Conversion of a Delayed Rectifier K⁺ Channel to a Voltage-Gated Inward Rectifier K⁺ Channel by Three Amino Acid Substitutions

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Summary

Single, double, and triple mutations progressively shift Shaker activation to more hyperpolarized potentials, resulting in an increase in the fraction of inactivated channels at negative resting voltages. The most negatively shifted mutation, the triple mutant, behaves like an inward rectifier. What is usually considered activation for an inward rectifier is, for the triple mutant, recovery from inactivation, and what is usually considered deactivation is inactivation. This conversion from outward rectifier to inward rectifier does not rely on a difference in sign or direction of charge movement of the voltage sensor, since activation of the Shaker outward rectifier is due to a different gate than activation of the triple mutant inward rectifier. Other voltage-dependent inward rectifiers in the Shaker family may work by a similar mechanism.

Introduction

K⁺ channels have been classified as either outward rectifiers or inward rectifiers based on their opening in response to either depolarization (outward rectifier) or hyperpolarization (inward rectifier) (Hille, 1992). Identification of the genes encoding channels in both classes has shown that a distinction also holds, in part, at the molecular level. Voltage-dependent outwardly rectifying K⁺ channels, such as Shaker (Chandy and Gutman, 1995), have six putative transmembrane segments per subunit, including a highly charged S4 segment, and an ion-conducting P region (Hartmann et al., 1991; MacKinnon and Yellen, 1990; Yellen et al., 1991; Yool and Schwarz, 1991). They exhibit voltage-dependent activation gating in response to positive voltage steps from resting potentials. One class of inward rectifier channels, the IRK-related family, is composed of channels with smaller subunits containing only two putative membrane-spanning regions in addition to the P region (Ho et al., 1993; Kubo et al., 1993). These channels activate in response to negative voltage steps from resting potential due to voltage-dependent relief of block by magnesium and polyamines (Picker et al., 1994; Lopatin et al., 1994; Nichols et al., 1994). Other inward rectifiers, however, have been found that possess an intrinsic voltage-dependent activation that is not due to relief of an extrinsic blocking particle (Hille, 1992; Hoshi, 1995; Silver and DeCoursey, 1990). The KAT1 family of plant inward rectifiers (Muller-Rober et al., 1995; Schachtman et al., 1992) and HERG (Sanguinetti et al., 1995; Trudeau et al., 1995; Warmke and Ganetzky, 1994), cloned from human hippocampus, both contain six putative transmembrane segments, including an S4 segment, and a P region and are thus more closely related to the Shaker family than to the IRK family. These S4-containing inward rectifiers raise an interesting question about the mechanism of voltage-dependent gating. How do channels with the same basic sequence topology and putative S4 voltage sensor (Liman et al., 1991; Papazian et al., 1991, 1995; Yang and Horn, 1995) open in response to changes in voltage of opposite polarity?

We have addressed this question by converting an outwardly rectifying Shaker channel into a family of voltage-gated inward rectifiers by combinations of three amino acid substitutions that progressively shift Shaker activation gating to more hyperpolarized voltages. In these inward rectifier channels, what would normally be described as activation gating upon negative voltage steps comes about from recovery of the channels from fast N-type inactivation through the open state (see Demo and Yellen, 1991; Gomez-Lagunas and Armstrong, 1994; Ruppersberg et al., 1991).

The essential features of gating of the fast inactivating Shaker channel can most simply be described by a kinetic scheme with three states (Hoshi et al., 1990):

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Depolarizing Step

C ─── O ─── I (l)
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Hyperpolarizing Step

The closed to open transition (C—O) corresponds to the Shaker activation gate, and the open to inactivated transition (O—l) corresponds to the Shaker inactivation gate. Detailed steady-state and kinetic data have shown that there are a number of transitions among closed states before opening (Bezanilla et al., 1994; Koren et al., 1990; Schoppa et al., 1992; Zagotta and Aldrich, 1990; Zagotta et al., 1994), which are represented in this scheme as a single C—O transition. At the resting potential, and at more negative voltages, Shaker channels will populate states to the left. After a step to more positive voltages, the channels will move from the closed state through the open state to the inactivated state.

