Gating Charge Immobilization Caused by the Transition between Inactivated States in the Kv1.5 Channel

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ABSTRACT Sustained Na^+ or Li^+ conductance is a feature of the inactivated state in wild-type (WT) and nonconducting *Shaker* and Kv1.5 channels, and has been used here to investigate the cause of off-gating charge immobilization in WT and Kv1.5-W472F nonconducting mutant channels. Off-gating immobilization in response to brief pulses in cells perfused with NMG^+_i/NMG^+_o is the result of a more negative voltage dependence of charge recovery ($V_{1/2}$ is −96 mV) compared with on-gating charge movement ($V_{1/2}$ is −6.3 mV). This shift is known to be associated with slow inactivation in *Shaker* channels and the disparity is reduced by 40 mV, or ~50% in the presence of 135 mM Cs^+_i. Off-gating charge immobilization is voltage-dependent with a $V_{1/2}$ of −12 mV, and correlates well with the development of Na^+ conductance on repolarization through C-type inactivated channels ($V_{1/2}$ is −11 mV). As well, the time-dependent development of the inward Na^+ tail current and gating charge immobilization after depolarizing pulses of different durations has the same time constant (τ = 2.7 ms). These results indicate that in Kv1.5 channels the transition to a stable C-type inactivated state takes only 2–3 ms and results in strong charge immobilization in the absence of Group IA metal cations, or even in the presence of Na^+_o. Inclusion of low concentrations of Cs^+_i delays the appearance of Na^+ tail currents in WT channels, prevents transition to inactivated states in Kv1.5-W472F nonconducting mutant channels, and removes charge immobilization. Higher concentrations of Cs^+_i are able to modulate the deactivating transition in Kv1.5 channels and prevent the residual slowing of charge return.

INTRODUCTION

In voltage-gated K^+ channels, the potential dependence of gating charge return after depolarization is bimodal. After small depolarizations, charge return is fast, similar to outward charge movement. However, in the absence of permeating ions, on repolarization after channel opening there is a rising phase to off-gating currents and slowed decay (Perozo et al., 1993; Stefani et al., 1994). Much of this slowing is because of the relative voltage independence of the concerted opening transition (Zagotta and Aldrich, 1990; Zagotta et al., 1994b; Ledwell and Aldrich, 1999), but in the absence of univalent cations small enough to permeate the channel, in mammalian cells and excised patches from oocytes, charge return is even slower so that it cannot be readily distinguished from the zero current level, and is then said to be "immobilized" (McCormack et al., 1994; Wang et al., 1999). This effect can be prevented by the presence of univalent or divalent metal cations (Chen et al., 1997; Hurst et al., 1997; Starkus et al., 1998) and studies suggest either that these cations are able to allosterically modulate K^+ channel deactivation, or alternatively, that the slowing is predominantly caused by an accelerated inactivation that can be partially prevented by these cations (Yellen, 1997; Chen et al., 1997). This latter explanation fits with ionic current data that have shown that C-type inactivation is accelerated when the extracellular cation concentration is changed (Lopez-Barneo et al., 1993) or univalent metal cations are removed altogether (Baukrowitz and Yellen, 1995; Kukuljan et al., 1995). An important additional feature of slow inactivation-induced charge immobilization in *Shaker* wild-type (WT) and W434F nonconducting mutant (NCM) channels is that the voltage dependence of charge return is shifted toward more negative potentials by ~−50 mV (Olcese et al., 1997).

Recently, new methods have become available to follow the movement of K^+ channels into inactivated states, and these can provide important additional information to gating and ionic current studies. They include changes in fluorescence and changes in the relative permeabilities to Na^+ and K^+ of the inactivated state(s). Fluorescence studies of slow inactivation have revealed the presence of at least two non-K^+ conducting states in the inactivation pathway (Loots and Isacoff, 1998), as had previously been suggested from ionic and gating current studies (Yang et al., 1997; Kiss et al., 1999). The first, or proximal, state reached after the open state has a higher Na^+ permeability and results from a relatively local conformational change in the outer pore mouth (Loots and Isacoff, 1998). The second, more distal state from the open state corresponds to the fully C-type inactivated state and shows both a lower Na^+ permeability (Kiss et al., 1999) and more extreme conformational changes (Loots and Isacoff, 1998). Na^+ conductance of the inactivated state(s) appears as a sustained current during prolonged depolarization, or as slow tails on deactivation (Starkus et al., 1997; Starkus et al., 1998). The amount of sustained Na^+ current through inactivated channels on depolarization varies depending on the channel type, but a consistent feature of the inactivated channels is a large Na^+ conductance on repolarization to negative potentials.

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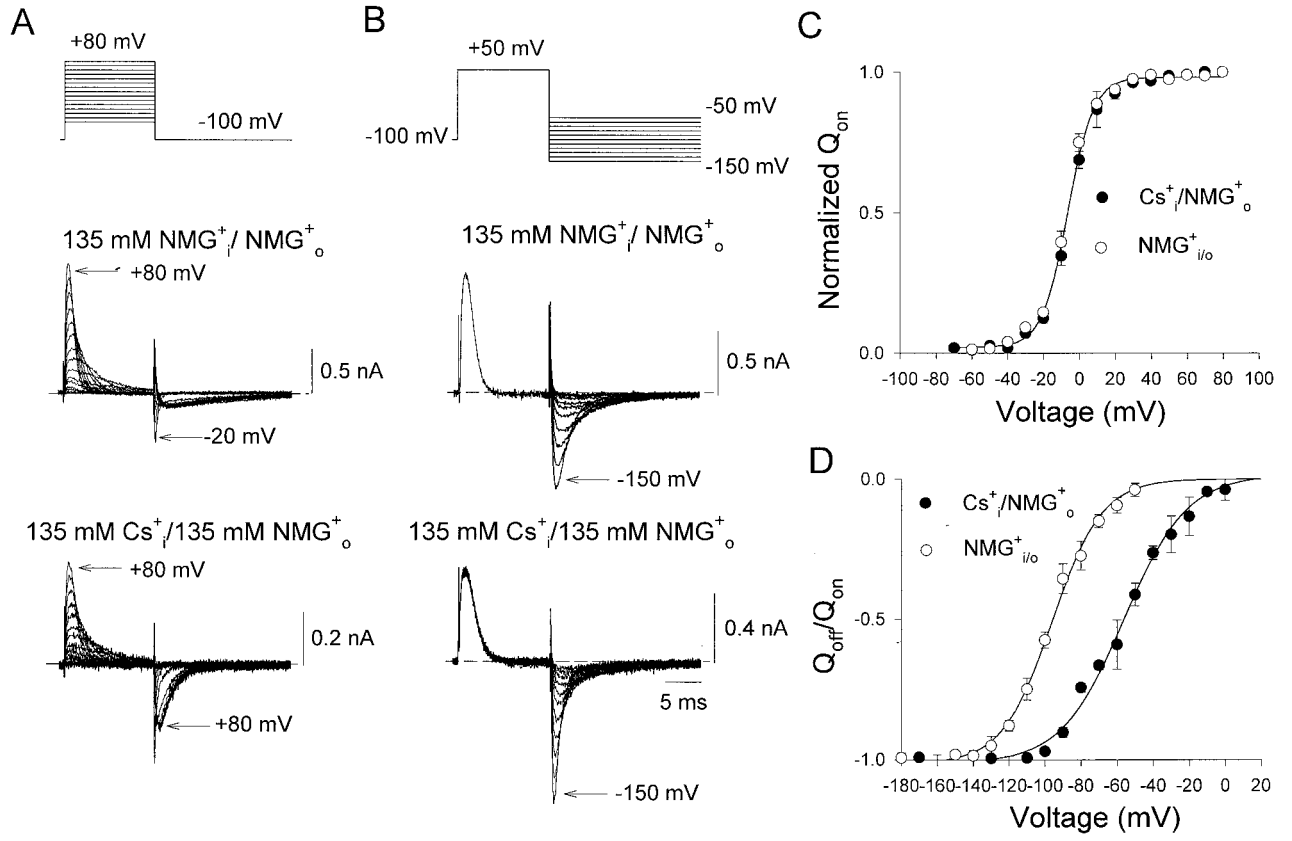


FIGURE 1 Gating charge immobilization on repolarization in the Kv1.5 NCM is caused by a rapid shift in the voltage dependence of charge return. (*A*) Off-gating currents after 12-ms depolarizations from −60 to +80 mV. Voltage protocol is shown at the top. Currents in the middle panel were recorded with symmetrical NMG^+_i/NMG^+_o solutions. Currents in the lower panel were recorded with 135 mM Cs^+_i substituted for the NMG^+_i. (*B*) Currents in the middle and lower panels were recorded on repolarization to a range of potentials between −50 and −150 mV, after a step depolarization to +50 mV, as illustrated by the protocol in the top panel, and under the same ionic conditions as in A. (*C*) Voltage dependence of Q_{on} for the experiments shown in *A*. The $V_{1/2}$ for the fitted curve was −6.3 ± 0.4 mV, with a slope factor *k* of 7.3 ± 0.3 mV ($n = 20$). (*D*) Voltage dependence of Q_{off} for the experiments shown in (*B*), for NMG^+_i/NMG^+_o (○) and Cs^+_i/NMG^+_o (●). For NMG^+_i/NMG^+_o, the $V_{1/2}$ was −96 ± 0.6 mV; *k* was 14 ± 0.6 mV ($n = 8$). For Cs^+_i/NMG^+_o, the $V_{1/2}$ was −56 ± 1.3 mV and *k* was 17 ± 1.2 mV ($n = 8$).

