Semisynthetic K⁺ channels show that the constricted conformation of the selectivity filter is not the C-type inactivated state

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Edited by Richard W. Aldrich, University of Texas at Austin, Austin, TX, and approved August 16, 2013 (received for review May 7, 2013)

C-type inactivation of K⁺ channels plays a key role in modulating cellular excitability. During C-type inactivation, the selectivity filter of a K⁺ channel changes conformation from a conductive to a non-conductive state. Crystal structures of the KcsA channel determined at low K⁺ or in the open state revealed a constricted conformation of the selectivity filter, which was proposed to represent the C-type inactivated state. However, structural studies on other K⁺ channels do not support the constricted conformation as the C-type inactivated state. In this study, we address whether the constricted conformation of the selectivity filter is in fact the C-type inactivated state. The constricted conformation can be blocked by substituting the first conserved glycine in the selectivity filter with the unnatural amino acid D-Alanine. Protein semisynthesis was used to introduce D-Alanine into the selectivity filters of the KcsA channel and the voltage-gated K⁺ channel K_vAP. For semisynthesis of the K_vAP channel, we developed a modular approach in which chemical synthesis is limited to the selectivity filter whereas the rest of the protein is obtained by recombinant means. Using the semisynthetic KcsA and K_vAP channels, we show that blocking the constricted conformation of the selectivity filter does not prevent inactivation, which suggests that the constricted conformation is not the C-type inactivated state.

The ability of K⁺ channels to selectively conduct K⁺ ions is accomplished by a structural unit called the selectivity filter (1). The selectivity filter consists of four K⁺ binding sites built using the main chain carbonyl oxygens and the threonine side chain from the protein sequence, which is typically T-V-G-Y-G (Fig. 1A) (2, 3). This sequence, referred to as the signature sequence, is highly conserved among K⁺ channels (2). The high degree of conservation of the signature sequence indicates a similar structure for the selectivity filter of all K⁺ channels, which is in fact observed in the K⁺ channel structures presently available (4).

In addition to ion discrimination, the selectivity filter participates in a gating process referred to as C-type inactivation, during which the channel transitions from the conductive state to a nonconductive state (5). C-type inactivation has been extensively investigated in voltage-gated K⁺ (K_v) channels and is observed on prolonged opening of K_v channels by a sustained membrane depolarization (4, 6). C-type inactivation is an effective mechanism to control K_v channel activity and to regulate action-potential frequency in an excitable cell (7). An inactivation process, which is similar to C-type inactivation, is also observed in K⁺ channels that do not belong to the K_v family, such as the bacterial K⁺ channel KcsA. The KcsA channel is gated by pH (8). A decrease in the intracellular pH causes channel opening by conformational changes at the bundle crossing of the pore lining helices. In the closed state, the bundle crossing of the pore lining helices acts as a barrier for the movement of ions across the membrane (9). Activation of the KcsA channel is followed by inactivation during which the current decreases (Fig. 1B) (10, 11). Inactivation in the KcsA channel is proposed

to be C-type as it shares a number of functional similarities with C-type inactivation in K_v channels (12–14). This similarity, coupled with the amenability of KcsA to structural studies, has made it an attractive system for elucidating the structure of the selectivity filter in the C-type inactivated state.

Models for the selectivity filter in the C-type inactivated state have been proposed based on structures of the KcsA channel at low K⁺ or in the open state. The selectivity filter of the KcsA channel undergoes a conformational change from the conductive state at high K⁺ to a nonconductive state at low K⁺ (Fig. 1C) (3, 15). In the low K⁺ conformation, there is a rotation around the Gly77–Val76 peptide bond that causes the α-carbon of Gly77 to twist inwards and constrict the pore. This rotation disrupts the second and third ion binding sites in the selectivity filter and renders the channel nonconductive (Fig. 1A, C, and D). As the rate of C-type inactivation increases at low K⁺, the conformation of the selectivity filter at low K⁺ was proposed to represent the C-type inactivated state (16). Recently, a series of structures with varying degrees of opening at the bundle crossing of the pore lining helices were obtained by using a constitutively open mutant of the KcsA channel (17). Higher degrees of opening at the bundle crossing (25–32 Å) were accompanied by a conformational change in the selectivity filter that was presumed to be nonconductive (Fig. 1D). This nonconductive conformation of the selectivity filter was proposed to represent the C-type inactivated state. The conformations of the selectivity filter in low K⁺ or in the open-channel structure are quite similar except

