The N-Terminus of the K Channel KAT1 Controls Its Voltage-Dependent Gating by Altering the Membrane Electric Field

Irene Marten and Toshinori Hoshi
Department of Physiology and Biophysics, College of Medicine, The University of Iowa, Iowa City, Iowa 52242 USA

ABSTRACT Functional roles of different domains (pore region, S4 segment, N-terminus) of the KAT1 potassium channel in its voltage-dependent gating were electrophysiologically studied in *Xenopus* oocytes. The KAT1 properties did not depend on the extracellular K^+ concentration or on residue H267, equivalent to one of the residues known to be important in C-type inactivation in *Shaker* channels, indicating that the hyperpolarization-induced KAT1 inward currents are related to the channel activation rather than to recovery from inactivation. Neutralization of a positively charged amino acid in the S4 domain (R176S) reduced the gating charge movement, suggesting that it acts as a voltage-sensing residue in KAT1. N-terminal deletions alone (e.g., Δ20–34) did not affect the gating charge movement. However, the deletions paradoxically increased the voltage sensitivity of the R176S mutant channel, but not that of the wild-type channel. We propose a simple model in which the N-terminus determines the KAT1 voltage sensitivity by contributing to the electric field sensed by the voltage sensor.

INTRODUCTION

The KAT1 potassium channel cloned from *Arabidopsis thaliana* belongs to the *eag* family of ion channel proteins (Anderson et al., 1992; Trudeau et al., 1995), which includes the human *eag*-related HERG channel involved in a genetic cardiac disorder (Curran et al., 1995; Sanguinetti et al., 1995). When the KAT1 channels are expressed in *Xenopus* oocytes, a time-dependent increase in the inward potassium currents is observed upon hyperpolarization (Anderson et al., 1992; Schachtman et al., 1992). Structurally, as with other voltage-gated *Shaker*-like K^+ channel α-subunits, each KAT1 subunit appears to contain at least six putative transmembrane segments, including the S4 segment with several positively charged amino acid residues.

The *Shaker*-like K^+ channels from the animal kingdom activate upon depolarization and subsequently inactivate via at least two biophysically and molecularly distinct inactivation mechanisms called N- and C-type inactivation. Recovery from these inactivation mechanisms is facilitated by hyperpolarization (Jan and Jan, 1997). Based on the numerous experiments on K^+ channels of the *Shaker* superfamily, several functional domains of ion channel proteins have been identified. The S4 domain, with its positively charged residues, is important for sensing changes in the membrane electric field (Sigworth, 1993; Larsson et al., 1996; Mannuzzu et al., 1996; Seoh et al., 1996). Triple mutations in the S4 domain of *Shaker* channels could also promote N-type inactivation, such that an apparent conversion of an outward to an inward rectifier is observed (Miller and Aldrich, 1996). The N-terminus contains structural motifs that support subunit assembly and N-type inactivation (Hoshi et al., 1990; Li et al., 1992; Aldrich, 1994; Rettig et al., 1994). Amino acids in the extracellular vestibule of the pore and in the S6 segment are known to control C-type inactivation (Hoshi et al., 1991; Lopez-Barneo et al., 1993; Ogielska et al., 1995). For instance, substitution of threonine at position 449 in *Shaker* K^+ channels with amino acids with a large hydrophobic side chain (T449V or T449Y) markedly disrupts C-type inactivation (Lopez-Barneo et al., 1993). Recovery from C-type inactivation accounts for the currents observed through the HERG channels upon hyperpolarization in part because certain mutations in the P-segment and external K^+ critically affect the HERG currents (Smith et al., 1996; Schönherr and Heinemann, 1996).

In our previous paper we demonstrated that the voltage sensitivity of the KAT1 channel expressed in *Xenopus* oocytes is modulated by its C terminus, with longer C-terminal deletions resulting in less voltage sensitivity (Marten and Hoshi, 1997). Thus, in addition to the putative voltage-sensing transmembrane segments, the C-terminal domain contributes to the voltage sensitivity of the KAT1 channel. Furthermore, we found that the N-terminus of the KAT1 channel regulates the voltage dependence of KAT1. This novel role of the N-terminus as a determinant of the voltage-dependent gating behavior is now beginning to be investigated, especially in the *eag* family of ion channels (Schönherr and Heinemann, 1996; Terlau et al., 1997).

In this work we electrophysiologically examined the function of different channel domains (pore region, S4 segment, and N-terminus) in voltage-dependent gating of the KAT1 channel. The data do not support the idea that N- and C-type inactivation as known from the *Shaker* superfamily contributes to the gating mechanism of KAT1. We further show that the KAT1 N-terminus has a novel role in determining the channel's voltage sensitivity. We propose a model in which the N-terminus of KAT1 may influence the

Received for publication 23 December 1997 and in final form 3 March 1998.

Address reprint requests to Dr. Toshinori Hoshi, University of Iowa College of Medicine, Department of Physiology and Biophysics, Bowen Science Building 5–452, Iowa City, IA 52242. Tel.: 319-335-7845; Fax: 319-335-7330; E-mail: toshinori-hoshi@uiowa.edu.

Dr. Marten's present address is Institut für Biophysik, Universität Hannover, Herrenhäuserstrasse 2, 30419 Hannover, Germany.

0006-3495/98/06/2953/10 $2.00

voltage-sensing function of the S4 segment by forming a part of the S4 canal.

MATERIALS AND METHODS

Molecular biology

KAT1 channel mutants were generated by the standard polymerase chain reaction-based cassette mutagenesis. Sequences of the polymerase chain reaction-amplified segments were verified by the DNA core facility of the University of Iowa. LaserGene programs (Madison, WI) were used to analyze DNA and protein sequences. The oocytes were prepared and injected with RNAs, essentially as described by Hoshi (1995).

Electrophysiology

Whole-oocyte recordings were performed with a two-electrode voltage-clamp amplifier (725B or 725C; Warner Instruments, Hamden, CT). Glass microelectrodes were filled with 3 M KCl, 10 mM HEPES (pH 7.2), and had a typical input resistance of less than 0.5 MΩ. The electrophysiological measurements were performed at 20–22°C in the presence of 50 mM KCl, 90 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.2). The pH was adjusted with *N*-methyl-D-glucamine (NMG). Other extracellular solutions used are noted in the figure legends.

Data were digitized with an ITC-16 interface card (Instrutech, Great Neck, NY) and stored for later analysis. Pulse/PulseFit (HEKA elektronik, Lambrecht, Germany), Igor Pro (WaveMetrics, Lake Oswego, OR), and DataDesk (Data Description, Ithaca, NY) running on Apple Power Macintosh computers were used for voltage stimulation, data acquisition, and analysis. Leak currents were determined by using a modified *P*/*n* procedure and subtracted from the macroscopic currents presented.

The analysis of the deactivation kinetics and determination of the $G(V)$ curves were performed as described by Marten and Hoshi (1997). The $G(V)$ curves were calculated using the tail current amplitude recorded at a constant deactivating voltage pulse after a series of hyper- and depolarizing voltage pulses. The macroscopic conductance data points were fitted with a simple Boltzmann distribution, as given by the following function: $G(V) = [1/(1 + \exp((V - V_{1/2})zF/RT)]$, where R, T, and F have the usual meanings, z represents the number of equivalent charges, and $V_{1/2}$ is the half-activation voltage, at which 50% of the maximum conductance level is reached.

If not otherwise mentioned, results are presented as mean ± standard deviation (n = number of experiments). Error bars in figures represent standard deviation.

