Molecular Basis of Ion Selectivity, Block, and Rectification of the Inward Rectifier Kir3.1/Kir3.4 K⁺ Channel

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The glycine-tyrosine-glycine (GYG) sequence in the p-loop of K⁺ channel subunits lines a narrow pore through which K⁺ ions pass in single file intercalated by water molecules. Mutation of the motif can give rise to non-selective channels, but it is clear that other structural features are also required for selectivity because, for instance, a recently identified class of cyclic nucleotide-gated pacemaker channels has the GYG motif but are poorly K⁺ selective. We show that mutation of charged glutamate and arginine residues behind the selectivity filter in the Kir3.1/Kir3.4 K⁺ channel reduces or abolishes K⁺ selectivity, comparable with previously reported effects in the Kir2.1 K⁺ channel. It has been suggested that a salt bridge exists between the glutamate-arginine residue pair. Molecular modeling indicates that the salt bridge does exist, and that it acts as a “bowstring” to maintain the rigid bow-like structure of the selectivity filter and restrict selectivity to K⁺. The modeling shows that relaxation of the bowstring by mutation of the residue pair leads to enhanced flexibility of the p-loop, allowing permeation of other cations, including polyamines. In experiments, mutation of the residue pair can also abolish polyamine-induced inward rectification. The latter effect occurs because polyamines now permeate rather than block the channel, to the remarkable extent that large polyamine currents can be measured.

How ion channels, particularly K⁺ channels, achieve high selectivity for one ion over a closely related one has been a mystery apparently solved with the crystallization of archetypal K⁺ channel pores (1, 2). The sequence glycine-tyrosine-glycine (GYG) within the extracellular loop of each of four subunits presents backbone carbonyls that line the narrowest part of the pore in such an orientation as to be perfectly positioned to coordinate a column of dehydrated K⁺ ions as they move through the channel. Larger ions are effectively excluded from entering the column; smaller ions are not effectively coordinated and, hence, dehydration and entry are energetically unfavorable (3, 4). Consistent with the notion that the GYG motif is necessary for K⁺ selectivity, mutations or deletions of the motif can result in loss of K⁺ selectivity (5), and many structural relatives of K⁺ channels are non-selective channels that lack the GYG motif in the p-loop (6). However, it is also clear that some additional structural feature is necessary for the GYG motif to confer K⁺ selectivity (7). For example, a recently identified class of cyclic nucleotide-gated (HCN) channels contains a GYG motif in the p-loop, but shows relatively poor selectivity for K⁺ over Na⁺ (8). In addition, several voltage-gated K⁺ channels, all of which have the GYG motif, exhibit differential selectivities for K⁺ over Na⁺ (7), and significant Na⁺ currents can be measured through some channels in the absence of K⁺ (7).

The defining feature of Kir channels is inward rectification that results from the voltage-dependent block by intracellular polyamines and Mg²⁺ (9). In Kir2.1, a number of residues in the second transmembrane helix (M2) and the proximal C terminus have been shown to be important in inward rectification: Asp-172, Glu-224, and Glu-299. Mutation of these residues leads to a drastic reduction in inward rectification (10–13) and, in the Kir2.1 channel, it is likely that they contribute to the stable binding of polyamines and Mg²⁺. As we show here, though, equivalent mutations of (Asp-172 and Glu-224 at least) in the Kir3.1/Kir3.4 channel have little effect on inward rectification, indicating that additional structural features are critical.

There is a glutamate residue that is intracellular to the GYG motif in the p-loop of Kir channels and is absent in voltage-dependent K⁺ channels. Yang et al. (14) showed that mutation of this residue (Glu-148) in Kir2.1 results in a non-functional channel. Because non-selective channel activity could be rescued by additional mutation of an arginine residue extracellular to the GYG motif, i.e. the double mutation E138R/R148E, Yang et al. (14) proposed that a salt bridge exists between the glutamate and arginine residues. We now show that mutation of the equivalent glutamate and arginine residues in Kir3.4 causes dramatic loss of ion selectivity and inward rectification in the Kir3.1/Kir3.4 channel. Molecular modeling indicates that these residues do indeed form a salt bridge and, furthermore, the modeling shows that the salt bridge acts as a “bowstring” to maintain the rigid structure of the p-loop and thereby restrict ion selectivity. Relaxation of the bowstring by disruption of the salt bridge leads to enhanced flexibility of the p-loop and increased permeation of other cations, including polyamines, explaining the loss of selectivity (and inward rectification) observed experimentally. We suggest that this principle may be common to all K⁺-selective channels and that in other K⁺ channels, a similar torsion of the p-loop may involve strongly hydrogen-bonded pairs of residues (15).

