeric KcsA band (~17 kDa, even lanes) and another sample is kept at room temperature to check the tetrameric stability (~60 kDa band, odd lanes). Lanes: 1, marker (Benchmark prestained protein ladder); 2 and 3, H25R/E118A/E120A; 4 and 5, wild-type KcsA; 6 and 7, E118Q/E120Q; 8 and 9, H25R; 10 and 11, background (E71A); 12 and 13, H25R/R117Q/R121Q/R122Q. Gels from different experiments were normalized by using the ladder (Benchmark). (B) Single-channel current amplitudes at ±100 mV (black bars) and ±100 mV (gray bars) at 100 mM symmetrical [K⁺] and pH 4 on the trans-side are graphed for: 1, wild-type KcsA; 2, background (E71A); 3, E118Q/E120Q; 4, E118A/E120A; 5, H25R; 6, H25R/E118Q/E120Q; 7, H25R/E118A/E120A; 8, H25R/R117Q/R121Q/R122Q; 9, E118A/E120A/H124R; 10, H25A/E118A/E120A. (C) I–V curves for two individual bilayers with H25R/E118A/E120A and background (E71A) KcsA (Inset) at pH 4 (trans-side) are shown for symmetric 100 mM [K⁺] with (red symbols) or without (black symbols) 10 mM intracellular Na⁺. Smooth lines have no theoretical meaning.