Two Types of Inactivation in Shaker K⁺ Channels: Effects of Alterations in the Carboxy-Terminal Region

Toshinori Hoshi, William N. Zagotta, and Richard W. Aldrich
Howard Hughes Medical Institute
Department of Molecular and Cellular Physiology
Stanford University School of Medicine
Stanford, California 94305

Summary

Shaker potassium channels inactivate and recover from inactivation with multiple exponential components, suggesting the presence of multiple inactivation processes. We describe two different types of inactivation in Shaker potassium channels. N-type inactivation can occur as rapidly as a few milliseconds and has been shown to involve an intracellular region at the amino-terminal acting as a blocker of the pore. C-type inactivation is independent of voltage over a range of −25 to +50 mV. It does not require intact N-type inactivation, but is partially coupled to it. The kinetics of C-type inactivation are quite different for channels with different alternatively spliced carboxy-terminal regions. We have localized the differences in C-type inactivation between the ShB and ShA variants to a single amino acid in the sixth membrane-spanning region. N- and C-type inactivation occur by distinct molecular mechanisms.

Introduction

Inactivation is a prominent characteristic of voltage-gated ion channels. Multiple inactivation time constants that range from a few milliseconds to several seconds have been described for voltage-gated sodium channels (Narahashi, 1974; Adelman and Palti, 1969; Chandler and Meves 1970; Peganov et al., 1973; Khodorov et al., 1974, 1976; Fox, 1976; Almers et al., 1983). Among different types of voltage-gated potassium channels, inactivation rates can vary over a wide range (Hagiwara et al., 1961; Ehrenstein and Gilbert, 1966; Adrian et al., 1970; Schwarz and Vogel, 1971; Connor and Stevens, 1971; Neher, 1971; Aldrich et al., 1979; Hoshi and Aldrich, 1988; Zagotta et al., 1988; Solomon and Aldrich, 1988). Shaker potassium channels exhibit a particularly rapid inactivation. At +50 mV and 20°C, inactivation of native Drosophila Shaker channels has time constants on the order of 3–4 ms (Zagotta and Aldrich, 1990). A slower inactivation process was suggested because of a slow decrease in the probability of channel opening with repeated voltage pulses (Zagotta and Aldrich, 1990). Although the presence of other potassium currents in Drosophila muscle makes the study of additional, slower components of current decay difficult, recordings of Shaker channels expressed in Xenopus oocytes allow determination of kinetics with little contamination from other channel types. Macroscopic currents from a number of alternatively spliced Shaker variants expressed in Xenopus oocytes decay with multiple exponential components, further suggesting other inactivation processes in addition to the fast inactivation (Timpe, 1988b; Iverson and Rudy, 1990).

The fast inactivation in ShB channels is voltage-independent, is coupled to activation, and involves the amino terminus of the channel protein by a “ball and chain” type mechanism, in which the amino-terminal domain acts as a tethered inactivation particle that can block the internal mouth of the channel (Zagotta et al., 1989, 1990; Zagotta and Aldrich, 1990; Hoshi et al., 1990; Choi et al., 1991; Demo and Yellen, 1991, Biophys. Soc., abstracts). Deletion and point mutations near the amino terminus of the Shaker polypeptide slow or disrupt the fast inactivation process, and the addition of a small, amino-terminal peptide restores inactivation in a manner consistent with the ball and chain inactivation mechanism (Hoshi et al., 1990; Zagotta et al., 1990). However, both the amino- and the carboxy-terminal domains have been implicated in the decay of macroscopic currents and in the time course of recovery from inactivation in paired-pulse experiments (Timpe et al., 1988a, 1988b; Iverson et al., 1988; Ivan and Rudy, 1990). Along with the multiexponential decay of macroscopic currents, the large differences in recovery time course between different carboxy-terminal variants has been taken as evidence that two different inactivation processes occur in Shaker potassium channels (Zagotta and Aldrich, 1990; Iverson and Rudy, 1990). These results raise several questions about Shaker channel inactivation: Does the multiexponential decay reflect multiple, functionally distinct processes? If so, how do the different inactivation processes relate to each other? Do they involve different regions of the channel molecule? Is the mechanism of the slower inactivation similar to the ball and chain mechanism of the faster inactivation?

