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Defining the Conductance of the Closed State in a Voltage-Gated K^+ Channel

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Summary

The opening and closing of the ion conduction pathway in ion channels underlies the generation and propagation of electrical signals in biological systems. Although electrophysiological approaches to measuring the flow of ions in the open state have contributed profoundly to our understanding of ion permeation and gating, it remains unclear how much the ion-throughput rate decreases upon closure of the ion conduction pore. To address this fundamental question, we expressed the Shaker Kv channel at high levels and then measured macroscopic K^+ currents at negative membrane voltages and counted the number of channels by quantifying the translocation of gating charge. Our results show that the conductance of the closed state is between 0 and 0.16 fS, or at least 100,000 times lower than for the open state of the channel, indicating that the flow of ions is very tightly regulated in this class of K^+ channels.

Introduction

The principal functional role of ion channel proteins is to provide a pathway for ions to cross the lipid membrane. In most instances, the ion conduction pathway is carefully regulated so that it opens and closes in response to a particular type of stimuli, a process widely referred to as gating (Hille, 2001). Voltage-gated potassium (Kv) channels are a large family of K^+-selective channels that open and close in response to changes in membrane voltage. These channels are tetramers, with each subunit containing six transmembrane (TM) segments, designated S1 through S6 (Figure 1A). Functional studies on the gating of Kv channels suggest that the S6 segment forms an activation gate located near the intracellular entrance to the ion conduction pore (Figure 1B; Armstrong, 1969, 1971; Armstrong and Hille, 1972; del Camino and Yellen, 2001; Hackos et al., 2002; Holmgren et al., 1997, 1998; Liu et al., 1997; Zhou et al., 2001a). More recently, comparisons between the structure of the KcsA K^+ channel (Doyle et al., 1998), which is thought to be closed (Jiang et al., 2002a; Roux et al., 2000; Zhou et al., 2001a), and the Ca^{2+}-activated MthK channel in the open state (Jiang et al., 2002a) have suggested that a gate is located near the intracellular entrance to the pore and that it is formed by the TM2 helix, a region that corresponds to S6 in Kv channels (Jiang et al., 2002b). In these prokaryotic K^+ channels, opening of the intracellular gate involves bending of the TM2 helices at a Gly residue that is conserved in all types of K^+ channels, greatly widening the intracellular entrance to the ion conduction pathway.

In spite of these groundbreaking functional and structural studies, it remains unclear how large a barrier to the movement of K^+ ions exists in the closed state of K^+ channels. In other words, how closed is a closed channel? This is a fundamental question that has important implications for the number of gates that are present in a given channel and for the mechanics by which these gates operate to regulate the flow of ions. Recently, del Camino and Yellen reported that the reactivity between Ag^+ and Cys residues at position 474 in the S6 gate region of the Shaker Kv channel decreases by about 700-fold when the channel closes (del Camino and Yellen, 2001). Since the size and diffusion properties of Ag^+ and K^+ are quite similar, these results raise the possibility that the S6 gate decreases the flow of K^+ ions by only about 10^3. Are these changes in the accessibility of ions for the S6 gate sufficient to explain ion gating in Kv channels? In order to answer this question, we need to know how large a change in the ion-throughput rate accompanies closure of the channel. From single channel recordings of the Shaker Kv channel, the unitary conductance (γ) for the main open state is $\sim$20 pS in symmetrical 100 mM K^+ (Heginbotham and MacKinnon, 1993). Because the detection limit for this technique is $\sim$1 pS at 5 kHz bandwidth (Sakmann and Neher, 1995), it cannot report on decreases in the ion-throughput rate that are greater than about 100-fold. The goal of the experiments described here was to estimate the decrease in γ using a macroscopic approach. Our results indicate that the closed state has a conductance for K^+ ions that is at least 100,000-fold lower than the open state.