RESULTS

Extracellular [K^+] and the H267S mutation do not markedly affect KAT1 kinetics

In *Shaker*-like K^+ channels, C-type inactivation has been shown to depend on extracellular K^+, external TEA (tetraethylammonium$^+$), and certain amino acid residues in the pore region (Choi et al., 1991; Hoshi et al., 1991; Lopez-Barneo et al., 1993; Levy and Deutsch, 1996). For instance, high extracellular [K^+] slows down C-type inactivation (Lopez-Barneo et al., 1993) and accelerates recovery (Levy and Deutsch, 1996). Although some mammalian channels in the *eag* family are known to conduct ionic current upon hyperpolarization, the hyperpolarization-induced HERG inward currents have been shown to represent recovery from fast C-type inactivation (Schönherr and Heinemann, 1996; Smith et al., 1996; Spector et al., 1996). Thus, if the hyperpolarization-induced KAT1 currents are also related to recovery from C-type inactivation, the time course of the KAT1 currents should depend on extracellular K^+ and be altered by mutations previously shown to affect C-type inactivation in *Shaker*-like channels, as found for the HERG channels.

We tested this hypothesis by comparing the time courses of KAT1 currents in the presence of different external K^+ concentrations. Fig. 1, *A* and *B*, shows KAT1 currents

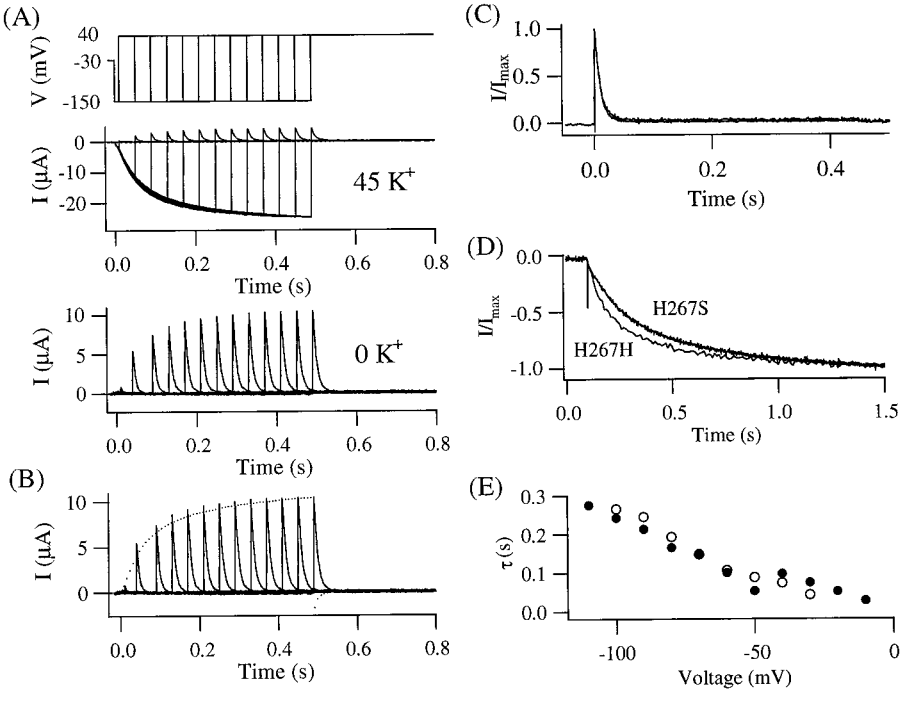

FIGURE 1 Dependence of KAT1 activation/deactivation on extracellular [K^+] and on the H267S mutation. (*A*) The envelope of peak tail currents was recorded in 45 mM K^+ and 0 mM K^+ in the bath solution at +40 mV after a series of KAT1-activating prepulses to −150 mV for different durations. The voltage pulse protocol is shown at the top. (*B*) The inward current trace from *A*, recorded with 45 mM K^+ by a 500-ms prepulse to −150 mV, was inverted, scaled (*dotted line*), and superimposed on the envelope of tail currents recorded in 0 mM K^+. (*C*) Tail currents obtained at +40 mV with 0 and 45 mM K^+ were normalized and superimposed. The bath solutions in *A–C* contained 45 mM KCl (or 45 mM NMG), 95 mM NMG, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2 adjusted with HCl. (*D*) Normalized inward currents induced by a voltage step to −150 mV are shown for the wild-type (H267H) and H267S channels. (*E*) Deactivation time constants of the wild-type (○) and H267S channel (●) are given as a function of voltage.

elicited by hyperpolarization pulses to −150 mV for different durations, with 0 mM K^+ (no K^+ added) and 45 mM K^+ in the extracellular medium. Because no appreciable inward currents were measured with 0 mM K^+, the time course of KAT1 current was estimated by the envelop of outward tail currents at +40 mV. We found that time courses of the KAT1 currents recorded with 0 mM and 45 mM K^+ upon hyperpolarization were indistinguishable (Fig. 1 *B*). If the hyperpolarization-induced K^+ currents represent recovery from C-type inactivation, then the apparent tail current recorded upon depolarization represents in part the time course of inactivation rather than deactivation. This hypothesis predicts that high extracellular K^+ should slow down the apparent tail current time course. We found that the KAT1 tail currents at depolarized voltage were not affected by external K^+ (Fig. 1 *C*). Similar results were obtained when extracellular pH was lowered to 5.6 to increase the likelihood that the histidine at position 267 in the external mouth of the pore segment was protonated. Thus, extracellular [K^+] does not regulate the apparent activation or deactivation time course of the KAT1 channel, suggesting that extracellular K^+-sensitive C-type inactivation may not be involved in the KAT1 channel gating.

In addition to the ionic current time course, high extracellular [K^+] is also known to increase the amplitude of the macroscopic outward current through channels with strong C-type inactivation by increasing the number of channels available to open by facilitating recovery, even though higher extracellular K^+ actually decreases the driving force (Pardo et al., 1992; Lopez-Barneo et al., 1993). Schönherr and Heinemann (1996) further showed that extracellular Cs^+ is even more effective than K^+ in enhancing the functional availability of the HERG channels that show very fast C-type inactivation. In contrast, as described by Véry et al. (1995) and shown in Fig. 1 *A*, the outward KAT1 tail current amplitudes did not depend on extracellular K^+ and changed with the K^+ driving force. When 140 mM KCl was replaced by 140 mM CsCl (data not shown), the outward tail current amplitudes were indistinguishable, suggesting that high extracellular [Cs^+] did not facilitate recovery from C-type inactivation (cf. Véry et al., 1995). These results suggest that the gating mechanisms of HERG and KAT1 may be different.

The amino acid position 449 in the *Sh*B channel regulates C-type inactivation (Lopez-Barneo et al., 1993). In particular, the T449S mutation accelerates C-type inactivation, decreasing its time constant from a couple of seconds to ~100 ms (Schlief et al., 1996), and slows down the rate of recovery from inactivation (Hoshi, unpublished observation). In contrast, C-type inactivation is almost removed in *Sh*BΔ6–46:T449H (Lopez-Barneo et al., 1993). Based on the amino acid sequence similarity, histidine at position 267 in KAT1 is equivalent to position 449 in *Sh*B. If the apparent activation of KAT1 channels upon hyperpolarization represents recovery from inactivation as found for HERG channels, the apparent deactivation of the KAT1 H267S channel upon depolarization should be faster, and its apparent activation upon hyperpolarization should be slower than in the KAT1 wild-type channel. To test this prediction, kinetics of the KAT1 wild-type and H267S channels were compared. The apparent activation time courses of the H267S and wild-type KAT1 channels did not markedly differ (Fig. 1 *D*). Deactivation time constants of the wild-type and mutant channels were similar at all of the voltages examined (Fig. 1 *E*).

