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Correlation between Charge Movement and Ionic Current during Slow Inactivation in Shaker K⁺ Channels

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ABSTRACT Prolonged depolarization induces a slow inactivation process in some K⁺ channels. We have studied ionic and gating currents during long depolarizations in the mutant Shaker H4-Δ(6–46) K⁺ channel and in the nonconducting mutant (Shaker H4-Δ(6–46)-W434F). These channels lack the amino terminus that confers the fast (N-type) inactivation (Hoshi, T., W.N. Zagotta, and R.W. Aldrich. 1991. Neuron. 7:547–556). Channels were expressed in oocytes and currents were measured with the cut-open-oocyte and patch-clamp techniques. In both clones, the curves describing the voltage dependence of the charge movement were shifted toward more negative potentials when the holding potential was maintained at depolarized potentials. The evidences that this new voltage dependence of the charge movement in the depolarized condition is associated with the process of slow inactivation are the following: (a) the installation of both the slow inactivation of the ionic current and the inactivation of the charge in response to a sustained 1-min depolarization to 0 mV followed the same time course; and (b) the recovery from inactivation of both ionic and gating currents (induced by repolarizations to −90 mV after a 1-min inactivating pulse at 0 mV) also followed a similar time course. Although prolonged depolarizations induce inactivation of the majority of the channels, a small fraction remains non–slow inactivated. The voltage dependence of this fraction of channels remained unaltered, suggesting that their activation pathway was unmodified by prolonged depolarization. The data could be fitted to a sequential model for Shaker K⁺ channels (Bezanilla, F., E. Perozo, and E. Stefani. 1994. Biophys. J. 66:1011–1021), with the addition of a series of parallel nonconducting (inactivated) states that become populated during prolonged depolarization. The data suggest that prolonged depolarization modifies the conformation of the voltage sensor and that this change can be associated with the process of slow inactivation.

KEY WORDS: gating current • potassium current • charge movement

INTRODUCTION

Upon depolarization, the macroscopic conductance increases and then shows a progressive decay. The reduction in conductance with time was referred to as inactivation by Hodgkin and Huxley (1952). Depending on the nature of the mechanism involved, the time course of the inactivation process ranges from a few milliseconds (fast inactivation) to several seconds (slow inactivation). To explain the fast inactivation process in Na⁺ channels from squid giant axon, Armstrong and Bezanilla (1977) proposed the “ball-and-chain” model. In this model, a tethered inactivating particle, the ball, is able to block the ion passage only after channel opening. In Shaker K⁺ channels, fast inactivation is mediated by the first 20 amino acid residues (the ball) that are tethered in the 60 amino acid residues that lie between the ball and the first transmembrane domain (Zagotta et al., 1989, 1990; Hoshi et al., 1990). Fast inactivation is induced by the binding of the amino terminus of the channel protein to the internal mouth of the pore. Because of the involvement of the amino terminus in this process, fast inactivation is also known as N-type inactivation. During N-type inactivation, the NH₂ terminus interacts with the voltage sensor and slows down the return of the gating charge to its resting position upon repolarization (Bezanilla et al., 1991). This slowdown of the charge return prompted by the inactivation process was first observed in Na⁺ channels and christened “charge immobilization” (Armstrong and Bezanilla, 1977). Shaker K⁺ channels with amino acid residues 6–46 deleted (Shaker H4-Δ), lacks fast inactivation (Hoshi et al., 1990) and charge immobilization (Bezanilla et al., 1991).

Slow inactivation, on the other hand, is less understood. Ehrenstein and Gilbert (1966) showed that prolonged depolarizations resulted in a slow reduction of the K⁺ conductance in squid giant axon. The mo-
The molecular mechanism of this process can be studied in Shaker K⁺ channels lacking fast inactivation (Shaker H4-Δ) since they show a relatively voltage insensitive slow decrease in channel open probability as a result of prolonged depolarizations (Hoshi et al., 1991; Choi et al., 1991; Yellen et al., 1994; Liu et al., 1996). Since point mutations in the carboxyl terminus of the channel (S6 transmembrane segment) affect slow inactivation, this process is commonly denominated C-type inactivation (Hoshi et al., 1991; López-Barneo et al., 1993) and is produced by a cooperative mechanism (Panyi et al., 1993; Ogielska et al., 1995). However, mutations in regions other than the S6 segment (for example, in the pore region) can also dramatically alter the inactivation time course (López-Barneo et al., 1993; De Biasi et al., 1993). These results strongly suggest the presence of more than one molecular mechanism in determining the rate of channel inactivation. In those cases in which pore (P) residues in K⁺ channels are involved in determining the inactivation kinetics, the process has been referred to as P-type inactivation (De Biasi et al., 1993).

