A Conserved Residue Cluster That Governs Kinetics of ATP-dependent Gating of Kir6.2 Potassium Channels

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Background: Kir6.2 potassium channels are regulated by ATP. Results: We measured Kir6.2 gating kinetics in response to rapid ATP concentration jumps. Mutations to Trp-68 and Lys-170 dramatically decelerate gating. Conclusion: Trp-68 and Lys-170 interact to form a cluster that enables rapid gating transitions. Significance: The Trp-68/Lys-170 cluster is highly conserved and may play a similar role in other Kir channels.

ATP-sensitive potassium (K\textsubscript{ATP}) channels are heteromultimeric complexes of an inwardly rectifying Kir channel (Kir6.\(x\)) and sulfonylurea receptors. Their regulation by intracellular ATP and ADP generates electrical signals in response to changes in cellular metabolism. We investigated channel elements that control the kinetics of ATP-dependent regulation of K\textsubscript{ATP} (Kir6.2 + SUR1) channels using rapid concentration jumps. WT Kir6.2 channels re-open after rapid washout of ATP with a time constant of \(~60\) ms. Extending similar kinetic measurements to numerous mutants revealed fairly modest effects on gating kinetics despite significant changes in ATP sensitivity and open probability. However, we identified a pair of highly conserved neighboring amino acids (Trp-68 and Lys-170) that control the rate of channel opening and inhibition in response to ATP. Paradoxically, mutations of Trp-68 or Lys-170 markedly slow the kinetics of channel opening (500 and 700 ms for W68L and K170N, respectively), while increasing channel open probability. Examining the functional effects of these residues using \(\phi\) value analysis revealed a steep negative slope. This finding implies that these residues play a role in lowering the transition state energy barrier between open and closed channel states. Using unnatural amino acid incorporation, we demonstrate the requirement for a planar amino acid at Kir6.2 position 68 for normal channel gating, which is potentially necessary to localize the e-amino of Lys-170 in the phosphatidylinositol 4,5-bisphosphate-binding site. Overall, our findings identify a discrete pair of highly conserved residues with an essential role for controlling gating kinetics of Kir channels.

Inwardly rectifying potassium (Kir)\textsuperscript{7} channels regulate cellular excitability in response to signaling pathways and metabolism (1). Binding of ligands to the cytoplasmic domain affects the stability of a gate in the pore-forming transmembrane domains (2, 3) and possibly an additional gating motif in the cytoplasmic G-loop (4–6). Although different Kir channel subtypes exhibit sensitivity to different classes of ligands (e.g. nucleotides and \(G\beta\) subunits), their strong sequence conservation suggests similar mechanisms are involved in transducing cytoplasmic ligand binding to changes in stability of the channel gate. One universally shared ligand of Kir channels is anionic phospholipids (typically PI\(P_x\)), which are required for channel function (7, 8). Amino acids potentially involved in PI\(P_x\) binding have been identified through mutagenesis and structure-function approaches, and the physical description of this binding site has been refined considerably by recent crystallographic studies (4, 9, 10).

K\textsubscript{ATP} channels are a unique subtype of Kir channels, formed by co-assembly of Kir6 family subunits with sulfonylurea receptors (11–13). These channels provide a useful model for ligand-dependent regulation of Kir channels because ATP can be rapidly and reversibly applied to influence channel activity. Binding of ATP to the cytoplasmic domain of Kir6.2 channels stabilizes the closed state of the channel. Mutations in Kir6.2 often diminish ATP sensitivity and underlie a monogenic form of neonatal diabetes with a dominant pattern of inheritance (14–16). Characterization of mutations identified in neonatal diabetics and insights from biophysical characterization of Kir6.2 mutants have generated a detailed description of essential residues that control ATP regulation of these channels.

The abbreviations used are: Kir, inwardly rectifying potassium; PI\(P_x\), phosphatidylinositol 4,5-bisphosphate; PNDM, permanent neonatal diabetes mellitus.
Mutations can impact ATP sensitivity by directly altering the ATP binding pocket or the coupling of ATP binding to the channel gate (usually accompanied by a change in intrinsic open probability, $P_o$, of the channel) (17). However, characterization of the effects of these disease-causing mutations on ligand-dependent gating is frequently restricted to their effects on steady-state ATP sensitivity.

An additional consideration is to address the energetic landscape that the channel must navigate while transitioning between closed and open states. What are the rates of Kir channel responses to their regulatory ligands? What structural elements control these rates? In this study, we investigated these questions by characterizing the kinetics of ATP-mediated gating of Kir6.2 and examining whether various disease-causing mutations affect the kinetics of Kir6.2 regulation. Our findings highlight a conserved residue cluster that controls the kinetics of Kir6.2 responses to ATP. These residues also make important contributions to the PIP$_2$-binding site, suggesting distinct roles of lipid interactions with specific residues in the PIP$_2$-binding site.

