Forced Gating Motions by a Substituted Titratable Side Chain at the Bundle Crossing of a Potassium Channel*

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Background: ATP-sensitive potassium (K<sub>ATP</sub>) channels translate cellular metabolism (generation of ATP) in an electrical signal. Results: Mutual repulsion between specific substituted titratable residues in the bundle crossing forces K<sub>ATP</sub> channels to open and changes their apparent ATP sensitivity. Conclusion: ATP-dependent gating involves conformational changes in the bundle crossing region of K<sub>ATP</sub> channels. Significance: This reflects an engineered method for control of ion channel activity by a non-natural mechanism.

Numerous inwardly rectifying potassium (Kir) channels possess an aromatic residue in the helix bundle crossing region, forming the narrowest pore constriction in crystal structures. However, the role of the Kir channel bundle crossing as a functional gate remains uncertain. We report a unique phenotype of Kir6.2 channels mutated to encode glutamate at this position (F168E). Despite a prediction of four glutamates in close proximity, Kir6.2(F168E) channels are predominantly closed at physiological pH, whereas alkalization causes rapid and reversible channel activation. These findings suggest that F168E glutamates are uncharged at physiological pH but become deprotonated at alkaline pH, forcing channel opening due to mutual repulsion of nearby negatively charged side chains. The potassium channel pore scaffold likely brings these glutamates close together, causing a significant pK<sub>a</sub> shift relative to the free side chain (as seen in the KcsA selectivity filter). Alkalization also shifts the apparent ATP sensitivity of the channel, indicating that forced motion of the bundle crossing is coupled to the ATP-binding site and may resemble conformational changes involved in wild-type Kir6.2 gating. The study demonstrates a novel mechanism for engineering extrinsic control of channel gating by pH and shows that conformational changes in the bundle crossing region are involved in ligand-dependent gating of Kir channels.

Inwardly rectifying potassium (Kir) channels comprise a transmembrane pore-forming domain conserved within the cation channel superfamily(1). In contrast to their voltage-dependent cousins (Kv, Nav, and Cav channel families), Kir channels do not possess a canonical voltage-sensing domain (2–5). Rather, their ligand sensitivities and gating behaviors are determined by a cytoplasmic ligand-binding domain, and apparent voltage sensitivity in the physiological milieu arises from blockade by intracellular cations (Mg<sup>2+</sup> and polyamines) (6). Kir channels exhibit diverse ligand dependence: some are regulated by G-proteins, others by intracellular nucleotides, and still others by intracellular pH, whereas all Kir channels are stimulated by phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) (7–9). The growing understanding of regulation of Kir channels continues to highlight their role as important integrators of signaling pathways and underscores the importance of understanding Kir gating mechanisms.

Kir<sub>x</sub> channels are the pore-forming subunits of ATP-sensitive potassium (K<sub>ATP</sub>) channels. They are distinguished from other Kir channels by their sensitivity to intracellular nucleotides (ATP and ADP), and the uniquely diverse pharmacology of K<sub>ATP</sub> makes these channels a valuable model for characterizing Kir channel gating (5, 10–13). Many Kir channel types (most notably the G-protein-coupled inwardly rectifying potassium (GIRK) and K<sub>ATP</sub> channels) possess a conserved aromatic residue at the helix bundle crossing (see Fig. 1, A and B), also the locus of the narrowest pore constriction in crystal structures of bacterial Kir homologs (Phe-146 in KirBac1.1 and Tyr-132 in KirBac3.1) (see Fig. 1B) (14). Although crystal structures suggest that this position is important for Kir channel closure, the notion of a functional gate at the Kir channel bundle crossing has been frequently questioned (15–18).

We undertook a mutagenic approach to investigate the functional importance of residues in the bundle crossing region and inner cavity of Kir6.2 (19). Through these studies, we have uncovered several mechanisms for extrinsic/non-natural control of Kir6.2 gating. We previously reported a point mutation that introduces voltage-dependent gating into normally voltage-independent Kir6.2 gating. We report the generation of Kir6.2 channels with a very pronounced pH-dependent gating mechanism that arises after introduction of a glutamate residue at the helix bundle crossing position (Phe-168). Our findings indicate that deprotonation of the F168E glutamate drives channel opening, likely due to a mutual repulsion of similar charges in the bundle crossing. Additionally, we demonstrate that conformational changes driven by the engineered pH
dependence are closely related to gating motions arising from intrinsic ATP/PIP2-dependent gating. These results demonstrate the importance of conformational motions in the helix bundle crossing of Kir channels and illustrate general principles that underlie a mechanism for extrinsic or "engineered" control of ion channel gating.

