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Glycine as a D-amino acid surrogate in the K^+-selectivity filter

Francis I. Valiyaveetil*, Matthew Sekedat†, Roderick MacKinnon*‡, and Tom W. Muir†‡

*Laboratory of Molecular Neurobiology and Biophysics and Howard Hughes Medical Institute, The Rockefeller University, Box 47, 1230 York Avenue, New York, NY 10021; and †Laboratory of Synthetic Protein Chemistry, The Rockefeller University, Box 223, 1230 York Avenue, New York, NY 10021

Contributed by Roderick MacKinnon, October 20, 2004

The K^+ channel-selectivity filter consists of two absolutely conserved glycine residues. Crystal structures show that the first glycine in the selectivity filter, Gly-77 in KcsA, is in a left-handed helical conformation. Although the left-handed helical conformation is not favorable for the naturally occurring L-amino acids, it is favorable for the chirally opposite D-amino acids. Here, we demonstrate that Gly-77 can be replaced by D-Ala with almost complete retention of function. In contrast, substitution with an L-amino acid results in a nonfunctional channel. This finding suggests that glycine is used as a surrogate D-amino acid in the selectivity filter. The absolute conservation of glycine in the K^+-selectivity filter can be explained as a result of glycine being the only natural amino acid that can play this role.

K^+ channels | semisynthesis

The selectivity filter of K^+ channels is formed from a very conserved stretch of amino acids called the K^+ channel signature sequence: T-X-X-T/S-X-G-Y/F-G (Fig. 1*a*) (1). All known K^+ channels have this sequence; it underlies nature's chemical solution to the problem of conducting K^+ ions selectively and rapidly across the membrane of cells (1, 2). What is special about this sequence, and why do K^+ channels require it?

The atomic structure of the KcsA K^+ channel revealed that the selectivity filter is a tube of ≈12 Å in length that is produced by four signature sequence peptides, one contributed by each of the four identical K^+ channel subunits (1, 3). These peptides direct four sequential main-chain carbonyl oxygen atoms (from the sequence T75-V-G-Y78 in the KcsA K^+ channel) toward the pore (Fig. 1*b*). Together with the side-chain hydroxyl oxygen atom of the T75 position, the directed carbonyl oxygen atoms create four K^+-binding sites in a row inside the filter. To create these binding sites, the peptide main chain has to adopt an unusual conformation in which the dihedral angles of the sequence T-V-G-Y alternate between the left-handed and right-handed α-helical regions of the Ramachandran plot (Fig. 1*c*) (4–6).

One way to achieve the unusual main-chain conformation observed in the K^+-selectivity filter is to use alternating L- and D-amino acids in the peptide sequence. In ribosome-synthesized proteins, D-amino acids are not an option, only L-amino acids are used, which strongly prefer right-handed α-helical dihedral angles (4). To create the K^+-selectivity filter, two left-handed helical conformations (at positions 75 and 77) from a sequence of four amino acids are required. We questioned how this unusual conformation is accomplished in the K^+-selectivity filter.

The structure of KcsA suggests the following. The first amino acid of the selectivity filter in the left-handed helical conformation is threonine at position 75. The hydroxyl group of Thr-75 forms a part of the fourth K^+-binding site in the selectivity filter. In protein structures, an amino acid is sometimes observed in an unfavorable conformation (7, 8). These instances occur overwhelmingly in protein active sites, and the protein structure in the vicinity of the active site helps to stabilize the amino acid in the unusual conformation. Such an effect must account for why Thr-75 is in a left-handed helical conformation. The other amino acid in the left-handed helical conformation in the selectivity filter is glycine at position 77. Glycine is the only amino acid in proteins synthesized on the ribosome that comfortably resides in the left-handed α-helical region of the Ramachandran plot (4). Being achiral, it makes perfect sense that glycine can act as life's surrogate D-amino acid.

