Decomposition of Slide Helix Contributions to ATP-dependent Inhibition of Kir6.2 Channels*

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Regulation of inwardly rectifying potassium channels by intracellular ligands couples cell membrane excitability to important signaling cascades and metabolic pathways. We investigated the molecular mechanisms that link ligand binding to the channel gate in ATP-sensitive Kir6.2 channels. In these channels, the “slide helix” forms an interface between the cytoplasmic (ligand-binding) domain and the transmembrane pore, and many slide helix mutations cause loss of function. Using a novel approach to rescue electrically silent channels, we decomposed the contribution of each interface residue to ATP-dependent gating. We demonstrate that effective inhibition by ATP relies on an essential aspartate at residue 58. Characterization of the functional importance of this conserved aspartate, relative to other residues in the slide helix, has been impossible because of loss-of-function of Asp-58 mutant channels. The Asp-58 position exhibits an extremely stringent requirement for aspartate because even a highly conservative mutation to glutamate is insufficient to restore normal channel function. These findings reveal unrecognized slide helix elements that are required for functional channel expression and control of Kir6.2 gating by intracellular ATP.

Inwardly rectifying potassium (Kir)3 channels are essential regulators of membrane excitability. Their functional hallmark is preferential conduction of ions in the inward direction due to voltage-dependent block by Mg2+ and polyamines at voltages larger than the K+ reversal potential (1, 2). This unique voltage dependence underlies the main functional difference between Kir channels and their voltage-gated (Kv) relatives because Kir channels exhibit activity near the potassium reversal potential and contribute significantly to regulation of the resting membrane voltage. Disruption of Kir channel function underlies numerous genetically inherited diseases of ion transport and membrane excitability, including Andersen syndrome (3, 4), Bartter syndrome (5), SeSAME (seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance)/EAST (epilepsy, ataxia, sensorineural deafness, tubulopathy) syndrome (6, 7), primary aldosteronism (8), neonatal diabetes (9, 10), and congenital hyperinsulinism (11, 12).

In addition to voltage-dependent blockade, some Kir channels (in the Kir3 and Kir6 families) are regulated by ligand-dependent gating that controls conformational changes around the helix bundle crossing (in the transmembrane domain) and, potentially, a second auxiliary gate in the cytoplasmic G loop (13–17). These ligand-dependent gating mechanisms act physiologically to influence or trigger cellular depolarization. One very well known example is regulation of heart rate by the sinoatrial node IK,ACh current (mediated by G protein-coupled inwardly rectifying potassium channels) (18). A second example is regulation of glucose-stimulated insulin secretion by KATP channels (the pancreatic KATP isoform comprises the Kir6.2 and SUR1 (sulfonylurea receptor 1) subunits and is the model for ligand-dependent gating used in this study) (19, 20). In this physiological system, glucose metabolism elevates intracellular ATP, leading to inhibition of KATP channels and membrane depolarization, triggering Ca2+ influx essential for insulin secretion (9, 21).

Kir channels comprise a canonical tetrameric pore-forming module similar to other K+-selective channels (15, 16, 22, 23). In place of the voltage-sensing domain that typifies the Kv channel family, the Kir channel pore module is coupled to a cytoplasmic domain that extends the ion permeation pathway and serves as a ligand-binding site for regulatory molecules like Gβγ subunits (in Kir3 channels) or ATP (in Kir6 channels) (16, 24). In addition, all Kir channels require phosphatidylinositol bisphosphate for channel activity, and recent crystallographic studies have demonstrated that the binding site for this essential phospholipid is formed predominantly by positively charged side chains in the C-terminal extension (“C-linker”) of the pore-lining M2 helix (15, 16).

This modular architecture necessitates a mechanism for transduction of ligand binding to the channel gate. The observation of a transverse interfacial helix (the “slide helix”) in Kir
channel structures has provoked considerable speculation regarding its role as a mediator of coupling between the cytoplasmic (“ligand-sensing”) and transmembrane (“gating”) domains (22, 25–27), with obvious analogies drawn to the S4–S5 linker as a coupling element for voltage sensor movement and channel gating in voltage-gated channels (28–30). We have taken a systematic structure-based approach to investigate the role of the slide helix and identify components that are essential for functional channel expression and ATP inhibition of Kir6.2 channels. Importantly, our experiments tackle a frequently encountered problem in structure-function studies of ion channels: mutations in particularly interesting channel motifs often lead to loss of function, impeding further analysis. We describe an effective targeted approach to rescue of loss-of-function mutants, enabling characterization of mutants that have been overlooked because of their loss-of-function phenotype.