This generates slow Na^+ tail currents with a very prominent initial rising phase followed by a slow decay process that reflects the deactivation of inactivated channels to closed-inactivated states in *Shaker* channels (Starkus et al., 1998) and in Kv1.5 (Wang et al., 2000).

In this study we used increased Na^+ or Li^+ conductance as an index of the presence of inactivated Kv1.5 channels, and correlated it with the onset of charge immobilization in Kv1.5-W472F NCM channels. We determined that there are two inactivated states in Kv1.5, one more proximal which has a relatively high Na^+ conductance, and a deeper distal, inactivated state with a low Na^+ conductance, recovery from which is responsible for the prominent charge immobilization seen in Kv1.5 when small metal cations are absent. We examined the Na^+ conductance and charge immobilization in the presence of intracellular Cs^+ to determine which transitions were responsible for the modulation of charge return induced by small univalent cations. Low Cs^+_i concentrations prevent the transitions to inactivated states in the NCM channel, and delay these transitions in WT channels, whereas higher concentrations of Cs^+_i modulate the deactivating transition in WT and NCM channels.

MATERIALS AND METHODS

Cells and solutions

Two forms of human Kv1.5 were used in the present experiments, the wild form of which we originally cloned from human fetal heart (Fedida et al., 1993). WT and NCM channels were expressed in human embryonic kidney cell lines. Kv1.5 was mutated in the plasmid expression vector, using the Stratagene Chameleon Kit (Stratagene; La Jolla, CA) to convert tryptophan 472 to phenylalanine (W472F). This NCM mutation is analogous to the ShH4-IR W434F (Perozo et al., 1993). HEK-293 cells were stably transfected with WT Kv1.5 or Kv1.5-W472F (NCM) cDNAs in pRC/CMV, using lipofectamine reagent (Canadian Life Technologies, Bramalea, ON) in a 1:10 (w:v) ratio. For recording from HEK cells, patch pipette solutions contained (in mM): N-Methyl D-glucamine (NMG^+), 135; EGTA, 5; $MgCl_2$, 1; HEPES, 10; and were adjusted to pH 7.2 with HCl. A concentration of 10 mM CsCl was added directly to this internal solution. The 135

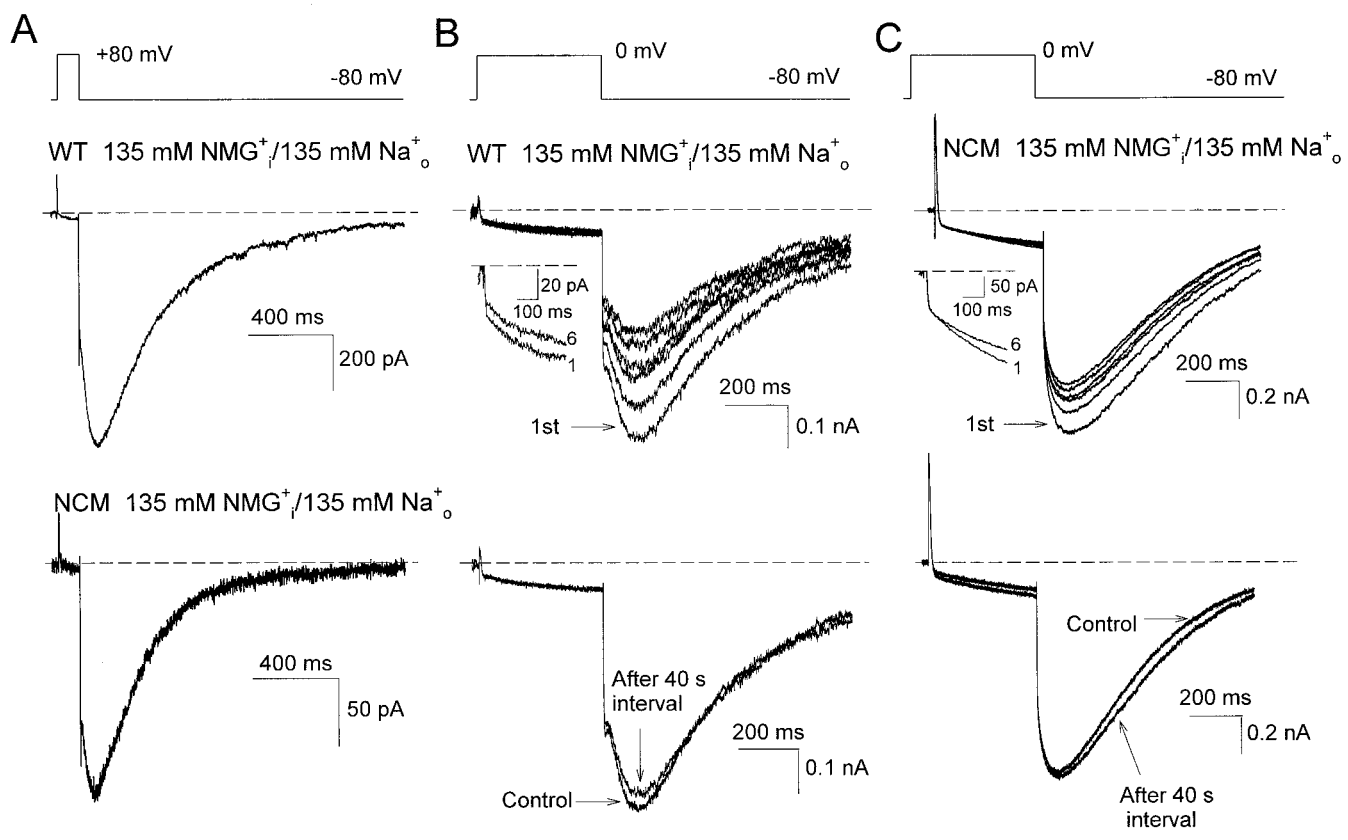


FIGURE 2 Na^+ currents show that NCM channels gate similarly to WT channels on repolarization. (*A*) WT Kv1.5 and NCM channel currents (*lower panel*) under identical ionic conditions with 135 mM NMG^+_i and 135 mM Na^+_o. Cells were rested for 60 s and then pulsed from −80 to +80 mV for 100 ms before repolarizing to −80 mV for 1.6 s. (*B*, *C*) failure of recovery from inactivation in both WT (*B*) and NCM (*C*) channels. Cells were rested for 60 s and then pulsed from −80 to 0 mV for 400 ms in *B* and *C*, upper panels, at a frequency of 0.2 Hz. Slow Na^+ tails were observed on repolarization to −80 mV, and decreased in amplitude from pulses 1–6. A decrease in current during the depolarization to 0 mV is shown in the insets of *B* and *C* at higher gain. The recovery of current is shown in the bottom panels after a 40-s rest, compared with the control current tracing before stimulation, same cells as above.

mM Cs^+_i solution was made by equimolar substitution of the NMG^+_i, and pH was adjusted to 7.2 with CsOH. The bath solution contained (in mM): NMG^+, 135; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 1; and was adjusted to pH 7.4 with HCl. When 135 mM Na^+_o solution was used, NMG^+ was omitted, and the pH was adjusted to 7.4 with NaOH. All chemicals were from Sigma Aldrich Chemical Co. (Mississauga, ON).

Electrophysiological procedures

Coverslips containing cells were removed from the incubator before experiments and placed in a superfusion chamber (volume 250 μl) containing the control bath solution at 22–23°C. Current recording and data analysis were done using an Axopatch 200A amplifier and pClamp 6 software (Axon Instruments, Foster City, CA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments; Sarasota, FL). Capacitance compensation and leak subtraction using a P/6 protocol from a holding potential of −100 mV were routinely used. Data were sampled at 10–50 kHz and filtered at 2–10 kHz. All Q_{on} and Q_{off} measurements were obtained by integrating the on- or off-gating currents until they returned to the baseline (25 ms for Q_{off}, which was the maximum duration of repolarizing pulses in Figs. 1 and 4). Membrane potentials have been corrected for the junctional potentials (<10 mV) that arose between pipette and bath solutions. Data are shown as mean ± S.E. throughout.