EXPERIMENTAL PROCEDURES

Channel Expression and Electrophysiology—Site-directed mutagenesis on Kir3.1 and Kir3.4 and preparation of cRNA was carried out as described previously (16). Double mutations in a single subunit were

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The abbreviations used are: HCN, cyclic nucleotide-gated; hD₂, human dopamine D₂; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDG, N-methyl-D-glucamine; Cx38, connexin38; ANOVA, analysis of variance.
Ion Selectivity and Rectification in Kir3.1/Kir3.4

Asp-173 of Kir3.1 and Glu-231 of Kir3.4 Do Not Play a Major Role in Inward Rectification—Fig. 1 illustrates currents around the reversal potential of the wild-type Kir3.1/Kir3.4 channel and two mutant channels. Endogenous currents from un.injected oocytes over the same potential range are shown for comparison. Because of strong polyamine-dependent inward rectification (9), only small outward currents were observed through the wild-type channel (Fig. 1). Kir2.1, mutation of Asp-172 and Glu-224 leads to a drastic reduction in inward rectification (10–13). An equivalent aspartate residue is only present in Kir3.1 (Asp-173), whereas an equivalent glutamate residue is only present in Kir3.4 (Glu-231). Fig. 1 shows the Kir3.1(D173Q)/Kir3.4 mutant channel still exhibiting strong inward rectification. As a summary of inward rectification, Fig. 2 shows the ratio of conductance at +40 mV to that at −100 mV in various mutant channels. This potential range was chosen because within it, endogenous currents in the oocyte are small (Fig. 1), whereas outside it, endogenous depolarization-activated delayed rectifier current or endogenous hyperpolarization-activated Cl− current can be significant in some oocytes. Fig. 2 shows that, like the conductance ratio for the wild-type channel, the conductance ratio for Kir3.1(D173Q)/Kir3.4, Kir3.1/Kir3.4(E231Q) and Kir3.1(D173Q)/Kir3.4(E231Q) mutant channels was low (in Fig. 2, compare i, iii, and iv with i), i.e. inward rectification was still strong in the case of these mutant channels. The role of these two residues in inward rectification is more important in the Kir2.1 channel. For comparison, Fig. 2 (xii and xiv) shows the equivalent conductance ratio for the Kir2.1 channel from Kubo and Murata (10). Whereas the ratio is low in the wild-type channel, it is drastically increased (i.e. inward rectification is drastically reduced) in the Kir2.1(D172N,E224Q) double mutant channel (Fig. 2).
mutant channel. It is concluded that, in the Kir3.1/Kir3.4 channel at least, additional structural features are required for inward rectification.

**Charged Residues Near the Selectivity Filter Control Inward Rectification**—Fig. 3 shows the sequence of Kir3.1 and Kir3.4 p-loops aligned with the corresponding region of KcsA. Proposed interactions between charged residues (see modeling below) are indicated by the connecting lines in Fig. 3. As suggested for Kir2.1 (14), a salt bridge is proposed between Glu-139/Arg-149 and Glu-145/Arg-155 in Kir3.1 and Kir3.4, respectively. Mutation of Glu-145 in Kir3.4 resulted in a reduction of inward rectification (Fig. 1), and the ratio of conductances at +40 and −100 mV was significantly increased for the mutant channel (Fig. 2a).

Inward rectification was also significantly reduced, though to a smaller extent, in Kir3.1/Kir3.4(R155E) and Kir3.1/Kir3.4(E145R,R155E) mutant channels (Fig. 2, x and xii).

Neutralization of the proposed salt bridge glutamate residue in Kir3.1 (E139Q) did not affect inward rectification (Fig. 2e), although mutation of the proposed salt bridge arginine residue in Kir3.1 (R149E) resulted in a non-functional channel. Why the mutation Kir3.1(R149E) but not Kir3.4(R155E) should result in a non-functional channel is unclear. Prediction is not trivial, because these residues participate in a complex salt network (Fig. 3), and it is unlikely that any two residues or charge pair reversals will be exactly equivalent. However, the Kir3.1(R149E)/Kir3.4(E145R) double mutant channel was active (why the effect of the mutation Kir3.1(R149E) should be rescued by the mutation Kir3.4(E145R) is also unclear). In the case of the Kir3.1(R149E)/Kir3.4(E145R) double mutant channel, the proposed salt bridges of both subunits will be disrupted, and inward rectification was strikingly reduced (Fig. 2a).

Mutation of other neighboring glutamate residues (E141 and Glu-145/Arg-155 in Kir3.1 and Kir3.4, respectively. Mutations of other neighboring glutamate residues (E141 and E147 in Kir3.1; E145R and E147 in Kir3.4; see Fig. 3) had no effect on inward rectification (Fig. 2, viii and ix).

