A Trapped Intracellular Cation Modulates K⁺ Channel Recovery From Slow Inactivation

Evan C. Ray and Carol Deutsch
Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104

Upon depolarization, many voltage-gated potassium channels undergo a time-dependent decrease in conductance known as inactivation. Both entry of channels into an inactivated state and recovery from this state govern cellular excitability. In this study, we show that recovery from slow inactivation is regulated by intracellular permeant cations. When inactivated channels are hyperpolarized, closure of the activation gate traps a cation between the activation and inactivation gates. The identity of the trapped cation determines the rate of recovery, and the ability of cations to promote recovery follows the rank order K⁺ > NH₄⁺ > Rb⁺ > Cs⁺ >> Na⁺, TMA. The striking similarity between this rank order and that for single channel conductance suggests that these two processes share a common feature. We propose that the rate of recovery from slow inactivation is determined by the ability of entrapped cations to move into a binding site in the channel’s selectivity filter, and refilling of this site is required for recovery.

INTRODUCTION

Voltage-gated potassium (KV) channels are elegantly designed to function as negative feedback regulators of membrane potential. This role is critical for repolarization of the action potential in excitable cells. Once opened by depolarization, many KV channels undergo a time-dependent decrease in conductance known as inactivation. Entry into, and recovery from, inactivated states determines the amount of K⁺ conductance available to maintain cells at a negative resting membrane potential.

At least two mechanisms contribute to K⁺ channel inactivation. N-type, or fast, inactivation occurs within milliseconds of activation as the channel’s cytosolic N terminus occludes the pore, blocking the passage of K⁺ ions through the channel (Hoshi et al., 1990; Zagotta and Aldrich, 1990). A second inactivation process occurs in response to sustained depolarization (Hoshi et al., 1991). It involves a localized rearrangement at the outer mouth of the channel (Choi et al., 1991; Yellen et al., 1994; Liu et al., 1996; Ogielska and Aldrich, 1999; Larsson and Elinder, 2000), including the outer portion of the channel’s selectivity filter (Starkus et al., 1997; Harris et al., 1998; Ogielska and Aldrich, 1999). Rates associated with this process are typically slower than N-type inactivation; thus it is commonly referred to as slow inactivation.

Several factors affect entry into the slow inactivated state. First, both the concentration and species of cations present influence the rate of entry. Increased concentrations of permeant cations decrease the rate of slow inactivation (Grissmer and Cahalan, 1989a; Lopez-Barneo et al., 1993; Baukrowitz and Yellen, 1995, 1996; Kiss and Korn, 1998; Fedida et al., 1999). The ability of different species of cations to decrease the rate of slow inactivation corresponds approximately to the ability of these cations to permeate the channel (Lopez-Barneo et al., 1993). Second, intracellular blockers accelerate entry into the slow inactivated state (Armstrong, 1969; Hoshi et al., 1991; Baukrowitz and Yellen, 1995; Rasmusson et al., 1995; Loots and Isacoff, 1998), suggesting that slow inactivation is facilitated by preventing the replacement of an outwardly exiting pore K⁺ with a K⁺ ion from the internal solution (Baukrowitz and Yellen, 1996). These observations attest to a relationship between permeation and inactivation gating and support the suggestion that occupancy of cation binding sites in the permeation pathway inhibits closure of the inactivation gate (Lopez-Barneo et al., 1993). This is a version of the “foot-in-the-door” mechanism invoked to explain the influence of monovalent cations on activation-gate closure (Swenson and Armstrong, 1981).

After inactivation, membrane hyperpolarization favors exit from the inactivated state via a gating process referred to as recovery. This process is at least as important as entry into the inactivated state in determining the number of conductive channels available to a cell. It is this availability that contributes critically to generation of an action potential, the frequency of action potential firing, and repolarization of the membrane following an action potential. The physiological importance of recovery from slow inactivation is underscored by the finding that in some channels, even when fast inactivation is

Abbreviations used in this paper: FR, fractional recovery; I, inactivated; NI, noninactivated; TMA, tetramethylammonium.
present, recovery from the slow inactivated state is still rate limiting in returning channels to a state from which they can be called upon to repolarize the cell (Rasmusson et al., 1995; Kurata et al., 2004). Although the detailed sequence of events involved in recovery from slow inactivation is not known, recovery, like entry into the inactivated state, depends on the interaction of the channel with permeant cations. Increased concentrations of extracellular cations speed the rate of recovery after slow inactivation (Pardo et al., 1992; Levy and Deutsch, 1996a,b; Rasmusson et al., 1998), and more conductive extracellular cations do so more effectively than less conductive cations (Levy and Deutsch, 1996a).

These precedents for modulation of gating kinetics by extracellular cations indicate that Kᵥ channels possess extracellular cation-binding modulatory sites. However, occupancy of these sites is not the sole determinant of inactivation rate. Indeed, cation binding to more intracellular sites along the axis of permeation also influences inactivation (Fedida et al., 1999; Ogilksa and Aldrich, 1999), an observation that has been suggested to occur as a result of electrostatic interaction between cations occupying inner and outer sites along the pore axis (Ogilksa and Aldrich, 1999). Thus, cations at internal and external sites along the permeation pathway may act in concert to modulate the rate and extent of slow inactivation. Just as entry into the inactivated state is governed by the occupancy of cations at multiple sites, so too might occupancy of internal and external sites conspire to modulate exit from the inactivated state. Although several studies have characterized the influence of extracellular cations on recovery (Pardo et al., 1992; Rasmusson et al., 1995; Levy and Deutsch, 1996a,b), little is known about the effects of intracellular cations on this process. In this study, we examine the influence of intracellular cations on recovery. We find that when the activation gate of an inactivated channel closes, a cation from the intracellular solution becomes trapped between the intracellular end of the selectivity filter and the activation gate. This trapped cation interacts with a modulatory site in the filter to govern recovery rate. The rate of recovery depends on the identity of the trapped cation and correlates with conductance of the cation through the open channel. It is the nature of the trapped cation and its ability to jump from one site to another in the selectivity filter that is common to both the recovery process and conductance through the open channel. These findings reveal a new role for K+ ions in occupying and stabilizing the conductive structure of a voltage-gated K+ channel.

**Materials and Methods**

**Cell Culture**

Human embryonic kidney cells transformed with SV40 large T antigen (tsA201) were grown in DMEM-high glucose supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin-g, and 100 μg/ml streptomycin (Invitrogen) at 37°C in a 9% CO₂ and 95% air humidified atmosphere. Cells were passaged twice per week after a 7-min incubation in Versene containing 0.2 g EDTA/L (Invitrogen).

**DNA Clones and Site-directed Mutagenesis**

Modified Shaker-IR in a G1W1-CMV mammalian expression plasmid, under the control of a highly expressing Kozak consensus promoter sequence (Kozak, 1991), was provided by R. Horn (Thomas Jefferson University, Philadelphia, PA) (Ding and Horn, 2002). This construct includes a deletion of amino acids 6–46 to remove N-type inactivation, and C301S and C308S point mutations (Holmgren et al., 1996). Amino acid substitutions at position 449 were introduced using a QuickChange site-directed mutagenesis kit (Stratagene). Mutants were sequenced at the University of Pennsylvania School of Medicine DNA Sequencing Facility using an ABI 3100 16 capillary sequencing apparatus with BigDye Taq FS Terminator V 3.1 chemistry. A calcium phosphate transfection kit (Invitrogen) was used to cotransfect CD8 carried in an EBO-pcD vector (Margolskee et al., 1988; Margolskee et al., 1993) with a Shaker-IR 449 construct using 6 or 8 μg of CD8 or Shaker-IR DNA, respectively, per 100-ml dish of tsA201 cells. Transfected cells were replated onto Corning 35-mm polystyrene cell culture dishes either pretreated with poly-l-ornithine (Sigma-Aldrich) to improve cell adhesion for pulling patches or untreated for whole-cell patching. 12–36 h following transfection, current was recorded from transfected cells, which were identified by decoration with anti-CD8 antibody-coated Dynabeads (Dynal Biotech) as described previously (Margolskee et al., 1993; Jurman et al., 1994).