Materials and Methods

Electrophysiology

For continuous inside-out patch recordings, data were filtered at 2 kHz, sampled at 10 kHz, and stored directly on a computer hard drive using Clampex software (Molecular Devices). Symmetrical recording solutions were used, with the following composition: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, and 4 mM K$_2$HPO$_4$ adjusted to pH 7.3 using KOH.

By pressure-driven flow (syringe pump) at room temperature, solutions were delivered through a microbarrel solution delivery turret, driven by an RSC-200 (BioLogic) solution exchanger to enable rapid solution jumps. Chemicals were purchased from Sigma or Asis Chem. The “Ind” Trp variant was synthesized in-house as described previously (19, 20).

Incorporation of unnatural amino acids was carried out as described in an earlier publication (18, 21–23). Briefly, unnatural amino acids were protected with nitroaryl-oxycarbonyl and activated as the cyanomethyl ester, which was then coupled to the dinucleotide pdCpA (custom synthesis by Dharmaco). This aminoacyl dinucleotide was subsequently ligated to a modified tetrahymena thermophile tRNA. The aminoacylated tRNA amino acid was deprotected by UV irradiation immediately before co-injection with the cRNA for Kir6.2 (Trp-68TAG) and SUR1. In a typical experiment, 80 ng of tRNA amino acid, 25 ng of Kir6.2(Trp-68TAG) cRNA, and 25 ng of SUR1 cRNA were injected in a 50-nl volume. In control experiments, the cRNA alone or the cRNA plus synthetic tRNA (for nonsense suppression, described below) was injected. After injection, oocytes were incubated for 12–48 h at 18 °C.

Fluorinated tryptophan derivatives and cyclohexylalanine were purchased from Sigma or Asis Chem. The “Ind” Trp variant was synthesized in-house as described previously (19, 20).

Data Analysis

Estimation of Open Probability by Stationary Noise Analysis—We used stationary noise analysis to estimate $P_o$ in long recordings using the following equation. $P_o = 1 - (\sigma^2/(i I)),$ where $\sigma^2$ is the variance; $i$ is the single channel current at $-50$ mV (we used a value of 4 pA, assumed to be identical for all channel mutants); and $I$ is the macroscopic current at $-50$ mV (24, 25). As a second index of open state stability, we also measured ATP sensitivity and found that increased $P_o$ was observed together with weaker ATP sensitivity in all mutants tested (see “Results”), consistent with previous reports (7, 26).

Segments of recordings (10–20 s) used for noise analysis were filtered at 2 kHz and sampled at 10 kHz. Recordings exhibited very little, if any, current rundown because all recordings were done in the presence of 1 mM EDTA (previously demonstrated to prevent rundown of K$_{ATP}$ channels) (27).
**Rate-limiting Step in ATP-dependent Gating of Kir6.2**

Derivation of $K_{eq}$ from Open Probability (Assumptions and Caveats)—We derived equilibrium constants for $\phi$ value analysis, using the estimated $P_o$ (based on stationary noise analysis), and making the simplifying assumption of a two-state model of channel gating (C $\leftrightarrow$ O) as shown in Equation 1.

$$K_{eq} = P_o/(1 - P_o)$$  \hspace{1cm} (Eq. 1)

We recognize that this is a simplifying assumption, as there are typically multiple time constants required to describe single channel gating kinetics of $K_{ATP}$ (Kir6.2 + SUR1) channels (28–30). Therefore, the estimated $K_{eq}$ are best interpreted to reflect relative changes in open state stability compared with channels made up of WT Kir6.2 + SUR1. Although this is an important shortcoming of the analysis, it provides a useful comparison to distinguish the kinetics of “rate-controlling” positions from other residues tested in Kir6.2. Also, we have confirmed that the most severe mutations that slow Kir6.2 channel gating kinetics have similar intraburst gating kinetics (“flicker” closings) relative to WT Kir6.2 (see under “Results”). Thus, changes in $P_o$ that are observed by stationary noise analysis are likely due primarily to changes in bursting behavior (burst length or interburst duration). Throughout the text, data for estimated rates and equilibrium constants are presented as mean ± S.E.

**Results**

**Rapid Solution Exchange in Patches**—Kir6.2 is a pore-forming subunit of the $K_{ATP}$ channel, and the functional Kir6.2 tetramer includes four identical ATP-binding sites at the interfaces of the N and C termini of neighboring subunits (31). In the absence of Mg$^{2+}$ ions (required for ATP interaction with the nucleotide binding folds of sulfonylurea receptor), ATP acts predominantly via the ATP-binding sites formed by Kir6.2, causing channel inhibition. In this study, we used rapid solution exchange to explore the kinetics of Kir6.2 channel gating in response to ATP concentration changes.

