Inwardly rectifying 

Structure and Dynamics of the Pore of Inwardly Rectifying K\textsubscript{ATP}

channels

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Inwardly rectifying K\textsuperscript{+} currents are generated by a complex of four Kir (Kir1–6) subunits. Pore properties are conferred by the second transmembrane domain (M2) of each subunit. Using cadmium ions as a cysteine-interacting probe, we examined the accessibility of substituted cysteines in M2 of the Kir6.2 subunit of inwardly rectifying K\textsubscript{ATP} channels. The ability of Cd\textsuperscript{2+} ions to inhibit channels was used as the estimate of accessibility. The distribution of Cd\textsuperscript{2+} accessibility is consistent with an \alpha-helical structure of M2. The apparent surface of reactivity is broad, and the most reactive residues correspond to the solvent-accessible residues in the bacterial KcsA channel crystal structure. In several mutants, single channel measurements indicated that inhibition occurred by a single transition from the open state to a zero-conductance state. Analysis of currents expressed from mixtures of control and L164C mutant subunits indicated that at least three cysteines are required for coordination of the Cd\textsuperscript{2+} ion. Application of phosphatidylinositol 4,5-diphosphate to inside-out membrane patches stabilized the open state of all mutants and also reduced cadmium sensitivity. Moreover, the Cd\textsuperscript{2+} sensitivity of several mutants was greatly reduced in the presence of inhibitory ATP concentrations. Taken together, these results are consistent with state-dependent accessibility of single Cd\textsuperscript{2+} ions to coordination sites within a relatively narrow inner vestibule.

Potassium channels are tetrameric assemblies of subunits, each of which contains a conserved extracellular hydrophobic P-loop, flanked by at least two transmembrane domains. The P-loop and the following transmembrane domain (S6 or M2) determine pore properties (1–12). In the crystal structure of the core region of KcsA (a two-transmembrane domain bacterial K\textsuperscript{+} channel), the P-loop forms the outer ion selectivity filter, and \alpha-helical M2 lines the long inner vestibule between the selectivity filter and the cytoplasm (13). No direct structural information is available for mammalian K\textsuperscript{+} channels. However, pore structure has been inferred by use of the substituted cysteine accessibility method using silver (14), cadmium (15, 16), or organic sulfhydryl reagents (17, 18) as cysteine-reactive probes. The study of Liu et al. (18) indicates no obvious structure of S6 in voltage-gated K\textsuperscript{+} (Kv) channels, and recent systematic mutageneses of M2 in Kir2.1 (IRK1) (19–21) question the general applicability of the KcsA crystal structure to the structure of mammalian K\textsuperscript{+} channels.

K\textsubscript{ATP} channels are tetramers of Kir6.2 subunits, and hallmark nucleotide inhibition results from an interaction of ATP with cytoplasmic regions of Kir6.2 (22–25). Each Kir6.2 subunit is normally associated with a sulfonylurea receptor (SUR1 or SUR2) (22, 26–28), which provides additional nucleotide and drug sensitivities (29–32). The similarity of Kir6.2 to other inwardly rectifying K\textsuperscript{+} (Kir) channels, the ability to control gating by nucleotides, and a relatively large single channel conductance (~80 picosiemens) make this an attractive model system in which to study the pore structure and dynamics of Kir channels. We introduced cysteines throughout M2 of Kir6.2 and examined their accessibility to Cd\textsuperscript{2+} ions. Our results are consistent with M2 being \alpha-helical and structurally and dynamically similar to M2 of the bacterial KcsA channel (13, 33).

EXPERIMENTAL PROCEDURES

Expression of K\textsubscript{ATP} Channels in COS7 Cells—COS7 cells were transfected with pCMV6b-Kir6.2 (with mutations as described), pECF-sur1A, and pGreenLantern (Life Technologies, Inc.) as described previously (24).