**EXPERIMENTAL PROCEDURES**

**K<sub>ATP</sub> Channel Constructs**—All mutant channel constructs were generated from a mouse WT Kir6.2 gene expressed in the pcDNA3.1(−) plasmid (Invitrogen). Point mutations were introduced by overlapping PCR-based methods, followed by subcloning of desired fragments into pcDNA3.1(−). Concatenated channel constructs were synthesized using PCR amplification of individual subunits with primers that introduced the desired flanking restriction sites, followed by subcloning into the pcDNA3.1(−) vector and verification by sequencing and digestion. Tetrameric channels were first constructed as two separate dimers to simplify sequencing and then combined into a final tetrameric construct. Channel subunits were subcloned between the Xbal-NotI (“slot 1”), NotI-EcoRI (“slot 2”), EcoRI-BamHI (“slot 3”), and BamHI-HindIII (“slot 4”) restriction sites. Gly<sub>6</sub> linkers were included at the C-terminal end of each subunit in the tetramer, followed by (two or three for NotI) amino acids corresponding to the introduced restriction sites. Start codons in slots 2–4 were removed to minimize spurious translation initiation within the tetrameric constructs. A C-terminal GFP tag was also included in the tetrameric channel constructs to track synthesis of full-length tetramers. We found empirically that currents from cells transfected with the tetrameric constructs matured more slowly than those from cells transfected with the monomeric constructs, and the GFP tag assisted significantly in finding strongly expressing cells.

**Channel Expression and Electrophysiology**—COSm6 cells were maintained in culture in a 5% CO<sub>2</sub> incubator at 37 °C in DMEM supplemented with 10% FBS and penicillin/streptomycin. Cells were transfected with channel constructs and hamster SUR1 using Lipofectamine 2000. For non-GFP-tagged constructs, cells were also transfected with a GFP plasmid to allow selection of transfected cells for recording.

Kir6.2ΔC36 constructs were expressed in *Xenopus laevis* oocytes. Ambion mMESSAGE T7 kits were used to synthesize mRNA from pcDNA3.1(−) constructs encoding either Kir6.2ΔC36 or Kir6.2(F168E)ΔC36, followed by injection (10–20 ng) into *Xenopus* oocytes 1 day after injection. Inside-out patches were excised from oocytes 1–3 days after injection.

Data were filtered at 1 kHz, digitized at 5 kHz, and stored directly on a computer hard drive using Clampex software (Axon Inc.). The standard pipette (extracellular) and bath (cytoplasmic) solution used in these experiments had the following composition: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, and 4 mM K<sub>2</sub>HPO<sub>4</sub>. The solution pH was adjusted with KOH or HCl to the desired level. Because the recording solutions are not very strongly buffered, the solution pH was verified each experimental day and verified after the addition of other solutes that can affect solution pH (namely Ba<sup>2+</sup> and ATP). Solutions were delivered at room temperature by pressure-driven flow into a chamber designed to prevent mixing of experimental solutions and to allow rapid solution exchange. Identical solutions were used for recordings from both mammalian cell lines and *Xenopus* oocytes. Chemicals were purchased from Sigma-Aldrich or Fisher.

**Western Blot Detection of K<sub>ATP</sub> Channel Surface Expression**—COSm6 cells in 6-well plates were transfected with Kir6.2 channel cDNA (600 ng) and FLAG-tagged SUR1 (1.2 μg in the pECE vector). After 3 days of incubation, cell surface proteins were isolated by biotinylation (Pierce cell surface protein isolation kit). Western blotting was carried out with samples of total cell lysates, as well as biotinylated fractions, using 7.5% SDS-PAGE, followed by transfer to nitrocellulose membrane and labeling with mouse anti-FLAG primary antibody (Sigma-Aldrich) and HRP-conjugated goat anti-mouse secondary antibody (ABM, Vancouver, Canada). Blots were visualized by ECL methods (Femto ECL detection kit, Pierce) using a FluorChem SP gel imager (Alpha Innotech).