Results and Discussion

The Shaker Kv channel predominantly adopts a closed conformation at negative membrane voltages but can be opened by membrane depolarization (Yellen, 1998). This gating behavior is illustrated in Figures 2A and 2B, where an oocyte expressing between 10^5 and 10^6 channels displays robust voltage-activated currents in response to membrane depolarization. In order to isolate currents originating from the expressed channel, membrane depolarizations were given before and after application of Agitoxin-2, a K^+ channel inhibitor that selectively interacts with Kv channels (Garcia et al., 1994). For the toxin-sensitive currents shown in Figures 2A and 2B, there is no detectable current at voltages more negative than -50 mV.

Our first objective was to see if we could detect a

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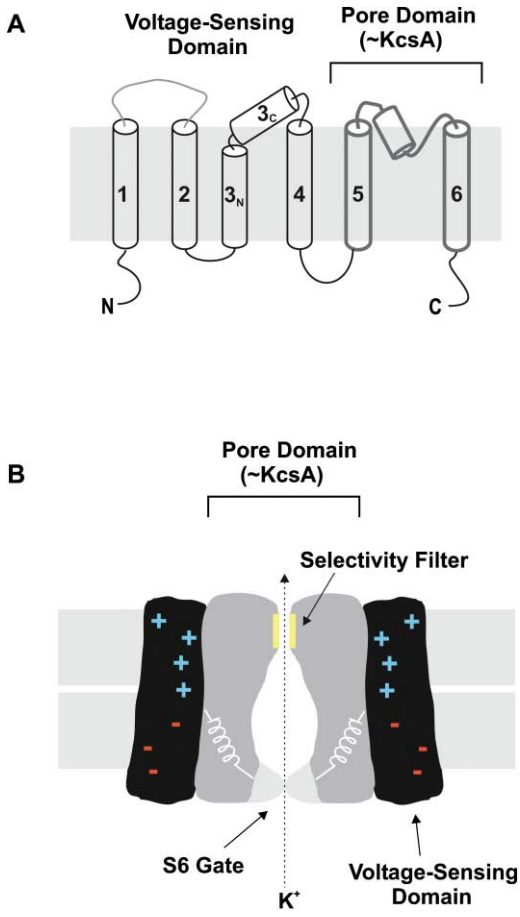

Figure 1. Architecture and Functional Parts of a Kv Channel

(A) Membrane folding diagram for a Kv channel drawn according to Li-Smerin et al. (2000) and Li-Smerin and Swartz (2001).

(B) Cartoon illustrating functional parts of a Kv channel. The central pore domain probably adopts a structure that is similar to the transmembrane portion of the KcsA K^+ channel (Doyle et al., 1998). This domain contains the permeation pathway and the intracellular S6 gate. Each peripheral voltage-sensing domain is formed by the S1 through S4 segments.

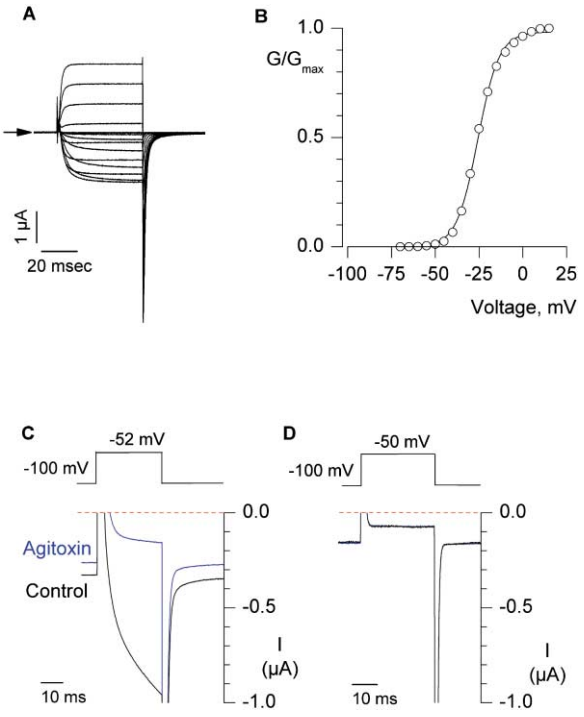

Figure 2. Identification of Kv Channel Current at Negative Voltages

(A) Family of Agitoxin-sensitive ionic currents for an oocyte expressing the Shaker Kv channels at moderate levels. Holding voltage was −90 mV, tail voltage was −60 mV, and depolarizations were from −60 mV to +20 mV in 5 mV steps. Linear capacity and background conductances were identified and subtracted by blocking the Shaker channel with Agitoxin-2. Black arrow corresponds to the zero current level.