The present study, previously presented in abstract form (Olcese et al., 1994, 1995), further investigated the nature of the effect of prolonged depolarization on the ionic conductance and correlates these effects on ionic current with effects on gating current in the Shaker H4-Δ K⁺ channel. Prolonged depolarization produced changes in the voltage dependence of the charge movement similar to the ones described by Bezanilla et al. (1982) for the Na⁺ channel in squid giant axon. Charge immobilization, as a consequence of long depolarization, has also been reported for the human K⁺ channel Kv1.5 (Fedida et al., 1996).

Materials and Methods

Molecular Biology and Oocyte Injection
cDNA encoding for Shaker H4 K⁺ channel (Kamb et al., 1987) lacking the amino acids 6–46 to remove the fast inactivation (Shaker H4-Δ) was used for measurements of ionic and gating currents (Hoshi et al., 1990). For gating current measurements in the corresponding nonconducting mutant, the mutant Shaker H4-Δ W434F (Perozo et al., 1993) was used.

24 h before cRNA injection, Xenopus laevis oocytes (stage V–VI) were treated with collagenase (200 U/ml; Gibco BRL, Gaithersburg, MD) in a Ca²⁺-free solution to remove the follicular layer. Oocytes were injected with 50 nl cRNA 1 μg/μl suspended in water using a “nano-injector” (Drummond Scientific Co., Broomall, PA) and maintained at 18°C in modified Barth’s solution containing (mM): 100 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 Na-HEPES (pH 7.6), and 50 mg/ml gentamicin.

Gating and Ionic Current Recording
Gating and ionic currents were recorded 1–7 d after injection using the cut-open oocyte Vaseline gap voltage clamp (COVG) (Stefani et al., 1994) and conventional cell-attached patch clamp technique. Methanesulphonic acid (MES)³ was the main anion in the recording external solutions. In the cut-open oocyte technique, the external solutions were (mM): 107 Na-MES, 2 Ca-(MES)₂, 10 Na-HEPES (isotonic Na-MES), 107 K-MES, 2 Ca-(MES)₂, 10 Na-HEPES (isotonic K-MES) or 110 N-methylglucamine-methanesulphonate (NMG-MES), 2 Ca-(MES)₂, 10 NMG-HEPES (isotonic NMG-MES Ca-MES 2). The internal solution contained (mM): 110 Kglutamate, 10 K-HEPES. The oocytes were K⁺ depleted by internal perfusion of the oocyte with a solution containing (mM): 110 NMG-MES, 10 NMG-HEPES, 10 (NMG)₂EGTA (isotonic NMG-MES). The perfusion (1 ml/h) was attained introducing a 20–50 μm glass pipette connected to a syringe pump in the lower part of the oocyte. Standard solution for the intracellular recording micropipette was (mM): 2,700 Na-MES, 10 NaCl. Low access resistance to the oocyte interior was obtained by permeabilizing the oocyte with 0.1% saponin. For the experiments in cell-attached configuration, after the removal of the vitelline membrane, the oocytes were K⁺ depleted in a solution containing (mM): 110 CsMES, 2 MgCl₂, 10 Cs-HEPES (isotonic Cs-MES). To speed up the intracellular K⁺ replacement by Cs⁺, the oocyte membrane was damaged at various places with a thin needle. Patch pipettes were filled with (mM): 110 Cs-MES, 2 CaCl₂, 10 Cs-HEPES (isotonic Cs-MES CaCl₂ 2). All recording and perfusion solutions were buffered at pH 7.0 with 10 mM HEPES. All experiments were performed at room temperature of 22–24°C.

In most cases, gating currents were recorded unsubtracted. Linear components were analogically compensated at positive potentials (20–40 mV) where the membrane capacity becomes voltage independent. This is also the case in slow-inactivated channels in which the charge–voltage curve was shifted to more negative potentials. P/–4 subtracting protocol (Bezanilla and Armstrong, 1977) from a positive holding potential (20 mV) was used for some of the experiments describing the time course of charge inactivation. The filter frequency was 1/5 the sampling frequency.

Modeling

The model-fitting procedure was implemented using the parameter optimization program SCoP (Simulation Resource, Inc., Bannen Springs, MI). We used a state kinetic model, expressed as a system of differential equations, with discrete transitions occurring between states. The transition rates between the horizontal lines of states (see Fig. 10 A) were exponential functions of the potential as predicted by the Eyring theory. The vertical transitions are assumed to be voltage independent.

