Research Paper

Moving the pH Gate of the Kir1.1 Inward Rectifier Channel

Mikheil Nanazashvili1
Hui Li1
Lawrence G. Palmer2
D. Eric Walters3
Henry Sackin1,*

1Department of Physiology and Biophysics; 2Department of Biochemistry; The Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064 USA; 3Department of Physiology and Biophysics; Weill Medical College of Cornell University, New York, New York USA

*Correspondence to: Henry Sackin; Department of Physiology and Biophysics; The University; New York, New York USA

Original manuscript submitted: 10/11/06
Revised manuscript submitted: 12/06/06
Manuscript accepted: 12/12/06

This manuscript has been published online, prior to printing for Channels, Volume 1, January/February 2007; ©2007 Landes Bioscience

ABSTRACT

Both structural and functional studies suggest that pH gating of the inward rectifier potassium (K) channel, Kir1.1 (ROMK), is mediated by the convergence of four hydrophobic leucines (one from each subunit) near the cytoplasmic bundle-crossing of the inner transmembrane helices. We tested this hypothesis by moving the putative leucine gate from L160-Kir1.1b to other positions along the inner transmembrane helix, and measuring inward current and conductance as functions of internal pH, using the Xenopus oocyte heterologous expression system. Results of these studies indicated that it was possible to replace the putative inward rectifier pH gate at L160-Kir1.1b by either a leucine or methionine at 157-Kir1.1b (G157L-L160G or G157M-L160G). Although both leucine and methionine gated the channel at 157-Kir1.1b, residues of similar hydrophobicity (tyrosine and valine) did not. Hence, hydrophobicity was a necessary but not a sufficient condition for steric gating at 157. This was in contrast to the 160-Kir1.1b locus, where side-chain hydrophobicity was both a necessary and sufficient property for steric gating. Homology models were constructed for all mutants that expressed significant whole-cell currents, using the closed-state coordinates of the prokaryotic inward rectifier, KirBac1.1. Models of mutants that retained pH gating were too narrow at the bundle crossing to permit hydrated K ion permeation in the closed-state. On the other hand, mutants that lost pH gating had ample space at the bundle crossing for hydrated K permeation in the closed-state. These results support our hypothesis that hydrophobic leucines at the cytoplasmic end of the inner transmembrane helices comprise the principal pH gate of Kir1.1, a gate that can be relocated from 160-Kir1.1b to 157-Kir1.1b.

INTRODUCTION

Previous studies1,2 have suggested that the putative pH gate of Kir channels is formed by the convergence of hydrophobic leucines, at the bundle crossing of the inner helices (see Fig. 1A). Replacing these leucines with residues having small (L160G) or polar (L160S, L160T) side chains produced mutant channels that did not close normally and continued to pass inward K current during cytoplasmic acidification.1 This suggested that L160-Kir1.1b was the structural locus of the Kir pH gate. Additional studies with cysteine-modifying reagents indicated that acidification prevented access of cytoplasmic-side sulphydryl reagents to an inner helix cysteine at C156-Kir1.1b, consistent with localization of the Kir1.1 gate at the helix bundle crossing.3

There is good evidence for gating at the K channel bundle crossing. EPR studies on KcsA have indicated significant movement of the inner helices in the region of the bundle crossing.4 Comparison of the putative KcsA closed with the MthK open configurations suggested a primary gate at the bundle crossing of the inner helices.5 Furthermore, electrophysiological studies have provided evidence that quaternary ammonium ions, cysteine modifying reagents, as well as Cd2+ and Ag+ are prevented from entering the Shaker Kv channel by state-dependent closure of a steric gate at the bundle crossing.6,7,8

In the inward rectifier family, MTS inaccessibility in the closed state implicated the bundle crossing as the relevant gate for ATP-dependent closure of Kir6.2 channels.9 Additional studies with spermine trapping in the N160D/L157C mutant of Kir6.2 elegantly demonstrated channel closure at the inner helical bundle crossing of this channel.10