**EXPERIMENTAL PROCEDURES**

*K_{ATP} Channel Constructs*—All mutant channel constructs were generated from mouse WT Kir6.2 expressed in the pcDNA3.1(-) plasmid (Invitrogen). All channel constructs had GFP fused to the Kir6.2 C terminus by a 6× glycine linker. Point mutations were introduced by overlapping PCR-based methods, followed by subcloning of the desired fragments into pcDNA3.1(-) and verification by restriction endonuclease digestion and Sanger sequencing (Genewiz). SUR1 (hamster) and FLAG-SUR1 (a gift from Show-Ling Shyng, Oregon Health Sciences University, Portland, Oregon) were also expressed in pcDNA3.1(-).

**Channel Expression and Electrophysiology**—COSm6 cells were maintained in culture in a 5% CO₂ incubator at 37 °C in DMEM supplemented with 10% FBS and penicillin/streptomycin. Cells were transfected with channel constructs and hamster SUR1 using either FuGENE6 (Roche) or jetPRIME (Polyplus). Recordings were done 2–3 days following transfection.

For continuous recordings, data were filtered at 1 kHz, sampled at 5 kHz, and stored directly on a computer hard drive using Clampex software (Axon Inc.). For voltage-step recordings, data were filtered at 2 kHz and sampled at 10 kHz. Symmetrical recording solutions with the following composition were used: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, and 4 mM K₃HPO₄. The pipette solution was adjusted to pH 7.3 using KOH. Bath solution pH was adjusted with KOH or HCl. The bath solution pH was verified each experimental day for each solution, with particular attention to the addition of solutes that can affect solution pH (such as ATP). Solutions were delivered at room temperature by pressure-driven flow (syringe pump) through a multibarreled solution delivery turret driven by the RSC-200 (BioLogic) rapid solution exchanger to enable solution jumps. Chemicals were purchased from Sigma-Aldrich or Fisher. Throughout the text, data are presented as mean ± S.E.

**Non-radioactive Rb Efflux Assay**—CosM6 cells were transiently transfected with SUR1 and various Kir6.2 mutants with FuGENE6 (Roche) or jetPRIME (Polyplus), and efflux assays were performed after 2 days. Cells were loaded for 1 h with Rb⁺ loading medium (5.4 mM RbCl, 150 mM NaCl, 2 mM CaCl₂, 0.8 mM Na₂HPO₄, 1 mM MgCl₂, 5 mM glucose, 25 mM HEPES (pH 7.4)), washed twice quickly with PBS, and incubated in assay buffer (118 mM NaCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 10 mM HEPES (pH 7.4)) supplemented with metabolic inhibitors (2.5 µg/ml oligomycin, 1 mM 2-deoxy-D-glucose) when indicated. Aliquots of the assay buffer were removed at multiple time points (5, 10, 20, and 40 min), and the Rb⁺ concentration was determined by flame atomic absorption spectroscopy using an Aurora Biomed ICR8000. Rb⁺ efflux was calculated as a fraction of total loaded Rb⁺ (determined as the sum of extruded Rb⁺ and Rb⁺ left in lysed cells at the end of the assay). For clarity of figure presentation, data were normalized to efflux from WT Kir6.2-transfected cells (normalized Rb⁺ efflux = (EffluxWT Kir6.2-transfected − EffluxUntransfected)/EffluxWT Kir6.2-transfected) (EffluxWT Kir6.2-transfected − EffluxUntransfected). However, raw efflux data were used for statistical tests. For simplicity, only data from the 40-min time point have been presented (as mean ± S.E.).

Western Blot Detection of K_{ATP} Channel Surface Expression—COSm6 cells in 6-well plates were transfected with mouse Kir6.2 channel cDNA (600 ng/well) and either FLAG-tagged hamster SUR1 (1.2 µg/well) or WT hamster SUR1 (1.2 µg/well) as indicated in the respective figures. After 3 days of incubation, Western blotting was carried out on total cell lysates, using 7.5% SDS-PAGE, followed by transfer to a nitrocellulose membrane. SUR1 protein was detected by labeling with mouse anti-FLAG primary antibody (for FLAG-SUR1, Sigma-Aldrich), or a monoclonal SUR1 antibody (University of California Davis/National Institutes of Health NeuroMab facility, clone N289/16, for WT SUR1) 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).