Significance

C-type inactivation is a conformational change at the selectivity filter, the ion binding site in a K⁺ channel that renders it non-conductive. C-type inactivation is important in modulating cellular excitability. Previous studies have suggested a “constricted conformation” for the selectivity filter in the C-type inactivated state. Here, we use protein semisynthesis to introduce unnatural amino acids into the selectivity filter to block it from attaining the constricted conformation. We show that blocking the constricted conformation does not affect C-type inactivation. This study therefore suggests that the constricted conformation of the selectivity filter is not the C-type inactivated state in a K⁺ channel. The study also highlights ways in which chemical synthesis can be used to manipulate large integral membrane proteins.

Author contributions: P.K.D., A.G.K., K.M., and F.I.V. designed research; P.K.D., A.G.K., C.A.C., J.J.D., K.M., and F.I.V. performed research; P.K.D., A.G.K., K.M., and F.I.V. analyzed data; and F.I.V. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1308699110/-DCSupplemental.

Fig. 1. The conductive and constricted conformations of the K^+ selectivity filter. (A) Close-up view of the selectivity filter of wild-type KcsA channel at high K^+ concentration $[K^+]$ (PDB ID code: 1k4c). Two diagonally opposite subunits are shown in stick representation. K^+ ions are shown as purple spheres. (B) Macroscopic currents of the wild-type KcsA channel elicited by a pH jump show inactivation. Currents were elicited at +100 mV by a rapid change of solution pH, at the arrow, from pH 7.5 (10 mM Hepes-KOH, 200 mM KCl) to pH 3.0 (10 mM succinate, 200 mM KCl). The selectivity filter of the KcsA channels at low $[K^+]$ (C, PDB ID code: 1k4d) and in the 32-Å open state (D, PDB ID code: 3f5w) show the constricted conformation. A rotation of the Val76–Gly77 bond causes constriction of the pore. The Gly77 C α –C α distance in the opposite subunits is 8.1 Å for the conductive conformation and 5.4–5.5 Å for the constricted conformation at low $[K^+]$ or in the 32-Å open state. (E) Structure of the selectivity filter of KcsA^{G77dA} at high $[K^+]$ (PDB ID code: 2ih3). (F) A hypothetical structure of the KcsA^{G77dA} selectivity filter in the constricted conformation. Two adjacent subunits are shown. The methyl side chain of D-Ala77 of one subunit and the carbonyl oxygen atoms of the Val76 and D-Ala77 in the adjacent subunit that clash are shown in van der Waals (VDW) representation. (G) Structure of the selectivity filter of KcsA^{G77dA} at low $[K^+]$ (PDB ID code: 2ih1). (H) Superposition of the selectivity filter of the KcsA^{G77dA} in high $[K^+]$ (blue) and low $[K^+]$ (red) shows that the D-Ala substitution in the selectivity filter blocks the constricted conformation.

for slight differences toward the lower half of the selectivity filter and the orientation of the Thr75 side chain. Due to their similarity, we jointly refer to these conformations as the “constricted” conformation of the selectivity filter. Changes in the conformation of the KcsA selectivity filter at low K^+ or low pH have also been detected by solution and solid-state NMR and are

consistent with the constricted conformation of the selectivity filter (18–20).

However, does the constricted conformation represent the selectivity filter in the C-type inactivated state? An important caveat of the structural studies is that the C-type inactivated state must be accurately captured by the conditions used for structure determination. Experimental validation is therefore necessary before the constricted conformation can be conclusively assigned as the C-type inactivated state. Here, we used unnatural amino acid mutagenesis to test whether the constricted conformation of the selectivity filter of the KcsA channel corresponds to the C-type inactivated state. We also used unnatural amino acid mutagenesis on the archaeobacterial K_v channel K_vAP, to test whether the constricted conformation is relevant during C-type inactivation in a K_v channel. We show that inactivation in the K_vAP channel is functionally similar to C-type inactivation in a eukaryotic K_v channel. To carry out unnatural amino acid mutagenesis, we developed a modular semisynthesis of the K_vAP channel that allowed us to use chemical synthesis to modify the selectivity filter. Our results on the KcsA and the K_vAP channels show that the constricted conformation of the selectivity filter is not the C-type inactivated state.

