Modulation of C-Type Inactivation by K^+ at the Potassium Channel Selectivity Filter

Laszlo Kiss and Stephen J. Korn
Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269 USA

ABSTRACT With prolonged or repetitive activation, voltage-gated K^+ channels undergo a slow (C-type) inactivation mechanism, which decreases current flow through the channel. Previous observations suggest that C-type inactivation results from a localized constriction in the outer mouth of the channel pore and that the rate of inactivation is controlled by the rate at which K^+ leaves an unidentified binding site in the pore. We have functionally identified two K^+ binding sites in the conduction pathway of a chimeric K^+ channel that conducts Na^+ in the absence of K^+. One site has a high affinity for K^+ and contributes to the selectivity filter mechanism for K^+ over Na^+. Another site, external to the high-affinity site, has a lower affinity for K^+ and is not involved in channel selectivity. Binding of K^+ to the high-affinity binding site slowed inactivation. Binding of cations to the external low-affinity site did not slow inactivation directly but could slow it indirectly, apparently by trapping K^+ at the high-affinity site. These data support a model whereby C-type inactivation involves a constriction at the selectivity filter, and the constriction cannot proceed when the selectivity filter is occupied by K^+.

INTRODUCTION

Most voltage-gated K^+ channels inactivate with prolonged depolarization. Inactivation proceeds by two mechanisms. N-type inactivation, which is present in some K^+ channels, is relatively fast ($\tau < 10$ ms) and utilizes a cytoplasmic blocking particle linked to the amino-terminal region of the channel (Zagotta et al., 1990). C-type inactivation, which in some channels coexists with N-type inactivation, involves a conformational change near the outer mouth of the pore (Grissmer and Cahalan, 1989; Yellen et al., 1994; Liu et al., 1996). Entry into and recovery from the C-type inactivated state are relatively slow (Hoshi et al., 1991; Marom and Levitan, 1994; Levy and Deutsch, 1996). Consequently, C-type inactivation appears to be most important under conditions where it can accumulate, such as during repetitive firing of action potentials and frequency-encoded events (Aldrich, 1981; Hsu et al., 1993, Marom and Abbott, 1994; Marom and Levitan, 1994).

C-type inactivation displays a wide range of rates in different channels (Stuhmer et al., 1989; Iverson and Rudy, 1990; Lopez-Barneo et al., 1993, Marom and Levitan, 1994). This diversity of inactivation rates among channels provides functional diversity for neurons that require different firing patterns and different maintenance of excitability with repetitive firing. Within a single neuron, modulation of inactivation rate by extracellular modulators would tailor the inhibitory influence of K^+ channels to the immediate situation, including neuronal firing pattern, stimulus history, and the activity and metabolism of neighboring cells. For example, elevation of extracellular $[K^+]$, in both physiological and pathological ranges, slows entry into the C-type inactivated state (Lopez-Barneo et al., 1993, Marom and Levitan, 1994; Baukrowitz and Yellen, 1996) and speeds recovery from the C-type inactivated state (Levy and Deutsch, 1996). Thus, events that elevate extracellular $[K^+]$ would be expected to reduce the fraction of inactivated channels and thus increase the inhibitory efficacy of K^+ channel activation.

The slowing of C-type inactivation by K^+ appears to be due to occupancy of the pore by K^+ (Baukrowitz and Yellen, 1996). Based on kinetic arguments, Baukrowitz and Yellen (1996) reasoned that the site at which K^+ controlled inactivation rate was one of the K^+ binding sites involved in permeation and that it was the dwell time of the last ion in the pore that controlled inactivation rate. Cysteine mutagenesis studies (Yellen et al., 1994; Liu et al., 1996), the cooperative interaction of all four channel subunits in the inactivation mechanism (Kavanaugh et al., 1992; Ogielska et al., 1995; Panyi et al., 1995), and the slowing of inactivation by external tetraethylammonium (TEA) and K^+ (Choi et al., 1991; Lopez-Barneo et al., 1993; Marom and Levitan, 1994; Baukrowitz and Yellen, 1996) are all consistent with the hypothesis that the channel undergoes a constriction near the outer mouth of the pore during inactivation. This conformational change appears to be relatively local and not at the outer extremity of the pore, as binding of a channel toxin to a site in the outer vestibule is relatively unaffected by inactivation (Liu et al., 1996).

A conserved, eight-amino-acid sequence in the P region of all voltage-gated K^+ channels (the signature sequence) is critical to the integrity of the selectivity filter (Heginbotham et al, 1994). K^+ channels support competition between K^+ and Na^+ at binding sites within the pore (Bezanilla and Armstrong, 1972; French and Shoukimas, 1985; Callahan and Korn, 1994; Korn and Ikeda, 1995; Kiss et al., 1998), and in Kv2.1, this competition contributes to the selectivity

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Address reprint requests to Dr. Stephen Korn, Department of Physiology and Neurobiology, Box U-156, University of Connecticut, 3107 Horsebarn Hill Road, Storrs, CT 06269. Tel.: 860-486-4554; Fax: 860-486-3303; E-mail: korn@oracle.pnb.uconn.edu.

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filter mechanism (Korn and Ikeda, 1995). The signature sequence is immediately adjacent to and apparently internal to the amino acids involved in the inactivation-associated conformational change (Liu et al., 1996). One hypothesis suggested by these observations is that the selectivity filter binding site is the site at which K^+ modulates inactivation. An alternative hypothesis, however, is that binding of K^+ to a site remote from the selectivity filter, presumably external to the selectivity filter, allosterically prevents the inactivation process from proceeding. This is supported by the observation that millimolar concentrations of extracellular K^+ are required to slow inactivation, whereas K^+ should have a much higher affinity for the selectivity filter binding site.

We have tested these hypotheses using a chimeric channel that has two advantageous properties. First, the channel conducts Na^+ in the absence of K^+. This allowed us to examine the competition mechanism between K^+ and Na^+ at two binding sites in the channel, one of which is presumably the selectivity filter. Second, the channel undergoes slow (apparently C-type) inactivation when carrying Na^+ current. This property allowed us to examine the influence of cation occupancy of these two binding sites on the inactivation process. Our results suggest that occupancy of the selectivity filter binding site by K^+ slows inactivation and that occupancy of the outer site by either K^+ or Na^+ influences inactivation indirectly by trapping K^+ at the selectivity filter binding site.

MATERIALS AND METHODS

Cell culture and channel expression

The chimera used in this study, obtained from Dr. Rod MacKinnon, consisted of the loop between S5 and S6 from Kv1.3 inserted into Kv2.1 (Gross et al., 1994). K^+ channel cDNA was subcloned into the pcDNA3 expression vector and expressed in the human embryonic kidney cell line HEK 293 (American Type Culture Collection, Rockville, MD). Briefly, cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum with 1% penicillin/streptomycin (maintenance media). Cells were co-transfected by electroporation (Invitrogen (San Diego, CA) Electroporator II; 71 μF, 375 V) with channel expression plasmid (15 μg/0.2 ml) and the CD8 antigen (1 μg/0.2 ml). After electroporation, cells were plated on coverslips coated with protamine (1 mg/ml; Sigma Chemical Co., St. Louis, MO) submerged in maintenance media. Electrophysiological recordings were made 18–30 h later. On the day of recording, cells were washed with fresh medium and incubated with Dynabeads M450 conjugated with antibody for CD8 (1 μl/ml; Dynal, Oslo, Norway). Cells that expressed CD8 were coated with beads, which allowed visualization of transfected cells (Jurman et al., 1994). Greater than 90% of cells that were coated with beads expressed K^+ channels.

