The Inactivation Gate of the Shaker K⁺ Channel Behaves Like an Open-Channel Blocker

Susan D. Demo and Gary Yellen
Howard Hughes Medical Institute
and the Department of Neuroscience
The Johns Hopkins School of Medicine
Baltimore, Maryland 21205

Summary

Following voltage-dependent activation, Drosophila Shaker K⁺ channels enter a nonconducting, inactivated state. This process has been proposed to occur by a "ball-and-chain" mechanism, in which the N-terminus of the protein behaves like a blocker tethered to the cytoplasmic side of the channel and directly occludes the pore to cause inactivation. To complement the ample evidence for the involvement of the N-terminus, we sought evidence that it blocks the pore directly. We found that inactivation exhibits several distinctive properties of pore blockade. First, recovery was speeded by increased external K⁺ concentrations, just as blockade can be relieved by trans-permeant ions. Second, single-channel experiments show that the channel reopens from the inactivated state upon repolarization. These openings were usually required for recovery, as though the blocking particle must exit the pore before the channel can close.

Introduction

Voltage-dependent K⁺ channels are found in many types of excitable and nonexcitable cells. They constitute a diverse class of channels that may be distinguished by their differences in gating, conductance, and pharmacology. These channels open upon depolarization and enter a long-lasting, nonconductive state (the inactivated state), from which they cannot be activated by depolarization. Among the different members of this class of channels, the rates of inactivation vary from milliseconds to seconds. Physiologically, this variation affects the generation, propagation, and integration of electrical signals in cells.

The transient K⁺ channels encoded by the Drosophila Shaker gene are among the most rapidly inactivating type of K⁺ channel. Like voltage-activated Na⁺ channels, they inactivate within a few milliseconds after opening. Recent experiments on Shaker K⁺ channels support a "ball-and-chain" mechanism for their fast inactivation, like the mechanism originally proposed by Bezanilla and Armstrong (1977) for Na⁺ channel inactivation. Both types of channels inactivate after opening, and the inactivation can be abolished by mild intracellular protease treatment (Armstrong et al., 1973; Hoshi et al., 1990). Protease treatment is thought to remove an inactivation particle that is normally tethered to the cytoplasmic side of the channel. In the ball-and-chain model, inactivation is caused by the reversible binding of this particle to the open state.

Genetic manipulation of Shaker K⁺ channels further supports the ball-and-chain model. Site-directed mutagenesis experiments have localized the critical cytoplasmic domain to the N-terminus of the Shaker protein; deleting 20 amino acids from this region removes inactivation (Hoshi et al., 1990). Furthermore, cytoplasmic application of a synthetic peptide corresponding to the deleted region can reconstitute inactivation of the mutated, noninactivating channels (Zagotta et al., 1990). The most attractive explanation for these results is that the N-terminus behaves like an "inactivation particle" and directly occludes the pore to cause inactivation. The only evidence that inactivation involves pore blockade, however, is that internal tetraethylammonium, which is itself thought to bind to the pore (Armstrong, 1966), appears to compete with the inactivation process (Choi et al., 1991).

We have further tested the hypothesis of direct pore blockade and found that rapid inactivation of Shaker K⁺ channels exhibits several properties that are considered diagnostic of direct pore blockade. For example, recovery from inactivation is speeded by increasing concentrations of permeant K⁺ ions on the opposite (external) side of the membrane, as previously described for other pore blockers (Armstrong, 1966; Yellen, 1984; MacKinnon and Miller, 1988; Neyton and Miller, 1988). Also, binding of the inactivation particle tends to keep the channel open. This effect is apparent from the unusually large tail currents, which result from channels passing through the open state in the process of recovering from inactivation. This finding is analogous to the ability of other blockers to prevent channel closing (Neher and Steinbach, 1978; Armstrong, 1971). These observations, taken together with the evidence for the involvement of an N-terminal domain, support the ball-and-chain model of inactivation.

Results

High External K⁺ and Hyperpolarization Speed Recovery from Inactivation

The effect of external K⁺ on recovery from inactivation of Shaker H4 K⁺ channels expressed in Xenopus oocytes was measured using a two-pulse protocol (figure 1A). During the first depolarizing pulse, the channels opened and inactivated. Following a recovery period of variable duration, a second test depolarizing pulse was applied to measure the fraction of channels that had recovered from inactivation. For short recovery periods, the current in response to the second depolarization was small because most of the channels were still inactivated. With longer recovery periods, the current was larger because more of the channels had a chance to recover. Figure 1B shows that
increasing concentrations of external K⁺ speed the rate of recovery. A simple interpretation of this result is that the external K⁺ ions are relieving blockade by an internal inactivation particle.