Generation of a Non-reactive Background and Introduction of Cysteines—Point mutations were prepared by gene extension at the junctions of relevant residues by sequential polymerase chain reaction as described (24). The starting wild-type construct had a deletion of 36 amino acids (ΔC36) from the C-terminal end (23). This ΔC36 mutation has no effect on the function of channels coexpressed with SUR1 (as in all present experiments), but facilitates expression of channel activity without SUR1, which may be useful for future experiments. Unless otherwise indicated, the control Kir6.2 and mutant constructs also contained an N160D substitution. This generated strongly rectifying channels in the presence of spermine, permitting estimation of zero current for mutants that are very insensitive to ATP. To examine the effects of thiol reagents on substituted cysteines, it is necessary to generate a non-reactive background. Trapp et al. (34) recently reported that inhibition of wild-type Kir6.2/SUR1 channels by the thiol reagent p-chloromercuriphenyl sulfonate results from interaction with cysteine 42 since this reactivity was not present in a C42S mutant. Preliminary experiments showed that both Cys-42 and C42S mutant constructs expressed channels that were sensitive to Cd\textsuperscript{2+}. Cd\textsuperscript{2+} is considerably smaller than p-chloromercuriphenyl sulfonate and may access and react with residues more deeply in the channel than can reagents like p-chloromercuriphenyl sulfonate. This suggested that Cd\textsuperscript{2+} reactivity may result from an interaction with cysteine 166 in M2. C166S mutant channels were not inhibited by 100 μM Cd\textsuperscript{2+} (see Fig. 1) unless the open state stability was substantially reduced by prior treatment with poly-L-lysine (see “Results”). In the background construct for the systematic mutagenesis (Kir6.2(C166S/N160D/ΔC36), referred to as control Kir6.2), the mutation C166S was therefore necessary to examine the accessibility of substituted cysteines in M2. However, additional cysteine replacements were not necessary as long as the open state stability was high enough to prevent interaction with Cys-42. Cysteine 42 is preserved since, in several introduced cysteine mutations, the additional C42S mutant rendered channels nonfunctional or caused run-
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FIG. 1. Cd$^{2+}$ inhibition of Kir6.2/ SUR1 channels. A, predicted transmembrane topology of Kir6.2. Significant residues discussed under “Results” are indicated. B, effect of Cd$^{2+}$ on inside-out patch currents isolated from COS7 cells cotransfected with SUR1 and either control Kir6.2 (CTRL) or S166C mutant subunits. In this and subsequent figures, inward currents at −50 mV are shown as upward deflections; zero current is indicated by a dashed line. Cd$^{2+}$ was applied as indicated. C, current in Cd$^{2+}$, relative to control (relative current) = [Cd$^{2+}$] relationships for experiments like those in B. Symbols and bars show mean ± S.E. for $n = 8$ patches in each case (CTRL, control; ○, S166C). Curves are linear least-square fits of the Hill equation: relative current $= 1/(1 + ([\text{Cd}^{2+}]/K_{i}))^{n}$, where $K_{i}$ (Eq. 1) is [Cd$^{2+}$] causing half-maximal inhibition.

FIG. 2. Cysteine substitutions in M2 cause large changes in $K_{\text{ATP}}$ sensitivity and open state stability. A, representative inside-out cysteine mutant/SUR1 channel currents (see “Experimental Procedures”). ATP was applied as indicated. B, relationship between $K_{i}$ (ATP) and $P_{\text{open}}$ for each expressed cysteine mutant (○, mean, $n = 3–13$) and for control Kir6.2 (CTRL, ○). The curve is the relationship predicted by changes in the open state stability (i.e. changes in the $K_{i}$ equilibrium constant) in the kinetic scheme of Shyng et al. (12) (Scheme 1).

down within a few seconds of patch isolation (cf. Ref. 34). Neither the starting wild-type construct (Kir6.2/SUR1) nor control Kir6.2/SUR1 channels were sensitive to 100 μM external cadmium (data not shown).

Patch-Clamp Measurements—Patch-clamp experiments were made at room temperature in a chamber that allowed the solution bathing the exposed surface of the isolated patch to be changed in <1 s (24). The standard bath (intracellular) and pipette (extracellular) solution used in these experiments had the following composition: 140 mM KCl, 10 mM K-HEPES, and 1 mM K-EGTA (pH 7.3) with the additions described. Cadmium was added as chloride salt and was expected to be present in solution as approximately equal parts Cd$^{2+}$ and CdCl$^{2-}$ ions. In Cd$^{2+}$-containing solutions, K-EGTA was omitted, and 20 mM KCl was replaced by KF (as a replacement Ca$^{2+}$ chelator).