Because external [K^+], external [Cs^+], and a mutation known to affect C-type inactivation did not markedly affect the KAT1 channel properties, distinct mechanisms are likely to be responsible for the hyperpolarization-induced currents in the HERG and KAT1 channels.

The S4 mutation R176S affects the voltage-dependent gating of KAT1

As in the *Shaker* channels, the S4 segment of KAT1 contains several positively charged residues, indicating that it could function as a voltage-sensing domain (Fig. 2 *A*). To further examine the voltage-dependent gating mechanism of KAT1, we introduced several mutations in the S4 segment of the KAT1 channel, based on the sequence comparison of the KAT1 and *Shaker* S4 segments. Representative data obtained from the R176S mutant in response to hyperpolarizing voltage pulses are shown in Fig. 2 *B*. Time-dependent macroscopic inward currents with fast deactivation kinetics were elicited, indicating functional expression of the R176S channel (Fig. 2 *B*). Normalized macroscopic conductance-voltage ($G(V)$) curves were estimated from the tail current measurements and fitted with a simple Boltzmann function (Fig. 2 *C*). We found that the R176S mutation, which removes one positive charge in the S4 segment, affected both the half-activation voltage and the steepness of the $G(V)$ curve. The R176S mutation shifted the half-activation voltage ($V_{1/2}$) to a more positive voltage by almost +40 mV ($V_{1/2\ WT} = -118.9 \pm 1.9$ mV; $V_{1/2\ R176S} = -80.5 \pm 13.1$ mV; two-sample *t*-test: $p = 0.0002$) and reduced the equivalent charge movement (z) by 40% from 1.7 to 1.0 ($z_{WT} = 1.71 \pm 0.17$, $n_{WT} = 5$; $z_{R176S} = 1.02 \pm 0.05$, $n_{R176S} = 5$; $p < 0.0001$). The results suggest that R176 may lie within the membrane electric field and participate in the voltage-sensing action of the KAT1 channel, and that the positively charged amino acid residues in the S4 segment of KAT1 contribute to determination of the voltage sensitivity.

The N-terminal deletions shift voltage dependence

In our previous work, we showed that the distal region of the C-terminus is important in determining the voltage sensitivity of KAT1 (Marten and Hoshi, 1997). Some C-terminal deletions reduced the equivalent gating charge movement, indicating a possible physical interaction between the C-terminus and the voltage-sensing domain, probably S4 (see above). We examined whether the N-

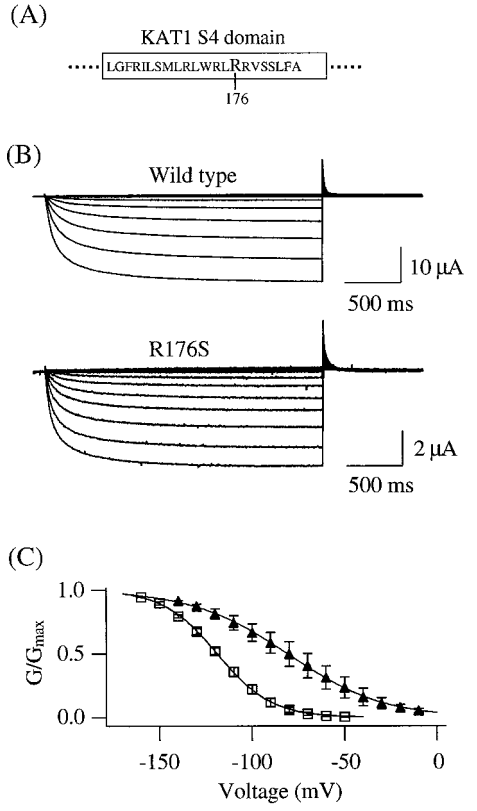

FIGURE 2 Effect of the mutation R176S in the S4 domain on the voltage dependence of KAT1. (*A*) Amino acid sequence of the putative voltage-sensing transmembrane S4 domain containing the residue R176, given in single-letter code. (*B*) Time-dependent macroscopic currents were observed in response to 2.5-s voltage pulses applied in 10-mV increments in the range of −140 to +10 mV. The decline of the steady-state currents was caused by a pulse to +10 mV (wild-type) or +30 mV (R176S). (*C*) Normalized macroscopic conductance-voltage ($G(V)$) curves determined for the wild-type (○, $V_{1/2} = -118.9 \pm 1.9$ mV, $z = 1.71 \pm 0.17$, $n = 5$) and the R176S channels (▲, $V_{1/2} = -80.5 \pm 13.1$ mV, $z = 1.02 \pm 0.05$, $n = 5$) are shown. The bath solution contained (in mM) 20 KCl, 120 NaCl, 2 $MgCl_2$, 10 HEPES, pH 7.2 (NMG).

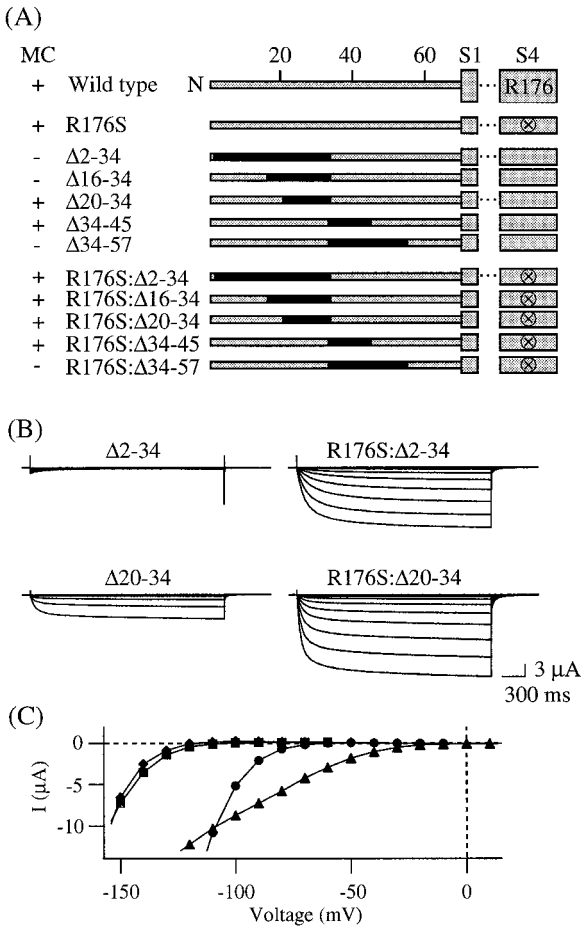

FIGURE 3 Effect of N-terminal deletions and/or the S4 mutation R176S on the ability of KAT1 in mediating macroscopic currents at hyperpolarized voltages. (*A*) Schematic representations of the N-terminus and the transmembrane segments S1 to S4 (*horizontal bars*) of wild-type and mutant KAT1 channels. Deletions in the N-terminus are marked by black bars. The point mutation R176S is marked by a circled cross in the S4 domain. MC + and MC − indicate that KAT1-like macroscopic currents were or were not recorded, respectively. (*B*) Representative current traces induced in the wild-type or mutant channel-expressing oocytes by pulses to the voltage range of −150 to −60 mV in 10-mV steps. Repolarization occurred at −50 mV for the R176S:Δ2–34 and the Δ2–34 channel, at −60 mV for the R176S:Δ20–34, and −80 mV for the Δ20–34 channel. (*C*) Steady-state current-voltage ($I(V)$) relationships are shown for the R176S (▲), the R176S:Δ16–34 (♦), the wild-type (●), and the Δ20–34 channels (■). Experiments on the R176S and R176S:Δ16–34 channels were performed in the presence of 140 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2 (NMG).

terminus has a similar role in regulating the voltage-dependent behavior by making systematic deletions in the N-terminus (Fig. 3 *A*). When the deletion mutants with the wild-type background (Δ2–34, Δ16–34, Δ20–34, Δ34–45, and Δ34–57) were examined, macroscopic currents were observed only from mutants with the two smallest deletions (Δ20–34 and Δ34–45). No KAT1-like macroscopic currents were observed from the mutants with other, larger deletions (Δ2–34, Δ16–34, and Δ34–57). Furthermore, we observed that pulses to more hyperpolarized voltages were required to activate the KAT1 Δ20–34 channels than the wild-type channels (Fig. 3 *C*). The apparent activation voltage for the Δ20–34 channels was approximately −130 mV, as compared with −80 mV for the wild-type channel.