RESULTS

C-type inactivation rapidly shifts the voltage dependence of gating charge return

It is known that C-type inactivation in *Shaker* homologs such as Kv1.5 is associated with a relative immobilization of off-gating charge (Q_{off}). On-gating currents from a holding potential of −100 mV appear as transient currents when the cells are depolarized to positive potentials, but when repolarized to −100 mV, the peak off-gating currents are very small, and the time constant of relaxation of off-gating current (τ_{off}) is slow (Fig. 1 *A*). As shown by Olcese et al. (1997) for *Shaker* channels expressed in oocytes, this immobilization is a voltage- and time-dependent phenomenon, as pulses to more negative potentials (down to −140 mV in their case) induce the gating charge to return more quickly. The same is true in Kv1.5, where at −150 mV charge returns rapidly and completely (Fig. 1 *B*). What is different in Kv1.5 expressed in mammalian cells, compared with *Shaker* chan-

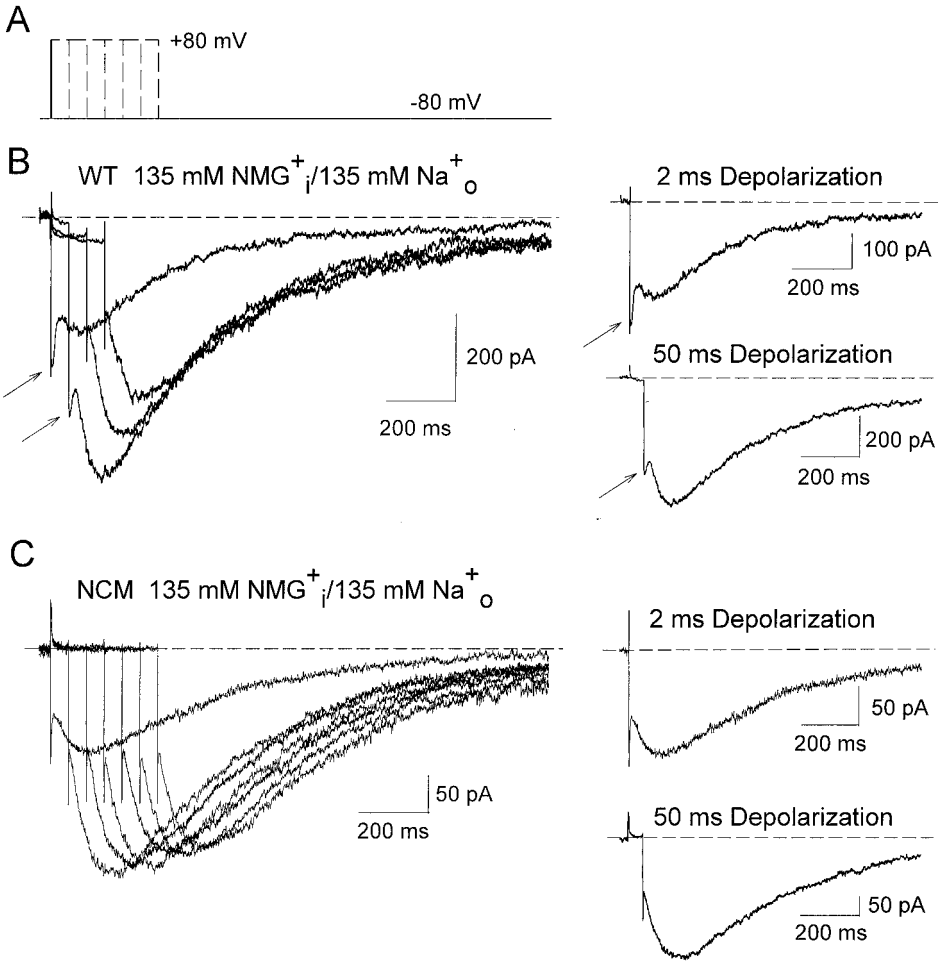


FIGURE 3 Both WT and NCM channels rapidly enter the inactivated state in the absence of internal univalent metal cations. (*A*) The pulse protocol used to collect data in *B* and *C*. Cells were pulsed from −80 mV to +80 mV for durations from 2 ms increasing to 50 ms for the second depolarization, and subsequently incrementing 50 ms each cycle. Pulses were given at a cycle interval of 20 s. (*B*, *C*) WT and NCM current data, respectively, obtained using the protocol described in *A*. Tracings for separate depolarizations have been superimposed in the left panels and those for 2-ms and 50-ms depolarizations are shown separately in the right panels. Note the absence of an initial inward tail transient in NCM channels after short depolarizations (*arrow* on WT data).

nels expressed in oocytes, is how rapidly this effect can be observed. In *Shaker* channels the effect took depolarizations of hundreds of milliseconds to develop, whereas in Kv1.5, the effect is clearly visible after 10 ms and, as we shall show later, occurs almost instantaneously on depolarization. Despite this, the voltage dependence of the charge immobilization in Kv1.5 is very similar to that seen in *Shaker*, such that charge is ~40% immobilized if off-gating current is measured at −100 mV as in Fig. 1 *A* (*upper*) and integrated to the baseline (point at −100 mV in Fig. 1 *D*).

We have previously shown that addition of small univalent metal cations to the NMG^+ solutions can modulate the timecourse of charge return (Chen et al., 1997; Wang et al., 1999), and that Cs^+ effectively prevents charge immobilization at −100 mV (Fig. 1 *A*, *lower*). We have now observed that this effect is caused by a shifting of the potential dependence of charge return, rather than any increase at one particular potential (Fig. 1 *B*, *lower*). In the presence of 135 mM Cs^+_i, charge return at −100 mV is almost complete, and this is accounted for by a 40–50 mV positive potential shift in the $V_{1/2}$ of charge return as shown in the charge-voltage relationships in Fig. 1 *D*. It should be noted that, although Cs^+_i modulates the voltage dependence of charge return, it does not affect the voltage dependence of on-gating charge movement (Fig. 1 *C*).

Na^+ tail currents through Kv1.5 allow the tracking of inactivated channels

Previous work has suggested that *Shaker* (Starkus et al., 1997) and a mammalian homolog Kv1.5 (Wang et al., 2000), as well as Kv2.1 (Kiss et al., 1999), undergo a change of permeability during inactivation such that

