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Peptide Backbone Mutagenesis of Putative Gating Hinges in a Potassium Ion Channel

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The dynamic movements that underlie the transitions between the closed and open states of a potassium ion channel are still not fully understood. Most potassium channels contain a highly conserved glycine residue in the second transmembrane domain (TM2) that is thought to act as a flexible gating hinge. However, inwardly rectifying (Kir) potassium channels also possess an additional invariant glycine at the base of TM2 near the helix-bundle crossing that has been proposed to contribute to TM2 flexibility during channel gating similar to the PVP motif in voltage-gated potassium channels. In this study we have addressed the relative contribution of these putative glycine gating hinges by using unnatural amino acid mutagenesis to introduce α-hydroxyacetic acid (αG) and thereby an amide-to-ester mutation into the backbone of Kir2.1 at the upper (G168) and lower (G177) glycine positions. This mutation is predicted to increase the flexibility of the TM2 backbone at these positions without altering the side chain. We show that introduction of αG at the upper gating hinge position produces channels that open more slowly at hyperpolarized potentials whereas αG at the lower glycine position does not affect channel gating. These results are consistent with a structural model in which K^+ channel gating involves bending of the inner pore helix (TM2) at or near the upper glycine, but where the lower glycine found in Kir channels is more likely to be required for tight packing of the TM2 helices at the helix-bundle crossing rather than acting as a gating hinge.

Understanding the conformational changes that occur as a potassium channel undergoes the reversible transition between the open and closed states still represents one of the major challenges in ion channel structural biology. Comparison of the X-ray crystal structures of the KcsA K^+ channel in the closed state with that of the MthK channel in the open state has led to a model of K^+ channel gating in which the physical gate is formed by the four pore-lining TM2 helices crossing over each other near its intracellular entrance to constrict (i.e., close) the pore. During channel opening it is proposed that the TM2 helices bend in the middle and splay outwards so that the gate at the lower "helix bundle crossing" widens thus forming an open pathway from the cytoplasm to the selectivity filter.[1,2]

In the open-state MthK crystal structure the TM2 helix is bent at a highly conserved glycine residue.[2] This glycine residue is conserved in >80% of K^+ channel sequences, and functional studies of the KCNQ1 and NaChBac channels support a model in which pivoted bending of this glycine hinge occurs during channel gating.[3–6] However, functional studies of the Kv *Shaker* family and the structure of Kv1.2 suggest that the helices are rigid at this point and instead bend at a highly conserved PVP motif lower down in S6(TM2) within the helix bundle crossing.[7]

Understanding the role of the putative TM2 hinge in Kir channels is yet more complex because these channels have two highly conserved glycine residues in TM2; one at the higher MthK glycine hinge position, the other lower down closer to the helix bundle crossing in a position which aligns with the *Shaker* PVP motif (Figure 1).[6,8,9]

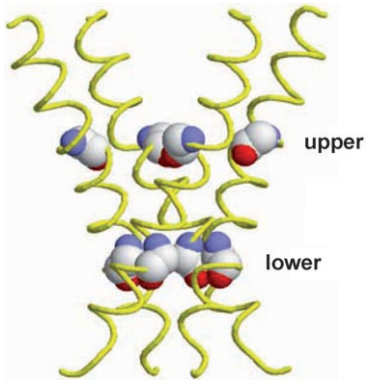

Figure 1. Homology model of the pore of a Kir channel with only TM2 α-helices shown in yellow for clarity. The position of the two highly conserved glycine residues in TM2 are shown as cpk spheres. The "upper" glycine hinge region is thought to bend during channel gating. However, the role of the "lower" glycine at the helix bundle crossing is unclear.

Previous studies have used traditional site-directed mutagenesis to investigate the role of these conserved glycines and other TM2 residues,[3–5,8–11] and suggest an important role for the upper glycine hinge in channel gating. However, these results can be difficult to interpret because changing the nature of the amino acid side chains at these positions does not simply change TM2 flexibility and often has secondary unintended effects on channel gating. Indeed, recent studies have shown that the side chain at the upper glycine hinge position

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in Kir3.4 interacts with residues within the P-loop and might alter channel gating indirectly through these interactions.[10,11]