Electrophysiology

Currents were recorded using either the whole-cell patch clamp technique or excised, outside-out patches. Patch pipets were fabricated from N51A glass (Garner Glass Co., Claremont, CA), coated with Sylgard, and fire polished. Series resistance ranged from 1 to 2.2 MΩ and was compensated by 80–90% (Axopatch 1D, Axon Instruments, Foster City, CA). Currents were filtered at 2–5 KHz (internal Axopatch filter) and sampled at 70–600 μs/point. The holding potential was always −80 mV. All summed experimental data are reported as mean ± SEM, and statistical significance was determined by unpaired Student's *t*-test (Sigmaplot, Jandel Scientific). All data analysis and curve fitting was done with Clampfit (Axon Instruments).

Electrophysiological solutions

Currents were recorded in a constantly flowing, gravity-fed bath. Solutions were placed in one of six reservoirs, each of which fed via plastic tubing into a single quartz tip (~100 μm diameter; ALA Scientific Instruments, Westbury, NY). The tip was placed within 10 μm of the cell being recorded before the start of the experiment. One solution was always flowing, and solutions were changed by manual switching (complete solution changes took 5–10 s). Control internal solutions contained (in mM): 140 XCl (X = K^+, Na^+, or $Tris^+$), 20 HEPES, 10 EGTA, 1 $CaCl_2$, 4 $MgCl_2$, pH 7.3, osmolality 285. Control external solutions always contained (in mM): 165 XCl, 20 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.3, osmolality 320. Additions and substitutions are listed in the figure legends.

RESULTS

Na^+ conductance and slow inactivation in the chimera

Kv2.1 conducts Na^+ in the absence of K^+ (Korn and Ikeda, 1995) but inactivates extremely slowly (De Biasi et al., 1993). Kv1.3 does not conduct Na^+, even in the absence of K^+ (not shown), but displays C-type inactivation (cf. Panyi et al, 1995). The chimera used in this study combined these properties. With 140 mM internal K^+ and 160 mM external Na^+, step depolarization produced voltage-activated, outward K^+ currents (Fig. 1 *A*). After repolarization to −80 mV, outward tail currents were observed, which demonstrates that the channel is highly selective for K^+ over Na^+. In the absence of internal or external K^+, the channel conducted Na^+ (Fig. 1 *B*). K^+ and Na^+ currents activated with similar voltage dependence ($V_{1/2} = -1.0 \pm 2.1$ mV ($n = 6$) and -4.66 ± 3.5 mV ($n = 8$), respectively). However, K^+ currents were sustained during the 100-ms depolarization, whereas Na^+ currents inactivated with a time constant of 30–100 ms.

With longer depolarizing steps, K^+ currents also displayed slow inactivation (Fig. 1 *C*). As in Shaker, inactivation was slowed by elevation of external K^+ (Fig. 1 *D*).

The faster inactivation of Na^+ currents compared with K^+ currents is consistent with the observation that occupancy of the pore by K^+ slows inactivation. This interpretation, however, relates K^+ occupancy to the mechanism of classical C-type inactivation. Consequently, it was of interest to determine whether the mechanism of slow inactivation of Na^+ currents was similar to that of C-type inactivation of K^+ currents or whether the inactivation mechanism was fundamentally different with these two permeant ions or in this chimera.

C-type inactivation of K^+ currents displays three characteristic properties: it is slowed by external TEA (Choi et al., 1991), slowed by extracellular acidification when the amino acid at the position corresponding to 401 in Kv1.3 is a histidine (Busch et al., 1991), and slowed by elevation of extracellular K^+ (Lopez-Barneo, 1993; Baukrowitz and Yellen, 1996). Consistent with the first property, application of 30 mM TEA, which blocked Na^+ currents by 48.4 ±