(B) Conductance (G) versus voltage (V) relations obtained by plotting normalized tail current amplitudes against the voltage of the preceding depolarization. Same cell as in (A). Smooth curve is a single Boltzmann fit to the data with $V_{50} = -26$ mV and $z = 4.1$.

(C) Agitoxin block of current at −100 mV in an oocyte expressing the Shaker Kv channel at high levels. Traces shown are averages from five consecutive traces obtained before (black) and after (blue) the addition of 1 μM Agitoxin-2.

(D) Effects of Agitoxin on current at −100 mV in an uninjected oocyte. Traces shown are averages from five consecutive traces obtained before (black) and after (blue) the addition of 1 μM Agitoxin-2.

toxin-sensitive current at negative membrane voltages where the Shaker Kv channel is thought to be predominantly closed. From the results above, it appears that there is no detectable toxin-sensitive current at −100 mV when the channel is studied at moderate expression levels. To increase channel expression, we injected oocytes with large quantities of Shaker cRNAs (50 ng per cell) containing both 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene (Liman et al., 1992), and we waited for 3 to 7 days for expression to reach maximal levels. The majority of cells expressing Shaker at high levels exhibited unstable holding currents when the membrane voltage was held at −100 mV, making further characterization of these cells impossible. However, a small percentage of cells (~1%) displayed holding currents at −100 mV that were stable enough to assess their sensitivity to Agitoxin-2. One example is illustrated in Figure 2C, where Agitoxin-2 was applied in an extracellular solution containing 100 mM K^+ to an oocyte expressing the Shaker Kv channel. Qualitatively, we can tell that this cell is expressing Shaker to high levels because even a weak depolarization to −52 mV evokes large voltage-activated currents. When Agitoxin was then applied to the cell, the toxin blocked about 65 nA of current at −100 mV before the test depolarization and completely blocked the voltage-activated current at −52 mV. The mean (± SEM) toxin-sensitive current at −100 mV for 16 oocytes examined in this manner was 43 ± 8.4 nA. When experiments were performed on uninjected oocytes, the toxin-sensitive current at −100 mV was 2.8 ± 2.2 nA (n = 3), an example of which is shown in Figure 2D. In other experiments we also assessed the toxin sensitivity of currents at −100 mV when cells expressing Shaker were bathed in an extracellular solution containing 10 mM K^+. In these instances, we observed relatively small toxin-sensitive currents (5.6 ± 0.2 nA; n = 3), suggesting that the toxin-sensitive current is carried by K^+. Taken together, these

results suggest that ionic currents observed at −100 mV are mediated by the Shaker Kv channel. A large body of evidence supports the notion that Agitoxin and related toxins interact with the external vestibule of the ion conduction pore in K^+ channels (Anderson et al., 1988; Goldstein and Miller, 1993; Goldstein et al., 1994; Gross et al., 1994; Gross and MacKinnon, 1996; Hidalgo and MacKinnon, 1995; Legros et al., 2000; MacKinnon et al., 1990, 1998; MacKinnon and Miller, 1988, 1989; Miller, 1988, 1995; Naranjo and Miller, 1996; Park and Miller, 1992; Ranganathan et al., 1996; Stocker et al., 1991). Thus, the Agitoxin sensitivity of the currents that we measured at −100 mV strongly suggest that they originate from the movement of K^+ ions through the ion conduction pore and not through some other pathway within the protein.