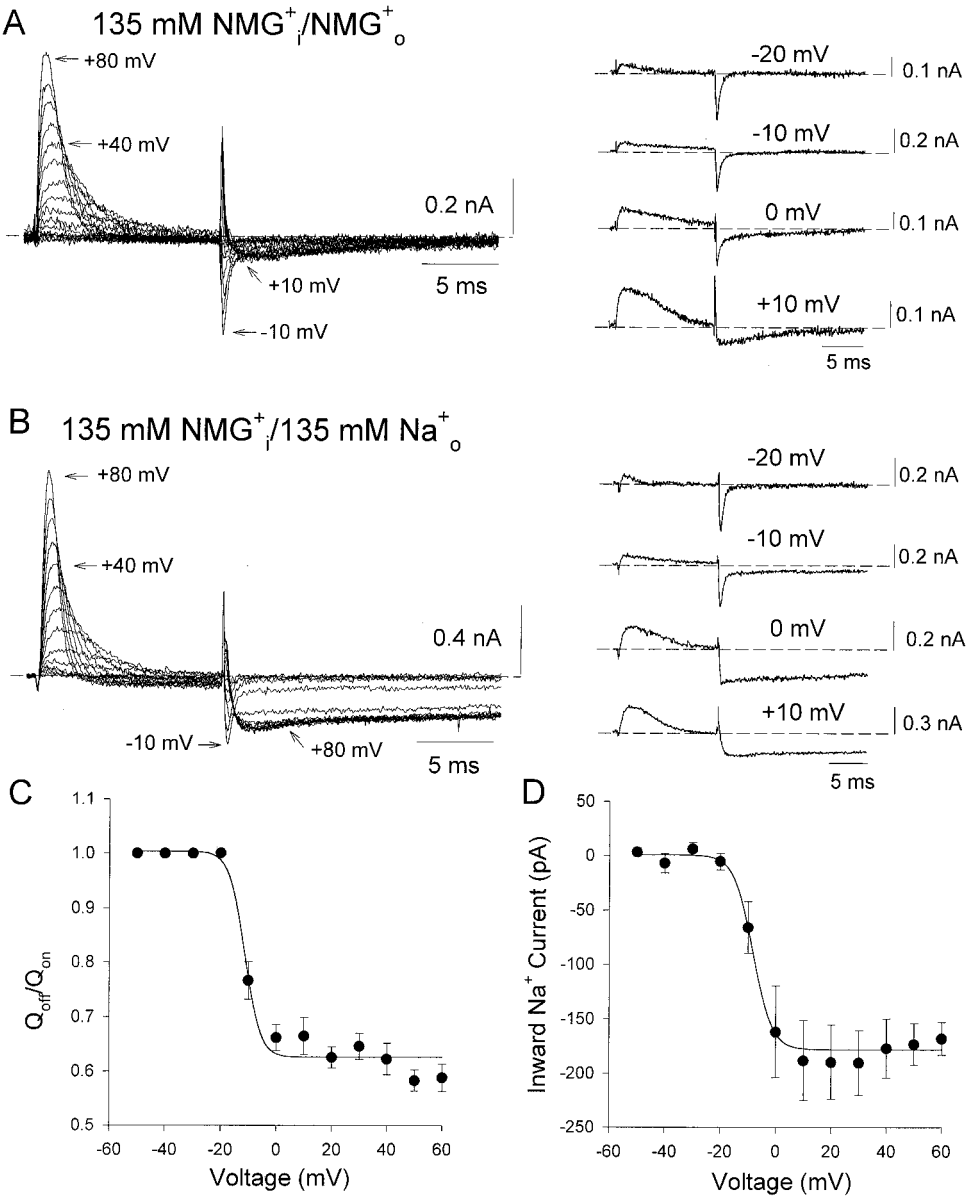


FIGURE 4 The voltage dependence of Q_{off} immobilization matches that of Na^+ currents through the NCM. (*A*, *B*) Gating current-voltage relationships induced by depolarizations from −100 mV to between −60 mV to +80 mV in 10 mV steps, with symmetrical NMG^+ (*A*), or with 135 mM Na^+_o (*B*). The right panels in each case illustrate selected pulse potentials between −20 and +10 mV. Note that in all cases, repolarization potential was −100 mV. (*C*) Voltage dependence of charge immobilization measured as Q_{off}/Q_{on} as a function of potential, matches the voltage dependence of inward Na^+ current (*D*), measured as the sustained current level at the end of repolarization. For NMG^+_i/Na^+_o the $V_{1/2}$ for sustained Na^+ current was −12.2 ± 0.9 mV and *k* was 3.4 ± 0.9 mV ($n = 6$). For NMG^+_i/NMG^+_o the $V_{1/2}$ for off-gating charge immobilization was −11.3 ± 1.1 mV and *k* was 2.8 ± 1.5 mV ($n = 7$).

channels lose their permeability to K^+ but become significantly more permeable to Na^+. This is not normally seen as an Na^+ conductance physiologically, as internal K^+ prevents the Na^+ from entering the selectivity filter of the K^+ channel (Starkus et al., 1997; Kiss et al., 1998), and can only be clearly examined in the absence of internal and external K^+. The main aim of the present study is to use this Na^+ conductance to measure whether channels are inactivated or not, and to relate the presence of significant Na^+ conductance to off-gating charge immobilization. Most of the experiments have been carried out on the Kv1.5 NCM to allow inclusion of Na^+ in the internal or external solutions, but only allow conduction through inactivated states. As a first result we show that NCM channels undergo gating and Na^+ conductance changes similar to those of WT channels during inactivation and recovery.

It is known that in K^+-free media both WT *Shaker* and Kv channels conduct Na^+ through the inactivated state, but at a lower level than the open state (Starkus et al., 1997;

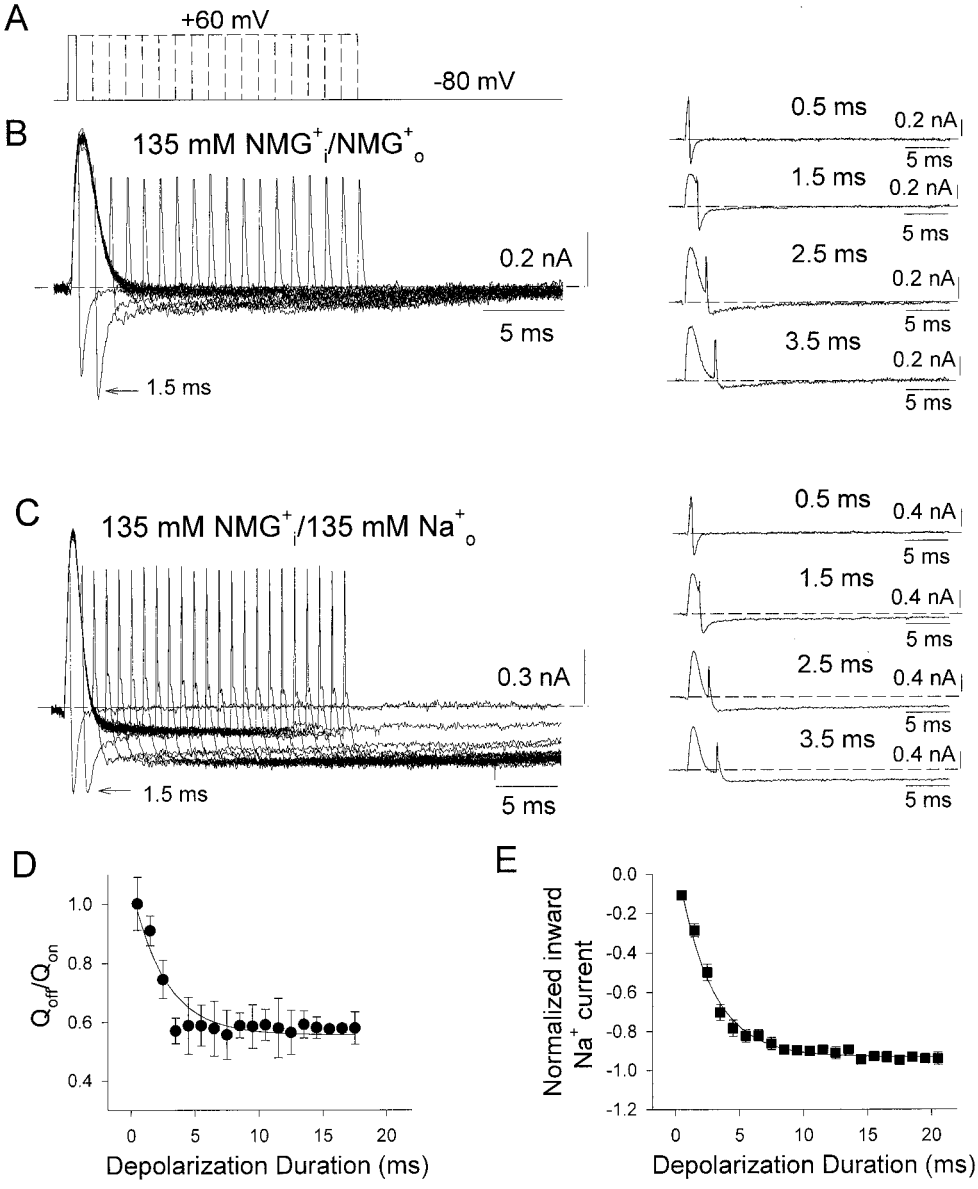


FIGURE 5 The time dependence of Q_{off} immobilization matches the time dependence of Na^+ current appearance through the NCM. (*A*) The pulse protocol used to collect data in *B* and *C*. (*B*) On- and off-gating currents from −80 to +60 mV, in response to increasing pulse duration. The initial duration was 0.5 ms and this was incremented by 1 ms for each cycle. The pulses were applied at 0.2 Hz. Envelopes of gating current were obtained with symmetrical NMG^+ (*B*), or with 135 mM Na^+_o (*C*). The right panels in each case illustrate selected on- and off-gating currents at increasing pulse durations as indicated. Note that the slowing of off-gating current return matches the increase in sustained inward Na^+ current (*B*, *C*, *right*). (*D*, *E*) Timecourse of charge immobilization (Q_{off}/Q_{on}) and inward Na^+ tail development with pulse duration. Charge immobilization was measured as the off-/on-gating charge at each pulse duration. The Na^+ current was measured at the peak inward current level. In each case data were from 9 cells, and for *E*, decay was fit with a single exponential ($\tau = 2.7 \pm 0.2$ ms), shown as a line through datapoints. The same fit was applied to data in *D*.

Wang et al., 2000). Channels can also close while still inactivated and this pathway is illustrated by deactivating Na^+ tail currents on repolarization, shown in Fig. 2. WT channels are inactivated by a strong prepulse to +80 mV and then repolarized to −80 mV to observe tail currents (Fig. 2 *A*). With 135 mM Na^+_o an instantaneous inward tail current reflects conduction through inactivated channels (a driving force effect). The tail increases substantially and then decays slowly as inactivated channels close. The rising phase of the tail current after repolarization reveals the transition through an intermediate higher Na^+ conductance state before deactivation. NCM channels show identical changes, as shown in the lower panel of Fig. 1 *A*, suggesting that they also follow the general scheme that we have recently used to model these changes in WT channels (Wang et al., 2000):