**RESULTS**

**Mutant Kir6.2 Activated by Intracellular Alkalization**—We observed an unexpected phenotype in Kir6.2 channels with a glutamate substitution at Phe-168 (at the helix bundle crossing). A reasonable prediction for this experiment was that close proximity of charged glutamate side chains might force the M2 helices apart and thereby bias the channels toward an open state. Surprisingly, we observed that Kir6.2(F168E) ion channels conducted very little current around pH 7. However, intracellular alkalization (using inside-out patch recordings) caused a very large current increase (Fig. 1D). This activation was rapid and completely reversible, and currents exhibited voltage-depen-
dent block by Ba\(^{2+}\) and spermine (Fig. 1D), demonstrating that the pH-dependent currents are carried by Kir6.2(F168E). A good measure of the pK\(_a\) of the effect was not possible because the response did not saturate without significant seal breakdown (usually above pH 9). Remarkably, the alkaline pH-dependent activation of Kir6.2(F168E) was completely reversed from the WT Kir6.2 property of inhibition by alkalization (Fig. 1C) (21).

**Mechanism for Alkalization-dependent Activation of Kir6.2(F168E)**—We speculated that glutamate substitution at position 168 results in a profound pK\(_a\) shift of the side chain carboxylic acid. When glutamates and/or aspartates are nearby, a pK\(_a\) shift of the carboxylic acid reduces the energetic penalty arising from close approach of like charges and can be especially dramatic if the side chains are in a hydrophobic environment (22). This often appears as two carboxylates sharing a proton or one behaving as a protonated (uncharged) species, thereby minimizing repulsive coulombic interactions. In studies of ion channels, the best known example of this arrangement appears in the KcsA selectivity filter, where the Glu-71 and Asp-80 side chains are apposed and are presumed to share a proton (Fig. 2C). A similar arrangement is apparent in KirBac1.1 (23) (Fig. 2C). A satisfactory explanation for the pH dependence of F168E channels might be that alkalization promotes deprotonation of the carboxylates and forces a conformational change of the helix bundle crossing due to mutual repulsion (Fig. 2, A and B).

To test whether alkalization-dependent activation of Kir6.2(F168E) requires mutual repulsion of F168E charges, we generated tetrameric constructs with one, two, or three F168E subunits. Significant alkalization-dependent activation was observed for channel constructs with two (FFEE or FEFE, with different orientations of F168E subunits in the tetramer) or three (FEEE) copies of F168E (Fig. 3, A–C and G). However, channels carrying a single F168E (FFFE) did not exhibit alkalization-dependent activation (Fig. 3, D and G). Additionally, 1:1 dimers of WT and Kir6.2(F168E) channels were activated by alkalization (Fig. 3G, ‘FE’+‘FE’). The degree of alkalization-dependent activation was notably weaker in the FE dimeric construct. We have not investigated this in great detail but suspect...
that this may be related to a higher intrinsic open probability of this construct (which would limit the extent of activation that could occur). A similar effect is discussed below for Kir6.2(F168E) mutant channels in Fig. 8.

Importantly, previous studies of tetrameric Kir6.2 channel constructs have reported a high intrinsic open probability (24, 25). To ensure that the absence of alkalization-dependent activation of FFFE channels did not arise because of an intrinsically high $P_o$ (from which little further channel activation could be observed), we also examined the effects of pH after polylysine-mediated rundown. After polylysine treatment resulting in ~60% current reduction, inhibition upon alkalization persisted, similar to WT Kir6.2 (Fig. 3, panels i and ii). Significant changes in single channel current magnitude were not observed, but openings were far more frequent at alkaline pH. Similar observations were made in four patches.

Position Dependence of Alkalization-dependent Activation—Alkalization-dependent activation conferred by the F168E mutation appears to be position-specific, as glutamate substitutions at other pore-lining positions did not exhibit similar pH-dependent activation (Fig. 5). There are also several titratable side chains in the vicinity of F168E (His-70, Lys-170, and His-175), raising the possibility of pH-dependent salt bridges (Fig. 6A). We ruled this out by systematically neutralizing His-70, Lys-170, and His-175 on a Kir6.2(F168E) background. Each double mutant channel exhibited alkalization-dependent activation comparable with Kir6.2(F168E) (Fig. 6, B–E). Importantly, His-175 has been identified as a determinant of intrinsic pH sensitivity in Kir6.2.

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FIGURE 4. Microscopic effects of pH-dependent activation of Kir6.2(F168E) channels. A, continuous record at ~50 mV of a tetrameric (FFE) construct (three copies of Kir6.2(F168E) + one copy of WT Kir6.2) with pH jumps between 6.5 and 8.5 as indicated. Downward deflections at pH changes are solution exchange artifacts and were minimized during figure preparation. The FEE tetramer was used because it exhibited similar pH dependence as F168E channels but took considerably longer to express currents and was very amenable to recording single channel currents. B, expanded view of channel openings in regions i, ii, and iii indicated in A. Significant changes in single channel current magnitude were not observed, but openings were far more frequent at alkaline pH. Similar observations were made in four patches.