However, some studies in both inward rectifiers11,12 and CNG channels13 have implied that the ligand gate is located at a site above (in an outward direction to) the bundle crossing, perhaps near the selectivity filter.
Therefore, we examined whether L160 was a unique locus for the Kir1.1 ligand gate or whether other positions along the inner transmembrane helix could also function as steric gates. We restricted our mutational analysis to residues between C156 (indicated by the yellow sulfur atoms in Fig. 1A) and A161 (the last residue on the inner helix within the membrane phase). Mutations above C156-Kir1.1b would be complicated to interpret because they might interfere with the putative motion of the inner helices during opening of the channel. Mutations below A161 are cytoplasmic and were assumed not to participate directly in gating at the bundle-crossing.

Our experimental protocol was to remove the putative wild-type leucine gate at L160-Kir1.1b and replace it with residues at one of six positions along the Kir1.1b inner transmembrane helix: 155-161 (excluding 160). The location of these residues is indicated in Figure 1B, along with their inward rectifier alignment. Mutations at each of these positions were examined for pH sensitivity by measuring whole-cell currents in *Xenopus* oocytes, using the two-electrode voltage clamp. Internal pH was controlled with permeant buffers and all titrations were corrected for external pH sensitivity.

Our results indicate that putative wild-type gate at L160-Kir1.1b could be replaced by either a leucine or methionine residue at 157+-Kir1.1b. Hydrophobic residues at other locations along the inner transmembrane helix did not rescue pH gating. These data were compared with homology models derived from the closed-state structure of the prokaryotic KirBac1.1.

**METHODS**

**Mutant construction and expression of channels.** Point mutations in Kir1.1b (ROMK2; EMBL/GenBank/DDBJ accession No. L29403) were engineered with a PCR QuickChange mutagenesis kit (Stratagene), using primers synthesized by Integrated Data Technologies (IDT, 1710 Commercial Park, Coralville, IA 52241). Nucleotide sequences were checked on an Applied Biosystems 3100 DNA sequencing machine at the University of Chicago Cancer Research Center.

Plasmids were linearized with Not I restriction enzyme and transcribed in vitro with T7 RNA polymerase in the presence of the GpppG cap using mMESSAGE mMACHINE kit (Ambion, Austin, TX). Synthetic cRNA was dissolved in water and stored at -70°C before use. Stage V-VI oocytes were obtained by partial ovarioectomy of female *Xenopus* laevis (NASCO, Ft. Atkinson, WI), anesthetized with tricaine methanesulfonate (1.5 g/L, adjusted to pH 7.0). Oocytes were defolliculated by incubation (on a Vari-Mix rocker) in Ca-free modified Barth’s solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, adjusted to pH 7.5 with NaOH) containing 2 mg/ml collagenase type IA (Cat# C9891, Sigma Chemical, St. Louis, MO) for 90 min, and (if necessary) another 90 min in a fresh enzyme solution at 23°C. Oocytes were injected with 0.5 to 1 ng of cRNA and incubated at 19°C in 2x diluted Leibovitz medium (Life Technologies, Grand Island, NY) for one to three days before measurements were made.

All experiments described were conducted at room temperature (21 ± 2°C) on ROMK2 (Kir1.1b) or mutants of ROMK2, expressed in *Xenopus* oocytes.

Prior to the start of experiments, all oocytes were preincubated in 100 mM K solutions (50 mM Cl + 50 mM acetate) for 2 hrs at pH 7.8. This compensated for prior exposure of oocytes to low external K during the period of exogenous expression and insured that both wild-type and mutant oocytes had reproducible responses to pH.

