Loss of *Shaker* K Channel Conductance in 0 K^+ Solutions: Role of the Voltage Sensor

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ABSTRACT In potassium-free solutions some types of K channels enter a long-lasting nonconducting or "defunct" state. It is known that *Shaker* K channels must open in K^+-free solutions to become defunct. Gating current studies presented here indicate an abnormal conformation in the defunct state that restricts S4 movement and alters its kinetics. Thus an abnormality initiated in the P region spreads to the gating apparatus. We find that channels most readily become defunct on repolarization to an intermediate voltage, thus prolonging occupancy of one of the several intermediate closed states. The state dependence of becoming defunct was further dissected by using the gating mutant L382A. Simply closing this channel at 0 mV (reversing the last activation step) does not make the mutant channel defunct. Instead, it is necessary to move further left (more fully closed) in the activation sequence. This was confirmed with *Sh*IR experiments showing that channels become defunct only if there is inward gating charge movement. Rapid transit through the intermediate states, achieved at very negative voltage, is relatively ineffective at making channels defunct. Several mutations that removed C-type inactivation also made the channels resistant to becoming defunct. Our results show that normal gating current cannot be stably recorded in the absence of K^+.

INTRODUCTION

Voltage-gated potassium channels are transmembrane proteins that regulate the flux of K^+ across the membrane in response to changes in the electrical field. Under normal physiological conditions potassium channels are very selective to K^+ over Na^+ (Hille, 1992). Although the channel is generally viewed as a pore with a gate that opens and closes the pore independently of the permeating ions, there are several reports demonstrating a direct effect of permeating ions on the gating apparatus of the channel. For example, changes in the concentration of extracellular K^+ alter the properties of the channels, such as the rate of deactivation (Swenson and Armstrong, 1981; Chen et al., 1997) and C-type inactivation (Hoshi et al., 1991; Lopez-Barneo et al., 1993; Kiss and Korn, 1998). Particularly dramatic changes in the properties of some channels are caused by complete removal of K^+ from the internal and external media—the channel loses its ability to conduct K^+ (becomes defunct). The channel can be protected against this loss of conduction by the presence either inside or outside of a readily permeant cation, or certain channel blockers (Chandler and Meves, 1970; Almers and Armstrong, 1980; Gomez-Lagunas, 1997). This phenomenon was originally discovered for squid potassium channels, which become irreversibly defunct in 10–20 min in the absence of K^+. It was hypothesized that a permeant cation counterbalances negative charges or oriented dipoles lining the pore, thus preventing a destabilizing electrostatic repulsion between them (Almers and Armstrong, 1980). Gomez-Lagunas (1997) has shown that conductance vanishes when the *Shaker* potassium channel is opened and closed in K^+-free solutions. Holding at a steady −80 mV with no pulsing, or reducing the holding potential from −80 to −50 mV with pulsing, prevents the loss of conduction. Once lost, conductance can be recovered completely by prolonged depolarization (tens of seconds to minutes) in the presence of external K^+, but does not return, even in the presence of K^+, if the membrane is held negative to −50 mV (Gomez-Lagunas, 1997).

These results clearly indicate the crucial role of membrane potential (V_m) in the loss of conductance. Here we investigate the role of V_m in making the channel defunct. Specifically, we wanted to know if voltage is important because it helps to drive K^+ from the channels, or if the loss of conductance depends in some way on the position of voltage-sensing parts of the gating apparatus.

MATERIALS AND METHODS

Preparation of ShIR mutant channels

Following the advice of Dr. Gary Yellen, the template used for PCR-directed mutagenesis was *Sh*IR cDNA subcloned in the pGW1-CMV expression vector (British Biotechnology, Oxford, England). *Sh*IR differs from the full-length *Shaker* cDNA (Kamb et al., 1988) in that it contains a deletion of amino acids 6–46 that removes the N-terminal inactivation domain (Hoshi et al., 1990). The following point mutations were introduced into the *Sh*IR cDNA: Leu382 to Ala (L2A), Thr449 to Val (T449V), Thr449 to Tyr (T449Y), and Thr449 to Ala (T449A).

Polymerase chain reaction (PCR) was performed using one mutagenic primer and the opposite template-matching primer. PCR products containing the desired mutations were flanked by unique restriction sites (Xba1/Bgl2 for Leu382 mutants and Bgl2/Sma1 for Thr449 mutants). The digested PCR fragments were subsequently ligated into the appropriately digested pGW1-CMV vector. All restriction enzymes and buffers were from New England Biolabs (Beverly, MA). Mutagenesis was verified by cDNA sequencing.

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Expression of WT and mutant channels in mammalian cells

The wild-type and mutant *Sh*IR channels subcloned into pGW1-CMV mammalian expression vector (British Biotech) were transiently expressed in tsA201 cells (HEK 293 cells, ATCC CRL 1573, stably transfected with SV40 large T antigen). Cells were transfected by electroporation as described previously (Jurman et al., 1994). The channel expression plasmid (5–20 μg DNA) was cotransfected with 1 μg of πH3-CD8 plasmid encoding the α subunit of the human CD8 lymphocyte antigen. Before recording, the cells expressing CD8 antigen were identified using antibody-coated beads (Dynal, Lake Success, NY) as described by Jurman et al. (1994). Approximately 90% of cells labeled with the beads expressed the channels.