**Statistical Analysis**—All experiments required multiple comparisons of mutants with WT Kir6.2 or Kir6.2[F168E] channels. After confirmation of normality, we used a one-way ANOVA multiple comparison test followed by Dunnett’s post hoc test to compare mutants with WT Kir6.2 or Kir6.2[F168E] background channels, as indicated in the appropriate figure legends.

**RESULTS**

Functional Scan of the Kir6.2 Slide Helix—The modular structure of Kir channels comprises a canonical pore-forming transmembrane domain (TMD) and a cytoplasmic domain (CTD) (Fig. 1A). The TMD forms an interface with the CTD via the N-terminal transverse slide helix and the C-terminal extension (C-linker) of the pore-lining M2 helix (Fig. 1A) (15, 16). We have designated them helix Sa and helix Sb on the basis of a “kink” apparent in recent eukaryotic Kir crystal structures (15, 16) (Fig. 1A). It is noteworthy that helix Sb is very highly conserved among the eukaryotic Kir channels, whereas helix Sa is more divergent (Fig. 1B). However the functional significance of the slide helix kink is not known.

We tested whether slide helix mutants could form functional channels by scanning their activity (coexpressed with SUR1) using a non-radioactive Rb⁺ efflux assay (Fig. 2A). Under conditions of metabolic inhibition (2-deoxy-D-glucose + oligomycin to activate K_{ATP} channels, Fig. 2A, black bars), robust Rb⁺ efflux was observed through seven of 10 helix Sa mutants, whereas six of 10 helix Sb mutants exhibited significant or com-
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FIGURE 1. Domain architecture and interface of Kir channels. A, Kir channel structure (Kir2.2 + phosphatidylinositol bisphosphate, PDB code 3SPI) highlighting the interface between the N-terminal half of one subunit (silver, with the interfacial slide helix highlighted in red and yellow) and the C-terminal half of an adjacent subunit (green). The magnified image illustrates the close approach of the interfacial slide helix with the C-terminal extension (C-linker) of the M2 helix and the cytoplasmic βC-βD loop. B, alignment of multiple Kir channel slide helix segments. The highlighted position corresponds to Kir6.2 residue Asp-58 at the kink between helices Sa and Sb.

We have developed an alternative approach to rescue channel function using a forced gating mechanism described recently (14). In this strategy, substitution of a glutamate in the
hydrophobic Kir channel bundle crossing (F168E mutation in Kir6.2) generates channels that open upon intracellular alkalization, likely because of mutual repulsion of the acidic glutamate side chains at the bundle crossing gate. A similar mechanism of mutual repulsion has been described for activating mutations in KirBac channels (17, 40). Despite perturbation of the helix bundle crossing, Kir6.2[F168E] channels remain well inhibited by ATP, with 80% of current inhibition in 1 mM ATP (Figs. 4, B and D, and 5B) and an IC50 for ATP (at pH 8.0) of 61 ± 15 μM. This differs modestly from the ATP sensitivity of WT Kir6.2 (32 ± 4 μM), especially when compared with alternative rescue methods such as the C166S background. However, an important difference from WT Kir6.2 channels is that Kir6.2[F168E] exhibits a “plateau” conductance at high ATP concentrations (Fig. 4D).

**Functional Rescue of Slide Helix Mutants by an engineered Forced Gating Mechanism**—Remarkably, when expressed on the background of the Kir6.2[F168E] mutant, all slide helix mutants generated large macroscopic currents in excised membrane patches (Fig. 5), including all deleterious slide helix mutants identified in Fig. 2. This suggests that the F168E background mutation effectively rescues loss of function slide helix mutants, consistent with our demonstration of intact surface expression of these mutants (Figs. 2 and 3). Thus, we employed this novel and highly effective rescue approach for systematic comparison of mutations at all slide helix positions.

We determined the IC50 for ATP inhibition of each slide helix mutant (on the Kir6.2[F168E] background at pH 8.0 because currents are considerably larger) (Fig. 5). It is noteworthy that activity and ATP sensitivity of Kir6.2[F168E] channels are pH-sensitive (14), so it was essential to meticulously adjust the pH in all solutions to ensure that effects did not arise from slight variations in pH. Exemplar sweeps measuring ATP sensitivity in various slide helix mutants are depicted in Fig. 5A, in which patches were formed and excised in pH 7.3, switched to pH 8.0 to demonstrate the F168E-mediated pH dependence, and stepped through several ATP concentrations at pH 8.
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8.0. Effects of pH and ATP were all rapid, stable, and fully reversible.