**Glu-145/Arg-155 Salt Bridge in Kir3.4 Controls Ion Selectivity**—We have shown previously that the mutation E145Q in Kir3.4 results in the loss of block by extracellular Ba$^{2+}$ (16). This mutation also causes a loss of high affinity block by extracellular Cs$^+$; whereas Cs$^+$ blocked the wild-type channel, it failed to block the Kir3.1/Kir3.4(E145Q) mutant channel at concentrations up to 100 mM (Fig. 4, A–C). Interestingly, the equivalent mutation (E139Q) in Kir3.1 once again had a dissimilar effect: it increased Cs$^+$ sensitivity by a small amount (Fig. 4D). Mutation of the neighboring glutamate residues (Glu-141 in Kir3.1 and Glu-147 in Kir3.4; see Fig. 3) again had a minimal effect on Cs$^+$ block (Fig. 4D).

The loss of sensitivity to both extracellular (Cs$^+$ and Ba$^{2+}$) and intracellular (polyamine-induced inward rectification) pore blockers with the mutation of Glu-145 in Kir3.4 could reflect two separate effects on affinity at extracellular and intracellular blocking sites. Alternatively, it could reflect an alteration of the permeability of the selectivity filter itself, so that rather than blocking the pore, these ions now permeate. Fig. 5 shows typical currents, and Fig. 6 shows mean currents (at −130 mV) through wild-type and mutant channels when the bathing solution contained 90 mM K$^+$ or when K$^+$ was substituted by 90 mM Rb$^+$, Na$^+$, Cs$^+$, Li$^+$, or Ba$^{2+}$. The wild-type channel conducted K$^+$ and smaller Rb$^+$ currents, but there was essentially no current carried by other monovalent ions or Ba$^{2+}$ (Figs. 5 and 6). Like inward rectification and Cs$^+$ block, ion selectivity was unaffected in Kir3.1(E141Q)/Kir3.4 or Kir3.1/Kir3.4(E147Q) mutant channels (Figs. 5 and 6). However, increased Rb$^+$ current was observed in the Kir3.1(E139Q)/Kir3.4 mutant channel, and an even more dramatic reduction of ion selectivity was observed in the Kir3.1(Kir3.4(E145Q) mutant channel: all ions tested with the exception of Ba$^{2+}$ produced substantial currents (Figs. 5 and 6). The Kir3.1(Kir3.4(R155E) mutant channel was also non-selective, and in this case all ions tested, even Ba$^{2+}$, produced sub-
stastial currents (Figs. 5 and 6). In general, a dramatic reduction of ion selectivity was observed in all channels in which Glu-145 or Arg-155 charges in Kir3.4 were neutralized or reversed: both Kir3.1/R149E/Kir3.4(E145R) and Kir3.1/Kir3.4(E145R, R155E) double mutant channels exhibited a dramatic lack of discrimination between K⁺ and other monovalent or divalent Ba²⁺ ions (Figs. 5 and 6), demonstrating that the salt bridge in Kir3.4 controls ion selectivity.

Even Polymyines Can Permeate Mutant Channels—Blockade of Kir channels by extracellular polyamines has not been reported previously, but as shown in Fig. 7, both divalent putrescine (Fig. 7A) and tetravalent spermine (Fig. 7B) actually cause a weak voltage-dependent block of the wild-type channel from outside the membrane, with δz of 0.32 and 0.44 for putrescine and spermine, respectively (Fig. 7C). As with Cs⁺ block, putrescine and spermine blocked Kir3.1/Kir3.4(E145Q) and Kir3.1/Kir3.4(R155E) mutant channels much less effectively (Fig. 7, A and B). Putrescine block of the Kir3.1/Kir3.4(E145Q) mutant channel was voltage-independent (Fig. 7C), whereas spermine block of the Kir3.1/Kir3.4(E145Q) mutant channel and putrescine and spermine block of the Kir3.1/Kir3.4(R155E) mutant channel showed a curious biphasic response on increasing the polyamine concentration: the degree of block increased and then decreased as the concentration was raised (Fig. 7, A and B). This result may be explained by a balance of block and permeation of the selectivity filter by the polyamine. Fig. 7D shows current at -130 mV in the presence of 30 mM putrescine or spermine. Whereas the polyamines blocked the wild-type channel, they blocked the Kir3.1/Kir3.4(E145Q) mutant channel less effectively and failed to block the Kir3.1/Kir3.4(R155E) mutant channel.