Electrophysiology

Ionic currents were recorded from inside-out excised patches (Hamill et al., 1981) 24–72 h after transfection. Membrane potential was controlled with custom-made software and hardware operated through an IBM PS-2 computer. Currents were recorded using glass pipettes prepared from Kimax-51 capillary tubes (Kimble), filtered at 10 kHz, and sampled between 20 and 100 kHz. Electrode resistance was in the range of 1–2 MΩ.

Gating currents (I_g) were recorded in whole-cell configuration (Hamill et al., 1981) 24 h after transfection. To ensure a good space clamp, only single round cells that lacked processes and did not form visible contact with other cells were chosen. Recordings were performed using the same pipettes and equipment as for the inside-out patches. Series resistance compensation was used. Currents after voltage jumps were corrected for leak and capacitance. Unmodified gating currents were recorded in K^+/Na^+-free NMG^+-containing solutions during the first 5–10 voltage jumps, before the shape of I_g was changed. It was possible to simultaneously measure ON I_g and $I_{\text{carried by Na}^+}$ in the presence of Na^+, since the amplitude of $I_{\text{carried by Na}^+}$ was much smaller than that of ON I_g at the time when the latter reached its peak value (see Starkus et al., 1997). Each piece of data presented in this work was confirmed in three or more experiments.

Solutions

For the ionic current (I_K) measurements, the composition of the solutions was (in mM): extracellular: 150 NaCl, 1 $MgCl_2$, 3 $CaCl_2$, and 10 HEPES/NaOH (pH 7.4); intracellular: 150 NaCl or 150 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES/NaOH (pH 7.4). For the experiments with L2A mutant channels, Mg^{2+}-free intracellular solutions were used. For I_g measurements the following solutions were used (in mM): extracellular: 150 NMG hydroxide neutralized with HCl, 1 $MgCl_2$, 3 $CaCl_2$, and 10 HEPES (pH 7.4); intracellular: 120 HCl, 20 HF, 10 EGTA, and 10 HEPES (pH 7.4) (adjusted with *N*-methyl-D-glucamine (NMG) hydroxide). In some whole cell experiments Na^+ was substituted for NMG^+. All of the reagents were from Sigma (St. Louis, MO). The experimental chamber was continuously perfused at a rate 1 ml/min. Full exchange of the solution in the chamber was achieved within ~30 s.

RESULTS

The loss of K channel function observed when K^+ is removed internally and externally has an interesting and complicated dependence on V_m, the membrane voltage (Gomez-Lagunas, 1997). Loss of conduction occurs only if the membrane is depolarized and then repolarized. Furthermore, the holding potential must be negative to −50 mV. A possible explanation is that repolarization to negative V_m helps to drive tightly bound K^+ ions from channels activated by a depolarization. To test this hypothesis, we varied V_m during the interval while the channels were closing (*inset*, Fig. 1 *D*). We applied 0 K^+ solution to both sides of the membrane for 1 min at −80 mV, followed by either 1) no pulse or 2) a single depolarization-repolarization sequence with return to −40, −80, or −120 mV for 45 ms as the channels closed, with subsequent return to −80 mV. Fig. 1 *A* shows I_K before and after 0 K^+, when there was no pulse during 0 K^+ exposure. I_K is almost the same, and the defunct fraction after 0 K^+ was less than 5%. The result of a single pulse with 45-ms repolarization to −40 mV is shown in Fig. 1 *B*. Eighty percent of the channels became defunct in response to a single pulse. To our surprise, the defunct fraction decreased when V_m was made more negative, as shown in Fig. 1 *C*. Only 30% of the channels became defunct when the channels closed at −120 mV, precisely the opposite of our initial hypothesis. The results for three repolarization voltages are summarized in Fig. 1 *D*. The duration of the activating pulse made little difference, provided it was long enough to activate all of the channels. The results suggest that the loss of function occurs as the channels are in the act of closing, and not when they are open or fully deactivated. They do not agree with the original hypothesis, that voltage helps drive bound K^+ ions from the channel.

A further test of the hypothesis that negative voltage drives K^+ from the channels was nonetheless performed,

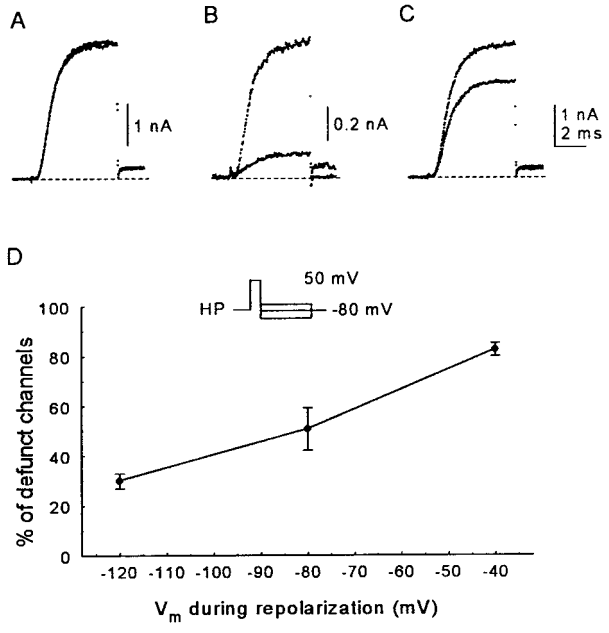

FIGURE 1 The proportion of channels made defunct by a single pulse in 0 K^+ depends on repolarization voltage. (*A–C*) I_K traces recorded before and after a 1-min interval in 0 K^+ solution. During the interval in 0 K^+, there was (*A*) no pulsing, (*B*) a single pulse with repolarization to −40 mV for 45 ms, or (*C*) a single pulse as in *B*, but with repolarization to −120 mV. The voltage protocol is shown in the inset in *D*. (*D*) Summary of results from experiments as in *B* and *C*. Each point represents an average ± SEM, calculated from three (−80 mV) or four (−40, −120 mV) different patches.

using a mutant Leu382Ala (commonly called L2A) that has a positive-shifted p_{open} curve, such that all channels are closed at 0 mV (McCormack et al., 1993). I_K from this mutant is shown in Fig. 2 *A*. The current activates when V_m is stepped from 0 to 150 mV, and deactivates very rapidly on return to 0 mV. The channel thus can close when there is no driving force at all to remove K^+ ions from the channel. As shown in Fig. 2 *B*, about half of the channels become defunct whether closing is at −80 mV, where there is a strong driving force, or at 0 mV, where there is none.