**Electrophysiology**

Standard methods were used to record Ca²⁺ currents in the whole-cell configuration and K+ currents in outside-out patches. Perfusion experiments were performed on inside-out patches. Data were acquired using an Axopatch 200B amplifier with 10 kHz filtration (Axon Instruments, Inc.), digitized with a Digidata 1322A analogue-to-digital converter, and recorded with a 20-kHz sampling interval using Clampex (Axon Instruments, Inc.) on a personal computer (Dell). For whole-cell and outside-out experiments, electrodes of 1.6–2.2 MΩ resistance were pulled from SG10 leaded glass (Richland Glass). For inside-out patches, 8–11 MΩ pipettes were pulled from lead-free 8520 glass (Warner Instruments). Pipettes were coated with R-6101 elastomer (Essex Group, Inc.) and fire polished. Adjustments for bath–pipette liquid junction potentials were made before current recording. Whole-cell recordings were performed after a 10-min dialysis period to ensure equilibration of pipette and intracellular solutions. All holding potentials were −100, and voltage errors were <3 mV after series resistance compensation. All experiments were performed at room temperature (20–24°C). Data were analyzed using Clampfit (Axon Instruments, Inc.) and Igor (WaveMetrics, Inc.). Unless otherwise stated, reported errors are SEM.

**Solutions**

Standard intracellular solutions contained 105 XF, 35 XC1, 10 EGTA, 10 HEPES (X is the relevant cation and concentrations are given in mM), titrated to pH 7.36–7.38 with XOH, for a final concentration of 160–165 mM and osmolarity of 285–295 mOsm. Standard extracellular solution was 20 mM KCl, 1.5 CaCl₂, 1.0 MgCl₂, and 10 HEPES. Osmolarity was brought to 296–300 mOsm with NMG, and pH was titrated to 7.36–7.38 with HCl. For solutions with reduced test cation concentrations, osmolarity was maintained by the addition of an appropriate concentration of NMG. All chemicals were obtained from Fisher Scientific or Sigma-Aldrich.
Recovery Measurements
To measure recovery, a standard two-step voltage protocol was used. Unless otherwise stated, voltage steps were to +50 mV. The duration of the first step used to inactivate channels and measure initial peak current amplitude was at least fivefold longer than the channel’s time constant for inactivation. For longer pulses, recovery rates did not change as a function of pulse duration (not depicted). After a recovery period at −100 mV, the second voltage step was applied, and peak recovered current amplitude was measured. No leak subtraction was used. Fractional recovery (FR) was calculated as $(I_{rec} - I_{ss})/(I_o - I_{ss})$, where $I_o$, $I_{rec}$, and $I_{ss}$ represent the peak current amplitudes during the first depolarizing step, the second depolarizing step, and the inactivated steady state, respectively (Levy and Deutsch, 1996a). In whole-cell recordings in Cs$^+$ (Figs. 1–3), because peak Cs$^+$ current amplitude was not fully restored within a reasonable recovery period (≤20 min), no depolarizing step was applied during the initial 10-min dialysis period, after which a single two-pulse protocol was administered, and the cell was discarded.

Perfusion Experiments
Inside-out patches were perfused with standard intracellular solutions at a rate of 1 ml/min. The pipette solution contained standard 20 mM K$^+$ with NMG. Reversal potentials were measured on each patch to confirm complete perfusion. Solution switches were performed using a RSC-160 perfusion apparatus (Bio-Logic Science Instruments). On average, perfusion was ≥95% complete within 90 ms. Voltage protocols were modified to compensate for small liquid junction potentials arising from solution switching. To ensure full restoration of current between recovery measurements, patches were maintained at −100 mV in K$^+$, for ≥4 min after depolarization in intracellular K$^+$. Statistical significance of differences in mean fractional recovery was determined using the Student-Newman-Keuls all pairwise multiple comparison procedure.

RESULTS
Intracellular Cs$^+$ Slows Recovery
To study the influence of intracellular permeant ions on recovery, we sought conditions in which recovery could be easily characterized, including conditions producing tractable inactivation and recovery rates. Although Shaker-IR inactivates, its relatively slow inactivation time constant $(\tau_{inact})$ of 1.3 s in 2 mM extracellular K$^+$ is too large for convenient recovery measurements. In contrast, the Shaker-IR 449A and 449K mutants have a $\tau_{inact}$ of 160 and 40 ms, respectively (Lopez-Barneo et al., 1993). Therefore, we compared recovery time courses for Shaker-IR (T449), and mutants Shaker-IR 449A and Shaker-IR 449K. We believe this allows us to study the same inactivation process because, although it is possible that a 449 mutation fundamentally alters the mechanism of slow inactivation, there is no evidence
which a complete recovery time course was recorded. For Cs at a single RP.

square minimization using data from all cells simultaneously. This was necessary because in some cells as few as one determination of recovery was made the number of cells contributing to the FR for each point on the x axes shown in Fig. 1. The parameters and their errors were estimated by weighted chi-

slow phases of the curve; and Aslow and Afast denote the weight coefficients for each of these phases. When was not significantly larger than 0, the time course was assumed to be monoexponential and was re-fit with the single exponential function FR

τ

chain. T449 recovers quickly in K

was slowed depended on the identity of the 449 side in intracellular K

and Table I, recovery consistently occurred more slowly in Cs

dilute extracellular K

was not significantly larger than 0, the time course was assumed to be monoexponential and was re-fit with the single exponential function FR = 1 − exp(−RP/τrec), where RP represents the recovery period, i.e., the time that channels were allowed to recover at the −100 mV holding potential; τfast and τslow represent the time constants for the fast and slow phases of the curve; and λfast and λslow denote the weight coefficients for each of these phases. When τrec and τfast did not differ significantly or λfast was not significantly larger than 0, the time course was assumed to be monoexponential and was re-fit with the single exponential function FR = 1 − exp(−RP/τrec), where τrec represents the single exponential recovery time constant. For K

i, values in parentheses represent the number of patches from which a complete recovery time course was recorded. For Cs

i, no cell contributed more than one FR measurement, and values in parentheses represent the number of cells contributing to the FR for each point on the x axes shown in Fig. 1. The parameters and their errors were estimated by weighted chi-square minimization using data from all cells simultaneously. This was necessary because in some cells as few as one determination of recovery was made at a single RP.

to support this conjecture. Furthermore, 449 mutants exhibit at least one hallmark of slow inactivation: a dependence of inactivation rate and recovery rate (unpublished data) on extracellular [K+]i. Initially, we studied 449 mutants using K

o or poorly permeant Cs

as the intracellular cation. The permeability ratio of these cations (P

o/P

K

i) is 0.10, and the conductance ratio (γ

o/γ

K

i) is 0.01 (Heginbotham and MacKinnon, 1993).

For these recovery measurements, 20 mM extracellular K

(K

o) was used because of the diminutive nature of Cs

i currents through 449K channels in more dilute extracellular K

solutions. As shown in Fig. 1 and Table I, recovery consistently occurred more slowly in the presence of intracellular Cs

(Cs

i) than in intracellular K

(K

i). The degree to which recovery was slowed depended on the identity of the 449 side chain. T449 recovers quickly in K

i, and an exponential function fits the T449 recovery time course well, with τrec = 3.27 ± 0.14 s. Recovery of T449 occurred slightly more slowly in Cs

i and is better fit with a double-exponential function.