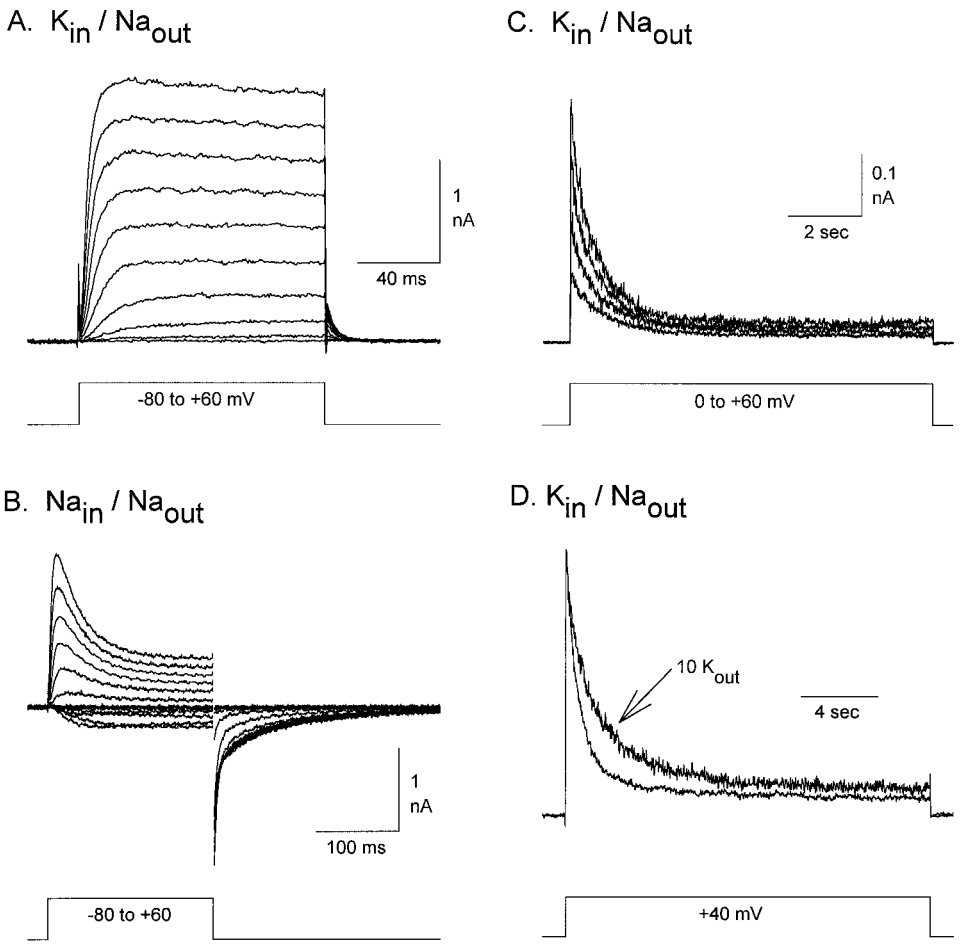

FIGURE 1 Potassium and sodium currents through the chimera. (*A*) Potassium currents evoked by 120-ms depolarizing steps between −80 and +60 mV. The intracellular solution contained 140 mM K^+, and the extracellular solution contained 160 mM Na^+. (*B*) Sodium currents evoked by 200-ms depolarizing steps between −80 and +60 mV. Solutions were identical to *A* except internal K^+ was replaced by Na^+. (*C*) K^+ currents evoked by 9.9-s depolarizing steps to 0, 20, 40, and 60 mV. (*D*) Normalized K^+ currents, evoked by 24-s steps to +40 mV in the absence of extracellular K^+ and presence of 10 mM extracellular K^+. Solutions in *C* and *D* were identical to those in *A*.

3.2% ($n = 5$), significantly slowed inactivation (Fig. 2 *A*). The chimera contains a histidine at position 380 contributed by Kv1.3, and as expected of a C-type inactivation process, Na^+ current inactivation in the chimera was also slowed by extracellular acidification (Fig. 2 *B*). Finally, application of extracellular K^+ produced a concentration-dependent slowing of Na^+ current inactivation (Fig. 2 *C*). These data suggest that this slow inactivation process in this chimera, with Na^+ as the charge carrier, is mechanistically similar to C-type inactivation.

Fig. 2 *C* demonstrates that extracellular K^+ slowed inactivation of Na^+ currents at submillimolar concentrations, which produced little or no detectable current in the absence of other permeant ions (not shown). The ability to study the effects of low [K^+] on Na^+-conducting K^+ channels provided us with the opportunity to examine whether K^+ slowed inactivation by interacting with either of two functionally identifiable sites, the selectivity filter binding site or a lower-affinity cation binding site external to the selectivity filter.

Selectivity filter binding site

As with Kv2.1, competition between K^+ and Na^+ plays an important role in the selectivity mechanism of the chimera. Addition of low concentrations of K^+ block Na^+ currents through the channel, and further elevation of K^+ results in production of K^+ currents (Kiss et al., 1998). In this chimera, extracellular K^+ blocked Na^+ currents with an IC_{50} of less than 30 μM (see Fig. 3). By definition, this site of competition between K^+ and Na^+, where low concentrations of K^+ block Na^+ current, directly forms part of the selectivity filter mechanism. In the experiments in Figs. 3 and 4, we asked the question of whether binding of K^+ to this selectivity filter binding site slows inactivation.

Fig. 3 illustrates the influence of low external [K^+] on inward Na^+ currents, carried by 165 mM Na^+ at 0 mV. This allowed us to examine the competition between K^+ and Na^+ entering the pore from the same direction, without complications from differential voltage-dependence of K^+ and Na^+ entry and/or binding. At a concentration of 10 μM, K^+ had little influence on current magnitude, which suggests that at this concentration, K^+ was not binding significantly to the selectivity filter (Fig. 3, *A* and *D*). Inactivation rate was also unaffected at this [K^+]. Elevation of external [K^+] to 30 μM significantly blocked Na^+ currents and markedly reduced the inactivation rate (Fig. 3, *B* and *D*). At 0.1 mM K^+, Na^+ currents were further inhibited, and the rate of inactivation was reduced to an unmeasurable rate over this duration (Fig. 3, *C* and *D*). These data demonstrate that the concentration dependence of K^+ binding to the selectivity

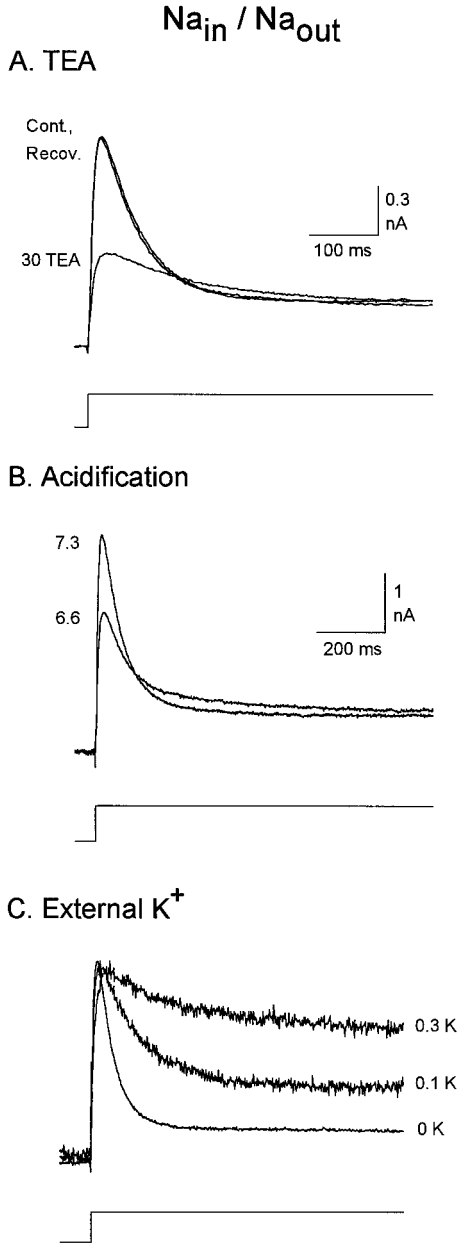

FIGURE 2 Slow inactivation of sodium currents. (*A*) Na^+ currents evoked by 500-ms depolarizations to +40 mV in the absence (control and recovery) and presence of 30 mM external TEA. The decay time constants were 49.0 ± 1.68 ms and 98.9 ± 5.6 ms (n = 5) in the absence and presence of TEA, respectively. (*B*) Na^+ currents evoked by a 1-s depolarization to +40 mV at extracellular pH values of 7.3 and 6.6. Decay time constants were 58 and 101 ms, respectively (n = 2). (*C*) Na^+ currents evoked by a 1-s depolarization to +40 mV in the presence of 0, 0.1, and 0.3 mM external K^+. Currents were normalized to the peak to illustrate the effect of K^+ on inactivation (external K^+ reduced outward currents by 75.9 ± 0.42 (n = 8) and 84.3 ± 0.36% (n = 7), respectively). In all panels, intracellular and extracellular solutions contained 140 mM and 160 mM Na^+, respectively. In *C*, K^+ was added to the extracellular solution in the concentrations shown.

filter (as measured by block of Na^+ current) was essentially identical to that required to slow the rate of inactivation. These data are consistent with the conclusion that K^+ slowed inactivation by binding to the selectivity filter.