Data Analysis—Offset-line analysis was performed using Axotape and Microsoft Excel programs. Wherever possible, data are presented as mean ± S.E. Microsoft Solver was used to fit data by least-square algorithm. For estimation of open probability in the absence of ATP ($P_{\text{open}}$) from macroscopic currents, single channel current ($I_{\text{z}}$) was assumed to be constant at 3.75 pA, corresponding to a single channel conductance of 75 pS. $P_{\text{open}}$, was then calculated by fitting the following equation: $P_{\text{open}} = 1 - (a^{n}b^{i}c^{j})$. The estimations of channel composition (see Fig. 5) utilized the analysis described in detail in Ref. 27. Briefly, $G_{rel}$ curves of homomeric channels (Fig. 5, B and C) were fitted with single Boltzmann functions. $G_{rel}$ curves of heteromeric channels were fitted with the sum of five Boltzmann functions (Equation 1),

$$G_{rel} = \sum_{i=1}^{5} A_{i} \cdot (1 + \exp((F/RT) \cdot z_{i} \cdot (V - V_{i})))^{-1}$$

where $A_{i}$, $V_{i}$, and $z_{i}$ are the amplitude, voltage of half-maximal inhibition, and effective valency, respectively, of the $i$th component, with the following constraints. 1) $V_{1}$ and $z_{1}$ and $V_{2}$ and $z_{2}$ are given by the average values for these parameters for L164C/N160D and control Kir6.2 (Asn-160) channels, respectively, such that $V_{1} < V_{2} < V_{3} < V_{4} < V_{5}$ and $z_{1} > z_{2} > z_{3} > z_{4} > z_{5}$. 2) The fitted amplitude ($A_{i}$) corresponds to the probability ($P_{i}$) of formation of each channel type, with each subunit being incorporated with equal probability following the binomial distribution (Equation 2),

$$P_{i} = \left(\begin{array}{c} 4 \\ i \end{array}\right) \cdot P^{i} \cdot (1 - P)^{4-i}$$

in which there are four subunits in a functional channel, $x$ is the number (zero to four) of wild-type subunits in a particular channel, $p$ is the probability of inclusion of a control Kir6.2 subunit, and $1 - p$ is the probability of inclusion of an L164C/N160D subunit. Fits were determined by allowing $p$ to vary.
In Fig. 5E, the estimated percentage of Cd^{2+} block is given by Equation 3,
\[
\% \text{Cd}^{2+} \text{ block} = 10 + 90 \cdot \sum_{y=0}^{4} \left( \frac{y}{y} \right) \cdot p^y \cdot (1-p)^{4-y} \quad (\text{Eq. 3})
\]
in which there are four subunits in a functional channel, \( y \) is the number (zero to four) of L164C/N160D subunits in a particular channel, and \( p \) is the fraction of L164C/N160D subunits in the mixture. This model assumes that at least \( x \) cysteine-substituted subunits are required in a channel for full inhibition, above a background inhibition of 10%.

To model the pore structure, the M2 sequence of Kir6.2 was threaded on the KcsA structure (13) according to the alignment of Kir6.2 with ROMK1, incorporated into a general alignment of \( K_1 \) channels (see Fig. 1 of Ref. 13). After minimization of side chains (Discover, MSI, San Diego, CA), the images in Fig. 7 display only the solvent-accessible surfaces (C and E) of the M2 helices between residues 144 and 175 (Insight, MSI).