Consistent with the idea that external K⁺ ions must enter deeply into the channel to expel a blocking particle, the recovery rate is quite voltage dependent (Figure 2). Hyperpolarization speeds recovery at all external K⁺ concentrations, with an e-fold change in 40 mV. This contrasts with the negligible voltage dependence in the onset of inactivation (Zagotta and Aldrich, 1990). Perhaps the voltage dependence of recovery results from hyperpolarization driving K⁺ ions into the pore and "pushing" out the inactivation particle, as has been suggested for K⁺ relief of charybdotoxin (CTX)

Figure 2. Hyperpolarization Speeds Recovery from Inactivation
The voltage dependence of the recovery rate measured as described in Figure 1 at the indicated external K⁺ concentrations. Each point represents the mean ± SEM of at least three determinations.
Table 1. Recovery in 2 mM External K⁺ Has a Double-Exponential Time Course

<table>
<thead>
<tr>
<th>V&lt;sub&gt;tail&lt;/sub&gt; (mV)</th>
<th>Fast</th>
<th>Slow</th>
<th>% Fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>−80 mV</td>
<td>13 ± 0.6</td>
<td>0.18 ± 0.06</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>−100 mV</td>
<td>17 ± 2</td>
<td>0.36 ± 0.1</td>
<td>0.77 ± 0.05</td>
</tr>
<tr>
<td>−120 mV</td>
<td>20 ± 2</td>
<td>1.1 ± 0.5</td>
<td>0.81 ± 0.04</td>
</tr>
</tbody>
</table>

Rate constants for recovery in 2 mM external K⁺ are listed for currents recorded as in Figure 1. The time course was fit with a double-exponential function. Paired depolarizing pulses were applied once every 60 s, and the voltage was held at −80 mV between applications. Data represent the mean ± SEM for at least three experiments.

block of the Ca²⁺-activated K⁺ channel (MacKinnon and Miller, 1988).

Recovery in Low K⁺ Follows a Double-Exponential Time Course

In high concentrations of external K⁺ (≥30 mM), recovery had a single-exponential time course. In low external K⁺ (0.5–2 mM), however, recovery was more complicated. In addition to the fast phase observed at higher K⁺ concentrations, there was a second, slower component. Table 1 shows the rates of these two components in 2 mM external K⁺. Both rates are voltage dependent and increase with hyperpolarization. The proportion of the fast component also increased slightly with hyperpolarization. We suspect that the fast component corresponds to the recovery process measured at higher external K⁺ concentrations, and, consequently, these rate constants are used in Figure 1B. Although we have not determined the mechanism underlying the slow component, we address several possibilities in the Discussion.

Single-Channel Experiments Show Reopenings from the Inactivated State upon Repolarization

In high concentrations of external K⁺, we were surprised to see prominent inward tail currents upon repolarization from a depolarizing step that inactivated most of the channels. Tail currents, as they were originally described by Hodgkin and Huxley (1952) for the delayed rectifier K⁺ channel, result from channels that were open at the end of the pulse. After repolarization, the current is initially large and then declines monotonically as the channels close.

In our experiments, since most of the channels were inactivated at the end of the pulse, we expected small, rapid tail currents. Instead, after repolarization, we observed large tail currents with slow kinetics. In some cases, the current actually increased before declining. This process is clearly different from that observed in the delayed rectifier. We also observed that the time course of the tail currents matched the time course of recovery (Figure 3). We propose that rather than returning "silently" to the recovered closed state,

Figure 3. The Time Course of the Tail Currents Matches the Time Course of Recovery

The time course of recovery is shown superimposed on the tail current. The two-pulse protocol described in Figure 1B was applied using a recovery voltage of −120 mV. Only the current recorded with the longest recovery duration is shown. The open rectangles mark the peak current in the second pulses for recovery periods of shorter duration. The open diamonds represent the peak currents vertically offset. Outward currents were recorded in the presence of 500 mM external K⁺ in the inside out patch configuration.

Figure 4. Single-Channel Records Show Reopenings from the Inactivated State upon Repolarization

Single K⁺ channel currents during and after a depolarizing pulse, recorded in 2 mM external K⁺. The records represent seven consecutive traces at 2 s intervals. Currents were digitally leak subtracted as described in the Experimental Procedures. Outside out patch with a single channel; 36 hr post-injection. The height of the voltage diagram corresponds to 2 pA in the current trace.
Figure 5. Increasing External K⁺ Concentration Speeds Reopenings from the Inactivated State

First latency distributions for single-channel openings in the tail, at 160 mM and 30 mM external K⁺. P0 is the fraction of channels opening at or before time t after repolarization. Activity of a single channel was recorded at a tail voltage of -120 mV after a 20 ms depolarizing pulse to +60 mV. Exponential fits of 9 ± 0.6 ms (n = 5) in 160 mM external K⁺ and 23 ± 2 ms in 30 mM external K⁺ (n = 3) were calculated from log-binned histograms.