In order to better understand the unitary properties underlying the macroscopic current that we measured at −100 mV, we need to know the number of channels (N). To determine N, we quantified the total gating charge (Q) per cell from the integral of gating currents (resulting from movement of the voltage-sensors [Armstrong, 1992; Bezanilla and Stefani, 1998]), using the previously determined charge per channel of 13.6 for the Shaker Kv channel (Aggarwal and MacKinnon, 1996; Schoppa et al., 1992; Seoh et al., 1996). Gating currents were elicited by depolarization in the presence of Agitoxin-2, as illustrated in Figure 3A. Both the On and Off components of gating current were then integrated to obtain the value of Q for different strength depolarizations (Figure 3B). Q-V relations obtained in this manner are shown for nine cells in Figure 3C. In each cell, we used the maximal value of Q, obtained by fitting the Q-V relation with a single Boltzmann function, to calculate channel number. For the example shown in Figures 3A and 3B, we obtained a Q value of 11.4 nC, which corresponds to 5.3×10^9 channels. Figure 4 shows results from a series of experiments where we measured the toxin-sensitive current at −100 mV and determined N in the same oocytes. Figures 4A and 4B show one example where the toxin-sensitive current at −100 mV was 70 nA and Q_{max} was 5.6 nC, from which we calculate a channel number of 2.6×10^9. When both of these measurements were performed on oocytes expressing different numbers of Shaker channels, ranging from 2×10^8 to near 6×10^9, we observed a strong correlation ($r = 0.78$; $p = 0.0003$) between the amplitude of the toxin-sensitive current at −100 mV and the number of channels (Figure 4C). This correlation further suggests that the toxin-sensitive current at −100 mV results from the conduction of K^+ through the Shaker Kv channel. Linear regression fit to the I versus N relation yields a slope of 1.6×10^{-17} A per channel (Figure 4C). If the toxin-sensitive current measured at negative voltages results from the flux of K^+ ions through the closed state of the channel, then the slope of the I versus N relation will be equal to the unitary current (i) for the closed state. In this case, the corresponding unitary conductance (γ) of the closed state would be 1.6×10^{-16} S.

Another possibility is that the toxin-sensitive current measured at −100 mV results from the flow of ions through channels that are open, albeit only rarely. Hypothetically, if the toxin-sensitive conductance (G) at −100 mV arises from channels that are open and coupled to the voltage sensors, the ratio of G/G_{max}, where G_{max} is calculated from the product of i, N, and $P_o$max, should be equal to the ratio of P_o at −100 mV to $P_o$max. Unitary measurements for the Shaker Kv channel at 2.5 kHz bandwidth predict that voltage sensor-coupled openings at −100 mV would have a $P_o < 10^{-9}$ and that the $P_o$max at positive voltages is ~0.8 (Islas and Sigworth, 1999). From the results presented above, G/G_{max} is 8×10^{-6}, a value that is over three orders of magnitude larger than the ratio of P_o/P_omax ($<10^{-9}$), suggesting that the toxin-sensitive currents measured at −100 mV cannot arise from open channels that are coupled to the voltage sensors. In addition, if the current we measured arises from voltage sensor-coupled openings, then the

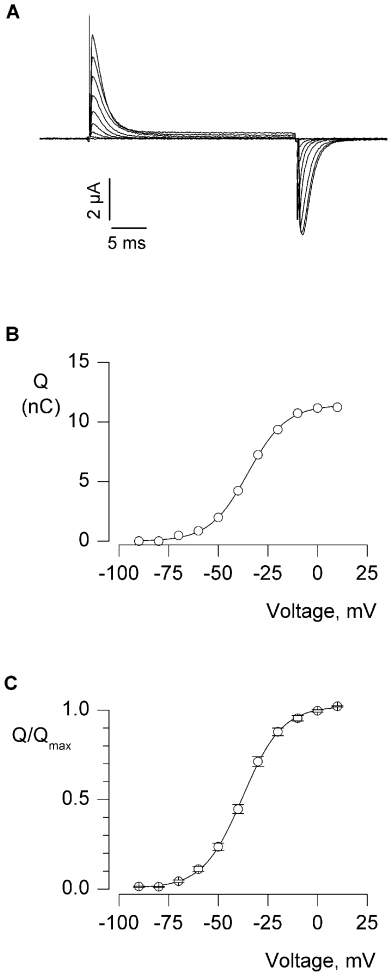

Figure 3. Measurement of Gating Currents for the Shaker Kv Channel

(A) Family of gating current records obtained in an extracellular solution containing NMDG instead of K^+ and the addition of 50 μM Agitoxin-2. Holding voltage was −100 mV and depolarizations were to voltages between −90 and 0 mV in 10 mV increments. A P/-4 protocol was used to subtract leak and linear capacitive currents.