The above results suggest significant polyamine permeation of the mutant channels. To confirm this possibility, we replaced the 90 mM K⁺ of the mutant channels. To confirm this possibility, we replaced the 90 mM K⁺ of the mutant channels with either K⁺ or polyamine (putrescine in Fig. 8A and spermine in Fig. 8B) as the charge carrier. Normalized current-voltage relationships are also shown in Fig. 8, A and B, and the mean amplitude of current at -130 mV is shown in Fig. 8C for the different channels with K⁺, polyamine, or NMDG as the charge carrier. In uninjected or water-injected oocytes, only very small endogenous currents (<0.8 μA at -130 mV) were observed with the different charge carriers (Fig. 8C). Remarkably, there was still substantial inward current through the wild-type channel (significantly larger than endogenous current under the same conditions; p < 0.05) with putrescine or spermine, but not NMDG, as the charge carrier (Fig. 8, A–C). Even more dramatically, putrescine and spermine carried very large currents through Kir3.1/Kir3.4(E145Q), Kir3.1/Kir3.4 (R155E), and Kir3.1/R149E/Kir3.4(E145R) mutant channels (Fig. 8, A–C). For example, in the case of the Kir3.1(R149E)/Kir3.4(E145R) mutant channel, at -100 mV, spermine current was on average 18× greater than K⁺ current. These results dramatically demonstrate that tetravalent polyamines not only permeate the selectivity filter of the wild-type channel, but in mutant channels, may do so at high rates.

Nature of the Spermine Current—We have previously shown that electrodifusitive spermidine efflux from Xenopus oocytes occurs through connexin38 (Cx38) hemi-channels (26); to check that the spermine current recorded in the present study was not through these channels, oocytes were coinjected with anti-sense oligonucleotide to Cx38 (17). This abolished the non-selective Cx38 inward current on perfusion with 0 Ca²⁺ (Fig. 9A) (26). Fig. 9B shows K⁺ and spermine currents, and Fig. 9C shows current-voltage relationships with K⁺ or spermine as the charge carrier from oocytes heterologously expressing the Kir3.1/Kir3.4(E145Q) mutant channel and oocytes not heterologously expressing the mutant channel (all oocytes injected with the Cx38 antisense oligonucleotide). The currents with spermine in Fig. 9, B and C are similar to those in Fig. 8B, demonstrating that Cx38 hemi-channels are not responsible for the spermine current.
Fig. 5. Ion selectivity of wild-type and mutant channels. Typical currents from the wild-type channel and various mutant channels when the bathing medium contained 90 K⁺, Rb⁺, Na⁺, Cs⁺, Li⁺, or Ba²⁺ are shown. Pulses are from −130 to +40 mV. Arrows, zero current.
In most oocytes expressing Kir3.1/Kir3.4(E145Q) and other non-selective mutant channels, there was a depolarization-activated (and Ca\(^{2+}\)-activated) transient Cl\(^{-}\) current following hyperpolarizing pulses (e.g. see the Kir3.1/Kir3.4(E145Q) current traces in Fig. 4). It is possible that the transient Cl\(^{-}\) current was prominent when a non-selective mutant channel was expressed, because the mutant channels allowed an influx of Ca\(^{2+}\). Although this current was prominent after pulses, it was unlikely to have contaminated current recordings during voltage pulses, because it is inactivated at the holding potential of 0 mV and is only activated on depolarization (to the holding potential) after relief of inactivation during a hyperpolarizing pulse (27). In addition, in one batch of oocytes, there was no transient Cl\(^{-}\) current, and the currents recorded during voltage pulses with the Kir3.1/Kir3.4(E145Q) mutant channel were not different (see the Kir3.1/Kir3.4(E145Q) current traces in Fig. 1). The recordings in Fig. 9, B and C were obtained in 0 Ca\(^{2+}\) (this is only possible in the presence of the Cx38 antisense oligonucleotide, because 0 Ca\(^{2+}\) opens the Cx38 hemi-channels). Under these conditions, all Ca\(^{2+}\)-activated Cl\(^{-}\) currents are abolished (as can be seen by the lack of transient Cl\(^{-}\) current after the pulses in Fig. 9B). Because the currents in Fig. 9, B and C are similar to those under our normal recording conditions (Fig. 8B), this is further evidence that Ca\(^{2+}\)-activated Cl\(^{-}\) current did not contaminate current under our normal recording conditions.