Results

Ionic Current Inactivation

Fig. 1, A and B shows the time course of the current for Shaker H4 and Shaker H4-Δ for a series of depolarizing pulses of 50-ms duration. Shaker H4 displays a fast decay of the ionic current with a time constant of a few milliseconds (Fig. 1 A). In the mutant Shaker H4-Δ, under the same experimental conditions and time scale, the ionic current is maintained during the pulse (Fig. 1 B). However, in the same deletion-mutant Shaker H4-Δ,

³Abbreviations used in this paper: COVG, cut-open oocyte Vaseline gap voltage clamp; G-V, conductance–voltage; HP, holding potential; MES, methanesulphonic acid; NMG-MES, N-methylglucamine-MES.
The experiments were done in isotonic K-MES. To reach the steady state for the slow inactivation process, oocytes were maintained for 1 min at the given holding potential (HP) before the pulse protocol. Fig. 2A shows that the current measured from an HP of −70 mV are larger than those measured at an HP of −30 mV, indicating that the fraction of inactivated channels increases as the holding potential becomes more positive. Fig. 2B illustrates conductance–voltage (G-V) curves obtained from the amplitude of the tail currents at a constant return potential (−50 mV). Membrane conductance (G) was calculated from $G = I_{(−50 \text{mV})}/(E−E_K)$, where $I_{(−50 \text{mV})}$ is the peak tail current at −50 mV, $E_{(−50 \text{mV})}$ is the return potential (−50 mV), and $E_K$ is the K+ reversal potential (0 mV in isotonic K-MES). At −50 mV return potential, the slow time course of the tail currents facilitated the peak current determination. Long membrane depolarizations strongly reduce the membrane conductance without significantly changing the voltage dependence of channel opening (Fig. 2, B and C). Fig. 2D shows the steady state slow inactivation curve. The relative conductance measured as peak tail current at −50 mV for a pulse to −1 mV was plotted as a function of the holding potential, and the data were fitted to a Boltzmann equation that gave a half inactivation voltage of −38.5 mV and an effective valence of 7.2. The holding potential was maintained for 1 min before the test pulse was applied. The curve represents the reduction in availability of K+ channels to open as a function of the holding potential.

**Figure 1.** N- and C-type inactivation in Shaker channels. (A) Shaker H4: pulses from −60 to 10 mV in 10-mV steps. Note the fast decay of the K+ current due to the N-type inactivation. (B) Shaker H4-Δ: pulses from −80 to 30 mV in 10-mV steps. The deletion of the amino acids 6–46 (Shaker H4-Δ) completely removes the fast inactivating properties of the channel. (C) Shaker H4-Δ: long pulses (18 s) from −40 to 20 mV in 10-mV steps. Long depolarizing pulses make evident the presence of a slow inactivation process. COVG technique, external isotonic Na-MES.

**Figure 2.** Steady state inactivation properties of Shaker H4-Δ. (A) Effect of the HP (−70 and −30 mV) on K+ currents recorded with the COVG technique in isotonic K-MES. Pulses between −70 and 17 mV in 3-mV steps from the tested HP were applied. Tail currents were measured at the end of these pulses at a repolarization potential to −50 mV. The oocyte was maintained at each holding potential for 1 min before running the stimulating protocol with low frequency stimulating pulses to prevent their effect on the inactivation (e.g., a pulse every 1 s at −70 mV and every 5 s at 0 mV HPs). Linear components were subtracted after the stimulating run with P/1 from a subtracting holding potential −70 mV more negative than the tested HP and were between −140 and −53 mV with 3-mV increments. (B) G-V curves with the protocol shown in A from the same oocyte at different HPs: ◆, −90; □, −70; ■, −60; △, −50; Δ, −40; ○, −30 mV. (C) Normalized G-V curves. (D) Steady state inactivation curve obtained from tail current amplitude at −50 mV after a depolarizing pulse to −1 mV. Experimental points were fitted to $G_m = G_{\text{max}} + G_{\text{min}}/[1 + \exp(-z(V_{1/2} − V_m)/F/RT)]$. The fitted values were: $G_{\text{max}} = 0.17 \text{ mS}$, $G_{\text{min}} = 0.007 \text{ mS}$, $V_{1/2} = −38.5 \text{ mV}$ and $z = 7.2$. 

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of the membrane depolarization and shows that the steady state inactivation curve is strongly dependent on the holding potential.

Charge Movement from “Slow Inactivated” Channels

Measurements of charge movement make it possible to explore the voltage-dependent characteristics of the different protein conformations that give origin to the closed states that lead to channel opening. We used gating current measurements as a tool to investigate how the activation pathway of the slow inactivated Shaker H4ΔK channels was altered. Using the cut-open oocyte voltage clamp and giant macropatch techniques in K+-depleted oocytes, we measured gating current uncontaminated by ionic currents. Oocytes expressing Shaker H4Δ were K+ depleted by internal perfusion with NMG-MES (see MATERIALS AND METHODS). Fig. 3, A and B show selected gating current traces evoked by the indicated pulse potentials, from different holding potentials (−90 mV, Fig. 3 A and 0 mV, Fig. 3 B). The charge moved during the voltage steps, calculated by integrating the ON gating current, is plotted as a function of the pulse potential (Q-V) in Fig. 3, C and D. Q-V curves for different holding potentials were constructed from the charge measured immediately after the voltage steps (ON gating current). These measurements should minimize some charge recovery that may occur during repolarizing pulses from depolarized holding potentials. To obtain a more direct comparison of the Q-V curves obtained with the different holding potentials, in Fig. 3 D we have plotted the absolute values of the charge. The total amount of charge that moves in control conditions (−90 mV HP) and after slow inactivation (0 mV HP) is the same. However, the Q-V curve generated by the channels in the inactivated state (0 mV HP) was shifted by ~50 mV to the left along the voltage axis when compared with the Q-V curve obtained from the noninactivated channels (−90 mV HP). The shift in the Q-V curve indicates that, when the charge returns from the inactivated state after prolonged depolarization, it sees a different energy landscape than the charge moving in normally polarized channels from closed states to the open state. In kinetic terms, this means that the charge movement from the inactivated state does not follow the same kinetic pathway as when the charge moves from the closed to the open state.