R176S mutation rescues the N-terminal deletions

Based on the observation that activation of the KAT1 Δ20–34 channel requires greater hyperpolarization, we hypothesized that other apparently nonfunctional mutants (Δ2–34, Δ16–34, and Δ34–57) do not show ionic currents because their activation curves are shifted out of our recording limits. We further hypothesized that the R176S mutation, which shifts the activation curve to a more positive direction by +40 mV, could rescue these apparently nonfunctional N-terminal deletion channels by shifting their activation curves back into our recording range. To test these hypotheses, the S4 mutation R176S was introduced in the apparently nonfunctional N-terminal mutant channels (Fig. 3). In the R176S background, macroscopic inward

currents were recorded not only with the Δ20–34 and Δ34–45 deletions, but also with the previously nonfunctional Δ2–34 (Fig. 3 *B*) and Δ16–34 mutants (Fig. 3 *C*). No appreciable time-dependent currents were recorded from cells injected with R176S:Δ34–57 RNA on depolarization or hyperpolarization (not shown). These results suggest that the Δ2–34 and Δ16–34 channels are functionally expressed in the oocytes, but are activated at extremely hyperpolarized voltages beyond our recording range.

R176S mutation does not rescue the C-terminal deletions

The R176S mutation in the S4 segment rescues the N-terminal deletion mutants by shifting their activation curves to more positive directions. We showed previously that some C-terminal deletion mutants do not show any time-dependent currents on hyperpolarization (Marten and Hoshi, 1997). We examined whether the R176S mutation could rescue the apparently nonfunctional C-terminal deletion mutants (Fig. 4). Representative records obtained from oocytes injected with double-mutant RNAs containing the S4 mutations and C-terminal deletions (R176S:Δ311–677, R176S:Δ467–677, S168R:Δ311–677, and S168R:Δ467–677; Fig. 4 *A*) are shown in Fig. 4 *B*. The S4 mutation S168R shifts the half-activation voltage of KAT1 to an even more positive voltage than R176S (by approximately +100 mV, data not shown; Dreyer et al., 1997). Unlike the N-terminal deletions, which were rescued by the S4 mutations, macroscopic inward currents were not detected in these S4- and C-terminal double mutant channels (Fig. 4 *B*). The nonfunctionality of these C-terminal channel mutants is probably linked to the loss of C-terminal motifs highly conserved among known plant K^+ channels, enabling the aggregation of the channel subunits (Daram et al., 1997).

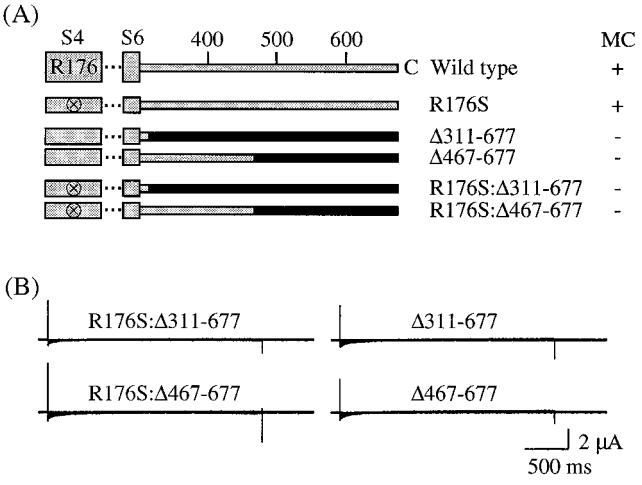

FIGURE 4 Effect of C-terminal deletions and/or the S4 mutation R176S on the ability of KAT1 in mediating macroscopic currents at hyperpolarized voltages. (*A*) Schematic representations of the C-terminus and the transmembrane segments S4 to S6 (*horizontal bars*) of wild-type and mutant KAT1 channels. Deletions and the point mutation R176S are illustrated as in Fig. 2 *A*. (*B*) Representative current traces elicited by 2.5-s voltage pulses to the voltage range of −150 to −30mV in 10-mV steps, followed by voltage steps to −50 mV.

The N-terminus of KAT1 affects the deactivation kinetics of KAT1

The *I*(*V*) curves obtained from the Δ20–34, R176S:Δ16–34, and R176S channels were shifted along the voltage axis when compared with the wild-type *I*(*V*) curve (Fig. 3 *C*). To infer the biophysical mechanisms responsible for the altered voltage dependence, we examined the activation and deactivation time courses. Fig. 5 *A* shows the activation time courses of the R176S, the Δ20–34, and the R176S:Δ20–34 mutant channels. The activation time courses of the R176S channel and Δ20–34 channels were indistinguishable from that of the wild-type channels (Fig. 5 *A*, *top* and *middle panels*). The R176S:Δ20–34 channels activated only slightly faster than wild-type channels (Fig. 5 *A*, *lower panel*).

In contrast, the deactivation time course was markedly different in the mutant channels (Fig. 5, *B* and *C*). In the R176S channel, the deactivation time course at +30 mV was markedly slower than that of the wild-type channel (Fig. 5 *B*, *top panel*). Mean time constant of the R176S deactivation at +30 mV was 63.1 ± 6.7 ms (n_{R176S} = 7), compared with 15.9 ± 4.3 ms (n_{WT} = 5) in the wild-type channel. The deactivation time course of the Δ20–34 channel was appreciably faster than that of the wild-type channel. Mean time constant of the inward tail current recorded from the Δ20–34 channels at −100 mV was more than four times faster than that from the wild-type channel ($\tau_{\Delta 20-34}$ = 23.8 ± 1.4 ms, $n_{\Delta 20-34}$ = 4; τ_{WT} = 102.3 ± 9.1 ms, n_{WT} = 6). A less pronounced decrease in the deactivation time constant at −100 mV was observed for KAT1 mutant channels in which the N-terminal deletion Δ20–34 was combined with the S4 mutation R176S ($\tau_{R176S:\Delta 20-34}$ = 55.4 ± 3.5 ms, $n_{R176S:\Delta 20-34}$ = 4; Fig. 5, *B* and *C*). Voltage dependence of the deactivation time constant measured from the mutant and wild-type KAT1 channels is shown in Fig. 5 *C*. Among the N-terminal deletions (Δ2–34, Δ16–34, Δ20–34, and Δ34–45), the Δ2–34 deletion most effectively restored the R176S-induced change in the KAT1 deactivation kinetics (Fig. 5 *C*). Thus the N-terminal deletions differentially accelerate the deactivation kinetics and counteract the effects of the S4 neutralization mutation on the deactivation kinetics.