**Whole-cell recordings and control of internal pH.** Whole-cell currents and conductances were measured in intact oocytes using a two-electrode voltage clamp (Model CA-1, Dagan Corp, Minneapolis, MN) with 16 command pulses of 30 ms duration between -200 mV and +100 mV, centered on the resting potential. Oocytes expressing ROMK2 or mutants of ROMK2 were bathed in permeant acetate buffers to control their internal pH as previously described. The composition of the bath for the whole-cell experiments was: 50 mM KCl, 50 mM K acetate, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM HEPES, and 1 mM SITS (4-acetamido-4-isothiocyanostilbene-2-
Moving the pH Gate

2'-disulfonic acid). SITS was used to minimize small endogenous chloride currents; however batches of oocytes exhibiting chloride currents larger than 1 μA were discarded, as were oocytes that did not exhibit at least a 40 mV shift in membrane potential for a 10-fold change in external [K].

The relation between intracellular and extracellular pH was calculated from a previous calibration with ROMK oocytes: pH\textsubscript{i} = 0.595 x pH\textsubscript{o} + 2.4.\textsuperscript{17} This calibration was recently verified by comparing pH titration curves determined with both permeant buffers and excised (inside-out) patches.\textsuperscript{1}

Use of permeant buffers to control oocyte pH has the disadvantage that both external and internal pH are changed simultaneously. Although wild-type ROMK exhibits no significant external pH dependence at the voltages used, some of the mutants did have measurable external pH dependence. Consequently, all of the two-electrode voltage clamp currents were corrected for external pH by first running complete pH cycles with impermeant buffers, in which only outside pH was changed. The same oocytes were then subjected to both internal and external pH changes using permeant acetate buffers. In this way, each oocyte served as its own control, assuming that external and internal pH responses are independent processes. Raw data on external and internal pH dependence of the pH-gated G157L-L160G mutant are shown in Figure 2 and explained in the Results section. Finally, to maximize the signal to noise, we elected to study only mutants whose inward current was greater than 4 microamps at -200 mV.

Whole-cell inward conductance was measured over a linear portion of the current-voltage curve, near the maximum inward current, and normalized for each oocyte to compensate for differences in expression efficiency among mutant channels. Based on our

Figure 2. Effect of external and internal pH on macroscopic whole-cell currents from G157L-L160G, measured with the two-electrode voltage clamp. External current was first determined at 9 voltage steps in 20 mV decrements between +60 mV and -100 mV using impermeant buffers at pH 10 (A) and pH 7 (B). The resulting external pH current-voltage relations are given in (C). The same pulse protocol was then repeated on the same oocyte, using permeant acetate buffers at pH 10 (D) and pH 7 (E). The resulting current-voltage relation in (F) represents the effect of a simultaneous change in both external and internal pH. As discussed in the methods, the dependence of current on external pH was used to scale the permeant buffer currents to reflect the effect of internal pH alone, assuming that internal and external pH effects are independent.
observations with ROMK2-17 we presumed that the pH dependence of normalized whole-cell inward conductance (at elevated negative cell potentials) primarily reflects changes in channel gating rather than changes in single channel conductance. However, we cannot be absolutely certain that there were no effects of pH on mutant single channel conductance because single-channel recordings were not done in this study.

**Homology modeling.** To facilitate interpretation of the electrophysiological results, we made a homology model of the Kir1.1b sequence along the inner transmembrane helices (from A137-Kir1.1b to I163-Kir1.1b) based on the X-ray crystal structure of the closed state of KirBac1.1 (Protein Data Bank entry 1P7B, ref. 16), near the putative pH gate, using the Molecular Operating Environment® software (MOE version 2005.06, Chemical Computing Group, Montreal Canada). Twenty models were generated, in order to identify regions of the structure that are robustly modeled and regions that are not. Models were evaluated using the MOE Protein Report module, which identifies steric contacts, bond lengths, bond angles, and dihedrals that are outside normal ranges. The final model was energy-minimized using the CHARMM22 force field. Individual mutants were then modeled from this structure. Side-chain conformations of the mutated residue and nearby residues were modeled from a library of side-chain rotamers. Cases of ambiguous side-chain conformation are discussed in the Results.

