The Relation between Ion Permeation and Recovery from Inactivation of *Shaker*B K^+ Channels

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ABSTRACT We have studied the relation between permeation and recovery from N-type or ball-and-chain inactivation of *Shaker*B K channels. The channels were expressed in the insect cell line Sf9, by infection with a recombinant baculovirus, and studied under whole cell patch clamp. Recovery from inactivation occurs in two phases. The faster of the two lasts for approximately 200 ms and is followed by a slow phase that may require seconds for completion. The fast phase is enhanced by both permeant ions (K^+, Rb^+) and by the blocking ion Cs^+, whereas the impermeant ions (Na^+, $Tris^+$, $choline^+$) are ineffective. The relative potencies are $K^+ > Rb^+ > Cs^+ > NH_4^+ \gg Na^+ \approx choline^+ \approx Tris^+$. Ion permeation through the channels is not essential for recovery. The results suggest that cations influence the fast phase of recovery by binding in a site with an electrical distance greater than 0.5. Recovery from fast inactivation is voltage-dependent. With Na^+, $choline^+$, or $Tris^+$ outside, about 15% of the channels recover in the fast phase (−80 mV), and the other 85% apparently enter a second inactivated state from which recovery is very slow. Recovery in this phase is not influenced by external ions, but is speeded by hyperpolarization.

INTRODUCTION

*Shaker*B K^+ channels have at least two types of inactivation: a fast or N-type and a slow or C-type (Iverson and Rudy, 1990; Hoshi et al., 1992). Fast inactivation is produced by the occlusion of the pore by a cytoplasmic domain or "ball" formed by the first 20 amino acids of the protein (Hoshi et al., 1990; Zagotta et al., 1990). This mechanism, known as the "ball-and-chain," was first proposed by Armstrong and Bezanilla (1977) to explain the inactivation of sodium channels.

Recently, Ruppersberg et al. (1991) and Demo and Yellen (1991) demonstrated that fast inactivating K channels reopen during recovery from inactivation. Demo and Yellen (1991) demonstrated that K^+ ions from the outside relieve the block by the ball peptide. Recovery is faster at negative voltage, and they concluded that the voltage dependence of recovery comes from the effect of the voltage on the driving force of K^+. Ruppersberg et al. (1991), studying the rates of block and unblock of the channels by synthetic ball peptides, concluded that recovery from inactivation is voltage-dependent even in 2.5 mM K^+ and that the recovery process is intrinsically voltage-dependent, independent of the influence of external cations.

We have extended the observations of Demo and Yellen (1991) about the relation between permeation and gating by testing the effects on inactivation recovery of both permeant and impermeant ions. We have also examined the controversial point about the voltage dependence of the process. Our results show that impermeant as well as permeant ions can speed recovery, demonstrating that permeation all the way through the pore is not required. Thus, cations coming from the external solution relieve the block by the inactivating ball not by flowing through the channel, but by binding to a site deep in the pore (electrical distance >0.5). Recovery from inactivation is voltage-dependent even in the absence of permeant ions. Finally, there is at least a second inactivated state associated with N-type inactivation, from which recovery is very slow, voltage-dependent, but uninfluenced by ions in the external medium.

MATERIALS AND METHODS

Cell Culture and *Shaker*B expression

The insect cell line Sf9, from the army-worm caterpillar *Spodoptera frugiperda* was kept in culture at 27°C in Grace's media supplemented with 10% fetal bovine serum, and with a combination of penicillin (50 units/ml), streptomycin (50 μg/ml), and gentamycin sulfate (50 μg/ml) (Summers and Smith, 1988). For the patch clamp experiments, the cells were split from confluent flasks and dispersed onto coverslips. The cells were then infected, with a multiplicity of infection of 10, with the recombinant baculovirus *A. californica* nuclear polyhedrosis virus, containing the *Shaker*H4 or *Shaker*B K^+ channel cDNA (Kimberly et al., 1990). The cells were kept at 27°C and used for experiments 2 days after the infection. The recombinant baculovirus was kindly provided by Dr. C. Miller.

Electrodes

The electrodes were pulled from borosillicate glass (KIMAX 51), fire-polished to a 0.7–1.3 MΩ resistance, and used without any further treatment.