Results

Blocking the Constricted Conformation of the K^+ Selectivity Filter with a D-Ala Substitution.

The key difference between the conductive and the constricted conformation of the selectivity filter in the KcsA channel is a rotation around the Gly77–Val76 peptide bond (Fig. 1 A, C, and D). In the conductive state, Gly77 has a left-handed helical conformation. The left-handed helical conformation is not favorable for L-amino acids but is favorable for D-amino acids (21). It has been demonstrated that Gly77 plays the role of a surrogate D-amino acid by showing that substituting Gly77 with D-Ala (in contrast, to any L-amino acid substitution) results in a functional channel (22). X-ray crystallographic analysis showed that the structure of the selectivity filter in the G77dA mutant is identical to the wild-type channel except for the additional methyl group (due to the D-Ala substitution) (Fig. 1E) (23). D-Ala, however, has restricted conformational freedom compared with Gly (21). Therefore, the D-Ala substitution limits the conformational flexibility at this position. Importantly, the D-Ala substitution prevents the selectivity filter from attaining the constricted conformation because the D-Ala77 side chain will clash with the backbone carbonyl oxygen atoms of the adjacent subunit (Fig. 1F). Preventing the constricted conformation of the selectivity filter by the G77dA substitution has been demonstrated by solving the crystal structure of the KcsA D-Ala77 mutant at low K^+ (1 mM) (Fig. 1G) (23). Under these conditions, the selectivity filter of the wild-type channel adopts the constricted conformation whereas the selectivity filter in the D-Ala mutant remains in the conductive conformation (Fig. 1H). The D-Ala substitution therefore allows us to test whether the constricted conformation of the selectivity filter corresponds to the C-type inactivated state. If the constricted conformation corresponds to the C-type inactivated state, we expect that the process of C-type inactivation should be blocked or substantially altered in the D-Ala mutant channel.

The Gly77→D-Ala Substitution in the KcsA Channel Does Not Block Inactivation.

We used semisynthesis to generate KcsA channels with a G77dA substitution (KcsA^{G77dA}) or the wild-type selectivity filter (KcsA^{WT}) (Fig. S1) (22, 23). Briefly, the semisynthesis uses native chemical ligation (NCL) to assemble the KcsA polypeptide from a recombinant N-terminal fragment (residues 1–69) and a synthetic C-terminal fragment (residues 70–123) with either the G77dA substitution or the wild-type sequence. In the NCL reaction, a peptide with a C-terminal thioester reacts with a peptide with an N-terminal Cys, resulting in linking the two

Fig. 2. Effect of D-Ala77 substitution on inactivation in the KcsA channel. (A) Macroscopic currents for the KcsA channels were elicited at +100 mV by a jump from pH 7.5 to pH 3.0. The initial 10 s of the decay phase following peak current was fit with a single exponential to obtain τ_{inact} . (B) Single KcsA channel currents recorded at +100 mV in steady-state conditions at pH 3.0. The KcsA WT, G77dA, and G77dA + E71A channels were obtained by semisynthesis whereas the KcsA-E71A channel was obtained by recombinant expression. The KcsA channels used also contain the following amino acid substitutions: S69A, V70C (at the ligation site), Q58A, T61S, R64D (to confer AgTx₂ sensitivity), and A98G (to increase the open probability of KcsA) (S2 Text, Table S1). The pH 3.0 solution is 10 mM succinate, 200 mM KCl whereas the pH 7.5 solution is 10 mM Hepes-KOH, 200 mM KCl.