The channels retrace their steps, passing back through the open state before closing.

Single-channel experiments show this reopening process clearly (Figure 4). After a depolarizing step that caused the channel to open and inactivate, we observed openings in the tail period. The distribution of latencies to first opening in the tail is single exponential and thus probably represents a sojourn in a single nonconducting state prior to reopening (see Figure 5). Because the channel was inactivated at the end of the pulse, the latency period is almost certainly spent in the inactivated state. Henceforth, we interpret the rate of channel reopening during the tail period as the rate of passing from the fast-inactivated (I) state to the open (O) state:

\[ C \xrightarrow{\delta} O \xrightarrow{\delta} I \]

(1)

where \( \delta \) is the voltage-dependent rate of closing from O. (Here the single O state represents the voltage-independent open and closed states of Zagotta and Aldrich [1990] lumped together.) At negative voltages, the rate of reopening from the closed state(s) C is negligible. According to this model, the overall process of recovery to the closed state involves two steps: reversal of inactivation (I\( \rightarrow \)O) and final closure (O\( \rightarrow \)C).

To study the mechanism of inactivation more directly, we examined the effects of external K⁺ and voltage on the I\( \rightarrow \)O step.

External K⁺ and Hyperpolarization Speed Reopening from the Inactivated State in the Tail

Figure 5 shows distributions of the latency to first opening in the tail at 30 and 160 mM external K⁺. Consistent with the effects on the overall recovery rate, higher external K⁺ concentrations increased the rate of reopening from the inactivated state. The reopening rate was also steeply voltage dependent (Figure 6). These observations show that the I\( \rightarrow \)O step has a similar permeant ion and voltage dependence as overall recovery. Although the O\( \rightarrow \)C step probably contributes to the voltage dependence of recovery, it probably does not contribute to the permeant ion dependence. Increased permeant ions most likely slow this transition rather than speed it (Swenson and Armstrong, 1981). Therefore, the permeant ion and voltage dependence of macroscopic recovery is probably determined by the rate of reopening from the inactivated state.

Although the I\( \rightarrow \)O step is steeply voltage dependent at negative voltages, it approaches a voltage-independent minimum value at positive voltages (Figure 6). We measured the reopening rate at positive voltages from the distribution of the nonconducting periods between bursts of openings in long depolarizing pulses. These periods have been shown to correspond to inactivation (Zagotta and Aldrich, 1990). The reopening rate was unaffected by voltage in this range. The simplest interpretation of this voltage de
Dependence is that it arises from the voltage-dependent occupancy of the pore by K+. The reopening rate in the negative range increases as hyperpolarization drives K+ into the pore.

In High External K+ Recovery Usually Occurs through the Open State

After repolarization, the single-channel tail currents frequently show reopenings from the inactivated state that appear to be tightly correlated with recovery. We therefore tested whether the channel must reopen to recover, by using a two-pulse protocol on single-channel patches. A depolarizing pulse (D1) that activated the channel was followed by a hyperpolarizing "tail" pulse (T) and a second depolarizing test pulse (D2) (Figure 7). We asked whether the channel must appear in the tail pulse (i.e., pass through the open state) to recover from inactivation before the D2 pulse; we only considered trials in which the channel opened and then inactivated during the D1 pulse; thus, there were four possible outcomes: T ∧ D2 equals opening in the tail and opening in the second pulse, T ∧ D2 equals opening in the tail and no opening in the second pulse, T ∧ D2 equals no opening in the tail and opening in the second pulse, and T ∧ D2 equals no opening in the tail and no opening in the second pulse. Two of these occurred most frequently: (T ∧ D2) and (T ∧ D2). This pattern confirms that channel opening in D2 (and thus recovery) is correlated with opening in T.

Occasionally, however, we did observe openings in the second pulse even when there was no opening in the tail (outcome T ∧ D2; missed events were corrected for as described in Experimental Procedures). There are two possible ways that this could occur: the channel may have recovered "silently" (without opening in I) and therefore would be in the closed state at the beginning of D2, or the channel may have remained inactivated throughout the duration of T (and thus would be in the inactivated state at the beginning of D2), but then opened from the inactivated state during D2. One way to distinguish between these two possibilities is by observing the latency to first opening in D2 for those trials with no opening in T (i.e., a conditional first latency histogram of the T trials). If the channel is opening from the closed state, the latencies will be brief, as they are in the first pulse. On the other hand, if the channel is opening from the inactivated state, the latencies will be longer and more distributed over the duration of D2.