ATP sensitivity of most slide helix mutants closely resembled the Kir6.2[F168E] background channel (Kir6.2[F168E][L56A]) as provided as an example in Fig. 5A). However, numerous slide helix mutations had statistically significant effects on ATP sensitivity relative to the Kir6.2[F168E] background channel (Fig. 5B). Many of these positions have been identified in early biophysical studies of Kir6.2 or in patients diagnosed with neonatal diabetes, so we focused on two prominent clusters with the most dramatic effects. The first set (I49A, R50A) has been described previously and lies close to the well described ATP binding site, likely contributing to binding the ATP γ-phosphate. Positions Ile-49 and Arg-50 are tolerant to mutation (Fig. 2A), and so both residues have been functionally characterized in earlier studies (41, 42). More interesting was the cluster of mutants (D58A, F60A) in the kink region of the slide helix (at the helix Sa and Sb junction, Fig. 1A). These positions are not predicted to contribute to the putative ATP binding site and are unlikely to make any direct contacts with ATP (41). Moreover, they are relatively intolerant of mutation (Fig. 2A), although an F60Y mutation has been identified previously in a neonatal diabetes patient and appears to play an important role for ATP inhibition (there is strong conservation for aromatic residues at this position (Fig. 1B), although we have not investigated their functional role any further in this study) (25). The Asp-58 position had the largest effect on ATP sensitivity in our scan, a novel effect impossible to explore previously because of the complete loss of function that accompanies this mutation. D58A mutant channels had profoundly reduced ATP sensitivity with very little inhibition observed even in 10 mM ATP (Fig. 5A, right panel). It is noteworthy that effects of the D58A mutation significantly outweigh effects of Kir6.2 mutations at positions identified in neonatal diabetic patients (Arg-50, Gln-52, Gly-53, Arg-54, Val-59, Phe-60, and Tyr-61) (9, 21). Also noteworthy is that these data discriminate different contributions of functionally “essential” residues to the channel gating mechanism. That is, not all loss-of-function mutations impact the ATP sensing mechanism in equivalent ways. By rescuing non-functional mutant channels, these experiments distinguish previously unrecognized components of the ATP-dependent gating mechanism.

Kinetic Features of Kir6.2[F168E] Channels and Impact of Slide Helix Mutations—Notably, in addition to rescuing loss-of-function mutants, the Kir6.2[F168E] mutation imparts a weakly voltage-dependent gating phenotype that is absent in WT Kir6.2 channels (43). Membrane hyperpolarization causes time-dependent opening of Kir6.2[F168E] channels, and these kinetic features persist clearly in inside-out patches after washout of endogenous polyamines (Fig. 6A). Therefore, this voltage dependence appears to be an intrinsic property of Kir6.2[F168E] mutant channels (rather than a consequence of block by residual polyamines) (2). These properties are similar to a polyamine-independent voltage-dependence in Kir6.2[L157E] channels reported previously, but with opposite polarity (43).
FIGURE 5. ATP sensitivity of Kir6.2 slide helix mutant channels. A, continuous inside-out patch clamp recordings at −50 mV for CosM6 cells expressing Kir6.2[F168E] and exemplar Kir6.2[F168E] slide helix mutants (all coexpressed with SUR1). Internal pH and ATP concentrations were switched as indicated with a rapid solution exchange device. B, IC_{50} for ATP inhibition of slide helix mutants on the Kir6.2[F168E] background. ATP inhibition was recorded at internal pH 8.0 (n = 8/construct). The broken bars for I49A and D58A indicate IC_{50} concentrations considerably higher than the largest ATP concentration (10 mM) tested. The gray filled bars highlight mutants that exhibited loss of function in Rb^{-} efflux assays (Fig. 2). One-way ANOVA followed by a post hoc Dunnett’s test for comparisons to the Kir6.2[F168E] control were used. *, p < 0.05 relative to Kir6.2[F168E].