Charge Movement after Prolonged Depolarization in the Nonconducting Shaker H4Δ-W434F

The shift toward more negative potentials of the Q-V curve, induced by long depolarizations, is also present in the nonconducting clone Shaker H4Δ-W434F. For comparison with the conducting clone Shaker H4Δ in the same ionic conditions, oocytes expressing the mutant channel were also K+ depleted, and the internal medium replaced with 120 mM NMG-MES. Fig. 4, A

Figure 3. Gating currents from −90 and 0 mV HPs in the conducting Shaker Δ. The oocyte interior was perfused with isotonic NMG-MES up to the complete elimination of K+ currents. The external solution was isotonic NMG-MES Ca-(MES)2. Unsubtracted gating currents; linear components were analogically compensated with the amplifier transient cancellation at 20 mV HP. (A and B) Current traces from −90 and 0 mV HP, respectively. The cell was maintained at 0 mV HP for 1 min; pulse stimulation was every 1 s at 0 mV and every 0.5 s at −90 mV HP. (C) Q-V curves obtained by integrating the ON gating currents to different test potentials from −90 (■) and 0 (●) mV HP. (D) Q-V curves from C replotted defining Q = 0 at the extreme negative potential for both HPs. The total charge moved is the same at both HPs, but holding the membrane potential at 0 mV induces an ~50-mV shift to more negative potentials of the Q-V curve.
and B show representative gating current traces for the Shaker H4-Δ-W434F channel. The traces were recorded during voltage steps to the indicated potentials from −90 (Fig. 4 A) and 0 (Fig. 4 B) mV holding potential. The Q-V curve of the slow inactivated channels (0 mV HP) is clearly shifted toward more negative potentials compared with that obtained from −90 mV HP (Fig. 4 C). The midpoints of the two Q-V curves are ~25 mV apart. Interestingly, in both the conducting and the nonconducting clones, the voltage dependence of their charge movements when at hyperpolarized potentials was very similar (Bezanilla et al., 1991; compare Figs. 3 D and 4 C) and both have a left-shifted Q-V curve after a long depolarization. In Shaker H4-Δ, the left shift of the Q-V obtained from a holding potential of 0 mV is ~20 mV more negative than in Shaker H4-Δ-W434F.

![Gating currents from -90 and 0 mV HP in the nonconducting mutant Shaker Δ-W434F. Same protocol and ionic conditions as in Fig. 3, A and B. Unsubtracted gating currents from -90 and 0 mV HP, respectively. (C) Q-V curve with the absolute charge values. The total charge moved is the same at both HPs. Holding the membrane potential at 0 mV induces an ~25-mV shift to the more negative potential of the Q-V curve.](image)

**Installation of Slow Inactivation**

The shift to more negative potentials of the Q-V curve in slow inactivated channels could be explained as the conversion of channels from a “permissive” form (available for activation) to a “reluctant” conformation. In the reluctant (inactivated) conformation, the voltage sensor moves under a different voltage dependence than the permissive conformation. We propose here that the gating charge conversion occurring during long depolarizations is related to the slow inactivation process. To test this hypothesis, we compared the time course of the charge conversion with the time course of the slow inactivation of the current at the same potential. In Fig. 5 A, the membrane potential was held at −90 mV for at least 1 min to fully recover all the channels from inactivation. Inactivating prepulses of different duration to 0 mV were applied before a test pulse from 0 to −60 mV. It is clear that the gating current at −60 mV is reduced as the prepulse is made longer (Fig. 5 A). To quantify the effect of the prepulse, the integral of the ON gating current normalized to the maximum charge (Fig. 5 C, black trace) was plotted as a function of the prepulse duration.

As the inactivating prepulse becomes longer, the Q-V curve progressively shifts to the left, approaching the position of the Q-V curve obtained after 1 min at 0 mV HP (steady state) (Fig. 4 C, black dot). Therefore, the charge reduction reflects the speed of the shift of the Q-V curve in depolarizing conditions. There is no charge reduction, but only a change in its voltage dependence.

To compare the establishment of the charge conversion with the conduction inactivation, we measured the slow decay of the K⁺ current due to slow inactivation during 1-min depolarizing pulse at 0 mV in the conducting clone (Fig. 5 B). At the end of the pulse, the ionic conductance was reduced by ~85% due to the slow inactivation. The normalized ionic current decay was plotted (Fig. 5 C, thick trace) along with the normalized charge in Fig. 5 C. Both processes can be reasonably well fitted simultaneously by the sum of two exponential functions with the same time constants (τ) for ionic and gating current inactivation of 4.1 and 24 s.