Electric recording

Macroscopic currents were recorded under whole cell patch clamp (Hamill et al., 1981), 2 days after the infection of the cells. *Shaker*B channels were the only K^+ conductance present in the plasma membrane of the infected Sf9 cells (Kimberly et al., 1990).

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The cell membrane was voltage-clamped, and the current was recorded with a home-made patch clamp amplifier with a 10 M feedback resistor. The output of the amplifier was low-pass-filtered through an 8-pole Bessel filter (Frequency Devises, Inc., Haverhill, MA) with a cutoff frequency of 10 KHz. The delivery of the voltage steps and the acquisition of the data were made through a home-made 16-bit interface connected to an IBM/PS2. The data were stored in the memory of the computer for later analysis.

About 80–100% of the series resistance was electronically compensated. The holding potential used throughout the work was −80 mV. No further increase in the K^+ current was observed by holding the membrane at more negative potentials. Leak current was measured with control pulses of −50 mV from the holding potential of −80 mV.

Solutions

The composition of the internal solution used in all the experiments was: 90 mM KF, 30 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES-K, pH 7.2. The composition of the external "normal" Na^+ solution was: 145 mM NaCl, 2 mM $CaCl_2$, 10 mM Mes-Na, pH 6.4. All of the other external solutions were made by substituting the appropriate amount of the chloride salt of the test cation, for an equiosmotic amount of NaCl in the normal solution, with the exception of the choline$^+$ and Tris$^+$ solutions (Fig. 7) in which all the Na^+ was isosmotically replaced by these ions. In the text, the external solutions will be referred by the concentration (mM) of the test cation (e.g., 100 K^+). All of the chemicals used were from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Permeation and recovery from inactivation

To study the relation between recovery from inactivation and permeation, we first compared the extent of recovery with a series of monovalent cations in the external solution, at −80 mV.

Fig. 1 compares the currents obtained using a two-pulse protocol with 100 mM K^+, Rb^+, $(NH_4)^+$, Cs^+, and isosmotic Na^+ (~150 mM). A first depolarization (+60 mV/40 ms) was applied to activate and inactivate the channels, then the membrane was repolarized to −80 mV for a variable period (numbers in the figure). A second depolarization was then applied to see the fraction of the channels that had recovered from inactivation during the interpulse period. The current during the interpulse, which is represented by a gap in the traces, was not sampled.

The traces in Fig. 1 show that (a) compared with K^+ or Rb^+, recovery with the impermeant Na^+ is very poor. Na^+ ions are quite inert in promoting recovery from inactivation; however, there is a considerable recovery with Cs^+, which is also impermeant (compare the traces in Cs^+ with those in Na^+); moreover, (b) Cs^+ is more effective than NH_4^+, which is permeant.