FIGURE 6. Slide helix effects on the unique kinetic features of Kir6.2[F168E] channels. A, inside-out patch recordings from CosM6 cells expressing Kir6.2[F168E] and various exemplar Kir6.2[F168E] slide helix mutants (all coexpressed with SUR1). Patches were pulsed between −150 mV and +50 mV (0 mV holding potential). B, activating components of current after voltage steps to −150 mV were fit with a single exponential equation to extract the time constant τ_{step} at pH 8.0 (n = 8/construct). The broken bar indicates that the time constant of channel opening was too rapid to accurately measure. The gray filled bars indicate mutants that exhibited loss of function in Rb^{-} efflux assays and required the F168E rescue approach to detect ionic currents. One-way ANOVA followed by a post hoc Dunnett’s test for comparisons to the Kir6.2[F168E] control were used. *, p < 0.05 relative to Kir6.2[F168E].
The voltage-dependent kinetic features of Kir6.2[F168E] offered a unique additional assay to compare the effects of slide helix mutants on channel function.

Most mutants had little or no effect on gating kinetics, with hyperpolarization-dependent channel opening remaining similar to Kir6.2[F168E] channels (Fig. 6A). Only two slide helix mutations near the helix Sa-Sb kink (D58A, T61A) markedly altered the kinetics of voltage-dependent channel opening (Fig. 6B). The Asp-58 position stood out in this comparison of slide helix positions because kinetics in Kir6.2[F168E][D58A] mutant channels were too rapid to resolve (Fig. 6, A and B). We estimate that in well formed inside-out patches, we can confidently resolve time constants of ~0.5 ms, and so 2 ms is indicated as a lower limit for 1/τstep of D58A channels. It is noteworthy that the ATP binding site mutations (I49A, R50A) did not alter the gating kinetics (Fig. 6A), suggesting that D58A changes ATP sensitivity by a distinct mechanism that does not involve disruption of the ATP binding site.

Effects of Asp-58 Mutations Are Independent of Intracellular pH—A well understood property of KATP channels is that ATP sensitivity is a variable channel parameter that changes with channel open probability. Manipulations that increase the open probability (Po; e.g. mutations or increased membrane phosphatidylinositol bisphosphate content) typically weaken ATP sensitivity (44). The F168E background channel has a unique property of pH-dependent Po, enabling rapid and reversible changes of channel Po by changing intracellular pH (14). We exploited this to ensure that the loss of ATP sensitivity in Asp-58 mutants did not result from a significant enhancement of channel Po relative to the F168E background channel. We tested inhibition of Kir6.2[D58A][F168E] by 10 mM ATP over a wide pH range, from pH 7.3 (where few channels are open) to pH 8.8 (often yielding 10- to 20-fold larger currents than pH 7.3) and found that channels remained highly insensitive to ATP over this broad range of channel activity (Fig. 7, A and B). These experiments indicate that the loss of ATP sensitivity in Kir6.2 D58A mutant channels does not arise from saturation of channel open probability.

Stringent Requirement for Slide Helix Residue Asp-58—Although multiple Kir6.2 slide helix mutations abolish functional expression (25, 45–48), systematic comparisons enabled by our rescue approach highlight a distinct role for Asp-58 among these functionally essential residues. In particular, Asp-58 appears to make essential contributions both to formation of conductive channels and to appropriate transduction of ATP binding to the channel gate. We further investigated residue Asp-58 by introducing numerous mutations at this position. Notably, Asp-58 exhibits an extremely stringent amino acid tolerance because even the most conservative mutations examined (D58E and D58N) were unable to generate functional channels by Rb+ efflux (Fig. 8A). To confirm that Asp-58 mutants were not affecting Rb+ permeability, we also tested whether Rb+ efflux of different Asp-58 mutants could be rescued with the F168E background (Fig. 8A). We also repeatedly tested the conservative D58E and D58N mutations (on a WT background) with patch clamp experiments but failed to detect any functional expression (data not shown). On the basis of the presence of an upper band on SDS-PAGE (with caveats as discussed previously with Figs. 2 and 3), Asp-58 mutants appeared to reach the plasma membrane (Fig. 8B), and this was also directly confirmed for D58A channels by surface biotinylation (Fig. 3). Furthermore, all Asp-58 mutants characterized by patch clamp were functionally rescued by F168E (demonstrating robust surface expression) and exhibited extremely severe disruption of ATP sensitivity and gating kinetics (Fig. 8A inset, C, and D).