**RESULTS**

**Cysteine Substitutions in M2 Alter the Stability of the Open State**—To examine the effects of thiol reagents on substituted cysteines, it is minimally necessary to generate a non-reactive background. With the naturally occurring cysteine at position 166, Kir6.2 channels expressed from Kir6.2/SUR1 subunits were inhibited by 100 \( \mu \text{M} \) Cd^{2+} (Fig. 1B, S166C (wild type)), but when this residue was mutated to serine, Kir6.2(C166S)/SUR1 channels were almost completely insensitive to 100 \( \mu \text{M} \) Cd^{2+} (Fig. 1B, CTRL). In this control Kir6.2 background (see “Experimental Procedures”), cysteines were individually substituted for each residue from Asp-153 to His-175. K\(_{\text{ATP}}\) currents were expressed by 18 of the 23 mutants, but with striking differences in nucleotide sensitivity (\( K_{1/2}(\text{ATP}) \), [ATP] causing half-maximal inhibition of channel activity) and \( P_0(\text{zero}) \) (Fig. 2). At different positions, cysteine substitution caused up to 2 orders of magnitude increase (e.g. Q173C and I162C) or decrease (e.g. H175C and L164C) in ATP sensitivity compared with control Kir6.2 channels. There is a correlation between \( K_{1/2}(\text{ATP}) \) and \( P_0(\text{zero}) \) (Fig. 2B). Such a correlation is predicted by kinetic models of the K\(_{\text{ATP}}\) channel (12) in which ATP does not directly close the channel, but stabilizes a closed state. Hence, the effects of M2 cysteine substitution mutations on ATP sensitivity and \( P_0(\text{zero}) \) may be explained by assuming that each mutation alters the intrinsic stability of the open state versus an ATP-accessible closed state (cf. Ref. 12).

Although control Kir6.2/SUR1 is intrinsically insensitive to Cd^{2+} (Fig. 1B), sensitivity is induced after treatment with

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**Fig. 3. Cysteine substitutions in M2 show an \( \alpha \)-helical pattern of Cd\(^{2+}\) reactivity.** A. representative inside-out mutant/SUR1 channel currents. ATP or Cd\(^{2+}\) was applied as indicated. For mutations with \( K_{1/2(\text{ATP})} < 1 \text{ mM} \), PIP2 was applied to bring sensitivity to at least 1 mM (shown for S166C and D160C) before the final test Cd\(^{2+}\) application. For the D160C mutant, the gap during PIP2 application was 5 min, and 1 mM MTSEA was applied after recovery from test Cd\(^{2+}\). Time bars represent 1 min. B. current remaining in 100 \( \mu \text{M} \) Cd\(^{2+}\) (mean ± S.E., \( n = 3–22 \) in each case) for cysteine mutants (solid bars). 0 indicates nonfunctional mutants. C. \( \alpha \)-helical projection of the M2 segment. Cd\(^{2+}\) reactivity of cysteine mutants is indicated by color coding, with gray indicating nonfunctional mutants. The \( K_{1/2(\text{ATP})} \) was estimated by fits of the Hill equation as in Fig. 1. CTRL, control; WT, wild-type.
poly-L-lysine (data not shown; cf. Ref. 35), which screens negative charges on the membrane and hence destabilizes the open state (24, 35). This raises one possibility that mutations will reveal an otherwise hidden cation reactivity, not directly due to reaction with the introduced cysteine, but due to lowered open state stability. Such possibilities are always a concern in this type of systematic mutagenesis, but in this particular case, the channel open state can be stabilized by exposure to phosphatidylinositol 4,5-diphosphate (PIP2), which increases $P_{\alpha(\text{iso})}$ and reduces ATP sensitivity, shifting the channel behavior along the relationship shown in Fig. 2B to the right (12, 24, 25). To reduce the possibility of uncovering Cd$^{2+}$ reactivity at another residue, the intrinsic $K_{\text{Ca}}$/$P_{\alpha(\text{iso})}$ relationship (Fig. 2B) was used to identify mutants with a lower intrinsic open state stability than control Kir6.2. For any such mutants, PIP$_2$ was applied to increase $K_{\text{Ca}}$ open state stability than control Kir6.2. For any such mutants, PIP$_2$ was applied to increase $K_{\text{Ca}}$open state stability than control Kir6.2. For any such mutants, PIP$_2$ was applied to increase $K_{\text{Ca}}$ open state stability than control Kir6.2. This prior treatment then permitted comparison of Cd$^{2+}$ reactivity at the same open state stability.