Solution exchange kinetics of inside-out patches are influenced by variable recession of the plasma membrane into the patch pipette (sometimes referred to as the patch $\Omega$) as suction is applied during seal formation. Slow solution exchange can affect the apparent kinetics of ligand interactions (32, 33). To control for this, we set a benchmark internal control that was used for all data presented. To ensure rapid kinetics of perfusion, we performed K$^+$ concentration jumps in parallel with experimental protocols, on every patch (see representative patch in Fig. 1A). The change in reversal potential from the concentration jump changes the K$^+$ current magnitude, and we used this as an index for the kinetics of solution exchange. We arbitrarily defined a cutoff for acceptable time constants of solution exchange of 30 ms. This cutoff was chosen because it was achievable yet still faster than gating kinetics observed for ATP-dependent gating of Kir6.2 (described below). Patches with slower kinetics were omitted from further analysis due to the likely confounding effects of restricted diffusion of ATP in the patch.

Many PNDM Mutations Dramatically Alter Open State Stability, with Mild Effects on Channel Opening Kinetics—We used rapid solution exchange to assess rates of Kir6.2 channel reopening after ATP washout. WT Kir6.2 channels exhibit fairly rapid recovery kinetics after ATP washout, with time constants of 57 ± 8 ms (Fig. 1B, black trace, solution exchange time constant 18 ± 2 ms). Please note that we have depicted currents in ATP washout experiments in the positive direction (to intuitively reflect channel activation); however, in reality the currents are inward and measured at a membrane potential of −50 mV. Time constants for WT Kir6.2 reopening were consistently slower than K$^+$ concentration exchange rates. Nevertheless, these rates were within the same order of magnitude, and we are cognizant that contamination of the measured reopening rate (by the kinetics of washout of ATP from the patch) is a possibility. To further gauge the practical limits of solution exchange, we measured reopening rates for various mutants at Kir6.2 Cys-166 (known to strongly affect channel $P_o$) (34). Mutations at this position had small but discernable effects on the rate of Kir6.2 channel reopening after ATP washout (Fig. 1B). The high $P_o$ mutant C166S slightly accelerated channel reopening, although a mutation with lower intrinsic $P_o$ (C166I) caused channels to re-open more slowly (Fig. 1B). Overall, these observations indicate that ATP unbinding is rapid and that channel reopening rates are resolvable, although often close to the limit of our capabilities for rapid solution exchange.

Sample recordings (Fig. 1B) illustrate the parameters we extracted from these data. After the ATP solution jump (into a 0 ATP solution), we fit the rise time of the current recovery to estimate a rate constant ($k_{open}$) for channel reopening (see under “Materials and Methods”). After patches reached a steady state in the absence of ATP, we used stationary noise analysis to estimate channel $P_o$ and derive $K_{eq}$ values based on the assumption of a two-state gating model (see under “Materials and Methods”). The inset of Fig. 1B depicts representative currents from patches with similar current size to illustrate the clear difference in stationary noise between WT Kir6.2 and high $P_o$ mutants like Kir6.2(C166S) (Fig. 1B, inset). Similar data were extracted for mutations in the slide helix and bundle crossing regions (Gln-52, Gly-53, and Val-59, Fig. 1C) linked to neonatal diabetes (PNDM) and known to alter open state stability. These parameters ($k_{open}$ and $K_{eq}$) were plotted for mutants at each position (Fig. 1, D–G), similar to previously described $\phi$ value analyses for ion channels and other proteins (35–37). In most cases, there was a modest positive correlation between the reopening rate and open state stability (Fig. 1, D–G), i.e. as $P_o$ increased, the channel re-opening rate became faster, although these effects were small. This is intuitively consistent because stabilization of the open state can be at least partly rationalized by acceleration of the channel opening rate.

Although these mutations exhibit similar trends (Fig. 1, D–G), some important details should be considered. First, the effects of these mutations on opening rate are mild and do not account for the apparent change in open state stability (if this were the case, a plot of $k_{open}$ versus $K_{eq}$ would yield a slope of 1, although the slopes of our plots are much smaller). Also, there is a practical upper limit to the rate of channel reopening that can be measured, due to the limits of solution exchange (Fig. 1A). Because many of the measured rates of channel reopening are close to our limit of solution exchange, it is possible that these
plots underestimate the steepness of the $k_{\text{open}}$ versus $K_{\text{eq}}$ relationship.