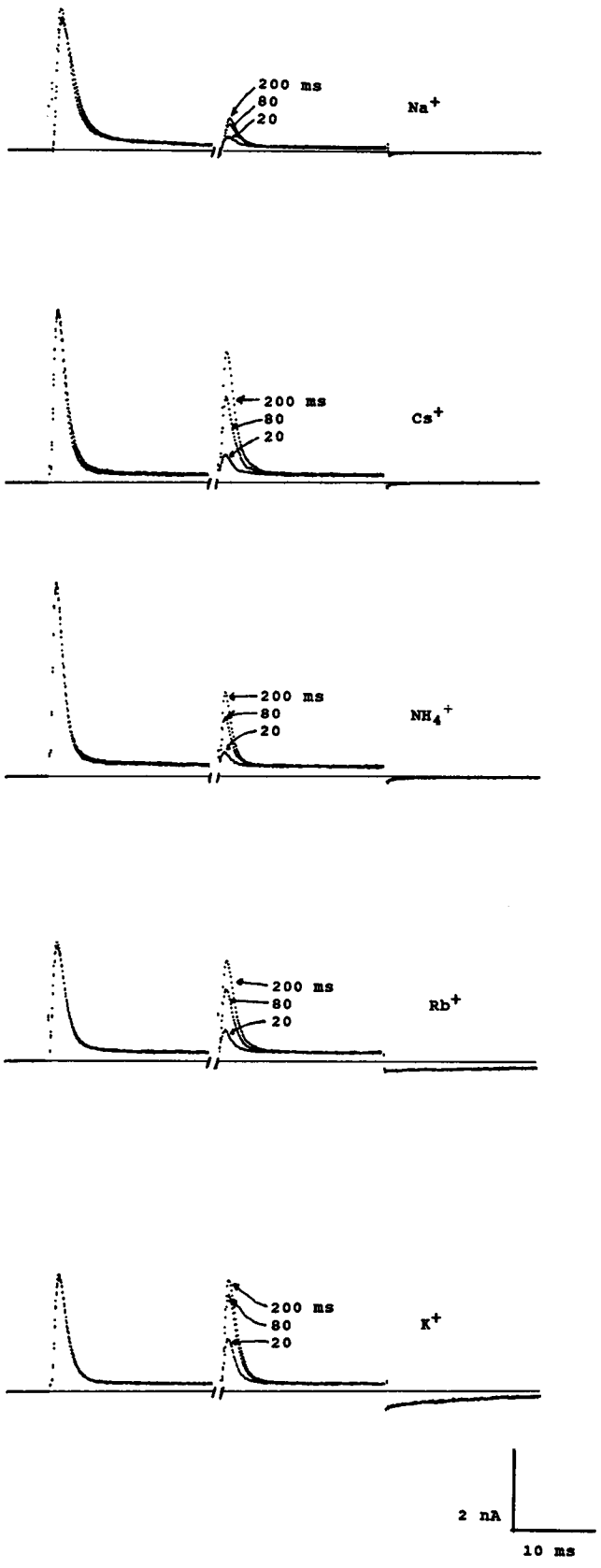

FIGURE 1 Recovery from inactivation at −80 mV. The cells were placed in an external solution containing 100 mM indicated cation in substitution of an isosmotic amount of NaCl in the "normal" Na^+ solution (see Materials and Methods). Recovery from inactivation was measured with a two-pulse protocol. A first +60 mV/40 ms pulse was applied to open and inactivate the channels (*left* traces), then the membrane was repolarized to −80 mV for a variable interpulse time. A second +60 mV/40 ms pulse was applied to see the recovery during the interpulse (*right* traces). The interpulse was not sampled; therefore, it is just represented with a gap at the end of the left traces. The horizontal line shows the zero current level. The scale is the same for all the panels. The "normal" Na^+ solution was ~145 mM NaCl, 2 mM $CaCl_2$, 10 mM MES-Na, pH 6.4 (300 mOsm). Experiment fe223b(1).

Notice also, in Fig. 1, the slow inward tails at the end of the second (test) depolarization in K^+ and Rb^+ (the ones after the first pulse were not sampled). These tails represent reopening from inactivation (Ruppersberg et al., 1991), and they have a time constant one order of magnitude bigger than those of deactivation after a short pulse (not shown). They will be called "slow tails" following Demo and Yellen (1991). In some experiments, an initial hook is seen in the slow tails, particularly when the density of the channels is high and the potential is −80 mV or less negative. As shown in Fig. 1, the slow tails are obviously not seen with Cs^+, although Cs^+ is almost as effective as Rb^+ at −80 mV (see below).

Fig. 2 compares the time course (at −80 mV) of recovery, which is clearly separable into two phases. The fast phase ends after about 200 ms and is followed by a slow phase that requires a second or more to be complete. With all of the ions tested the time course of the fast phase is similar. At −80 mV, it is well described by a single exponential of the form $f_{max} \cdot (1 - \exp(-t/\tau))$. The main difference among ions is in the value of both parameters, f_{max} and τ. f_{max} is notoriously different, in comparison, and τ is more similar for the various ions. Both parameters change with the voltage (see below). The similarity in time course suggests that the mechanism of the fast phase of recovery is the same with all the ions. In particular, it suggests that in the fast phase the channels reflux through the open state even when the "inert" ion Na^+ (which neither permeates nor blocks) is outside. Figs. 1 and 2 also show that the order of potency in enhancing recovery during the fast phase is $K^+ > Rb^+ > Cs^+ > NH_4^+ \gg Na^+$. Clearly, some impermeant ions as well as permeant ones can speed removal of the ball from the inner pore mouth.

