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External Pore Collapse as an Inactivation Mechanism for Kv4.3 K^+ Channels

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Abstract. Kv4 channels are thought to lack a C-type inactivation mechanism (collapse of the external pore) and to inactivate as a result of a concerted action of cytoplasmic regions of the channel. To investigate whether Kv4 channels have outer pore conformational changes during the inactivation process, the inactivation properties of Kv4.3 were characterized in 0 mM and in 2 mM external K^+ in whole-cell voltage-clamp experiments. Removal of external K^+ increased the inactivation rates and favored cumulative inactivation by repetitive stimulation. The reduction in current amplitude during repetitive stimulation and the faster inactivation rates in 0 mM external K^+ were not due to changes in the voltage dependence of channel opening or to internal K^+ depletion. The extent of the collapse of the K^+ conductance upon removal of external K^+ was more pronounced in NMG^+-than in Na^+-containing solutions. The reduction in the current amplitude during cumulative inactivation by repetitive stimulation is not associated with kinetic changes, suggesting that it is due to a diminished number of functional channels with unchanged gating properties. These observations meet the criteria for a typical C-type inactivation, as removal of external K^+ destabilizes the conducting state, leading to the collapse of the pore. A tentative model is presented, in which K^+ bound to high-affinity K^+-binding sites in the selectivity filter destabilizes an outer neighboring K^+ modulatory site that is saturated at ~2 mM external K^+. We conclude that Kv4 channels have a C-type inactivation mechanism and that previously reported alterations in the inactivation rates after N- and C-termini mutagenesis may arise from secondary changes in the electrostatic interactions between K^+-binding sites in the selectivity filter and the neighboring K^+-modulatory site, that would result in changes in its K^+ occupancy.

Key words: K channel — Inactivation — Kv4 — Kv4.3 — C-inactivation — Ito — Channel inactivation

Introduction

K^+ channel activity is modulated by external and internal K^+ ions. This general property of K^+ channels was early observed by Almers and Armstrong (1980) in the squid giant axon K^+ channels. K^+ channels lost their function when K^+ was eliminated from both sides of the membrane. It was concluded that K^+ channels were normally occupied by K^+ and/or other small monovalent cations and that they become nonfunctional after the removal of internal and external K^+ ions (Almers & Armstrong, 1980). These initial observations were the basis for understanding the mechanism(s) of action of K^+ ions and other cations in several classes of K^+ channels (Pardo et al., 1992; Lopez-Barneo et al., 1993; Yellen et al., 1994; Baukrowitz & Yellen, 1995; Liu, Jurman & Yellen, 1996). Since the effect of removing external K^+ depends on the K^+ affinity of the K^+ channel site, some channels can function properly in "zero" mM external K^+ (contaminant K^+ ~10 μM) due to their high affinity to K^+. This is the case for the large-conductance, voltage-dependent and Ca^{2+}-activated K^+ (MaxiK, BK) channels. In this channel, reducing further the external K^+ concentration with a K^+ chelator ((+)-18-Crown-6-tetracarboxylic

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acid), causes them to enter a long-lasting nonconductive state (Vergara et al., 1999).

Shaker K^+ channels inactivate through two distinct molecular mechanisms, N- and C-type inactivation. N-type inactivation involves the N-terminal domain with the already classical "ball and chain" mechanism, while C-type inactivation is due to structural modifications of the external mouth of the pore (Armstrong & Bezanilla, 1977; Zagotta et al., 1990; Hoshi et al., 1990; Hoshi, Zagotta & Aldrich, 1991; Yellen et al., 1994; Panyi, Zheng & Deutsch, 1995; Liu et al., 1996; Basso et al., 1998; Loots & Isacoff, 1998; Zhou et al., 2001). The following observations in Shaker type (Kv1) K^+ channels suggest that external K^+ and small cations modulate C-type inactivation and channel recovery from the inactivated state: 1. External tetraethylammonium (TEA^+) reduces C-type inactivation (Grissmer & Cahalan, 1989*a*; Choi, Aldrich & Yellen, 1991). 2. C-type inactivation rate depends on external K^+; decreasing K^+ accelerates inactivation, suggesting that K^+ ions occupying the outer region of the pore prevent its collapse and compete with the inactivation process. The relative potency of external cations in accelerating C-type inactivation is $NMG^+ > Na^+ \sim Cs^+ \sim$, $NH4^+ > K^+ \sim Rb^+$ (Pardo et al., 1992; Lopez-Barneo et al., 1993). 3. Internal K^+ channel blockers increase C-type inactivation rate, as they prevent outward K^+ flux (Choi et al., 1991; Baukrowitz & Yellen, 1995; Baukrowitz & Yellen, 1996). 4. Increasing external K^+ speeds recovery from C-type inactivation via a voltage-dependent binding of K^+ to an extracellular site (Levy & Deutsch, 1996). Thus, as a general mechanism, the inactivation time constant of C-type inactivation depends on the occupancy by K^+ of an external modulatory site. In other words, K^+ channels need external K^+ to function properly, and removal of external K^+ favors C-type inactivation by promoting a constriction or partial collapse of the external region of the pore (Yellen, 1998).

More recently, it has been demonstrated that the occupancy of the external modulatory site does not only depend on the external K^+ concentration and/or K^+ flux, but also on the K^+ affinity of a site within the channel pore. A decrease in the affinity of a site within the channel pore would reduce electrostatic interactions between the K^+ ions in the pore and in the external modulatory site and, as a consequence, the occupancy of the modulatory site would increase, slowing down C-type inactivation. Conversely, an increase in K^+ affinity of the site within the pore will have opposite affects, destabilizing the occupancy of the modulatory site and favoring C-type inactivation (Ogielska & Aldrich, 1999).

The role of external K^+ has been extensively studied in Shaker-type K^+ channels; however, a detailed study on the effect of removing external K^+ on Kv4 channels is lacking. Kv4 channels are one of the molecular components of the outward K^+ currents that shape the early repolarization phase of the action potential (commonly referred to as "A-type" or "Ito" current, (Serodio & Rudy, 1998; Isbrandt et al., 2000). The rate of inactivation is one of the important intrinsic properties of Kv4 channels controlling the action potential shape. Kv4 inactivation can be described with two or three components of decay and the predominant mechanism seems to be the population of inactivation from pre-open closed states (Jerng & Covarrubias, 1997; Jerng, Shahidullah & Covarrubias, 1999; Bähring et al., 2001; Beck & Covarrubias, 2001). In Kv4 channels, an increase of the external K^+ concentration produces an acceleration of their inactivation process (Jerng & Covarrubias, 1997; Bähring et al., 2001; Eghbali et al., 2001). This is in contrast with C-type inactivation in Shaker K^+ channels, in which raising external K^+ causes a reduction in the rate of inactivation, likely due to an increased occupancy of an external modulatory K^+ site (Lopez-Barneo et al., 1993). In addition, the recovery from inactivation in Kv4 channels is slower in high external K^+, which is also opposite to what is found in Kv1 channels where the recovery of C-type inactivation is faster in high external K^+ (Levy & Deutsch, 1996; Jerng & Covarrubias, 1997).

In Kv4 channels, molecular maneuvers to examine its inactivation mechanism show that an N-terminus deletion removes or greatly slows down the fast component of inactivation ($\tau = 16$ ms) without affecting a slower inactivating component ($\tau \sim 1000$ ms) associated with closed state inactivation. In addition, mutations in the inner vestibule of the Kv4 pore drastically slowed inactivation rates and reduced Kv4 4-aminopyridine blockade. Based on all the above results in Kv4 channels, it has been proposed that Kv4 channels lack typical N- and C-type inactivation as described for Shaker K^+ channels, but possess an inactivation mechanism that may involve voltage-associated conformational changes of the internal vestibule in a concerted action involving N- and C-termini (Jerng & Covarrubias, 1997; Jerng et al., 1999; Bähring et al., 2001). Nevertheless, removing external K^+ using Na^{2+} or NMG^+ as the main cations, a landmark maneuver to investigate C-type inactivation, has not been addressed for Kv4 channels (Yellen, 1998). In this work we now characterize the inactivation process of Kv4.3 channels upon removal of external K^+. We found that external K^+ removal increased the inactivation rate and facilitated cumulative inactivation by repetitive stimulation. This effect was more pronounced when external Na^+ was replaced by NMG^+. The faster inactivation rate and current reduction during cumulative inactivation by repetitive stimulation occurred without changes of the voltage dependency of channel opening or of the internal K^+ concentration.