As K^+ readily permeates K^+ channels, one would expect that, even at low concentrations, K^+ ions that bind to the selectivity filter could exit the channel by passing through the channel. This throughput of the highly permeant K^+ would result in a shift in reversal potential. Fig. 4 illustrates that, indeed, these very low concentrations of K^+ significantly shifted the reversal potential in the presence of high concentrations of Na^+. Fig. 4 *A* illustrates tail currents at different repolarization potentials in the presence of 140 mM internal and 165 mM external Na^+, after channel activation by depolarization to +40 mV. The current reversed at +4.8 ± 0.4 mV (n = 7; Fig. 4 *C*, open circles, and Fig. 4 *D*), which is at the calculated Na^+ equilibrium potential (+4.1 mV). Fig. 4 *B* illustrates tail currents from the same cell as in Fig. 4 *A*, except that 0.3 mM K^+ was added to the extracellular solution. In addition to blocking the Na^+ current, addition of K^+ shifted the current reversal potential in the positive direction (Fig. 4 *C*, filled circles), which demonstrates that, at these low concentrations, K^+ was passing through the channel. Indeed, as little as 0.1 mM K^+ resulted in a statistically significant shift in reversal potential, and increasing [K^+] progressively shifted the reversal potential to more positive potentials (Fig. 4 *D*).

An external intrapore binding site

The data in Figs. 3 and 4 are consistent with the conclusion that occupancy of the selectivity filter by K^+ slowed inactivation. However, two other conclusions are possible. First, K^+ could bind to two different intrapore binding sites with the same concentration dependence, and binding to one site (the selectivity filter) could block Na^+ currents whereas binding to a separate site could influence inactivation. Although this seems unlikely due to the low concentration of K^+ necessary to produce both effects, it nonetheless cannot be dismissed. Second, there could be more than one site at which K^+ directly slows inactivation. Thus, the fact that K^+ influences inactivation by binding to the selectivity filter does not exclude the possibility that binding of K^+ to a different site could also influence inactivation rate. The experiments in Figs. 5-7 address these possibilities.

Addition of extracellular Na^+ produced a concentration- and voltage-dependent block of K^+ currents (Fig. 5, *A* and *B*). Fig. 5 *C* illustrates currents evoked by depolarization for 1.2 s. Despite a 63% block of current, application of extracellular Na^+ did not affect inactivation kinetics (Fig. 5, *C* and *D*). The observation that block by Na^+ was voltage dependent suggests that the site of action was inside the pore. Consequently, these experiments indicate that Na^+ blocked K^+ currents by over 50% at a cation binding site in the permeation pathway and that reduction of K^+ occupancy at this site did not influence inactivation kinetics. Our hypothesis suggests that this site was not the selectivity filter binding site. However, these experiments did not address the identity of this site.

Useful data on the voltage dependence of K^+ block of Na^+ currents could not be obtained because of the high

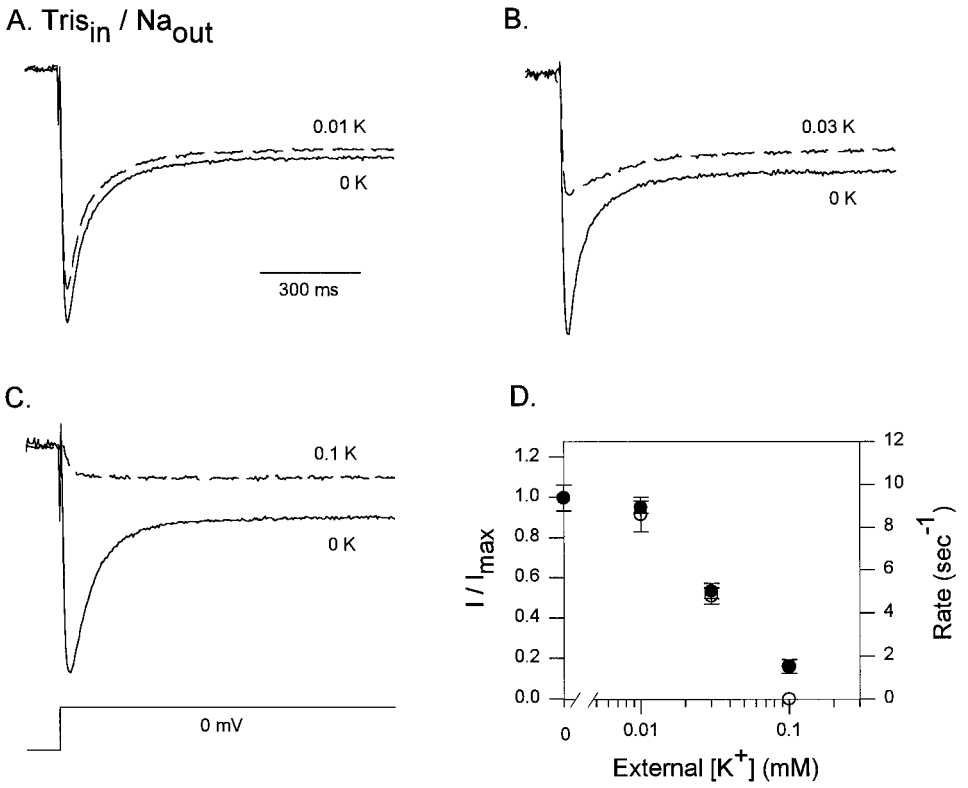

FIGURE 3 Concentration dependence of K^+-induced block of Na^+ currents and slowing of inactivation rate. (*A*, *B*, and *C*) Inward Na^+ currents, evoked by 2-s depolarizations to 0 mV, in the presence of 0 external K^+ (*solid line*), 0.01 mM (*A*, *dashed line*), 0.03 mM (*B*, *dashed line*), and 0.1 mM (*C*, *dashed line*) external K^+. The extracellular solution contained 165 mM Na^+, and the intracellular solution contained 140 mM Tris. (*D*) Superimposed plots of fractional current (●) and rate of inactivation (○) as a function of [K^+]. Values represent mean ± SEM of 6–11 cells. Plots were scaled on the ordinate such that the inactivation rate at 0 mM K^+ corresponded to the fractional current at 0 mM K^+. Inactivation at 0.1 mM K^+ was unmeasurable over this time scale and was assigned a value of 0.

throughput of K^+ through the channel. Consequently, comparing the voltage dependence of K^+ block of Na^+ and Na^+ block of K^+ currents was not informative. However, as Na^+ is capable of conducting through this chimera, even in the presence of 140 mM internal K^+ (not shown), it is reasonable to postulate that, if Na^+ were binding to the selectivity filter to block K^+ current, Na^+ would pass through the channel. The experiment in Fig. 6 tested this possibility.

Fig. 6 *A* illustrates tail currents obtained at different repolarization potentials in the presence of 3 mM internal and external K^+, after activation of the channels at +40 mV. Currents reversed at 0 mV, the K^+ equilibrium potential (Fig. 6, *A* and *C*). Addition of 100 mM external Na^+ produced a voltage-dependent block of the current but had absolutely no effect on the reversal potential (Fig. 6, *B* and *C*; $n = 4$). These data demonstrate that, under conditions where Na^+ significantly blocked currents carried by K^+, Na^+ did not pass through the channel. As Na^+ is capable of passing through the channel, even in the presence of high internal K^+ (not shown), these data further suggest that Na^+ was not reaching the selectivity filter. Consequently, these data are consistent with the interpretation that Na^+ blocked K^+ at a site distinct from and external to the selectivity filter.