The concentration dependence of the effect of Cs^+ on the fast phase of recovery at −80 mV is shown in Fig. 3 *A* where, for reference, we also plotted the corresponding values with

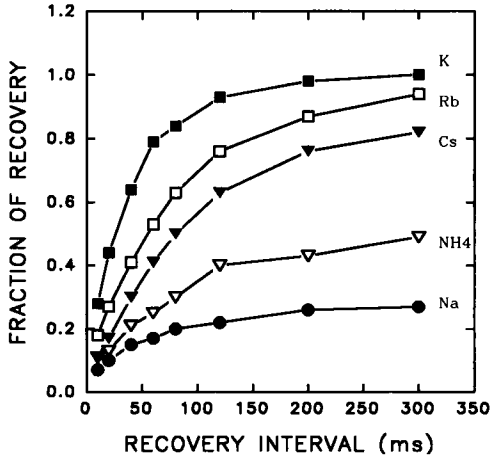

FIGURE 2 Time course of the fast phase of recovery at −80 mV. The fraction of recovery as a function of the interpulse duration was calculated from the complete series of traces in Fig. 1 as the relative peak current: $(I_{peak,2} - I_{40,2})/(I_{peak,1} - I_{40,1})$, where I_{peak} is the peak current in the second or the first pulse respectively, and I_{40} is the current at the end of the corresponding 40 ms pulse. The lines in the figure were drawn by eye.

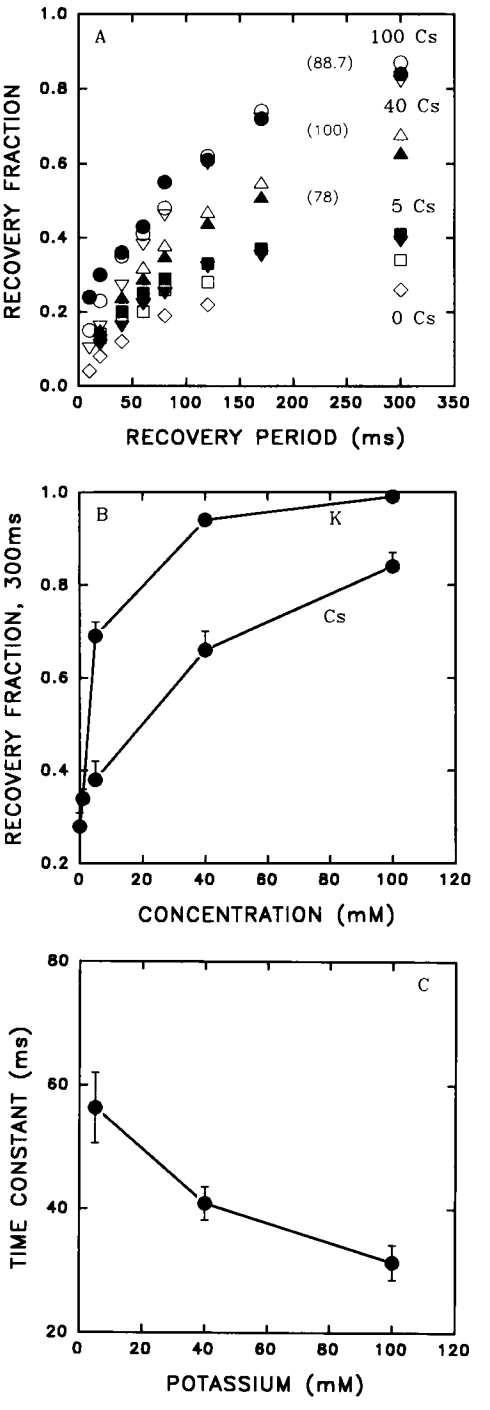

FIGURE 3 Recovery from inactivation as a function of the external concentration of Cs^+. (*A*) Recovery was measured as in Fig. 1, at −80 mV. The different symbols correspond to experiments with different cells. The numbers in parentheses are the time constants (in ms), obtained by fitting an exponential to the average of the points in the figure. The trace labeled 0 Cs corresponds to a normal Na^+ solution, as in Fig. 1. (*B*) Percentage of recovery in 300 ms as a function of the concentration of Cs^+ or K^+. The points in Cs^+ were read from the data in a at 300 ms (they are not extrapolated values). The points in K^+ were determined in at least three separate experiments. The points at 0 mM were taken in Na^+. The lines were drawn by eye. (*C*) Time constant of the fast phase of recovery as a function of the potassium concentration. The time constants are the average of at least three experiments. They were obtained either from fitting an exponential to the slow tails or to the results of the two-pulse protocol. The lines were drawn by eye.