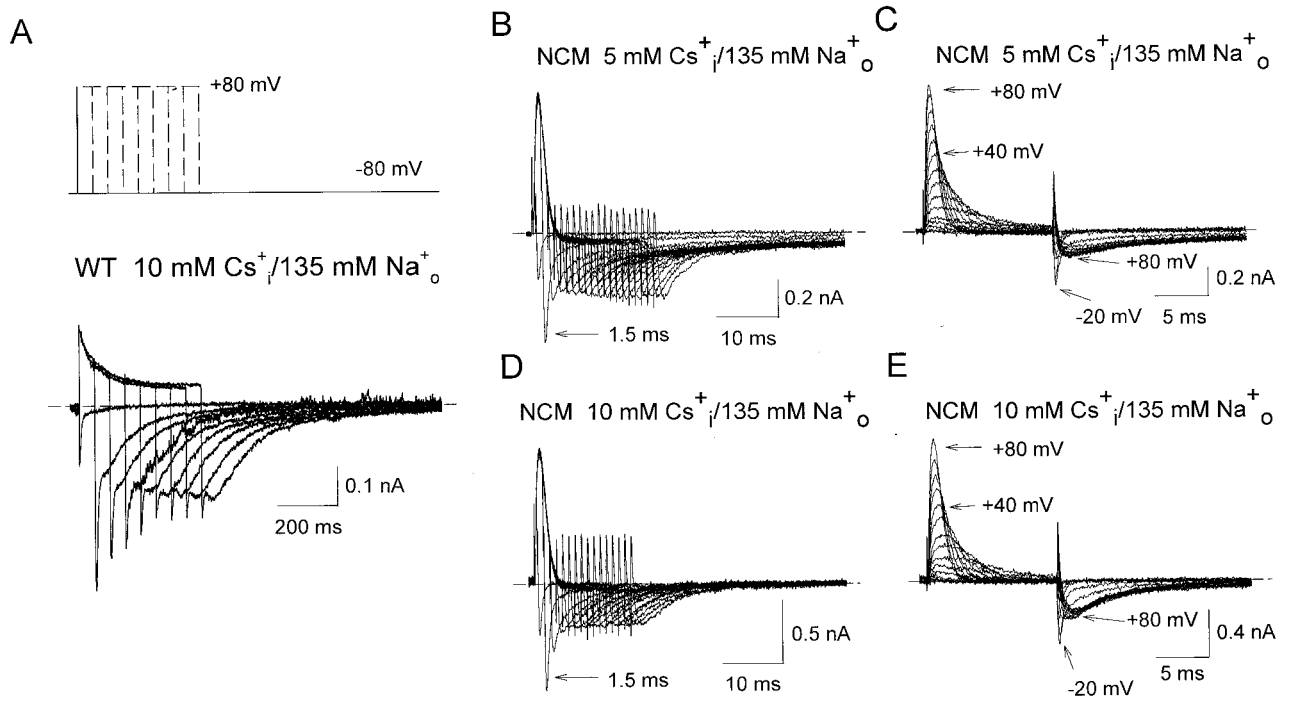
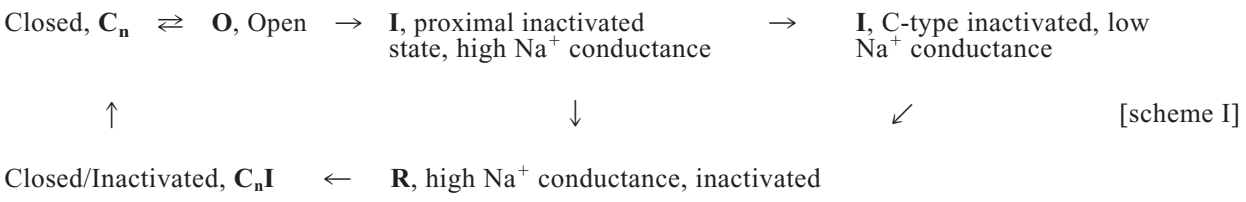


FIGURE 6 Cs^+_i affects the appearance of Na^+ tail currents in NCM channels. Solutions contained 135 mM Na^+_o/135 mM NMG^+_i plus 5 or 10 mM Cs^+_i as stated. (*A*) Control experiment carried out on WT channels using a protocol as in Fig. 3 *A*. Data show a delay in appearance of the Na^+ tail current when 10 mM Cs^+_i is included in the pipette solution. In remaining panels, the experiments shown in Fig. 5 *C* and Fig. 4 *B* are repeated in NCM channels, but with the addition of 5 mM mM Cs^+_i in *B* and *C* and 10 mM Cs^+_i in *D* and *E*. (*B*, *D*) Data are for increasing pulse durations from 0.5–15.5 ms. (*C*, *E*) Gating I-V relations are shown with Cs^+_i. Cells were held at −100 mV and pulsed to a range of potentials between −80 and +80 mV in 10-mV steps.



In the presence of external Na^+ alone, the proximal inactivated state is not detected during the onset of inactivation as it is traversed too rapidly, but its presence can be revealed by adding 1–2 mM concentrations of internal K^+ (Kiss et al., 1999; Wang et al., 2000), or other cations, as shown below in Figs. 6–8. Note that inactivated WT channels generally recover through the higher Na^+ conductance state, to close in the inactivated form, and return back to resting closed states via a slow vertical transition. The time constant of this slower recovery to resting closed states in WT channels is of the order of 3–4 s (Wang et al., 2000). Consequently, repetitive depolarizations at faster rates than those allowing complete recovery are expected to cause a progressive decrease in the tail current amplitude, as channels are "trapped" in closed-inactivated states. Such a decrease is clearly seen in WT channels in Fig. 2 *B* during stimulation at 0.2 Hz. There is a decrease in both the inward current during the depolarizing pulse (shown at higher gain in the *inset*) and the tail current on repolarization. A longer interstimulus interval, for 40 s in this case (Fig. 2 *B*, *lower*) results in full recovery of the WT tail current amplitude compared with the pre-control. We can test the availability, and thus the recovery pathway of NCM channels in a similar way by examining the amplitude of the slow tail current in response to repetitive depolarizations. At a rate of 0.2 Hz, the amplitude of the slow Na^+ tail decreases substantially by the sixth pulse (Fig. 2 *C*), but is fully available after a 40-s interval as shown by the recovery to the control level in the lower panel. These changes seen in both WT and NCM channels suggest that they traverse similar pathways during the recovery from inactivation, and that NCM channels are able to occupy non-inactivated closed states at rest.

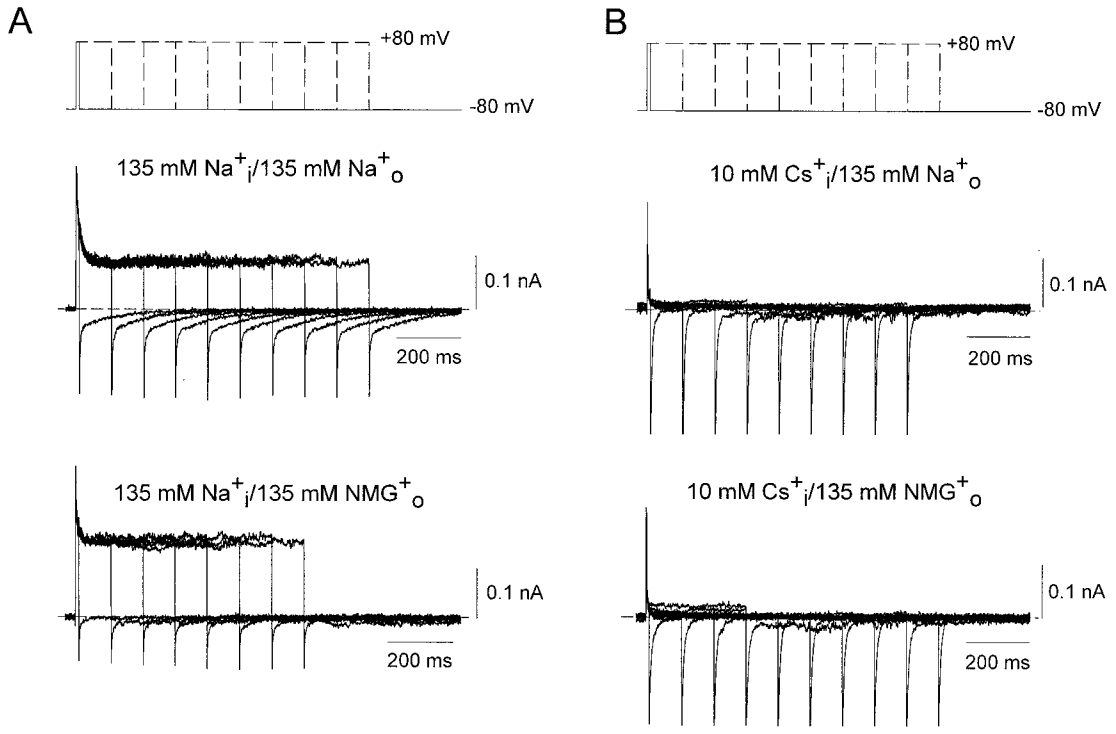


FIGURE 7 Effects of 135 mM Na^+_i or 10 mM Cs^+_i on Na^+ tail currents in NCM channels. (*A*) Pulse protocol is shown at the top, with an initial 10-ms depolarization and 100-ms increments. The pulses were applied at 0.2 Hz. *Middle*, membrane currents obtained using top panel protocol with symmetrical 135 mM Na^+ as indicated. *Bottom*, the same experiment as in the middle but with NMG^+_o to show the disappearance of Na^+ tail currents. Data in the middle and bottom were obtained from the same cell. (*B*) Pulse protocol as in *A* (*top*). *Middle*, experiment carried out on NCM channels with 10 mM Cs^+_i and 135 mM Na^+_o. Data show that with 10 mM Cs^+_i, the Na^+ tail currents were prevented, which is supported by the control experiment at the bottom with 10 mM Cs^+_i/135 mM NMG^+_o. Data shown at the middle and bottom were obtained from the same cell.