L2A, like L2V (Leu382Val), has almost normal gating current for steps to 0 mV, indicating that the initial steps in the activation process occur in a nearly normal way, even though the channel gate does not open (McCormack et al., 1993). Using this mutant it is possible, then, to separate the influence on loss of function of the early activation steps from that of the last step, which opens the channel. In Fig. 3 the channels were opened and closed by pulses to 150, from 0 mV (*left*) or −80 mV (*right*). In both cases the channels opened and closed, but the holding potential was different. None of the channels became defunct when the holding potential was 0 mV, although they opened and closed. About 60% of them became defunct during a second 0 K^+ exposure when the holding potential was −80 mV. This shows that merely closing the gates is not sufficient to cause the loss of function, and implies that it is necessary to drive the channels through the intermediate closed states, as would occur on return to −80 mV.

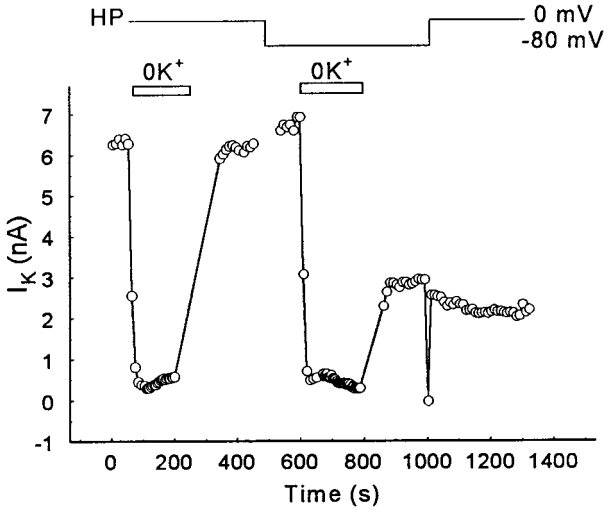

FIGURE 3 Gate closing in 0 K^+ is not sufficient to make channels defunct. Peak I_K (○) through L2A channels is plotted as a function of time. I_K was induced by 15-ms pulses to 150 mV from the HP = 0 (*left*) or −80 mV (*right*; the voltage protocol is shown at the *top of the plot*).

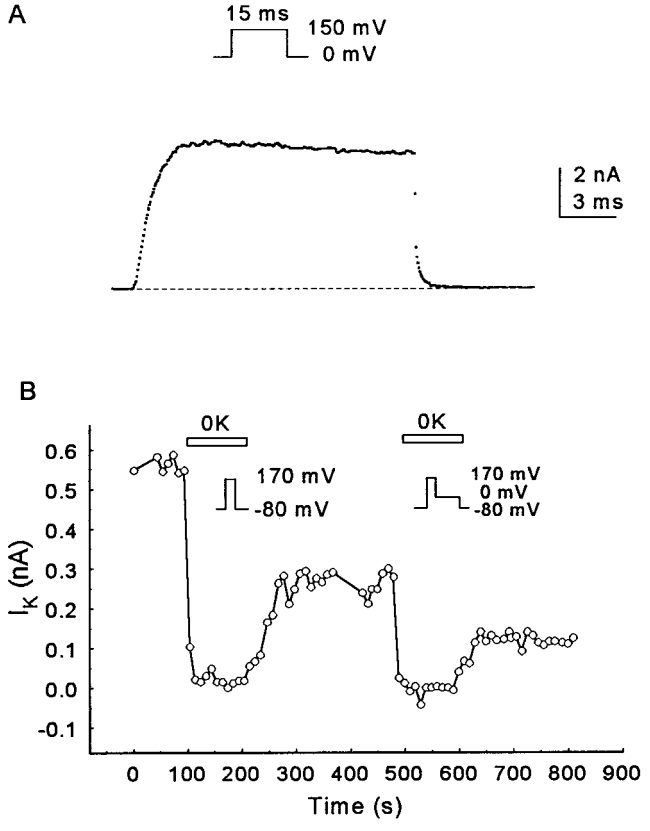

FIGURE 2 L2A channels become defunct in 0 K^+ even when closed in the absence of an electrical field. (*A*) I_K of L2A channels induced by a 15 ms-voltage step to 150 mV from HP = 0 mV. On return to 0 mV, the channels deactivate with a time constant of 0.3 ms. (*B*) Peak I_K (*open circles*) as a function of time. During the first application of 0 K^+ (*open bar*), 10 15-ms pulses to 170 mV were applied from HP = −80 mV. During the second application of 0 K^+, each of 10 pulses was followed by a 45-ms repolarization to 0 mV.