Na^+ (0 Cs^+). Cs^+ increases recovery even at 5 mM, the lowest concentration that we tested. Fig. 3 *B* compares the percentage of recovery after 300 ms at −80 mV as a function of the concentration of Cs^+ or K^+; clearly, the extent of recovery depends on the concentration of both ions. On the other hand, the time constant of the fast phase in Cs^+ does not have a clear dependence on the concentration (numbers in parentheses, in ms, in Fig. 3 *A*). All three curves with Cs^+ in Fig. 3 *A* can be well fitted with a time constant of 89 ms, whereas f_{max}, the fraction of recovery at 300 ms, varies: 0.38 for 5 Cs^+, 0.66 for 40 Cs^+, and 0.84 for 100 Cs^+. Fig. 3 *C* shows that the time constant of recovery decreases modestly as the concentration of K^+ increases from 5 to 100 mM. In contrast to the extent of recovery, the time constant does not vary much with the concentration (see Discussion).

Cs^+ blocks K channels from the outside, whereas Na^+ does not (Bezanilla and Armstrong, 1972; Adelman and French, 1978; French and Shoukimas, 1985). This suggests that the ability of Cs^+, as compared with Na^+, to modify the block by the internal ball originates in its capacity to equilibrate, at negative potentials, in a blocking site in the conduction pore. We have estimated that with 40 mM K^+ outside, 20 mM Cs^+ blocks *Shaker*B with an electrical distance *d* of 0.9. Also, the bigger extent of recovery in Cs^+ as compared with $(NH_4)^+$, further demonstrates that it is not the flow of ions through the channel that enhances recovery, but the equilibrium occupancy of a site in the pore.

To have an idea of how far in the pore an ion has to bind to enhance recovery, we examined the effect of high Ca^{2+}. It is known that external Ca^{2+} blocks the inward current through *Shaker*B channels with a *d* of 0.5 (Gómez-Lagunas and Armstrong, 1993). During reopening from inactivation, the block by Ca^{2+} causes a reduction in the amplitude of the slow tails seen in 100 mM K^+, without affecting recovery from inactivation. This is shown in Fig. 4 *A*, where currents obtained with the two-pulse protocol in 2 and 40 mM Ca^{2+} are superposed; the figure shows the control currents and those that followed them after 80 ms at −80 mV. Notice that the extent of recovery is the same even though Ca^{2+} reduces the amplitude of the slow tails (signaled with arrows in the figure; the tails at the end of the first pulse are not shown). The lower part of the figure shows the slow tails at −60 and −120 mV in 2 and 40 mM Ca^{2+}, after scaling to show that their time course is the same. Even in a Na^+-external solution, in the absence of K^+, Ca^{2+} does not affect recovery at any potential, as shown in Fig. 4 *B* at −80 mV. We have observed the same results in another four cells.

Therefore, neither the reduction in the current during the slow tails nor the occupancy by Ca^{2+} of a site with a *d* of 0.5 affects recovery from inactivation. This indicates that to enhance the dissociation of the ball an ion needs to bind in a site with an electrical distance greater than 0.5, as does Cs^+.

From the experiments with Cs^+, it is clear that an ion need not carry current through the channel to aid in expelling the inactivating ball. This point is reinforced by comparing the effects of Rb^+ and K^+ on recovery. In Fig. 5, the graph at the upper right shows recovery curves at −60 mV in K^+ and