The existence of an additional inactivation process involving, at least in part, a different region of the channel molecule is supported by experiments showing that internal tetraethylammonium (TEA) competes with the fast inactivation, whereas external TEA competes with the slow inactivation seen in ShB channels that have been mutated in the amino-terminal region to remove fast inactivation (Choi et al., 1991). These results suggested a conformational change near the external mouth of the channel for the slow inactivation process.

In this study, we have investigated the properties of the slower inactivation process by kinetic analysis and site-directed mutagenesis. We have focused on the relationship between the two types of inactivation and the involvement of the alternatively spliced carboxy-terminal domain in the slow inactivation process.
Results

At least two distinct types of inactivation, occurring on vastly different time scales, can be seen in recordings from single ShB channels during voltage pulses (Figure 1). As shown in Figure 1A, the channel opens rapidly after a voltage jump, often in a burst of one or two openings separated by closed times on the order of 0.3 ms. These closures probably represent the channel briefly returning to the last closed state in the opening pathway (Zagotta and Aldrich, 1990). The bursts are terminated by a rapid form of inactivation that occurs by a mechanism involving the amino-terminal domain (Zagotta and Aldrich, 1990; Hoshi et al., 1990; Zagotta et al., 1990). With longer pulses it is clear that the initial bursts of openings are often followed by additional bursts occurring after long-lasting closures (Figure 1B). Since these reopenings occur in single channel patches after initial openings, they represent channels that have returned from inactivation, as opposed to channels with first openings late in the pulse. These returns from inactivation, however, occur with a decreased frequency later in the pulse. This demonstrates a slower inactivation process that exists in these channels.

The rate of return from slow inactivation is very slow compared with that of rapid inactivation. Figure 1C shows the activity of a single ShB channel for the first 60 s after a voltage step to 0 mV. Closed times with two distinctly different classes of durations can be seen. On this time scale, the short clusters of openings separated by closed times on the order of 40–70 ms represent the channel moving between the open state and the rapid inactivated state. These clusters are separated by very long closures, on the order of 3–4 s, in which the channel is in a second, slower inactivated state. The closed durations between clusters reflect the amount of time it takes the channel to recover from slow inactivation.

Relationship between Fast and Slow Inactivation

Slow inactivation could be related to rapid inactivation in several ways. One possibility, as proposed by Iverson and Rudy (1990), is that the slow inactivation process must be preceded by the rapid inactivation process, as formalized by the following sequential kinetic scheme:

\[ O^* \overset{\text{IF}}{\rightarrow} I_F \overset{\text{IS}}{\rightarrow} I_S \]

where \( O^* \) represents any state from which the channel can undergo rapid inactivation, including the open state as well as one or more closed states along the activation pathway (Zagotta and Aldrich, 1990). This scheme would be expected if, for example, the slow inactivation was a result of an inactivation particle shifting to a second, more stable position in its receptor, or if a slow inactivation gate could close behind the fast inactivation particle, locking it into position. This scheme makes the simple prediction that mutant channels without the ability to undergo the fast, amino terminus–mediated inactivation should not be able to undergo slow inactivation. ShBΔ6–46 channels, which have a deletion of amino acids 6–46, and do not exhibit fast inactivation, still exhibit a slower inactivation process (Figure 2A; see also Hoshi et al., 1990). This inactivation persisted in channels with many different mutations in the amino terminus, as well as in ShB channels that have inactivation disrupted by internal trypsin, and is very similar in time course to the inactivation seen in many delayed rectifier-type potassium channels (Hoshi and Aldrich, 1988, Zagotta et al., 1988; Sole and Aldrich, 1988; Stühmer et al., 1988, 1989; Frech et al., 1989; Koren et al., 1990; Wei et al., 1990). Recently, Choi et al. (1991) have shown that the inactivation remaining in this deletion mutant is pharmacologically distinct from the rapid inactivation in wild-type ShB channels.
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Figure 2. Inactivation in ShB and ShBA6-46

(A) Openings of a single ShBA6-46 channel in a outside-out patch. Voltage pulses (9.1 s) to +50 mV from the holding voltage of −100 mV were applied every 20 s. The data were filtered at 300 Hz and digitized at 333 Hz.