Our homology models were based on the closed (rather than open) state coordinates because gating by steric occlusion would occur when the diameter of a hydrated K+ ion exceeds the minimum closed-state inner channel diameter. Furthermore, only closed state coordinates have been determined (to date) for the Kir family.16

**RESULTS**

The putative pH gate can be moved from 160 to 157. For clarity, the pH titration data for each of the mutants is described together with the corresponding homology model for that mutant. In agreement with previous findings,2 pH gating was abolished by the L160G mutation. However, gating in the L160G mutant could be rescued by replacing the normally occurring glycine at G157-Kir1.1b by a hydrophobic leucine (Fig. 2).

In contrast to wild-type Kir1.1, which shows no external pH dependence, some of the mutants, including G157L-L160G, had a significant dependence on external pH. As discussed in the Methods, we corrected for external pH effects by running two consecutive pH cycles on each oocyte; first with impermeant buffers to gauge the magnitude of the external pH effect and then with permeant acetate buffers, which alter both external and internal pH. Representative data for the G157L-L160G mutant are shown in Figure 2, where decreasing external pH from 10 to 7 reduced inward whole-cell conductance by 32% (Fig. 2C); whereas reducing both external and internal pH from 10 to 7 decreased whole-cell conductance by 92% (Fig. 2F). Operationally, the external pH data were used to renormalize the permeant acetate data to determine the effect of changes in internal pH alone, assuming that external and internal pH effects are independent processes. Since the internal pH sensor for Kir1.1 is now presumed to reside in the cytoplasmic C-terminal region of the channel,20 it seems reasonable to assume that external and internal pH effects are independent processes.

Leucine was not the only side chain that could reconstitute gating in L160G-Kir1.1b mutants (magenta line, Fig. 3). A methionine side chain at G157 could also function as a pH gate (green line, Fig. 3).

The sigmoidal dependence of G157L,M-L160G conductance on pH implies a gating mechanism similar to that of wild-type ROMK (blue line, Fig. 3). However, the G157L-L160G and G157M-L160G mutants did have alkaline shifted pKas relative to Kir1.1 (ROMK2 pKa = 6.6 ± 0.02 compared to G157L-L160G = 7.5 ± 0.04 and G157M-L160G = 8.1 ± 0.1). Presumably, these shifts occurred because flexible glycines at 157 had been replaced by less flexible Leu or Met residues. Similar alkaline shifts occurred with the single point mutants: G157L and G157M (orange and yellow curves, Fig. 3).

Homology models for the G157L-L160G and G157M-L160G mutants are shown in Figure 4. The ion pore is viewed from the cytoplasmic side at a plane near the bundle crossing, and the 160-Kir1.1b and 157-Kir1.1b residues are depicted in space-filling format. In both figures, the transparent blue circle represents the hydrated diameter of a K+ ion, assuming eight waters of hydration. Operationally this diameter was computed as 2.63 times the ionic (Pauling) diameter of a K+ ion. As indicated in the figures, a hydrated K+ ion would be too large to traverse the closed-state helical bundle crossing. Since dehydration of the K+ ion is extremely unlikely because of the energetic cost, these models are consistent with closure of both G157L-L160G and G157M-L160G at low pH (Fig. 3).

pH gating at 157-Kir1.1b and side-chain hydrophobicity. Although 157-Kir1.1b appears to be an alternative locus for Kir1.1 gating, only leucine (.94 relative hydrophobicity) or methionine (.74 relative hydrophobicity) side-chains were able to form functional gates at this position. Residues of similar hydrophobicity, alanine (.62 relative hydrophobicity), tyrosine (.88 relative hydrophobicity), and valine (.83 relative hydrophobicity) did not gate the channel at 157, as indicated by persistence of the open state at low pH in the mutants: G157A-L160G, G157Y-L160G and G157V-L160G (Fig. 5A). (Scaled hydrophobicity values were compiled by Dr. Shaun...
Figure 4. Bottom view, closed-state, homology models at the bundle crossing of: (A) G157L-L160G-Kir1.1b and (B) G157M-L160G-Kir1.1b, in which the putative gate at L160 has been translocated to 157-Kir1.1b. In both homology models, a hydrated K ion (transparent blue circle) would be sterically occluded from the permeation path at the level of the bundle crossing. Hence, these mutants should be gated closed at low internal pH.