The N-terminus of KAT1 influences the voltage sensitivity of KAT1

To quantitatively compare the voltage sensitivity of the N-terminal and the S4 mutant channels, we constructed macroscopic *G*(*V*) curves from the measured tail currents. The *G*(*V*) curves were fitted with a simple Boltzmann function (Figs. 2 and 6 *A*). The estimated half-activation

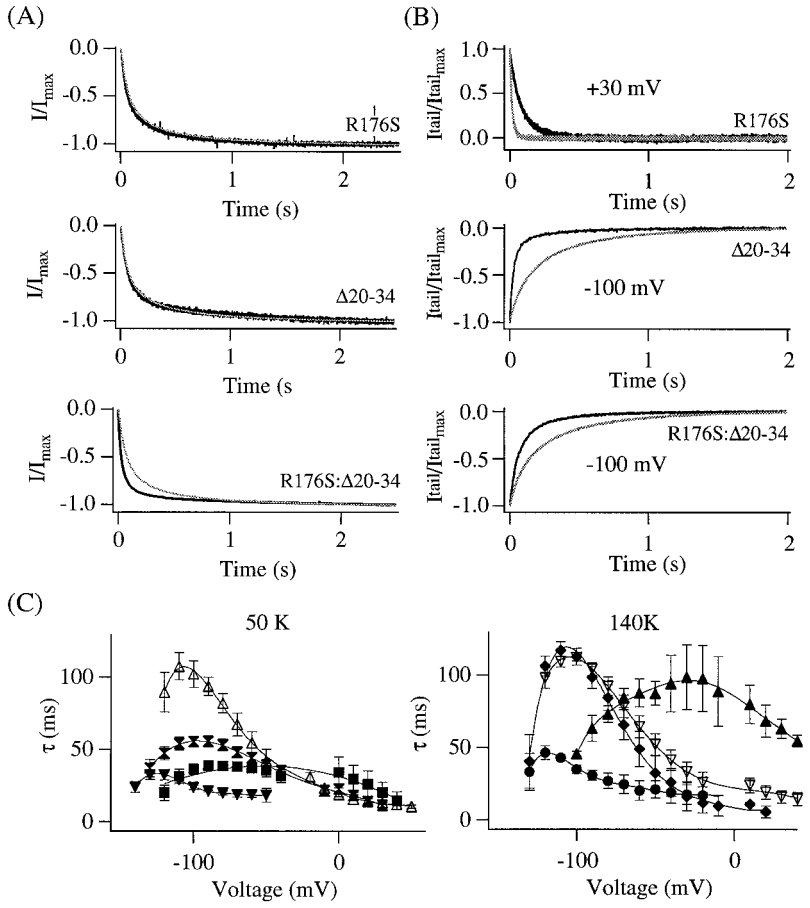

FIGURE 5 Effects of the S4 mutation R176S and the N-terminal deletions on the activation and deactivation kinetics of the KAT1 channel. (*A*) Macroscopic inward currents elicited at −140 mV in oocytes expressing the wild-type channels (*gray traces*) or mutant channels (R176S, Δ20–34, R176S:Δ20–34; *black traces*). Currents recorded 5 ms after onset of the pulse were normalized to the maximum inward current amplitude at the end of the 2.5-s pulse. The bath solution used for the measurements on the Δ20–34 channel and the wild-type channel consisted of 50 mM KCl, 90 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM HEPES, pH 7.2 (NMG). In others, the solutions contained 20 mM KCl, 120 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2 (NMG). (*B*) Comparison of the deactivation time courses in the wild-type (*gray traces*) and mutant KAT1 (*black traces*) channels. Tail currents were normalized and superimposed. The tail currents of the R176S and wild-type channels were compared at +30 mV in the presence of 140 mM extracellular KCl. The currents of the Δ20–34, R176S:Δ20–34 and wild-type channels were recorded at −100 mV in the presence of 50 mM KCl and 90 mM NaCl in the bath. The bath solution used for the experiments on the R176S:Δ20–34 channels also contained 0.5 mM $CaCl_2$. (*C*) Voltage dependence of the fast component in the tail current time course. Tail currents were fitted with a sum of two exponentials, and the fast time constant values were plotted against the voltage. The experiments were performed in 50 mM KCl (*left*) and 140 mM KCl (*right*). The bath solution used for the experiments on R176S:Δ20–34 channels (⧗, *left*) also contained 0.5 mM $CaCl_2$. Symbols have the following meanings: △, wild-type channel (n = 6); ▽, wild-type channel (n = 5); ▲, R176S channel (n = 7); ▼, Δ20–34 channel (n = 4); ⧗, R176S:Δ20–34 channel (n = 4); ◆, R176S:Δ2–34 channel (n = 5); ●, R176S:Δ16–34 channel (n = 6); ■, R176S:Δ34–45 channel (n = 8). The smooth lines represent polynomial fits to the data only to facilitate comparison.

voltages ($V_{1/2}$) and the number of equivalent gating charges (z) are shown by box plots in Fig. 6 *B*. The deletions in the N-terminus (Δ2–34, Δ16–34, Δ20–34) shifted $V_{1/2}$ toward more negative voltages. In contrast, the S4 mutation R176S shifted the $G(V)$ curve toward more positive voltages. Limiting slope measurements, which could be used to estimate the equivalent charge movements in a model-independent manner (Zagotta et al., 1994), were not performed because the very small tail current amplitudes at depolarizing potentials prevented reliable calculations of G/G_{max} values ($\ll$0.001) at the voltages where the open probability was very small. To examine whether the effect of the N-terminal deletions (Δ2–34, Δ16–34, Δ20–34) on $V_{1/2}$ is related to the loss of a putative phoshorylation site for protein kinases C- or cGMP-dependent protein kinases (Pearson and Kemp, 1991), we generated the mutant channel S26A:S27A. However, the electrophysiological properties of the S26A:S27A channel such as kinetics, $V_{1/2}$, and z were similar to those of the wild-type channel (data not shown), indicating that the putative phosphorylation site in the N-terminus is not likely to be involved in regulating the gating behavior of KAT1.

The S4 mutation R176S reduced the equivalent gating charge movement, consistent with the hypothesis that this amino acid residue is involved in voltage sensing. However, the N-terminal deletions, Δ20–34 and Δ34–45, did not alter the number of equivalent gating charges in a statistically significant manner (Figs. 2 and 6; Marten and Hoshi, 1997). Because the R176S mutation decreased the voltage sensi-