(B) ShB macroscopic current recorded from an inside-out patch in response to a 9.1 s pulse from −100 mV to +50 mV (top). The macroscopic currents from ShB and ShBA6-46 were scaled so that the peak amplitude of the slowly decaying component of the ShB current is approximately equal to the peak amplitude of the ShBA6-46 current (bottom).

(C) Box plots of the time constants of the decay of the ShB slow component and of ShBA6-46 currents recorded at +50 mV. ShB and ShBA6-46 macroscopic currents were elicited by eye with single exponentials. The central outlined box shows the middle half of the data, between approximately the 25th and 75th percentiles. The horizontal line in the middle of the box marks the median of the data. The “whiskers” extending from the top and bottom of the box show the main body of the data. Outlying or extreme values, if present, are plotted individually with circles or stars. The shaded region illustrates the 95% confidence interval of the median (Tukey, 1977; Velleman and Hoaglin, 1981). Sample sizes were 6 and 5 for ShB and ShBA6-46, respectively.

These results indicate that slow inactivation in the mutant channels is not simply residual fast inactivation that has not been removed by the deletion. Instead, it is a distinct process from fast inactivation and likely corresponds to the slow process observed in ShB channels with intact fast inactivation. We will refer to this type of inactivation as C-type inactivation, and the inactivation that is removed by the amino-terminal deletions as N-type inactivation. Because the sequential model predicts that removal of N-type inactivation should remove C-type inactivation as well, this model is unlikely to explain the relationship between N-type and C-type inactivation.

Another way that N-type and C-type inactivation may be related is that they represent exclusive processes; the channel can undergo N-type inactivation or C-type inactivation but not both. This exclusive model can be formalized by the following kinetic scheme:

\[
\begin{align*}
O^* & \rightleftharpoons l_n \\
\uparrow & \\
l_c & \\
\end{align*}
\]

where O* represents the set of states from which the channel can undergo rapid inactivation. This model would be expected if, for example, C-type inactivation resulted from a conformational change, such as the binding of another inactivation particle that competes with the N-type inactivation particle for its receptor. It predicts that the slow phase of decay seen in wild-type ShB channels should be markedly slower than the decay in channels with disrupted N-type inactivation (ShBA6-46), because wild-type channels are partially protected from C-type inactivation by N-type inactivation. The slow component of decay in ShB channels is compared with the decay of current in channels with disrupted N-type inactivation in Figure 2B. The decay time course of currents from channels without N-type inactivation (ShBA6-46) was slower than the slow component of decay of current from ShB, with intact N-type inactivation. This is opposite to what would be predicted for an exclusive mechanism. That the slow decay in ShB is faster than the decay in ShBA6-46 suggests that C-type inactivation is partially coupled to N-type inactivation. It can occur regardless of whether N-type inactivation has occurred, but occurs faster from the N-type inactivated state:

\[
\begin{align*}
O^* & \rightleftharpoons l_n \\
\uparrow & \\
l_c & \\
\end{align*}
\]