The mutants exhibit more dramatic phenotypes. 449A recovers with biphasic kinetics (Fig. 1; see also Meyer and Heinemann, 1997). This is true regardless of the identity of the intracellular cation (unpublished data). However, in the presence of Cs

i, the biphasic nature of the recovery time course becomes more pronounced, with τslow (~10-fold larger than τfast. In 449K, recovery is bi-exponential and even more dependent on the nature of the intracellular cation. When K

o is present on both sides of the membrane (K

o/K

i), the time constants of the two recovery phases differ by less than fivefold. Replacement of K

o with Cs

i produces a dramatic slow phase with a weight coefficient, Afast of 0.60 ± 0.03, and a 100-fold difference in τfast and τslow. The prominent slow phase observed in Cs

i suggests that replacement of K

i with Cs

i causes a substantial fraction of channels to enter a more stable inactivated state in response to a sustained depolarizing voltage step.

Because binding of extracellular K

o to inactivated K channels promotes recovery (Levy and Deutsch, 1996a,b), we asked whether slow recovery occurs merely as a consequence of removal of K

o, or as a result of the

| Mutant | Intracellular Cation | λfast or τrec (s) | Afast or τslow (s) |
|--------|---------------------|-----------------|------------------|
| T449 (WT) | K

i (6) | – | 3.27 ± 0.14 | – |
| Cs

i (3–4) | 0.88 ± 0.06 | 3.46 ± 0.45 | 0.13 ± 0.06 | 62 ± 50 |
| 449A | K

i (5) | 0.47 ± 0.14 | 7.4 ± 2.0 | 0.53 ± 0.14 | 322 ± 4.9 |
| Cs

i (3–6) | 0.72 ± 0.12 | 26.6 ± 4.9 | 0.28 ± 0.12 | 240 ± 190 |
| 449K | K

i (3) | 0.87 ± 0.09 | 3.98 ± 0.87 | 0.13 ± 0.11 | 24 ± 16 |
| Cs

i (3–7) | 0.40 ± 0.03 | 4.8 ± 1.1 | 0.60 ± 0.03 | 530 ± 160 |

Time courses were fit with the double exponential function: FR = 1 − [λfast*exp(−RP/τfast) + λslow*exp(−RP/τslow)], where RP represents the recovery period, i.e., the time that channels were allowed to recover at the −100 mV holding potential; τfast and τslow represent the time constants for the fast and slow phases of the curve; and λfast and λslow denote the weight coefficients for each of these phases. When τrec and τfast did not differ significantly or λfast was not significantly larger than 0, the time course was assumed to be monoexponential and was re-fit with the single exponential function FR = 1 − exp(−RP/τrec), where τrec represents the single exponential recovery time constant. For K

o, values in parentheses represent the number of patches from which a complete recovery time course was recorded. For Cs

i, no cell contributed more than one FR measurement, and values in parentheses represent the number of cells contributing to the FR for each point on the x axes shown in Fig. 1. The parameters and their errors were estimated by weighted chi-square minimization using data from all cells simultaneously. This was necessary because in some cells as few as one determination of recovery was made at a single RP.

Figure 2. Reduction of intracellular Cs

i speeds recovery. 449A current traces in response to a step to +50 mV are shown before (1) and after (2) a 90-s RP at −100 mV. Dashed gray line represents zero-current level. Reduction of Cs

i increased FR from 0.69 (in 164 mM Cs

i) to 0.90 (in 10 mM Cs

o). Addition of NMG

+ to the 10 mM Cs

o solution maintained osmolarity. K

i was 5 mM to maintain an outward driving force with both intracellular solutions. Decreased outward driving force in 10 mM Cs

o results in smaller ionic currents, allowing gating currents (*) to be readily observed. For the current traces shown, the inactivation time constants obtained in 164 and 10 mM Cs

o are 164 and 47 ms, respectively. The average time constants (±SEM) are 180 ± 17 ms (n = 3) and 47.3 ± 2.9 ms (n = 4), respectively.
presence of Cs\(^{+}\). If recovery is slower in Cs\(^{+}\), simply due to K\(^{+}\) removal, then fractional recovery (FR) in the absence of K\(^{+}\) should not vary with Cs\(^{+}\) concentration. In contrast, if slow recovery were due to binding of Cs\(^{+}\) to the channel, then a reduction in Cs\(^{+}\) would speed recovery. We therefore compared fractional recoveries for 449A in 164 and 10 mM Cs\(^{+}\), each in the absence of K\(^{+}\). Fig. 2 shows current recordings during both pulses of a two-pulse recovery protocol in 164 mM Cs\(^{+}\) (left) and 10 mM Cs\(^{+}\) (right). In 164 mM Cs\(^{+}\), a significantly smaller fraction of channels recovered than in 10 mM Cs\(^{+}\) after 90 s at −100 mV. Reduction of Cs\(^{+}\) increased FR from 0.68 ± 0.01 (in 164 mM Cs\(^{+}\), \(n = 3\)) to 0.91 ± 0.02 (in 10 mM Cs\(^{+}\), \(n = 5\)). The simplest explanation for this finding is that intracellular Cs\(^{+}\) binds to a site within the channel and, in so doing, promotes slow recovery.

### The Recovery Modulatory Site Resides in the Permeation Pathway

To determine whether the binding site lies in the permeation pathway, we examined the relationship between fractional recovery and direction of current flow in 449K using K\(^{+}\)/Cs\(^{+}\). This mutant, as demonstrated by Fig. 1 and Table I, was the mutant of choice for all subsequent experiments. If the binding site lies in the permeation pathway, large outward currents that flood the pathway with Cs\(^{+}\) would tend to retard recovery; large inward currents that flood the pathway with K\(^{+}\) would speed recovery. In contrast, if the binding site for the modulatory cation resides outside the permeation pathway, recovery should be independent of current direction.

To manipulate current direction, the potential at which channels were inactivated was varied and the dependence of FR on inactivating potential was measured (Fig. 3). Cation concentrations (20 K\(^{+}\)/80 Cs\(^{+}\)) were chosen to render a positive reversal potential (\(E_{\text{rev}} \approx 29\) mV) so that FR could easily be measured at multiple test potentials on either side of \(E_{\text{rev}}\). Potentials significantly more positive than \(E_{\text{rev}}\) resulted in outward Cs\(^{+}\) currents with small FR (0.37 ± 0.03 at 70 mV). This FR resembled the value previously observed in 20 K\(^{+}\)/164 Cs\(^{+}\), at +50 mV (0.35 ± 0.01, Fig. 1). Fractional recovery increased after inactivation at potentials closer to \(E_{\text{rev}}\). At potentials less positive than \(E_{\text{rev}}\), large inward K\(^{+}\) currents resulted, and FRs at 10 mV (0.75 ± 0.06) approached a maximum, similar to that observed in the 20 K\(^{+}\)/80 K\(^{+}\) control (0.77 ± 0.02 at 10 mV). Fractional recovery in 20 K\(^{+}\)/80 K\(^{+}\) showed no sensitivity to changes in inactivation potential, with similar FR after inactivation at 10 and 90 mV (0.76 ± 0.02 at 90 mV). Thus, FR is voltage dependent under bi-ionic conditions, consistent with a modulatory binding site within the permeation pathway. Alternatively, the recovery rate could be set intrinsically by the depolarized voltage that causes inactivation. However, this alternative interpretation fails to explain (1) the invariance of FRs obtained for channels bathed in bilateral K\(^{+}\) (K\(^{+}\)/K\(^{+}\)) and depolarized to different potentials, (2) the striking similarity between FRs obtained for channels bathed in K\(^{+}\)/K\(^{+}\) and those bathed in bi-ionic solutions (20 K\(^{+}\)/80 Cs\(^{+}\)) at potentials allowing large inward currents, and (3) the presence of the reversal potential within the voltage range through which fractional recovery crosses between its extremes. We therefore favor the conclusion that the binding site (or sites) governing recovery resides within the permeation pathway.