Residue Asp-58 as an Important Coupling Element between the CTD and TMD—We propose that ATP sensitivity of Kir6.2 channels requires both an appropriate ATP binding site and an intact interface between the CTD and TMD (Fig. 9A). Our findings indicate that this interface depends significantly on Asp-58, a residue we refer to as the “aspartate anchor.” A model to explain our findings is that when the Asp-58 TMD-CTD anchor is in place, gating kinetics are slow, possibly because conformational changes of the TMD and CTD are coupled (16, 17, 49), resulting in an energetic barrier to opening. Mutations in the vicinity of the ATP binding site (i.e. I49A or R50A) attenuate ATP sensitivity but preserve slow gating kinetics similar to the F168E background channel (Fig. 9B) because this Asp-58 “coupling element” is still intact. This interpretation is generally consistent with previous reports describing Kir6.2 I49 and Arg-50 mutants, which alter ATP sensitivity but do not affect
gating properties of the channel in ATP-free conditions (42, 50). In contrast, disruption of the TMD-CTD interface by mutation of Asp-58 abolishes both ATP inhibition and slow gating kinetics (Fig. 9). We describe the effects of these mutants as uncoupling the TMD and CTD so that conformational changes of the CTD are no longer linked to the TMD (Fig. 9).

Complexity of Contacts between Residue Asp-58 and the CTD—Kir6.2 position Asp-58 stands out as a functionally important element that lies at the interface of the CTD and TMD of Kir channels. We also investigated residues within the complimentary CTD in the vicinity of Asp-58. Inspection of recent eukaryotic Kir channel structures reveals that the Asp-58 side chain is positioned especially close to residue Arg-206, a highly conserved residue among Kir channels (Fig. 10). However, the organization of the CTD-TMD interfacial region varies considerably between different published structures, and contacts may change in different channel states. In addition to Arg-206, Asp-58 is located in a dense region of positively charged side chains that might also form important interactions (namely, Arg-206 and Lys-207 in the CTD and His-175, Arg-176, and Arg-177 in the C-linker extension of the M2 helix) (Fig. 10).

We investigated this interfacial region by mutating these positive charges and examining their functional effects. We first neutralized each cluster of positive charge (R206A/R207A double mutant and H175A/R176A/R177A triple mutant). Both of these compound mutants ablate channel function in Rb<sup>+</sup> efflux assays (Fig. 10B), consistent with previous reports that the R206A and R177A mutations abolish Kir6.2 function (37). This was also consistent with Rb<sup>+</sup> efflux assays for individual mutants. R177A and R206A had no detectable activity, H175A and R176A exhibited ~20% efflux relative to WT Kir6.2, and K207A exhibited efflux comparable with WT Kir6.2 (Fig. 10B).

We exploited the F168E background to rescue activity and characterize non-functional CTD mutants. Surprisingly, despite the predicted close proximity of Asp-58 and Arg-206, neutralization of Arg-206 (and/or Lys-207) does not reproduce features of Asp-58 mutants. Notably, Kir6.2[F168E][D58E][K207A] channels exhibit only slightly perturbed ATP inhibition and slow gating kinetics (Fig. 10C and D). We also examined positions Arg-206 and Lys-207 individually with more disruptive mutations. Of these, only a highly disruptive charge reversal mutation at R206D altered ATP inhibition significantly to a degree comparable with Asp-58 mutations (Fig. 10D). However, severe mutations at position 207 (e.g. K207D) did not alter ATP sensitivity. All Arg-206 mutations exhibited accelerated gating kinetics relative to F168E alone, but they were nevertheless slower than the Asp-58 mutants (Figs. 8A, inset, and 10E). Kinetics in Lys-207 mutant channels were indistinguishable from the F168E background (Fig. 10E). These findings suggest that a salt bridge between Asp-58 and Arg-206, apparent in recent crystal structures, does not contribute significantly to the mechanism of ATP inhibition.

The Kir6.2[H175A][R176A][R177A] triple mutant (on the F168E background) exhibited dramatically reduced ATP inhibition (Fig. 10C and D). This finding indicates that this charge cluster in the C-linker plays a more important functional role in ATP inhibition compared with Arg-206. We also analyzed these effects with individual point mutations on the F168E background.
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On the basis of these assays, we attributed the loss of ATP sensitivity of the triple mutant almost entirely to the R177A mutation because both H175A and R176A channels were well inhibited by ATP (Fig. 10D). Kir6.2[F168E][H175A][R176A][R177A] channels exhibited accelerated gating kinetics, although again not as rapid as the Asp-58 mutants (Fig. 10E). Both R176A and R177A individual mutations (on the F168E background) exhibited accelerated gating kinetics, whereas H175A had minimal effects (Fig. 10E).