Properties of C-type Inactivation

The ability to eliminate N-type inactivation by mutagenesis has allowed us to study the properties of C-type inactivation in isolation from N-type inactivation. Unlike fast inactivation, which occurs on a time scale similar to that of activation, slow inactivation kinetics can be measured from the decay in macroscopic current. Like N-type inactivation, C-type inactivation occurs with a rate that does not depend on voltage over the range −25 to +50 mV (Figure 3). Scaling the macroscopic currents resulting from voltage steps to between −25 and +50 mV illustrates that the macroscopic slow inactivation rate is independent of
Figure 3. Properties of Inactivation in ShBA6-46
(A) ShBA6-46 macroscopic currents were recorded in an inside-out patch in response to 9.1 s voltage pulses to -50, -25, 0, +25, and +50 mV from the holding voltage of -100 mV every 18 s (top). The currents recorded at -25, 0, +25, and +50 mV were scaled so that their peak amplitudes are approximately equal (bottom).
(B) Box plots of the time constants of the ShBA6-46 macroscopic current inactivation at different voltages. Sample sizes were 3, 9, 3, and 10 for -25, 0, +25, and +50 mV, respectively.
(C) Box plots of the mean cluster durations of ShBA6-46 at 0 and +50 mV. Single-channel openings were elicited essentially as described in Figure 2A. Sample sizes were 8 and 5 for 0 and +50 mV, respectively. A cluster was defined as openings separated by closures of less than 30 ms.

Voltage (Figure 3A). Box plots of the macroscopic time constants at -25, 0, +25, and +50 mV demonstrate that voltage-independent C-type inactivation was a consistent feature of a large number of patches (Figure 3B). The rate of C-type inactivation can also be estimated by measuring the durations of the clusters of openings before the channel inactivates into the long lived inactivated state. The box plots of Figure 3C show that the C-type inactivation rate measured in this manner is also voltage-independent and similar to the rate measured from macroscopic currents. These results demonstrate that the conformational change associated with C-type inactivation does not involve significant movement of charge through the electric field across the membrane.

The time course of inactivation is different among carboxy-terminal variants of Shaker (Timpe et al., 1988a, 1988b; Iverson et al., 1988; Iverson and Rudy, 1990). The ShB and ShA alternatively spliced variants are identical in sequence for all of their amino-terminal domain and the core region encompassing the S1 through S5 proposed transmembrane segments (Figure 4A). However, their sequences begin to diverge just before the last putative transmembrane segment, S6, with a single amino acid difference at position 453 between S5 and S6, 2 amino acid differences at positions 463 and 464 in S6, and many differences in the putative cytoplasmic carboxy-terminal domain (Figure 4B; Schwarz et al., 1988; Pongs et al., 1988; Kamb et al., 1988). These sequence differences cause the currents from ShB channels to decay more rapidly than those from ShA channels (Figure 4C; Timpe et
The difference in decay rates probably indicates that N-type inactivation is somewhat slower in ShA. More dramatically, though, these sequence differences cause ShA channels to recover from inactivation much more slowly than ShB channels (Timpe et al., 1988a, 1988b; Iverson et al., 1988; Iverson and Rudy, 1990). This could arise from a difference in recovery rates from either N-type inactivation or C-type inactivation. To examine the rates of C-type inactivation directly, we generated the identical deletion in the amino-terminal region (Δ6–46) to remove the inactivation mediated by this region (see text). Sample sizes (top to bottom) were 6, 14, 4, 1, 6, 28, and 3.

The carboxyl deletions were made in the carboxy-terminal cytoplasmic domain of ShB and ShA. No expression was seen from ShBΔ6–46, ShAΔ6–46, or 9.1 s voltage pulses to +50 mV from the holding voltage of –100 mV. Sample sizes (top to bottom) were 9, 3, 2, 4, 6, and 2.

(C) Box plots of the time constants of inactivation of the macroscopic currents from

the point mutants in the S6 region. Mutations were made at the 3 residue positions where ShA and ShB differ (positions 453, 463, and 464). Amino acids at positions 453, 463, and 464 are indicated. In addition to the mutations indicated, these mutants also contained deletions in the amino-terminal region (Δ6–46) to remove the inactivation mediated by this region (see text). Sample sizes (top to bottom) were 6, 14, 4, 1, 6, 28, and 3.
termine which of the 3 amino acid differences are necessary for the faster, more stable form of C-type inactivation in ShA, we have mutated each residue in ShAA6-46, in turn, from the ShA form to the ShB form. The box plots show that two of these mutations, ShAA6-46,F453V and ShAA6-46,V4641, still exhibit fast C-type inactivation like ShAA6-46, but ShA channels with the ShB residue at the second of the three positions (ShAA6-46,V463A) exhibits slow C-type inactivation like ShAA6-46. The converse mutation, ShBB491-615, exhibits C-type inactivation like ShAA6-46. It therefore appears that a valine at position 463 in S6 is responsible for the rapid and more stable form of C-type inactivation and is sufficient to transform the C-type inactivation in ShB to the rapid and more stable form.