**Recovery from Slow Inactivation**

The recovery of the charge movement and ionic current were measured after a 1-min preconditioning pulse to 0 mV that drove most of the channels into a slow inactivated state. Then, repolarizing pulses of different duration to −90 mV were delivered, allowing different times of recovery at this potential. The recovery of the charge and the ionic current as a function of the duration of the −90 mV pulse was measured with a test pulse from −90 to +20 mV. The recovery of the slow inactivated charge was measured by integrating the ON
gating current during the test pulse. ON gating current increased progressively depending on the duration of the recovery interval (Fig. 6A). In this case, the charge increase reflects the shift towards the right along the voltage axis of the Q-V curve. An identical protocol was used to monitor the recovery of the ionic current (Fig. 6B). As it was described for the installation of slow inactivation, a tight correlation between the time courses of charge and ionic current recovery was found. Experimental points describing the recovery of both charge

Figure 6. Correlation between the time course of recovery of slow inactivation and of changes in charge movement. (A) Shaker H4-Δ-W434F. Upper trace is the pulse protocol, remaining traces are unsubtracted gating currents. Linear components were analogically compensated with the amplifier transient cancellation at 20 mV HP. Numbers preceding the traces are the duration of the conditioning pulse to 0 mV. The −90 mV HP was held for 1 min between each stimulating pulse to fully recover from the inactivation. Note that increasing the duration of the conditioning pulse to 0 mV induces a reduction in the charge movement measured for a pulse from 0 to −60 mV. (B) Shaker H4-Δ. Time course of the ionic current during 1-min depolarizing pulse to 0 mV. (C) Shaker H4-Δ. Time course of ionic current during a pulse to 0 mV (as shown in B). Data are normalized to their minima and maxima and fitted to the sum of two exponential functions (narrow trace) constraining the two time constants to be the same for ionic and gating current data: $\tau_{fast} = 4.1$ s, $\tau_{slow} = 24$ s. The ratios between the fast and slow components for charge movement and ionic current were 2.15 and 3.7, respectively. Data points are normalized to their minima and maxima.

Figure 5. Correlation between the time course of installation of C-type inactivation and time course of changes in charge movement. (A) Shaker H4-Δ-W434F upper trace is the pulse protocol, remaining traces are unsubtracted gating currents. Linear components were analogically compensated with the amplifier transient cancellation at 20 mV HP. Numbers preceding the traces are the duration of the conditioning pulse to 0 mV. The −90 mV HP was held for 1 min between each stimulating pulse to fully recover from the inactivation. Note that increasing the duration of the conditioning pulse to 0 mV induces a reduction in the charge movement measured for a pulse from 0 to −60 mV. (B) Shaker H4-Δ. Time course of the ionic current during 1-min depolarizing pulse to 0 mV. (C) Shaker H4-Δ. Time course of ionic current during a pulse to 0 mV (as shown in B). Data are normalized to their minima and maxima.

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and ionic current could be well fitted simultaneously to the sum of two exponential functions with the same time constants. The two $t$ obtained by the fit were 0.01 and 1.1 s (Fig. 6C), indicating that the recovery from slow inactivation at 290 mV in Shaker H4-D channel is a much faster process than the installation at 0 mV.

We measured the recovery of charge movement and ionic conductance simultaneously in the conducting clone Shaker H4-D to give further support to the hypothesis that the changes in charge movement are correlated to the slow inactivation of the channel. In this case, we took advantage of the low permeability of Shaker H4-D channels to Cs$^+$ to record at the same time gating and ionic current of the same order of magnitude. Cs$^+$ currents were recorded in cell attached patches under symmetrical isotonic Cs-MES in permeabilized oocytes. We confirmed that in external Cs$^+$ slow inactivation is still present (López-Barneo et al., 1993). The complete exchange of the internal oocyte medium with the bath solution (isotonic Cs-MES) was checked measuring the reversal potential of the ionic current. Fig. 7A shows the currents elicited under these conditions and Fig. 7B shows the current to voltage (I-V) curve, confirming the symmetry between the internal and the pipette solutions containing isotonic Cs-MES with 2 mM CaCl$_2$. Pulsing to the reversal potential for Cs$^+$ (0 mV). The channels were inactivated at 0 mV for 1 min and recovered with different pulse duration to $-90$ mV. Note that increasing the duration of the conditioning pulse to $-90$ mV produces an increase of the charge movement measured for a pulse from $-90$ to 0 mV, and a parallel increase in the tail current at the repolarizing potential ($-90$ mV). (B) Time course of the recovery of ionic current and gating charge. Simultaneous fit to normalized charge movement (from $-90$ to 0 mV as in A) and peak tail current during the repolarization to $-90$ mV. Data (ionic current $\bullet$ and charge $\Delta$, normalized to their minima and maxima) were fitted to the sum of two exponential functions (continuous line) with the same time constants and different amplitudes: $t_{fast}=0.28$ s, $t_{slow}=6.47$ s. The ratios between the fast and slow components for charge movement and ionic current were 2.33 and 1.35, respectively.