The experiment just described suggests that the gating charge must move toward the fully deactivated state, passing through the intermediate states, if the loss of function is to occur. Gating charge movement can be prevented by prolonged depolarization (Olcese et al., 1997), as shown in Fig. 4 *A*, which used *Sh*IR. The traces labeled Control are unmodified I_g for a depolarization to 0 mV from a holding potential of −80 mV (Control protocol). The lower traces in the three panels of Fig. 4 *A* were recorded with the Test protocol, in which V_m was held at 0 mV and stepped to −80 mV, and I_g was measured on depolarizing again to 0 mV (*arrows* in the protocol diagram). After 10 ms at −80 mV, there is no detectable gating current. After 200 ms it is ~20% of normal, and ~75% of normal after 3 s.

These observations were used in Fig. 4 *B* to ascertain how the loss of function depended on gating charge movement. The initial points give the amplitude of control I_K. After they were recorded, the HP was switched to 0 mV, and the internal solution was changed to 0 K^+ (with 0 K^+ outside throughout). Near the end of the 0 K^+ exposure, the test protocol was applied five times, with a 10-ms interval at −80 mV. On return to 150 K^+ solution and HP −80 mV, I_K was almost the same as before 0 K^+. During the second exposure to 0 K^+ the repolarization interval was 200 ms, and a slight decrease in I_K was observed on return to 150 K^+. Finally, after the third 0 K^+ exposure, with 10 3-s intervals at −80 mV, 80% of the channels were defunct.

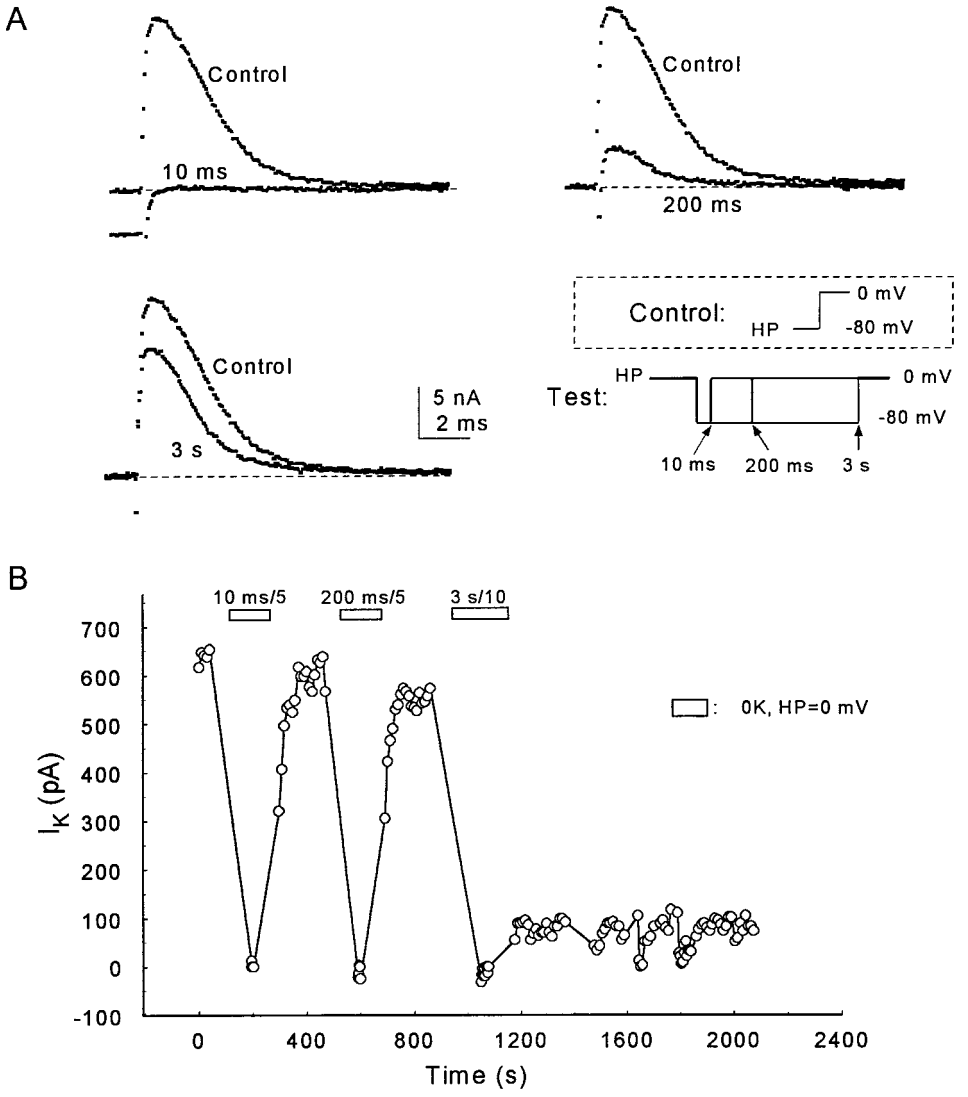

FIGURE 4 Most of the gating charge must be moved from the open state to closed states to render a channel defunct. (*A*) For the test traces (10 and 200 ms and 3 s), *Sh*IR channels were slow-inactivated by holding at 0 mV, and gating currents were recorded on stepping to 0 mV after the indicated interval at −80 mV (Test protocol). For the control traces the holding potential was −80 mV (Control protocol). It takes several seconds for gating charge movement to recover after slow inactivation. (*B*) Peak I_K (*open circles*) from *Sh*IR channels induced by voltage steps to 0 mV from the HP = −80 mV as a function of time. A series of 10-, 200-, or 3000-ms pulses to −80 mV was applied during exposure to 0 K^+ (*open bars*). Numbers above the bars indicate the pulse duration/number of pulses. In each case the HP was switched to 0 mV 1 min before changing to 0 K^+.