One way to circumvent this problem is to use unnatural amino acid mutagenesis to mutate the peptide backbone of the TM2 α-helix in order to change its flexibility. The introduction of amide-to-ester backbone mutations preserves the nature of the side chain, but the mutation cannot serve as a hydrogen bond donor as the critical -NH is missing. Therefore, substitution of a glycine with α-hydroxyacetic acid will have two major affects. Firstly, it will remove a backbone hydrogen bond that stabilizes the α-helix. Secondly, it will greatly lower the rotation barrier around the ester (formerly amide) bond. These combined effects will allow greater twisting and flexibility of TM2 at these specific positions,[12] and this approach has successfully been used to study the relationship between TM flexibility and gating in the nicotinic acetylcholine receptor.[13]

In this study we have used nonsense codon suppression and synthetic aminoacyl-tRNAs to introduce α-hydroxyacetic acid (and thereby an amide-to-ester mutation) into both the upper and lower conserved glycine positions in Kir2.1 channels expressed in *Xenopus* oocytes. A nonsense suppressor tRNA containing a CUA anticodon was synthesized and chemically charged with α-hydroxyacetic acid (αG). This charged suppressor tRNA was co-injected into *Xenopus* oocytes with Kir2.1 mRNA mutated to contain a UAG stop codon at either the upper (G168X) or the lower (G177X) glycine positions in TM2.

In both cases, currents several μA in amplitude were obtained within 24–36 h. This technique is potentially subject to a variety of artifacts caused either by readthrough of the stop codon, or reacylation of the tRNA with a natural amino acid.[13] However, for both G168X and G177X, injection of up to 10 ng of mRNA alone failed to generate any current even after 48 h. Likewise co-injection of mutant mRNA and uncharged tRNA failed to generate currents different from uninjected controls (not shown).

Co-injection of G177X mRNA and charged suppressor αG-tRNA yielded whole-cell currents which appeared identical to wild-type Kir2.1. By contrast, the currents generated by αG-tRNA suppression of G168X mRNA were markedly different (Figure 2); the currents showed stronger inward rectification (ratio of inward to outward current) at positive potentials, and a much slower time-dependent activation at hyperpolarizing potentials; whereas wild-type Kir2.1 currents reach a steady-state rapidly at −100 mV ($\tau = 367 \pm 76$ ms), channels with αG at position 168 (αG168) were markedly slower ($\tau = 1970 \pm 60$ ms).

We also recorded single channel currents for αG168 and αG177 mutant channels in cell-attached patches from *Xenopus* oocytes. As shown in Figure 3, the single channel amplitude for both mutants appeared unaffected, and the open probability (P_O) for αG168 channels (0.25 ± 0.02) and αG177 channels (0.26 ± 0.03) was also not different to wild-type Kir2.1 (0.23 ± 0.02).

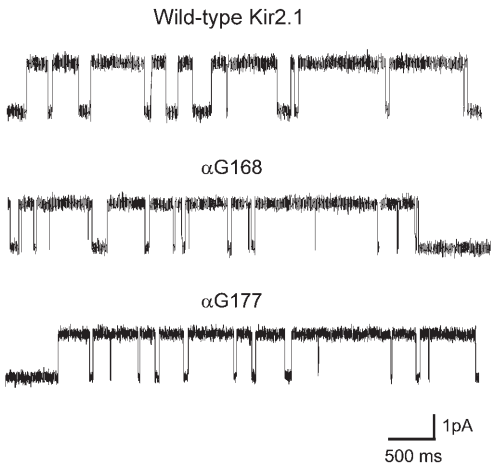

Figure 3. Cell-attached single channel recordings of wild-type Kir2.1 compared to αG168 and αG177. Recorded at −100 mV.

The lack of any effect of introducing αG at the lower conserved glycine (G177) in the helix-bundle crossing is highly significant as this demonstrates that increasing TM2 flexibility at this position does not affect Kir2.1 channel gating. This result is consistent with our previous study[9] which indicated that