**Figure 7.** Cs$^+$ currents in Shaker H4-D. (A) Membrane currents in cell-attached mode from an oocyte in which the internal medium was equilibrated with isotonic Cs-MES. The pipette was filled with isotonic 2 mM Cs-MES CaCl$_2$, $-90$ mV HP. Pulse protocol (top): pulses were from $-80$ to 60 mV in 20-mV increments. Linear components were subtracted with $P/4$ protocol from $-90$ mV subtracting HP. (B) I-V relationship from the experiment in A. The reversal potential for Cs$^+$ is 0 mV.

**Figure 8.** Ionic and gating current of Shaker H4-D recover from slow inactivation with the same course. Same conditions as in Fig. 7. (A) Current traces in isotonic Cs-MES obtained pulsing to the reversal potential for Cs$^+$ (0 mV). The channels were inactivated at 0 mV for 1 min and recovered with different pulse duration to $-90$ mV. Note that increasing the duration of the conditioning pulse to $-90$ mV produces an increase of the charge movement measured for a pulse from $-90$ to 0 mV, and a parallel increase in the tail current at the repolarizing potential ($-90$ mV). (B) Time course of the recovery of ionic current and gating charge. Simultaneous fit to normalized charge movement (from $-90$ to 0 mV as in A) and peak tail current during the repolarization to $-90$ mV. Data (ionic current $\bullet$ and charge $\Delta$, normalized to their minima and maxima) were fitted to the sum of two exponential functions (continuous line) with the same time constants and different amplitudes: $t_{fast}=0.28$ s, $t_{slow}=6.47$ s. The ratios between the fast and slow components for charge movement and ionic current were 2.33 and 1.35, respectively.
show that both charge movement (transient outward current) and membrane conductance (tail current) recover as the recovering time to −90 mV increases. The time course of the recovery for the charge and the ionic current, plotted in Fig. 8 B, follow a very similar time course. Both recoveries were fitted simultaneously to the sum of two exponential functions with different weights (τ_{fast} = 0.28 s, and τ_{slow} = 6.47 s).

Relative Proportion of the Reluctant and Compliant Components in the Q-V Curves Is a Function of the Holding Potential

We explored the effect of intermediate depolarizations on the voltage dependence of the charge movement. Holding potentials ranging between −90 and 0 mV were applied to evaluate their effects on the charge movement. The holding potential was maintained for at least 1 min before voltage stepping. Fig. 9 A shows Q-V curves obtained in the same oocyte at different holding potentials. The gating charge movement was quantified by fitting two Boltzmann distributions (Q_1 and Q_2).

\[
Q(V) = \frac{Q_1}{1 + \exp \left( \frac{V - V_1}{z_1 R T} \right)} + \frac{Q_2}{1 + \exp \left( \frac{V - V_2}{z_2 R T} \right)},
\]

where V_1 and V_2 are the midpoints and z_1 and z_2 are the effective valences for gating charge components Q_1 and Q_2, respectively. All the Q-V curves could be simultaneously fitted with the same effective valences, z_1 and z_2, and with different relative amplitudes for the two components.
ponents of the Q-V curves: the best fit gave $z_1 = 2.94$, $z_2 = 4.43$. At hyperpolarized holding potentials more negative than $-60$ mV, the Q-V curves show a smaller component that corresponds to the movement of $Q_1$ ($\sim$15–20% of the total charge) with a shallower voltage dependence ($z_1 = 2.94$) and a second larger component ($Q_2$) with higher voltage dependence ($z_2 = 4.43$) (Stefani et al., 1994). Raising the HP more positive than $-50$ mV (i.e., increasing the population of the slow inactivated channels), the Q-V curves start shifting toward the left along the voltage axis. The half activation potential ($V_{1/2}$) of $Q_1$ was $\sim-70$ mV for all holding potentials showing a very small sensitivity to the HP, while for $Q_2$ half activation potential ($V_{1/2}$) was less negative for holding potentials more positive than $-40$ mV (Fig. 9 B). The changes in the Q-V curves with different holding potentials was mainly due to changes in the relative amplitudes of the components $Q_1$ and $Q_2$. $Q_1$ increased as the HP was made more positive and it reached almost 100% of the amplitude of the total charge movement for HP more positive than $-30$ mV (Fig. 9 C). At the intermediate holding potentials, between $-30$ and $-50$ mV, $\sim$50% of the channels are inactivated (Fig. 2 D), and in this voltage range a clear separation between $Q_1$ and $Q_2$ is observed with $\sim$0.5 relative amplitude. The change in relative amplitudes of $Q_1$ and $Q_2$ as the HP is made more positive is most economically explained on the basis of an increase in the population of channels that are slow inactivated.