D. Black at the Univ. of Texas Health Center http://psyche.uhct.edu/shaun/SBlack/aagrease.html). These results are in contrast to findings with the L160-Kir1.1b locus, where all of the hydrophobic residues tested (Leu, Met, Val, Phe) gated the channel.2 Hence, hydrophobicity alone was not the sole determining factor for side-chain gating at 157-Kir1.1b.

The pH titrations of Figure 5A were consistent with the homology models of G157A-L160G (Fig. 6A), G157Y-L160G (Fig. 6B) and G157V-L160G (not shown), that indicated enough space for hydrated K ions to permeate the bundle crossing in the closed state. The G157F-L160G mutant had too low an expression to evaluate its pH dependence.

In contrast, the single-point mutants: G157A, G157Y, and G157V, all of which had leucine (gates) at 160-Kir1.1b, retained pH gating and closed at low pH (Fig. 5A). The alkaline shifts in apparent pKa associated with these mutations were similar to what was observed with the G157L and G157M mutations (Fig. 3), and would be consistent with replacement of flexible glycine side chains by less flexible residues.

Polar residues (Ser and Thr) cannot function as gates at the 157 position. Mutants in which the Gly at G157-Kir1.1b was replaced by residues having the polar side chains Ser or Thr did not exhibit gating and were unable to close at low pH (Fig. 5B). The Ser results are consistent with the homology model of G157S-L160G (Fig. 6C), which indicated sufficient space for permeation by a hydrated K ion in the closed state.

On the other hand, we found two alternative homology models for the G157T-L160G closed state. In the first, the gamma carbons of the threonine side-chains partially occluded the permeation path at 157-Kir1.1b (not shown). In the alternative side-chain configuration, having equivalent likelihood, the threonine hydroxyls faced into the pore (Fig. 6D). This provided just enough clearance for a hydrated K to permeate the closed-state at the bundle crossing, consistent with an absence of pH gating in this mutant (orange line of Fig. 5B). In fact, Figure 6D suggests that favorable interactions between the threonine hydroxyls and the K hydration shell would foster K permeation at this site.

Figure 5. (A) Hydrophobic G157L-L160G mutants that don’t gate. Side-chain hydrophobicity at G157-L160G does not completely determine pH gating. G157A-L160G (green open squares) and G157V-L160G (red open squares) are three examples where hydrophobic side-chains at 157-Kir1.1b cannot replace the putative leucine gate at L160. The corresponding single mutants (with intact L160) retained their pH gating, but with altered pKa: G157A (pKa = 7.13 ± 0.01, brown open circles), G157Y (pKa = 6.76 ± 0.02, magenta open squares), and G157V (pKa = 6.85 ± 0.02, cyan solid circles). Wild-type ROMK (blue inverted triangles) and L160G (purple inverted triangles) are shown for comparison. All data represent normalized, whole-cell, inward conductance plotted as a function of internal pH, as in Figure 3. (B) Polar side-chain, G157L160G mutants that don’t gate. G157S-L160G (brown solid circles), and G157V-L160G (orange open circles) are two examples of polar side-chains at 157-Kir1.1b that did not gate the channel closed. However, the corresponding single mutants (with intact L160) retained pH gating, but with altered pKa’s: G157S (pKa = 6.87 ± 0.04, cyan circles), and G157V (pKa = 6.85 ± 0.06, red open circles). Wild-type ROMK (pKa = 6.6 ± 0.02, blue inverted triangles) and L160G are shown for comparison. All data represent normalized, whole-cell, inward conductance plotted as a function of internal pH, as in Figure 3.
For completeness, the titration curves for the single-point mutants, G157S and G157T, are also shown in Figure 5B. Their titrations are consistent with having leucine side-chains at L160 that could function as steric gates.