As shown in Figure 8, the conditional second pulse latency histogram had two distinct components, slow and fast. The slow component corresponds to reopenings from the inactivated state. The fast component had a rate similar to the rate of latencies in the first pulse, and thus it probably represents opening from a resting closed state after recovery by a silent pathway.

To assess further the amount of silent recovery, we used the following equation, which breaks down the conditional probability of observing an opening in D2, given that there was no opening in T (P(D2|T)), into its component probabilities based on the channel's state (C or I) at the beginning of D2:

\[
P(D2|\overline{T}) = P(D2|C) \cdot P(C|\overline{T}) + P(D2|I) \cdot P(I|\overline{T})
\]

P(D2|\overline{T}) equals the conditional probability of opening in D2 given no openings in T. This value is measured.
Table 2. Recovery Usually Occurs through the Open State

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>160 mM K⁺...</th>
<th>-80 mV</th>
<th>-120 mV</th>
<th>30 mM K⁺...</th>
<th>-120 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>T+D2</td>
<td>40%</td>
<td>56%</td>
<td>32%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T+D2</td>
<td>17%</td>
<td>9%</td>
<td>13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T+D2</td>
<td>12%</td>
<td>17%</td>
<td>21%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T+D2</td>
<td>31%</td>
<td>18%</td>
<td>34%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction recovering through open state</td>
<td>0.92</td>
<td>0.83</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probability of recovery during a silent 20 ms tail</td>
<td>0.09</td>
<td>0.40</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate of silent recovery (s⁻¹)</td>
<td>4.6</td>
<td>25.2</td>
<td>19.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The outcomes of single-channel recovery experiments as in Figure 7 are listed for several recording conditions, together with the inferred statistics of recovery. Experiments performed under the same condition were pooled, and the corrected percentage of a particular outcome was determined (see Experimental Procedures). In 160 mM external K⁺ and V_mem = -120 mV, data represent five pooled experiments in which there was a total of 504 trials (ranging from 21-270 trials per experiment). In 30 mM external K⁺, four experiments were pooled for a total of 811 trials (24-340 trials per experiment). The probability of recovery during a silent 20 ms tail pulse (P(T|I)) was calculated according to text equation (2). The fraction recovering through the open state and the rate of silent recovery were calculated as described in the Experimental Procedures.

directly from the single-channel trials by calculating P(D2+T)/P(T), P(D2|C) equals the probability of opening from a (recovered) closed state. This value is estimated from the probability of opening in D1 from the resting closed state. P(C|T) equals the probability of reaching the closed state during a silent tail pulse (this is what we wished to determine). P(D2|T) equals the probability of making an I→O transition during D2. The probability of seeing these openings is estimated from measurements of reopening from the I state during prolonged pulses to the D2 voltage. P(T|I) equals the probability of remaining inactivated during a silent tail pulse rather than recovering. This is equal to 1 - P(C|T).

As shown in Table 2, the probability P(C|T) of recovering through a closed state in 160 mM external K⁺ is 0.09. Since the duration of the tail was 20 ms, this translates to a first order rate of recovery through the silent pathway of 4.6/s, which is much slower than the rate of recovery through the open state (64/s). Consistent with these rates, we find that 92% of the time the channel recovers through the open state. Thus, open-state recovery is the favored pathway.

**Hyperpolarization Increases the Probability of Recovering through a Closed State**

To determine the effect of voltage on the pathway of recovery, we repeated the single-channel two-pulse experiments with a more negative tail voltage (-120 mV). The hyperpolarization increased the probability of recovering during a silent tail pulse to 0.4, which translates to a rate of 25/s (Table 2). The ability of hyperpolarization to speed this process is similar to the voltage dependence of channel closing, perhaps because closing from the inactivated state is analogous to closing from the open state.

Varying the external K⁺ concentration did not appear to affect the rate of silent recovery (Table 2), at least in the range between 30 and 160 mM (single-channel tail currents cannot be well resolved in less than 30 mM external K⁺).

**Discussion**

The inactivation of Shaker K⁺ channels has been proposed to occur by a ball-and-chain mechanism, in which an internal tethered blocker binds to the pore and blocks K⁺ flow. In support of this model, we found that inactivation exhibits some of the properties of pore blockade. These properties include the following: binding only to the open channel; sensitivity to permeant ion concentration, with a characteristic voltage dependence; and slowing the channel closing rate. All of our observations can be interpreted simply if we assume that the inactivated state is an open channel blocked by an internal particle. Openings from this state reflect the exit of the blocking particle from the pore.