This is consistent with the idea that gating charge must be able to move upon polarization for the loss of function to occur. Simply stepping to −80 mV, even for 200 ms, is not sufficient to cause the loss of function. Instead it is necessary that gating charge be able to move when the step to −80 mV is applied.

What happens when channels become defunct? The channel has two major parts, the pore region and the gating apparatus. Certainly the pore stops conducting, but are there alterations in the gating region as well? Fig. 5 *A* shows gating current recorded from functional and defunct channels in 0 K^+. The "Before" recording was made immediately after changing to 0 K^+ before the channels had time to become defunct. I_g ON in the defunct state is reduced to about one-third of normal. On repolarization the I_g tail in the unmodified case has an obvious rising phase that lasts about a millisecond, and then it decays very slowly. The defunct gating current tail has a rapidly decaying component followed by a small slow component. Clearly, the abnormalities that begin with the absence of K^+ from the pore region propagate to the gating apparatus.

It has been noted that Na^+ permeability of *Shaker* K channels increases upon removal of K^+ (Starkus et al., 1997; Ogielska and Aldrich, 1997); this effect is apparent in Fig. 5 *B*. In this experiment, the solutions were changed from 147 Na^+ 3 K^+//150 Na^+ (out//in) to 150 Na^+//150 Na^+ at ~190 s on the time axis. About 10 s later, an outward current began to appear during pulses, reaching 3 nA before declining to near zero at 400 s. This current was not present in symmetrical NMG, and we conclude that it is carried by Na^+. The period of high Na^+ permeability coincided with a decline in I_g that is evident in the graph. I_g dropped to ~20% of its normal amplitude, coincident with the decline of Na^+ current.

What residues within the channel are involved in the loss of function? Some mutations have been found that either prevent entry into, or facilitate recovery from, the defunct state. K channels of type Kv2.1 naturally have a tyrosine at the position equivalent to T449 in *Shaker* (Frech et al., 1989), and are immune to becoming defunct in 0 K^+ (Korn and Ikeda, 1995). This led us to examine T449Y in *Sh*IR. As shown in Fig. 6 *A*, T449Y recovers quite quickly after 100

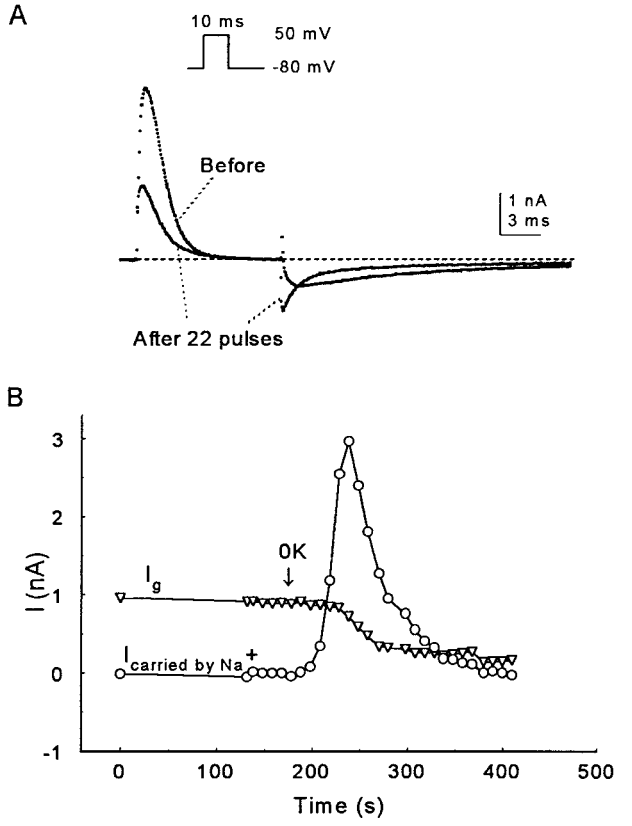

FIGURE 5 The gating current of defunct channels is altered. (*A*) I_g of unmodified (Before; see Materials and Methods) and defunct (After 22 pulses) *Sh*IR channels induced by a 10-ms voltage step to 50 mV from HP −80 mV. The modification was complete in less than 20 pulses in $0K^+$. (*B*) Peak I_g (▽) and $I_{carried\ by\ Na^+}$ (○) as a function of time. The arrow indicates the switch to perfusion with K^+-free solution. Note that $I_{Na} = 0$ in the presence of K^+.