peptides with a native peptide bond (24). The ligation product obtained by the NCL reaction is folded to the native tetrameric state using lipid vesicles. We purified the semisynthetic KcsA channels and reconstituted them into lipid vesicles for recording channel activity. For measurements of inactivation, we elicited macroscopic currents by a rapid change in the intracellular pH (Fig. 2A) (10). On a pH jump, the KcsA^{WT} channels quickly activate as indicated by a rapid increase in current and then slowly inactivate as indicated by the decay in current. The activation and inactivation phases can be fit with single exponentials to get an activation time constant of 140 ± 67 ms ($n = 12$) and an inactivation time constant (τ_{inact}) of 2.76 ± 1.04 s for KcsA^{WT}. The KcsA^{G77dA} channels behaved similarly to KcsA^{WT} and activated with a time constant of 69 ± 69 ms ($n = 10$) followed by inactivation with a τ_{inact} of 2.2 ± 0.8 s. In the steady state at low intracellular pH, KcsA has a low open probability (P_o) as it resides mainly in the inactivated state (10). With the intracellular pH at 3.0, single-channel measurements on the KcsA^{WT} channel indicated a P_o of 0.17 ± 0.04 ($n = 4$) and a mean open time (τ_o) of 23 ± 11 ms ($n = 3$) at +100 mV (Fig. 2B). The higher P_o observed in this study, compared with previous reports, is due to the presence of the A98G substitution (25). The KcsA^{G77dA} channels had a P_o of 0.25 ± 0.1 ($n = 7$) and a τ_o of 21 ± 15 ms ($n = 3$). These macroscopic and single-channel measurements show similar functional properties for KcsA^{WT} and KcsA^{G77dA} channels, indicating that the D-Ala77 substitution does not affect inactivation.

Inactivation in KcsA is substantially reduced by substitution of Glu71 with Ala (10). We observed that incorporation of the E71A substitution into both the KcsA^{WT} and KcsA^{G77dA} channels resulted in a decrease in the rate of inactivation (Fig. 2A). Single-channel measurements showed a substantial increase in P_o for both the KcsA^{WT} and the KcsA^{G77dA} with the E71A substitution (0.65 ± 0.12 , $n = 2$ for KcsA^{E71A} and 0.57 ± 0.22 , $n = 6$ for KcsA^{E71A+G77dA} at +100 mV) (Fig. 2B). The similar effect of the E71A substitution indicates a similar mechanism of inactivation in the KcsA^{WT} and KcsA^{G77dA} channels.

Recovery from inactivation in the KcsA channel takes place after closure of the activation gate by increasing the intracellular pH (26). To study recovery from inactivation, we used a double pH pulse protocol (Fig. S2). The recovery from inactivation profiles observed for the KcsA^{WT} and KcsA^{G77dA} channels is similar (time constants for recovery 15 ± 5.0 s, $n = 4$ for KcsA^{WT} and 17.5 ± 8.0 s, $n = 3$ for KcsA^{G77dA}) (Fig. S2), which indicates that the G77dA substitution does not affect recovery of the channel from the inactivated state.

Our experiments do not reveal any significant differences in the inactivation process in the KcsA^{G77dA} and the KcsA^{WT} channels. As the selectivity filter in the KcsA^{G77dA} channels cannot attain the constricted conformation, our results demonstrate that the constricted conformation of the selectivity filter does not correspond to the C-type inactivated state in the KcsA channel.

Inactivation in the K_vAP Channel. Next, we investigated whether the constricted conformation of the selectivity filter is relevant during inactivation in a K_v channel. For these studies, we used K_vAP, an archaebacterial K_v channel (27). K_vAP channels activate on depolarization and then undergo inactivation (Fig. 3A) (27). To confirm involvement of the selectivity filter of the K_vAP channel (Fig. 3B) in inactivation, we investigated the effect of permeant ions and amino acid substitutions. We observed that the rate of inactivation in the K_vAP channel depends on K⁺ concentration and that τ_{inact} decreases from 428 ± 123 ms ($n = 26$) to 161 ± 38 ms ($n = 10$) when the K⁺ concentration is reduced from 150 to 8 mM (Fig. 3C). In contrast, changing the permeant ion from K⁺ to Rb⁺ slows the rate of inactivation and τ_{inact} increases to $1,802 \pm 556$ ms ($n = 7$) (Fig. 3C). Inactivation is also influenced by amino acid substitutions around the selectivity