WT and NCM channels can enter the C-type inactivated state rapidly in the absence of univalent metal cations

Further changes in Na^+ conductance occur in both WT and NCM channels when longer depolarizations are applied to the cells in a 135 mM Na^+_o solution (Fig. 3). In WT channels very brief depolarizations of 1–2 ms do not allow all channels to enter the inactivated states, so that on repolarization a number of channels deactivate from the open state. The rapid tail current that results can be seen in Fig. 3 *B* (*arrows*), but in NCM channels only the slower tail is observed (Fig. 3 *C*). These slow tails develop in the inward direction in both types of channels, before decaying again slowly to the baseline. This suggests that both types of channels enter a fully developed C-type inactivated state, and transit through a higher Na^+-conductance state on repolarization, before deactivating to closed-inactivated states. It is clear that both WT and NCM channels enter the C-type state extremely rapidly on depolarization, and that extracellular Na^+ only slightly impedes this process.

Correlation between the voltage and time dependence of off-gating current immobilization and the appearance of sodium conductance in NCM channels

The appearance of Q_{off} immobilization with increasing prepulse potential is shown in Fig. 4 *A*. In NCM channels, on-gating currents appear during depolarizations positive to -70 mV and increase in amplitude with larger depolarizations, then decay more rapidly at positive potentials. When repolarized to -100 mV, off-gating currents reach a peak rapidly and decay monoexponentially after depolarizations to <-10 mV. After depolarizations to more positive potentials, the peak off-gating current is reduced and the time constant of relaxation of off-gating current (τ_{off}) slows dramatically as it develops a second phase of decay (see 0 mV tracing in Fig. 4 *A*). A clear threshold for off-gating current slowing occurs at ~ -10 mV, where the pore would normally be open for the channel to conduct ions (Fedida et al., 1993). This can be seen when individual records are plotted in the right panels of Fig. 4 *A*. When 135 mM Na^+_o

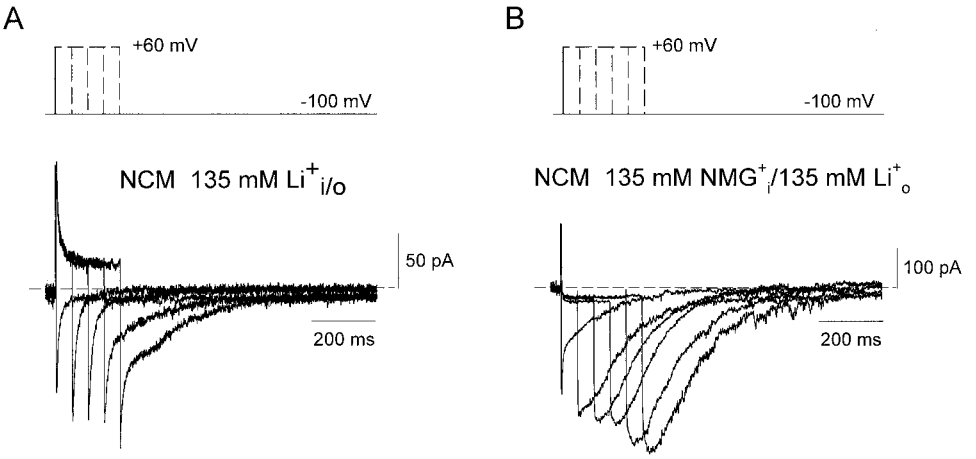


FIGURE 8 The appearance of Li^+ tail currents through NCM channels in symmetrical or extracellular Li^+. Pulse protocol is shown at the top of *A* and *B*. Depolarization was to +60 mV for 2 ms incremented by 50 ms for each pulse, applied at 0.2 Hz. (*A*, *below*), current data from protocol carried out on NCM channels with symmetrical Li^+. Data show that Li^+ tail currents are similar to Na^+ tail currents obtained with symmetrical Na^+ (Fig. 7 *A*, *middle*). (*B*, *bottom panel*) Experiment as in *A*, but with 135 mM NMG^+_i/135 mM Li^+_o. Li^+ tail currents show the same rising phase and the slow decay as the Na^+ tail (in Figs. 2 and 3) with longer depolarizations.

is substituted for NMG^+_o, as soon as potentials are reached where the channels open (Fig. 4 *B*), a sustained inward current is recorded. This is clearly seen after a rapid initial transient of returning gating current at −10 mV in the right panel. At more positive potentials, the fast transient of charge return disappears (+10 mV), and is replaced by a slower rising phase of current. This is accompanied by a sustained inward current that saturates in amplitude at ~+20 mV (Fig. 4, *B* and *D*). When the voltage dependence of this sustained current is plotted on an I-V relation (Fig. 4 *D*, $V_{1/2} = -11.3$ mV) and compared with the Q_{off}/Q_{on} ratio (Fig. 4 *C*, $V_{1/2} = -12.2$ mV) as an index of charge immobilization, it is clear that the increased inward Na^+ current on repolarization matches the voltage dependence of immobilized charge.

The timecourse of charge immobilization also matches the timecourse of increased Na^+ conductance (Fig. 5). In the presence of NMG^+_o, depolarizing pulses to +60 mV are given for increasing durations, starting at 0.5 ms, to create an envelope of on- and off-gating currents. The off-gating currents after the shortest depolarizations of 0.5 and 1.5 ms are fast and of significant amplitude, but at later times, including the tracing at 2.5 ms (Fig. 5 *B*, *right*), charge is largely immobilized on repolarization. Replacement of NMG^+ with 135 mM Na^+_o reveals an inward Na^+ current during the depolarizations after the on-gating current has decayed, and on repolarization a sustained inward current is present after the second depolarization. After the shortest depolarization, there is no sustained current, indicating that channels have either not opened or are not yet inactivated and consequently unable to conduct Na^+. A graph of the timecourse of charge immobilization (Fig. 5 *D*) closely matches the timecourse of the rise of Na^+ conductance (Fig. 5 *E*). In both cases lines through datapoints have been fitted with a time constant of 2.7 ms, illustrating the close correlation between the rapid establishment of C-type inactivation and charge immobilization.

Intracellular Cs^+ delays the onset of Na^+ conductance and reduces charge immobilization

The aims of these experiments are to show that Cs^+ can delay the onset of inactivation in both WT and NCM channels and reduce charge immobilization (Fig. 6). In Fig. 6 *A*, 10 mM Cs^+_i is included in the pipette solution, and a protocol similar to that used in Fig. 3 applied to cells expressing WT channels. From a holding potential of −100 mV, WT channels are pulsed to +80 mV for increasing durations from 1 ms to 400 ms (Fig. 6 *A*). As before, the shortest depolarization opens few channels, and those that open do not inactivate, so a predominantly rapid tail current is observed on repolarization. During longer depolarizations, a decaying outward Cs^+ current is observed. The outward current and the rapid tail current decline in amplitude over an identical timecourse, inversely correlated with the rising amplitude of the slow Na^+ tail current. These inward current changes on repolarization are similar to those seen when no Cs^+_i was present, but they occur over a much longer time scale (compare with Fig. 3 *B*). The rising phase of the slow Na^+ tail current so clearly seen in Fig. 3 is much reduced, and only becomes apparent after 200-ms or longer depolarizations in Fig. 6 *A*. The result shows that the presence of 10 mM Cs^+_i is not enough to block the inward Na^+ tail current, but the onset of inactivation, indexed by outward current decay, the decrease in rapid tail

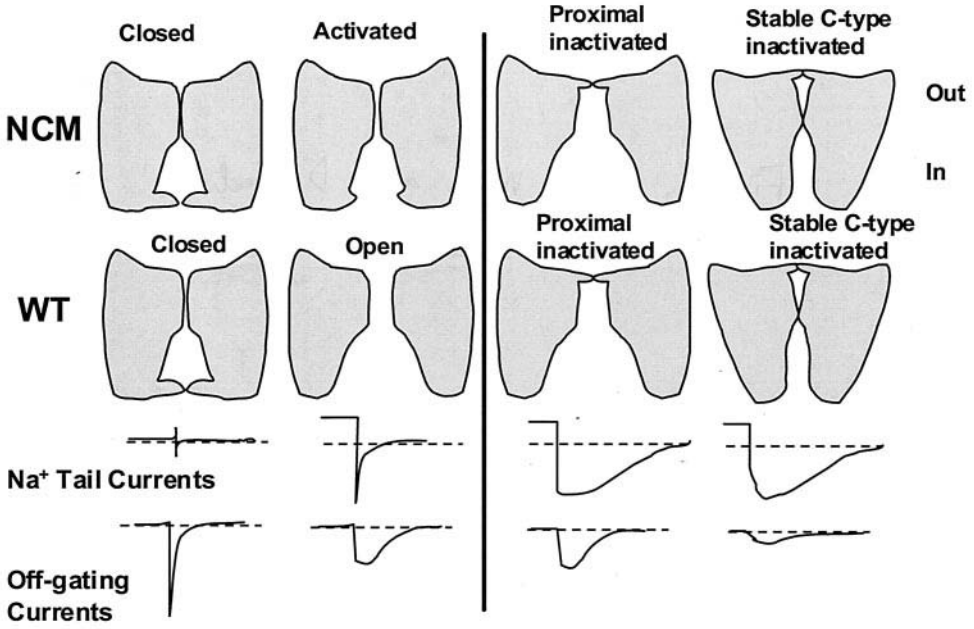


FIGURE 9 Summary of proposed conformations of Kv1.5 and Kv1.5-W472F (NCM) during inactivation determined from gating currents and Na^+ conduction studies. The vertical line denotes limit of transitions reachable from the left in the presence of 10 mM Cs^+_i/135 mM Na^+_o. Note that Na^+ tail currents through the open state are seen only in WT channels.

amplitude, and the development of the slow Na^+ tail, is greatly slowed.