FIGURE 5. Position-specific requirements for alkalization-dependent activation of Kir6.2. Glutamate mutants at inner cavity-lining positions were tested for pH dependence. Representative currents at ~50 mV with pH jumps as indicated are presented for each pore-lining mutant channel. Only Kir6.2(F168E) channels (boldface arrow, lower right) exhibited activation at alkaline pH.
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A Kir bundle crossing region

B

C [F168E][H70A]/SUR1

D [F168E][K170A]/SUR1

E [F168E][H175A]/SUR1

FIGURE 6. Neutralization of titratable residues near F168E. A, a molecular model of the bundle crossing region of Kir6.2. B–E, double mutants Kir6.2(H70A/F168E) (n = 7), Kir6.2(F168E/K170A) (n = 7), and Kir6.2(F168E/H175A) (n = 5) were examined for pH dependence. All double mutants exhibited alkalization-dependent activation comparable with Kir6.2(F168E) channels.

(28). Persistence of alkalization-dependent activation in Kir6.2(F168E/H175A) double mutant channels indicates that the pH dependence of the F168E mutation does not arise from a modulation of the intrinsic pH sensor of the channel. Overall, these data indicate that the F168E mutation is sufficient for alkalization-dependent activation and that pH changes likely trigger a conformational change in the vicinity of the F168E substitution.

Surface Expression of Alkalization-dependent Channel Mutant—We also determined the cell surface expression level of Kir6.2(F168E/H175A) by assessing the presence of a mature glycosylated form of SUR1 (indicated by arrows in Fig. 7). Interestingly, we were unable to detect functional currents from Kir6.2(F168D) channels despite the similarity of aspartate and glutamate side chains, so cell surface expression of F168D channels was also examined, along with WT Kir6.2. For all channel + FLAG-SUR1 combinations examined, two FLAG-SUR1 bands were detected by Western blotting (Fig. 7A). F168E channels consistently generated smaller protein levels relative to WT or Kir6.2(F168D) channels and required longer exposures to convincingly resolve bands (Fig. 7A, boxed). FLAG-SUR1 migration on SDS-PAGE is interpreted with the lower band corresponding to a core-glycosylated immature form and the upper band corresponding to a mature glycosylated form expressed at the cell surface (29, 30). We confirmed the validity of this interpretation by demonstrating that FLAG-SUR1 expression in the absence of Kir6.2 failed to generate the mature glycosylated form (Fig. 7A) and that cell surface biotinylation isolated only the upper band (Fig. 7B). These findings demonstrate that Kir6.2(F168E) and Kir6.2(F168D) channels are trafficked with efficiency similar to WT Kir6.2. However, Kir6.2(F168D) channels are not sufficient to generate functional currents over the pH range tested in our experiments.

Alkalization-dependent Activation Is Influenced by Channel Open Probability—We tested whether the pH-influenced activity of Kir6.2(F168E) is related to the intrinsic channel gating mechanism. First, we introduced the F168E mutation together with a gain-of-function Kir6.2 mutation (I296L) that acts by increasing channel open probability (31, 32). The Kir6.2(F168E/I296L) double mutant exhibited large currents at acidic pH and was weakly potentiated upon alkalization (Fig. 8, A and C). In many patches, pH-activated currents from Kir6.2(F168E/I296L) appeared to saturate at alkaline pH (Fig. 8A). These data suggest that the Kir6.2(F168E/I296L) channels exhibit a high open probability at low pH. In a complementary experiment, we used the R176A mutation (which disrupts PIP2 sensitivity) to generate channels with low open probability (Fig. 8B) (33). In Kir6.2(F168E/R176A) double mutant channels, very little current activation was observed until the most extreme alkaline pH, consistent with a greater stimulus required to force these channels open. These findings further support that alkalization of Kir6.2(F168E) channels modulates the native gating mechanism.