**Outward Permeation of Polyamine Through the Kir3.1/Kir3.4 Channel**—The data described so far demonstrate permeation of polyamines through the Kir3.1/Kir3.4 channel from the extracellular solution. Of course, in the native environment, it is primarily intracellular polyamines that interact with the channel. To test whether intracellular polyamines can also permeate the Kir3.1/Kir3.4 channel, the efflux of \([^{3}H]spermidine\) (radio-labeled trivalent polyamine) from oocytes expressing the wild-type Kir3.1/Kir3.4 channel (Fig. 10) was measured. Oocytes expressing the voltage-gated K\(^{+}\) channel, Kv1.4, and un.injected oocytes were used as controls. Efflux of \([^{3}H]spermidine\) was first measured in 3 mM K\(^{+}\) solution (90 mM K\(^{+}\) solution with equimolar substitution of K\(^{+}\) by Na\(^{+}\)). In 3 mM K\(^{+}\), the conductance of the Kir3.1/Kir3.4 channel is very low, and little or no efflux of polyamine through the Kir3.1/Kir3.4 channel is expected. Indeed, this was the case. In 3 mM K\(^{+}\), the efflux of \([^{3}H]spermidine\) from oocytes expressing the Kir3.1/Kir3.4 channel, oocytes expressing the Kv1.4 channel and uninjecte oocytes was small and not different (Fig. 10; n = 7–13; one way ANOVA, not significant). In 90 mM K\(^{+}\), the conductance of the Kir3.1/Kir3.4 channel is high, and efflux of polyamine through the Kir3.1/Kir3.4 channel is expected. In oocytes expressing Kv1.4 and uninjecte oocytes, efflux of \([^{3}H]spermidine\) in 90 mM K\(^{+}\) was not different from that in 3 mM K\(^{+}\) (Fig. 10). In contrast, in oocytes expressing the Kir3.1/Kir3.4 channel, efflux was significantly increased in 90 mM K\(^{+}\) compared with that in 3 mM K\(^{+}\) (Fig. 10; n = 10; ANOVA, p < 0.001). Furthermore, this efflux was partially and significantly blocked by 10 mM Ba\(^{2+}\) (Fig. 10; Ba\(^{2+}\) block was not complete, but at −0 mV and in high K\(^{+}\), Ba\(^{2+}\) block is weak). These data demonstrate that **intracellular** polyamines permeate the Kir3.1/Kir3.4 channel in the outward direction.

**Structural Consequences of Disrupting the Salt Bridge**—The present data demonstrate dramatically altered selectivity as a result of mutations to charged residues that lie near to the selectivity filter without changing the GYG motif in an otherwise highly selective K\(^{+}\) channel. To gain further insight into the likely role of these charged residues in channel function, we examined comparative models of the Kir3.1/Kir3.4 channel. A sequence alignment of Kir3.1/Kir3.4 M1-p-M2 domains with KcsA fragment 23–119 was generated (see “Experimental Procedures”). This alignment is identical to the one proposed by Doyle et al. (1) for Kir1.1 with KcsA, and the hydropathy profile of each sequence was superimposable over the entire length. The Kir3.1 and Kir3.4 sequences were threaded over the KcsA structure according to the selected alignment and optimized (see “Experimental Procedures”) to generate a heterotrameric channel from two Kir3.1/Kir3.4 dimers (Fig. 11). Whereas G53 and T82 are two facing residues in KcsA, this homology modeling positions the side chains of the analogous Cys-123/Cys-129 and Cys-155/Cys-161 residues of Kir3.1/Kir3.4 to form a disulfide bridge, in accordance with the demonstrated disulfide bridge between the two homologous cysteine residues in the Kir2.1 channel (28).

Fig. 11A shows different views of the modeled channel that give immediate insight into the likely role of Glu-139/Glu-145 and Arg-149/Arg-155 charges. Tight intra-subunit salt bridges are predicted between each Glu/Arg pair. Each Glu/Arg pair effectively spans the gap behind the GYG loop, forming a bowstring in each subunit (Fig. 11A). Close inspection (Fig. 11B) reveals additional weak electrostatic inter-subunit interactions between Arg-149 (in Kir3.1) and Glu-159 (in Kir3.4), and intra-subunit interactions between Arg-155 and Glu-131 (in Kir3.4), but with no involvement of the Glu-141 (Kir3.1) or Glu-147 (Kir3.4) charges (Fig. 3) in these salt bridges, consistent with the lack of effect of mutation of these residues on channel properties (see above).

A salt bridge between the glutamate and arginine residues was first proposed for Kir2.1 based on the observation that mutation of either residue results in a loss of channel function, whereas swapping the two residues does not (14). This study is the first to use modeling to address the issue of the salt bridge, and the modeling confirms that the salt bridge does exist.