Fig. 3. Inactivation in the K_vAP channel. (A) K_vAP currents elicited by depolarization to +100 mV in 150 mM KCl and 10 mM Hepes-KOH (pH 7.5) show inactivation. (B) Close-up view of the selectivity filter of the K_vAP channel (PDB ID code: 1orq). Two diagonally opposite subunits are shown in stick representation. K⁺ ions are shown as purple spheres. Residues V192 and Y199 in the K_vAP channel (equivalent to V438 and Y445 in the Shaker B K⁺ channel) are indicated. (C) Normalized currents for the wild-type K_vAP channel elicited by depolarization to +100 mV in 8 mM and 150 mM KCl with 10 mM Hepes-KOH (pH 7.5) and 150 mM RbCl with 10 mM Hepes-RbOH (pH 7.5). (D) Normalized currents for the wild-type, Y199F, and V192A K_vAP channels by depolarization to +100 mV in 150 mM KCl. All of the recordings were carried out in (3:1) POPE (1-palmitoyl-2-oleoylglycerol-3-phosphoethanolamine):POPG (1-palmitoyl-2-oleoylglycerol-3-phosphoglycerol) lipid bilayers with a holding potential of −100 mV.

filter, with significantly faster inactivation in K_vAP mutants with a Y199F ($\tau_{inact} = 95 \pm 25$, $n = 16$) or a V192A ($\tau_{inact} = 73 \pm 11$, $n = 11$) substitution (Fig. 3D). The effects of permeant ions and amino acid substitutions establish the involvement of the selectivity filter of the K_vAP channel in inactivation. Further, they mirror the effects of permeant ions and amino acid substitutions on C-type inactivation in eukaryotic K_v channels, like the Shaker channel, indicating that the inactivation in K_vAP is similar to C-type inactivation in a eukaryotic K_v channel (28–30).

Modular Semisynthesis of the K_vAP Channel. To test the role of the constricted conformation in inactivation of the K_vAP channel, we need to introduce D-Ala into the selectivity filter (Fig. S3). This endeavor requires the ability to use chemical synthesis to modify the selectivity filter. The size of the K_vAP channel (282 residues) and the location of the selectivity filter (residues 196–200) precluded the use of the two-fragment strategy that we used for the semisynthesis of the KcsA channel, as this calls for the synthesis of an ~90- to 100-aa peptide. Chemical synthesis is only efficient for peptides ~50–60 aa in length (31). Accordingly, we devised a three-fragment modular approach for the semisynthesis of the K_vAP channel. In our synthetic design, the selectivity

filter is obtained by chemical synthesis whereas the remainder of the protein is made by recombinant means (Fig. 4A). The K_vAP polypeptide is assembled from a synthetic selectivity filter peptide and the two recombinant peptides by two sequential NCL reactions. Following assembly, the K_vAP polypeptide is purified and then folded to the native tetrameric state to provide semisynthetic K_vAP channels. In this approach, using peptide synthesis for the selectivity filter allows us to incorporate a wide variety of unnatural amino acids and peptide backbone modifications into the selectivity filter whereas using recombinant means for obtaining the N- and C-peptides liberates us from the size limits of peptide synthesis and allows us to tackle relatively large integral membrane proteins like the K_vAP channel.

To test the synthetic approach, we assembled K_vAP channels with the wild-type selectivity filter sequence. We selected residues 191 and 211, which flank the selectivity filter, as the ligation sites because the Cys substitutions that are required for the NCL reaction are well tolerated at these positions. The K_vAP polypeptide was assembled from a recombinant thioester peptide (residues 1–190, S1-5 peptide), a synthetic filter peptide (residues 191–210, P), and a recombinant N-terminal Cys peptide (residues 211–282, S6 peptide). For the NCL reactions, the