### Location of the Binding Site for Intracellular Cations

Where in this pathway does intracellular Cs\(^{+}\) bind? On the intra- or extracellular side of the slow inactivation gate? For simplicity, we refer to the location of cation–channel interaction as a single binding site, although...
this interaction may occur through multiple binding sites. To address this question, inside-out patches were subjected to pulses of Cs\(^{+}\) at various times during inactivation and recovery. If the cation binding site resides on the extracellular side of the inactivation gate, Cs\(^{+}\) must access that site before closure of the gate. Application of Cs\(^{+}\) before inactivation will slow recovery, whereas Cs\(^{+}\) application after inactivation will have no effect on recovery. If the binding site is present on the intracellular side of the inactivation gate and still accessible to Cs\(^{+}\), a Cs\(^{+}\) pulse may suffice to slow recovery.

Three technical issues were considered in these experiments: (1) the solution exchange time for the pulsed applications, (2) the junction potentials induced by solution changes, and (3) ensuring complete recovery between protocols. The first two issues were addressed by
direct measurement (Fig. 4 A; Materials and Methods). Complete exchange (>-95%), determined by $E_{\text{rev}}$, occurred within 90 ms, and voltage protocols included corrections for all junction potentials. The third issue was particularly relevant because multiple FR measurements were made with each inside-out patch. To allow channels to fully recover between protocols, patches were maintained at the holding potential (~100 mV) in $K^+$ for a minimum of 4 min. Regardless of whether $Cs^+$ was applied, the second test depolarization of the two-pulse protocol (applied in the presence of $K^+$) followed by a 4-min “reset” period (also in the presence of $K^+$), restored the original current amplitude. With these issues resolved, we proceeded to carry out the experiments shown in Fig. 4 (B and C) (Protocols 1–23).

When $K^+$ was continuously applied to channels during a two-pulse protocol, a fraction of inactivated channels equal to 0.89 ± 0.01 recovered in 9 s (Protocol 1). However, when $Cs^+$ perfusion was initiated 21 ms after the start of the depolarizing pulse and maintained for the majority of the recovery period, FR decreased to 0.49 ± 0.01 (Protocol 2). This observation confirms that an exposure of the channel to intracellular $Cs^+$ is sufficient to slow recovery, even when the peak current recorded is carried by $K^+$. A $Cs^+$ pulse applied to open channels as their inactivation gates close, but terminated before the end of the depolarizing pulse (Protocol 3), resulted in FR of 0.87 ± 0.01, statistically indistinguishable (P = 0.45) from the FR obtained when $K^+$ is present continuously. This finding suggests that the modulatory binding site does not reside on the extracellular side of the inactivation gate and is more likely to lie on the intracellular side. Moreover, if channels bind $Cs^+$ during the depolarization in Protocol 3, from either the open or the inactivated state, they rapidly release it at the end of the $Cs^+$ pulse.

Indeed, if the modulatory site is on the intracellular side of the slow inactivation gate and accessible, then we expect application of $Cs^+$ to inactivated channels to slow recovery. To test this hypothesis, we applied $Cs^+$ to inactivated channels as they recovered from inactivation. In Protocols 4–6, $Cs^+$ pulses were administered to inactivated channels during the initial, middle, or final third of a 9s recovery period. No significant difference in FR occurred when $Cs^+$ was applied during the middle (FR = 0.88 ± 0.01) or final (FR = 0.86 ± 0.01) thirds of the recovery period (P = 0.33 and 0.10, respectively). However, FR was slightly, but significantly, smaller when $Cs^+$ was applied during the first third of the recovery period (FR = 0.80 ± 0.01, P < 0.01).

These results suggest that a period of sensitivity to $Cs^+$ occurs soon after inactivated channels are returned to a hyperpolarized holding potential. To identify the specific time interval that is ion sensitive, we used overlapping $Cs^+$ pulses of equal (~400 ms) duration applied before, during, and after the end of the first depolarizing pulse in a two-pulse protocol (Fig. 4 C). $Cs^+$ pulses ending before the termination of the depolarizing pulse failed to reduce FR (Protocols 8–10), consistent with the results shown in Fig. 4 B. When $Cs^+$ was present as channels returned to their holding potential, FR decreased significantly (Protocols 11–18), whereas later in the recovery period (>100 ms after the end of the depolarizing pulse), channels became insensitive to $Cs^+$ (Protocols 19–23). Assuming that slow inactivation effectively bars access of intracellular $Cs^+$ to binding sites on the extracellular side of the inactivation gate, these results provide additional evidence that the modulatory binding site resides on the intracellular side of this gate.

To explain the decreased recovery that is manifest many seconds after a brief $Cs^+$ pulse, we propose that an intracellular cation is trapped in the permeation pathway after return of a channel to its holding potential. In support of this hypothesis, the original current amplitude was completely restored between consecutive two-pulse protocols (allowing repeated FR measurements to be made on the same patch), suggesting that trapped $Cs^+$ can be released by the second depolarization in a two-pulse protocol. To explicitly test whether depolarization releases the trapped $Cs^+$, we modified Protocol 14 shown in Fig. 4 C to include a 10-ms step to +50 mV after the $Cs^+$-to-$K^+$ switch. This 10-ms step was sufficient to fully restore the FR to levels seen in the absence of a $Cs^+$ pulse (Fig. 5 A). This suggests that a trapped $Cs^+$ continued to slow recovery, even after intracellular $Cs^+$ was washed away, and a liberation step during the recovery period released the trapped $Cs^+$. Consistent with this conclusion, a 10-ms step in the continued presence of $Cs^+$ does not restore FR, eliminating the possibility that depolarization itself restores FR.

A straightforward interpretation of this observation would be that depolarization opened the channel’s activation gate, allowing trapped $Cs^+$ to be replaced with $K^+$ from the internal solution. If release of $Cs^+$ is dependent on opening of the activation gate, then recovery should be a function of activation-gate open probability ($P_a$) and the kinetics of $Cs^+$ inhibition should correlate with the kinetics of activation. To test this hypothesis, we compared recovery after liberation voltage steps to two different voltages, 0 and +80 mV. At these voltages, the channel has markedly different activation kinetics, activating more slowly at 0 mV than at +80 mV. We measured FR as a function of liberation-step duration and compared this to activation rates of the channel from a holding potential of −100 mV to 0 or +80 mV. At +80 mV, $Cs^+$ liberation kinetics bore a striking resemblance to those of channel activation at the same potential, even faithfully recapitulating the sigmoidal delay in Shaker’s activation time course (Hoshi et al., 1994; Zagotta et al., 1994a; Zagotta et al., 1994b). This agreement suggests that the rate-limiting step in $Cs^+$ liberation
The rate of opening of the activation gate. The same experiment performed with a liberation voltage step to 0 mV confirms this conclusion. At 0 mV, activation occurred significantly more slowly than at +80 mV, as did the kinetics of Cs$^+$ liberation. The correlation between Cs$^+$ liberation and channel opening kinetics provides strong evidence that a depolarizing pulse releases trapped Cs$^+$ from inactivated channels by opening the channel’s activation gate.

These results suggest that slow recovery occurs when Cs$^+$ is trapped between the channel’s activation and inactivation gates. In contrast, K$^+$ does not slow recovery, either because it does not occupy this site or because, when in this site, it influences recovery differently. We next asked how other intracellular monovalent cations influence recovery.

Selectivity of the Modulatory Binding Site

The channel’s permeation pathway includes both the selectivity filter and the central cavity (Doyle et al., 1998; Hille, 2001; Zhou et al., 2001), a 10-Å-wide aqueous vestibule located halfway through the lipid bilayer and thought to be accessible to the intracellular solution in the channel’s open state (Jiang et al., 2002b). Both the
selectivity filter and the central cavity exhibit ion selectivity (Neyton and Miller, 1988; Hille, 2001; Nimigean and Miller, 2002; Y. Zhou and MacKinnon, 2004). Because the binding site for the intracellular cation is within the permeation pathway (Figs. 3–5), we expect the modulatory binding site to be ion selective as well.