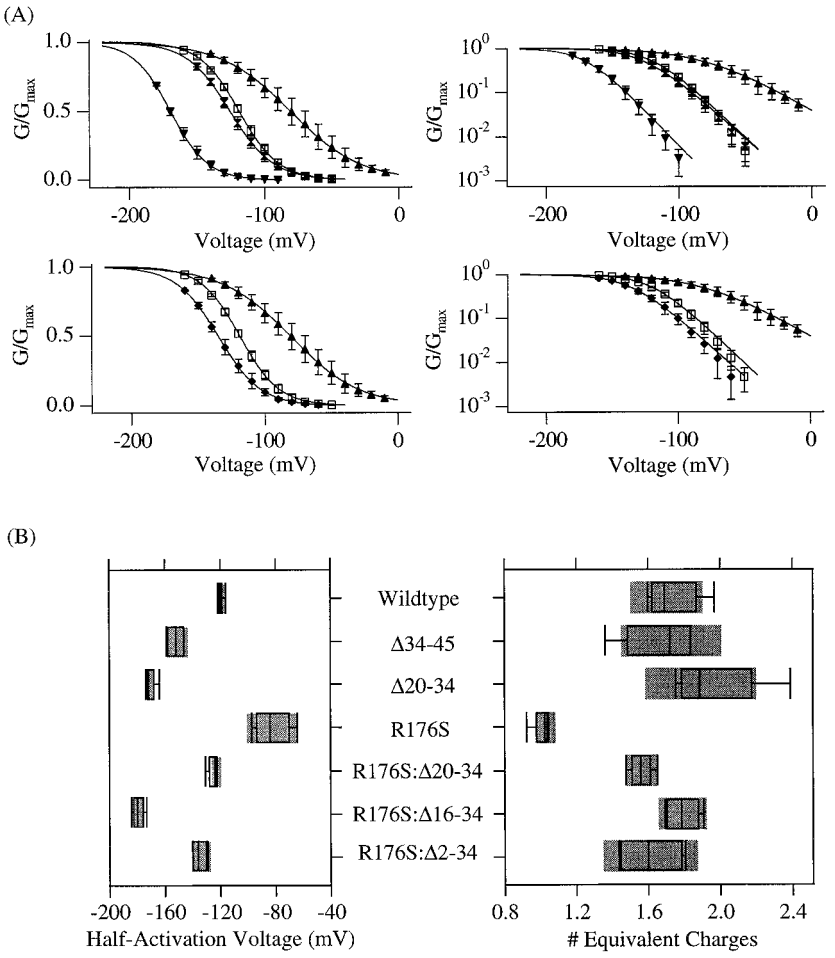

FIGURE 6 Effect of N-terminal deletions and the S4 mutation R176S on the voltage dependence and sensitivity of KAT1. (*A*) Normalized $G(V)$ curves determined for the wild-type and the mutant KAT1 channels are plotted on a linear (*left*) or semilogarithmic (*right*) axis. Along with the wild-type (□, $n = 5$) and R176S (▲, $n = 5$) channels, $G(V)$ curves from the Δ20–34 (▼, $n = 4$) and the R176S:Δ20–34 (⧗, $n = 5$) channels are shown at the top, and those from the R176S:Δ2–34 channel (◆, $n = 6$) are shown in the bottom panel. (*B*) Half-activation voltages and number of equivalent charges estimated for wild-type and mutant channels by fitting $G(V)$ curves with a simple Boltzmann function are shown as box plots. The vertical lines of the central box represent the 25th percentile, the median, and the 75th percentile of the data set. The highest connected data values are given by whiskers. Shaded areas represent the 95% confidence intervals of the medians. Except for the Δ34–45 ($n = 5$), the Δ20–34, and the R176S:Δ16–34 ($n = 5$) channels, the experiments were performed in the presence of 20 mM KCl, 120 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2 (NMG). The bath solution used for the Δ34–45, the Δ20–34, and the R176S:Δ16–34 channels contained 140 mM KCl and 0 mM NaCl. The bath solution for the Δ20–34 and the R176S:Δ16–34 channels contained 50 mM KCl and 90 mM NaCl.

tivity whereas the N-terminal deletions did not affect the sensitivity, it was expected that the double-mutant channels, R176S:Δ2–34, R176S:Δ16–34, and R176S:Δ20–34, would have decreased equivalent gating charges similar to that of the R176S channels. However, the number of equivalent gating charges estimated for these S4 and N-terminal double-mutant channels was surprisingly indistinguishable from that estimated for the wildtype channels rather than for the R176S channels (e.g., $z_{R176S:\Delta2-34} = 1.61 \pm 0.15$, $z_{wild\text{-}type} = 1.71 \pm 0.17$, $z_{R176S} = 1.02 \pm 0.05$; $n_{R176S:\Delta2-34} = 6$, $n_{wild\text{-}type} = 5$, $n_{R176S} = 5$). The results indicate that the N-terminal deletions can restore the equivalent gating charges reduced by the S4 mutation, although the N-terminal deletions alone do not affect the gating charge movement. A simple model that accounts for this paradoxical observation is presented below.

DISCUSSION

Voltage-dependent activation and deactivation of ion channels in the *Shaker* superfamily were thought to be mainly determined by the transmembrane channel domains. In the KAT1 channel, the N- and C-termini domains also contribute to the voltage-dependent gating. We recently reported the influence of the C-terminus on the voltage sensitivity of KAT1, suggesting an interaction of the C-terminus and the voltage-sensing domain (Marten and Hoshi, 1997). The results presented here show that in addition to the C-terminus, the N-terminus of KAT1 contributes to the voltage sensitivity of KAT1. Our results further show that the inward rectification of KAT1 is not likely to represent recovery from inactivation, as shown for the HERG channels (Smith et al., 1996; Schönherr and Heinemann, 1996).

Inward rectification of KAT1 is not related to inactivation

The apparent inward rectification of HERG channels has been linked to fast C-type inactivation and hyperpolarization-dependent recovery from inactivation (Warmke and Ganetzky, 1994; Smith et al., 1996; Schönherr and Heinemann, 1996). Despite the primary sequence similarity between HERG and KAT1, our results argue against the hypothesis that the gating mechanisms of HERG and KAT1 are similar. The KAT1 gating depends neither on the extracellular K^+ and Cs^+ concentration (Fig. 1, *A–C*; Véry et al., 1995) nor on the amino acid at position 267 (Fig. 1, *D* and *E*), which is equivalent to position 449 in the *Sh*B channel, known to be important in C-type inactivation. If N-type inactivation (Hoshi et al., 1990; Miller and Aldrich, 1996)

were involved, then truncation of the N-terminus should result in slower deactivation of KAT1. However, KAT1 deactivated faster when N-terminal residues were removed (cf. in Fig. 5: Δ20–34 channels versus wild-type channels; R176S:Δ2–34 and R176S:Δ16–34 channels versus R176S channels). Thus N-type inactivation is not likely to be directly involved in the KAT1 channel's rectification. Thus the hyperpolarization-induced inward currents are probably related to KAT1 activation rather than to recovery from inactivation.

The S4 segment may function as a voltage sensor of KAT1

For voltage-gated channels from the animal kingdom, the S4 segment is proposed as the major voltage sensor of the channel protein (Papazian et al., 1995; Larsson et al., 1996; Seoh et al., 1996). Neutralization of a positively charged residue in the S4 domain of KAT1 (R176S) caused a rightward shift in the midpoint of activation and, more importantly, a decrease in the equivalent gating charge movement, identifying Arg^{176} as a voltage-sensing residue of KAT1 (Figs. 2 and 6). Similar observations were made for the KAT1-homologous K^+ channel KST1 when its S4 residue R181, an equivalent to the KAT1 residue R177, was neutralized (R181Q; Hoth et al., 1997). Judging from the 40% reduction in the gating charge movement, the KAT1 residue R176 only partially traverses the transmembrane field (cf. Fig. 7). When an additional positive charge (S168R) was introduced in the S4 domain (Dreyer et al., 1997), the $G(V)$ curve was steeper compared to that of the wild-type channel, indicating that the residue S168 moves within the membrane electric field during the activation process. Thus, as with the *Shaker* superfamily, the S4 segment of plant K^+ channels such as KAT1 and KST1 functions as a voltage sensor.

The N-terminus of KAT1 controls its voltage-dependent gating

Amino acid residues outside the S4 segment are also known to influence the voltage-dependent gating of the *Shaker* channel. For instance, residues in other transmembrane segments (S2 and S3) in the *Shaker* channels, and leucines in the S4-S5 linker segment of the *Shaker*, Na^+ and Ca^{2+} channels contribute to voltage-sensing function (Papazian et al., 1995; Larsson et al., 1996; Seoh et al., 1996; Tiwari-Woodruff et al., 1997; Auld et al., 1990; McCormack et al., 1991; Garcia et al., 1997). Furthermore, we recently demonstrated that the putative cytoplasmic C-terminus of the KAT1 channel influences the voltage sensitivity (Marten and Hoshi, 1997). In addition, the results presented here reveal that the N-terminus also influences the voltage-sensing process of KAT1 (Fig. 6). N-terminal deletions caused a leftward shift of the half-activation voltage along the voltage axis, primarily because of faster deactivation kinetics (Figs. 5 and 6). The deletions Δ2–34 and Δ16–34 even shifted the activation curve out of the recording range. In comparison, some deletions in the N-terminus of the outward rectifier r-*eag* shifted the activation curve to more negative voltages (Terlau et al., 1997). The deactivation and activation kinetics of these r-*eag* mutant channels slowed, whereas a N-terminal deletion in HERG channels accelerated deactivation, as in KAT1 channels (Schönherr and Heinemann, 1996; Fig. 5). The N-terminus of KAT1 contains a motif (lysine-glutamine-serine, at position 24–26) for a protein kinase C (PKC)- and cGMP-dependent phosphorylation site (Pearson and Kemp, 1991). Removal of the putative phosphorylation site (S26A) did not alter the electrophysiological properties of KAT1, indicating that the effect of the N-terminus on the activation curve may not depend on its phosphorylation status.