Overall, these findings indicate that the effects of mutating the essential Asp-58 position cannot be replicated by a single structurally rationalized CTD/C-linker residue, suggesting that Asp-58 interactions with the CTD may be more complex than a salt bridge interaction with a single residue (see “Discussion”). Nevertheless, within the cluster of positively charged residues at the domain interface, Arg-177 stands out as an important determinant of ATP sensitivity.

DISCUSSION

Inwardly rectifying potassium channels translate metabolic and signaling pathways into changes in membrane voltage. This function relies on transduction of ligand binding from the cytoplasmic domain to changes in the stability of the channel gate in the transmembrane domain. Despite diversity in the signaling molecules that regulate various Kir channels (52), conservation of domain architecture and primary sequence suggests that aspects of channel gating will be similar among different Kirs. Thus, we hope that our findings will provide insights that translate to ligand-dependent gating mechanisms in other Kir channels, such as Gβγ regulation of G protein-coupled inwardly rectifying potassium channels.

In this study, we developed a novel forced gating approach to systematically evaluate the functional importance of each Kir6.2 slide helix residue and other residues in the TMD-CTD interface. In considering this forced gating approach, we highlight several important features of F168E relative to WT Kir6.2 (14). Firstly, the F168E mutation imparts pH sensitivity and enables forced opening of channels (allowing rescue of loss-of-function mutants). Secondly, Kir6.2[F168E] channels exhibit an intrinsic voltage dependence by a mechanism that remains unclear. Most importantly, the Kir6.2[F168E] ATP IC_{50} is quite close to WT Kir6.2 (Fig. 4D), unlike rescue mechanisms described previously that employ Cys-166 mutations (with an IC_{50} of 5–10 mM) (39). In this regard, it is clear that a robust mechanism for ATP inhibition persists in Kir6.2[F168E] channels. Consistent with this conservation of the ATP inhibition mechanism in Kir6.2[F168E] channels, mutations of numerous residues known to affect ATP sensitivity in WT channels (e.g. Ile-49, Arg-50, Glu-52, Gly-53, Val-59, Phe-60, and Tyr-62) (9, 10) also diminish ATP sensitivity on the F168E background.

Non-equivalence of Loss Of Function Mutations—Our findings highlight positions that are highly intolerant of mutation and would be impossible to characterize without a functional rescue approach. From the perspective of a genetic disease mechanism, loss-of-function K_{ATP} mutants are expected to cause similar phenotypes (12). However, when considering detailed mechanisms of channel function, these data highlight previously untestable features because they distinguish functional differences between loss-of-function mutations, particularly in terms of contributions to ligand-dependent gating. For instance, although residue Asp-58 is essential and intimately involved in the CTD-TMD transduction of ATP binding, other positions (despite being essential for functional expression), appear to be less involved in interdomain communication (e.g. Phe-55, Tyr-61, and Asp-65 mutants all retain significant ATP sensitivity, Fig. 5). We were also surprised to observe large effects on ATP sensitivity clustered to a very small subset of slide helix residues. Mutations that perturb ATP sensitivity have been identified at numerous slide helix positions in patients with neonatal diabetes (10). However, our rescue and functional screen highlight that many of these disease-causing mutations (although clearly severe from a physiological perspective) are not especially severe perturbations of interdomain communication relative to mutations of Asp-58.

Paradoxical Effects of Asp-58 Mutations—It is noteworthy that Asp-58 mutations exhibit complete loss of function (P_o of zero) despite being essentially insensitive to ATP. This might be considered paradoxical because ATP insensitivity is normally associated with channel gain of function (high P_o) (9, 53). However, Asp-58 appears to be essentially insensitive to ATP and, while the direct effect of mutation is ATP insensitivity (high IC_{50}), the indirect effect of mutation is ATP sensitivity (high P_o). The combination of ATP insensitivity (high IC_{50}) and ATP sensitivity (high P_o) suggests a more complex effect that is not easily explained by the simple model of ATP binding to Kir channels.
ever, in the case of position Asp-58, we suspect that this apparent paradox relates to its essential function at the domain interface for both ATP sensitivity and functional expression. Consistent with this essential role for Asp-58 in channel functional expression, a previous study has demonstrated interactions between slide helix residues and the CTD in Kir2.1 and their disruption by certain Andersen syndrome (loss of Kir2.1 function) mutations (51). These findings highlight a generally important role for CTD-TMD interactions in functional channel expression (including an essential role for Kir2.1 residue Asp-71, equivalent to Kir6.2 Asp-58). However, these experiments could not distinguish relative contributions of different slide helix residues to the channel gating mechanism because no functional recordings were possible from loss-of-function mutant channels. Our study overcomes this experimental difficulty and identifies a subset of residues at the Kir channel domain interface that are essential for both channel activity and appropriate transduction of ligand-dependent gating.