(B) Q-V relation for the cell in (A). Smooth curve is a single Boltzmann fit to the data with $V_{50} = -35$ mV and $z = 2.6$. Q_{max} for this cell was 11.4 nC, corresponding to 5.3×10^9 channels.

(C) Normalized Q-V relation for a population of nine cells. Error bars are SEM. Smooth curve is a single Boltzmann fit to the data with $V_{50} = -38$ mV and $z = 2.6$ for the population.

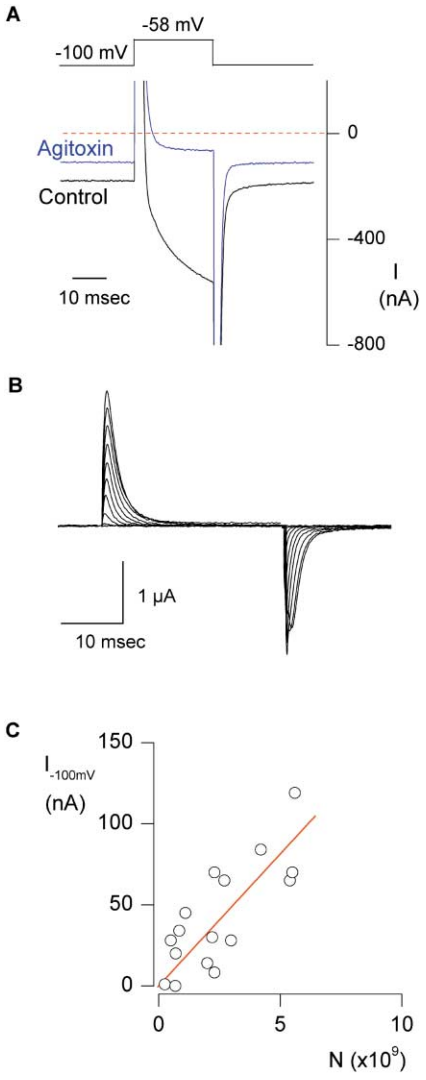

Figure 4. Measurement of Toxin-Sensitive Current and Counting Channels

(A) Averages of five consecutive traces obtained before and after the addition of 1 μM Agitoxin-2 to an oocyte expressing the Shaker Kv channel at high levels.

(B) Family of gating currents records from the same cell in (A) after changing to a solution containing NMDG instead of K^+ and the addition of 50 μM toxin. Holding voltage was −100 mV and depolarizations were to voltages between −90 and 0 mV in 10 mV increments. A P/-4 protocol was used to subtract leak and linear capacitive currents. Q_{max} for this cell was 5.6 nC, corresponding to 2.6×10^9 channels.

(C) Plot of toxin-sensitive current (I) versus channel number (N). Toxin-sensitive current was measured at −100 mV as in (A) and $N = Q_{max}/(1.6 \times 10^{-19}\ Ce^{-1} \times 13.6\ e\ \text{channel}^{-1})$ with Q_{max} determined as in (B) and in Figure 3. Red line is a linear regression fit to the data where $I = iN$ with $i = 1.6 \times 10^{-17}$ A per channel.