To probe the selectivity of the modulatory site, we measured recovery as a function of intracellular cation. Pulses of various intracellular cations were applied to inactivated 449K channels either at +50 mV, during the transition from +50 to −100 mV, or late in the recovery phase at −100 mV (Fig. 6 A). As with Cs⁺, the other monovalent cations, Rb⁺, NH₄⁺, or Na⁺, reduced FR when the cation pulse coincided with the transition from +50 to −100 mV, but had no effect when applied earlier or later in the protocol. Pulses of NMG⁺ did not change FR (Fig. 6 B). To examine the entire recovery time course for each test cation, we used a protocol similar to Protocol 2 in Fig. 6 A. The results are plotted in Fig. 6 C, along with the fits to a double-exponential function containing a fast and slow time constant (τ_fast and τ_slow) and weight coefficients, A_fast and A_slow for the respective fast and slow phases of recovery. For permeant cations, K⁺, NH₄⁺, Rb⁺, or Cs⁺, regardless of the intracellular cation present when inactivated channels are returned to the hyperpolarized holding potential, τ_fast, A_fast, and A_slow for the recovery time courses are similar (Table II). The simultaneous fit to the time courses of all of the permeant cation pulse recoveries shown in Fig. 6 C demonstrates that the variation of a single parameter, τ_slow, is sufficient to account for each time course (χ² = 0.007).
The invariance of $A_{\text{slow}}$ indicates that the identity of the applied permeant cation does not influence the fraction of channels that recover slowly. However, the variation in the rate of recovery, $1/\tau_{\text{slow}}$, with type of intracellular cation is consistent with the hypothesis that a modulatory cation is trapped following return of channels to a hyperpolarized potential and that the nature of this trapped cation determines the recovery rate. Entrapment is supported by the fact that all of the non-K$^+$ permeant cations’ slow recovery long after the intracellular solution is switched back to K$^+$. Slow recovery can be explained by one of two possible scenarios: the presence of a particular cation in the modulatory site, or by an empty modulatory site. The latter model can be eliminated based on the following considerations. First, raising Cs$^+$ slows the recovery rate (Fig. 2), exactly the opposite result expected if empty modulatory sites promote slower recovery. Second, a model in which empty modulatory sites underlie slow recovery predicts that the rate of the slower component would be the same for all ion species, a prediction that is also in conflict with our data. This suggests that the fast recovery observed in the presence of K$^+$ occurs not because K$^+$ fails to occupy the modulatory binding site, but because K$^+$, when trapped at this site, catalyzes recovery more effectively than do other cations. Consistent with this hypothesis, we interpret the similarity between the kinetics of recovery from channels bathed in bilateral K$^+$ ($\tau_{\text{rec}} = 4.3$ s) and the fast phase of recovery after the application of other intracellular cations ($\tau_{\text{fast}} = 2.5–3.8$ s) as representing the fraction of channels containing a trapped K$^+$ at the modulatory site.

The effect of intracellular impermeant cations, NMG$^+$ and Na$^+$, on recovery differs from that seen for permeant cations. The recovery time course after a pulse of NMG$^+$ displays monoeponential kinetics ($\tau_{\text{rec}} = 4.01 \pm 0.02$ s, Fig. 6 C) that closely resemble the recovery seen from channels bathed in K$^+$. These results indicate that NMG$^+$ does not stabilize a long-lived nonconducting state. Either a trapped NMG$^+$ fails to bind to the modulatory site or NMG$^+_{\text{i}}$ does not become trapped behind the activation gate. However, tetramethylammonium (TMA), a cation that can enter the channel from the intracellular compartment, but cannot permeate further into the selectivity filter, prevents recovery. When 160 mM TMA is applied to inactivated 449K channels using Protocol 2 shown in Fig. 6 A, FR is 0.64 ± 0.03 at 90 s and 0.62 ± 0.02 at 180 s (Fig. 6 C). When Na$^+$ is applied, only 0.83 ± 0.01 of the original current amplitude is restored in the 180-s period during which recovery was examined. This time course is best fit with a bi-exponential function with $\tau_{\text{fast}}$ similar to that seen when K$^+$ is applied (3.93 ± 0.43 s) and a very large $\tau_{\text{slow}}$ (980 ± 650 s). $A_{\text{slow}}$ is small (0.20 ± 0.02) compared with that observed for permeant cations, suggesting that 80% of channels trap a K$^+$ and 20% trap Na$^+$ in the modulatory site. In the latter case, the channel does not recover (see Discussion, Fig. 8).

Our working hypothesis to explain these data is that inactivated channels, when returned to a hyperpolarized holding potential, trap a cation behind the activation gate. The ability of channels to return to a noninactivated state depends on the identity of the trapped cation, in the following rank order: K$^+$ > NH$_4^+$ > Rb$^+$ > Cs$^+$ >> Na$^+$, TMA.

**DISCUSSION**

Although the processes of permeation and gating occur on time scales that typically differ by more than five orders of magnitude, gate movement may be highly sensitive to permeant ions that transiently visit binding sites of the permeation pathway. The regulation of gating by permeant ions is highlighted in our study of the recovery from slow inactivation in *Shaker*. First, the rate of recovery depends upon the species of permeant ion present on the intracellular side of the channel. The recovery from inactivation at a hyperpolarized voltage is relatively rapid in the presence of internal K$^+$ and is slower if the channels are hyperpolarized in the presence of intracellular NH$_4^+$, Rb$^+$, Cs$^+$, Na$^+$, or TMA. Second, the ion dependence of recovery derives from entrapment of a cation within the permeation pathway, specifically between the channel’s slow inactivation gate and the intracellular activation gate. This trapping is caused by activation

### Table II

| Perfused Cation | Independent Fit | Global Fit |
|----------------|----------------|------------|
|                | $A_{\text{slow}}$ | $\tau_{\text{fast}}$ (s) | $\tau_{\text{slow}}$ (s) | $A_{\text{slow}}$ | $\tau_{\text{fast}}$ (s) | $\tau_{\text{slow}}$ (s) |
| Permeant Cations | | | | | | |
| K$^+$ | – | 4.30 ± 0.03 | – | 0.37 ± 0.01 | 3.18 ± 0.16 | 6.78 ± 0.63 |
| NH$_4^+$ | 0.33 ± 0.01 | 3.25 ± 0.10 | 56 ± 3.2 | 0.37 ± 0.01 | 3.18 ± 0.16 | 47 ± 4.5 |
| Rb$^+$ | 0.38 ± 0.01 | 2.54 ± 0.19 | 201 ± 27 | 0.37 ± 0.01 | 3.18 ± 0.16 | 290 ± 28 |
| Cs$^+$ | 0.36 ± 0.01 | 3.81 ± 0.12 | 358 ± 32 | 0.37 ± 0.01 | 3.18 ± 0.16 | 355 ± 62 |
| Impermeant Cations | Na$^+$ | 0.20 ± 0.02 | 3.93 ± 0.43 | 980 ± 650 | – | – | – |
| NMG$^+$ | – | 4.01 ± 0.02 | – | – | – | – |

Time courses were fit as described in Table I. See Fig. 6 C for description of global fit.
gate closure upon repolarization of the channel. Fig. 7 proposes a mechanism involving cation movement between modulatory and exchangeable sites within the permeation pathway. This mechanism is largely consistent with our data and constitutes a framework for the following discussion.