Moving leucines from 160 to 158 or 161 abolishes gating. Moving the putative leucine gate from L160 to either 158-Kir1.1b or 161-Kir1.1b abolished pH gating, where both A158L-L160G (orange line, Fig. 7) and A161L-L160G (purple line, Fig. 7) failed to close at low internal pH, similar to what was seen with L160G (red triangles, Fig. 7). This was consistent with the A158L-L160G and A161L-L160G closed-state models, which indicated that hydrated K ions (transparent blue circles) could easily traverse the bundle crossing of both A158L-L160G (Fig. 8A) and A161L-L160G (Fig. 8B) because the leucine side-chains of these mutants faced away from the permeation path.

Titrations of the single point mutations A158L and A161L are also shown in Figure 7. Both of these single-point mutants retained pH gating, consistent with having leucine side-chains at L160. However, both mutations showed alkaline shifts in their pKa’s. For A158L, the shift was 0.75 ± 0.06 pH units (relative to wild-type ROMK); but for A161L, the shift was ambiguous since a maximum current could not be obtained for this mutant (Fig. 7).

Mutants that produced insufficient current for evaluation. Three leucine mutants (M155L-L160G, C156L-L160G, and I159L-L160G) and one phenylalanine mutant (G157F-L160G) expressed whole-cell currents that were too small to construct reliable pH titration curves. Homology models indicated that the leucines in both M155L-L160G and I159L-L160G would be oriented obliquely and would be unable to block the permeation path in the closed state. However, the homology model of C156L-L160G would be consistent with pH gating. Unfortunately, none of these predictions could be tested because expression levels of these mutants were too low to construct reliable pH titrations.

**DISCUSSION**

Structural studies of the prokaryotic KirBac1.1 have implied that inward rectifier channels close via the convergence of four hydrophobic residues at the bundle crossing of the inner transmembrane helices.15,16 In Kir1.1, this putative gate is thought to consist of four leucines (one from each subunit) that occlude the permeation path at low internal pH. In the present study, we examined this hypothesis for pH gating by attempting to move the L160-Kir1.1b gate to another position along the inner helix.

Previous studies had demonstrated that Kir1.1 channels were strongly sensitive to internal pH, with an apparent pKa of 6.6 ± 0.011 and a Hill coefficient between 3 and 5.1,2 Similar pKa values have been reported for Kir1.1 by a number of different investigators, although the phosphorylation state of the channel can produce minor variations in pH sensitivity.2,18,19,21-25 In addition, it is generally agreed that the pH gating of Kir1.1 occurs via a decrease in the number of active channels, without a significant change in the kinetics of the active state.5

Although our previous studies suggested a physical pH gate at L160-Kir1.1b,2 they did not completely rule out the possibility that the L160G mutation simply stabilizes the open state without itself actually being a steric gate. The results of the present study, in which the gate was reconstituted by replacing the Gly at 157 by Leu or Met on top of the L160G mutation suggests that a leucine side-chain at either L160 or L157 can function as a steric gate. However, even our new results do not rule out the possibility that the L160G-Kir1.1b mutation may allosterically stabilize the open state of the channel.

Generally, the Kir1.1 bundle crossing mutants of the present study fell into two general classes: (1) mutants that were gated by internal pH (G157L, G157L-L160G, G157M, G157M-L160G, G157A G157S, G157T, G157V, G157Y, A158L, A161L) and (2) mutants that were not (L160G, G157A-L160G, G157S-L160G, G157T-L160G, G157V-L160G, G157Y-L160G, A158L-L160G, and A161L-L160G). Some of these pH-insensitive mutants nonetheless showed a (non-sigmoidal) decline in whole-cell conductance at low values of internal pH. This varied from less than 5% for A158L-L160G, G157Y-L160G to as much as 25% for G157T, G157T-L160G, and G157V-L160G. Low expression levels in these latter mutants precluded examination of single channels at low pH. However, previous studies with the L160G mutant indicated that the decline in whole-cell conductance at low pH was matched by a similar decline in single-channel activity (see Figs. 5A and 6 of ref. 2). This small whole-cell conductance decrease in some non-pH
gating mutants may involve an additional gating process at a location separate from the bundle crossing. None of our results preclude the possibility of other gates in the permeation path, perhaps at the selectivity filter.