When 5 or 10 mM Cs^+_i was included in the pipette solution, and recordings were made from NCM channels, the immobilization of off-gating charge was much reduced. In Fig. 6 *B* after pulse durations of 3 ms or longer the fast return of off-gating charge is lost, but slowing and immobilization of charge is much less than that observed without Cs^+_i (Fig. 5 *B*). With 5 mM Cs^+_i the Na^+ conductance still develops but to a lesser degree, and more slowly compared with Fig. 5 *C* (without Cs^+_i). In the presence of 10 mM Cs^+_i and extracellular Na^+ (Fig. 6 *D*), the overall Q_{off}/Q_{on} ratio is preserved at 1.1 ± 0.1 ($n = 8$ cells). A charge ratio >1.0 indicates that a small amount of Na^+ tail is still present when 10 mM Cs^+_i is included, although this does not result in the sustained inward current observed in 5 mM, or the absence of Cs^+_i (Fig. 5 *C*). The charge ratio is subsequently maintained at this level for all subsequent depolarizations. Not only is the timecourse of immobilization slowed, but also the voltage-dependent immobilization of charge return is diminished (Fig. 6, *C* and *E*). After depolarizations to 0 mV and more positive potentials, the slowing of off-gating charge return is much attenuated compared with NMG^+_i alone (Fig. 4 *A*). There seems to be a concentration-dependent effect of Cs^+_i, as 5 mM still allows some Na^+ conductance (Fig. 6 *C*), whereas 10 mM prevents charge immobilization completely, the overall Q_{off}/Q_{on} ratio is 1.12 ± 0.05 ($n = 8$ cells), and little or no Na^+ conductance is seen (Fig. 6 *E*).

The data suggest that inclusion of a small amount of internal cation, Cs^+_i in this case, can delay or even prevent the transition of channels into inactivated states. This is indicated by the delay or failure of the Na^+ tail currents to appear on repolarization, and by a concentration-dependent prevention of off-gating charge immobilization. These results also indicate a difference in behavior between the two channel forms. In the presence of 10 mM Cs^+_i, the WT channel still shows significant Na^+ conductance on repolarization, even at the shortest intervals tested in Fig. 6 *A*. In contrast, NCM channels fail to show significant Na^+ conductance in the presence of 10 mM Cs^+_i (Fig. 6, *D* and *E*).

This difference in behavior between NCM and WT channels is confirmed by two experiments on NCM channels shown in Fig. 7. In the presence of symmetrical 135 mM Na^+_i/Na^+_o, the protocol of Fig. 6 *A* is repeated in which the cell is depolarized for increasing lengths of time. In the presence of intra- and extracellular Na^+, an outward Na^+ current is seen, and the tails on repolarization decay slowly but have no rising phase. The data indicate that the channels are trapped in an early or proximal inactivated state (Scheme I), in that they are Na^+ permeant in both the outward and inward directions. However, the tails lack a rising phase, and this does not develop over time, which indicates that under these experimental conditions they are unable to progress to the distal C-type state. The data show that NCM channels can occupy the proximal state, and also illustrate the nature of the Na^+ tail currents in this state. The control experiment is shown in the bottom panel of Fig. 7 *A*

where the Na^+ tail current is abolished when Na_o^+ is omitted, demonstrating its Na^+ dependence. When 10 mM Cs_i^+ is included in the pipette solution instead of Na_i^+ (Fig. 7 *B*) the slowly decaying Na^+ tail current is abolished, and tail currents (capacitance artifacts and gating currents only) are identical in the presence (*middle panel*) or absence (*lower panel*) of Na_o^+. The results in this figure suggest that 10 mM Cs_i^+ prevents the entry of NCM channels into either the proximal or stable C-type inactivated states, unlike its action on WT channels (Fig. 6).

Li^+ shows a similar permeation pattern to Na^+ through NCM channels

We were interested to discover whether other ions could permeate the inactivated states of Kv1.5 as they can through *Shaker* channels (Starkus et al., 1998), and how they modulate the movement of NCM channels into the two inactivated states that we have observed in the presence of Na^+. Li^+ data are shown in Fig. 8. In Fig. 8 *A*, currents were obtained in symmetrical Li^+ conditions, and can be compared with the Na^+ experiment in Fig. 7 *A*. Depolarizing pulses starting at 2 ms and increasing in duration reveal outward Li^+ current and slowly decaying tails on repolarization at all pulse durations. The data show the channels staying in proximal inactivated states. In Fig. 8 *B*, only extracellular Li^+ is present and the experiment is repeated. Here, only the tail current after the briefest depolarization looks like those observed in Fig. 8 *A*. After longer depolarizations, tails progressively adopt a rising phase before the slow decay to the baseline. Under these conditions Li^+ tail currents adopt the rising phase and slow decay of Na^+ tail currents under similar conditions (Figs. 2 and 3).

DISCUSSION

Rapid onset inactivation in Kv1.5 in the absence of univalent metal cations

A number of experimental paradigms used in this study have clearly demonstrated the rapid onset of an inactivation process in both WT and NCM Kv1.5 channels. The −40 mV negative shift in the voltage dependence of charge return described in NCM channels after 12-ms pulses (Fig. 1) is reminiscent of the much slower changes in *Shaker* channels expressed in oocytes (Olcese et al., 1997). Using increased Na^+ or Li^+ conductance as an index of channel inactivation (Figs. 3 and 8), rapid inactivation is established after depolarizations as short as 2 ms (Fig. 3) in both NCM and WT channels in the absence of univalent cations. At the end of a depolarization long enough to move all the on-gating charge, the development of the inward Na^+ current is complete, suggesting that only a few milliseconds are required to move almost all channels into a stable C-type inactivated state (Fig. 5 *C*). The rapidity with which these changes occur compared with *Shaker* channels expressed in oocytes might be related to the channels themselves or the expression system. However, we note that charge immobilization in excised patches from oocytes can apparently be as fast as we have observed in HEK cells (McCormack et al., 1994).

Both the voltage dependence and timecourse of charge immobilization closely match the appearance of the inward Na^+ conductance and establish an association between charge immobilization and the stable C-type inactivated state (Figs. 4, *C* and *D*, 5, *D* and *E*). In NCM channels, the rapidity of these events and the close correlation of charge immobilization and Na^+ conductance may reflect the transition from one inactivated state to a deeper C-type state, because these channels are thought to be already inactivated (Yang et al., 1997). Still, most of the events are identical and just as rapid in WT channels (Figs. 2, 3, and 6). The main difference observed between the two types of channel are the fast rising and rapidly decaying Na^+ currents through normally deactivating or open WT channels described in Fig. 3 *B*. Taken together, the data provide good evidence that the inactivation process proceeds extremely rapidly in both WT and NCM channels on depolarization when only Na_o^+ is present, and the charge immobilization that occurs is directly caused by the occupancy of a stable C-type inactivated state.

The NCM channel is inactivated at depolarized potentials

NCM channels have generally been thought to exhibit very similar voltage-dependent gating behavior to WT channels, but with the advantage that ion conduction through the open state can be prevented (Perozo et al., 1993), which makes them effective tools for the study of gating currents. When NMG_i^+/135 mM Na_o^+ solution was used, we observed inward Na^+ currents as has been reported for C-type inactivated WT and W434F *Shaker* mutant channels (Starkus et al., 1997, 1998). The activation of currents during depolarizing prepulses was slow (Fig. 2 *C*), and the tail currents on repolarization were stable once channels were fully activated (Figs. 3 *C*, 5 *C*). This suggests that NCM channels either inactivate very rapidly on depolarization (Starkus et al., 1998) or are permanently inactivated (Yang et al., 1997).