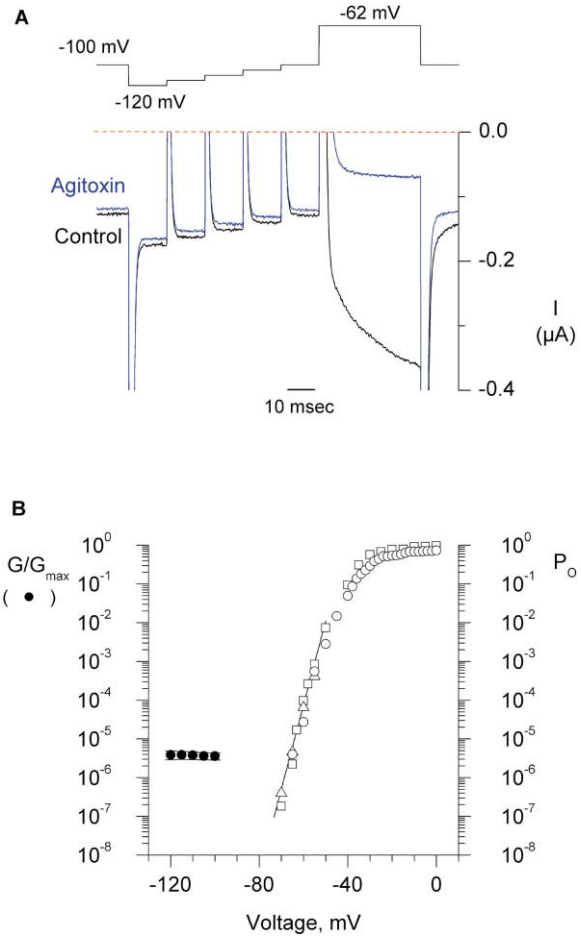

Figure 5. Voltage Independence of Toxin-Sensitive Conductance at Negative Voltages

(A) Toxin block of current at various negative voltages. Averages of five consecutive traces obtained before and after the addition of 1 μM Agitoxin-2 to an oocyte expressing the Shaker Kv channel at high levels.

(B) Plot of G/G_{max} versus voltage for determinations made in three cells. $G = I/V$ where I is the toxin-sensitive current measured between −120 and −100 mV and $G_{max} = (NiP_o max)/V$, where N is calculated from Q as described in Figures 3 and 4, $i = 2$ pA at −100 mV, and $P_o max = 0.8$. P_o data (open symbols) from three patches, generously provided by Islas and Sigworth (1999), are shown for comparison. Smooth line, shown between P_o of 10^{-7} and 10^{-2}, corresponds to a single Boltzmann function with $V_{50} = -41$ mV and $z = 12.7$.

corresponding conductance should be steeply voltage dependent because P_o is steeply voltage dependent (valence ~12) at least down to values of 10^{-9} (Figure 5B; Islas and Sigworth, 1999). To examine the voltage dependence of the toxin-sensitive current, we made measurements at voltages ranging from −120 to −100 mV (Figure 5A) and calculated G/G_{max}. As shown in Figure 5B, the toxin-sensitive conductance measured at negative voltages has no significant voltage dependence, indicating that it does not originate from rare openings that are coupled to the voltage sensors.

Even though the conductance we measured cannot be explained by the type of open events that have been observed in single channel recordings of the Shaker Kv channel (Islas and Sigworth, 1999), it could result from undetected, very brief open events that are independent of the voltage sensors. Although voltage sensor-independent openings have not been observed in the wild-type Shaker K^+ channel, either because they are very brief or because the coupling between the voltage sensors and the gate is exceedingly tight in this channel,

they have been observed in Ca^{2+}-activated K^+ channels (Horrigan and Aldrich, 2002; Horrigan et al., 1999) and in mutants in the activation gate region of the Shaker Kv channel (Sukhareva et al., 2003). If these opening events occur in the wild-type Shaker channel, they could contribute to the macroscopic conductance that we measured here. We therefore conclude that the conductance of the closed state lies between 0 and 0.16 fS.