Location of the Trapped Cation
Slow inactivation involves a rearrangement of the outer mouth of the channel and a portion of the selectivity filter (Grismer and Cahalan, 1989b; Choi et al., 1991; Yellen et al., 1994; Liu et al., 1996; Starkus et al., 1997; Harris et al., 1998; Kiss et al., 1999; Ogielska and Aldrich, 1999; Larsson and Elinder, 2000; Wang et al., 2000). This rearrangement prevents the passage of ions across the selectivity filter. The conducting conformation of the selectivity filter includes five adjacent cation binding sites numbered 0 (outermost) through 4 (innermost) (Morais-Cabral et al., 2001). Although some of these sites may be disrupted when the channel inactivates, the conformational changes associated with slow inactivation appear to be localized to the more external binding sites (Ogielska and Aldrich, 1999; Zhou et al., 2001). Thus, selectivity filter binding sites such as site 4, and possibly site 3, may persist in the inactivated state (Harris et al., 1998; Jiang and MacKinnon, 2000; Lenaeus et al., 2005) and constitute the outermost possible site(s) for the location of entrapped modulatory cations. At the other end, the innermost boundary for the site of entrapment is the closed activation gate (Fig. 7C). This is inferred from two observations. First, liberation of Cs$^+$ from inactivated channels at depolarized potentials tracks activation gate opening kinetics. Second, repolarization of the inactivated channel to $-120$ mV closes the activation gate with a time constant of $\sim 23$ ms (Panyi, G., and C. Deutsch, 2006, *Biophys. J.* 90:243a), consistent with our observation that the period during which channel recovery is maximally sensitive to intracellular cations lasts no more than 200 ms after return of depolarized channels to their holding potential. Thus, activation gate transitions are likely to be responsible for both the entrapment and the release of the cation.

The activation gate is formed by the bundle crossing of each of the four subunit’s S6 transmembrane helices at the intracellular side of the channel’s pore domain (Liu et al., 1997; Doyle et al., 1998; Jiang et al., 2002a). After closure of the gate, the channel retains a 10-Å-wide aqueous cavity between the selectivity filter and the S6 helix bundle (Doyle et al., 1998). Near the center of this cavity is a site for monovalent cations, including Tl$^+$, K$^+$, Rb$^+$, Cs$^+$, and Na$^+$ (Y. Zhou and MacKinnon, 2004). A cation residing in this cavity site is expected to be readily exchangeable when the activation gate is open. Cs$^+$ has the additional ability to occupy a position between site 4 and the central cavity site, though occupancy of this site is likely to be mutually exclusive with both site 4 and the central cavity site (Y. Zhou and MacKinnon, 2004). Because this site is located near site 4 (but more intracellular), we refer to it as site 4’ (M. Zhou and MacKinnon, 2004). Site 4’ may be the site through which Cs$^+$, and Rb$^+$, but not K$^+$, compete with the binding of the intracellular channel blocker, TBSb...
(Thompson and Begenisich, 2003). We propose that the modulatory site is at 4 or 4’ and that the exchangeable site is either here or at a more intracellular site, perhaps in the center of the cavity (Fig. 7 A). Cations in either of these sites become trapped when the activation gate closes.

**Mechanism of Ion-sensitive Recovery**

Upon inactivation, channels bind K⁺ in the modulatory site (Fig. 7 A). K⁺ occupancy of this site promotes recovery. When Cs⁺ is applied to channels with an open activation gate, replacement of K⁺ with Cs⁺ requires departure of K⁺ from the modulatory site. A closed inactivation gate presumably precludes outward movement of a K⁺ ion through the selectivity filter. Therefore, the exchange of Cs⁺ for K⁺ must occur through the open activation gate. We propose that the exit rate of K⁺ from the modulatory site is significantly slower in the inactivated channel than in the noninactivated channel due to a higher K⁺ affinity in the inactivated channel. This explains the observed biphasic recovery shown in Fig. 6 C. After repolarization of inactivated channels during a non-K⁺ permeant cation pulse, ~60% of channels recover quickly, with a rate nearly identical to that seen in K⁺. The other 40% of channels recover with a rate that depends on the identity of the cation applied during the hyperpolarization transition. The fast recovery route taken by 60% of the channels is depicted by the right column of steps in Fig. 7. The slower route for the remaining 40% is depicted by the left column of transitions. The slow, rate-limiting exit rate for K⁺ from the modulatory site explains why a fixed fraction of channels successfully exchange K⁺ with a perfused permeant cation, and why this fraction (0.4) is independent of the species of non-K⁺ permeant cation applied.

Although the rate of K⁺ exit from the channel may be slow relative to activation gate transitions, the rate of Cs⁺ exit is not, as suggested by our finding that the rate-limiting step in Cs⁺ liberation is opening of the activation gate. The faster rate of exit for Cs⁺ may be explained by its low affinity for the modulatory site. For example, Cs⁺ may prefer to occupy a site similar to site 4’ or the central cavity site, rather than site 4. Regardless of which site Cs⁺ occupies, washout from that site is fast relative to opening of the activation gate (Fig. 5 and Fig. 7 D).

Further support for an intimate relationship between permeation and recovery is evident from the monotonic relationship between rate of recovery and open channel conductance (Fig. 8 A). We speculate that a permeant ion in the modulatory binding site of an inactivated channel (I) moves from the modulatory site to a deeper location (in state NI [noninactivated]) within the selectivity filter for the channel to recover (Fig. 8 B). I’ is a transient state that resembles a conductive state with respect to the structure of the selectivity filter. Recovery in this model occurs when the ion in state I’ hops further into the selectivity filter, thus stabilizing the NI state. In an open channel, the relative ability of an ion to move easily from one site to another is manifest as its conductance. The correlation between the recovery rate and conductance suggests that the I’-to-NI transition is common to both processes. Thus, a highly conductive cation like K⁺ is expected to promote more rapid recovery than a less conductive ion. If an ion does not move rapidly enough from the modulatory site in I’ to the second site in NI, the channel will rapidly return to state I. In this model, therefore, the cation selectivity sequence for recovery should correlate with conductance. Our model (Figs. 7 and 8) suggests that an ion like Cs⁺, when bound in the modulatory site, dissociates more rapidly than K⁺ into the cavity, but slower than K⁺ into the selectivity filter (the I’-to-NI transition). This is simply explained if K⁺ has a higher affinity for the modulatory site than Cs⁺, but that the energy barrier for Cs⁺ to move deeper (more extracellular) into the selectivity filter is much higher than the comparable barrier for K⁺. This is consistent with the higher K⁺ selectivity of sites deeper into the selectivity filter (Aqvist and Luzhkov, 2000; Berneche and Roux, 2001; Noskov et al., 2004).

Moreover, the model shown in Fig. 8 B can explain the results obtained with internal Na⁺ (Fig. 6 C). Only ~20% of the channels trap Na⁺ ion when hyperpolarized in the presence of Na⁺, due to the relatively poor selectivity of the most intracellular selectivity filter sites, for example, sites 3 and 4 (Aqvist and Luzhkov, 2000; Berneche and Roux, 2001; Noskov et al., 2004). Once in this modulatory site, Na⁺ does not move readily into the more extracellular site of state NI. This is consistent with the fact that sodium is nearly impermeant, having a conductance in Shaker that is indistinguishable from leak (Heginbotham and MacKinnon, 1993). Thus, there is no recovery on the time scale of our measurements. The rapid 80% recovery is from inactivated channels that have trapped a K⁺ ion. Similarly, the lack of recovery by inactivated channels that have trapped a TMA ion during a TMA-pulse is consistent with the model proposed in Fig. 8 B.

Occupancy of the selectivity filter by permeant cations is likely to be necessary for the maintenance of a conductive conformation. The presence of permeant cations in the selectivity filter has been suggested to be necessary to counterbalance the electrostatic repulsion experienced by opposing selectivity filter oxygen atoms as a consequence of their negative dipoles (Almers and Armstrong, 1980; Shrivastava and Sansom, 2000; Zhou et al., 2001). At low permeant cation concentrations, the selectivity filter binding sites are not fully occupied and the selectivity filter is distorted (Shrivastava and Sansom, 2000; Morais-Cabral et al., 2001; Zhou et al.,...
A possible consequence of removal of permeant cations from the channel, therefore, is entry into a stable nonconducting conformation (Melishchuk et al., 1998; Loboda et al., 2001). Underpopulation of at least some selectivity filter binding sites may also be characteristic of the slow inactivated state (Lopez-Barneo et al., 1993; Baukrowitz and Yellen, 1995; Rasmusson et al., 1995; Baukrowitz and Yellen, 1996; Levy and Deutsch, 1996a,b; Molina et al., 1997; Kiss and Korn, 1998; Molina et al., 1998; Fedida et al., 1999; Ogielska and Aldrich, 1999), and refilling of these sites may be required for recovery (Berneche and Roux, 2005).