The above explanation for the role of glycine in the K^+-selectivity filter is reasonable, but how would one demonstrate it experimentally? In this study, we synthesized a K^+ channel with D-Ala in the position of Gly-77 and showed that it produces a selective K^+ channel with wild-type-like pharmacological properties. L-Ala at this same position fails to produce functional channels, and many previous attempts to produce functional K^+ channels with other L-amino acids at this equivalent position have failed (9). D-Ala satisfies the dihedral angle requirements of Gly-77 in the K^+-selectivity filter, but it does not provide the same minimal steric clash or dynamical possibilities that are intrinsic to glycine. Therefore, success with D-Ala leads us to conclude that, above all, glycine is used in the selectivity filter to fulfill specific dihedral angle requirements, and thus, it serves as a D-amino acid surrogate.

Methods

Protein Expression and Purification. The recombinant KcsA protein used in this study contained the following amino acid substitutions: P2→A, S69→C, and A98→G (10). For ease of semisynthesis, a truncated form of KcsA (missing the C-terminal 35 aa) was used in these studies. The P2→A substitution was introduced during the cloning of KcsA, the S69→C substitution was required for the ligation chemistry, and the A98→G substitution was required for single-channel measurements to be carried out on the truncated form of KcsA that was used in these studies (10). The L-Ala mutant contained the G77→A substitution in addition to the substitutions mentioned above. KcsA proteins were expressed and purified according to established procedures (1). The C-terminal 35 aa (residues 126–160) were removed by chymotrypsin digestion, and the truncated protein was purified by gel-filtration chromatography (1).

Semisynthesis of G77→D-Ala KcsA. By manual solid-phase peptide synthesis, we synthesized the C-peptide corresponding to residues 69–122 of KcsA with the substitutions S69→C, G77→D-Ala, and A98→G, and we purified it by using the protocols described in refs. 10 and 11. We generated the KcsA polypeptide (1–122) with the D-Ala-77 substitution by expressed protein ligation of the synthetic C-peptide with a recombinantly expressed N-peptide α-thioester (corresponding to residues 1–68). The protocols for the expression, purification of the N-peptide α-thioester, and the ligation reaction were used without modification (11). The KcsA polypeptide synthesized was folded to

Abbreviation: TEA^+, tetraethylammonium.

‡To whom correspondence may be addressed. E-mail: muirt@mail.rockefeller.edu or mackinn@mail.rockefeller.edu.

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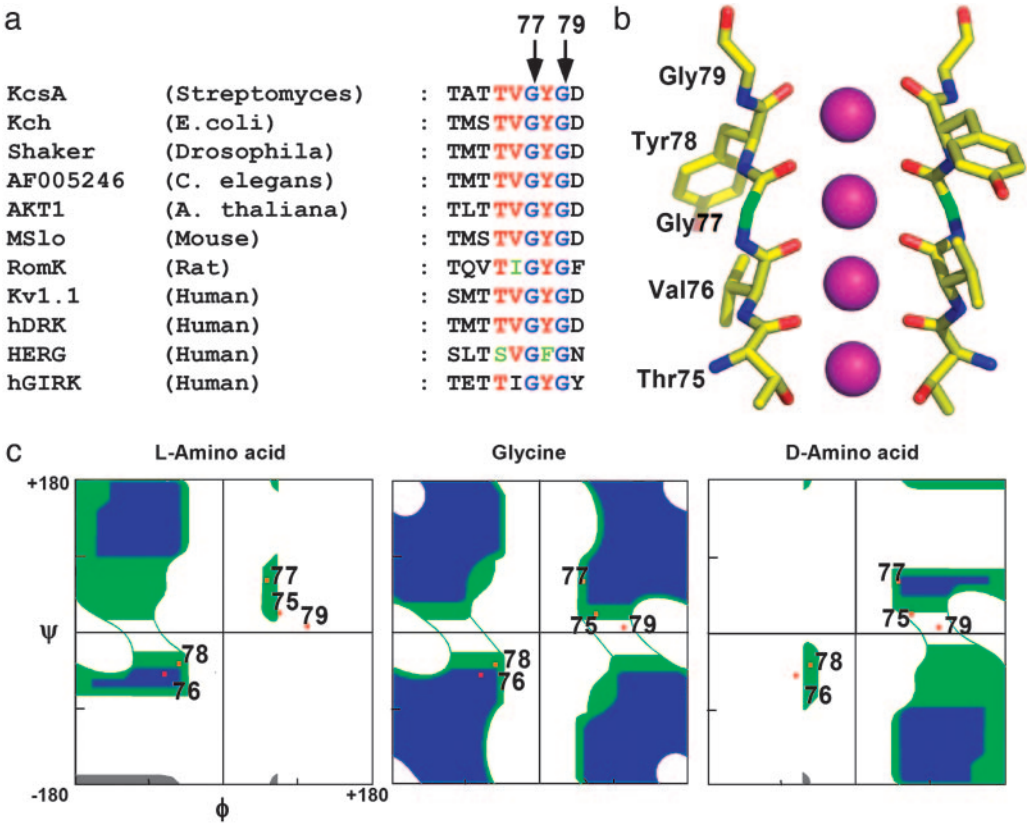