Indirect influence of the outer binding site on inactivation rate

The experiments in Fig. 5, *C* and *D*, indicate that the rate of inactivation of inward currents was insensitive to whether K^+ or Na^+ was bound to the outer binding site. However, these data do not address the question of whether inactivation rate could be influenced at all by cation binding to the outer binding site. Occupancy of the outer binding site would be expected to trap K^+ at the selectivity filter and slow inactivation. The observation that millimolar external concentrations of K^+ prolong K^+ currents (Fig. 1 *D*; Baukrowitz and Yellen, 1996) is consistent with such an effect, and inconsistent with an action of K^+ at a binding site that has a micromolar affinity for K^+. The experiments in Fig. 5 would not detect such a trapping mechanism, as both the blocker and permeant ion entered the channel from the same direction. The experiments in Fig. 7 tested the possibility that binding of an ion to the outer binding site could slow inactivation by trapping K^+ at the selectivity filter.

Fig. 7 *A* shows outward currents carried by 3 mM internal K^+, evoked by depolarization to +40 mV. Substitution of extracellular Na^+ for Tris blocked the current and markedly slowed inactivation. Consistent with an action of Na^+ inside the pore, prolongation was voltage dependent (Fig. 7 *B*). These data can be explained by two possible actions of Na^+. First, switching to extracellular Na^+ would significantly increase the occupancy of the outer binding site and slow inactivation by trapping K^+ at the selectivity filter. Alternatively, occupancy of this site may have slowed inactivation not by trapping K^+ at the selectivity filter but as a direct result of the increased occupancy of this outer site. We tested these possibilities by examining the influence of external Na^+ on inactivation of outward Na^+ currents (Fig. 7 *C*).

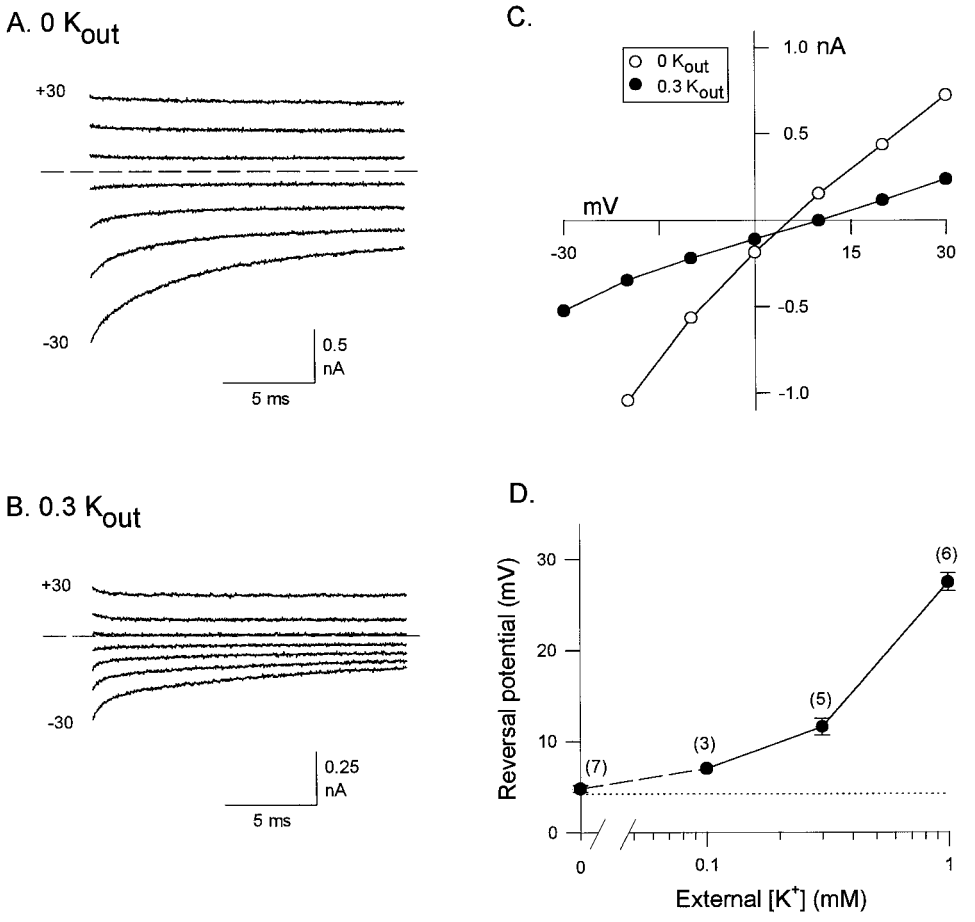

FIGURE 4 Shift in Na^+ current reversal potential by low potassium concentrations. (*A* and *B*) Tail currents at repolarization potentials between −30 and +30 mV, after current activation by a 132-ms voltage step to +40 mV. The dashed line represents 0 current level. Internal Na^+ was 140 mM and external Na^+ was 165 mM, producing a calculated Na^+ equilibrium potential of +4.1 mV. Currents were recorded in the presence of 0 K^+ (*A*) and 0.3 mM external K^+ (*B*). The extrapolated reversal potential for the currents in *A* (0 K^+) was +5.2 mV (*C*, ○). In seven cells tested, the mean extrapolated reversal potential in 0 K^+ was 4.8 ± 0.4 mV (*D*). The extrapolated reversal potential for the currents in *B* (0.3 mM K^+) was 10.0 mV; in five cells tested, the mean extrapolated reversal potential was 11.6 ± 0.9 mV (*D*). (*D*) Plot of reversal potential as a function of external [K^+]. Numbers in parentheses represent the number of cells tested. Values at all [K^+] were statistically different ($p < 0.05$ or better) from the reversal potential value at 0 K^+.

Fig. 7 *C* illustrates outward currents carried by 140 mM internal Na^+, in the presence of external Tris and Na^+. Na^+ had a very slight but significant effect on the inactivation rate at all membrane potentials between +20 and +80 mV (Fig. 7 *D*). Clearly, however, addition of external Na^+ did not prolong Na^+ currents similarly to the prolongation of K^+ currents. There are two possible interpretations to these data. The first possibility is that addition of external Na^+ did, indeed, increase the occupancy of the outer site. The second possibility is that the external cation binding site was already fully saturated due to the Na^+ traversing the channel from the inside. Consequently, addition of external Na^+ would not change occupancy of this site and would be expected to do nothing to the inactivation rate. This seems unlikely to us, as current magnitude was either unchanged or increased in the presence of external Na^+ (Fig. 7 *C*). This is consistent with an increased conductance expected from loading the pore with more permeant ion. Were occupancy unaffected by addition of external Na^+, one would expect currents to be reduced due to the large shift in Na^+ equilibrium potential. Regardless of which interpretation is correct, as Na^+ currents decayed faster than K^+ currents in the presence of external Na^+ (compare the filled squares to the filled circles in Fig. 7 *D*), these data support the conclusion that occupancy of this site by Na^+ slowed inactivation only by trapping K^+ at a more internal site. Combined with the data in Fig. 5, these data are inconsistent with the hypothesis that occupancy of the outer site by Na^+ directly slowed the inactivation process.