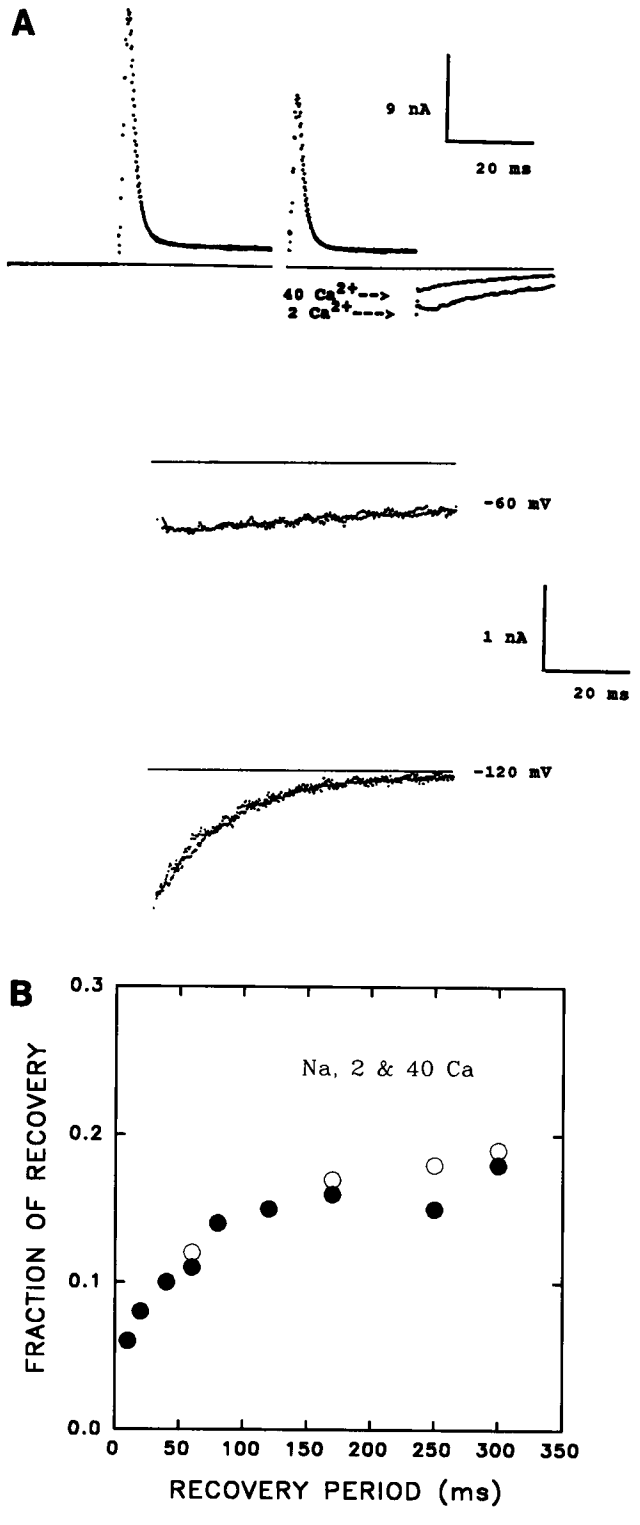

FIGURE 4 Recovery from inactivation in high extracellular Ca^{2+}. (*A*) The upper part of the figure shows the currents obtained with a −80 mV/80 ms interpulse in 100 K^+ + 2 Ca^{2+}, and 100 K^+ + 40 Ca^{2+}. The currents are superposed. Notice that the outward currents in the second pulse are identical at the two Ca^{2+} concentrations, indicating that the fraction of recovery reached during the interpulse was the same. On the other hand, notice the reduction in the amplitude of the slow tail in 40 Ca^{2+}. The lower part of the figure shows the slow tails in 2 and 40 Ca^{2+} at the two extreme voltages of −60 and −120 mV. The tails are superposed to show that their time course is the same. (*B*) Recovery from inactivation in Na^+ with 2 (●) and 40 mM Ca^{2+} (○).