Cd$^{2+}$ Reactivity Indicates an α-Helical Pattern in M2—Sensitivity to 100 μM Cd$^{2+}$ was systematically examined in the 18 expressed mutations (after pretreatment with PIP$_2$ in mutants with low open state stability, e.g. S166C) (Fig. 3, A and B). In addition to the potential caveat that any mutation might uncover a hidden reactivity at other residues, it is possible that additional, non-cysteine side chains may contribute to coordination of Cd$^{2+}$ ions (36). We cannot fully exclude such possibilities, so bearing them in mind, we tentatively conclude that residues that induce Cd$^{2+}$ sensitivity when substituted by a cysteine are likely to be interacting with Cd$^{2+}$ and to face the pore. Fig. 3C represents the mutated residues on an α-helix projection, with color-coded contours corresponding to the estimated $K_{\text{Ca}}$ for Cd$^{2+}$ inhibition. Although the inability to assay five non-expressed mutants limits interpretation, the distribution of Cd$^{2+}$ sensitivity is most consistent with an α-helical structure, with a broad surface of reactivity and a particularly sensitive “hot” region at residues 164, 168, and 172. The α-helix seems to continue at least to residue 173, longer than previously expected from hydrophathy analyses (22, 37, 38). Moreover, the orientation of the surface of reactivity is not parallel to the axis of the helix, but is tilted at $-40^\circ$ from the axis.

Nature of the Cd$^{2+}$ Inhibition Process at Position 164—The rate of inhibition by Cd$^{2+}$ was variable, but the recovery rate was relatively constant, between mutants (Fig. 4). In addition, even though raising the open state stability with PIP$_2$ did not alter the overall pattern of Cd$^{2+}$ reactivity (data not shown), the reactivity of all mutants between residues 153 and 166 was slowed after PIP$_2$ treatment (Fig. 4A). Variability of cysteine accessibility could reflect the need for multiple thiol groups to coordinate a Cd$^{2+}$ ion within the pore (16) and for the pore to “collapse” or “close down” onto the ion during coordination. If so, then stabilizing the open state with PIP$_2$ might slow down the coordination process. We performed detailed experiments to gain more insight into the nature of the Cd$^{2+}$ inhibition of the very sensitive L164C mutant (Fig. 5). Since each residue in the homotetrameric channel contains a cysteine at position 164, it is conceivable that a Cd$^{2+}$ ion might react with each one independently, each “hit” causing partial reduction of current (cf. Ref. 20). However, it is clear from Fig. 5A that the Cd$^{2+}$ hit occurred as a full-conductance event, and subconductances were not observed during Cd$^{2+}$ application or removal. Full-conductance transitions were also observed in patches containing one or just a few L157C, Q173C, and A174C channels (data not shown), consistent with a single Cd$^{2+}$ ion inhibiting the channel in each case.

We next examined how many thiol groups contribute to Cd$^{2+}$ coordination at Cys-164. Fig. 5 (B–D) shows control Kir6.2(Asn-160), mutant L164C/N160D, and mixed currents in macroscopic patches. L164C/N160D channels were inhibited by 100 μM Cd$^{2+}$ due to the L164C mutation (upper panel) and also rectified strongly in 20 μM spermine due to the N160D mutation (middle panel). Conversely, control Kir6.2(Leu-164/Asn-160) channels were insensitive to Cd$^{2+}$ (−10% inhibition) and, lacking the aspartate at position 160, were insensitive to spermine. The striking difference in spermine sensitivity with a neutral or negatively charged residue at position 160 can be used in mixed expression to determine the stoichiometry of the channels (27, 39). Such analyses clearly demonstrate that four subunits contribute to generation of the pore. Once the stoichiometry is known, spermine sensitivity can be used to determine the relative fraction of each mutant subunit in channels formed from a mixture of the two. Mixed expression of the two constructs (in an ~1:1 DNA ratio) generates channels with differing spermine sensitivity. During a voltage ramp in the presence of spermine, there are several phases to the rectification (Fig. 5D). These different phases are quantitatively predicted by assuming that 51% of the L164C/N160D subunits contribute to channel assembly (Fig. 5D). In this case, almost 95% of the channels contained at least one cysteine at residue 164, and almost 70% contained at least two. Nevertheless, the channels were still insensitive to Cd$^{2+}$ (Fig. 5D, upper panel). Fig. 5E shows results obtained from multiple such experiments, with the ratios of expressed subunits determined as described for Fig. 5D. The Cd$^{2+}$ sensitivity is consistent with the assumption that three or more cysteines are required for inhibition (Fig. 5E), suggesting that single Cd$^{2+}$ ions gain access to the open pore and are then coordinated by three or four cysteine residues (Fig. 5F).