Kinetic Control by a Conserved Tryptophan in the Bundle Crossing Region—We have demonstrated that WT Kir6.2 channel reopening occurs near the limit of our detection ability and that mutations with increased $P_o$ modestly increase the observed rate of channel reopening. Interesting divergent findings emerged with further investigation of PNDM-linked positions in the bundle crossing region. Notably, in Trp-68 mutants, we observed markedly slower channel reopening (Fig. 2A), in contrast to the rapid opening of mutants in Fig. 1. For example, W68L exhibited a reopening time constant of 510 ± 40 ms (solution exchange time constant of 10 ± 3 ms). This result was unexpected, because none of the mutations examined previously had exhibited such a dramatic slowing of reopening, and because Trp-68 mutations have been reported to increase open state stability (38). We extended this observation by determining $K_{\text{eq}}$ and $k_{\text{open}}$ values for multiple Trp-68 mutants. This analysis consistently revealed that the re-opening rate became markedly slower in mutants despite higher $P_o$ (Fig. 2, A and B). In mutants tested in detail, there was no obvious relationship between side chain properties and the gating effect, as slow rates of channel reopening were observed for acidic (Asp and Glu) and hydrophobic (Leu) side chains (Fig. 2B). The other aromatic side chain tested (Phe, open symbol in Fig. 2B) was an outlier from the overall trend, yielding somewhat faster reopening rates relative to other mutants, but still slower than the native Trp. A Tyr substitution could not be tested because W68Y is extremely resistant to ATP inhibition (up to 10 mM) (38). The extremely slow gating of Trp-68 mutants is counterintuitive, because it suggests that despite a more stable open state, these mutants open much more slowly than WT Kir6.2. Thus, the native Trp at position 68 appears to be essential for rapid gating transitions during channel re-opening.

Interactions of Trp-68 with Nearby Side Chains—A previous study describing the effects of Kir6.2 Trp-68 mutations hypothesized that Trp-68 might interact with Thr-171, Lys-170, or Ile-167 in the M2 helix (38). The other aromatic side chain tested (Phe, open symbol in Fig. 2B) was an outlier from the overall trend, yielding somewhat faster reopening rates relative to other mutants, but still slower than the native Trp. A Tyr substitution could not be tested because W68Y is extremely resistant to ATP inhibition (up to 10 mM) (38). The extremely slow gating of Trp-68 mutants is counterintuitive, because it suggests that despite a more stable open state, these mutants open much more slowly than WT Kir6.2. Thus, the native Trp at position 68 appears to be essential for rapid gating transitions during channel re-opening.
of Ile-167 (Fig. 3A) and Thr-171 (Fig. 3B) behaved similarly to most positions tested around the bundle crossing (Fig. 1, D–G), exhibiting mild acceleration of channel reopening in mutants with enhanced open state stability. However, Lys-170 mutants closely mimicked Trp-68 mutants, whereby channel recovery from ATP inhibition became markedly slower despite significantly higher open state stability (Fig. 3, C and D). Among the mutants tested in detail, K170N or K170S caused the most extreme slowing of channel reopening. For example, K170S channels reopened with a time constant of 700–1000 ms (solution exchange kinetics 8–2 ms), and often exhibited a prominent slow component causing channels to take 10–20 s to completely recover from ATP inhibition (Fig. 3D). K170R channels also exhibited slow reopening kinetics, consistent with reports of this mutation in neonatal diabetic patients (indicating similar functional effects as other mutations of Lys-170) (39, 42). This finding suggests that a positive charge at position 170 is not itself sufficient for rapid channel gating kinetics.

Our findings indicate that mutations of Lys-170, but not Ile-167 or Thr-171, closely recapitulate the slow reopening
observed in Trp-68 mutants. Kinetics of reopening in Trp-68 and Lys-170 mutant channels are often ~100-fold slower than the kinetics of solution exchange, increasing our confidence in these kinetic measurements. Given the close proximity of these residues in the three-dimensional channel structure, together with their unique effects, our findings indicate that Trp-68 and Lys-170 interact to form a cluster that minimizes the energy barrier for transitions between open and closed states.