Although unitary current recordings offer unique insight into the gating and permeation of ion channels (Sakmann and Neher, 1995), the amplitude of currents in the open states is often less than 100-fold larger than the noise. Consequently, little is know about the movement of ions in the closed state, except that flux is decreased by at least 10- to 100-fold. Our results using a macroscopic approach place an upper limit of 0.16 fS on the value of γ for the closed state of the channel. In the case of Shaker, since γ for the open state is $\sim$20 pS, this result suggests that the rate at which K^+ ions permeate is decreased by at least 100,000-fold when the channel closes. Where does this sizable energy barrier ($\sim$7 kcal mol^{-1}) exist in the closed state of the channel? In Kv channels, previous work has shown that the S6 transmembrane segment forms an activation gate located near the intracellular entrance to the ion conduction pore (Armstrong, 1969, 1971; Armstrong and Hille, 1972; del Camino and Yellen, 2001; Hackos et al., 2002; Holmgren et al., 1997, 1998; Liu et al., 1997; Zhou et al., 2001a). In the X-ray structure of the KcsA K^+ channel, the inner helices (equivalent to S6 in Kv channels) form a bundle crossing near the intracellular extent of the protein. Although it remains unclear how large a barrier is produced by this bundle crossing, molecular dynamic simulations of KcsA suggest that a barrier of $\sim$20 kcal mol^{-1} exists at the intracellular entrance to the pore, arising largely from the need to dehydrate K^+ ions before they can enter the pore (Roux et al., 2000). Thus, our estimates might be explained by constriction of the internal pore along the lines of what is seen in the X-ray structure of KcsA (Doyle et al., 1998; Zhou et al., 2001b). In the Shaker Kv channel, the accessibility of Ag^+ for Cys residues at position 474, a residue located within the S6 gate region, differs by only 700-fold between closed and open states (del Camino and Yellen, 2001). These changes in accessibility for the S6 gate region cannot account for the greater than 100,000-fold decrease in γ that we estimate for the entire channel. It is possible that the difference in Ag^+ accessibility between open and closed states has been underestimated because Ag^+ has a higher accessibility than K^+ for the closed state of the channel or because Cys substitutions at 474 make the gate leaky. Alternatively, the difference between these two measurements may indicate that other regions of the channel, perhaps the selectivity filter (Chapman et al., 1997; Zheng and Sigworth, 1997, 1998; Zhou et al., 2001b), act together with the S6 gate to minimize the flux of ions in the closed state.

Experimental Procedures

Molecular Biology and Channel Expression

Experiments were performed using the Shaker H4 Kv channel (Kamb et al., 1988) in the pGEM-HE vector (Liman et al., 1992) with deletion of residues 6 through 46 to remove fast N-type inactivation (Hoshi et al., 1990; Zagotta et al., 1990). Shaker cDNA was linearized with *HindIII* and transcribed using T7 RNA polymerase. *Xenopus laevis* oocytes were removed surgically and incubated with agitation for 1–1.5 hr in a solution containing (mM) 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 5 HEPES, and 2 mg/ml collagenase (Worthington Biochemical Corp.) (pH 7.6) with NaOH. Defolliculated oocytes were injected with $\sim$0.5 to 50 ng of cRNA and incubated at 17°C in a solution containing (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, and 50 μg/ml gentamicin (Invitrogen/GIBCO-BRL) (pH 7.6) with NaOH.

Electrophysiological Recording

Macroscopic ionic and gating currents were recorded using two-electrode voltage clamp recording techniques between 1 and 7 days after cRNA injection using an OC-725C oocyte clamp (Warner Instruments). For ionic current measurements, the extracellular solution contained (mM) 100 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, and 5 HEPES (pH 7.6) with KOH. In all experiments, ionic currents mediated by the Shaker K^+ channel were assayed as the component of membrane current that is sensitive to Agitoxin-2, a specific Shaker channel pore-blocking toxin (Garcia et al., 1994). The saturating concentrations of toxin (1 μM) used in these experiments and the voltage dependence exhibited by this class of toxins (tighter binding at negative voltages) (Anderson et al., 1988; MacKinnon and Miller, 1988; Park and Miller, 1992) assure us that at negative voltages, blockade of the channel is effectively complete. Gating currents were recorded after measurement of toxin-sensitive ionic currents using an extracellular solution in which 100 mM N-Methyl-D-Glucamine (NMDG) was substituted for KCl and 50 μM Agitoxin-2 was added. In the case of gating currents, both linear capacity and background currents were subtracted using a P/-4 protocol (Armstrong and Bezanilla, 1974). Data were filtered at 2 kHz (8-pole Bessel) and digitized at 10 kHz. Microelectrode resistances were between 0.2 and 1.2 MΩ when filled with 3 M KCl. All experiments were performed at room temperature ($\sim$22°C).

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