These observations meet the criteria for a typical C-type inactivation, as removal of external K^+ destabilizes the conducting state, leading to the collapse of the pore. A tentative model is presented, in which K^+ bound to high-affinity K^+-binding sites in the selectivity filter destabilizes an outer neighboring K^+ modulatory that is saturated at 2 mM K^+. The extent of inactivation would depend on the K^+ occupancy of the modulatory site. In this model, high affinity K^+ binding sites in the pore can hold K^+ when the channel is in the closed state, thus destabilizing the modulatory site and allowing inactivation from pre-open closed states as shown for Kv4 channels (Jerng et al., 1999). We conclude that Kv4 channels have a C-type inactivation mechanism and that previously reported alterations in the inactivation rates after N- and C-termini mutagenesis may arise from long-range effects on the electrostatic interactions between K^+ binding sites in the selectivity filter and the neighboring K^+ modulatory site, which would result in changes in its K^+ occupancy and Kv4.3 inactivation rates.

Materials and Methods

HEK293 Cells Stably Expressing KV4.3 Channels

A stable HEK293 (human embryonic kidney) cell line with robust expression of Kv4.3 channel long form was developed. HEK293 cells maintained in Dulbecco's Modified Eagle Medium with L-Glutamine (supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, GibcoBRL) were transfected with the Kv4.3 long form in pcDNA3 using the calcium phosphate method (Graham & Van der, 1973; Song et al., 2001). Two days after transfection, the cells were washed and placed in medium containing 0.4 mg/ml G418 (Geneticin, a neomycin analogue). Three to four weeks after transfection, individual colonies were harvested and transferred to 96-well dishes. Two hundred and fifty colonies were screened for Kv4.3 expression using immunocytochemistry. The cell colony with the highest membrane labelling was selected for this study.

HEK293-Kv4.3-expressing cells were usually split once a week, plated on glass coverslips precoated with CELL-TAK (Becton Dickinson), and maintained in a 37°C incubator with 5% CO_2. In early experiments, electrophysiological recordings were performed 2–4 days after plating. Under this condition, the majority of the cells were forming confluent cumuli, and the measurements were performed in the remaining single cells. Current density and stability in these isolated cells was variable. We increased our experimental success by mechanically dissociating confluent cultures 2–4 days after plating cells. Cells were mechanically dissociated in PBS (mM): 10 Na_2HPO_4, 2.3 NaH_2PO_4, 138 NaCl, and 2.7 KCl, pH 7.4, and directly plated on to the experimental glass chamber. Healthy cells attached 2–5 min after plating and gave robust Kv4.3 currents.

Electrophysiology

Patch clamp was used in the whole-cell configuration at room temperature (20–23°C) using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled from borosilicate glass and fire-polished. The pipette solution contained (mM): 134 K-Methanesulphonate (K-MES), 6 KCl, 10 HEPES and 10 EGTA. The normal bath solution contained: 140 Na-MES, 2 KCl, 2 $CaCl_2$, 10 Glucose, 10 HEPES. Solutions without added K^+ (0 mM external K^+) had the same composition but without KCl and with Na^+ or N-Methyl-D-glucamine (NMG^+) as the main cations. The level of K^+ contamination in 0 mM K^+ solutions is ~10 μM (Vergara et al., 1999). All solutions were titrated to pH 7.35. The volume of the recording chamber was 0.3 ml and solution replacement was achieved in 30–60 sec. Measurements in 0 mM external K^+ solutions were performed under continuous flow of 1 ml/min.

Under whole-cell clamp configuration, cell passive properties were measured at 0 mV holding potential (HP) with 20-mV pulses. Typically, cells had an input capacity of 20 ± 3 (n = 8) pF with capacity transient time constants of 40–60 μsec. The calculated access resistance values of 2–3 MΩ agreed with the range of patch electrode resistance values. Access resistance was analogically compensated to about 70% that gave a 50–100 μsec clamp settling time. Tail currents were measured ~0.5 msec after the end of the test pulse. Membrane linear components were digitally subtracted using negative (20–30 mV) scaled constant pulses from −90 mV HP. Data were acquired with a PC44 board (Innovative Technologies, Moorpark, CA) that interfaced with an IBM compatible computer. The data were sampled at 10 kHz and filtered at 1/5 of the sampling frequency. The acquisition and analysis programs were developed in the laboratory.

Results

Removal of External K^+ Increases the Inactivation Rate

To investigate whether Kv4.3 channels undergo C-type inactivation, we studied the effect of removing external K^+ (to ~10 μM contaminant K^+, and referred to as 0 mM K^+) on the inactivation properties of Kv4.3 channels. Outward K^+ currents elicited to + 50 mV from a −90 mV holding potential (HP) were first recorded in 2 mM external K^+ (Fig. 1*A*, black trace). In this condition, K^+ currents were very stable during repetitive stimulation for long periods (140-msec pulses to + 50 mV every 4–10 sec, *see* Fig. 1*D*, •). When the external solution was changed to 0 mM K^+ solution with Na^+ as the main action, the inactivation rate became faster and, as expected for the increase in the K^+ driving force, the current peak amplitude increased (Fig. 1*A*, red trace).

Kv4 channel inactivation has been described by the sum of two or three exponential components (*see* Fig. 2) (Jerng & Covarrubias, 1997; Beck & Covarrubias, 2001). In this work, for comparative purposes, an estimate of the speed of the inactivation process was obtained by fitting the first 50 msec of the decaying currents to a single exponential. Figure 1*A* shows two traces with superimposed fits (thick lines) in 2 mM K^+ (black) and in 0 mM K^+ (red). The inactivation rate increased from 27 ± 3.2 sec^{-1} in

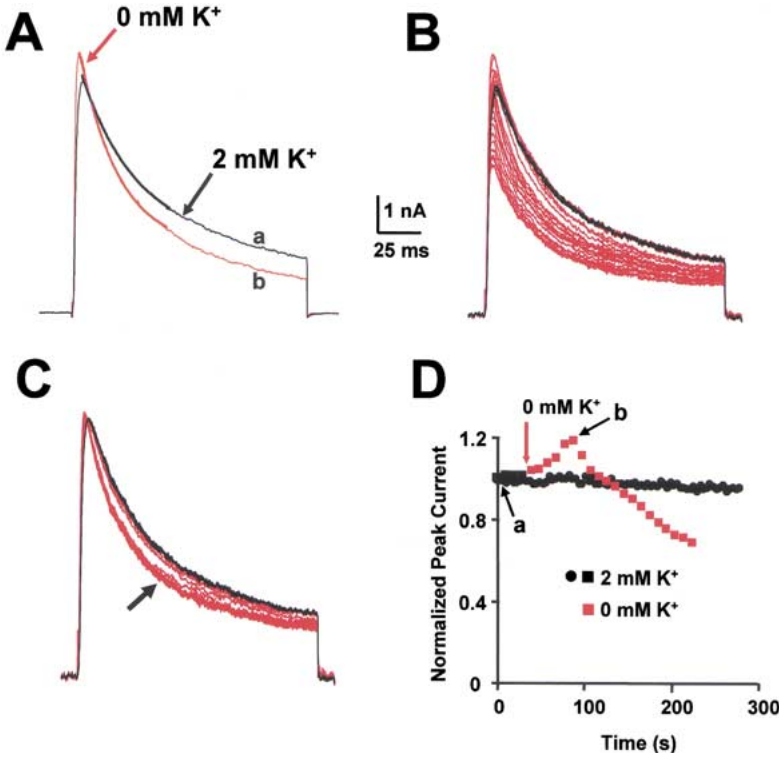

Fig. 1. Removal of external K^+ increases the inactivation rate and favors cumulative inactivation by repetitive stimulation. (*A*) Superimposed current traces during 140-msec depolarization pulses from −90 mV HP to +50 mV in 2 mM (black traces) and 0 mM external K^+ (red traces). The first 50 msec of the current decay were fitted with a single exponential; corresponding fits are shown as thick lines. Traces *a* and *b* were recorded at the times marked with arrows *a* and *b* in panel 1*D*. (*B*) Twentyfive superimposed traces recorded during 140-msec depolarization steps from −90 mV HP to 50 mV with 0.1/sec pulse stimulation frequency in 2 mM external K^+ (black traces) and after perfusion of 0 mM external K^+ (red traces). (*C*) The traces in *B* are scaled to the maximum peak current and are superimposed. (*D*) Peak currents in *B* were normalized to the peak current at time zero in 2 mM external K^+ and plotted as a function of time with 0.1/sec pulse stimulation frequency; black squares in 2 mM K^+ and red squares after replacement of external K^+ with Na^+. The red arrow shows when the solution was changed to 0 mM K^+. Black circles show peak current amplitude in another cell during repetitive stimulation in the presence of 2 mM K^+. Note the stability of the current in 2 mM external K^+ even at a higher stimulation frequency (0.25/sec).