**Stringency and Sensitivity of the TMD-CTD Interface**

—The demonstration of the essential role for the Asp-58 position adds important functional context to recently reported structures of Kir2.2 and Kir3.2 (15, 16). These structures suggest that

**FIGURE 10. Functional assessment of potential Asp-58 interaction partners.** A, structural model of the TMD-CTD interacting surface in Kir6.2 channels based on the Kir3.2 structure. B, multiple potential interface residues (His-175, Arg-176, Arg-177, Arg-206, and Lys-207) near Asp-58 were assessed in combination or individually by Rb- efflux. C, continuous inside-out patch clamp recordings at −50mV for CosM6 cells expressing Kir6.2[F168E] with mutations of cytoplasmic domain residues as indicated (all coexpressed with SUR1). D, IC_{50} for ATP inhibition of mutant channels was determined using the F168E mutation to rescue loss-of-function mutations. The R177A mutation dramatically weakens ATP inhibition. E, gating kinetics of multiple interface residues expressed on the Kir6.2[F168E] background. In each panel, ANOVA followed by a Dunnett’s post hoc test were used for comparisons to WT Kir6.2 or Kir6.2[F168E] channels (A, inset). *, p < 0.05 relative to control.
Asp-58 equivalent residue closely approaches a nearby arginine in the βC–βD loop (Kir6.2 residue Arg-206), possibly forming a salt bridge. However, our functional results indicate that this is likely not the only important interaction in this vicinity because neutralization of Arg-206 (and/or its neighbor Lys-207) has little effect on ATP sensitivity, except for the severe charge reversing R206D mutation. In addition, neutralization of Arg-177 had the most significant effect on ATP sensitivity of the charged CTD residues tested, although no crystal structures to date have suggested a salt bridge between Asp-58 and Arg-177. In recent G protein-coupled inwardly rectifying potassium channel structures, the Arg-177 equivalent side chain closely approaches residue Asp-204, suggesting an additional interaction in this interfacial region by which Arg-177 mutations could exert their effects. Lastly, residue Asp-58 lies at a slide helix kink that was not apparent in prokaryotic Kir channel structures but has emerged in the most recent Kir2.2 and Kir3.2 structures. The functional significance of this kink is not yet known, nor have we been able assess whether Asp-58 mutations disrupt the kink. Overall, our approach highlights a subset of mutation-intolerant residues (particularly Asp-58 and Arg-177) as previously unrecognized contributors to ATP-dependent inhibition. However, their specific interactions with nearby charged residues remain undefined.

As described above, recent Kir structures suggest numerous close contacts of charged side chains, raising many possibilities for functionally relevant salt bridge interactions. However, we must clarify that these hypothetical contacts have been difficult to convincingly demonstrate experimentally. Multiple approaches, including targeted metal bridges and complimentary charge reversal mutations (e.g. [D58R][R206D] and [D58R][R177D]), have been attempted in our laboratory. However, none of these manipulations regenerate WT channel function (data not shown). We suspect that this may be due in large part to the extreme stringency of position Asp-58. Recall that even a highly conservative D58E mutation could not recapitulate WT channel function (Fig. 8). Thus, it is perhaps not surprising that other manipulations of this region also severely perturb channel function and ATP sensitivity. At present, our approach enables characterization of functional contributions of individual residues in the TMD-CTD interface. However, further investigation of potential state-dependent contacts between residues in this region will be required to identify dynamic changes that underlie interdomain communication of ATP binding.

Conclusions—A forced gating approach has yielded novel insights into the ATP-dependent gating mechanism of Kir6.2 by identifying unique functional contributions of residues that are highly sensitive to mutation. Our findings reveal that a highly conserved slide helix aspartate plays a central role in the transmission of ligand binding to the channel gate. Additionally, Arg-177 in the TMD-CTD interface is highlighted as an essential residue for ATP-dependent gating. Lastly, the F168E forced gating rescue mechanism has worked very efficiently, enabling rescue of every loss-of-function mutation we tested. We anticipate that this will be a useful tool to probe other channel motifs that are particularly sensitive to mutation, such as the cytoplasmic “G loop.”

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