Fig. 4. Modular semisynthesis of the K_vAP channel. (A) The K_vAP polypeptide is assembled by two sequential NCL reactions. In the first NCL reaction, a recombinantly expressed N-Cys peptide is ligated to a chemically synthesized filter peptide thioester to yield the intermediate peptide. The Thz protecting group (pink sphere) on the N-terminal Cys of the intermediate peptide is removed; the deprotected intermediate peptide is purified and then ligated to a recombinantly expressed thioester peptide to yield the K_vAP polypeptide. The K_vAP polypeptide is purified and folded in vitro to the native state. The protein segment obtained by chemical synthesis is colored red whereas the protein segments obtained by recombinant means are colored gray. The ligation sites are represented by yellow circles or spheres. (B) SDS/PAGE of the first NCL reaction between the recombinant N-Cys peptide (S6, residues 211–282) and the filter peptide (P, residues 191–210) to form the intermediate peptide (P+S6, residues 191–282) at 0 min (lane 1) and 2 h (lane 2). (C) SDS/PAGE of the second ligation reaction between the S1-5 thioester (residues 1–190) and the intermediate peptide to form the K_vAP polypeptide at 0 min (lane 1) and 1 h (lane 2). (D) Size-exclusion chromatography of the semisynthetic K_vAP channel. Inset shows glutaraldehyde cross-linking of the peak fraction: lane 1, without cross-linker; lane 2, cross-linked with 0.1% glutaraldehyde. The oligomeric nature (1X, 2X, and 4X) of the cross-linked bands is indicated. (E) Single-channel trace for the semisynthetic K_vAP channel recorded at +100 mV. The single-channel current as a function of voltage for the semisynthetic (triangles, $n = 16$) and native (circles, $n = 11$) K_vAP channels. Data are represented as mean $\pm$ SD. (F) Voltage-activated macroscopic currents from the semisynthetic K_vAP channel recorded using the voltage protocol shown (Inset). (G) Voltage-dependent gating of the semisynthetic (triangles) and native (circles) K_vAP channels. Tail currents were recorded after the test voltage pulse by stepping to -100 mV. The fraction of the maximal current observed was plotted as a function of the test potential. The smooth line corresponds to a Boltzmann function with a $V_{0.5}$ of -40 mV and a z of 1.65. The recordings shown in E and F were conducted on K_vAP channels reconstituted into planar lipid bilayers composed of 1, 2-diphytanoylglycero-3-phosphocholine using symmetrical 150 mM KCl and 10 mM Hepes-KOH (pH 7.5).

synthetic filter peptide bears both an N-Cys and a C-terminal thioester. The K_v AP polypeptide was assembled in the C to N direction by two sequential NCL reactions (Fig. 4B and C and Fig. S4). Following assembly, the K_v AP polypeptide was purified and then folded in vitro at elevated temperatures using lipid vesicles as previously described (32). The folded semisynthetic K_v AP channels were purified (Fig. 4D) and reconstituted into planar lipid bilayers for measurement of channel activity. Single channel openings for the semisynthetic K_v AP channels are shown (Fig. 4E). The single-channel conductance (125 ± 7 pS, $n = 16$ for the semisynthetic channel versus 126 ± 8 pS, $n = 11$ for the native channel at $+100$ mV in 150 mM K^+) and the current-voltage curve for the semisynthetic K_v AP channel are similar to the native channel (Fig. 4E). We examined the K^+/Na^+ selectivity of the semisynthetic K_v AP channel by measuring the reversal potential of macroscopic tail currents with 200 mM K^+ in the internal solution and 20 mM K^+ and 180 mM Na^+ in the external solution. The measured reversal potential for the semisynthetic K_v AP channels is -43.0 ± 4.5 mV ($n = 4$), which is similar to the value of -43.8 ± 3.8 mV ($n = 3$) determined for the native K_v AP channels, indicating that selectivity of the semisynthetic channels is similar to the native channels. Voltage dependence of channel opening for the semisynthetic K_v AP channel resembled the native channel, with similar values for $V_{0.5}$, the voltage for half maximal opening, and z , the apparent gating charge ($V_{0.5} = -40.0 \pm 9.4$ mV, $z = 1.7 \pm 0.1$, $n = 4$ for the semisynthetic channel and $V_{0.5} = -40.8 \pm 5.2$ mV, and $z = 1.8 \pm 0.3$, $n = 4$ for the native channel) (Fig. 4F and G). These measurements indicate that the semisynthetic K_v AP channel is essentially similar to the native channel.