We have included additional data related to the trends described above. First, ATP sensitivity of all Trp-68 and Lys-170 mutants is weaker than WT Kir6.2 (Fig. 4, A and B). This trait is commonly observed in Kir6.2 mutants with higher open state stability (43), and it corroborates our conclusion based on stationary noise analysis. The higher $P_o$ that we calculated is also consistent with previous reports describing Trp-68 mutations in neonatal diabetes, although there are some quantitative differences. We consistently observed a higher $P_o$ of WT Kir6.2 channels (~0.6) than is often reported by other groups (0.4–0.5), along with slightly weaker ATP sensitivity, and we suspect this may be due to differences in cell culture conditions leading to altered membrane PIP$_2$ levels (7, 26, 38, 43, 44). Our $P_o$ estimates for Trp-68 mutants are also somewhat higher than a previous report using single channel measurements (38). We measured $P_o$ of W68A as 0.89 ± 0.02 (versus 0.77 ± 0.06) and W68F as 0.95 ± 0.01 (versus 0.83 ± 0.01), although there is a clear and consistent trend that Trp-68 mutations increase $P_o$ relative to WT Kir6.2 (our use of noise analysis rather than single channel recordings also likely contributes to these differences). Finally, it should be noted that exemplar current traces presented for mutants (Figs. 1B, 2A, and 3D and later in Fig. 9) are taken from different patches with different current magnitudes and scaled to highlight changes in gating kinetics, so differences in variance may not be immediately apparent. To illustrate the prominent difference between Kir6.2 and Trp-68 or Lys-170 mutants, we have graphed the relationship between current magnitude and variance in WT Kir6.2 patches along with selected Trp-68 and Lys-170 mutants, highlighting the consistently lower variance relative to WT Kir6.2 in these mutants (Fig. 4C).

Conservation of Trp-68 and Lys-170 in Kirs—Kir6.2 Trp-68 is among the most highly conserved residues in the Kir channel family. We have summarized a large multiple sequence alignment comprising 732 sequences. Fig. 5A depicts the % identity at each position, and Fig. 5B highlights positions above an arbitrarily defined cutoff of >90% identity (color-coded and mapped onto a model Kir channel in Fig. 5C). Not surprisingly, clusters of high conservation are found in the selectivity filter region and in the core of the Kir cytoplasmic domain. In addition, a subset of residues in the CTD-TMD interfacial region exhibits extremely high conservation, including an essential “aspartate anchor” at the kink of the slide helix (17), and position Trp-68 that is a landmark for the start of the M1 helix (38).

Lys-170 fell just short of the arbitrary 90% threshold for highlighting in Fig. 5B, although it is also strongly conserved (Fig. 5A). This slightly lower conservation in the alignment is because Lys-170 is frequently an Arg in prokaryotic Kir channels but a Lys in virtually all eukaryotic Kir channels (Fig. 3E). The strong conservation of Lys-170 in eukaryotic Kir channels is likely related to its role as a PIP$_2$-interacting residue, seen in recent structures of Kir2.2 and Kir3.2 in complex with PIP$_2$ (4, 10), although this role was not anticipated by previous functional studies in Kir6.2 (9, 45). Among residues highlighted in recent PIP$_2$-bound Kir structures (equivalent to Kir6.2 residues Lys-170, His-175, Arg-176, and Arg-177), Lys-170 appears to be the most conserved PIP$_2$ interactor (Figs. 3 and 5). Moreover, of the residues in close proximity to Trp-68 (Fig. 3F), Ile-167 and Thr-171 exhibit <35% conservation in our alignment. Overall, the conservation, proximity, and similar effects of

FIGURE 4. Parameters of ATP sensitivity and channel function in Trp-68 and Lys-170 mutants. A and B, inhibition of current relative to control was determined for a range of ATP concentrations applied to inside-out patches from indicated Trp-68 and Lys-170 mutant channels ($n$ values are in parentheses in the symbol legend). C, current variance and mean macroscopic current are presented for numerous cells transfected with each indicated mutant ($n$ values were 8 (WT), 7 (W68A), 6 (W68L), 7 (K170N), 7 (K170Q)). Note the consistently higher variance in WT Kir6.2 relative to channel mutants across a range of current magnitudes.
mutation are consistent with Trp-68 and Lys-170 functioning together to control reopening kinetics in Kir6.2 channels.

**Discrimination of Ligand Binding Effects from Gating Effects**—Experiments thus far do not distinguish whether Trp-68 and Lys-170 mutations decelerate ATP unbinding from the channel or slow a conformational change associated with reopening (after unbinding of ATP). We suspected the latter was more likely because ATP unbinding from WT Kir6.2 channels is relatively fast (Fig. 1B) and Trp-68 and Lys-170 mutations decrease ATP affinity (and are therefore unlikely to cause tighter binding of ATP). We reasoned that if Trp-68/Lys-170 controls a conformational change associated with reopening, then gating kinetics should be independent of the affinity of the inhibitory ligand. Therefore, we tested a series of adenine-derived inhibitory ligands with weaker potency (ATP, ADP, AMP, Fig. 6). The rate of reopening is similar for all three nucleotides, in WT Kir6.2, W68L, and K170N mutants (Fig. 6, A–C). When channels are inhibited by either ADP or AMP, slow gating persists in Trp-68 and Lys-170 mutants despite weaker potency of inhibition (particularly AMP). These data indicate that affinity or unbinding rate of the ligand does not influence the rate of channel reopening in Trp-68 or Lys-170 mutants. Rather, these residues control an intrinsic rate of reopening after ligand unbinding (Fig. 6E).