**The Bowstring Controls p-Loop Flexibility and Hence Determines Ion Selectivity**—What is the structural role of the bowstring generated by the salt bridge, and what are the functional consequences of breaking it? The intra-subunit salt bridges (Glu-139/Glu-149 in Kir3.1 and Glu-145/Arg-155 in Kir3.4) will reduce the degree of freedom of backbone torsional angles in the 10-residue closed loop that is formed within each subunit. The GYG motif is thus sterically constrained at the narrowest part of the pore, providing rigidity to the selectivity filter structure. To examine the likely rigidity of this structure in Kir channels, we made 10 independent dynamic simulations of the
modeled channel using randomly seeded starting atomic motions. Fig. 12A shows the variation of backbone inter-carbonyl distances during a representative trajectory of isothermal dynamics; differences from one simulation to another were in the noise range. From $t = 0$–100 ps, the system was energized under p-loop backbone constraints; from $t = 100$–110 ps the

Fig. 7. Extracellular putrescine and spermine block of wild-type and mutant channels. A and B, typical currents during pulses from $-130$ to $+40$ mV for the wild-type and mutant channels are shown under control conditions and in the presence of 30 mM putrescine (A) or spermine (B). Current-voltage relationships (corrected for junction potentials) in the absence and presence of various concentrations of putrescine or spermine (concentrations in mM given) are also shown (means ± S.E.; $n = 5$–6). C, plot of the dissociation constant, $K_D$, for putrescine or spermine block against the membrane potential for the wild-type channel and the Kir3.1/Kir3.4(E145Q) mutant channel (means ± S.E.; $n = 5$–6). The data for the wild-type channel are fitted with $K_D = K_D^{0mV} \exp(\delta z E/FRT)$, where $K_D^{0mV}$ is the dissociation constant at 0 mV, $\delta z$ is the equivalent valence, $E$ is the membrane potential and $F$, $R$, and $T$ have their usual meanings. The data for the mutant channel are fitted with a line to guide the eye. D, current at $-130$ mV for the wild-type channel and two mutant channels in the absence of polyamine and in the presence of 30 mM putrescine or spermine (means ± S.E.; $n = 5$–6). Currents are normalized to the current in the absence of polyamine.
Fig. 8. Putrescine and spermine permeation of wild-type and mutant channels. A and B, typical currents during pulses from −130 or −100 (Kir3.1[R149E]/Kir3.4[E145R] with spermine) to +40 mV are shown in the presence of 90 mM K⁺ (top) or 90 mM putrescine or spermine (bottom). Current-voltage relationships (corrected for junction potentials) in 90 mM K⁺, 90 mM putrescine or spermine, and in some cases 90 mM NMDG are also shown (means ± S.E.; n = 5–14). C, currents at −130 mV (corrected for junction potentials) in 90 mM K⁺, putrescine, spermine, and NMDG for the wild-type channel, various mutant channels, and also uninjected or water-injected oocytes (means ± S.E.; n = 5–23).
p-loop constraints were removed, but α-carbons of M1 and M2 were constrained; and from t = 110–200 ps, all constraints were removed. The variation of the distances along the same trajectory were very small, indicating little lateral and longitudinal motions (<1 Å). Similar local motions described in simulations of the KcsA channel (25) might be responsible for the energy flattening necessary to facilitate sliding of cations between the K⁺-binding positions (s1–s4) that line the selectivity filter, as they are pushed by entering cations from s0 (outside) or from the inner cavity. We explored the variation between wild-type and mutant channel three-dimensional models (Fig. 12A). Although there was little change in diameter in the 110- to 200-ps time-window at Thr-143/Thr-149 and Tyr-146/Tyr-152 residues, at other critical residues (Ile-144/Ile-150, Gly-145/Gly-151, Gly-147/Gly-153, and Tyr-149/Tyr-154), clear widening was possible in the mutant channels.

To investigate the consequences of such flexibility in the pore structure for ion binding within the pore, we sequentially modeled the channel with an ion placed every 0.5 Å along the channel axis and minimized, keeping the cation immobile. Then, we solved the Poisson-Boltzmann equation to determine the electrostatic potential contribution to the energetics of cation association at every position along the permeation pathway of wild-type and mutant Kir3.1(R149E)/Kir3.4(E145R) channels (Fig. 12B). All monovalent and divalent ions, as well as the polyaniones putrescine and spermine, are predicted to reach or enter the selectivity filter of wild-type channels from inside or outside (Fig. 12B). The likely position of binding sites for non-permeant alkali metal ions match those predicted from crystal structures of KcsA in the presence of Na⁺ (29) or Ba²⁺ (30) (Fig. 12B; data for Ba²⁺ not shown). This calculation indicates relatively small wells and barriers (<2 kcal/mol) for single monovalent ions (Fig. 12B) and does not give any clear insight into the enhanced monovalent permeability in the mutant channels (but see “Discussion”). However, we did the same with spermine and putrescine and obtained dramatically different results. The molecule was aligned along the channel axis, and the “head” (outermost) amine nitrogen was constrained to stay immobile. Importantly, as predicted from the very steep voltage-dependence of Kir channel block by intracellular polyanimes (31), polyanimes are expected to enter the pore deeply (to s2 or s3) from the inside of the membrane, and to enter s0 or s1 from the outside (Fig. 12B). There are large central energy barriers (4–6 kcal/mol) to both putrescine and spermine in the wild-type channel which should significantly limit permeation of these polyanimes (Fig. 12B). Strikingly, enlargement of the pore-size in the mutant channel abolished these barriers (Fig. 12B). Under the influence of an electric field, the polyanimes should readily permeate the mutant channel consistent with the experimental data in Figs. 8–10.