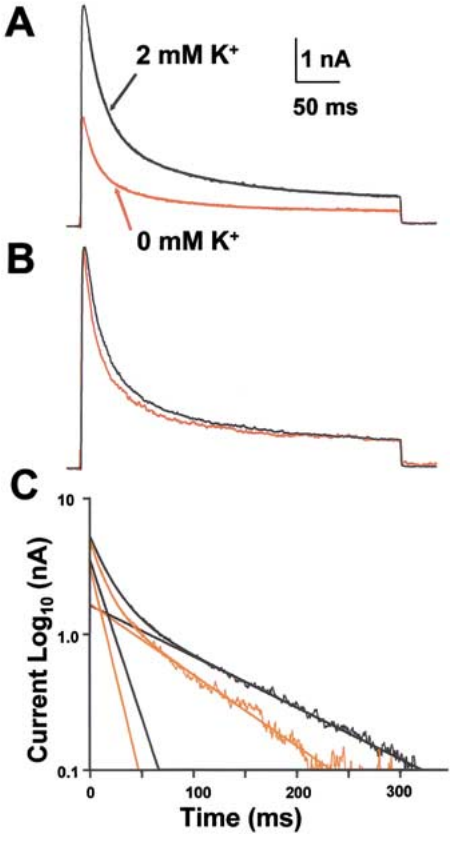

Fig. 2. Both slow and fast components of inactivation become faster upon removal of external K^+. (*A*) Superimposed current traces during 350-msec pulses from −90 mV HP to +50 mV in 2 mM (black traces) and 0 mM external K^+ with Na^+ (red traces). The decay phase of the current was fitted with a sum of two exponential functions, which are shown as thick lines. (*B*) Currents scaled to the peak value. (*C*) The scaled current traces in *B* are shown in logarithmic scale, with the corresponding fits (thick lines), including the fast and slow components, individually shown. Both fast and slow components became faster after removal of external K^+.

2 mM K^+ to 38 ± 1.7 sec^{-1} in 0 mM K^+ solution (n = 5, $p < 0.01$, *see* Fig. 7*F*). The faster inactivation rate in 0 mM external K^+ was maintained throughout the experiment (*see* Fig. 7*E*).

Progressive Reduction of the Peak Current Amplitude during Repetitive Stimulation in 0 mM External K^+

Figure 1*B* shows superimposed current traces evoked with pulses from −90 mV to +50 mV delivered every 10 sec in 2 (black) and 0 (red) mM external K^+. The traces in 2 mM K^+ were indistinguishable. In contrast, after switching to 0 mM external K^+ (with Na^+ as the main action), the peak current initially increased reaching a maximum in about 50 sec, and thereafter, progressively decreased (Fig, 1*B* red traces, Fig. 1*D* red squares). The slow time course in current increase after K^+ removal is likely due to the diffusion time of solution exchange (Fig. 1*D* red squares). In order to compare the time course of inactivation during repetitive pulses, the traces in 2 mM K^+ (black) and in 0 mM external K^+ (red) were scaled to the maximum peak current. Scaled traces after solution equilibration illustrate the faster inactivation rate after external K^+ removal (Fig. 1*C*, arrow). Peak current amplitudes as a function of time during repetitive stimulation are shown in Fig. 1*D*. After the initial increase in the peak current amplitude (arrow *b*) following the solution change (red arrow), peak current amplitudes progressively decayed in 0 mM K^+. The same figure shows in another cell the steadiness of peak current amplitudes in 2 mM external K^+ over an equivalent time period (•). The action of 0 mM K^+ on current amplitude and decay rate during repetitive stimulation can be reversed after changing back the solution to 2 mM K^+ (*see* Fig. 6). In other experiments, increasing the external K^+ concentration from 2 to 7 mM did not modify the inactivation rates ($n = 4$). In the absence of external K^+, repetitive stimulation progressively increases the population of channels that remain in a nonconducting absorbing state. The channels that enter the nonconducting state do not recover at the stimulation frequency used (140-msec pulses to +50 mV every 10 sec). During the repetitive stimulation after each pulse, a constant fraction of channels of about 0.05 of the preceding pulse do not reopen. This fraction was calculated from the ratio $(I_{P1}-I_{P2})/I_{P1}$, where I_{P1} and I_{P2} are the peak currents evoked by the preceding and subsequent pulses, respectively. These initial observations strongly suggest that removing external K^+ favors C-type inactivation by facilitating the collapse of the pore.

Both Slow and Fast Components of Inactivation Become Faster upon Removal of External K^+

As stated before, Kv4.1 and Kv4.3 inactivation phases can be described by the sum of two exponential components for 400-msec pulses (Jerng & Covarrubias, 1997; Beck & Covarrubias, 2001). We thus investigated the effect of removing external K^+ on both inactivation components. Fig. 2*A* shows superimposed current traces in 2 mM external K^+ (black trace) and in 0 mM external K^+ (with Na^+) (red trace) with the corresponding curves fitted to the sum of two exponential components. Pulses of 350 msec to 50 mV from HP = −90 mV were delivered at a stimulation frequency of 0.25/sec. As previously described (Fig. 1), Kv4.3 currents were constant in 2 mM K^+ and progressively decayed after external K^+ removal. By scaling both traces to the peak current amplitude, the faster inactivation in 0 mM external K^+ becomes evident (Fig. 2*B*). Both fast and slow components became faster after external K^+ removal; from 19 msec to 14 msec and from 116 to 82 msec, respectively. This is better seen when the scaled current and fitted components are displayed in semilog scale (Fig. 2*C*).

The Faster Inactivation Rate in 0 mM External K^+ is not due to a Voltage Shift of the Activation Curve

The inactivation rate of Kv4.1 and Kv4.3 channels becomes faster after raising external K^+ (Jerng & Covarrubias, 1997; Bähring et al., 2001). One of the proposed mechanisms underlying this action is the negative shift of the activation curve induced by high external K^+ (Eghbali et al., 2001). In view of the action of high external K^+ on the voltage dependence of channel opening, we tested whether lowering external K^+ may shift the activation curve, since a negative shift of the activation curve could also explain the faster inactivation in 0 mM external K^+. Fig. 3 illustrates such an experiment. The patch was repetitively stimulated with 140-msec pulses to +50 mV every 10 sec and activation curves were obtained in 2 and 0 mM K^+ solutions. The first set of peak (pulses from −90 to +60 mV every 10 mV) and tail (test pulse to +50 mV and postpulses from −110 to 60 mV every 10 mV) current traces were obtained in 2 mM K^+ (Fig. 3*A*,*C*). Thereafter, the external solution was exchanged to 0 mM K^+. After a three-minute period of repetitive stimulation in this solution, which induced a reduction in the peak current amplitude and a faster inactivation rate (Fig. 1), a second set of peak and tail current traces was obtained (Fig. 3*B*,*D*). Since this protocol in 0 mM external K^+ induced a small reduction in the +50 mV test pulse, current amplitudes were normalized to the first test pulse. Activation curves (Fractional P_O-V) (Fig. 3*G*), were obtained by dividing the peak I-V curve (Fig. 3*E*) by the instantaneous I-V curve (Fig. 3*F*) at each potential. As expected, due to the asymmetric K^+ concentrations, the instantaneous I-V curves rectify at negative potentials and the reversal potential in 0 mM K^+ becomes more negative than in 2 mM K^+ (Fig. 3*F*). In 2 mM K^+, only pulses more