The Inactivation Particle Binds to the Open Channel

A common property of pore blockade is that the blocking ion can bind only to the open channel. For example, tetraethylammonium must wait until the channel opens before it can block the delayed rectifier K⁺ channel from squid axon (Armstrong, 1966). Presumably, the binding site must be exposed before the blocker can bind. We tested whether the channel inactivates only from the open state or if it can also inactivate from a closed state. Although we cannot directly observe inactivation from a closed channel, we can infer this occurrence in single-channel patches. If the channel inactivates directly from the closed state upon depolarization, the trace will be blank. In 30 and 160 mM external K⁺, we see a very low frequency of blank records (5%–10%), most of which can be accounted for by our failure to detect brief
openings. This suggests that the channel can inactivate only from the open state, which is consistent with the pore blockade model of inactivation.

Zagotta and Aldrich (1990) reported a higher frequency of blank records in their study of Shaker gating in cultured Drosophila myotubes. There are three possible explanations for the difference in our observations. First, because Zagotta and Aldrich studied the channel behavior in low concentrations of external K⁺ (7 mM), it is possible that under these conditions the channel more readily inactivates from the closed state. Second, in low external K⁺, recovery from inactivation is very slow. If the frequency of pulse presentation is too high, then some of the time the channel will still be inactivated at the beginning of the pulse. This will appear as a blank record even though the channel had inactivated from the open state. Finally, it is possible that Shaker K⁺ channels in myotubes behave differently from channels expressed in oocytes.

Permeant Ions and Hyperpolarization Enhance the Exit Rate of the Inactivation Particle

We found that increasing external K⁺ concentration and hyperpolarization speed recovery from inactivation. Although this result is consistent with pore blockade, its interpretation is complicated because recovery not only reflects the exit of the blocker from the pore, but also includes closing of the channel.

In single-channel experiments, we observed openings in the tail that were correlated with recovery. These openings occurred when the channel was inactivated at the end of the pulse and exhibited a single exponential latency to first opening. This suggests that the channel opens directly from the inactivated state. The influence of permeant ions on the rate of opening is suggestive of internal pore blockade: increasing external K⁺ increases the rate of opening, as though K⁺ ions coming from the outside cross the channel and “push” the blocking particle from the pore.

The ability of permeant ions to enhance the exit rate of blockers added to the opposite side of the membrane has been observed in many channels. For example, blockade of the squid axon K⁺ channel by internal nonyltriethylammonium (C9) ions can be relieved by raising the external K⁺ concentration (Armstrong, 1971). Similarly, external K⁺ relieves internal Na⁺ block of the large conductance Ca²⁺-activated K⁺ channel (BK) by increasing the exit rate of Na⁺ (Yellen, 1984). Also in the BK channel, external K⁺ can speed the dissociation of externally applied CTX (MacKinnon and Miller, 1988), and external K⁺ can enhance the exit rate of a blocking Ba²⁺ ion (Neyton and Miller, 1988).

Hyperpolarization also increased the rate of opening from the inactivated state. At negative voltages, the voltage dependence was very steep. At more positive voltages, however, the opening rate approaches a voltage-independent minimum. This contrasts with the voltage independence normally associated with inactivation (Zagotta and Aldrich, 1990). We believe that inactivation is an inherently voltage-independent process and that our observations arise from the action of K⁺ in the pore. At more negative voltages, external K⁺ is driven into the pore, clearing the channel of the inactivation particle. The minimum value obtained at positive voltages probably reflects the inherent exit rate of the particle from the pore.

A similar voltage dependence was observed for CTX block of the BK channel (MacKinnon and Miller, 1988). These authors also proposed that this was due to the voltage-dependent occupancy of the pore by K⁺ and not the voltage-dependent interaction of the blocker with the channel. In support of this interpretation, they found that, in the absence of internal K⁺, the dissociation rate is voltage independent. In our experiments, although we cannot measure the voltage dependence of the opening rate in zero external K⁺, our results in 30 mM K⁺ show a trend that suggests a similar phenomenon (Figure 6).

The exact mechanism by which K⁺ speeds the exit rate is unknown. One hypothesis is that a K⁺-binding site in the pore is located near a positively charged group on the surface of the inactivation particle and that electrostatic repulsion between K⁺ and the particle leads to an increase in the exit rate. Based on the voltage dependence, we calculate the location of this binding site to be about halfway though the membrane field (assuming that a single K⁺ ion is responsible for this effect). This mechanism was proposed by MacKinnon and Miller (1988) for CTX block of the BK channel.