**DISCUSSION**

This study has revealed a number of novel findings concerning inward rectifier K⁺ channels. First, the modeling confirms that a salt bridge, first proposed by Yang et al. (14), does exist...
behind the selectivity filter. Second, the modeling suggests a role for the salt bridge: to maintain the structure of the p-loop and the GYG motif and, therefore, K⁺ selectivity (confirmed by experiments). Finally, this study suggests that the selectivity filter is the site of polyamine-induced inward rectification: both modeling and experiments show that polyamines are able to enter the selectivity filter, and experiments show that disruption of the selectivity filter disrupts inward rectification.

**Ion Selectivity**—The present study has shown that disruption of the salt bridges in the Kir3.1/Kir3.4 channel affected the selectivity of the channel. Mutation of Glu-139 in Kir3.1 resulted in a modest decrease in selectivity, whereas mutation of either Glu-145 or Arg-155 in Kir3.4 resulted in a marked loss (Figs. 5 and 6). On disruption of the salt bridge in Kir3.4, extracellular Ca²⁺ and polyamine block was reduced or lost (Figs. 4 and 7), consistent with increased permeation of the channel by these ions. In the Kir2.1 channel, disruption of the equivalent salt bridge by mutation of Glu-148 results in a non-functional channel (14). This result is not inconsistent with the present study: the Kir2.1 channel is a homotetramer unlike the Kir3.1/Kir3.4 channel. With Kir2.1, channel activity can be rescued by expressing wild-type Kir2.1 with Kir2.1(E138Q) (14), whereas expression of Kir3.1(E139Q) and Kir3.4(E145Q) results in a non-functional channel (unpublished observations). With Kir2.1, channel activity can also be rescued by swapping the glutamate and arginine residues involved in the salt bridge, i.e. the double mutation E138R/R145E (14), although this double-mutant channel is non-selective (14).

In the present study, swapping the glutamate and arginine residues involved in the salt bridge in Kir3.4 also resulted in a non-selective channel (in Fig. 5, see the traces for the Kir3.1/Kir3.4(E145R,R155E) mutant channel).

All other eukaryotic Kir channels, with the exception of Kir7.1, have the equivalent residue pair and are predicted to contain salt bridges. In the case of Kir7.1, which lacks the arginine residue involved in the salt bridge and has a methionine residue (Met-125) at the equivalent position, the substitution of an arginine residue for the methionine residue increases ion selectivity; the Rb⁺ conductance of the wild-type channel, which is ∼8× greater than the K⁺ conductance, is reduced ∼40× (32). This result is analogous to the results from the present study, in which the selectivity against Rb⁺ is reduced in the mutant Kir3.1/Kir3.4(R155E) channel (Fig. 6).

How does disruption of the salt bridge affect ion selectivity? Molecular modeling indicates that mutations which disrupt the Kir3.4 Glu-145/Arg-155 salt bridge increase the selectivity filter diameter (Fig. 12A). The molecular dynamics modeling clearly shows how this can lead to an increase in the permeation of polyamines through the selectivity filter, but not how it leads to the permeation of smaller ions such as Na⁺ (Fig. 12B). How does such a widening lead to permeation of Na⁺ (Figs. 5 and 6)? K⁺ permeation through the wild-type selectivity filter requires coordination with two water molecules and four carbonyls to maintain a hexahydration shell. The possibility arises that Na⁺ permeation in a wider pore occurs with the involvement of four water molecules, such that only two backbone carbonyls are required to maintain the hydration shell. This possibility is consistent with the hypothesis that the source of K⁺/Na⁺ selectivity is the energetic cost of monovalent metal cation dehydration. Berneche and Roux (25) proposed that carbonyl motions within the selectivity filter during isothermal dynamics should be greater than the difference between Na⁺, K⁺, or Ba²⁺ radii, and that small local arrangements should therefore be sufficient to permit effective coordination of any monovalent cation and, hence, filter geometry alone may not be enough to explain monovalent cation selectivity of KcsA-like channels. If the narrowest part of the wild-type selectivity filter allows only for coordination of two water molecules (one on each side of the monovalent cation along the channel axis), the cost of substitution of four water molecules oxygens (partial charge = −0.60 e) by four carbonyl oxygens (partial charge = −0.40 e) is higher for Na⁺ (a smaller cation; radius = 0.96 Å) than for K⁺ (radius = 1.36 Å) as defined by Coulomb’s law. This is consistent with the experimental free energy of Na⁺ and K⁺ solvation in water (−101 and −80 kcal/mol) versus carbonate (−59 and −65 kcal/mol; partial charge of oxygen = −0.53 e), which suggests a less favorable transfer from water to carbonyl environment for Na⁺ than for K⁺. Enlargement of the filter, as suggested in Fig. 12A for the mutant channels, could allow diffusion of Na⁺ with up to four coordinated water oxygens, only two of six being exchanged for backbone carbonyls. The energy cost of the electrostatic potential loss is in the lower range and might explain the diffusion of Na⁺.