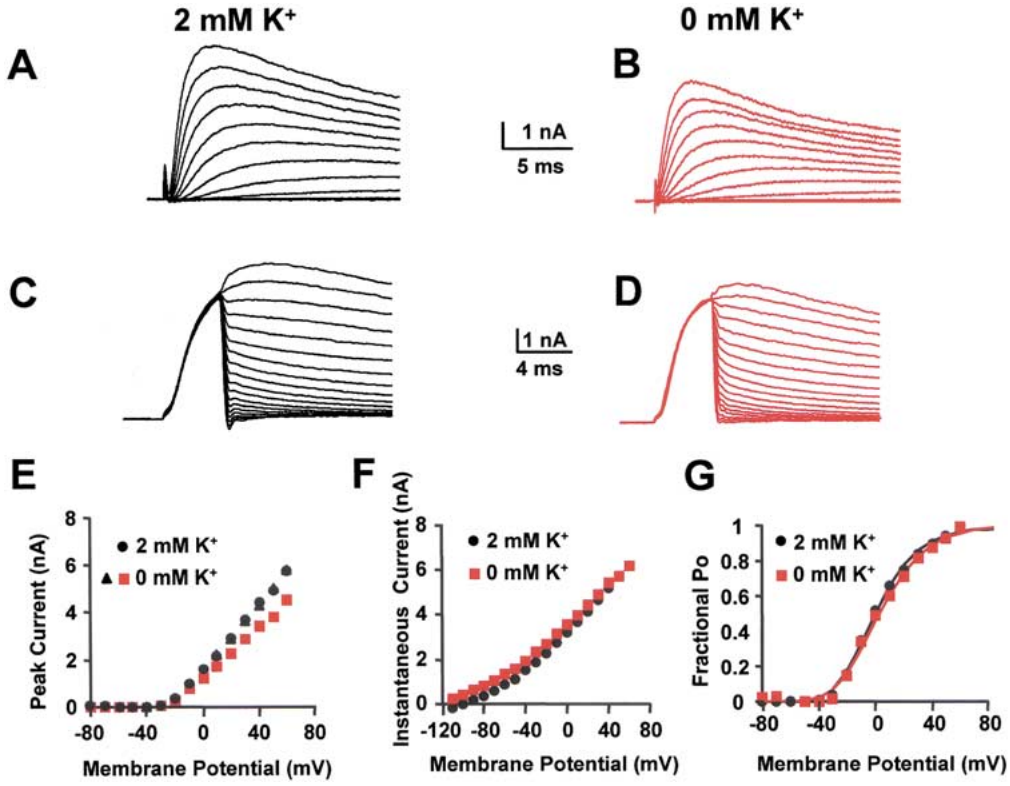

Fig. 3. Removal of external K^+ does not produce a shift in the activation curve. K^+ currents recorded during 140-msec depolarization steps from −80 to 80 mV in 10-mV increments from −90 mV HP. External solution contained 2 mM K^+ (*A*) or 0 mM K^+ (*B*) with Na^+. (*C*, *D*) Currents in response to constant 5-msec pulses to 50 mV followed by repolarization potentials between −110 to 60 mV in 10-mV increments in 2 mM K^+ (*C*) or 0 mM K^+ (*D*). Recordings in (*A*), (*B*), (*C*) and (*D*) were from the same cell. (*E*) Peak current-voltage relationship in 2 (black •) and in 0 (red ■) mM external K^+. The peak current in 0 mM external K^+ was scaled to the peak current in 2 mM external K^+ at +60 mV (black ▲). (*F*) Instantaneous *I-V* curves in 2 mM (black •) and 0 (red ■) mM external K^+. Instantaneous currents were measured 0.6 msec after the termination of the 50-mV pulses. (*G*) Fractional P_O in 2 (black •) and 0 (■, red) mM K^+. As the instantaneous *I-V* curve in 2 and 0 mM K^+ is not linear, the fractional P_O was calculated by dividing the peak current (*E*) by the corresponding instantaneous current (*F*) at each potential. Both curves were fitted by a fourth-order Boltzmann distribution ($V_{half} = -31$ mV, $z = 1.38$ in 2 mM K^+; $V_{half} = -31$ mV, $z = 1.26$ in 0 mM K^+).

negative than −105 mV elicit inward currents that were otherwise absent in 0 mM external K^+. Figure 3*E* demonstrates that peak *I-V* curves in 0 (black circles) and 2 mM K^+ (black triangles) are indistinguishable after normalization, already suggesting a lack of a shift in the activation curve. This is confirmed in the fractional P_O-*V* curves (Fig. 3*G*).

Data points were fitted to a fourth-order Boltzmann distribution

$$P_O = P_{O(max)}\left[\frac{1}{1+e^{\left(\frac{zF(V_{half}-V)}{RT}\right)}}\right]^4$$

where P_O is the fractional open probability, $P_{O(max)}$ is the limiting probability, V_{half} is the midpoint of activation; *V* is the test voltage, *z* is the effective valence, and *F*, *R* and *T* are the usual thermodynamic constants. Data points and fitted curves were practically identical in 2 and 0 mM external K^+ solutions with $V_{half} = -31$ mV and $z = 1.38$ in 2 mM K^+, and $V_{half} = -31$ mV and $z = 1.26$ in 0 mM K^+.

These results demonstrate that the faster inactivation in 0 mM external K^+ solutions are not due to changes in the voltage dependency of channel opening, and favor the view that removal of external K^+ promotes the installation of an inactivation mechanism with properties similar to C-type inactivation (Lopez-Barneo et al., 1993; Yellen, 1998).

Peak Current Amplitude Reduction after External K^+ Removal is not due to Internal K^+ Depletion

Figure 1*B–D* showed that repetitive stimulation (0.1/sec) in 0 mM external K^+ induces a progressive reduction in peak current amplitude. One may argue that this peak current reduction could be due to internal K^+ depletion. This is unlikely since whole-cell clamp records were performed in relatively small cells (20 ± 3 pF) with 1–2-megohm patch electrodes filled with isotonic K^+. However, during the experiment, intracellular K^+ concentration could be lowered in a