The Gly198→D-Ala Substitution in the K_v AP Channel Does Not Block Inactivation. Using the modular semisynthesis, we substituted the first conserved Gly in the selectivity filter of the K_v AP channel (Gly198) with D-Ala. For efficient folding of the K_v AP^{G198dA} mutant channels, it was necessary to substitute Val192 with Ala to prevent a steric clash between the D-Ala198 and Val192 side chains (Fig. S5). The semisynthesis provided the K_v AP^{G198dA} channels in good yields, and the D-Ala mutant channel had similar biochemical properties to the wild-type channel (Fig. S5). Single-channel activity and the current voltage curve for K_v AP^{G198dA} and the wild-type control channel (with the two Cys substitutions V191C and V211C for the ligation sites along with the V192A substitution) are shown in Fig. 5A. Reversal potential measurements for K_v AP^{G198dA} with 150 mM K^+ in the internal solution and 15 mM K^+ and 135 mM Na^+ in the external solution gave a value of -39.3 ± 2.3 mV ($n = 3$), similar to the native channel, indicating that the G198dA substitution does not affect K^+/Na^+ selectivity. Voltage-dependent gating of the K_v AP^{G198dA} channels was also similar to the native channels (Fig. S5). These results indicate that the G198dA substitution in the K_v AP channel does not affect ion conduction or voltage-dependent gating.

To test for inactivation, we elicited macroscopic currents for K_v AP^{G198dA} by depolarization to $+100$ mV from a resting potential of -100 mV (Fig. 5B). We observed inactivation in K_v AP^{G198dA} channels indicating that the D-Ala substitution in the selectivity filter does not block inactivation. Analysis of the inactivation profile gave a τ_{inact} of 162 ± 44 ms ($n = 28$) for K_v AP^{G198dA} compared with 71 ± 12 ms ($n = 22$) for the wild-type control channel. We investigated recovery from inactivation using a paired pulse protocol and observed similar recovery profiles for K_v AP^{G198dA} and the control channels (Fig. 5C and D). D-Ala substitution in the selectivity filter blocks the constricted conformation (Fig. S3) but does not block inactivation. Further, the inactivation properties of K_v AP^{G198dA} are quite similar to the wild-type control, indicating that the constricted conformation of the selectivity filter does not correspond to the inactivated state in the K_v AP channel.

Fig. 5. Effect of D-Ala198 substitution on inactivation in the K_v AP channel. (A) The single-channel current as a function of voltage for the K_v AP^{G198dA} (circles, $n = 13$) and the control (squares, $n = 3$) K_v AP channels. (Inset) A single-channel trace for the K_v AP^{G198dA} channel recorded at $+100$ mV. The control K_v AP channels contain the V191C, V192A, and V211C amino acid substitutions, and K_v AP^{G198dA} contains the G198dA substitution in addition. (B) Normalized currents for the K_v AP^{G198dA} and the control channels elicited by a depolarization to $+100$ mV from a holding potential of -100 mV. (C) Recovery from inactivation. Macroscopic currents for K_v AP^{G198dA} were elicited by two 5-s pulses (black first, gray second) to 100 mV, separated by holding at -100 mV for a variable duration (from 1 to 120 s). The fractional recovery was measured by the ratio of the peak current in the second pulse to the peak current in the first pulse. (D) Fractional recovery plotted as a function of the interpulse duration for K_v AP^{G198dA} (circles, $n = 5$) and the control channel (squares, $n = 5$). Solid lines represent single exponential fits used to obtain the time constant for recovery from inactivation (39.9 s for K_v AP^{G198dA} and 29.0 s for the wild-type control). The recordings shown in A–C were conducted on K_v AP channels reconstituted into planar lipid bilayers composed of (3:1) POPE:POPG using symmetrical 150 mM KCl and 10 mM Hepes-KOH (pH 7.5). For A and D, data are presented as mean $\pm$ SD.

Discussion

C-type inactivation is a gating mechanism at the selectivity filter of K^+ channels that results in shutting off the flow of ions through the channel. The conformational changes in the selectivity filter during C-type inactivation have not yet been fully understood. Structures of the KcsA channel determined under conditions that favor inactivation show a constricted conformation, which has been proposed to represent the C-type inactivated state. In this study, we evaluated whether this constricted conformation represents the C-type inactivated state. We blocked the constricted conformation of the selectivity filter by substituting the first conserved Gly with D-Ala and determined the effect of this substitution on inactivation.