Fig. 1. The selectivity filter of K^+ channels. (*a*) Sequence alignment of the selectivity filter sequence of several of K^+ channels. GenBank accession nos. are as follows: KcsA (*Streptomyces lividans*), CAA86025; Kch (*Escherichia coli*), P31069; Shaker (*Drosophila melanogaster*), P08510; *Caenorhabditis elegans*, AF005246; AKT1, (*Arabidopsis thaliana*), AAB95299; Mslo (*Mus musculus*), NP_034740; RomK (*Rattus norvegicus*), P35560; K_v 1.1 (*Homo sapiens*), NP_000208; hDRK (*H. sapiens*), X68302; HERG (*H. sapiens*), AAN05415; and hGIRK (*H. sapiens*), AAB07269. (*b*) Structure of the selectivity filter (residues 75–79) of the KcsA K^+ channel (PDB ID code 1K4C). Two subunits are shown in ball-and-stick representation, and the K^+ ions are shown as pink spheres. (*c*) The dihedral angles of the selectivity filter of KcsA (residues 75–79) are plotted on a Ramachandran diagram for an L-amino acid, glycine, and a D-amino acid. The favorable regions are shown in blue, and the allowed regions are shown in green.

the native tetrameric state by using lipid vesicles, and the folded tetrameric protein was purified from the unfolded monomeric protein as described (10, 12).

Electrophysiology. Recombinant and semisynthetic KcsA was reconstituted into lipid vesicles composed of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE, 7.5 mg/ml) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG, 2.5 mg/ml) at a protein-to-lipid ratio of 2.5 μg/mg as described (12). Planar lipid bilayers composed of POPE (15 mg/ml) and POPG (5 mg/ml) in decane were painted over a $\approx$50-μm hole in overhead transparency film separating the internal and external solutions (13). To induce fusion of channel-containing vesicles, the solution on the side (external) to which vesicles were added contained 150 mM KCl and 10 mM Hepes (pH 7.0), whereas the opposite (internal) side contained 15 mM KCl and 10 mM succinate (pH 4.0). After channel fusion, the solution on the internal side was exchanged for solution containing 150 mM KCl and 10 mM succinate (pH 4.0) by perfusion. Membrane voltage was controlled, and current was recorded with an Axopatch 200B amplifier with a Digidata 1322A analog-to-digital converter and AXOPATCH software (Axon Instruments, Union City, CA). Current was sampled at 10 kHz and low-pass filtered at 1 kHz. Before analysis, the data were filtered digitally at 500 Hz. As reported (10), two conductance states were observed for both the recombinant and the semisynthetic proteins. Data reported in this article are for the larger conductance state.

Tetraethylammonium (TEA^+)- and Na^+-Inhibition Studies. For these inhibition studies, channel activity was recorded with an external solution consisting of 150 mM KCl and 10 mM Hepes (pH 7.0) and an internal solution consisting of 150 mM KCl and 10 mM succinate (pH 4.0). For TEA^+-inhibition studies, TEA^+ was added to the external side and the residual current amplitude at +100 mV was recorded, whereas for Na^+-inhibition studies, Na^+ was added to the internal side and the residual current amplitude at +150 mV was recorded. The fraction of residual current was plotted against blocker concentration and fitted with the following equation to determine the equilibrium dissociation constant (K_d): fraction unblocked = $1/(1+[\text{blocker}]/K_d)$.