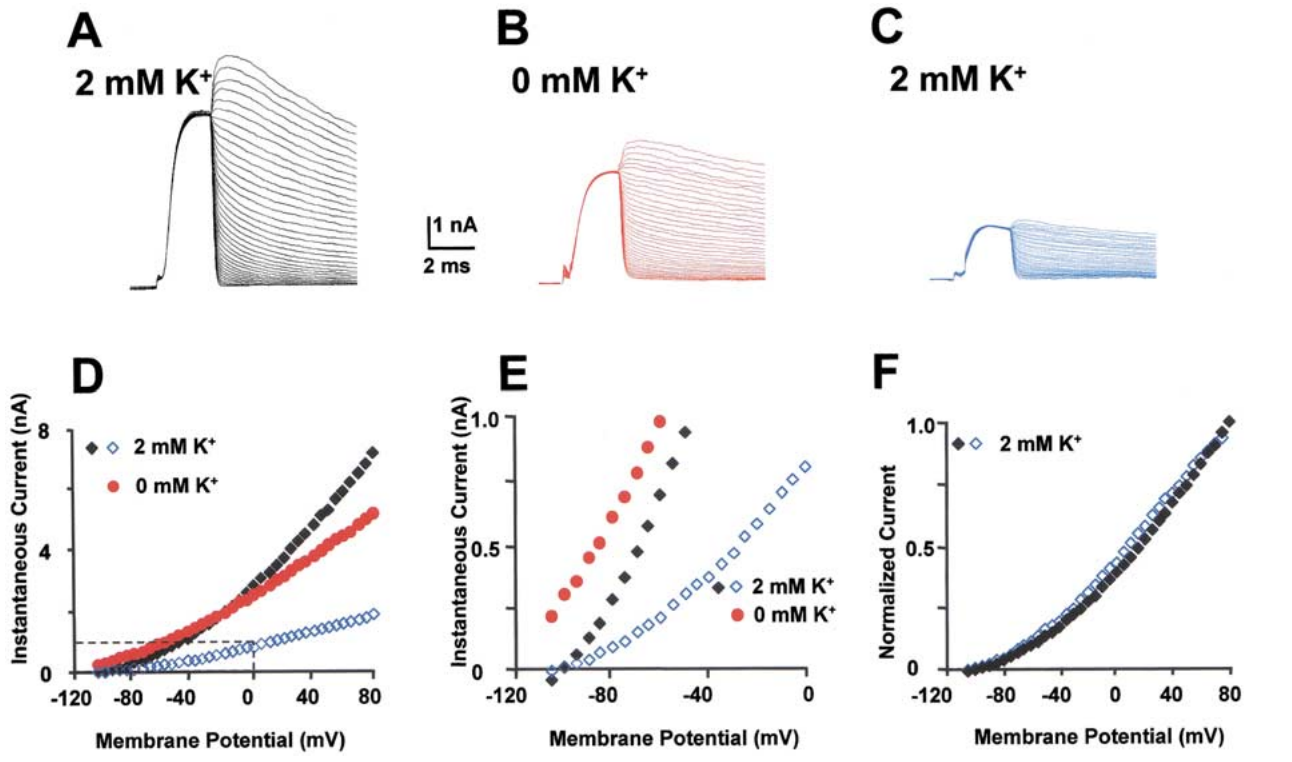

Fig. 4. The reduction in the current amplitude in 0 mM external K^+ is not due to internal K^+ depletion. Ionic currents in response to constant 5-msec pulses to 50 mV followed by repolarization potentials between −110 to 80 mV in 10-mV increments in 2 mM K^+ (*A*), 0 mM K^+ (*B*) and back to 2 mM K^+ (*C*). (*D*) Corresponding instantaneous *I-V* curves, initial measurement in 2 mM K^+ (black ♦), 0 mM K^+ (red •) and back to 2 mM K^+ (blue ◊). Tail currents were measured ~0.5 msec after the end of the initial 50-msec pulse. (*E*) Enlarged dotted box in *D*. (*F*) Normalized instantaneous *I-V* curves at the beginning of the experiment in 2 mM external K^+ (black ♦) and at the end of the experiment in 2 mM K^+ after a period of repetitive pulses in 0 mM K^+ as shown in *D*.

restrictive diffusional space at the inner side of the membrane. To directly evaluate this possibility, the patch was repetitively stimulated with 140-msec pulses to +50 mV every 10 sec and instantaneous *I-V* curves were obtained at different times. Instantaneous *I-V* curves were first obtained in 2 mM K^+ (Fig. 4*A*), then in 0 mM K^+ after a current amplitude reduction of ~40% (Fig. 4*B*), and finally immediately after returning to 2 mM K^+ after an additional 300-sec stimulation period in 0 mM K^+ that further reduced current amplitude by 70% (Fig. 4*C*). Figure 4*D* shows the corresponding instantaneous *I-V* curves, and Fig. 4*E* shows the dotted inset in *D* enlarged. The reversal potential measured initially in 2 mM K^+ (black ♦) was −105 mV and became more negative in 0 mM K^+ (red •). Thus, at this time of the stimulating protocol, the reduction in peak current by 40% cannot be explained by a reduction of the K^+ driving force. This conclusion is further supported by measuring the K^+ reversal potential immediately after returning to 2 mM K^+ following an additional 100-sec stimulation that further reduced current amplitude (blue ◊). In spite of the dramatic reduction of the peak current amplitude, the reversal potential in 2 mM external K^+ remained at a constant value of −105 mV (Fig. 4*DE* black ♦, blue ◊). This can be clearly seen in Fig. 4*F* after normalizing both instantaneous *I-V* curves in 2 mM K^+, in control conditions (black ♦) and after the period of stimulation in 0 mM external K^+ solution (blue ◊). This set of experiments, in which the peak current amplitude becomes dramatically reduced during repetitive stimulation in 0 mM K^+ without detectable changes in the K^+ reversal potential, rules out the possibility that the reduction in current amplitude could be due to internal K^+ depletion.

The Inactivation Process is Faster in External NMG^+ than in External Na^+

Previous work in Shaker K^+ channels has demonstrated that after replacement of external Na^+ by the large impermeant cation NMG^+, C-type inactivation becomes much faster (Lopez-Barneo et al., 1993). Thus, to further investigate the similarity between C-type inactivation and the inactivation process in Kv4.3 channels, we analyzed the effect of removing external K^+, having NMG^+ as the main external cation. Figure 5*A,B* shows that following removal of external K^+, the reduction of peak current ampli-

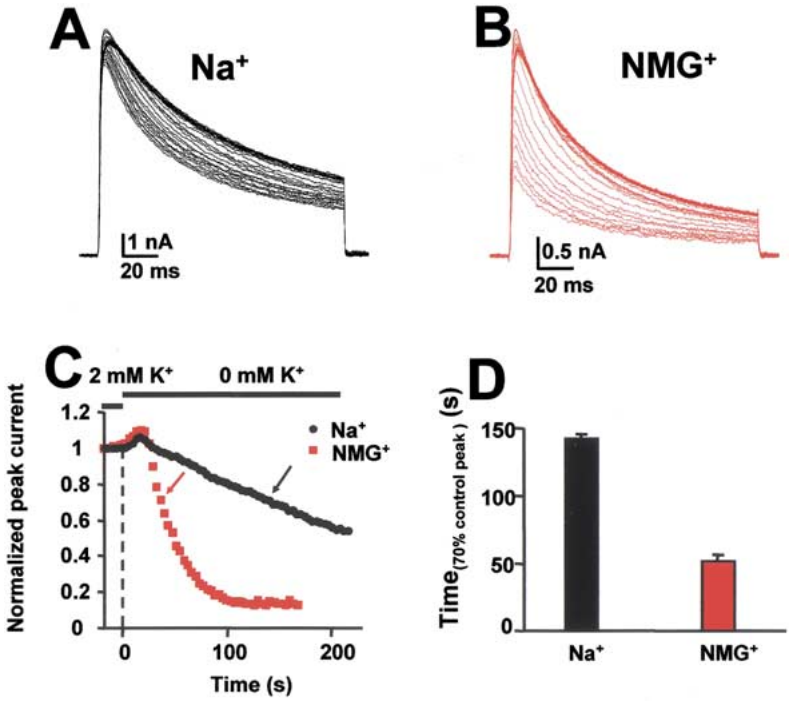

Fig. 5. The collapse of the K^+ conductance is much faster in NMG^+ than in Na^+ at 0 mM external K^+. Superimposed traces (32) recorded during 140-msec pulses from −90 mV HP to 50 mV at 0.25/sec stimulation frequency in 2 mM K^+ and in 0 mM external K^+ in the presence of 140 mM Na^+ (*A*) or 140 mM NMG^+ (*B*). (*C*) Normalized peak current amplitudes plotted as a function of time in external Na^+ (black •) or NMG^+ (red ■). (*D*) Time of the peak current to reach 70% of the peak value in 0 mM external K^+ in the presence of Na^+ (black bar) or NMG^+ (red bar). Note the much faster current decay in external NMG^+ when compared with external Na^+.

tudes for the same repetitive stimulation (140-msec pulses from −90 to +50 mV at 0.25/sec) was much faster in external NMG^+ (*B*) than in Na^+ (*A*). This is plotted in Fig. 5*C*, where normalized peak current amplitudes were graphed as a function of time in external Na^+ (black •) and external NMG^+ (red ■). In both external Na^+ and NMG^+, current amplitudes remain unchanged during the initial repetitive stimulation period in 2 mM K^+. After the solution change (time 0), the time course of peak current decay is much faster in NMG^+ than in Na^+, suggesting that in NMG^+ a larger fraction of channels per pulse populate an absorbing nonconducting state. Upon removal of the external K^+, the peak current amplitude diminishes to 70% of its control in 141 ± 4 sec ($n = 5$) in external Na^+, and in 50 ± 5 sec ($n = 3$) in external NMG^+ (Fig. 5*D*). In these experiments, the fraction of channels that do not recover after each pulse is higher in external NMG^+ (0.1) than in external Na^+ (0.05). The results indicate that the population of the absorbing nonconductive state is dependent on the ion content of the external solution. NMG^+ is less effective than Na^+ in maintaining the K^+ conductance active in the absence of K^+.

Collapse of KV4.3 Channels Induced by Cumulative Inactivation is Reversible

Since "defunct" K^+ channels in 0 mM K^+ can be recovered during stimulation in normal external K^+ (Gomez-Lagunas, 1997; Vergara et al., 1999), we investigated whether an equivalent recovery process occurs after cumulative inactivation by repetitive stimulation in 0 mM external K^+. Figure 6 shows that the K^+ conductance that has collapsed in the absence of K^+ (NMG^+-containing solution) could be restored after adding back 2 mM K^+. Panel (*A*) shows current traces and superimposed fits at the beginning of the stimulation (0.25/sec, 140-msec pulses to 50 mV from −90 mV HP) in 2 mM K^+ (*a*) and at different times after external K^+ removal (*b–e*). As previously shown, external K^+ removal induced a progressive current reduction associated with a faster time constant of decay (*b–e*). The decay time constant was reduced from 29 msec in 2 mM K^+ to 22–23 msec in 0 mM external K^+. Finally, a small current with a barely detectable inactivation phase remained (*f*, nonfitted). After the reinstallation of 2 mM K^+ for about 800 sec, peak current amplitude and time constant recovered (*g*).