We used protein semisynthesis to introduce D-Ala into the selectivity filter of the KcsA channel. To test whether the constricted conformation was relevant in C-type inactivation of a K_v channel, we carried out a similar D-Ala substitution in the K_v AP channel. For both the KcsA and the K_v AP channels, we observed that blocking the constricted conformation of the selectivity filter with the D-Ala substitution does not block inactivation. Further, the rates of inactivation and recovery in the D-Ala mutants of the KcsA and K_v AP channels were similar to the corresponding wild-type control channels. Our results therefore demonstrate that the constricted conformation of the selectivity filter does not correspond to the inactivated state in the KcsA and K_v AP channels.

Structural studies on K^+ channels that undergo C-type inactivation (other than the KcsA channel) also question whether the constricted conformation is the C-type inactivated state. The crystal structures reported for the K_v AP channel were determined under conditions that favor the open-inactivated state (33,

34). Correspondingly, these structures show that the inner helix bundle crossing in the pore is in an open conformation but do not show a constricted conformation for the selectivity filter. Similarly, the $K_v1.2$ and the chimeric $K_v1.2-2.1$ channels were crystallized under conditions that favor the open-inactivated state, but the structures do not show the constricted conformation for the selectivity filter (35, 36). The Ca^{2+} -activated K^+ channel MthK has been shown to undergo a gating process at the selectivity filter similar to C-type inactivation (37, 38). The structures of the selectivity filter of the MthK channel in the open state or in the absence of K^+ also do not show the constricted conformation (39). These studies do not support the constricted conformation as representing the inactivated state and also question whether the constricted conformation seen in the KcsA channel is observed in other K^+ channels. We speculate that the constricted conformation of the selectivity filter corresponds not to the C-type inactivated state but to the long-lived non-conducting state, referred to as the defunct state that has been described in certain K^+ channels on removal of K^+ (7).

What then is the structure of the selectivity filter in the C-type inactivated state? Our experiments indicate that a D-Ala substitution in the selectivity filter does not affect inactivation. The D-Ala substitution reduces the conformational freedom at the first Gly in the K^+ selectivity filter. The lack of a significant effect of the D-Ala substitution on inactivation suggests that the selectivity filter must have only modest conformational changes at this Gly on inactivation. One possibility is that the C-type inactivated state is an intermediate between the conducting conformation of the selectivity filter (observed in the 17-Å open

structure of KcsA) and the constricted conformation (observed in the 25-Å open structure) (17). Further structural studies, complemented by functional measurements, will be required to unequivocally define the conformation of the selectivity filter in the C-type inactivated state.

This study demonstrates the utility of semisynthesis for protein structure function investigations. The synthetic strategy used in this study for modifying the selectivity filter of the K_v AP channel can be easily extended to manipulate other functionally important regions such as the S4 helix or the S4-5 linker. We also anticipate that this modular semisynthesis approach will be applicable to other integral membrane proteins.

Materials and Methods

Semisynthesis of the KcsA channels was carried out using the NCL reaction as described (40). Electrophysiological measurements on the KcsA channels were carried out using giant liposome patch clamp (41). The semisynthetic K_v AP channels were assembled from a recombinant S1-5 thioester peptide, a synthetic filter peptide, and a recombinant N-Cys containing S6 peptide by NCL reactions. The semisynthetic K_v AP channels were folded in vitro at elevated temperatures using lipid vesicles as previously described (32). Functional measurements on the K_v AP channels were carried out using planar lipid bilayers as previously described (27). Detailed descriptions of materials and methods are provided in *SI Materials and Methods*. Data are presented as mean $\pm$ SD.

ACKNOWLEDGMENTS. We thank Drs. David Dawson and Eric Gouaux for advice on the manuscript. This research was supported by National Institutes of Health (NIH) Grant GM087546, American Heart Association Scientist Development Grant 0835166N, and a Pew Scholar Award (to F.I.V.). A.G.K. was supported by NIH National Research Service Award GM087852.

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