**Implications for Ion Selectivity in Other K⁺ Channels**—Voltage-gated K⁺ channels do not contain an ion pair at the equivalent positions to Glu-145 and Arg-155 in Kir3.4, and neither does the highly K⁺ selective (33) KcsA channel (Fig. 3). Although KcsA has an equivalent glutamate residue (Glu-71), it has a leucine residue (Leu-81) at the Arg-155 position and an aspartate residue (Asp-80) at the preceding position (Fig. 3). There has been debate regarding the likely ionization state of...
FIG. 12. Dynamic simulation of wild-type and mutant channel pores. A, isothermal molecular dynamic simulation of the pore in the presence of water molecules and no cations from the three-dimensional models of wild-type and mutant channels. Snapshots every picosecond of the average of the four distances between two equivalent backbone carbonyl oxygens from two neighboring subunits at six positions 143–148 (Kir3.1) along the narrowest part of the selectivity filter are shown. B, free energy profiles of ligand-channel interaction for K⁺, Na⁺, putrescine, and spermine. The positions of K⁺-binding positions in the channel model are indicated by the spots. Each point (every 0.5 Å) represents the result of an independently optimized model. The free energy reported is the difference between the complex and the free components (as described under "Experimental Procedures"). The distance along the channel is indicated in the model in each case. For putrescine and spermine, the “head” (outermost) amine nitrogen was held at the indicated positions. Two spermine molecules in the outer and inner binding sites in the channel model are shown on the right.
the Glu-71 residue in KcsA because the side chain was not resolved in the original KcsA crystal (1). At 2.0 Å resolution, the side chain is clearly not ionized, and the protonated carboxylic acid generates a hydrogen bond with Asp-80 as shown in Fig. 3(4). Molecular dynamics simulations indicate that this strong hydrogen bond is necessary to ensure that the channel does not deviate significantly from the crystallographic structure (25); the structure of the selectivity filter of the KcsA channel is significantly disrupted when these side chains are fully ionized on each of the four monomers (15). This is analogous to the function of the salt bridge in the Kir3.1/Kir3.4 channel indicated by the modeling in the present study (Fig. 12). Although there is no Glu-71 equivalent residue, an aspartate residue is present in almost all other extended K+ channel family members at the Asp-80 equivalent position, and we suggest that this residue may also be bonded to the 70/71 equivalent residue to maintain the bowstring arrangement and provide torsion to the GYG loop in these channels. HCN channels contain the GYG motif but are non-selective. Why is K+ so important for polyamines (Figs. 1 and 2)? Additional data from this study are consistent with polyamines having ready access to a common blocking site (presumably the selectivity filter) across both intracellular and extracellular polyamines. The present study unequivocally demonstrates that such permeation occurs (Figs. 8–10), with non-selective mutant channels being even more permeable to polyamines than wild-type channels (Figs. 7–9). The molecular modeling indicates this to be the result of enhanced flexibility of the mutant pore, with lowered energy barriers in the selectivity filter (Fig. 12B). The increased polyamine permeability of the mutant channels explains reduced inward rectification of these channels: there is polyamine permeation rather than block.

With one exception, all Kir channel subfamily members express the conserved Glu-145 and Arg-155 (Kir3.4) charges and are expected to contain the same intra-subunit salt bridge. However, Kir7.1 lacks the conserved arginine residue and has a histidine equivalent at the equivalent position (32). As predicted from the current study, the Kir7.1 channel has relatively weak inward rectification (32). It is interesting that KirBac1.1 also lacks the conserved arginine residue and has a histidine residue at the equivalent position (2); it is predicted that the KirBac1.1 channel will also have relatively weak inward rectification.

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