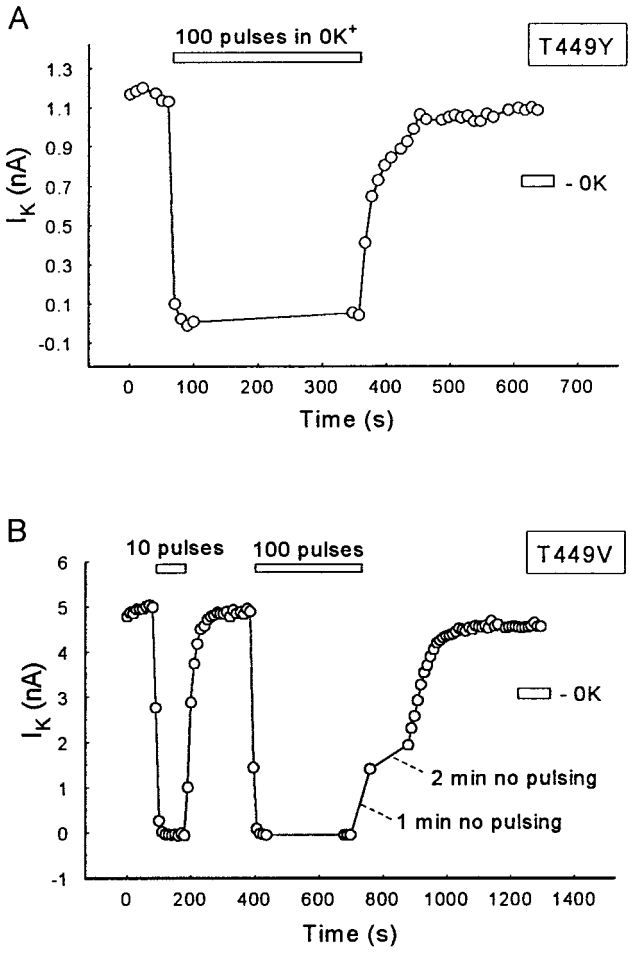

FIGURE 6 Mutations T449Y and T449V prevent transition into the defunct state. (*A* and *B*) Peak I_K of T449Y (*A*) and T449V (*B*) channels as a function of time (*open circles*). The indicated number of pulses, 15 ms to 0 mV from HP = −80 mV, was applied during perfusion with 0 K^+ (*open bar*). (*B*) The patch was perfused with K^+-free solution twice (*open bars*). The first application of 10 pulses (*left*) produced no conductance loss. The second application of 100 pulses (*right*) reduced the conductance by 70%. Recovery in the presence of K^+ was rapid compared to *Sh*IR, but required pulsing.

pulses in 0 K^+, rather than entering a long-lived defunct state. The time course of the recovery in Fig. 6 *A* is slower than the solution exchange, as can be judged from the fall of the current when K^+ is washed away. Thus at least some of the channels enter a nonconducting state in 0 K^+, but they recover rapidly compared to *Sh*IR. This point is made clearer in Fig. 6 *B*, using the mutant T449V. After the first exposure to 0 K^+, pulsing was continuous (at 0.1 Hz), and recovery was rapid and complete: none of the channels entered a long-lived defunct state. After the second exposure to 0 K^+, recovery was greatly retarded when pulsing was suspended for 2 min. The first data point after return to K^+ suggests that ~30% of the channels retain conductance during 0 K^+ exposure. This shows that the majority of the channels have entered a nonconducting state, but one that is rather rapidly reversible if pulsing is applied.

The nonconducting state assumed by T449Y and V can be distinguished from the fully defunct state by examining the gating currents of these mutants in 0 K^+ (Fig. 7). I_g for both mutants changes only slightly after extensive pulsing in the absence of K^+. When compared to I_g of *Sh*IR (Fig. 5 *A*), I_g ON is not significantly reduced in amplitude, and I_g OFF retains a rounded time course with a pronounced rising phase, rather than decaying monotonically. These mutants thus seem to enter a nonconducting state in which the abnormalities do not extend to the gating apparatus.

DISCUSSION

We studied the mechanism by which *Sh*IR channels exposed to 0 K^+ lose their ability to conduct (become defunct), and the relation between gate activity and the loss of conduction. As previously shown (Gomez-Lagunas, 1997), *Shaker* K channels must be opened and closed in 0 K^+ for the loss of conduction to occur. Why does this loss of conduction, which is initiated by occupancy changes in the P region, depend on gating and the membrane voltage?

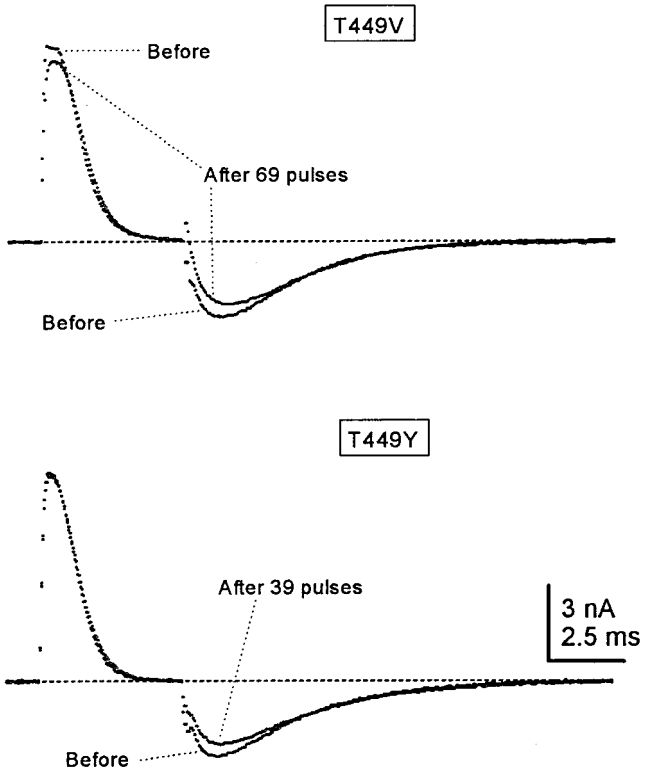

FIGURE 7 I_g of T449V and T449Y mutants, before and after the indicated number of pulses in 0 K^+ solutions. Currents were induced by 5-ms steps from HP −80 mV to 50 mV with repolarization to −120 mV. Note: most of the pulses in 0K^+ were applied with the repolarization to HP = −80 mV. Since OFF I_g's in these mutants are shallow at −80 mV, for illustration purposes the recording was made at −120 mV.