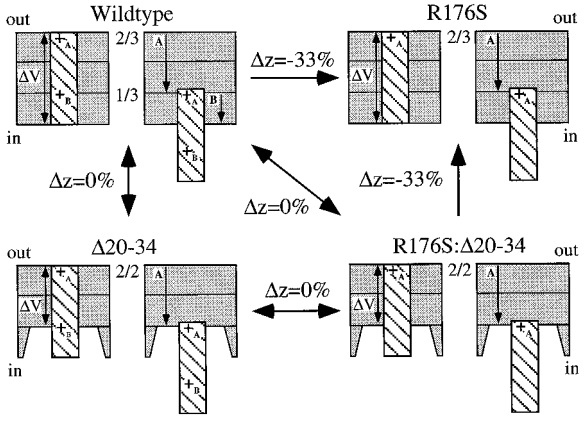

FIGURE 7 A simple model to account for the effects of the N-terminus and the S4 domain on the KAT1 voltage sensitivity. The diagram shows the S4 domain of one subunit as a striped bar, with two positively charged residues, $+_A$ and $+_B$. The gray background represents the dielectric body assembled by the channel protein itself and/or the lipid bilayer, and determines the magnitude of the electric field controlling the charge movement in the S4 domain. To describe charge movement through a fraction of the transmembrane potential (V), the dotted region is subdivided into three areas. The partial displacements of $+_A$ through two-thirds and of $+_B$ through one-third of the transmembrane potential result in the total charge movement of 1 in the wild-type channel during the steady-state activation process. When $+_B$ is neutralized (R176S), the gating charge movement decreases by 33%, leaving only $+_A$ to contribute the overall charge movement. When the N-terminal residues 20–34 are removed, the electric field strength increases, resulting in the movement of $+_A$ through the total transmembrane potential. The charge $+_B$ does not contribute to the gating charge movement, and the total number of gating charges moved in the Δ20–34 channel is unaltered with respect to the wild-type channel ($\Delta z = 0\%$). No changes in the gating charge movement occur when the S4 mutation is present in the Δ20–34 channel (Δ20–34 versus R176S:Δ20–34: $\Delta z = 0\%$).

The KAT1 N-terminus controls voltage sensing in the S4 segment by forming a part of the S4 canal

Our experiments on the S4 mutation, N-terminal deletions, and the double mutations combining the S4 and N-terminal mutations produced a paradoxical result. The S4 mutation

R176S decreased the equivalent gating charge movement in the wild-type background, but not in the N-terminal deletion mutants (Fig. 6). The model presented in Fig. 7 accounts for the paradoxical effect of the N-terminal deletions and the S4 mutation on the voltage sensitivity by postulating that the N-terminal deletions affect the fraction of the electric field traversed by the S4 gating charges.

The number of equivalent gating charges (z) estimated is the sum of the products of individual charges (z_i) and the fractions of the membrane field traversed (d_i), as given by $z = \Sigma z_i d_i$. If fewer charges are involved, but the fraction of the electric field that each charge traverses increases proportionally, the apparent gating charge movement z may not change. For the sake of simplicity, it is assumed that the number of equivalent gating charges of the wild-type channel is one per subunit, as estimated using the fourth power of a Boltzmann function (Hoshi, 1995). In our model, we postulate that two charges in the S4 segment, $+_A$ and $+_B$, move two-thirds and one-third through the electric field, corresponding to the total charge movement of one per subunit. The $+_A$ charge moves from 0% to 67% of the way through the electric field. The $+_B$ charge moves from 67% to 100% of the way through the electric field. When charge $+_B$ is neutralized, as in the R176S channel, only charge $+_A$ contributes to the gating charge movement by moving from 0 to 67% of the membrane field, decreasing the total charge movement by one-third ($\Delta z = 33\%$).

We propose that the KAT1 N-terminal segment forms a cytoplasmic part of the vestibule that the S4 segment traverses (67% to 100% of the field in Fig. 7). In the wild-type channel, charge $+_B$ traverses the membrane field contributed by the N-terminal segment. Deletion of the N-terminus removes one-third of the internal vestibule structure (67% to 100% in Fig. 7), and the transmembrane voltage drop occurs entirely within the segment normally traversed by charge $+_A$ (0 to 67% in Fig. 7). With the N-terminus removed, only charge $+_A$ senses the transmembrane field, and charge $+_B$ no longer acts as a voltage sensor. This model nicely accounts for the observation that the R176S mutation or neutralization of $+_B$ decreases the equivalent gating charge by 33% in the wild-type background, but does not alter the charge movement in the N-terminal deletion mutants. In the N-terminal deletion mutants, the transmembrane field is narrower and the R176 residue ($+_B$) no longer senses the membrane voltage. The remaining charge ($+_A$), acting as the only voltage-sensing residue, now traverses a larger fraction of the membrane electric field, producing the same equivalent gating charges whether the R176 residue ($+_B$) is present or not. According to this model, the N-terminus is probably tightly bound to the remaining dielectric body. This might explain that despite nine putative N-terminal cleavage sites (at positions 8, 12, 25, 41, 47, 49, 50, 59, 61), intracellular trypsin ($\leq$1% g/w) did not affect the deactivation kinetics of KAT1 (Marten and Hoshi, unpublished observation).

The N-terminus of K^+ channels was implicated mostly in inactivation and subunit assembly (Jan and Jan, 1997). Our data presented here from the KAT1 channel and those obtained recently from HERG and r-*eag* channels (Schönherr and Heinemann, 1996; Terlau et al., 1997) suggest that the N-terminus may also determine activation/deactivation kinetics, voltage dependence, and voltage sensitivity, possibly by interacting with the voltage-sensing S4 segment. Given the organizational similarities found among different voltage-gated ion channels, it is likely that the N-termini of other channels, such as voltage-dependent Na^+ and Ca^{2+} channels, may have similar roles in their voltage-dependent gating behavior.

We thank V. B. Avdonin for discussions, and J. Thommandru and J. Kabat for technical assistance. This paper is dedicated to the late J. Wells.

IM was supported by the Human Frontier Science Program (LT380/95), and TH was supported in part by the National Institutes of Health (GM51474).

REFERENCES

Aldrich, R. W. 1994. Potassium channels. New channel subunits are a turn-off. *Curr. Biol.* 4:839–840.

Anderson, J. A., S. S. Huprikar, L. V. Kochian, W. J. Lucas, and R. Gaber. 1992. Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 89:3736–3740.

Auld, V. J., A. L. Goldin, D. S. Krafte, W. A. Catterall, H. A. Lester, N. Davidson, and R. J. Dunn. 1990. A neutral amino acid change in segment IIS4 dramatically alters the gating properties of the voltage-dependent sodium channel. *Proc. Natl. Acad. Sci. USA.* 87:323–327.