Further Implications for \( K_v \) Channel Gating

Cation trapping during the repolarization transition has important implications for the nature of activation gate movement in inactivated channels. First, the findings that depolarization liberates a cation trapped by the activation gate and that Cs\(^+\) liberation tracks activation kinetics of the noninactivated channel suggests that activation gate opening at voltages between 0 and +80 mV is not influenced strongly by either inactivation or a trapped Cs\(^+\) ion. Our results are inconsistent with the previous speculation that large cations, including Cs\(^+\), must exit the central cavity before activation gate closure (Melishchuk and Armstrong, 2001). However, our results are consistent with crystal structures of KcsA in which a channel with a closed activation gate retains enough room in the central cavity for a permeant cation, including Cs\(^+\) (Y. Zhou and MacKinnon, 2004), as well as larger cationic blockers (Lenaeus et al., 2005). We conclude that slow-inactivated channels can accommodate permeant cations behind the closed activation gate.

A second implication from our studies pertains to the role of the 449 side chain in K\(^+\) channel gating. This side chain influences both entry into (Lopez-Barneo et al., 1993; Molina et al., 1997) and recovery from (Rasmusson et al., 1995; Meyer and Heinemann, 1997) the inactivated state. We find that the influence of the trapped cation on recovery rate also depends on the 449 side chain. It is possible that recovery reflects a ternary interaction in which the 449 side chain governs cation binding to an extracellular site, which in turn affects the interaction between trapped intracellular cations and the selectivity filter. Another possibility is that the 449 side chain determines how inactivation-associated rearrangements at the outer mouth of the channel influence the stability of ion binding sites within the selectivity filter. Although the mechanism remains unclear, it is certain that 449 plays an important role in governing both entry into and recovery from inactivation. The lack of conservation of this side chain among K\(^+\) channels contributes to the physiological diversity of K\(^+\) channel function.

Recovery from the slow inactivated state is a critical gating process. It determines the number of K\(^+\) channels available to maintain excitable cells at a negative resting potential, ultimately dictating cellular excitability. Maintenance of a conducting conformation appears to require bound permeant cations and, because K\(^+\) channels have evolved to coordinate K\(^+\) ions, these cations are the most effective at accomplishing this task. In addition to this prerequisite for permeation, we now

---

**Figure 8.** Conductance vs. recovery rate. (A) Rate constants from the slow phase of recovery derived from Fig. 6 C are plotted as a function of the single-channel conductance ratio for each of the indicated cations. Conductance data are from Heginbotham and MacKinnon (1993), measured in T449 Shaker-IR. Data for K\(^+\), Rb\(^+\), or NH\(_4\)\(^+\) are from single channel currents. Cs\(^+\) conductance was calculated using nonstationary fluctuation analysis because the small conductance of Cs\(^+\) (0.32 pS) precluded single channel current recording. We used nonstationary fluctuation analysis to confirm that mutation of T449 to A or V does not change \( \gamma_{cs}/\gamma_{k} \) (not depicted). \( \gamma_{cs} \) in 449K could not be measured due to its rapid inactivation and entry into the long-lived nonconducting state in the presence of Cs\(^+\). (B) A model describing how a trapped permeant cation may promote recovery. In the inactivated channel (I), a cation is bound to a modulatory site. The inactivated channel transiently reverts to the noninactivated conformation (I'), a state that is unstable in the absence of a cation bound in the selectivity filter. Only when the trapped cation moves into the selectivity filter the NI state become stable, resulting in recovery. The relative rates for these transitions are indicated by the length of the arrows.
include one for gating, specifically for recovery. Binding of permeant cations to sites in the permeation pathway stabilizes rearrangements in the channel protein that appear to be necessary for recovery from the slow-inactivated state.

We thank Dr. R. Horn for critical reading of the manuscript and technical advice, Dr. G. Panyi for thoughtful discussion and help with analysis, and Drs. C. Miller and B. Roux for suggestions.

This work was supported by National Institutes of Health grant GM 069837 and National Research Service Award HL-07027.

Olaf S. Andersen served as editor.

Submitted: 14 April 2006
Accepted: 30 June 2006

REFERENCES

Aquist, J., and V. Luzhkov. 2000. Ion permeation mechanism of the potassium channel. Nature. 404:881–884.

Almers, W., and C.M. Armstrong. 1989. Survival of K+ permeability and gating currents in squid axons perfused with K-free media. J. Gen. Physiol. 75:61–78.

Armstrong, C.M. 1969. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. J. Gen. Physiol. 54:553–575.

Baukrowitz, T., and G. Yellen. 1996. Use-dependent blockers and current by Armstrong, C.M. 1969. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. J. Gen. Physiol. 54:553–575.

Baukrowitz, T., and G. Yellen. 1996. Use-dependent blockers and exit rate of the last ion from the multi-ion pore of a K+ channel. Science. 271:653–656.

Berneche, S., and B. Roux. 2001. Energetics of ion conduction through the K+ channel. Nature. 414:73–77.

Berneche, S., and B. Roux. 2005. A gate in the selectivity filter of potassium channels. Structure. 13:591–600.

Choi, K.L., R.W. Aldrich, and G. Yellen. 1991. Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K+ channels. Proc. Natl. Acad. Sci. USA. 88:5092–5095.

Ding, S., and R. Horn. 2002. Tail end of the S6 segment: role in permeation in Shaker potassium channels. J. Gen. Physiol. 120:87–97.

Doyle, D.A., J. Morais Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K+ conduction and selectivity. Science. 280:69–77.

Fedida, D., N.D. Maruoka, and S. Lin. 1999. Modulation of slow inactivation in human cardiac Kv1.5 channels by extra- and intra-cellular permeant cations. J. Physiol. 515:315–329.

Grisser, S., and M. Cahalan. 1989a. Divalent ion trapping inside potassium channels of human T lymphocytes. J. Gen. Physiol. 93:609–630.

Grisser, S., and M. Cahalan. 1989b. TEA prevents inactivation while blocking open K+ channels in human T lymphocytes. Biophys. J. 55:203–206.

Harris, R.E., H.P. Larsson, and E.Y. Isacoff. 1998. A permanent ion binding site located between two gates of the Shaker K+ channel. Biophys. J. 74:1808–1820.

Heginbotham, L., and R. MacKinnon. 1993. Conduction properties of the cloned Shaker K+ channel. Biophys. J. 65:2089–2096.

Hille, B. 2001. Ion Channels of Excitable Membranes. Third edition. Sinauer Associates, Inc., Sunderland, MA. 814 pp.

Holmgren, M., M.E. Jurman, and G. Yellen. 1996. N-type inactivation and the S4-S5 region of the Shaker K+ channel. J. Gen. Physiol. 108:195–206.

Hoshi, T., W.N. Zagotta, and R.W. Aldrich. 1990. Biophysical and molecular mechanisms of Shaker potassium channel inactivation. Science. 250:533–538.

Hoshi, T., W.N. Zagotta, and R.W. Aldrich. 1991. Two types of inactivation in Shaker K+ channels: effects of alterations in the carboxy-terminal region. Neuron. 7:547–556.

Hoshi, T., W.N. Zagotta, and R.W. Aldrich. 1994. Shaker potassium channel gating. I: Transitions near the open state. J. Gen. Physiol. 103:249–278.

Jiang, Y., and R. MacKinnon. 2000. The barium site in a potassium channel by X-ray crystallography. J. Gen. Physiol. 115:269–272.

Jiang, Y., A. Lee, J. Chen, M. Cadene, B.T. Chait, and R. MacKinnon. 2002a. Crystal structure and mechanism of a calcium-gated potassium channel. Nature. 417:515–522.