Finally, the hypothesis that inactivation was slowed by occupancy of a cation binding site by K^+ predicts that, as long as the channel isn't saturated by K^+, increasing internal [K^+] should result in progressively slower inactivation. Fig. 7, *E* and *F*, show that this, indeed, is the case.

DISCUSSION

Occupancy of the selectivity filter and inactivation rate

Baukrowitz and Yellen (1996) postulated that the rate of C-type inactivation reflected the off-rate of the last K^+ ion from a binding site in the permeation pathway. The primary result of this paper is that this site is the selectivity filter binding site. These findings provide information regarding both the location and mechanism of C-type inactivation.

C-type inactivation involves a cooperative movement of all four subunits (Panyi et al., 1995; Ogielska et al., 1995). Cysteine mutagenesis studies indicated that this movement changes the exposure pattern of at least three amino acids (Shaker positions 448–450) near the outer mouth of the pore (Yellen et al., 1994; Liu et al., 1996). During inacti-

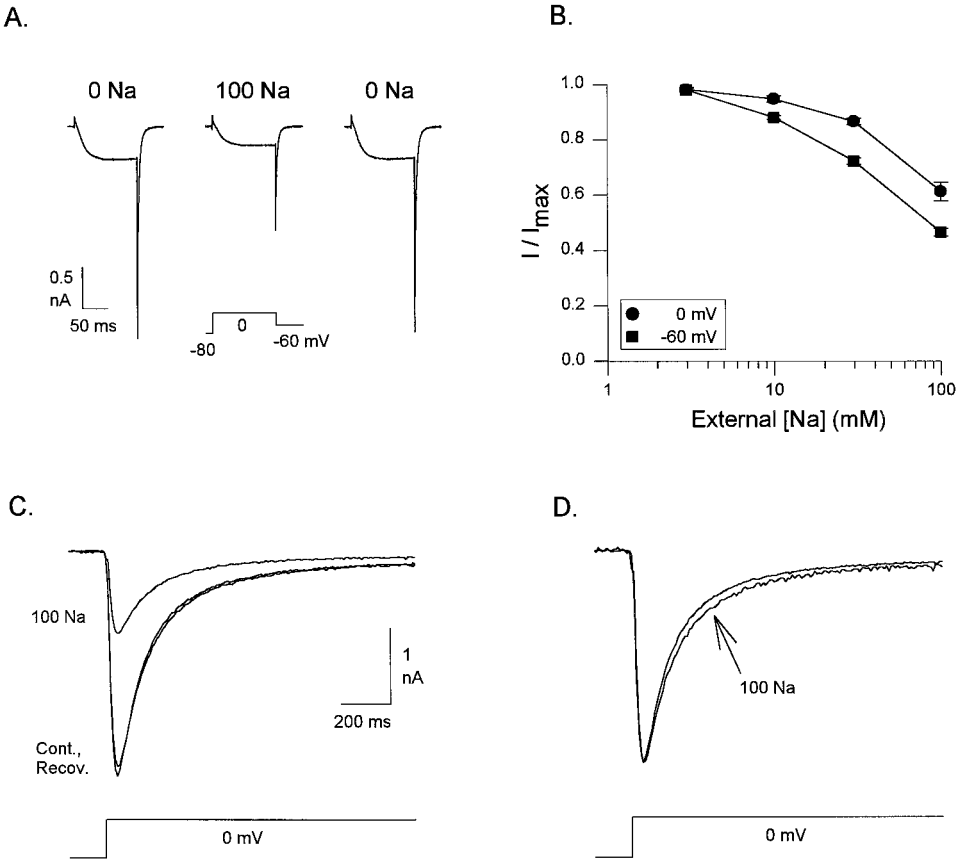

FIGURE 5 Block of inward potassium currents by external sodium. (*A*) Currents carried by 3 mM external K^+ (165 mM Tris plus 3 mM K^+ out/140 mM Tris in), evoked every 5 s, in the presence of 0 Na^+ and 100 mM Na^+ (Na^+ was added to the external solution; qualitatively similar results were obtained when Na^+ was substituted isosmotically for Tris). (*B*) Fractional current as a function of external [Na^+], measured at the end of the depolarization to 0 mV (●) and at the peak of the tail current (−60 mV; ■). Tail currents were measured 210 μs after repolarization (data were digitized at 70 μs/point). Symbols represent mean ± SEM of 4–10 cells, with at least two Na^+ concentrations tested on each cell. All error bars are plotted. (*C*) Currents carried by 3 mM K^+ (same solutions as in *A*), evoked every 15 s by depolarization to 0 mV for 1.2 s, in the absence (Cont., Recov.) and presence of 100 mM Na^+ (substitution of Na^+ for Tris and addition of Na^+ to the Tris-containing solution yielded identical results). In four cells, Na^+ blocked K^+ currents by 57.9 ± 1.9%. (*D*) Control and 100 mM Na^+ currents from *C*, normalized. Currents were well fit by two exponential functions, with time constants (in ms) of 148 ± 14 and 613 ± 69 (0 Na) and 176 ± 21 and 704 ± 111 (100 mM Na) (n = 4).

vation, cysteines at Shaker position 448 can become cross-linked, which suggests that the molecular movement during C-type inactivation brings these amino acids closer together (Liu et al., 1996). Finally, external TEA, which binds in the pore to Shaker position 449, slows the rate of C-type inactivation (Choi et al., 1991; Grissmer and Cahalan, 1989). Taken together, these data are consistent with the hypothesis that C-type inactivation involves a constriction of the outer mouth of the pore (Liu et al., 1996). This constriction appears to be localized, in that toxin binding to the outer mouth of the channel is relatively unaffected by inactivation (Liu et al., 1996).

The amino acids that were shown to move during inactivation are just one to two positions away from the signature sequence (Shaker positions 439–446), which contributes to the selectivity filter (Heginbotham et al., 1994). Our finding that occupancy of the selectivity filter binding site by K^+ slows inactivation is consistent with the conclusion that the molecular movement associated with inactivation directly involves the selectivity filter. More specifically, our data suggest a model in which inactivation is caused by constriction of the selectivity filter.