**Rate-controlling Mutants Prolong Burst and Interburst Intervals**—We used single channel recordings to determine whether mutations in the Trp-68/Lys-170 rate-controlling cluster were perturbing other channel properties. We compared single channel behavior of WT Kir6.2 with a dramatic rate-controlling mutant (K170N), co-expressed with SUR1. Single channel conductance was not significantly changed (Fig. 7A). Kir6.2 channels exhibit bursting behavior with rapid flicker openings and closings within each burst, which dominate the overall number of events in a single channel record (Fig. 7, A and B). Rapid closures (<10 ms in duration) are not remarkably changed by the K170N mutant (Fig. 7, A and B), as is commonly observed in Kir6.2 mutants with high P_o. However, intermediate closures (10–200 ms) were much less frequent in the K170N mutant (Fig. 7B). We interpret these findings to indicate that mutations in the rate-controlling Trp-68/Lys-170 cluster do not markedly change pore properties or rapid gating kinetics. Therefore, we restricted our analysis to events delineated by longer lived closings. In Fig. 7, C and D, we binned closing events 10 ms or longer (interbursts), along with the times elapsed (bursts containing many flickery closures) between these long closures. It is apparent that WT Kir6.2 channels exhibit frequent transitions between burst and interburst states, with neither reaching 1 s in duration except on very rare occasions (Fig. 7, C and E). In contrast, Kir6.2 (K170N) can open for extremely long lived bursts, making it challenging to collect sufficient numbers of burst/interburst events. Nevertheless, these channels exhibit a high proportion of closures that are 1 s or longer (Fig. 7, D and E), and we commonly observed closures lasting 10 s or more (Fig. 7D). These data suggest that K170N...
channels can access long-lived closed states even in the absence of ATP, and this may reflect the very slow reopening kinetics observed in macropatches.

**Interactions in the Rate-controlling Cluster Probed with Unnatural Amino Acids**—We aimed to investigate the rate-controlling Trp-68/Lys-170 cluster by identifying the mechanism of interaction between these conserved residues. We used unnatural amino acid mutagenesis to manipulate the properties of Trp-68 in very subtle ways. We were specifically interested to investigate whether Trp-68 and Lys-170 form a cation--π interaction (46). We employed nonsense suppression in *X. laevis* oocytes, whereby Kir6.2 (with an introduced TAG stop codon)
and SUR1 mRNA were co-injected with a synthetic aminoacylated tRNA carrying the desired amino acid. During protein synthesis, the tRNA can direct incorporation of the unnatural amino acid at the TAG stop site, leading to synthesis of functional channels (Fig. 8A).

This method has not been used for KATP channels before, so we first demonstrated that Kir6.2/SUR1 channels were amenable to nonsense suppression by rescuing WT Kir6.2(Trp-68TAG)/SUR1 function by co-injecting with tRNA loaded with various Trp analogs. In whole oocyte recordings, we elicited WT KATP currents using metabolic inhibition by 3 mM azide, resulting in large currents that could be inhibited by glibenclamide (Fig. 8B). In contrast, oocytes co-injected with unloaded tRNA (pdCpA control, with no conjugated amino acid), along with Kir6.2(Trp-68TAG)/SUR1, failed to generate glibenclamide-sensitive currents (Fig. 8C). Co-injection with tRNA conjugated to various Trp analogs was sufficient to rescue KATP activity, with large glibenclamide-sensitive currents elicited by metabolic inhibition (Fig. 8D). Using this system, we proceeded to measure kinetic behavior as described earlier, with excised inside-out membrane patches from injected oocytes.

Kir6.2(Trp-68TAG) channels rescued with the naturally occurring Trp residue exhibited rapid channel reopening comparable with the WT Kir6.2 measured previously (Fig. 9A). This demonstrates that data from inside-out patches from Xenopus oocytes were comparable with data from transfected cells, indicating that the measured reopening rate is a reproducible property. We also tested variants of Trp that alter π-electron distribution (F4-Trp, Fig. 9B) or ablate hydrogen bonding (by altering the position of the indole nitrogen, Fig. 9C, Ind). In both cases, fairly rapid channel re-opening rates persisted, although indole substitution decelerated reopening rate to some degree. Comparison with data generated from conventional mutagenesis (Fig. 2B, included in Fig. 9E for comparison) illustrates that the planar Trp variants (Trp, F4-Trp, and indole) are quite similar to WT Kir6.2 channels. However, Trp-68 substitution with a nonplanar (but cyclic) analog cyclohexylalanine, caused marked deceleration of re-opening kinetics (Fig. 8, D–F), similar to the W68L substitution. This suggests a requirement for a planar amino acid at Trp-68, and this may explain why W68F causes anomalously fast channel reopening (distinct from the trend of other substitutions in Fig. 2B). Apart from indole and F4-Trp, Phe is the only other planar substituent that we could test with rapid perfusion experiments (because W68Y abolishes inhibition by ATP). These data suggest a planar side chain is required at position 68 for fast gating kinetics, but a cation-/π or hydrogen bond interaction (with Trp-68) is not involved.