Figure 6*B* shows the peak current amplitude as a function of time (arrows correspond to records in *A*). Initially, peak current in 2 mM K^+ was stable; after removing K^+, peak current transiently increased followed by a steep reduction reaching a steady level in about 100 sec. The reinstallation of 2 mM K^+ induced a current recovery that was fitted to the sum of two exponential functions with amplitude factors and time constants of 1.4 nA and 40 msec, and 1.1 nA and 407 msec. Note that towards the end of the recovery process the cell was not continuously stimulated. These results show that Kv4 and Shaker K^+ channels are similarly modulated by external K^+, and favor

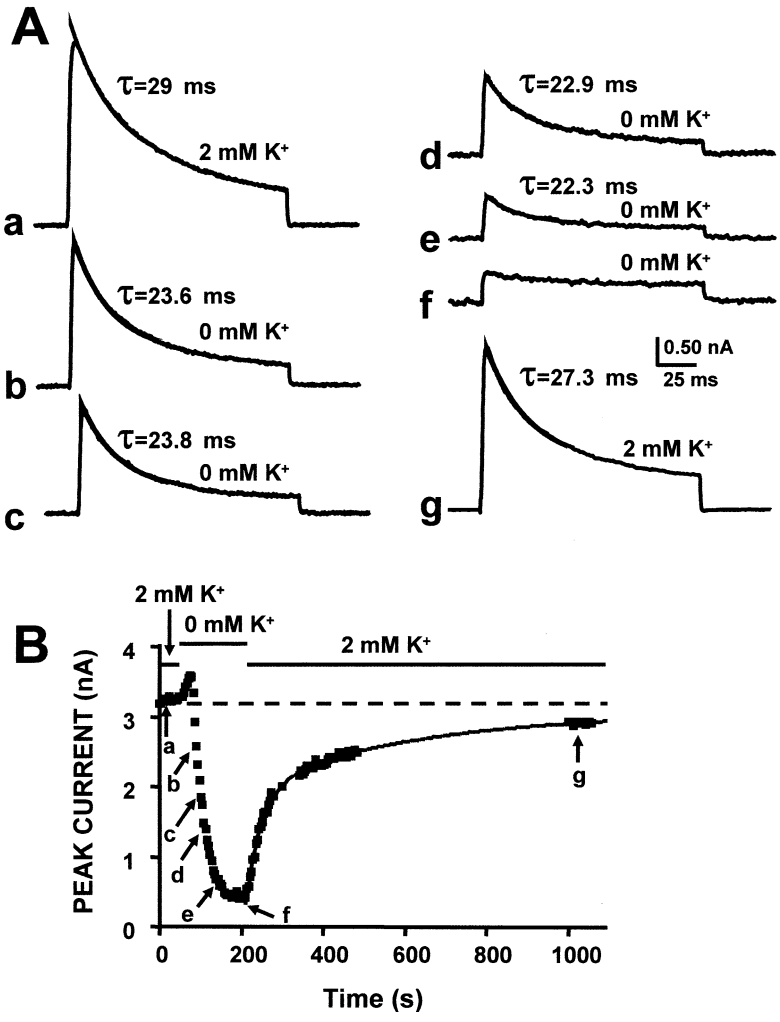

Fig. 6. Ionic current recovery in 2 mM external K^+ after exposure to 0 mM external K^+. Kv4.3 currents were recorded during 140-msec pulses from −90 mV HP to 50 mV at 0.25/sec in 2 and 0 mM external K^+ in the presence of NMG^+. (*A*) Traces and superimposed fits (not in *f*) in 2 mM K^+ (*a*), at different times in 0 mM K^+ (*b–f*) and after recovery in 2 mM external K^+ (*g*). The initial 50 msec of the decay phase was fitted to a single exponential (values next to the traces). (*B*) Time course of peak current amplitude in 2, 0 and 2 mM external K^+. The arrows in the graph are pointing to the traces in (*A*). Solid curve is the fit to the sum of two exponential functions with amplitude factors and time constants of 1.4 nA and 40 msec, and 1.1 nA and 407 msec. During the recovery process the cell was not continuously stimulated. The dashed line corresponds to the mean peak current amplitude at the beginning of the experiment in 2 mM external K^+.

the view that a common C-type inactivation mechanism may be present in Kv4 channels.

Current Amplitude Decline during Repetitive Stimulation after Removal of External K^+ Can Be Explained by a Reduction in the Number of Functional Channels

We have shown that repetitive pulses in 0 mM external K^+ cause a progressive reduction in the peak current amplitude (Figs. 1, 5 and 6). If the set of K^+ channels that enter the nonconductive state does not reopen from another state, one would expect that the remaining channels would open with the same kinetics. Figure 7*A*,*B* shows selected superimposed traces of decaying amplitude during repetitive stimulation at different times in 0 mM K^+ with external Na^+ (*A*) or NMG^+ (*B*) (pulses from −90 mV to +50 mV at 0.25/sec). To compare their time course of inactivation, traces were scaled as shown in Fig. 7*C*,*D*. Scaled traces in both Na^+ and NMG^+ are indistinguishable, indicating that the reduction in current size is not associated with significant kinetic changes. This is also shown by plotting as function of time the inactivation rate constant in 2 and 0 mM K^+ in external Na^+ and NMG^+ (Fig. 7*E*). The graph shows that in 2 mM K^+ the inactivation rates are faster in NMG^+ than in Na^+ and that after the solution change from 2 to 0 mM K^+ they become faster and reach a steady state in about 30 sec after solution exchange. Average values of inactivation rates in 2 and in 0 mM external K^+ in Na^+ and NMG^+ are summarized in the bar graph in Fig. 7*F*. In agreement with the view that NMG^+ is more effective than Na^+ in establishing inactivation, the inactivation rates were always faster in NMG^+ than in Na^+ (Fig. 5). In 2 mM K^+, the inactivation rates were 27 ± 3.2 sec^{-1} (n = 5) and 36 ± 2.8 sec^{-1} (n = 3) in Na^+ and NMG^+, respectively. Following removal of external K^+, they increased to 38 ± 1.7 sec^{-1} and 51 ± 1.3 sec^{-1}, respectively. These results strongly suggest that the reduction in current amplitude in the absence of external K^+ is due to a reduction of the number of