The first question we asked was whether an electrical driving force is necessary to thoroughly dekalify (remove K^+ from) the channel. Opening channels is important for making channels defunct (Gomez-Lagunas, 1997), probably because it allows the escape of K^+ from the pore lumen. Repolarization is also essential for driving channels into the defunct state, and might be necessary because it supplies a driving force to sweep tightly bound K^+ out of the pore. Two experiments addressed this question. In Fig. 1 it is shown that making the driving force stronger during the period when the channels are closing (making V_m more negative) actually decreases the likelihood of becoming defunct. This suggests that slow closing of the channels, i.e., relatively prolonged occupancy of intermediate closed states, is more important than the effect of the driving force. At first sight this may seem incompatible with the results of Gomez-Lagunas, who showed that more negative holding potential in the range −80 to −50 mV increased the likelihood of the loss of function. In our experiment, however, the holding potential was always −80 mV, and we changed only the voltage during the 45-ms interval at pulse end, while the channels were closing.

Why this difference in experimental protocol should be important will become apparent shortly, when the importance of gating charge movement to the loss of function is discussed.

The second test of the importance of driving force made use of the L2A mutant, whose gate closes at 0 mV (McCormack et al., 1993), a voltage at which there is no driving force at all. We find that the loss of function occurred equally well when the channels are closed at −80 mV (gates close, strong driving force) or at 0 mV (gates close, no driving force). Thus a strong driving force during the interval when channels are closing is not necessary.

Is dekalification alone sufficient to cause the loss of function? The L2A mutant does not become defunct in some conditions, even though it is clearly dekalified. When L2A is held at 0 mV (in 0 K^+), pulsed periodically to +150 mV to open the channels, then returned to 0 mV to close them, no loss of function occurs (Fig. 3). It is certain that the channels are dekalified because the Na^+ permeability of the channels increases. This is a signpost of dekalification; and it seems likely that Na^+ flux through the channels would sweep away any K^+ ions. Thus dekalification alone is not sufficient.

Is closing of the gate in dekalified channels sufficient to cause the loss of function? The experiment just cited shows this cannot be the case for the L2A mutant: the gates in this case opened at +150 mV and closed quickly at 0 mV (τ = 0.3 ms).

From the foregoing, with the L2A mutant in 0 K^+, 1) the loss of function does not occur for pulses from 0 to +150 mV if V_m never goes negative to 0 mV; but 2) it does occur if V_m is returned to a holding potential of −80 mV, after the gates are closed at 0 mV. This shows without question that a return to negative voltage is necessary for the loss of function to occur. How can this be explained? With the L2A mutant, as with L2V (McCormack et al., 1993), most of the gating charge movement occurs in the range between −80 mV and 0 mV, even though the channel gates do not open at all. The remaining fraction of the gating charge moves positive to 0 mV, and causes opening of the gate. The results with L2A show that loss of function only occurs after movement of that part of the gating charge that redistributes in the range from 0 mV to −80 mV. The results in Fig. 4 support this idea. When gating charge is "immobilized" by prolonged depolarization, stepping to −80 mV for intervals as long as 200 ms does not produce defunct channels. Only when the interval at −80 mV is long enough to restore much of the gating current does the loss of function occur. This strongly suggests that the loss of function occurs as the S4 units are forced into their closed conformation.

At this point it becomes clear that the results in Fig. 1 are consistent with the hypothesis that the transition into the defunct state occurs through one or several intermediate closed states that are more populated at −40 mV than at −80 or −120 mV. Apparently, from the intermediate closed state(s), the channel without K^+ can close normally, or it can enter an alternative state, inaccessible in the presence of K^+, that leads to the defunct state. Hyperpolarization minimizes the period of occupancy of the intermediate states

and minimizes the tendency to become defunct. When first repolarized to −40 mV, the channels can accumulate in the alternative state, and become defunct upon subsequent repolarization to −80 mV.

Are the changes that occur with the loss of function confined to the P region? The gating current traces in Fig. 5 *A* show that they are not. I_g ON is cut by approximately two-thirds, showing that gating charge movement is restricted in defunct channels. The time course of I_g OFF is strongly altered and resembles the tail gating current of an unmodified channel after depolarizations that are too small to open the channel gates. Apparently the channel is restricted to closed states, probably abnormal ones. The absence of the slow tail component that is characteristic of unmodified channels returning from the fully open state (Stefani et al., 1994) suggests that defunct channels cannot open.

Results regarding the loss of function of the channels are summarized in Fig. 8. A section of an unmodified, K^+-occupied closed channel is shown diagrammatically in Fig. 8 *A*, following the structural results of Doyle et al. (1998). The P region is in white, and the negative charges of the narrow selectivity filter are due to the carbonyl dipoles that line the P region (Doyle et al., 1998). Two of the four S4 helices are shown, each containing seven positive charges. Each S4 is connected to a gate segment that is pulled open when the S4's have been pushed outward by depolarization. In 0 K^+, the occupying ions have a better chance of escaping from the channel when its gate is open. This is probably because internal Na^+ ions can move outward through the channel to displace K^+, an idea that is consistent with the finding that loss of function is more rapid with Na^+ than with TMA^+ or NMG^+ internally (Gomez-Lagunas, 1997), because the latter two are not permeant. When the K^+ ions have escaped, we postulate that the two sites in the selectivity filter are empty much of the time, because affinity for Na^+ is relatively low. Under these circumstances, the electronegative carbonyl oxygens known to line the pore repel each other (*arrows*), destabilizing the structure, and leading to a conformational change in the P region (Fig. 8, *C* and *D*). An early stage of this process is an increase in Na^+ permeability in 0 K^+ (Fig. 5 *B*). The permeability increase to Na^+ is transient, and disappears as a channel becomes fully defunct. In the cartoon, the P regions lean laterally in the defunct state. They are prevented from doing so in Fig. 8 *B* by the S4 units, which are in activated position. On repolarization the S4's move inward, allowing the P region to deform (Fig. 8 *C*; evidence regarding the requirement for S4 movement is in Figs. 3 and 4). Once deformed, the P region interferes with movement of the S4 segments, accounting for the decrease in total gating charge movement. The diagram also helps to explain the effectiveness of depolarization on the restoration of function: when the S4's are biased outward by depolarization, they tend to push the P region back into normal conformation.