Cysteine mutagenesis studies, in both K^+ channels and cyclic nucleotide-gated channels (which have a P region highly homologous to that of K^+ channels) suggest that the narrow part of the pore is very short, perhaps 1 ionic diameter in length (see Goldstein, 1996). Single-point mutations in the signature sequence can alter the selectivity characteristics of the channel, or make the channel completely nonselective (Heginbotham et al., 1994), which suggests that just a single site may underlie selectivity in K^+ channels. Recently, we provided evidence that the presence of just a single high-affinity binding site could account for many of the permeation characteristics of K^+ channels (Kiss et al., 1998). Taken together, these data suggest that ionic selectivity may be determined by a structure that contains just a single high-affinity binding site. The cooperativity of all four subunits in the inactivation process,

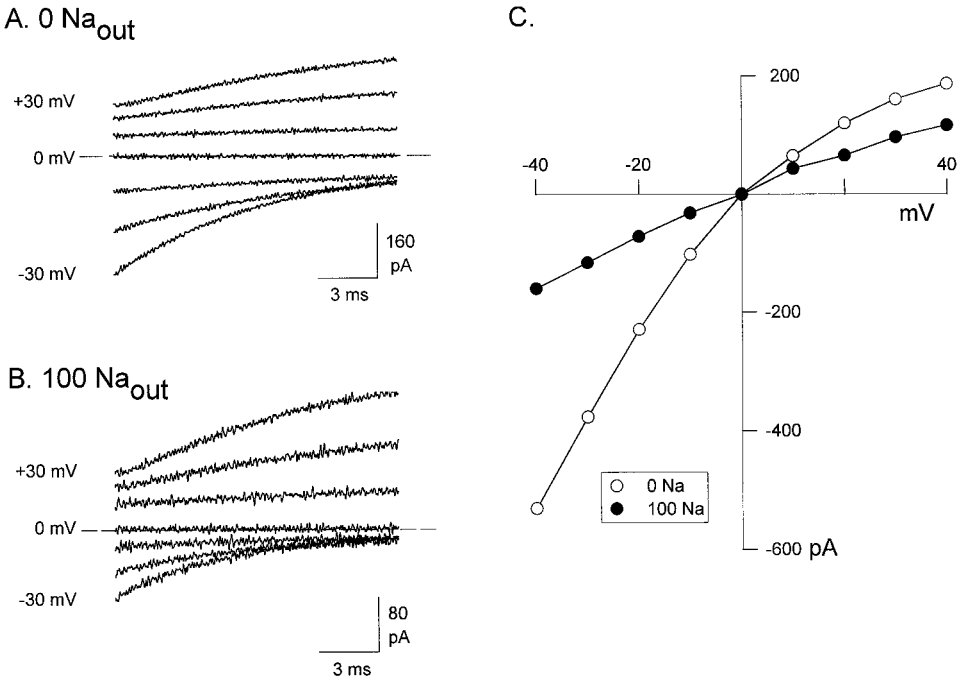

FIGURE 6 Potassium current reversal potentials in the presence and absence of sodium. The experiment was the same as that described in Fig. 4, except that currents were recorded in the presence of 3 mM internal and external K^+ (Tris as the additional cation) in the absence (*A*) and presence (*B*) of 100 mM external Na^+. Dashed lines indicate 0 current level. *C*. Instantaneous *I-V* plots of currents in *A* (○) and *B* (●). In four cells tested, measured reversal potentials were -0.25 ± 0.25 mV (0 Na^+) and -0.75 ± 0.47 mV (100 mM Na^+).

combined with these results, is consistent with a picture of the selectivity filter as a dynamic aperture. We imagine the inactivation mechanism to proceed as follows. In the open state, this aperture is fully open. During inactivation, the inner diameter of this aperture is reduced, thus decreasing conductance through the channel. While a K^+ or other cation is bound to (within?) this aperture, the constriction cannot take place. When this site is unoccupied, the constriction, and inactivation, proceeds.

Effects of external [K^+] on K^+ current inactivation

Previous observations (Lopez-Barneo et al., 1993; Marom and Levitan, 1994; Baukrowitz and Yellen, 1996), as well as our own (Fig. 1 *C*), demonstrated that elevation of external K^+ in the millimolar range slowed the rate of K^+ current inactivation. These results are consistent with a mechanism by which elevation of external K^+ increased the occupancy of K^+ on the external, low-affinity binding site and thereby trapped K^+ on the selectivity filter as K^+ traversed the channel from the inside. The external low-affinity site is relatively nonselective for K^+ and Na^+, as judged by the ability of Na^+ between 10 and 30 mM to block currents carried by 3 mM K^+ (Fig. 5). As the external site is typically exposed to ~150 mM Na^+, this suggests that the concentration dependence of this K^+ effect is derived from a competition between K^+ and Na^+ at this site. More generally, the dwell time of K^+ on the selectivity filter under physiological conditions will depend on the affinity of this outer site for K^+ and Na^+. Whereas occupancy of this site by either K^+ or Na^+ will slow inactivation, the extent of this slowing will depend on which ion, with its associated affinity, is bound. Consequently, variation among channels in the rate of C-type inactivation may depend not only on the affinity of K^+ for the selectivity filter binding site but also on the relative affinity of the external site for K^+ and Na^+.

Voltage dependence of inactivation

As observed in Figs. 1 *B* and 7, the rate of Na^+ current inactivation in this chimera was voltage dependent. As in other K^+ channels (Hoshi et al., 1991), the rate of K^+ current inactivation in the chimera was voltage independent (not shown, but see Fig. 1 *C*). This does not imply a fundamentally different mechanism of inactivation for Na^+ and K^+ currents but suggests that, as K^+ is increased, the inactivation process slows sufficiently to mask the faster, voltage-dependent event. This is consistent with the proposal of Baukrowitz and Yellen (1996) that the rate of inactivation depends on the dwell time of the last cation in the pore. Based on the trapping experiments in Fig. 7, *C* and *D*, binding of Na^+ to the selectivity filter does indeed have a small but significant effect on inactivation rate. Based on the dramatically lower affinity of Na^+ than K^+ for this site, the dwell time of Na^+ on the binding site would be predicted to be short. The observation that inactivation is voltage dependent in 100% Na^+ or very low [K^+] (where occupancy of the high affinity site is relatively low; Fig. 7) suggests that the off-rate of Na^+ from the binding site is fast relative to the intrinsic rate of the conformational change that underlies inactivation.