This scheme could also accommodate inward rectifier behavior if the channels were to occupy the inactivated (l) state at rest. If the voltage range for activation were much more negative, the equilibrium distribution among states at the resting potential would be shifted toward the inactivated state. Hyperpolarizing voltage steps would then cause the channels to move from the inactivated state through the open state, resulting in a relatively large current, and then to the closed state. Conversely, a positive voltage step would result in further inactivation and little or no current, since the channels would already reside in the rightmost inactivated state. Such a channel will thus show inward rectification. The above considerations suggest that an outward rectifier could be converted into an inward rectifier by shifting...
Results and Discussion

We tested this hypothesis in Shaker K⁺ channels by combining amino acid replacements that cause negative shifts in activation gating. Two point mutations in Kv1.1 (a noninactivating outward rectifier) and one point mutation in Shaker have been shown to shift the midpoint of activation to more hyperpolarized voltages (Liman et al., 1991; Lopez et al., 1991; Tyt gat and Hess, 1992). These mutations (R365N, R371I, and L366A; see Figure 1A), all in the S4 region, were combined into two double mutants (L366A/R371I and R365N/R371I) and one triple mutant. Figure 1B shows a representative conductance–voltage plot for each of the six constructs. Figure 1C shows their midpoints of activation. The effects of the mutations were cumulative, with progressive negative shifts of the midpoints of activation in the double and triple mutants.

Figure 2A shows a family of current traces recorded in symmetrical K⁺ solutions during voltage steps from a holding potential of −80 mV for the six channel types, illustrating the progressive change from an outward rectifier (wild type, top) to an inward rectifier (triple mutant, bottom). As the midpoint of activation shifts to more hyperpolarized voltages, more channels populate the inactivated state at the holding potential of −80 mV. The wild-type Shaker channels rarely populate the inactivated state at −80 mV (all channels are closed), and thus there is no current change in response to a hyperpolarizing step. With sufficiently positive voltage steps, the channels open and then inactivate, resulting in outward rectifying behavior. On the other hand, the triple mutant channels significantly populate the inactivated state at −80 mV, and thus a hyperpolarizing voltage step from −80 mV results in recovery from inactivation through the open state (Demo and Yellen, 1991; Gomez-Lagunas and Armstrong, 1994; Ruppersberg et al., 1991) and currents that resemble inward rectifiers (Muller-Rober et al., 1995; Schachtman et al., 1992). More positive voltage steps further populate the inactivated state, resulting in a smaller outward current relative to the inward current observed at hyperpolarized voltages. Thus, the triple mutant behaves like an inward rectifier. The single and double mutants have different relative amounts of inactivated channels at −80 mV resulting in current traces with characteristics intermediate to the outwardly rectifying wild-type channel and the inwardly rectifying triple mutant. As the peak conductance–voltage curve shifts to more negative voltages, the amount of inward rectification increases. These six constructs thus represent a family of K⁺ channels with different degrees of inward rectification.

The kinetic scheme used to describe the transition from an outward rectifier to an inward rectifier requires an inactivated state. Removal of the inactivated state in the Shaker wild-type channel by deletion of the N-terminal region results in a noninactivating outward rectifier (Hoshi et al., 1990). Without the inactivation mechanism, shifting the midpoint of activation should not generate an inward rectifier. N-terminal deletions were generated for all six constructs, and the same protocols used in Figure 2A for the constructs with intact inactivation are shown in Figure 2B for the corresponding constructs without an N-terminal inactivation domain. In the absence of inactivation, there is no transition from an outward rectifier to an inward rectifier, but rather, as the midpoint of activation is shifted to more hyperpolarized...
Conversion of Shaker to an Inward Rectifier