In contrast to the permeant ion effect on the exit rate, we found that increasing external K⁺ only slightly affected the rate of onset of inactivation (the burst durations in the pulse increased 1.5-fold with an increase from 2 mM to 160 mM external K⁺ concentration; data not shown). The pore blockade model of inactivation offers a simple explanation for this discrepancy. When the particle physically occludes the pore, there is a true equilibrium of K⁺ ions between the external solution and the binding site in the pore that is important for relieving blockade. Increasing K⁺ in the external solution increases the occupancy of this site. On the other hand, in the unblocked channel, K⁺ flows through the pore. At positive voltages, it is likely that its occupancy will be dominated by the K⁺ concentration in the internal solution (which remained constant in our experiments), and thus there would not be a large effect of external K⁺ on the inactivation rate. This mechanism has been proposed previously to explain the effects of internal K⁺ on CTX blockade of the BK channel (MacKinnon and Miller, 1988) and the effects of K⁺ on Ba²⁺ blockade of the same channel (Neyton and Miller, 1988).

The Presence of the Inactivation Particle in the Pore Interferes with Channel Closing

Single-channel two-pulse experiments showed that,
relevant to our study is the effect of internal quaternary ammonium ions on the N-terminal-deleted form of this Shaker K' channel (Choi and Yellen, 1991, Biophys. J., abstract). These ions block the mutated channel in a way that remarkably resembles inactivation, and blockade prevents channel closing.

We do observe some recovery through a silent pathway, implying that the channel can sometimes close while inactivated. We have not determined the nature of this closed inactivated state; however, we do know that hyperpolarization increases the rate of silent recovery. Since this parallels the voltage dependence for closing of the noninactivated channel, perhaps the conformational changes required for closing while inactivated are similar to that of closing from the open state.

Single-Channel Tail Currents Predict the Time Course of the Macroscopic Tail Currents and Recovery

To test our model for recovery, we measured the behavior of the single-channel tail currents in high concentrations of external K' and used these measurements to predict the time course of the macroscopic tail currents and recovery. Frequently we observed multiple bursts of openings in the tail, suggesting that the channel sometimes reenters the inactivated state instead of closing to a recovered closed state (see, for example, the fifth trace in Figure 4). The probability of this "reinactivation" was voltage dependent; hyperpolarization decreased the frequency of reinventing, probably by increasing the closing rate. Using the following model

\[ C \bowtie C \bowtie C \bowtie C \bowtie C \bowtie (C \bowtie O) \]

we reconstructed the time course of the macroscopic tail currents based on four measured single channel parameters: the mean open time, \( \tau_{\text{open}} \); the mean closed time, \( \tau_{\text{closed}} \); the mean burst duration, \( \tau_{\text{burst}} \); and the probability of "reinactivating," \( P_{\text{reinact}} \). As shown in

**Table 3. Single-channel Parameters Predict the Time Course of Macroscopic Recovery**

<table>
<thead>
<tr>
<th>160 mM K'_{out}</th>
<th>30 mM K'_{out}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>160 mM K'_{out}</td>
</tr>
<tr>
<td></td>
<td>-80 mV</td>
</tr>
<tr>
<td>( \tau_{\text{open}} ) (ms)</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>( \tau_{\text{closed}} ) (ms)</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>( \tau_{\text{burst}} ) (ms)</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Bursts per tail</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>I\rightarrow O (s^-1)</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>Predicted recovery rate (s^-1)</td>
<td>31 ± 3</td>
</tr>
</tbody>
</table>

The values for single-channel parameters used to determine the rate constants shown in text equation (3) are listed for several recording conditions. The parameters were obtained from idealized single-channel current records (Figure 5). \( \tau_{\text{open}}, \tau_{\text{closed}}, \tau_{\text{burst}} \) and the average number of bursts per tail record were used to calculate the rate constants \( a, b, \bar{b}, \) and \( \lambda \) (see Experimental Procedures). The \( I\rightarrow O \) rate was determined from the tail latencies. The bottom two rows show a comparison between the observed macroscopic recovery rate and that predicted from the single-channel behavior. The observed recovery rates were determined as in Figure 1, and the predicted rate was calculated from the \( I\rightarrow O \) rate and the probability of reinactivation, which equals 1 - \( 1/\text{average bursts per record} \).
Figure 9, the model predicts the shape of the observed tail current. A rising phase (or "hook") in the tail current is seen when the depolarizing pulse is long enough to inactivate most of the channels and the exit rate of the ball is sufficiently slower than the closing rate. Such a rising phase was also observed in the delayed rectifier K⁺ channel when blocked by internal C9 (Armstrong, 1971).

We were also able to predict the time course of the macroscopic recovery from two parameters of the single-channel "tail currents": latency to first opening in the tail and the probability of "reinactivation" (Table 3). Our ability to predict macroscopic recovery rates entirely from the properties of channel openings in the tails confirms that recovery through the open state is the dominant pathway for recovery.

Possible Mechanisms for the Slow Component of Recovery in Low K⁺

Recovery in low external K⁺ has two components: a fast phase, which we suspect reflects the exit of the inactivating particle from the pore, and a slow phase. We will consider several possible mechanisms for this slow phase; however, none is completely consistent with the experimental characteristics exhibited by the slow component.