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1 The abbreviations used are: PIP$_2$, phosphatidylinositol 4,5-diphosphate; MTSEA, (2-aminoethyl)methane thiosulfonate; MTS, methane thiosulfonate.
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**Fig. 5. Nature of Cd²⁺ inhibition at L164C.** A: representative record of an inside-out L164C/SUR1 patch, containing two channels. 10 mM ATP and 100 μM Cd²⁺ were applied as indicated. Both channels were eventually inhibited in Cd²⁺ by full-conductance transitions. Following Cd²⁺ removal, one channel opened, but the patch was lost before the second reopening. B–D: upper panels, representative records of the Cd²⁺ response of channels formed from control Kir6.2(Asn-160), L164C/N160D subunits, or mixtures. Middle panels, currents in response to 300-ms voltage ramps (+100 to −100 mV) versus membrane potential (Vm) for each patch. Currents are shown in the presence and absence of 20 μM spermine (spm). Lower panels, relative conductance (Grel/Gmax) versus the data shown above. Smooth lines in B and C are best fits of single Boltzmann functions to the data. In D (lower panel), the smooth line superimposed on the data is the sum of five Boltzmann functions corresponding to different combinations of Asn-160 and N160D in a tetramer, with fitted probability of Asn-160 incorporation (p) = 0.49 (see "Experimental Procedures"). E, percent inhibition by 100 μM Cd²⁺ versus the apparent fraction of L164C/N160D subunits in each channel. Data points are for individual experiments like those shown in D. Solid lines indicate the relationships expected for the simple assumption that the channel will be inhibited if only one (line 1), at least two or three (lines 2 and 3), or all four subunits (line 4) contain a cysteine residue (see Equation 3 under "Experimental Procedures"). F, schematic indicating the hypothesized interaction with cysteine from different subunits. Cd²⁺ enters the open channel, and then multiple cysteine side chains are required to coordinate the ion.

**ATP Prevents Cd²⁺ Inhibition**—Although structurally homologous to other Kir channels, Kir6.2 channels are uniquely sensitive to inhibition by micromolar ATP. Fig. 6A shows experiments that examined the interaction of ATP and Cd²⁺ inhibition in mutant N162C. The recovery from Cd²⁺ inhibition (Fig. 6A, left panel) was much slower (τRecovery > 20 s) (Fig. 4B) than the recovery from ATP inhibition (middle panel). When ATP was applied first and then Cd²⁺ was applied in the maintained presence of ATP, simultaneous removal of both agents resulted in a biphasic recovery with fast (i.e., τRecovery < 5 s) and slow (τRecovery > 20 s) components (Fig. 6A, right panel). The significant fast component indicates partial protection from Cd²⁺ inhibition by ATP. Similar results were obtained with other mutants (Fig. 6B). Based on the above experiments (Figs. 5 and 6), we suggest that Cd²⁺ coordination may result from the ion entering the open channel and then the pore essentially closing onto the Cd²⁺ ion. ATP stabilizes closed channels (12, 24), so subsequently applied Cd²⁺ ions cannot access the pore.