**Discussion**

Using rapid solution jumps to characterize ligand-mediated gating transitions of Kir6.2, we identified Trp-68 and Lys-170 as a unique cluster that controls the transition rate between closed...
and open states. In contrast, most other mutations tested had minor effects on the reopening rate (Figs. 1, D–G, and 3, A and B), causing a small acceleration of channel opening that correlates with stabilization of the channel open state. Mutations at both Trp-68 and Lys-170 stood out because of their unexpected combination of marked slowing of channel reopening, together with a significantly increased $P_o$ (consistent with decreased ATP sensitivity).

The relative orientation of Trp-68 and Lys-170 led us to suspect a cation-$\pi$ interaction, in which the charged Lys side chain forms an electrostatic interaction with the negative surface potential of the aromatic Trp-68 (47). However, direct testing of this possibility with unnatural amino acid substitution (F-Trp) ruled out a significant contribution of a cation-$\pi$ interaction to ATP sensitivity or gating kinetics. This finding highlights the importance of explicitly testing the nature of side chain interactions, even when there is a strong structure-based rationale suggesting a cation-$\pi$ interaction. In another important example, the orientation of voltage sensor charges relative to the “charge transfer center” Phe residue in recent Kv structures hinted at a cation-$\pi$ interaction. However, this could not be demonstrated functionally with the native Phe residue, although a cation-$\pi$ interaction could be engineered into this site by substitution of a Trp (48, 49).

**Unusual Consequences of Mutations of Lys-170, a PIP$_2$-binding Residue**—Trp-68 and Lys-170 are very close to the crystallographically identified PIP$_2$-binding site (4, 10). Based on homology with Kir2.2 and Kir3.2 structures, it is predicted that Lys-170, His-175, and Arg-176 will directly interact with PIP$_2$. In addition, the Arg-177 side chain contributes a positive charge to this region and may interact with PIP$_2$ in some states (although this has not been demonstrated in a structure). Because the specific chemical properties of Trp (H bonding or cation-$\pi$ propensity) are not essential for rapid channel gating, we speculate that Trp-68 may play a structural role as a guide or “cap” to correctly orient Lys-170 relative to PIP$_2$. An interesting observation was that the indole substitution of Trp (which abolishes H bonding of the Trp side chain) also decelerated channel reopening, although not as dramatically as most other Trp-68 mutations. Among integral membrane proteins, there is a high frequency of aromatic side chains at the lipid-aqueous interface, where Trp hydrogen bond interactions with lipids have been suggested to play a role in orienting helices relative to surrounding lipids (50, 51). Thus, a possible mechanism for the slight deceleration of ATP-dependent gating in Trp-68-Ind channels is that the Ind side chain (and hence Lys-170) is less constrained and incorrectly oriented relative to PIP$_2$. 

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**FIGURE 9. **Unnatural amino acid mutagenesis demonstrates the importance of a planar amino acid at position Trp-68. A–D, unnatural amino acid mutagenesis was used to introduce subtle Trp variants at Kir6.2 position 68. Chemical structures and the kinetics of channel reactivation after jumps from 1 mM ATP into 0 ATP solutions are depicted, using inside-out patches at a membrane voltage of −50 mV (inward currents are depicted in the positive/upward direction and are normalized to illustrate the time course of channel reopening). E, plot of reopening rates ($k_{open}$) versus equilibrium constant ($K_{eq}$) for unnatural mutations at position Trp-68 (colored symbols). Data from Fig. 2 have also been included for comparison (black symbols). For each mutant, $n$ values were as follows: F-Trp(6), indole (Ind)(6), cyclohexylalanine (CHA)(5), WT Trp(5). F, mean rates of reactivation for the unnatural amino acid substitutions at Kir6.2 residue Trp-68 (Trp, data in E). The reopening rate is markedly reduced by substitution of cyclohexylalanine. G, magnified view of the M1–M2 helical interface where Trp-68 and Lys-170 come into close contact, illustrating the en face arrangement of these side chains.
Rate-limiting Step in ATP-dependent Gating of Kir6.2