**Discussion**

Yellen et al. (1994) and Liu et al. (1996) have shown that slow inactivation involves a structural rearrangement of the outer mouth of the Shaker $K^+$ channel. Changes in the protein structure are likely expected to modify the position and the mutual interactions of the charged domains inside a folded protein. We have shown in this work that long depolarizations modify the voltage dependence of the gating charge movement and that this change appears to be related to the process of slow inactivation. From a molecular point of view, these results can be interpreted as a conformational change of the voltage sensor induced by the slow inactivation process.

We have shown the correlation between the time course of the changes in voltage dependence of the charge movement and the time course of the inactivation during a long depolarization, as well as the recovery of the ionic current and the recovery of the voltage dependence of the charge after a long depolarization. These data support the hypothesis that slow inactivation involves relatively slow changes in the protein structure under depolarizing potentials that modify the “close $\rightarrow$ open” pathway, enhancing the energy barrier that the protein must overcome to assume a conduct-
there are two distinct slow inactivation processes: P- and C-type and that only C-type inactivation is associated with modifications in the voltage sensor conformation that induces shifts in the Q-V curve.

**Kinetic Model for the Slow Inactivation**

Most of the kinetic and steady state properties shown here for *Shaker* H4-Δ and *Shaker* H4-Δ-W434F can be reproduced by a simple model (Fig. 10 A) based on the one presented by Bezanilla et al. (1982) and that accounts for slow inactivation and Q-V shift for the squid axon Na\(^+\) channels.

The model is based on an eight-states sequential model (Bezanilla et al., 1994) to which an equal number of inactivated states (I) were added (Fig. 10 A). The elementary rates connecting the upper and the lower rows of states, \(\gamma\) and \(\delta\), are voltage independent and much smaller than the rates connecting the states of the normal and inactivated modes. Thus, the channel gates with voltage in two modes: the normal mode (Fig. 10 A, upper row), and the inactivated mode (Fig. 10 A, lower row). The main assumption of this model is that the inactivated states are more stabilized as the channel progresses toward the open state. Specifically, this stabilization has been modelled as an interaction with energy \(W = w/kT\) that is the same in each of the transitions in the lower row. This interaction energy increases the forward rates between the inactivated states by a factor equal to \(\exp(W)\), and the resulting equilibrium constants are represented in the figure as \(K_i\exp(W)\), where \(i\) is the state index. Microscopic reversibility requires that the return rates between the inactivated and normal states will be multiplied by \(\exp(\sum W_i)\), stabilizing the inactivated states in proportion to the proximity of the open state. It is then easy to see that a positive holding potential will stabilize the rightmost inactivated state, and that under these conditions a short repolarization will have a leftward shift of the Q-V curve because the forward rates are increased by a factor exponentially dependent on the interaction energy.

When the membrane is extremely hyperpolarized, the channels will be preferentially in the normal mode (\(\delta \exp[7W] \gg \gamma\)). The relatively small value of the interaction energy (\(\approx 2.4\, kT\)) could be interpreted in molecular terms as a small and slow conformational change that affects the energy profile encountered by the voltage sensor. Thus, depolarized potentials maintained for prolonged periods of time make the return of the voltage sensor to the resting position more unlikely.

For long and strong depolarizations, the relatively slow population of the inactivated state connected to the open state produces the slow decay of the ionic current. From the inactivated state connected to the open state, the transition back to the open state becomes very unlikely due to the small backward rate constant of this transition. After the channel has reached the slow inactivated state, fast repolarizations produce charge movement that reflects the transition among the inactivated states. The transition \(I \rightarrow C\) becomes energetically more probable at hyperpolarized potentials. The new voltage dependence of the slow inactivated charge (experimentally corresponding to the left shifted Q-V curve at depolarized holding potentials) corresponds to the charge movement related to the transitions among the inactivated states in the lower line of the proposed model. For hyperpolarized holding potentials, where no significant slow inactivation is present, the Q-V
curve occurring between the closed states.

The kinetic model shown in Fig. 10 A was sufficient
for describing the features of the G(V) curves from
different holding potentials (Fig. 10 B) and it predicts
the position of the Q-V curves measured from −90, 0, and
−41-mV holding potentials. Fig. 10 D shows the time
course of the ionic current after a depolarization step

to 0 mV (thick line) and the prediction of the kinetic
scheme given in Fig. 10 A. The model fails to give an
adequate description of the second slow component of
the recovery from slow inactivation. This is an indica-
tion that the inactivation process may occur in more
than one transition. This would add another parallel
line of inactivated states that would make the model
complicated and difficult to test experimentally.

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REFERENCES

Armstrong, C.M., and F. Bezanilla. 1977. Inactivation of the sodium
channel. II. Gating current experiments. J. Gen. Physiol. 70:567–590.