**DISCUSSION**

**M2 of Kir6.2 Is a Pore-lining α-Helix**—As with all substituted cysteine accessibility method approaches, we must acknowledge potential caveats to interpretation of the results. As discussed above, induction of Cd²⁺ sensitivity could conceivably result from uncovering reactivity with hidden cysteines or even non-cysteine residues. It is not possible to fully examine such potential complications, so bearing them in mind, we tentatively conclude that residues that induce sensitivity to Cd²⁺, when substituted by a cysteine, are likely to directly interact with Cd²⁺ and to face the pore. Previous studies of Kv and Kir channels using scanning mutageneses have variously indicated either helical structures of M2 (21, 40) or no obvious periodicity of M2 (19, 20) or the equivalent S6 region of Kv channels (18). KcsA is a distantly related K⁺ channel consisting of only two α-helical transmembrane domains, but with higher primary sequence homology to mammalian Kv channels. The pattern of Cd²⁺ sensitivity of Kir6.2 mutants (Fig. 3) is most consistent with an α-helical structure of M2, which prompts the question, how similar is the reactive face to the pore-lining face of the equivalent domain in the KcsA structure? Fig. 7A shows an alignment of Kir6.2 with KcsA in this region based on the multisequence alignment generated by Doyle et al. (15), although it should be noted that alternative alignments have been proposed (21). We threaded the Kir6.2 sequence onto the KcsA structure as described under "Experimental Procedures." Fig. 7C (right) is a space-filling representation of one helical M2 domain (d) turned to expose the pore-lining face. The amino acids have been color-coded to indicate Cd²⁺ reactivity of cysteine-substituted mutants. Other views show the remaining three helices (a–c) along the pore axis (Fig. 7, B and C), and all four helices viewed into the plane of the membrane from the outside (Fig. 7, D and E). These space-filling representations show that all residues in Kir6.2 that correspond to pore-lining residues in the KcsA inner vestibule are Cd²⁺-sensitive. These include residues 157, 164, 165, 168,
169, 172, and 173, which also correspond to those KcsA residues that show strong spectral broadening (indicating close proximity to one another) when probed by EPR (33). In contrast, Cd$^{2+}$-insensitive residues are predicted to be at the very bottom of the helix (residues 174 and 175) or facing away from the central axis of the tetramer (residues 158, 163, 167, and 171). Comparison with KcsA also gives probable insight to the reason for the hot spot of Cd$^{2+}$ reactivity at residues 164, 168, and 172; these residues are predicted to lie at the very narrowest part of the inner vestibule (Fig. 7, C and E) (13). The oblique angle ($\sim 40^\circ$) of the helices relative to the pore axis in the KcsA structure is further consistent with the angled face of reactivity of Kir6.2 (Fig. 3C). The surface of reactivity does seem to be particularly broad, especially between residues 164 and 168. Even after treatment with PIP$_2$ to increase open state stability, we observed reactivity for the wild-type mutant S166C. This residue, being equidistant between the very reactive residues 164 and 168, is predicted to face away from the central axis.
One possibility is that this mutation induces a hidden reactivity. Alternatively, considerable helix motion may occur (see below), bringing this residue into position to react with Cd\(^{2+}\).

Lu et al. (20) recently used a substituted cysteine accessibility method to analyze the homologous region of Kir2.1 and observed a slightly more restricted pattern of MTSEA or 2-(tri-methylammoniumethyl)methane thiolsulfonate (MTSET) sensitivity than that reported here for Cd\(^{2+}\) sensitivity. Minor et al. (21) utilized a yeast rescue screen to examine amino acid tolerance in M2. Both studies reached the general conclusion that the KcsA structure may not be a suitable model for Kir channel structure. The underlying assumption in both the present study and that of Lu et al. (20) is that thiol reagent sensitivity reflects direct interaction with the introduced cysteine, from the aqueous medium. There are reports of mutations uncovering otherwise silent reactivities (34), of Cd\(^{2+}\) sensitivity reflects direct interaction with the introduced cysteine. We speculate that the KcsA crystal contains channels as adopting only the static crystal structure may be inappropriate. We find and attempted to control for the first possibility. The second possibility complicates, but does not exclude, the assumption of an interaction with the introduced cysteine. Acknowledging these potential caveats, we must nevertheless seek a unifying explanation for the divergent overall results of these studies. At position 164 in Kir6.2, where a single Cd\(^{2+}\) ion seems to be coordinated by three or four cysteines (Fig. 5), the pore should not be wider than the coordinated complex, i.e. a few angstroms. At the same residue (Cys-176) in Kir2.1, the pore can apparently accommodate quite large MTS reagents, implying that it will be considerably wider (20). However, there is movement of M2 helices during gating of KcsA (33), possibly involving both rotation and separation, so to consider the pore as adopting only the static crystal structure may be inappropriate. We speculate that the KcsA crystal contains channels trapped in the closed state (helices close together) (33) and that this may be similar to the structure of Kir6.2 with a trapped Cd\(^{2+}\) ion, at least at position 164. Conversely, MTSEA modification is likely to introduce a tethered “blocker” of an open channel. Similarly, rescue of K\(^{+}\) transport-deficient yeast would require substitutions that maintain open channels, so the picture to emerge from the studies of Kir2.1 (20, 21) may be of an open channel.