ShA and ShB also differ somewhat in their N-type inactivation rate, though this effect is much less pronounced than their difference in C-type inactivation (Figure 4C). To determine whether this difference is also conferred by the amino acid at position 463 or by differences at other positions, we have examined some of the carboxy-terminal deletions and point mutations in channels with an intact amino-terminal domain. The carboxy-terminal mutations with the intact amino-terminal domain of ShB (identical to ShA) were tested for their ability to undergo N-type inactivation. The carboxy-terminal deletions and point mutations all exhibited rapid N-type inactivation, indicating that most of the putative carboxy-terminal cytoplasmic domain is not required for N-type inactivation (Figure 6A). The time constant for inactivation, however, is somewhat altered in ShBB491-615, suggesting that this domain may play a small, indirect or modulating role in N-type inactivation. The differences in N-type inactivation between ShB and ShA appear to be largely or exclusively conferred by the amino acid difference at position 463, the same amino acid responsible for the differences in C-type inactivation. ShB-A463V exhibits N-type inactivation like ShA, and ShAA463A exhibits N-type inactivation like ShB. These effects on macroscopic N-type inactivation rates are also reflected in the mean open durations (Figure 6B). ShA, ShB-A463V, and ShBB491-615 all have longer open durations than ShB or ShAA463A, indicating that the slower macroscopic N-type inactivation rates are at least partially due to a decreased rate of inactivation from the open state.

The rate of recovery from inactivation is the property most strikingly different in ShA and ShB (Timpe et al., 1988a, 1988b; Iverson et al., 1988; Iverson and Rudy, 1990). Although we have shown that when N-type inactivation is removed, the rate of C-type inactivation is dramatically different in ShA and ShB, with N-type inactivation intact this difference is masked because the most rapid inactivation process is the one most readily observed. However, the difference in the stability of the C-type inactivated state can still be seen in the recovery from inactivation. Figure 7 shows the time course of recovery from inactivation as determined by a two pulse recovery protocol. ShB recovery is rapid and nearly complete within 1 s at -100 mV, whereas ShA has recovered only about 20% in this time (see also Timpe et al., 1988a, 1988b; Iverson et al., 1988; Iverson and Rudy, 1990). This difference in recovery rates can be attributed completely to the amino acid difference at position 463. ShB-A463V recovers slowly like ShA, and conversely, ShAA463A recovers rapidly like ShB. In addition, ShAA6-46 and ShBB6-46, both with N-type inactivation removed, recover slowly like their N-type inactivation-containing counterparts, ShA and ShBB-A463V. This suggests that most or all of the slow time course of recovery from inactivation in channels containing the ShA carboxyl terminus is due to recovery from C-type inactivation and not from differences in the stability of the N-type inactivated state.

Discussion

Shaker potassium channels exhibit a number of different types of closed conformations. In addition to the closed states that the channel progresses through during activation, they also exhibit multiple inacti-
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Figure 7. Time Courses of Recovery from Inactivation of Shaker Mutants

Two 90 ms pulses to +50 mV from the holding voltage of -100 mV separated by the time intervals shown on the abscissa were given. Fractional recovery is defined as the difference in amplitude between the peak current and the current at the end of the second 90 ms pulse divided by the corresponding value during the first pulse.

vated states. These inactivated states produce closed events that are much longer than the latencies to first opening and therefore could not reside in the opening pathway. The most prominent form of inactivation in ShB is a rapid inactivation that is voltage-independent and coupled to the activation processes. We have suggested that this rapid inactivation occurs by a process whereby the amino-terminal domain serves as a tethered inactivation particle that binds to a receptor site in the pore and occludes the channel (Iosif et al., 1990). The rate of this inactivation is significantly affected by alternative splicing of the amino terminus (Zagotta et al., 1989; Aldrich et al., 1990; Timpe et al., 1988b; Iverson et al., 1988; Iverson and Rudy, 1990).