The main conclusion of the present study is that it is possible to relocate the putative L160-Kir1.1b pH gate three residues above the bundle crossing (toward the selectivity filter). Hydrophobic leucines or methionines at 157-Kir1.1b effectively replaced the wild-type leucines at L160-Kir1.1b that are thought to function as steric gates for Kir1.1.

Homology models of G157L-L160G and G157M-L160G were consistent with retention of pH gating in these mutants, although the pK\textsubscript{a}'s of both were alkaline shifted relative to ROMK (Fig. 3). We believe this is caused by the removal of flexible G157 glycines that normally allow the inner helix to bend open at alkaline pH.\textsuperscript{1,15} This explanation is supported by the finding that the G157L and G157M single-point mutants also have similar alkaline shifts in their pH titrations. Analogous results were seen with Shaker K\textsubscript{v} mutants, in which replacement of conserved inner helix glycines stabilized the closed conformation of the channel.\textsuperscript{26} Finally, all the Kir1.1 mutants that retained pH gating had consistently lower Hill coefficients than wild-type ROMK. Our homology models did not shed light on this finding, and we have no theoretical explanation for this.

Another interesting result of the present study was that side chain hydrophobicity was a necessary, but not a sufficient, condition for pH gating in the 157-L160 Kir1.1b mutants. Although the hydrophobic residues Leu and Met gated the channel at 157-Kir1.1b, residues of similar hydrophobicity like Tyr and Val did not. This differs from results with L160-Kir1.1b, where side-chain hydrophobicity was both a necessary and a sufficient condition for gating (Fig. 7 of ref. 2). Homology models of the 157-L160G mutants were consistent with their respective pH gating and indicated that side-chain orientation was more important than hydrophobicity in predicting gating.

The 157-L160G mutants also showed a dependence of gating on side-chain size since large residues like Met and Leu gated the channel, but the smaller Ala residue (G157A-L160G) did not. This is in contrast to previous results, where L160A-Kir1.1b retained pH gating at the 160-Kir1.1b locus.\textsuperscript{2} However, the inability of Tyr to gate at 157 suggests that having a polar group at the 157 locus may be more important than side-chain size or overall hydrophobicity.

Not surprisingly, residues with highly polar side-chains (Ser and Thr) were unable to gate the channel closed at the 157 locus. This is similar to what was observed when leucines at L160-Kir1.1b were replaced by serines or threonines.\textsuperscript{2} Presumably, polar side-chains can interact favorably with the first hydration shell of a permeating K ion, allowing it to traverse the inner helical bundle-crossing of a closed-state channel. In addition, the homology model of G157S-L160G indicated ample space for permeation by a hydrated K ion in the closed state (Fig. 6C), consistent with the observation that this mutant does not close down at low pH (Fig. 5B).

However, the G157T-L160G mutant exhibited two alternative conformation models. In the first, the closed state permeation path was
occluded by carbon atoms of the $Tbr$ side-chains. This was inconsistent with the experimental data that G157T-L160G remained (80%) open at low internal pH (orange line, Fig. 5B). The second model for G157T-L160G depicted a more open configuration, where the hydroxyl groups on the $Tbr$ side-chains faced the permeation path (Fig. 6D). Presumably, these $Tbr$ hydroxyls could replace the OH groups on the water molecules surrounding the K ion, allowing permeation in the closed state, consistent with the orange line of (Fig. 5B). This is analogous to what had been proposed to explain differences in organic cation permeability through voltage-gated Na channels via different interactions with oxygen atoms lining the selectivity filter.27

Finally, attempts to relocate the putative L160-Kir1.1b gate to positions along the inner helix other than 157-Kir1.1b were largely unsuccessful. Leucines at 158 and 161-Kir1.1b yielded robust inward currents, but both of these mutants were unable to close at low pH. This was consistent with homology models for A158L-L160G and A161L-L160G, in which the Leu side-chains at 158 and 161 both point away from the pore, thereby allowing hydrated K ions to permeate the channel in the closed state. As such, the homology modeling (described in the Methods) proved to be an important confirmation of the electrophysiological data that elucidated (in the cases examined) the structural reasons underlying pH gating, both for mutants that gated and those that didn’t.