Figure 2. Current Families of Constructs with and without an N-Terminal Domain

(A) Mutants with an intact N-terminal domain show a gradual change from an outward rectifier to an inward rectifier. The six constructs are the same as in Figure 1, except that they all contain an intact N-terminal domain. From top to bottom: ShB, R365N, R371I, R365N/R371I, L366A/R371I, R365N/L366A/R371I. For all constructs except R365N/L366A/R371I, the oocyte was held at −80 mV, stepped through a range of voltages from −160 mV to +60 mV in increments of 20 mV for 300 ms, and then stepped to −40 mV for 100 ms (see inset voltage protocol at top). For R365N/L366A/R371I, the most positive voltage is −50 mV instead of 60 mV; all else is the same as for the other constructs; n values: ShB (6), R365N (7), R371I (6), R365N: R371I (4), L366A: R371I (4), R365N:L366A: R371I (9).

(B) Mutants lacking the N-terminal domain show no switch from an outward rectifier to an inward rectifier. The six constructs are the same as in (A), except that a 40 amino acid segment from amino acid 6 to 46 has been deleted to remove inactivation (Hoshi et al., 1990). From top to bottom: ShB Δ4-46, R365N, R371I, R365N/R371I, L366A/R371I, R365N/L366A/R371I. For all constructs, the oocyte was held at −80 mV, stepped through a range of voltages from −160 mV to +60 mV in increments of 20 mV for 300 ms, and then stepped to −40 mV for 100 ms (see inset voltage protocol at top of [A]); n values: ShB Δ4-46 (3), R365N (3), R371I (3), R365N: R371I (5), L366A: R371I (4), R365N:L366A: R371I (6).

The difference in rectification between the constructs with or without the N-terminal region is further illustrated in Figure 3, which shows representative peak current–voltage plots for all 12 constructs, normalized to the peak current at +60 mV. When the N-terminal inactivation domain is present (Figure 3A), there is a gradual change in the amount of inward rectification as the peak conductance–voltage curve shifts to more negative voltages. This change is absent when the N-terminal region is deleted (Figure 3B) and there is no inactivated state. For all 12 channels, C-type inactivation (Hoshi et al., 1991) is slow relative to the pulse lengths considered here and can be ignored. However, a channel with a negative activation range and fast C-type inactivation could also behave as an inward rectifier.

The demonstration that small changes in sequence can convert the Shaker channel into an inward rectifier provides a partial explanation for sequence similarities between Shaker, HERG, and the KAT1 family of plant inward rectifiers. The strong voltage-dependent inward rectification of the triple mutant is similar to that seen in KAT1. HERG channels can activate in response to depolarizations from rest, but behave as inward rectifiers after a prepulse to positive potentials due to a fast C-type inactivation (Sanguineti et al., 1995; Trudeau et al., 1995; Smith et al., 1996). With a similar pulse protocol, the behavior of the R371I channels is strikingly similar to that of HERG. However, in contrast with HERG, R371I channels rectify by an N-type inactivation mechanism. Figure 4 shows a prepulse protocol for ShB, R371I, and the two double mutants. Under these conditions, all four channels, including the wild-type ShB channel, show large inward currents upon hyperpolarization from +40 mV. As the Shaker activation range becomes more negative, there is a progressive increase in the rate of
recovery from inactivation (l—O) relative to the rate of
deactivation (O—C), thus generating currents similar to
HERG when challenged with the same pulse protocols
(Sanguinetti et al., 1995; Trudeau et al., 1995). HERG
currents can also display outward rectification if the
prepulse is negative (−70 mV) (Sanguinetti et al., 1995).
This dependency of rectification on prepulse potential
is also observed for the R371I mutant, which shows
outward rectification when the prepulse is negative (−80
mV, see Figure 2A). Differences in kinetic and steady-
state properties between HERG and R371I could be
accounted for by a difference in the relative rates of
activation (α, scheme I) and inactivation (γ, scheme I),
even though the molecular mechanisms of inactivation
are different between the two channels.