Bezanilla, F., R.E Taylor, and J.M., Fernandez. 1982. Distribution
and kinetics of membrane dielectric polarization. I. Long-term
inactivation of gating currents. J. Gen. Physiol. 29:21–40.

Bezanilla, F., E. Perozo, D.M. Papazian, and E. Stefani. 1991. Molecular
bases of gating charge immobilization in Shaker potassium channel.
Science (Wash. DC). 254:679–683.

Bezanilla, F., E. Perozo, and E. Stefani. 1994. The gating of Shaker
K+ channels. II. The components of gating currents and a model
of channel activation. Biophys. J. 66:1011–1021.

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

De Biasi, M., H.A. Hartmann, J.A. Drewe, M. Taglialetela, A.M.
Brown, and G.E Kirsh. 1993. Inactivation determined by a single
site in K+ pores. Pflugers Archiv. Eur. J. Physiol. 422:355–363.

Enzenstein, G., and D.L. Gilbert. 1966. Slow changes of potassium
permeability in the squid giant axon. Biophys. J. 6:553–566.

Fedida, D., R. Bouchard, and F.S.P. Chen. 1996. Slow gating charge
immobilization in the human potassium channel Kv 1.5 and its
prevention by 4-aminopyridine. J. Physiol. (Camb.). 494:377–387.

Hodgkin, A.L., and A.F. Huxley. 1952. A quantitative description of
membrane current and its application to conduction and excita-
tion in nerve. J. Physiol. (Camb.). 117:500–544.

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

Hoshi, T., W.N. Zagotta, and R.W. Aldrich. 1991. Two types of inac-
tivation in Shaker K+ channels: effects of alterations in the car-
boxyl-terminal region. Neuron. 7:547–556.

Kamb, A., L.EI. Iverson, and M.A. Tanouye. 1987. Molecular charac-
terization of Shaker, a Drosophila gene that encodes a potassium
channel. Cell. 50:405–413.

Labarca, P., and R. MacKinnon. 1993. Permeant ions influence the
rate of slow inactivation in Shaker channels. Biophys. J. 61:A578.

Liu, Y., M.E. Jurman, and G. Yellen. 1996. Dynamic rearrangement
of the outer mouth of a K+ channel during gating. Neuron. 16:
859–867.

López-Barneo, J., T. Hoshi, S.F. Heinemann, and R.W. Aldrich.
1993. Effects of external cations and mutations in the pore re-
gion on C-type inactivation of Shaker potassium channels. Recep-
tors Channels. 1:61–71.

Noceti, F., P. Baldelli, X. Wei, N. Qin, L. Toro, L. Birnbaumer, and
E. Stefani. 1996. Effective gating charges per channel in voltage
dependent K+ and Ca2+ channels. J. Gen. Physiol. 108:143–155.

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

Olcese, R., L. Toro, E. Perozo, F. Bezanilla, and E. Stefani. 1994.
Prolonged depolarization changes charge movement properties
in Shaker-IR W434F K+ channel. Biophys. J. 66:A107.

Olcese, R., L. Toro, F. Bezanilla, and E. Stefani. 1995. Correlation
between charge movement and ionic current during C-type inac-
tivation in Shaker-IR potassium channels. Biophys. J. 68:A33.

Panyi, G., Z. Sheng, L. Tu, and C. Deutsch. 1993. C-type inactiva-
tion of voltage gated K channel occurs by a cooperative mecha-
nism. Biophys. J. 69:896–906.

Perozo, E., R. MacKinnon, F. Bezanilla, and E. Stefani. 1993. Gating
currents from a non-conducting mutant reveal open-closed
conformations in Shaker K+ channels. Neuron. 11:353–358.

Seoh, S.-A., D. Starace, D.M. Papazian, E. Stefani, and F. Bezanilla.
1996. D447N and W434F mutations in the pore of Shaker B K+
channels prevent ion conduction but restore conducting states
by combined mutation with T449Y. Biophys. J. 70:A190.

Stefani, E., L. Toro, E. Perozo, and F. Bezanilla. 1994. The gating of
Shaker K+ channels. I. Ionic and gating currents. Biophys. J. 66:
996–1010.

Yan, Y., Y. Yang, and F.J. Sigworth. 1996. How does W434F block
Shaker channel current? Biophys. J. 70:A190.

Yellen, G., D. Sodickson, T.S. Chen, and M.E. Jurman. 1994. An en-
gineered cysteine in the external mouth of a K+ channel allows
inactivation to be modulated by metal binding. Biophys. J. 66:
1064–1075.

Zagotta, W.N., T. Hoshi, and R.W. Aldrich. 1989. Gating of single
Shaker K channels in Drosophila muscle and in Xenopus oocytes
injected with Shaker mRNA. Proc. Natl. Acad. Sci. USA. 86:7245–
7247.

Zagotta, W.N., T. Hoshi, and T. Aldrich. 1990. Restoration of inacti-
vation in mutants of Shaker potassium channels by a peptide de-
uced from ShB. Science (Wash. DC). 250:568–571.