Shaker potassium channels also undergo another type of inactivation that we have termed C-type inactivation. C-type inactivation manifests itself in many ways. In macroscopic currents from a number of Shaker variants in which N-type inactivation is incomplete, C-type inactivation can be seen as a slow decay in the current remaining after the rapid phase of inactivation. In single-channel recordings this appears as a decrease in the frequency of occurrence of bursts during the pulse. The onset of C-type inactivation varies over 2 orders of magnitude between the different alternatively spliced carboxy-terminal variants. It is very slow in channels containing the ShB carboxy-terminal region, but is much faster in channels containing the ShA carboxy-terminal region. The recovery rate from C-type inactivation can be very slow, as it is for variants that contain the carboxy terminal of ShA (Timpe et al., 1988a, 1988b; Iverson et al., 1988; Iverson and Rudy, 1990). This often produces an accumulation of channels in the C-type inactivated state and a rundown of the current with successive pulses. The multieponential time course of recovery in ShB also represents the recovery from both N- and C-type inactivation and perhaps other inactivated states as well.

In Shaker channels from naturally occurring variants, both N- and C-type inactivation can occur over a wide range of time courses. Among the known amino-terminal variants, ShB supports the fastest N-type inactivation, whereas ShH37 (ShH of Pongs) and ShD (Pongs nomenclature) amino-terminal regions support very slow or no N-type inactivation (Iverson and Rudy, 1990; Stocker et al., 1990). For C-type inactivation, the ShB carboxy-terminal domain, which includes an alanine at position 463 in S6, supports very slow C-type inactivation, whereas the ShA carboxy-terminal domain, which includes a valine at position 463 in S6, supports C-type inactivation, with time constants on the order of 10–20 ms. Because of the overlap in the ranges of kinetics for the two different inactivation processes, it is inappropriate to think of N-type inactivation as fast inactivation and C-type inactivation as slow inactivation. During voltage pulses of up to about 100 ms in duration, the ShB variant inactivates primarily by the N-type mechanism, whereas the ShA variant inactivates by both mechanisms.

We have studied the rate of the C-type inactivation process in channels with amino-terminal deletions that remove N-type inactivation. It occurs with a rate that is independent of voltage between -25 and +50 mV. If this voltage independence is maintained at lower voltages, then C-type inactivation must be at least partially coupled to activation and occur less readily from resting states. Because C-type inactivation still occurs in mutants without intact N-type inactivation particles, it is not absolutely coupled to N-type inactivation, as suggested by Iverson and Rudy (1990). Furthermore, because the rate of macroscopic C-type inactivation is not slowed when N-type inactivation is present, N- and C-type inactivation are not exclusive processes as would be expected if C-type inactivation occurred by a ball and chain mechanism that competed for a receptor site with N-type inactivation. Our results suggest that C-type inactivation is partially coupled to N-type inactivation, occurring more readily from the N-type inactivated state than from the combination of bursting states. This coupling may represent an allosteric decrease in the activation energy for the C-type inactivation transition when the amino-terminal ball is bound to its receptor.

The rate of entry into the C-type inactivated state in ShA carboxy-terminal variants is significantly faster than that in ShB carboxy-terminal variants. In addition, the rate of recovery from C-type inactivation is significantly slower in ShA as compared with ShB carboxy-terminal variants. These differences in C-type inactivation between ShA and ShB can be attributed to their differences in a single amino acid in the putative transmembrane segment S6. When the amino acid at position 463 is a valine, as it is in ShA, the C-type
inactivated state is more stable than when it is an alanine, as it is in ShB. Large deletions that span much of the putative cytoplasmic carboxyl end of ShA and ShB had little effect on the rate of C-type inactivation, suggesting that this cytoplasmic domain has little or no role in C-type inactivation.