Two mutant channels, T449Y and T449V, showed conduction abnormalities in 0 K^+, but the gating current measurements of Fig. 7 make it clear they were not completely defunct (compare the I_g traces of Fig. 7 with those of Fig. 5

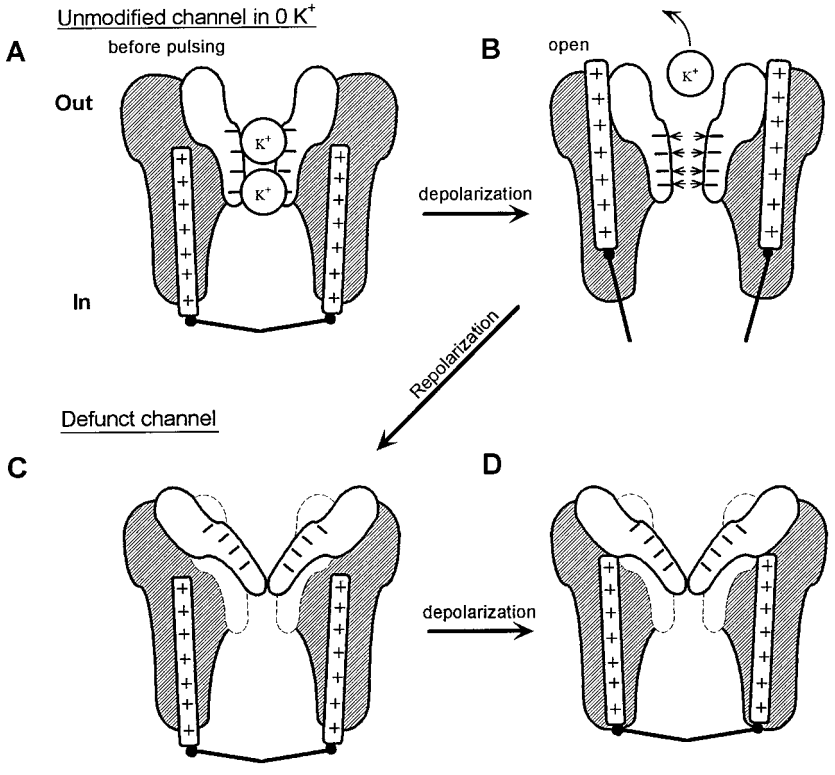

FIGURE 8 Summary and model of the transition to the defunct state in 0 K^+. (*A*) A section of an unmodified channel in the closed state at the resting potential and (*B*) in the open state at a depolarized potential. K^+ ions bound to carbonyl dipoles in the P region leave the pore when the gate opens. In the absence of K^+ the negative ends of the carbonyl oxygens repel each other, destabilizing the structure. Refolding, represented as tilting of the P regions, can only occur upon movement of S4's toward their closed state position (*C*). In the defunct channel (*C* and *D*), movement of S4 is restricted, accounting for reduced and altered gating current.

A). That is, the abnormalities in the P region did not spread to the gating apparatus.

Mutations at position 449 have effects that may be relevant to the phenomena we report here. According to the crystallographic data on the KcsA channel (Doyle et al., 1998), the residue equivalent to *Shaker* residue 449 is near the outer mouth of the pore, and a fairly long distance from the pore axis. Both T449Y and V show relatively little C-type inactivation. Interestingly, the mutant I470C also shows little C-type inactivation, and does not become defunct. Thus in all three cases where data are available, the two properties, C-type inactivation and susceptibility to becoming defunct, seem to go together. Whether this is generally true remains to be seen. Another intriguing connection is that some T449 mutants (T449A, E, K, and possibly N) rapidly lose the ability to conduct when external K^+ is removed (Lopez-Barneo et al., 1993; Jäger et al., 1998). This nonconducting state in the absence of external K^+, with internal K^+ at its normal level, is quite distinct from the defunct state, which occurs only in the absence of both external and internal K^+. Furthermore, recovery from the defunct state on K^+ restoration requires prolonged depolarization, which is not the case for mutants T449A, E, K recovering from removal of external K^+. Mutant T449A C-type inactivates and becomes defunct on removal of internal and external K^+. The other mutants were not tested.

Perhaps the most interesting conclusion from our work is that changes in the P region, initiated by the absence of K^+, can propagate to the gating apparatus of the channel, leading the channel into the defunct state, and that the movement of the voltage sensor is necessary for this transition to occur. These functional properties are located in distinct parts of the sequence. The pore is composed of transmembrane segments S5 and S6, whereas voltage-dependent gating is initiated by the S4 segment and some or all of the other three transmembrane segments. Our results point to a close connection between the gating and conduction functions.

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