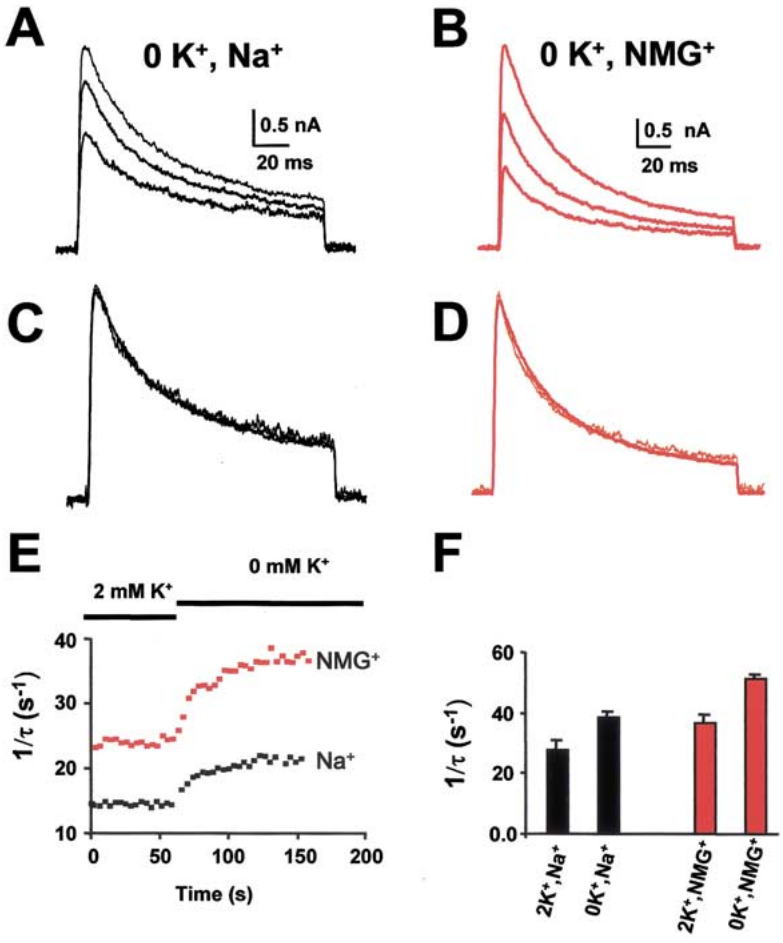

Fig. 7. The reduction in the current amplitude during repetitive pulsing is due to a reduction in the number of activatable channels. Three representative superimposed current traces during repetitive stimulation recorded in 0 mM external K^+, when either Na^+ (*A*) or NMG^+ (*B*) was present in the external solution. Pulses to 50 mV at 0.25/sec stimulation frequency. (*C*, *D*) Superimposed scaled currents in external Na^+ (*C*) or in external NMG^+ (*D*). (*E*) Rate constants as a function of time in 2 mM K^+/140 NMG^+ and 0 mM K^+/140 NMG^+ (red ■), and in 2 mM K^+/140 Na^+ and 0 mM K^+/140 Na^+ (black ■). (*F*) Mean rate constants ± SE in the same solutions as in *E* (n = 6–8). All values were significantly different ($p < 0.001$).

functional channels, and that the remaining functional channels gate with the same kinetics.

Discussion

Jerng and Covarrubias (1997) and Bähring et al. (2001) proposed that the inactivation mechanism in Kv4 channels is different from the classical N- and C-type inactivation described for Shaker K^+ channels (Jerng & Covarrubias, 1997; Jerng et al., 1999; Bähring et al., 2001). Based on mutagenesis experiments, it was concluded that the inactivation mechanism in Kv4 channels is due to a concerted action of the cytoplasmic N- and C-terminal regions of the pore-forming subunit and interactions between the S4-S5 loop with the distal section of the S6 loop (Jerng & Covarrubias, 1997; Jerng et al., 1999). In this work, we propose that a pore collapse mechanism similar to C-type inactivation described for Shaker K^+ channels also takes place in the inactivation process of Kv4 channels. It is possible that long-range effects of N- and C-terminal regions modify the pore structure, explaining the reported changes in inactivation after mutagenesis.

To gain further insight into the mechanism of inactivation of Kv4 channels, we have characterized the inactivation properties of Kv4.3 channels in 2 and in 0 mM K^+ solutions. We speculated that if Kv4 channels have an inactivation mechanism similar to C-type inactivation of Shaker K^+ channels, 0 mM external K^+ should enhance Kv4 inactivation. This was indeed the case, since 0 mM external K^+ speeds up Kv4.3 channel inactivation. In general, the effect of K^+ removal on Kv4.3 channel inactivation is similar to what has been extensively described for C-type inactivation in Shaker-related K^+ channels (Grissmer & Cahalan, 1989*b*; Pardo et al., 1992; Lopez-Barneo et al., 1993; Baukrowitz & Yellen, 1995). The main findings in this work are: 1. Removal of external K^+ increases the inactivation rate, whereas increasing K^+ from 2 to 7 mM has no effect. 2. The faster inactivation rate upon removal of external K^+ is not due to a shift of the activation curve. 3. Cumulative inactivation by repetitive stimulation becomes evident in 0 mM external K^+ solutions. 4. The reduction in current amplitude in 0 mM external K^+ during repetitive stimulation cannot be explained by internal K^+ depletion. 5. The inactivation rate is faster in NMG^+ than in Na^+, in both 2 and 0 mM

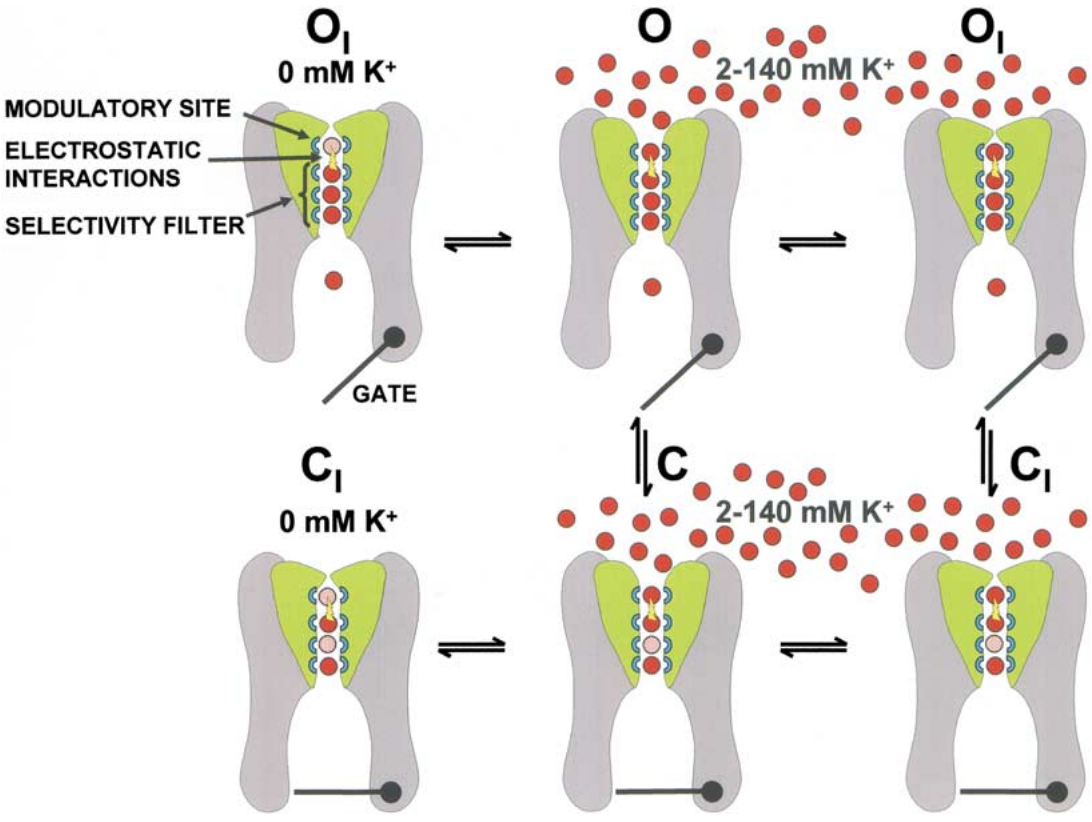

Fig. 8. Model of C-type inactivation in Kv4.3 channels. The channel is shown in the closed (*C*), open (*O*), closed inactivated (C_I) and open inactivated (O_I) states in 0 mM and 2–140 mM K^+ concentrations. K^+ ions are depicted as red filled circles. To explain that K^+ concentrations higher than 2 mM K^+ do not slow down inactivation, the model shows that the occupancy of the modulatory site is saturated in 2 mM K^+, thus its occupancy is similar in 2–140 mM K^+. Since in this saturated state, Kv4.3 channels show significant inactivation, it is proposed that the outer pore region with it modulatory site occupied by K^+ may intermittently collapse bringing the channel to the inactivated states (O_I and C_I). On the other hand, in 0 mM K^+, the K^+ occupancy of the modulatory is reduced (lighter red circles), favoring the C-type inactivation process. The model also shows that K^+ bound to high-affinity K^+ binding sites in the selectivity filter can destabilize the K^+ modulatory site by electrostatic interactions (yellow spark).

K^+ external solutions. 6. Cumulative inactivation by repetitive stimulation in 0 mM K^+ is more prominent in NMG^+ than in Na^+. 7. Channel inactivation during repetitive stimulation in 0 mM K^+ is reversible. 8. The reduction in the current amplitude during cumulative inactivation by repetitive stimulation is not associated with kinetic changes, suggesting that it is due to a diminished number of functional channels with unchanged gating properties.