Coupling of the Bundle Crossing Gate and ATP-binding Site—To test stringently whether pH-dependent activation of F168E channels resembles channel gating, we examined whether pH affects ligand sensitivity. To preface this experiment, note that the ATP-binding site is allosterically coupled to the channel gate, and therefore, manipulations (e.g. mutations, changes in lipid conditions) that change the stability of open versus closed states also alter the apparent Kir6.2 channel ATP sensitivity (Fig. 9E) (7, 8, 34). For instance, as membrane PIP2 content (and consequently, channel Po) is increased, ATP inhibition of Kir6.2 grows progressively weaker. If motions triggered by alkalization affect opening and closing transitions at the same gate that is operated by the native ATP regulatory mechanism, then pH-induced changes in the closed-open equilibrium of Kir6.2(F168E) channels may translate into changes in apparent ATP sensitiv-
ity, similar to the effects of increased membrane PIP$_2$. We tested this possibility by measuring ATP sensitivity at various internal pH values (Fig. 9, A–D). In WT Kir6.2 channels, internal pH had little effect on ATP inhibition (Fig. 9, A and B), ruling out a nonspecific effect of pH on ATP binding. In contrast, Kir6.2(F168E) channels exhibited significantly weakened ATP inhibition at alkaline pH (Fig. 9, C and D). Thus, Kir6.2(F168E) channels are reasonably well inhibited by ATP but with a sensitivity that is modified by internal pH. This indicates that pH-dependent conformational changes in Kir6.2(F168E) channels are likely coupled to the ATP-binding site and resemble gating motions in WT Kir6.2 channels.

Another interesting feature of these data is that ATP inhibition of Kir6.2(F168E) did not eliminate macroscopic currents, resulting in a plateau of conductance (asterisk in Fig. 9D, pH 7.3). We have confirmed that the plateau persists at very high ATP concentrations and that currents can be inhibited by spermine (Fig. 10), indicating that the plateau current is carried by Kir6.2(F168E) channels. However, the mechanistic basis for this plateau is unclear. One intriguing possibility is that the glutamate substitution results in a “leaky” closed state, in which channels are in a quasi-closed ATP-bound conformation, but the pore is not completely occluded due to ablation of the Phe-168 aromatic residue. Such partial closings were not apparent in single channel records, but it is noteworthy that the plateau current was best resolved (Fig. 10) at pH 7.3 (where $P_o$ is quite low). Therefore, the relative magnitude of the plateau to control (0 ATP) current would not represent the true conductance ratio of a leaky closed state to a fully open state because all closed channels could contribute to the plateau conductance, whereas at pH 7.3, only a small fraction of channels would be likely to open in 0 ATP.

**DISCUSSION**

**Targeted pH-dependent Conformational Changes**—Most Kir channel crystal structures have captured a presumed closed state with a narrow constriction in the bundle crossing region (14, 35). KirBac3.1 is the only Kir channel with structural data that may represent multiple channel states: a cryo-EM study modeled substantial rearrangements of the inner M2 helices during channel opening (comparable with the differences between the KcsA and MthK structures) (36), whereas a more...
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![Diagram](image)

**FIGURE 10. ATP inhibition does not cause complete closure of Kir6.2(F168E) channels.** *A*, ATP inhibition of Kir6.2(F168E) channels was measured at pH 7.3, resulting in a clear plateau conductance that persisted even at 5 mM ATP, with considerably reduced current fluctuations relative to the 0 ATP condition. The presence of a plateau conductance was validated by blocking currents completely with a pulse from −50 to +50 mV in the presence of spermine. *B*, currents of similar magnitude through WT Kir6.2 channels exhibited virtually complete inhibition by ATP.

recent report of multiple crystal structures of KirBac3.1 implies far more subtle rearrangements around the helix bundle crossing and favors a model in which the selectivity filter regulates channel conduction (18). These disparate reports mirror earlier uncertainties arising from functional characterization of ligand-dependent “gated access” of cysteine-reactive probes. Specifically, several studies have reported no gating-dependent changes in accessibility of the Kir channel inner cavity (15, 17), whereas other reports have described the kinetic details for cysteine modification by methanethiosulfonate reagents and demonstrated that gated access can indeed be observed at sufficiently low channel *P*0 (37, 38).

**Relationship to WT Kir6.2 Gating**—We cannot claim to have resolved these lingering uncertainties completely, although we would argue that the peculiar Kir6.2(F168E) phenotype sheds light on mechanisms of Kir channel gating and coupling to ligand-binding sites. We have demonstrated that it is possible to force channel opening with extrinsic stimuli that induce a conformational change around the helix bundle crossing. This finding does not provide direct evidence that the helix bundle crossing forms a functional gate in Kir6.2 but certainly indicates that conformational changes in this region can promote channel opening and are functionally coupled to the intrinsic ATP-binding site. Also, this induced gating motion likely resembles ATP-dependent conformational changes because forced channel opening changes the ATP sensitivity of the channel. Thus, conformational rearrangements of the bundle crossing are, at the very least, an important step in transducing ligand binding to the channel gate.