One important but expected difference between NCM and WT channels was illustrated in Fig. 3. In the data in Fig. 3 *B*, a brief but rapid initial spike of tail current was present in WT channels before the onset of the slow tail (*arrow*) that have been described previously (Starkus et al., 1997; Wang et al., 2000). This indicates Na^+ conduction through WT channels that did not have time to inactivate during the 2-ms depolarization. The rapid tail is absent after depolarizations longer than 50 ms. No such tail is observed in NCM channels (Fig. 3 *C*) after short depolarizations, which shows that it is functionally unable to conduct ions in the activated

conformation. Again, this might be because the NCM inactivates even more rapidly than WT channels, or because it is permanently inactivated in some manner.

It has been previously questioned how a time-dependent shift of the voltage dependence of charge movement observed in W434F (Olcese et al., 1997) and the present experiments can reflect inactivation if the channel is permanently inactivated (Yang et al., 1997). We note that if channels are permanently inactivated, it is also not clear why off-gating currents after small depolarizations are fast, and not shifted to more negative potentials, or why on-gating currents are not slowed if activation of inactivated channels is slow (Starkus et al., 1998). One piece of evidence suggests that NCM channels are not permanently in closed-inactivated states although repolarized. Na^+ tail currents through both the WT and NCM channels decrease in amplitude if pulses are given at 0.2 Hz (Fig. 2, *B* and *C*). This result was expected in WT channels as we have previously shown, along with others (Starkus et al., 1997), that the slow decay of the Na^+ tail current during a single pulse represents irreversible deactivation to closed-inactivated states (Wang et al., 2000). The sole recovery pathway back to normal closed states for WT Kv1.5 channels is a slow recovery from closed-inactivated to closed states with a time constant of ~4 s. The progressive decrease in tail current amplitude is caused by the trapping of channels in closed-inactivated states so that they are unavailable on subsequent depolarization. The same thing happens to NCM channels (Fig. 2 C) and indicates that they probably follow the same recovery pathway as WT channels, and eventually recover from closed-inactivated states to normal closed states.

Two inactivated states in the NCM and WT channels

Our data can be best interpreted in terms of more than one inactivated state as indicated by the summary in Fig. 9, which is informed by the studies of Loots and Isacoff (1998), and others' suggestions of multiple inactivated states in the inactivation pathway (Yang et al., 1997; Olcese et al., 1997; Kiss et al., 1999). Once activated, the NCM channel resides in a proximal inactivated state where changes in the outer pore conformation do not allow K^+ permeation, but do allow significant Na^+ or Li^+ conductance. Within a few milliseconds of depolarization, channels progress to a distal state that is energetically more stable, and from which return of gating charge is slowed. This distal, more stable C-type inactivated state has a lower Na^+ or Li^+ conductance than the proximal inactivated state (through which the channel may return during recovery from inactivation). The existence of these conformations can be deduced from the effects of intracellular Na^+ or Li^+ (Figs. 7 and 8), and internal Cs_i^+ on tail currents. Slow tail currents after depolarization in the presence of both intra- and extracellular Na^+ or Li^+ in the NCM, or after short depolarizations in WT channels in the presence of Cs_i^+ (Fig. 6 *A*), lack a significant rising phase. This indicates occupation of a higher Na^+ conductance state earlier in the inactivation pathway before the channel progresses to the lower Na^+ conductance, stable C-type state. The exit from the earlier, higher Na^+ conductance state is too rapid to be observed when only external Na^+ is present (Fig. 3). WT channels gate through a very similar pathway to NCM channels except for the addition of an open state through which the channel may conduct ions, seen in our data as rapid Na^+ tails on repolarization after brief depolarizations (Fig. 3 *B*).

Role of univalent cations in the prevention of charge immobilization

We have previously postulated that much of the slowing of off-gating charge return on repolarization, in the absence of univalent metal cations, is caused either by an allosteric modulation of K^+ channel deactivation, or an accelerated process of C-type inactivation (Chen et al., 1997). This statement was based on the observation that metal cations, and particularly Cs^+, are able to completely prevent this slow charge immobilization. Subsequently, we have found that the action of Cs^+ can be mediated by its inclusion in the intracellular or extracellular medium (Wang et al., 1999), and that the prevention of charge slowing by intracellular Cs^+ has a 50% effective concentration (EC_{50}) of 20 mM. None of the previous experiments was able to conclusively demonstrate whether the cations were modulating deactivation, preventing inactivation, or both. In these experiments, we have combined the Cs_i^+ experiment with studies of the slow Na^+ tail currents. Our initial observation was that inclusion of 135 mM Cs_i^+ reverses the voltage-dependent shift in off-gating charge characteristic of inactivation (Fig. 1, *B* and *D*). It is also of note that on-gating charge is unaffected, so the effect on off-gating charge cannot be attributed to a charge screening action of the cations different from that of NMG^+. This alone suggests an ability of Cs_i^+ to impede inactivation.

For Na^+ tail experiments we lowered internal Cs^+ to 5 or 10 mM to prevent blockage of the inwardly directed Na^+ tail. Data from WT channels (Fig. 6 *A*) show that the slow tail is not blocked, but its onset is greatly delayed. The fast component of the tail, representing Na^+ conduction through channels deactivating from the open state to normal closed states, persists for >200 ms. Its decay coincides with the decrease of outward Cs^+ current (macroscopic inactivation) and the rise of the slower Na^+ tail. The slower Na^+ tail lacks an initial rising phase until the rapidly decaying tail has disappeared. This suggests that Kv1.5 channels are retained in a proximal inactivated state for much longer under the Cs_i^+ conditions, but they can eventually progress to the stable C-type state. The experiment also clearly

indicates that small concentrations of intracellular Cs^+ do not block inward Na^+ tail currents in Kv1.5 channels. Under similar ionic conditions, in NCM channels, the time-course of charge immobilization is greatly slowed (Fig. 6, *B* and *D*) compared with when Cs^+_i is absent (Fig. 5), and the amount of charge immobilization is significantly reduced. There is little or no Na^+ inward tail current for more than the 20-ms duration of the experiments in Fig. 6 *D* (indicated by the $Q_{off}/Q_{on} \sim 1.0$), which suggests that in NCM channels inactivating transitions are largely prevented by 10 mM Cs^+_i. This is confirmed by data in Figs. 7 and 8. NCM channels are able to occupy the proximal inactivated state in the presence of symmetrical Na^+ or Li^+, as evidenced by the slowly decaying tail currents without a rising phase (Figs. 7 *A* and 8 *A*). However, these Na^+ tail currents are absent with Cs^+_i/Na^+_o (Fig. 7 *B*). The voltage-dependent charge immobilization in Fig. 6, *C* and *E*, is also much reduced with 5 or 10 mM Cs^+_i. The lack of significant Na^+ conductance for the duration of the experiments in Fig. 6, *D* and *E*, at 10 mM Cs^+_i suggests that the slowing of off-gating current that remains in this situation reflects reversal of the physiological slowing of the concerted opening transition, as has been suggested by many others (Zagotta et al., 1994a; Bezanilla et al., 1994). This finding also implies that NCM channels are not necessarily permanently inactivated. They do seem to rest in normal closed states (Fig. 2), and they can occupy activated states on depolarization that do not pass any cations.

Because 10 mM Cs^+_i virtually prevents the Na^+ tail currents (but does not block them, Fig. 6 *A*), it seems that low concentrations can substantially prevent both inactivation transitions. High concentrations of Cs^+_i (135 mM, Fig. 1) prevent any charge slowing on repolarization (Wang et al., 1999), and this is caused by an acceleration of the channel-closing transition. This seems to be a unique action of Cs^+ among univalent cations, as we have previously shown that other univalent cations, including K^+, are unable to accelerate charge return to this extent (Wang et al., 1999). Interestingly, this property of Cs^+ is shared by the divalent cation, Ba^{2+} (Hurst et al., 1996).

CONCLUSION

The data indicate that stable C-type inactivation in the Kv1.5 channel is associated with prominent charge immobilization. There is a large shift in the voltage dependence of recovery of gating charge on repolarization and parallel changes in both the voltage and time dependence of charge immobilization with the development of Na^+ conductance on repolarization through C-type inactivated channels. The WT and NCM channels both gate in a very similar manner during inactivation to a stable C-type inactivated state and during the recovery from inactivation. Extracellular Na^+ is not able to inhibit the development of the inactivation process in a significant way, so changes in the Na^+ conductance can be used to track the presence of inactivated channels. A concentration of 10 mM of Cs^+_i can slow the development of charge immobilization, and either delay the appearance of Na^+ tail currents in WT channels, or prevent them in NCM channels. The NCM, but not the WT, channel occupies a proximal, inactivated state as soon as it is activated. This state is nonpermeant to K^+ but permeant to Na^+. With sustained depolarizations, channels then move rapidly in the absence of univalent metal cations, and even in the presence of extracellular Na^+ to a deeper, more stable C-type inactivated state that is associated with prominent charge immobilization. The transitions to inactivated states in the NCM can be prevented by small amounts of intracellular Cs^+, which in turn prevents charge immobilization.

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