Reversal Potential Measurements. To determine K^+/Li^+ selectivity, single-channel currents were measured at various membrane voltages with 10 mM succinate/150 mM KCl (pH 4.0) as the internal solution and 10 mM Hepes/30 mM KCl/120 mM LiCl (pH 7.0) as the external solution. The single-channel traces were digitally filtered at 110 Hz to estimate the small-amplitude channel openings. The reversal potential was determined from a linear fit of the data between 0 and −80 mV. Experiments to determine K^+/Na^+ selectivity were carried out with 10 mM succinate/300 mM KCl (pH 4.0) as the internal solution and 10 mM Hepes/30 mM KCl/270 mM NaCl (pH 7.0) as the external solution.

Results and Discussion

Construction of the Diastereomeric Gly-77 KcsA Mutants. We replaced Gly-77 of KcsA by D-Ala by using the expressed protein

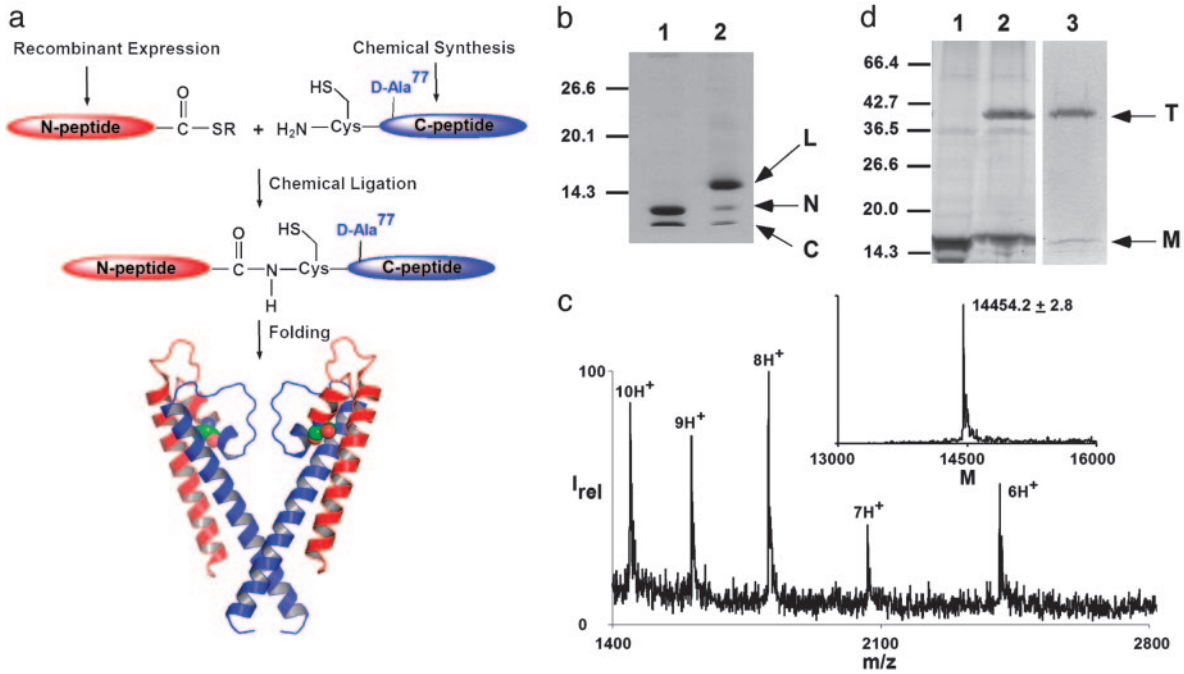

Fig. 2. Semisynthesis of the D-Ala mutant of KcsA. (*a*) Schematic representation of the semisynthesis of KcsA with the G77→D-Ala substitution. The KcsA polypeptide was assembled by expressed protein ligation of a recombinant N-peptide (residues 1–68, red) and a synthetic C-peptide (residues 69–122, with the G77→D-Ala substitution, blue). The product was folded into the native tetrameric state by using lipid vesicles. The ligation site at position 69 is shown. (*b*) SDS/PAGE gel (15%) of the ligation reaction at 0 min (lane 1) and 24 h (lane 2) showing the C-peptide (C), N-peptide (N), and the ligation product (L). (*c*) Electrospray MS of the ligation product. (*Inset*) Reconstructed spectrum (expected mass, 14,452.0 Da). (*d*) SDS/PAGE (12%) of the semisynthetic channel before (lane 1) and after (lane 2) the addition of lipid vesicles. The tetrameric semisynthetic channel was purified by cobalt-affinity chromatography and gel-filtration chromatography (lane 3).T, tetramer; M, monomer.