Jiang, Y., A. Lee, J. Chen, M. Cadene, B.T. Chait, and R. MacKinnon. 2002b. The open pore conformation of potassium channels. Nature. 417:523–526.

Jurman, M.E., L.M. Boland, and G. Yellen. 1994. Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. Biotechniques. 17:876–881.

Kiss, L., and S.J. Korn. 1998. Modulation of C-Type inactivation by K+ at the potassium channel selectivity filter. Biophys. J. 74:1840–1849.

Kiss, L., J. LoTurco, and S.J. Korn. 1999. Contribution of the selectivity filter to inactivation in potassium channels. Biophys. J. 76:253–263.

Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 266:19867–19870.

Kurata, H.T., Z. Wang, and D. Fedida. 2004. NH2-terminal inactivation peptide binding to C-type-inactivated Kv channels. J. Gen. Physiol. 123:505–520.

Larsson, H.P., and F. Elinder. 2000. A conserved glutamate is important for slow inactivation in K+ channels. Neuron. 27:573–583.

Lenaeus, M.J., M. Vamvouka, P.J. Focia, and A. Gross. 2005. Structural basis of TEA blockade in a model potassium channel. Nat. Struct. Mol. Biol. 12:454–459.

Levy, D.L., and C. Deutsch. 1996a. Recovery from C-type inactivation is modulated by extracellular potassium. Biophys. J. 70:798–805.

Levy, D.L., and C. Deutsch. 1996b. A voltage-dependent role for K+ in recovery from C-type inactivation. Biophys. J. 71:3157–3166.

Liu, Y., M.E. Jurman, and G. Yellen. 1996. Dynamic rearrangement of the outer mouth of a K+ channel during gating. Neuron. 16:859–867.

Liu, Y., M. Holmgren, M.E. Jurman, and G. Yellen. 1997. Gated access to the pore of a voltage-dependent K+ channel. Neuron. 19:175–184.

Loboda, A., A. Melishchuk, and C. Armstrong. 2001. Dialed and de-funct K channels in the absence of K+. Biophys. J. 80:2704–2714.

Loots, E., and E.Y. Isacoff. 1998. Protein rearrangements underlying slow inactivation of the Shaker K+ channel. J. Gen. Physiol. 112:377–389.

Lopez-Barneo, J., T. Hoshi, S.H. Heinemann, and R.W. Aldrich. 1993. Effects of external cations and mutations in the pore region on C-type inactivation of Shaker potassium channels. Receptors Channels. 1:61–71.

Margolskee, R.F., P. Kavathas, and P. Berg. 1988. Epstein-Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. Mol. Cell. Biol. 8:2837–2847.

Margolskee, R.F., B. McHendry-Rinde, and R. Horn. 1993. Panning transfected cells for electrophysiological studies. Biotechniques. 15:906–911.

Melishchuk, A., and C.M. Armstrong. 2001. Mechanism underlying slow kinetics of the OFF gating current in Shaker potassium channel. Biophys. J. 80:2167–2175.

216 Modulation of Recovery by a Trapped Cation
Melischuk, A., A. Loboda, and C.M. Armstrong. 1998. Loss of Shaker K channel conductance in 0 K+ solutions: role of the voltage sensor. *Biophys. J.* 75:1828–1835.

Meyer, R., and S.H. Heinemann. 1997. Temperature and pressure dependence of Shaker K+ channel N- and C-type inactivation. *Eur. Biophys. J.* 26:433–445.

Molina, A., A. Castellano, and J. Lopez-Barneo. 1997. Pore mutations in Shaker K+ channels distinguish between the sites of tetraethylammonium blockade and C-type inactivation. *J. Physiol.* 499:361–367.

Molina, A., P. Ortega-Saenz, and J. Lopez-Barneo. 1998. Pore mutations alter closing and opening kinetics in Shaker K+ channels. *J. Physiol.* 509:327–337.

Morais-Cabral, J.H., Y. Zhou, and R. MacKinnon. 2001. Energetic optimization of ion conduction rate by the K+ selectivity filter. *Nature* 414:37–42.

Neyton, J., and C. Miller. 1988. Discrete Ba2+ block as a probe of ion occupancy and pore structure in the high-conductance Ca2+-activated K+ channel. *J. Gen. Physiol.* 92:569–586.

Nimigean, C.M., and C. Miller. 2002. Na+ block and permeation in a K+ Channel of Known Structure. *J. Gen. Physiol.* 120:323–335.

Noskov, S.Y., S. Berneche, and B. Roux. 2004. Control of ion selectivity in potassium channels by electrostatic and dynamic properties of carbonyl ligands. *Nature* 431:830–834.

Ogielska, E.M., and R.W. Aldrich. 1999. Functional consequences of a decreased potassium affinity in a potassium channel pore. Ion interactions and C-type inactivation. *J. Gen. Physiol.* 113:347–358.

Pardo, L.A., S.H. Heinemann, H. Terfau, U. Ludewig, C. Lorra, O. Pongs, and W. Stühmer. 1992. Extracellular K+ specifically modulates a rat brain K+ channel. *Proc. Natl. Acad. Sci. USA.* 89:2466–2470.

Rasmusson, R.L., M.J. Morales, R.C. Castellino, Y. Zhang, D.L. Campbell, and H.C. Strauss. 1995. C-type inactivation controls recovery in a fast inactivating cardiac K+ channel (Kv1.4) expressed in Xenopus oocytes. *J. Physiol.* 480(Pt 3):709–721.

Rasmusson, R.L., M.J. Morales, S. Wang, S. Liu, D.L. Campbell, M.V. Brahmajothi, and H.C. Strauss. 1998. Inactivation of voltage-gated cardiac K+ channels. *Circ. Res.* 82:739–750.

Shrivastava, I.H., and M.S.P. Sansom. 2000. Simulations of ion permeation through a potassium channel: molecular dynamics of KcsA in a phospholipid bilayer. *Biophys. J.* 78:557–570.

Starkus, J.G., L. Kuschel, M.D. Rayner, and S.H. Heinemann. 1997. Ion conduction through C-type inactivated Shaker channels. *J. Gen. Physiol.* 110:539–550.

Swenson, R.P., Jr., and C.M. Armstrong. 1981. K+ channels close more slowly in the presence of external K+ and Rb+. *Nature* 291:427–429.

Thompson, J., and T. Begenisich. 2003. Functional identification of ion binding sites at the internal end of the pore in Shaker K+ channels. *J. Physiol.* 549:107–120.

Wang, Z., J.C. Hesketh, and D. Fedida. 2000. A high-Na (+) conduction state during recovery from inactivation in the K(+)+ channel Kvl.5. *Biophys. J.* 79:2416–2433.

Yellen, G., D. Sodickson, T.Y. Chen, and M.E. Jurman. 1994. An engineered cysteine in the external mouth of a K+ channel allows inactivation to be modulated by metal binding. *Biophys. J.* 66:1068–1075.

Zagotta, W.N., and R.W. Aldrich. 1990. Voltage-dependent gating of Shaker A-type potassium channels in *Drosophila* muscle. *J. Gen. Physiol.* 95:29–60.

Zagotta, W.N., T. Hoshi, and R.W. Aldrich. 1994a. Shaker potassium channel gating. III: Evaluation of kinetic models for activation. *J. Gen. Physiol.* 103:321–362.

Zagotta, W.N., T. Hoshi, J. Dittman, and R.W. Aldrich. 1994b. Shaker potassium channel gating. II: Transitions in the activation pathway. *J. Gen. Physiol.* 103:279–319.

Zhou, Y., and R. MacKinnon. 2003. Ion binding affinity in the cavity of the KcsA potassium channel. *Biochemistry* 43:4978–4982.

Zhou, Y., J.H. Morais-Cabral, A. Kaufman, and R. MacKinnon. 2001. Chemistry of ion coordination and hydration revealed by a K+ channel-Fab complex at 2.0 Å resolution. *Nature* 414:43–48.