Intriguingly, the presence of a hydrophilic glutamate at position 168 also prevented complete channel inhibition even at very high ATP concentrations. This resulted in a discrete plateau conductance with little fluctuation noise (Fig. 10), indicating a possible important role for hydrophobic residues for tight closure of the bundle crossing gate. An earlier study characterizing ATP inhibition in a variety of Kir6.2(F168X) mutants suggested the importance of large hydrophobic side chains in this position to achieve strong ATP sensitivity, although a plateau conductance was not explicitly reported (39). Our findings differ from this report because the ATP sensitivity of Kir6.2(F168E) is only modestly altered relative to that of WT Kir6.2 (Fig. 9, B and D) unless alkaline pH is imposed.

We emphasize that a rightward shift of ATP sensitivity (as reported in Ref. 39) does not necessarily indicate that channel closure is prevented, just that the closed state is destabilized relative to the open state. However, a plateau conductance (especially in the absence of any significant shift in ATP sensitivity) likely indicates that ATP-bound “closed” channels retain some permeability. Given the conservation of hydrophobic residues at the bundle crossing (Fig. 1B), it may be surprising that the presence of a hydrophilic glutamate is not more disruptive. For instance, single channel records from Kir6.2(F168E) exhibit well defined closures (as well as we are able to resolve) (Fig. 4), and macroscopic currents can be reduced considerably by ATP (Fig. 9) or internal acidification (Figs. 1 and 10). Together, these observations suggest that any conductance arising from “quasi-closed” F168E channels must be quite small and barely distinguishable from the closed state in single channel records.

**Mechanism of Engineered pH Dependence**—This study demonstrates a simple but novel mechanism to control channel gating rapidly and reversibly by introducing a protonatable side chain at a position that is important for channel opening and closing. A likely mechanism underlying this phenotype is pH-dependent mutual repulsion of glutamate side chains at F168E driving movement of the M2 helices and opening the channel pore. It should be noted that there are likely many factors influencing the engineered (non-native) pH-dependent gating. For example, despite carrying a similar carboxylic acid side chain, aspartate is not a suitable surrogate to observe the pH-dependent gating mechanism. No functional currents were detected from Kir6.2(F168D) channels, which could be due to poor tolerance of mutations in this functionally important region or possibly because aspartate undergoes a *pK* shift that pushes the pH dependence beyond a practical range for patch clamp experiments. Additionally, other potentially interesting protonatable side chains (His and Lys) have not provided useful data, and this may be because the protonated state that would lead to channel opening by mutual repulsion would also carry a ring of positive charge around the mouth of the inner cavity. Prior experiments involving introduction of positive charges (by cysteine modification) in the bundle crossing region resulted in dramatic reduction of macroscopic conductance (40, 41), likely by introducing a significant electrostatic barrier for ion permeation.

**Hydrophobic Bundle Crossing of Kir Channels**—In most Kir channels, one or more pore-lining residues in the narrow bundle crossing are occupied by large hydrophobic side chains, and this has been highlighted as potentially important for channel closure by the formation of a hydrophobic seal (14, 42). It is noteworthy that this arrangement differs from other common models of gating (Kv channels and KcsA) where the bundle crossing comprises side chains with much smaller volumes (e.g. valines in Kv channels) (43, 44). In eukaryotic Kir channels strongly regulated by cytoplasmic ligand binding (i.e. *K*ATP and GIRK pore-forming subunits), a phenylalanine is conserved in the final turn of the M2 helix (Fig. 1B). There appears to be less selective pressure for a bundle crossing aromatic in Kir channels that
physiologically are predominantly open and regulated by polyamine block (Kir2 subfamily) or pH (Kir1 and Kir4 subfamilies). This dichotomy may reflect a stringent physiological requirement for tight channel closure in the ligand-gated subgroup, although we suspect that this may relate to the conservation of a gating mechanism that requires transduction from a cytoplasmic ligand-binding site. The role of aromatic side chains in the Kir channel bundle crossing will be clarified with further exploration of the ligand-dependent gating mechanisms of Kir channels.

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Note Added in Proof—After acceptance of this article, a crystal structure of Kir2.2 in complex with PIP2 appeared in press (Structural basis of PIP2 activation of the classical inward rectifier K+ channel Kir2.2, epub August 28, doi 10:1038/nature10370), demonstrating conformational changes in the helix bundle crossing region that are likely associated with Kir channel opening.

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