ligation (14) semisynthesis approach. Reports of site-specific D-amino acid incorporation into proteins have relied on total synthesis and/or the use of the tRNA suppressor methodology (15, 16). We have reported (11) the semisynthesis of a truncated form of the KcsA channel (see Fig. 2*a*). Also, we have demonstrated that single-channel measurements can be carried out on the truncated KcsA channel by incorporation of the A98→G substitution (10). The synthesis involves chemical ligation of a recombinantly expressed peptide α-thioester (residues 1–68, referred to as the N-peptide) with a chemically synthesized peptide (residues 69–122, referred to as the C-peptide). The resulting polypeptide is then folded to the native tetrameric state by using lipid vesicles. These protocols provide chemical access to the selectivity filter of KcsA, and they were used to introduce the Gly-77→D-Ala substitution into the protein (Fig. 2 *b* and *c*). The D-Ala substitution was well tolerated as the folded protein migrated as a tetramer on SDS/PAGE (Fig. 2*d*).

We substituted Gly-77 of KcsA by L-Ala by using standard recombinant-mutagenesis methods. The L-Ala mutant is also tetrameric on SDS/PAGE, indicating that the substitution of a methyl group is well tolerated structurally (data not shown). The stability of the diastereomeric proteins is in accord with modeling studies, which indicates that replacement of either the pro-*R* or the pro-*S* hydrogen in Gly-77 does not create apparent steric clashes with residues in, or around, the selectivity filter.

Channel Activity of the Diastereomeric Gly-77 Mutants of KcsA. Channel activity was tested after the diastereomeric KcsA mutant proteins were reconstituted into planar lipid bilayers. On repeated attempts, we were unable to detect any single-channel activity for the L-Ala mutant indicating that this substitution results in nonconducting channels (data not shown). Lack of ion conductance in the Gly-77→L-Ala mutant of KcsA has been

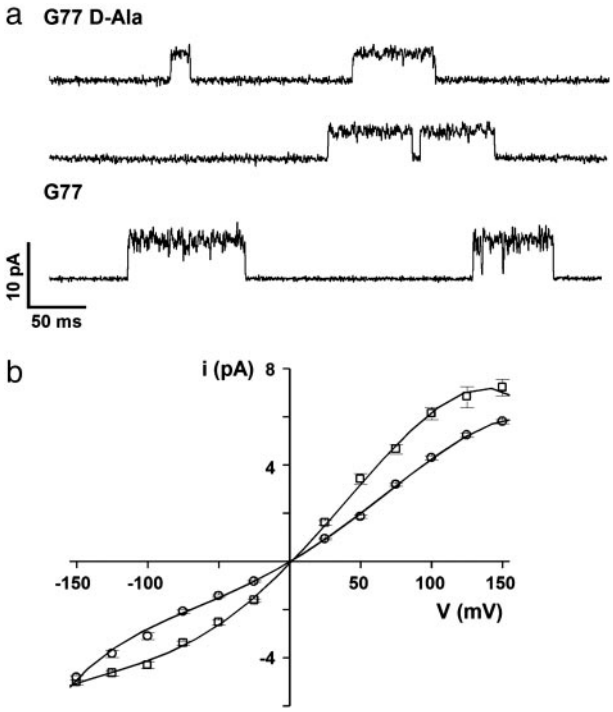

Fig. 3. Ion conduction by the D-Ala mutant of KcsA. (*a*) Representative single-channel traces for the D-Ala mutant or wild-type channels recorded at +100 mV in 10 mM succinate/150 mM KCl (pH 4.0) inside and 10 mM Hepes/150 mM KCl (pH 7.0) outside. The single-channel traces depicted are digitally filtered at 500 Hz. (*b*) Single-channel current as a function of membrane voltage in the above solutions for wild-type KcsA (□) and the D-Ala mutant (○). Lines have no theoretical meaning. Error bars represent the SEM of three experiments.