One interesting result is that the Cd\(^{2+}\) sensitivity of the Cys-160 mutant is rather low (Fig. 3). As with the corresponding result in Kir2.1 (E172C; see Ref. 20), this residue is sensitive to modification by MTSEA (Fig. 3A). An acidic residue at this or the equivalent position is the major determinant of polyamine-induced strong inward rectification in K\(_{ATP}\) (12) and other Kir channels (6, 8–10), consistent with the residue being an important determinant of pore properties. However, neutralization of this residue does not necessarily affect single channel conductance (12), so strong electrostatic interactions with permeant ions seem unlikely. A plausible explanation may be that 160 residues actually are quite distant from one another, just in the limit of the pore-lining area, but with their carboxylic groups accessible to solvent molecules (Fig. 7). In the KcsA crystal, the corresponding residue is phenylalanine 103 and lies just at the border between the pore-facing and lipid-facing zones (13). Even though a blocking polyamine in the pore might sense the aspartate negative charges at this distance, the side chain separation may be too great to affect K\(^{+}\) permeation or for effective coordination of a Cd\(^{2+}\) ion when cysteine is substituted. Lu et al. (20) have provided evidence that modification of a single Cys-172 residue by MTSET causes only a partial block (25% for each cysteine) of Kir2.1 channels. The conclusion that this residue lies in the groove at the edge of the pore-lining face might be consistent with these results since each MTS moiety might lie in and fill the groove, reducing the pore cross-sectional area by 25%.

**Cd\(^{2+}\)** Inhibition and Channel Gating—Our analyses indicate single transitions from the open state to the Cd\(^{2+}\)-inhibited state, without induction of conductances (e.g. Fig. 5A), and that at least three cysteines at position 164 are necessary for inhibition (Fig. 5E). These observations suggest that a single Cd\(^{2+}\) ion is coordinated in the central axis of the pore by multiple cysteines (Fig. 5F). Coordination of a Cd\(^{2+}\) ion by multiple cysteines would require that each thiol side chain be sufficiently close to the central axis of the pore. The data of Lu et al. (20) indicate that residues in M2 are well separated when probed by MTS reagents, and it is possible that helix motion is required to bring thiol groups into close enough proximity for Cd\(^{2+}\) coordination. EPR spectroscopy of M2 of KcsA (33) suggests that significant helix rearrangement can occur; KcsA channel closure involves M2 of each subunit coming closer together. Specifically, Perozo et al. (33) found that spectral broadening associated with spin labels at residues 108, 112, 115, and 116 (corresponding to Cd\(^{2+}\)-reactive residues 165, 169, 172, and 173 in Kir6.2) increases when channels are closed. Hence, we may envision a Cd\(^{2+}\) ion entering the open channel and then helices collapsing or closing onto the ion in the process of coordination (Fig. 5F). This might then explain why PIP\(_2\), which stabilizes the open (i.e. helix-separated) conformation of the channel, consistently slows down Cd\(^{2+}\) reaction rates (Fig. 4A). Similarly, the marked protection from Cd\(^{2+}\) in the ATP-inhibited state (Fig. 6) may result from ATP locking the channel in a closed state (12, 24) that blocks access of Cd\(^{2+}\) to the pore.

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Cysteine Scanning of the Pore of $K_{\text{ATP}}$ Channels


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