First, Armstrong (1971) observed a similar slow phase of recovery from internal C9 block of the delayed rectifier K⁺ channel from squid axon. Hyperpolarization slowed the rate and increased the proportion of this component. Since this voltage dependence parallels the voltage dependence of the closing rate, Armstrong proposed that the hyperpolarization was forcing channels to close around the blocking ion and "trap" it in the pore. Similarly, the BK channel can trap a Ba²⁺ ion in the pore with a voltage dependence that follows channel closing (Neyton and Miller, 1988). In our experiments, however, the time constant and the fractional decrease of the slow phase decrease with hyperpolarization. This voltage dependence is opposite that of the closing rate and, therefore, is not simply consistent with a trapping mechanism.

Alternatively, the slow phase may be related to the slow inactivation exhibited by this channel, which is distinct from the fast-inactivation mechanism that we have been discussing. The fast mechanism is termed N-type because of its association with the protein N-terminal region (Hoshi et al., 1991). Slow or C-type inactivation appears to be associated with an external region of the protein (Choi et al., 1991) and somehow involves the C-terminus (Hoshi et al., 1991). However, for slow inactivation to be responsible for the slow phase of recovery, its rate of onset must be much faster than that previously observed in the absence of fast inactivation.

Finally, it is tempting to postulate that the slow phase corresponds to recovery through a closed state, since the voltage dependence of these two processes match. However, the rate of silent recovery (measured in high external K⁺) is much faster than the rate of the slow component.

Although the slow phase of recovery is a complicated process, it is a prominent feature in the physiological range of external K⁺ concentration that is likely to be important in cell signaling and therefore deserves further study.

Comparison with Inactivation of Other Voltage-Dependent Channels

Taken together with evidence that the N-terminal region of the protein is involved in inactivation, our results strongly support the ball-and-chain model of inactivation for Shaker H4 K⁺ channels. Some characteristics of this mechanism are shared with other voltage-dependent channels. Na⁺ channel inactivation, for which the model was originally proposed, exhibits many of the same properties; however, the mechanism must be slightly different from that in the Shaker K⁺ channels because the absence of large tail currents suggests that Na⁺ channels do not reopen upon repolarization (Armstrong and Bezanilla, 1977). This implies that Na⁺ channels recover primarily through a closed state, perhaps by a process analogous to the silent recovery observed in the Shaker channel. Inactivation of some Ca²⁺ channels may also be described by a ball-and-chain mechanism. A recent report on Ca²⁺ channels in mouse cerebellar granule cells (Slesinger and Lansman, 1991) states that the channels sometimes reopen with some delay after repolarization, in a manner similar to that observed in Shaker K⁺ channels. This suggests that inactivation of these channels may also occur by pore blockade.

Experimental Procedures

Expression of Shaker K⁺ Channels in Oocytes

The plasmid containing the cDNA for the Shaker H4 K⁺ channel (Kamb et al., 1988) was linearized with EcoRI, and mRNA was synthesized in vitro from the linear cDNA template by standard methods using T7 polymerase as described (Mackinnon and Yellen, 1990). Digoxygenin triphosphate (0.5 mM) was included in the synthesis reaction to produce transcripts "capped" at the 5' end. Plasmid DNA was then digested by incubation with RNase-free DNase.

Oocytes were harvested from mature Xenopus laevis females previously injected with human chorionic gonadotropin (Xenopus One, Ann Arbor, MI) and dissociated in 1 mg/ml collagenase (Type 1A, Sigma). Isolated, follicle-free, stage V and VI oocytes were injected with about 50 nl of synthetic mRNA in distilled water (0.1-0.5 mg/ml). Injected oocytes were maintained at 18°C in modified frog Ringer's solution containing sodium pyruvate, theophylline, and penicillin, streptomycin, and glutamine, until time for recording (Mackinnon et al., 1988).

Recording Methods

Macroscopic and single-channel recordings were made from excised patches in the inside-out or the outside-out configuration (Hamill et al., 1981). The patches were voltage clamped, and currents were recorded with an Axopatch 200 patch-clamp amplifier (Axon Instruments, Burlingame, CA). The output of the amplifier was filtered at 3 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 10 kHz, unless otherwise indicated.

In all experiments, the internal solution contained the following: 160 mM KCl, 1 mM EGTA, 0.5 mM MgCl₂, 10 mM HEPES
(pH 7.4 with KOH). High external K⁺ solutions contained the following: 160 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES (pH 7.4). For solutions with lower concentrations of external K⁺, KCl was replaced with an equimolar amount of NaCl to maintain constant ionic strength. The 500 mM external K⁺ solution was prepared by adding KCl to the 160 mM K⁺ solution.