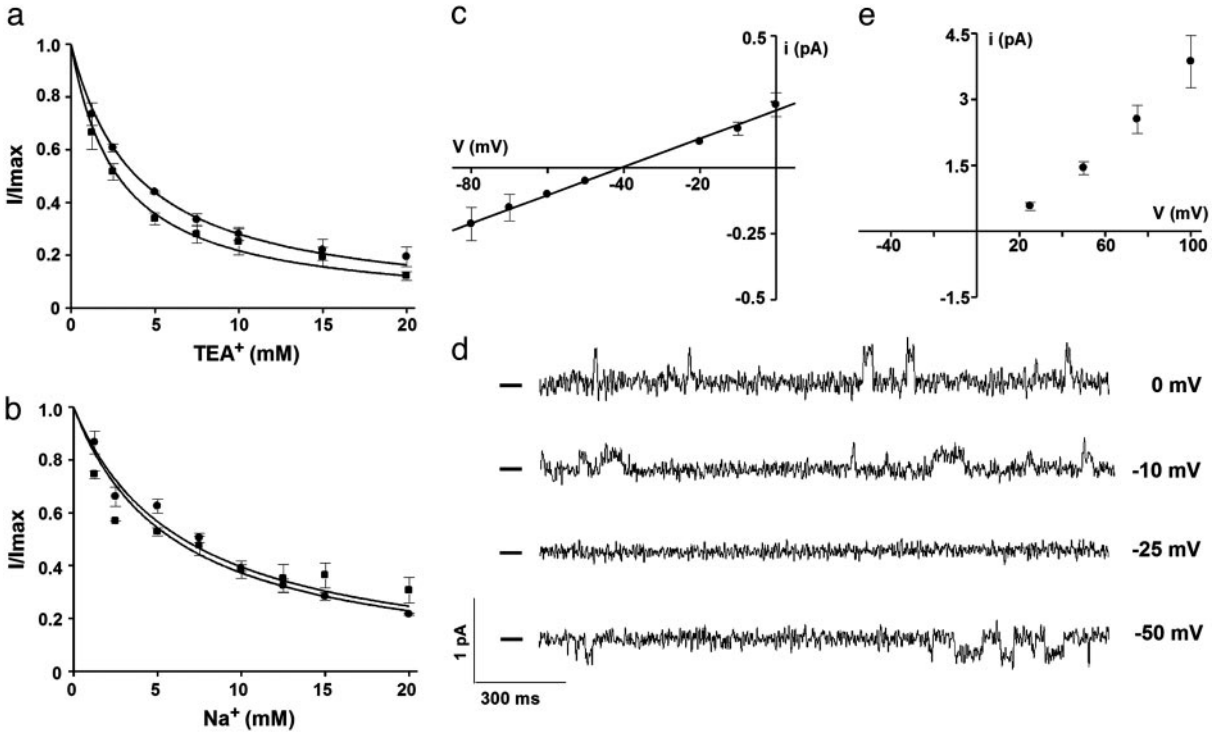

Fig. 4. Properties of the ion-conduction pathway in the D-Ala mutant of KcsA. (*a* and *b*) Inhibition of the D-Ala mutant (●) and wild-type KcsA (■) by external TEA^+ (*a*) and internal Na^+ (*b*) are shown. Fraction of unblocked current at +100 mV for external TEA^+ block and +150 mV for internal Na^+ block is plotted as a function of blocker concentration. Lines represent a fit to the following equation: fraction unblocked = $1/(1+[\text{blocker}]/K_d)$ where K_d is the equilibrium dissociation constant. (*c* and *d*) Single-channel currents were measured at various membrane voltages in 10 mM succinate/150 mM KCl (pH 4.0) inside and 10 mM Hepes/30 mM KCl/120 mM LiCl (pH 7.0) outside. The reversal potential was determined from a linear fit to the data points between 0 and −80 mV, and it is −38.9 ± 1.7 mV ($n = 3$). The single-channel traces were digitally filtered at 500 Hz. (*e*) Single-channel currents were measured at various membrane voltages in 10 mM succinate/300 mM KCl (pH 4.0) inside and 10 mM Hepes/30 mM KCl/270 mM NaCl (pH 7.0) outside. Error bars represent the SEM of three or four experiments.