Functional experiments aimed toward investigating PIP$_2$-binding residues have generally been designed with the rationale that PIP$_2$ augments Kir channel function (7, 26, 45). Therefore, PIP$_2$-binding residues were sought out by mutating positively charged residues in the membrane interface region and identifying mutants with significantly reduced function. In these experiments, the Lys-170 side chain was not identified (because Lys-170 mutations cause gain-of-function) and thus not included in subsequent models of PIP$_2$ binding in Kir6.2 (9, 45). However, recent structural studies have significantly refined the description of PIP$_2$ binding, highlighting the interaction with Lys-170. Although several channel/IPP$_2$ contacts indeed reduce channel function when mutated (17, 45), effects of Lys-170 mutations are very different, resulting in channel gain-of-function (and neonatal diabetes) (39, 42), although it should be noted that equivalent mutations in other some other Kir channels (e.g. Kir2.1 residue Lys-182) abolish channel activity (52). Our findings demonstrate that Lys-170 plays a unique role in controlling the rate of Kir6.2 channel response to changes in ATP concentration. These unexpected effects of a PIP$_2$-binding site mutant raise the interesting possibility that PIP$_2$ may have multiple distinct functional effects that can be mapped to specific elements of the binding site. However, our current findings cannot rule out that Trp-68 and Lys-170 mutations have functional effects that are independent of PIP$_2$. We currently prefer the idea that appropriate Lys-170 interactions with PIP$_2$ (guided by Trp-68) are essential to facilitate motion of the bundle crossing gate region as the channel opens and closes. This role would contrast with other PIP$_2$-interacting residues (e.g. Arg-176) that appear to be involved in stabilizing an interface between cytoplasmic and transmembrane domains (10).

Considerations Related to $\phi$ Value Analysis and Ligand-dependent Gating—The application of $\phi$ value analysis in this study should be considered together with recognition of the potential drawbacks of our approach. $\phi$ value analysis is typically applied to infer details of the transition energy “landscape” as proteins isomerize between states (e.g. folded $\leftrightarrow$ unfolded, open $\leftrightarrow$ closed), based on $\phi$ value slopes that normally fall between 0 and 1. As mentioned, for many of the mutants tested, the reopening rate is close to the practical limit for solution exchange in our system (Fig. 1, D–G). Therefore, slopes measured from these data could underestimate an acceleration of channel reopening in high $P_n$ mutants. For this reason, we are cautious to use these data for conventional applications of $\phi$ value analyses (to map spatial or temporal sequences of conformational changes at specific residue positions) (35, 37). The geometric challenge of a recessed membrane patch is a problem that is inherent to the recording technique. As a result it may be difficult to extend this approach for in-depth $\phi$ value analysis of Kir6.2, similar to what has been done in detail for some other channels (35, 53). It is also important to note our simplifying assumption (to estimate $K_{eq}$) that Kir6.2 channel gating can be described by a two-state equilibrium. More complex gating models are generally used to describe these channels (30, 43). For this reason, although there is little doubt that Lys-170 and Trp-68 mutations increase channel $P_n$, the calculated increase in $K_{eq}$ is best taken as a qualitative measure of open state stability.

With these considerations in mind, the $\phi$ value plots effectively demonstrate the unique role of Trp-68 and Lys-170 in Kir6.2. Based on the extremely slow reopening kinetics (relative to much faster solution exchange; Figs. 2 and 3), we are confident that mutation of either of these positions causes an unusual combination of increased $P_n$ together with slower channel reopening. These features differ starkly from other mutations tested and cause the unusual steeply negative $\phi$ value slope (Figs. 2 and 3), which to our knowledge has not been previously reported in an ion channel gating process. There are varying interpretations of the significance of $\phi$ values that fall outside the typical range of 0 to 1 (54). Based on our current understanding of $K_{ATP}$ channel gating, we feel it is informative to describe the Trp-68 and Lys-170 cluster as a “catalytic” site for gating, because these residues seem to minimize the transition state energy barrier for reopening of the channel. Thus, we speculate that the Trp-68/Lys-170 cluster controls the rate of reopening of the channel after ATP comes unbound, and mutation of these residues introduces a rate-limiting step in $K_{ATP}$ channel gating.

Conclusion—Using rapid solution exchange, we demonstrate that Trp-68 and Lys-170 form a cluster that controls the transition rate between open and closed states of Kir6.2. Although these side chains seem ideally oriented to form a cation–π interaction in crystal structures, no significant functional contribution of a cation–π interaction could be detected in terms of ATP sensitivity or kinetics of Kir6.2 channel gating. Rather, the planar nature of the Trp-68 side chain appears to be essential, and it may serve a structural role to correctly orient Lys-170 in the PIP$_2$-binding site.

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