These results demonstrate that small alterations in
amino acid sequence can change a Shaker-delayed rect-
fier channel into a voltage-gated inward rectifier. Mechan-
istically, this can be explained by a shift in activation
gating to more negative potentials so that the channels
populate inactivated states at rest. What is traditionally
considered activation for an inward rectifier is, in the
case of the triple Shaker mutant, recovery from inactiva-
tion, and what is traditionally considered deactivation
for an inward rectifier is inactivation. Since activation
of the two channels is due to two different gating mecha-
nisms, it is not necessary to alter the charge on the
voltage sensor or the direction it moves across the elec-
tric field to convert one into the other (Suchyna et al.,
1993; Verselis et al., 1994). These results compliment
both early work on anomalous rectification in squid axon
by relief of internal TEA block (Armstrong and Binstock,
1965) and recent work suggesting an inactivation-medi-
ated inward rectification for the HERG channel (San-
guinetti et al., 1995; Shibasaki, 1997; Trudeau et al., 1995;
Smith et al., 1996).

Experimental Procedures

Electrophysiology

All data were collected without leak subtraction, since the hyperpo-
larized shifted mutants are open over a large voltage range. Approx-
imately 0.8–1 ms at the beginning of each step was blanked. The

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Figure 3. Peak Current-Voltage Relationships

Representative normalized peak current-voltage for all constructs, with (A) and without
(B) an N-terminal domain. The current was
normalized to the value at +60 mV. For clarity,
the wild-type data (ShB and ShB:4-46) and
that from the triple mutant (with and without
N-terminus) are shown with lines connecting
the points. Leak current is not subtracted.
The following are n values and corresponding
symbols for the constructs with the N-termi-
nal domain: ShB (6) (asterisks), R365N (7)
(open squares), R371I (6) (closed circles),
R365N:R371I (4) (open circles), L366A:R371I
(4) (closed triangles), R365N:L366A:R371I (9)
(closed double triangles). The following are n
values and corresponding symbols for the con-
structs without the N-terminal domain:
ShB:4-46 (3) (asterisks), R365N (3)
(open squares), R371I (3) (closed circles),
R365N:R371I (5) (open circles), L366A:R371I
(4) (closed triangles), R365N:L366A:R371I
(6) (closed double triangles).

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Figure 4. Prepulse Protocols to Inactivate the Channels Maximally

Prepulse protocols to +40 mV for ShB, R371I,
R365N:R371I, and L366A:R371I, all with an
intact N-terminal inactivation domain. The
oocyte was held at −80 mV stepped to +40
mV for 100 ms and then through a range of
voltages from −160 mV to +60 mV in incre-
ments of 20 mV for 190 ms; n values: ShB (6),
R371I (7), R365N:R371I (6), L366A:R371I (8).
cut-open oocyte clamp (Dagan, Minneapolis, MN) (Tagliatela et al., 1994) was used for all experiments. The oocyte was permeabilized with 0.3% saponin; the external solution contained 110 mM KOH, 2 mM MgCl₂, 5 mM HEPES, pH 7.1 with methanesulfonic acid; the internal solution contained 110 mM KOH, 2 mM MgCl₂, 0.1 mM CaCl₂, 11 mM EGTA, 10 mM HEPES, pH 7.2 with methanesulfonic acid. The microelectrodes used had resistances less than 1 MΩ and were filled with 3 M KCl. The agar bridges contained 1 M Na-methanesulfonic acid, with platinum-iridium wires placed in the agar. All recordings were done at approximately 20°C.

Molecular Biology

Five sets of two oligos with the appropriate mutations were synthesized (Millipore, Bedford, MA) and inserted into the ShB:3.4-46 background to generate the five constructs lacking the N-terminal domain. These five mutants were then subcloned into the ShB gene to generate the five mutants with an intact N-terminal inactivation domain.

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References