These results begin to narrow down the possible physical mechanisms for C-type inactivation. C-type inactivation seems to occur by a mechanism distinctly different from that determining N-type inactivation. Whereas N-type inactivation involves a cytoplasmic domain of the channel, C-type inactivation is not markedly affected by deletions or alterations in the carboxy-terminal cytoplasmic domain and is instead dramatically altered by a single amino acid change in the transmembrane segment S6. It is unlikely, therefore, that C-type inactivation involves a cytoplasmic ball and chain mechanism like that implicated in N-type inactivation. This distinction in the physical regions of the channel involved in these inactivation conformational changes has also been shown by pharmacological studies. Choi et al. (1991) have shown that internal TEA competes with the N-type inactivation conformational change, whereas external TEA competes with C-type inactivation. C-type inactivation may therefore involve a constriction of the channel’s extracellular mouth that is made faster and more stable when S6 contains the larger amino acid valine than when it contains the smaller alanine.

The same alteration in amino acid 463 that is responsible for the large difference in C-type inactivation between ShA and ShB is also responsible for their smaller difference in N-type inactivation. ShB-A463V, with amino acid 463 mutated to the SHA form, exhibits slower N-type inactivation and faster C-type inactivation and becomes indistinguishable from ShA. Conversely, SHA-V463A exhibits faster N-type inactivation and slower C-type inactivation like ShB. The rate of N-type inactivation is only marginally influenced by large deletions in the putative cytoplasmic carboxyl terminus. Therefore all of the reported differences in gating between ShA and ShB can be attributed to a single amino acid at position 463 in S6, and it is unlikely that the carboxy-terminal cytoplasmic domain plays a key role in inactivation, as does the amino terminus.

There are several ways an amino acid in S6 might influence the rate of N-type inactivation. It seems unlikely that amino acid 463 is in the receptor site for the amino-terminal inactivation particle. The amino acid is located nearer the putative extracellular end of S6, and since N-type inactivation is not voltage-dependent, the amino terminal inactivation particle is not thought to move appreciably across the membrane. However, the residue at position 463 may indirectly influence the structure of the receptor. Alternatively, amino acid 463 may influence the permeation properties of the channel, which in turn influence the rate of N-type inactivation. For example, if a valine at position 463 increased the duration of ion occupancy of the pore and the rate of inactivation was slower in channels containing an ion, then the rate of N-type inactivation would be decreased. Consistent with this explanation is the observation that channels with a valine at position 463 have a somewhat larger single-channel conductance than channels with an alanine (data not shown). Furthermore, extracellular potassium has been shown to accelerate the rate of recovery from inactivation in a manner consistent with a mechanism in which ion occupancy affects inactivation (Demo and Yellen, 1991, Biophys. Soc., abstract).

Because of the different molecular mechanisms of the two types of inactivation, it would be inappropriate to suppose that all relatively fast inactivation in potassium channels is due to an amino-terminal ball and chain type mechanism. The slower C-type inactivation seen with the ShB carboxy-terminal region occurs with kinetics that are in the range of delayed rectifier channel inactivation kinetics (Hoshi and Aldrich, 1988; Zagotta et al., 1988; Solc and Aldrich, 1988; Stühmer et al., 1988, 1989; Frecch et al., 1989; Koren et al., 1990; Wei et al., 1990). It is possible that it shares mechanistic features with the inactivation of these other channels. The inactivation of dkrI potassium channels, which occurs with a time constant of several seconds, has been found to be affected by deletions in both the amino- and the carboxy-terminal cytoplasmic domains (VantDyken et al., 1990). It remains to be seen whether inactivation in this and other delayed rectifier channels occurs by a C-type mechanism.