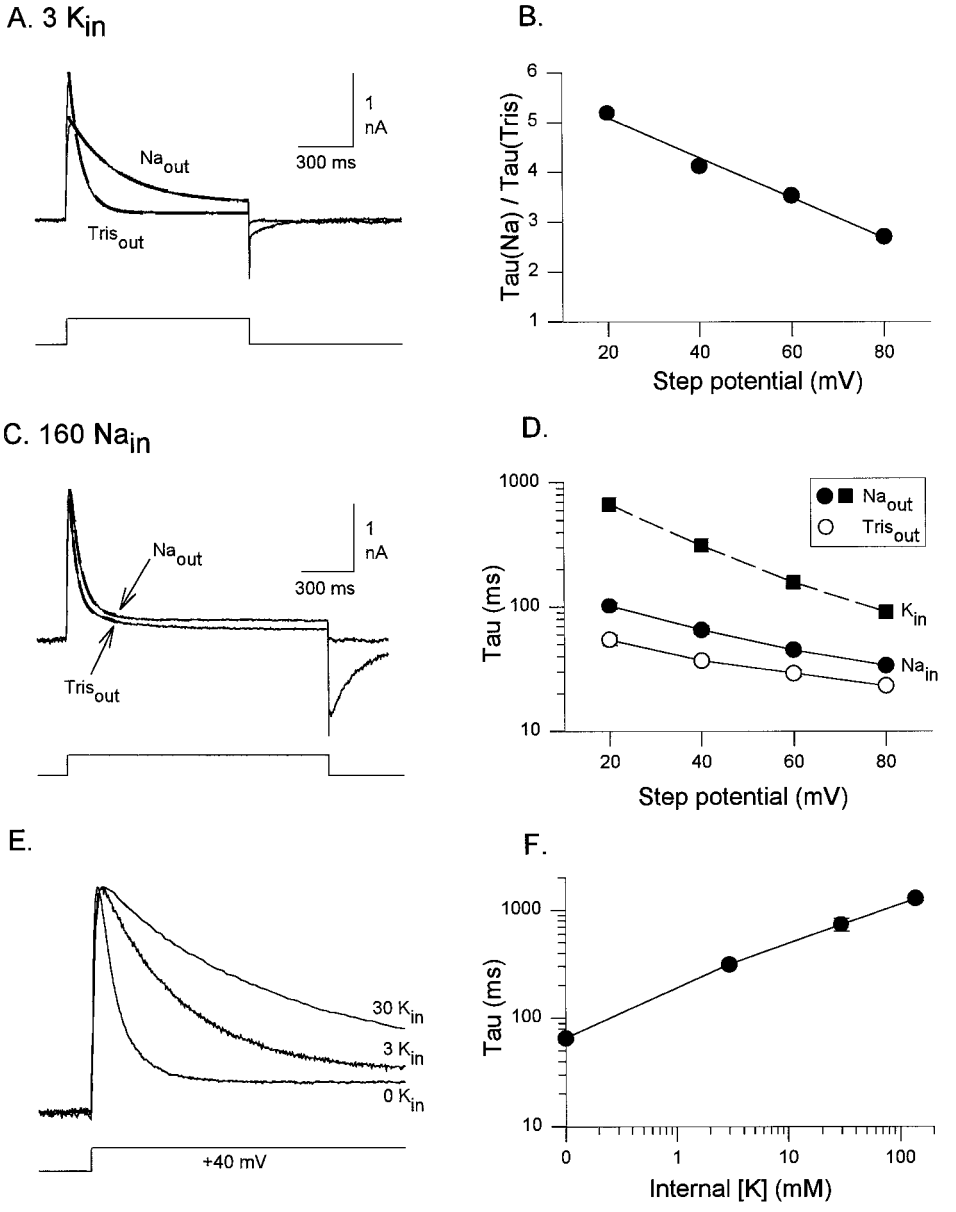

FIGURE 7 Effects of external Na^+ on inactivation of outward currents. (*A*) Outward K^+ currents, carried by 3 mM K^+, in the presence of 165 mM external Tris or Na^+. Single exponential fits are superimposed on the traces. (*B*) Voltage dependence of prolongation of outward K^+ currents by Na^+ ($n = 5$ in the presence of Tris; $n = 8$ in the presence of Na^+). (*C*) Outward Na^+ currents, carried by 140 mM Na^+, in the presence of 165 mM external Tris or Na^+. In three of four cells, currents were potentiated by 18.3 ± 0.2% ($n = 3$; $p < 0.05$; current magnitude changed by +1.7% in the other cell). Single exponential fits to the fast current component (peak to time marked by arrows) are superimposed on the curves. (*D*) Time constants of Na^+ currents in the presence of external Tris (○; $n = 7$) or Na^+ (●; $n = 6$). The filled squares represent the K^+ current time constants in the presence of external Na^+ (from the data in *A* and *B*; $n = 8$). (*E*) Outward currents evoked by a 1-s depolarization, with 140 mM Na^+ in the pipet and the three different K^+ concentrations noted. (*F*) Time constants of outward currents, as in *E* ($n = 5$–8). All error bars are plotted in *B*, *D*, and *F*.

Physiological implications

Our data suggest that inactivation rate is directly related to the occupancy of the selectivity filter by K^+. Consequently, events that alter occupancy at this site would be expected to influence inactivation rate. One possible mechanism to alter inactivation rate would be to change the affinity of this site for K^+. For example, functional diversity in K^+ channels may be achieved by subtle alterations in the amino acid composition of the pore, which could change the inherent affinity of the selectivity filter for K^+, alter the proximity of multiple K^+ binding sites in the pore (and thus influence electrostatic repulsion between K^+ bound at the selectivity filter and adjacent sites), or alter the affinity of cations for the putative low-affinity binding sites that flank the selectivity filter (Kiss et al., 1998). Events that modulate the affinity of intrapore binding sites for K^+ could similarly influence inactivation rate. Alternatively, inactivation rate could be decreased by a reduction in exit rate of K^+ from the channel and increased by a reduction of entry rate of K^+ into the channel.

Exit rate

Our data indicate that binding of a cation to a site external to the selectivity filter traps K^+ at the selectivity filter and thus increases its occupancy at this site. Consequently, inactivation rate would be expected to vary depending on the affinity of this outer site for Na^+. Furthermore, as K^+ and Na^+ compete for occupancy of this outer site, the relative affinities of K^+ and Na^+ for this site will influence the inactivation rate. Consistent with this hypothesis, elevation of external [K^+] in the millimolar range can slow the

inactivation rate of K^+ currents in physiological solutions (Lopez-Barneo, 1993; Baukrowitz and Yellen, 1995). The concentration dependence of this effect, whereby K^+ slowed inactivation with an EC_{50} of 2 mM in the presence of 150 mM external Na^+ (Baukrowitz and Yellen, 1995), is consistent with the approximate relative affinities of K^+ and Na^+ for this site suggested by the experiments of Fig. 5.

Entry rate

Decreasing the entry rate of K^+ into the pore apparently increases the inactivation rate. For example, the blocking particle involved in N-type inactivation speeds C-type inactivation, (Hoshi et al., 1991; Yellen et al., 1994). Similarly, internal TEA also speeds inactivation by decreasing K^+ entry into the pore (Baukrowitz and Yellen, 1996). The experiments in Fig. 7 *C*, in which elevation of internal $[K^+]$ slows inactivation rate, are consistent with these findings. Elevation of internal Na^+ has long been known to block K^+ channel currents (cf. Bezanilla and Armstrong, 1972). Recently, Knutson et al. (1997) suggested that glutamate reduced outward K^+ currents in oligodendrocyte progenitor cells due to block by intracellular Na^+ after glutamate-induced Na^+ influx. These results suggest the possibility that changes in internal $[Na^+]$ produced by physiological or pathological events could influence the inactivation rate by blocking entry of K^+ into the pore.

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