reported (17). Also, amino acid substitutions at the equivalent Gly position in the *shaker* K^+ channel have been analyzed. In all cases, the mutant channels were found to be dysfunctional in that the channels were nonconductive (or nonexpressing) or nonselective (9). Similarly, the replacement of the equivalent Gly by Ser in the $K_{ir}3.2$ channel results in a nonselective channel, whereas the replacement by Cys in the K_v 2.1 channel results in a nonconducting channel (18, 19). Based on these results, we conclude that selective ion conduction is not retained when this glycine residue is replaced by an L-amino acid.

In contrast to the L-Ala mutant, single-channel activity for the D-Ala mutant was readily detected (Fig. 3*a*). Comparison with the wild-type control indicates that the conductance for the D-Ala mutant is slightly lower; cord conductance of 43 pS at +100 mV for the D-Ala mutant compared with 61 pS for the wild-type protein (Fig. 3*b*).

Pharmacology of the Gly-77→D-Ala KcsA. TEA^+ ions block K^+ channels by binding to the mouth of the channel just external to the selectivity filter (1, 20). We find that TEA^+ inhibits both the D-Ala mutant and the wild-type protein with very similar dissociation constants (4.0 ± 0.2 mM for the D-Ala protein compared with 2.9 ± 0.4 mM for the wild-type protein) (Fig. 4*a*). Sodium ions also block KcsA, but they do so from the internal side because of the inability of Na^+ to permeate through the selectivity filter (13). Accordingly, Na^+ was found to inhibit both the D-Ala and wild-type proteins to similar degrees (at +150 mV, K_d = 6.6 ± 0.3 mM for the D-Ala mutant compared with 6.0 ± 0.8 mM for the wild type (Fig. 4*b*). Therefore, at the level of functional detail provided by these inhibition experiments, the D-Ala mutant of KcsA is similar to the wild-type protein.

The introduction of D-Ala into the selectivity filter is not without effect. The conductance for the D-Ala mutant is lower than the wild-type channel. This effect is most probably due to structural perturbations in the selectivity filter as a result of the Gly→D-Ala substitution. These differences are likely to be quite small because the mutant channel is still capable of conducting K^+.

Ionic Selectivity of the Gly-77→D-Ala KcsA. One of the hallmarks of K^+ channels is the exquisite selectivity for K^+ over other monovalent cations (21). We used reversal potential measurements to investigate whether the D-Ala substitution affects selectivity for K^+ over other monovalent cations. The reversal potential for single-channel currents of the D-Ala mutant was measured in a 5-fold gradient of K^+ with 150 mM K^+ on the internal side and 120 mM Li^+ and 30 mM K^+ on the external side (Fig. 4 *c* and *d*). The reversal potential was measured to be −38.9 ± 1.7 mV, which is close to the Nernst potential of −41.3 mV for a perfectly K^+ selective pore under these experimental conditions. Therefore, D-Ala mutant is capable of selecting K^+ over Li^+. In similar experiments using Na^+ instead of Li^+ as the test ion, the reversal potential could not be determined (Fig. 4*e*). It appears that the presence of Na^+ on the external side affects the open probability of the D-Ala channel dramatically, and thus, reversal of current was not detected. This behavior of the D-Ala mutant is different from the wild-type protein for which the presence of external Na^+ does not have such extreme effects on the open probability (22). The effect of external Na^+ on the D-Ala mutant is probably due to the structural changes in the

selectivity filter caused by the D-Ala substitution. The key notable observation here is that the D-Ala substitution results in a functional and selective (as measured for K^+ over Li^+) channel.

Conclusion

Glycine residues are absolutely conserved in the selectivity filter of K^+ channels. Here, we show that most of the functional characteristics of ion conduction through the KcsA K^+ channel could be recovered by replacement of Gly-77 with D-Ala, whereas replacement with L-Ala resulted in a nonfunctional channel. Based on these results, we conclude that the evolutionary driving force for glycine in the K^+-selectivity filter is for the maintenance of dihedral angles. These experiments support the idea that glycine functions as a surrogate D-amino acid in the signature sequence of K^+ channels.

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