In Shaker K^+ channels, C-type inactivation involves conformational changes in the outer region of the pore (Yellen et al., 1994; Liu et al., 1996). The occupancy of an outer modulatory binding site, as shown in Fig. 8, regulates the structural rearrangements that take place during C-type inactivation. The occupancy of this modulatory site is regulated by the external K^+ concentration and outward K^+ flow that concentrates K^+ ions in the outer pore regions (Choi et al., 1991; Lopez-Barneo et al., 1993; Baukrowitz & Yellen, 1995). In fact, the reduction of K^+ outward flow by internal blockers favored C-type inactivation by impeding the occupancy of the modulatory site (Baukrowitz & Yellen, 1995). Our results in Kv4.3 channel inactivation can be interpreted along the same hypothesis. The faster inactivation rate and a more prominent cumulative inactivation by repetitive stimulation in 0 mM external K^+ can be explained by a lower K^+ occupancy of an external modulatory binding site favoring the constriction of the outer pore region as in C-type inactivation. In line with this view, and as previously described in Shaker K^+ channels (Lopez-Barneo et al., 1993), the inactivation rate of Kv4.3 channels is significantly increased when external Na^+ is replaced by the large impermeant cation NMG^+. Furthermore, in external NMG^+ the cumulative inactivation by repetitive stimulation is greatly enhanced. Thus, the studies in 2 and 0 mM K^+ solutions support the existence of a C-type inactivation mechanism in Kv4.3 channels. We can propose that the process of lack of conduction in MaxiK channels at $K^+ < 4$ μM, and in Shaker K^+- and Kv4.3-channels at nominally 0 mM K^+ (Gomez-

Lagunas, 1997; Vergara et al., 1999; Loboda et al., 2001) is a general property of K^+ channels and underlies C-type inactivation. This process is governed by the K^+ occupancy of sites in the channel pore. When these sites are unoccupied, the external mouth of the pore collapses, closing the inactivation gate.

Shaker and Kv4 K^+-channels have some differences in the action of TEA^+ on their inactivation properties. TEA^+ is a common blocker of K^+ channels that can act both on extracellular and intracellular sites, and can be used to distinguish between N- and C-type inactivation in voltage-gated K^+ channels (Choi et al., 1991). Internal TEA^+ blockade of Shaker K^+ channels produces a significant slowdown of the N-type fast inactivation rate that is due to the competition for the same binding site of the inactivation particle and internal TEA^+. When 46 amino acids of the amino terminal are deleted (Shaker H4 Δ6–46), the fast N-type inactivation is removed, revealing the much slower C-type inactivation (Zagotta et al., 1990; Hoshi et al., 1991). In this condition, internal TEA^+ acts in a similar way in both Shaker H4 Δ6–46 and Kv4 channels by reducing the current amplitude during the pulse without affecting the inactivation rates (Choi et al., 1991; Jerng & Covarrubias, 1997). The fact that internal TEA^+ acts similarly in Shaker H4 Δ6–46 indicates the absence in Kv4 channels of an N-type inactivation mechanism but does not discard the existence of a C-type inactivation mechanism as previously suggested (Choi et al., 1991; Jerng & Covarrubias, 1997).

External TEA^+ in Shaker H4 Δ6–46 reduces the current amplitude and slows down the inactivation rate, suggesting that TEA binds in the outer pore region, preventing inactivation by impeding pore constriction, which would have normally occurred in the absence of TEA^+. In contrast to Shaker K^+ channels, Kv4 channels are not affected by external TEA^+ and replacement of external Na^+ by TEA^+ has no significant effect on current amplitude and inactivation (Dixon et al., 1996; Jerng & Covarrubias, 1997). Since Kv4 channels lack an external TEA^+ binding site, this lack of action of external TEA^+ does not rule out a C-type inactivation mechanism.

Recent studies have shown that the occupancy of the external modulatory site that regulates C-type inactivation can be influenced by the relative affinities of neighboring K^+ sites in the K^+ channel pore (Ogielska & Aldrich, 1999). The A463C mutation in Shaker K^+ channel decreases the affinity of an internal pore binding site reducing its occupancy and leading to reduced ion-ion repulsive interactions with neighboring sites. As a consequence, the external modulatory site is more frequently occupied resulting in a decreased rate of C-type inactivation. Conversely, an increased affinity of the K^+ site in the channel selectivity filter, as may occur in the A463V mutation, will increase K^+ occupancy of that site leading to higher ion-ion repulsion that would result in a decreased occupancy of the external modulatory site and a faster C-type inactivation (Ogielska & Aldrich, 1999). Figure 8 illustrates a model of the K^+ channel pore following Ogielska and Aldrich (1999), which could explain qualitatively the C-type inactivation mechanism in Kv4 channels. This model is a simplified version of the Kv4 allosteric models previously proposed for Kv4 channels (Beck & Covarrubias, 2001; Bähring et al., 2001). We initially consider a multi-ion conduction pore as inferred from pioneering studies of Ba^{2+} block in MaxiK channels and recent work in Shaker K^+ channels (Neyton & Miller, 1988*a*; 1988*b*; Harris et al., 1998). This assumption is consistent with the recently resolved crystal structure of the bacterial KcsA channel pore that has highly homologous sequence to most K^+ selective channels; thereby, the model assumes that K^+ channels have homologous ion binding sites and that K^+ ions in Kv4 channels can bind at four locations in the selectivity filter as in KcsA channels (Doyle et al., 1998; Jiang & MacKinnon, 2000; Morais-Cabral et al., 2001; Zhou et al., 2001). The channel is shown as having four pore binding sites; the more external site is the modulatory site regulating C-type inactivation. Since the Kv4 channel inactivates in 2 mM external K^+ and the inactivation process is saturated at that concentration, we propose that the channel can transit from the open state (O) to the open inactivated state (O_I) with the modulatory site saturated and occupied by K^+. Therefore, this model predicts that external K^+ concentration higher than 2 mM will not significantly affect inactivation, as observed experimentally. On the other hand, when the external K^+ concentration is reduced to 0 mM, the occupancy of the modulatory site will also be diminished, leading to faster inactivation rates (model 1, Fig. 8). In another model that also explains the lack of action of high external K^+ (>2 mM) on the inactivation rates, the occupancy of the modulatory site depends on the existence of an additional more external K^+ site that senses the bulk K^+ concentration (model 2, *not shown*). In model 2, the K^+ occupancy of the modulatory site also sets the extent of inactivation but depends on the K^+ occupancy of the more external site that becomes saturated in 2 mM external K^+. As in model 1, lowering the external K^+ concentration will reduce the K^+ occupancy of the modulatory site leading to faster inactivation rates, but in model 2 it is due to the reduced K^+ occupancy of the more external site. In both models, high-affinity K^+ binding sites in the pore can hold K^+ when the channel is in the closed state (C), thus, destabilizing K^+ from the modulatory site by electrostatic interactions (Fig. 8, *yellow spark*), and allowing inactivation from the closed state (C_I). In conclusion, both models will have external binding sites that are saturated at 2 mM external K^+ (modulatory site in

model 1 or a more external site in model 2) and can explain the faster inactivation rates after reducing external K^+ from 2 to 0 mM and the lack of inactivation slowdown in high external K^+ (> 2 mM). The slight increased rate of inactivation in isotonic K^+ (Jerng & Covarrubias, 1997; Eghbali et al., 2001; Bähring et al., 2001) can be explained by our recent finding of a *ca.* 10–20 mV negative shift of the activation curve in high external K^+. The shift of the activation curve to more negative potentials was associated with an equivalent shift of the steady-state inactivation curve, and of the rate *vs.* potential curve leading to a faster inactivation rate at a given potential (Eghbali and Stefani, *unpublished*).

In summary, we favor the idea that Kv4 channels can inactivate with the C-type inactivation modulatory site saturated at 2 mM external K^+. Removal of external K^+ decreases the occupancy of the modulatory site, resulting in an increase in the inactivation rates and cumulative inactivation by repetitive stimulation. Furthermore, a high-affinity K^+ binding site in the pore can hold K^+ when the channel is in the closed state, thus destabilizing the modulatory site by electrostatic interactions and allowing inactivation from closed states (Jerng et al., 1999). The degree of inactivation upon removal of external K^+ also depends on the ionic composition of the external solution. In the presence of external NMG^+, the modulatory site cannot be occupied, leading to a faster inactivation process, but when Na^+ ions are present in the external solution, Na^+ can occupy to some extent the modulatory site, reducing the inactivation process. Thus, the modulation by external ions of Kv4.3 channel inactivation is consistent with the existence of a C-type inactivation mechanism.

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