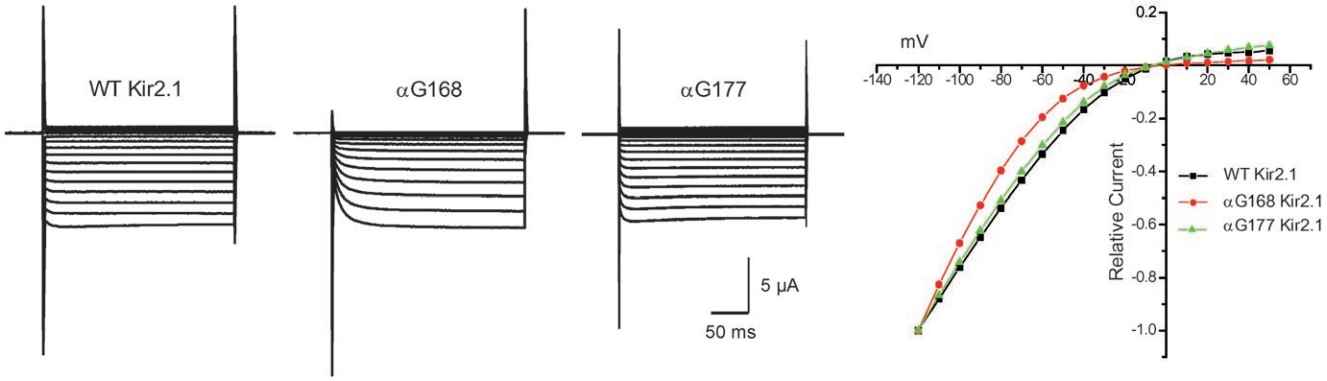

Figure 2. Representative whole cell current traces of wild-type Kir2.1, αG168, and αG177 recorded at potentials between −120 and +40 mV. Introduction of α-hydroxyacetic acid into the upper hinge position (αG168) causes some increased rectification at positive potentials and a much slower time-dependent activation at negative potentials. The increased rectification of αG168 currents is more clearly visible in the steady-state current–voltage (*I/V*) plot in the right hand panel ($n = 6$).

the size of the glycine side chain at this position is probably more important for tight-packing of the TM2 helices at the helix-bundle crossing, rather than allowing it to act as a flexible "gating hinge". All previous studies of mutations at this highly conserved lower glycine position have either produced nonfunctional channels or shown various effects on channel gating which have been difficult to dissect.[8,9] We now show that these effects on gating are likely to have arisen indirectly from unintended changes in TM2 packing at the helix-bundle crossing, rather than as result of changing TM2 flexibility per se.

By contrast, the changes observed in macroscopic gating kinetics upon introduction of αG into the upper glycine hinge position (αG168) are consistent with an important role of this putative hinge residue in Kir channel gating. However, simply increasing TM2 flexibility at this position does not cause an increase in open channel probability as might be expected. Instead, αG168 channels exhibit an increase in their time-dependent activation at hyperpolarizing potentials; this indicates that they might be more difficult to open. However, the open-probability of these mutant channels is not markedly different to wild-type Kir2.1; this indicates that any such effect is transient before currents reach the steady state level. These changes in time-dependent activation could either be due to the fact that increasing TM2 flexibility at this upper hinge position actually impairs, rather than eases, channel opening. This would mean that a certain degree of TM "rigidity" at this point in the α-helix is important. Alternatively, it could be due to a stronger interaction of Kir2.1 with intracellular polyamines such as spermine which bind deep within the pore[14] and which would unblock more slowly at hyperpolarizing potentials thus contributing to the time-dependent activation. The increased rectification of αG168 channels would be consistent with this, and similar effects of polyamines have also been proposed to underlie the time-dependent activation in Kir3.0 channels.[15]

In terms of a structural explanation, it is possible that an increase in TM2 flexibility at this upper hinge position allows the TM helices, and thus the inner pore, to open slightly wider thereby allowing polyamines to penetrate more deeply into their binding site. Alternatively, the αG168 mutation might affect the principal binding site for polyamines which resides one α-helical turn below G168 (D172).[14] The amide-to-ester mutation at G168 also reduces the electronegativity of the carbonyl oxygen in the ester linkage and it is this carbonyl which H-bonds with the –NH group of D172.[16–18] Thus, in addition to any effects on intrinsic channel gating, the αG168 substitution might also influence the relative position of the D172 rectification control site and influence the block by intracellular polyamines.

In conclusion, our results demonstrate that unnatural amino acid mutagenesis is a viable approach to study the influence of backbone flexibility in Kir channel gating in a way not possible with traditional forms of side-chain mutagenesis. Although we have only investigated changing TM2 flexibility at two positions, our results indicate that that the small nature of the glycine side chain at the lower putative glycine "hinge" position is likely to be more important for tight packing of TM2 at the helix bundle-crossing rather than allowing it to act as a gating hinge. Furthermore, although the effects of increasing TM2 flexibility at the upper hinge position are complex, it does not simply make the channel easier to open. It is therefore likely that a degree of backbone rigidity in the α-helix at this position is essential for correct channel function. This study therefore presents the possibility of a more detailed analysis of TM2 flexibility in potassium channels by using unnatural amino acid mutagenesis at other positions within TM2 by incorporation of a range of α-hydroxy acids. Such an ability to combine high-resolution structural mutagenesis and functional analysis with the increasingly detailed 3D structural data now available will become an important tool in our understanding of the dynamic changes which occur during K^+ channel gating.