In summary, our finding that the putative leucine gate (L160) of Kir1.1b could be repositioned (three residues toward the selectivity filter) to the 157 locus supports our original hypothesis that normal gating occurs by a convergence of leucines at L160-Kir1.1b, and that the bundle crossing is the principal (but not necessarily the exclusive) site for the inward rectifier gate.

References
1. Sackin H, Nanazashvili M, Palmer LG, Li H. Role of conserved glycines in pH gating of Kir1.1 (ROMK). Biophys J 2006; 90:3582-9.
2. Sackin H, Nanazashvili M, Palmer LG, Krambs M, Walters DE. Structural locus of the pH gate in the Kir1.1 inward rectifier channel. Biophys J 2005; 88:2597-606.
3. Zhang Y, Sackin H, Palmer LG. Localization of the pH gate in Kir1.1 channels. Biophys J 2006; 91:2901-9.
4. Liu YS, Sompongpiip R, Perozo E. Structure of the KcsA channel intracellular gate in the open state. Nature 2001; 8:883-7.
5. Jiang Y, Lee A, Chen J, Cadene M, Chalt B, MacKinnon R. The open pore conformation of potassium channels. Nature 2002; 417:523-6.
6. Holmgren M, Smith P, Yellen G. Trapping of organic blockers by closing of voltage-dependent K channels: Evidence for a trap door mechanism of activation gating. J Gen Physiol 1997; 109:527-35.
7. Yellen G. The moving parts of voltage-gated ion channels. Quarterly Reviews of Biophysics 1998; 31:239-95.
8. del Camino D, Yellen G. Tight steric closure at the intracellular activation gate of a voltage-gated K channel. Neuron 2001; 32:649-56.
9. Phillips LR, Enketchakul D, Nichols C. Gating dependence of inner pore access in inward rectifier K channels. Neuron 2003; 37:955-62.
10. Phillips LR, Nichols CG. Ligand-induced closure of inward rectifier Kir6.2 channel traps spermine in the pore. J Gen Physiol 2003; 122:795-804.
11. Proks P, Antcliff JF, Ashcroft FM. The ligand-sensitive gate of a potassium channel lies close to the selectivity filter. EMBO Rep 2003; 4:70-5.
12. Xiao J, Zhen XG, Yang J. Localization of PIP2 activation gate in inward rectifier K channels. Nat Neurosci 2003; 6:811-8.
13. Flynn GE, Zagotta WN. Conformational changes in S6 coupled to the opening of cyclic nucleotide-gated channels. Neuron 2001; 30:689-98.
14. Domene C, Doyle D, Vénien-Bryan C. Modeling of an ion channel in its open conformation. Biophys J 2005; 89:L1-L3.
15. Kuo A, Domene C, Johnson L, Doyle D, Vénien-Bryan C. Two different conformational states of the KirBac3.1 potassium channel revealed by electron crystallography. Structure 2005; 13:1-10.
16. Kuo A, Golbis J, Antcliff J, Rahman T, Lowe E, Zimmer J, Cuthbertson J, Ashcroft E, Eazaki T, Doyle D. Crystal structure of the potassium channel KirBac1.1 in the closed state. Science 2003; 300:1922-6.
17. Choe H, Zhou H, Palmer LG, Sackin H. A conserved cytoplasmic region of ROMK modulates pH sensitivity, conductance, and gating. Am J Physiol 1997; 273:F316-29.