**Data Analysis**

**Macroscopic Recovery**

Recovery from inactivation was measured in patches that contained many channels (100–1000) using a two-pulse protocol. Each trace was subjected to a linear, digital leak subtraction using a hyperpolarizing pulse from −80 to −100 mV. The integral of the first 15 ms of the 20 ms pulses was measured (excluding any remnant capacitive current) using the steady-state current as the integration baseline. The rate of recovery was determined from the ratio of the integral of the second pulse to the average integral of the first pulses plotted as a function of recovery duration. Fits were obtained using a least squares criterion.

**Single-Channel Analysis**

To determine if a patch contained only a single channel, we applied repeated pulses to a strongly depolarized voltage (+80 mV). At this potential, the open probability of a single channel was high (the frequency of blank records was less than 5%). Patches with no overlapping openings in at least 64 traces were used for single-channel analysis.

Current records were digitally leak subtracted using periods with no channel openings, and idealized records were generated with a 50% amplitude criterion to detect opening and closing transitions (Colquhoun and Sigworth, 1985). Distributions histograms were log binned (Sigworth and Sine, 1987) and fit with a simplex search algorithm (Press et al., 1986) using a maximum likelihood estimate (Jackson, 1986; Colquhoun and Sigworth, 1983).

The criterion for separating periods of inactivation from closings within a burst was determined from the double-exponential closed-time histogram. A minimum interburst duration (τ₀) was calculated that equalized the number of closings falsely attributed to inactivation with the number of inactivation events falsely assigned as closing. Bursts were then considered terminated by closures longer than τ₀.

**Correction for Missed Events**

To analyze the single-channel conditional two-pulse data, we scored the trials as T × D₂, T × D₂, T × D₂, or T × D₂. It was necessary to correct these values for the effects of missing bursts in T and D₂. Given the measured distribution of burst duration, open duration, and number of openings per burst at each voltage, we estimated the probability of burst detection (Pₛₜ) in two ways. First, we calculated the probability of detecting single long openings with the same distribution as the burst durations, based on the empirically measured filter function; this is a slight underestimate. Second, we calculated the probability of detecting each burst to be the probability of detecting any one of its openings; this is a slight underestimate. The two methods did not give significantly different results; the detection probabilities ranged from 0.85–0.95.

After determining the detection probability Pₛₜ at the tail and depolarizing pulse voltages, we corrected the observed likelihood of T × D₂, T × D₂, T × D₂, or T × D₂ using a matrix method. The matrix relates the observed outcomes (Rₒₘ) with the actual outcomes (Rₚₙ) such that

\[ Rₚₙ = Mₙₕ Rₒₘ \]

where, for example, the matrix element

\[ Mᵢₙ = Pₛₜ Vᵢₙ × [1 - Pₛₜ Vᵢₙ (T × D₂)] \]

gives the probability of observing T × D₂ for an actual outcome of T × D₂. To determine the actual values from the observed, we multiplied the observed values by the matrix inverse.

**Conditional Probability Analysis**

To find the fraction of channels recovering through the closed state, we determined the parameters in text equation (2), as described briefly in the text. P(D₂|C) was calculated from the frequency of nonblank trials in D₁ (corrected for missed events). P(D₂|I) was determined from the rate of opening from the inactivated state (I) measured from the interburst durations during 5 s depolarizing pulses to +80 mV. The probability of observing an opening from the inactivated state during a second depolarizing pulse of duration D is

\[ P(D₂|I) = \frac{1}{5} \int_{-80}^{+80} e^{-6} dt \]

After solving text equation (2) for P(C|T), which gives the probability of reaching the closed state during a silent tail pulse of duration T, we determined the first order rate constant for silent recovery (f) from the equation

\[ P(C|T) = \frac{1}{T} \int_{-80}^{+80} e^{-6} dt \]

The fraction of channels that recovered through the open state is P(T|C), which is calculated from the other conditional probabilities.

**Reconstruction of Tail Currents from Single-Channel Parameters**

To reconstruct the macroscopic tail currents we used the kinetic scheme shown in the Discussion (text equation (3)). The four underlying rate constants around the open state were calculated from four single-channel parameters: the mean open time, τₒₐₙ; the mean closed time, τᵣₜₑₙ; the mean burst time, τₑₙ; and the probability of reactivating, Pₑₙ, determined from the average number of bursts per tail period. The relationship between these parameters and the rate constants in text equation (3) can be shown from basic kinetic principles to be:

\[ τₒₐₙ = (β + δ)^{-1} \]
\[ τᵣₜₑₙ = (α + δ)^{-1} \]
\[ τₑₙ = \frac{α + β}{α + δ} \]
\[ Pₑₙ = \frac{1}{1 + δ \beta} \]

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