Choi, K. L., R. W. Aldrich, and G. Yellen. 1991. Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K^+ channels. *Proc. Natl. Acad. Sci. USA.* 88:5092–5095.

Curran, M. E., I. Splawski, K. W. Timothy, G. M. Vincent, E. D. Green, and M. T. Keating. 1995. A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell.* 80:795–803.

Daram, P., S. Urbach, F. Gaymard, H. Sentenac, and I. Chérel. 1997. Tetramerization of the AKT1 plant potassium channel involves its C-terminal cytoplasmic domain. *EMBO J.* 16:3455–3463.

Dreyer, I., S. Antunes, T. Hoshi, B. Müller-Röber, K. Palme, O. Pongs, B. Reintanz, and R. Hedrich. 1997. Plant K^+ channel α-subunits assemble indiscriminately. *Biophys. J.* 72:2143–2150.

Garcia, J., J. Nakai, K. Imoto, and K. G. Beam. 1997. Role of S4 segments and the leucine heptad motif in the activation of an L-type calcium channel. *Biophys. J.* 72:2515–2523.

Hoshi, T. 1995. Regulation of voltage dependence of the KAT1 channel by intracellular factors. *J. Gen. Physiol.* 105:309–328.

Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1990. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science.* 250:533–538.

Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1991. Two types of inactivation in *Shaker* K^+ channels: effects of alterations in the carboxy-terminal region. *Neuron.* 7:547–556.

Hoth, S., I. Dreyer, and R. Hedrich. 1997. Mutational analysis of functional domains within plant K^+ uptake channels. *J. Exp. Bot.* 48:415–420.

Jan, L. Y., and Y. N. Jan. 1997. Cloned potassium channels from eucaryotes and procaryotes. *Annu. Rev. Neurosci.* 20:91–112.

Larsson, H. P., O. S. Baker, D. S. Dhillon, and E. Y. Isacoff. 1996. Transmembrane movement of the Shaker K^+ channel S4. *Neuron.* 16:387–397.

Levy, D. I., and C. Deutsch. 1996. Recovery from C-type inactivation is modulated by extracellular potassium. *Biophys. J.* 70:798–805.

Li, M., Y. N. Jan, and L. Y. Jan. 1992. Specification of subunit assembly by the hydrophilic amino-terminal domain of the *Shaker* potassium channel. *Science.* 257:1225–1230.

Lopez-Barneo, J., T. Hoshi, S. H. Heinemann, and R. W. Aldrich. 1993. Effects of external cations and mutations in the pore region on C-type inactivation of *Shaker* potassium channels. *Receptors Channels.* 1:61–71.

Mannuzzu, L. M., M. M. Moronne, and E. Y. Isacoff. 1996. Direct physical measure of conformational rearrangement underlying potassium channel gating. *Science.* 271:213–216.

Marten, I., and T. Hoshi. 1997. Voltage-dependent gating characteristics of the K^+ channel KAT1 depend on the N and C termini. *Proc. Natl. Acad. Sci. USA.* 94:3448–3453.

McCormack, K., M. A. Tanouye, L. E. Iverson, J. W. Lin, M. Ramaswami, T. McCormack, J. T. Campanelli, M. K. Mathew, and B. Rudy. 1991. A role for hydrophobic residues in the voltage-dependent gating of Shaker K^+ channels. *Proc. Natl. Acad. Sci. USA.* 88:2931–2935.

Miller, A. G., and R. W. Aldrich. 1996. Conversion of a delayed rectifier K^+ channel to a voltage-gated inward rectifier K^+ channel by three amino acid substitutions. *Neuron.* 16:853–858.

Ogielska, E. M., W. N. Zagotta, T. Hoshi, S. H. Heinemann, J. Haab, and R. W. Aldrich. 1995. Cooperative subunit interactions in C-type inactivation of K channels. *Biophys. J.* 69:2449–2457.

Papazian, D. M., X. M. Shao, S. A. Seoh, A. F. Mock, Y. Huang, and D. H. Wainstock. 1995. Electrostatic interactions of S4 voltage sensor in Shaker K^+ channel. *Neuron.* 40:1293–1301.

Pardo, L. A., S. H. Heinemann, H. Terlau, U. Ludewig, C. Lorra, O. Pongs, and W. Stühmer. 1992. Extracellular K^+ specifically modulates a rat brain K^+ channel. *Proc. Natl. Acad. Sci. USA.* 89:2466–2470.

Pearson, R. B., and B. E. Kemp. 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.* 200:62–81.

Rettig, J., S. H. Heinemann, F. Wunder, C. Lorra, D. N. Parcej, J. O. Dolly, and O. Pongs. 1994. Inactivation properties of voltage-gated K^+ channels altered by presence of β-subunit. *Nature.* 369:289–294.

Sanguinetti, M. C., C. Jiang, M. E. Curran, and M. T. Keating. 1995. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{Kr} potassium channel. *Cell.* 81: 299–307.

Schachtman, D. P., J. I. Schroeder, W. J. Lucas, J. A. Anderson, and R. F. Gaber. 1992. Expression of an inward-rectifiying potassium channel by the *Arabidopsis* KAT1 cDNA. *Science.* 258:1654–1658.

Schlief, T., R. Schonherr, and S. H. Heinemann. 1996. Modification of C-type inactivating Shaker potassium channels by chloramine-T. *Pflügers Arch.* 431:483–493.

Schönherr, R., and S. H. Heinemann. 1996. Molecular determinants for activation and inactivation of HERG, a human inward rectifier potassium channel. *J. Physiol. (Lond.).* 493:635–642.

Seoh, S. A., D. Sigg, D. M. Papazain, and F. Bezanilla. 1996. Voltage-sensing residues in the S2 and S4 segments of the *Shaker* K^+ channel. *Neuron.* 16:1159–1167.

Sigworth, F. J. 1993. Voltage gating of ion channels. *Q. Rev. Biophys.* 27:1–40.

Smith, P. L., T. Baukrowitz, and G. Yellen. 1996. The inward rectification mechanism of the HERG cardiac potassum channel. *Nature.* 379: 833–836.

Spector, P. S., M. E. Curran, A. R. Zou, M. T. Keating, and M. C. Sanguinetti. 1996. Fast inactivation causes rectification of the I_{Kr} channel. *J. Gen. Physiol.* 107:611–619.

Terlau, H., S. H. Heinemann, W. Stühmer, O. Pongs, and J. Ludwig. 1997. Amino terminal-dependent gating of the potassium channel rat eag is compensated by a mutation in the S4 segment. *J. Physiol. (Lond.).* 502:537–543.

Tiwari-Woodruff, S. K., C. T. Schulteis, A. F. Mock, and D. M. Papazian. 1997. Electrostatic interactions between transmembrane segments mediate folding of *Shaker* K^+ channel subunits. *Biophys. J.* 72:1489–1500.

Trudeau, M. C., J. W. Warmke, B. Ganetzky, and G. A. Robertson. 1995. HERG, a human inward rectifier in the voltage-gated potassium channel family. *Science.* 269:92–95.

Véry, A.-A., G. Gaymard, C. Bosseux, H. Sentenac, and J.-T. Thibaud. 1995. Expression of a cloned plant K^+ channel in *Xenopus* oocytes: analysis of macroscopic currents. *Plant J.* 7:321–332.

Warmke, J. W., and B. Ganetzky. 1994. A family of potassium channel genes related to *eag* in *Drosophila* and mammals. *Proc. Natl. Acad. Sci. USA.* 91:3438–3442.

Zagotta, W. N., T. Hoshi, J. Dittman, and R. W. Aldrich. 1994. Shaker potassium channel gating. II. Transitions in the activation pathway. *J. Gen. Physiol.* 103:279–319.