Experimental Section

Synthesis of tRNA acylated by α-hydroxyacetic acid (αG-tRNA): The nitroveratryl (NV) protected cyanomethyl (CM) ester of α-hydroxyacetic acid (αG) was synthesized as done previously.[19] Briefly, NV-αG-OH (1.0 g, 79% yield) was synthesized by mixing *tert*-butyl bromoacetate (1.4 g) and 4,5-dimethoxy-2-nitrobenzyl alcohol (1.0 g) in a solution of 40% tetra-*n*-butylammonium hydroxide (0.8 mL) in 50% aqueous NaOH (4.5 mL) at 0 °C for 15 min. NV-αG-OH (0.95 g) was then treated with chloroacetonitrile (1.3 mL) in triethylamine (1.9 mL) to afford an activated ester, NV-αG-CM (0.8 g, 75% yield). The dinucleotide, 5′-*O*-phosphoryl-2′-deoxycytidylyl-adenosine (dCA), was synthesized by using the solution-phase phosphoramidite method originally reported by Robertson et al.[20] and later modified by Kearney et al.[21] The key phosphoramidites; 5′-dimethoxytrityl-*N*-benzoyl-2′-deoxycytidine,3′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite as well as bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite were purchased from Glen Research (Sterling, Virginia, USA) and Cambio (Cambridge, UK), respectively. The phosphoramidites were coupled successively to the substrate, tetrabenzoyladenosine,[20] to afford fully protected dCA. After deprotection with concentrated NH_4OH, dCA was purified with reversed-phase HPLC (SUPELCO PLC-18, 250 mm × 21.2 mm; 7 mL min^{-1}; 5–90% CH_3CN in 25 mM NH_4OAc over 60 min; UV 260 nm; $t_R = 12$ min) and identified with ESI-MS (the molecular ion $[M-H]^-$ of dCA was detected at *m/z* 635). NV-αG-CM (17 mg) was coupled to the dinucleotide dCA (12 mg) in DMF (1 mL) and purified by reversed-phase HPLC (the same conditions as above, $t_R = 37$ min). Formation of NV-αG-dCA was confirmed with ESI-MS (the molecular ion $[M-2H]^{2-}$ of NV-αG-dCA was detected at *m/z* 443.5). Truncated 74-mer THG73 $tRNA_{CUA}$ was prepared by in vitro transcription with the Mega-Short Script kit (Ambion, Austin, TX). NV-αG-dCA was then enzymatically ligated to truncated 74-mer THG73 $tRNA_{CUA}$ as detailed previously.[22] Completion of the ligation was ensured by using 15% TBE-Urea PAGE; Samples (0.5 mg) were run for 3 h at 150 V and stained with 0.2% Stains-All (Sigma) in 50% formamide for 5 min, destained in water for 15 min, following which 74-mer and 76-mer tRNAs are distinguishable. Immediately before coinjection with mRNA, NV-αG-tRNA was deprotected by photolysis to give αG-tRNA. 2 ng mRNA and 20 ng αG-tRNA were injected into the oocytes in a total volume of 50 nL. For control experiments, mRNA was injected in the absence of tRNA, and with the full length THG73 76-mer tRNA. Electrophysiological experiments were performed 18–36 h after injection.

Electrophysiology: Macroscopic whole-cell currents were recorded by standard two-electrode voltage clamp analysis as done previously.[23] For cell attached single-channel currents the pipette solution contained (in mM); KCl 120, $CaCl_2$ 1.8, and HEPES 10 (pH 7.2). The bath solution contained (in mM): KCl 120, EGTA 2, Tetrasodiumpyrophosphate 1, and HEPES 10 (pH 7.2). Under these conditions wild-type Kir2.1 exhibits a relatively low Po (~0.25) thus making any potential increase in open-probability easier to observe.[24] Single-channel activity was recorded by using an Axopatch 200B amplifier (Axon Instruments) at a voltage of −100 mV, filtered at 1–2 kHz (Frequency Devices 900), sampled at 5–10 kHz, and stored directly into the computer's hard disk through the Digidata 1322 A interface (Axon Instruments). All measurements were made at room temperature (21–23 °C). Analysis was carried out with Clampfit 9.2 (Axon Instruments).

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