Experimental Procedures

Generation of Mutant cDNAs

The ShBA6–46 deletion was generated as described previously (Hoshi et al., 1990). ShΔA6–46 was generated by splicing the amino-terminal end of ShBA6–46 onto SHA using the SalI site. The deletion mutants ShBA6–46,477–615 and ShBA6–46,491–615 were prepared by a modification of the procedure for the erase-a-Base System (Promega, Madison, WI). An altered pS72 plasmid (Promega) with the PvuII site removed and containing the ShBA6–46 cDNA insert was linearized by cutting with PvuII (which cuts at two different sites in the carboxy-terminal domain of ShB) at positions 1752 and 1843. The PvuII site at position 1752 is not predicted from the published sequence (Schwarz et al., 1988) and results from an apparent sequencing error in ShB at positions 1752 and 1753. This error also changes the 2 amino acids at positions 584 and 585 from histidine/valine in the published sequence (Schwarz et al., 1988) to glutamine/leucine, as it is in the other published reports of this sequence (Pongs et al., 1988; Kamb et al., 1988). Deletions were generated by digesting the linearized plasmid with ExoI for 1 min at 33°C. The ends of the digested plasmids were made blunt by treatment with S1 nuclease and then with DNA polymerase I (Klenow fragment). The deleted Shaker 5' ends were ligated to a PvuII–BglII fragment from the ShB plasmid. The deletion mutants ShBA6–46,584–646 and ShBA6–46,584–651 were prepared in a similar manner, but the ExoI deletion was done at 22°C and the deleted Shaker 3' ends and plasmid were ligated to a BglII–PvuII fragment from the ShBA6–46 insert. The ShBA6–46,557–628 deletion was generated by cutting ShBA6–46 at the PvuII sites, digesting the linearized plasmid with ExoII, and recircularizing the blunt-ended vector. For all of the mutations, single isolates were selected and sequenced to determine the length of the deletion and to ensure that they were in the correct reading frame.

The SHA deletions, chimeric channels, and point mutations were all generated by a method involving the polymerase chain
reaction. Briefly, oligonucleotides were synthesized containing the mutation or deletion and extending on their 5' ends to a nearby restriction site in the Shaker cDNA. These oligonucleotides were used in combination with another oligonucleotide in a polymerase chain reaction amplification of a fragment of the relevant Shaker cDNA. The product of the reactions was cut with two different restriction enzymes, and the fragment containing the mutation was gel-purified. This cassette was then ligated into the Shaker insert cut with the same two restriction enzymes. For all of the mutations, single isolates were selected, and the entire region of the amplified cassette was sequenced to check for the mutation and ensure against second-site mutations.

All of the above mutations in the carboxy-terminal region were first generated in Shaker cDNAs containing a deletion in their amino termini (ShBA4-46 or ShAA6-46). Many of these mutations were then cloned into a construct containing an intact amino terminus using the Sall site.

Expression of Channels in Xenopus Oocytes
Shaker channel RNA was transcribed and injected into Xenopus laevis oocytes as previously described (Zagotta et al., 1989).

Electrophysiology and Data Analysis
Macroscopic and single-channel recordings were done as previously described (Hoshi et al., 1990). Most of the experiments were done in cell-free configurations. Macroscopic currents were recorded using pipettes with initial resistances <1 MΩ, and no series resistances compensation was made. Recordings were made at 20°C. The internal solution contained 140 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 11 mM HEPES, and 10 mM HEPES (pH 7.4). The external solution contained 140 mM NaCl, 6 mM MgCl₂, 2 mM KCl, and 5 mM HEPES (pH 7.4). Data were analyzed essentially as described (Hoshi et al., 1990).

Note on Terminology
In this paper we use the terminology for the alternatively spliced domains that was proposed by the Jans' laboratory, from which we obtained the cDNA clones (Schwarz et al., 1988). Other laboratories use different terminology. Our ShB carboxy-terminal variant is designated the type 2 carboxyl variant in papers from the laboratories of 0. Pongs (Stocker et al., 1990) and the type 4 carboxyl variant in papers from the Jans' laboratory (van Guren et al., 1990). The ShA carboxyl region variant corresponds to the type 1 carboxyl variant in papers from the laboratories of M. Tanouye and B. Rudy (Kamb et al., 1988; Iveryson et al., 1988; Iveryson and Rudy, 1990). This nomenclature has been stabilized over time, and efforts have been made to use it consistently.

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