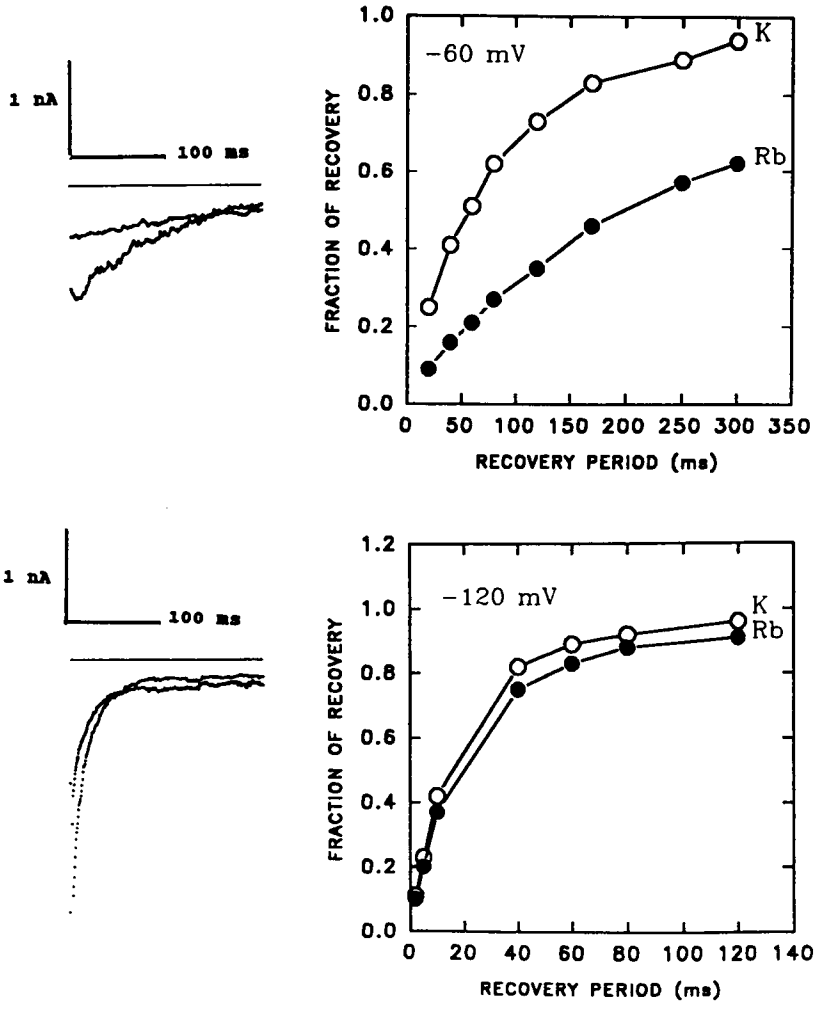

FIGURE 5 Recovery from inactivation in 100 K^+ and 100 Rb^+. The left side shows the slow tails obtained by repolarizing the membrane to either −60 mV (*top*) or −120 mV (*bottom*), after a +60 mV/40 ms pulse (not shown). The slow tails were scaled so the current at the end of the depolarization was the same with both ions (not shown). The tails with the biggest amplitude at all of the voltages are those in K^+. The tails are slower in Rb^+ than in K^+, but the difference in speed is clearly bigger at −60 than at −120 mV. The right are plots of the time course of recovery calculated as in Fig. 2. Experiment: mr123b.

Rb^+, and recovery is slower in the latter ion. The Rb^+ tail current is smaller than the one in K^+ at this voltage (traces at *left*), and it might be argued that smaller current is related to slower recovery. At −120 mV, however, recovery is almost equally fast in the presence of either ion, even though the tail current in Rb^+ is only half as large as in K^+. Thus, recovery rate is not simply related to the ability of an ion to carry current, as was also clear with Cs^+, which carries no current.

Voltage dependence of recovery with impermeant extracellular cations

It is of interest to know whether all of the voltage dependence of recovery arises from alterations of the voltage driving ions into the pore. This question was examined by measuring the voltage dependence of recovery with several impermeant ions. Fig. 6 shows that when all of the extracellular K^+ is replaced by an isosmotic amount of the impermeant Na^+, recovery has a clear voltage dependence. Because there is no evidence that Na^+ enters the pore, either to carry current or to block, this result strongly indicates that recovery from inactivation is intrinsically voltage-dependent.

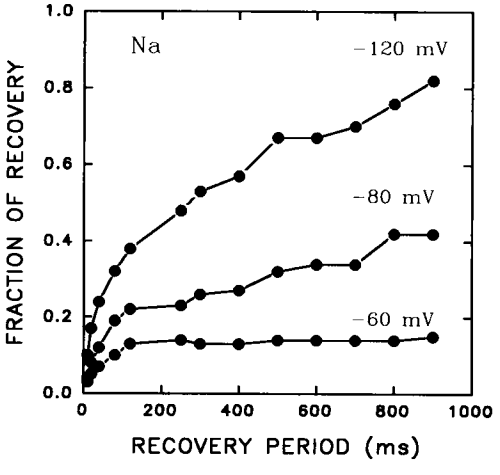

FIGURE 6 Voltage dependence of recovery from inactivation in Na^+. Recovery was measured with the two-pulse protocol at the three indicated voltages during the interpulse. Notice that recovery with only the impermeant Na^+ has a marked voltage dependence. External solution: ~145 mM NaCl, 2 mM $CaCl_2$, 10 mM MES-Na, pH 6.4. Experiment: ju112a.

This point was further tested by replacing Na^+ by an isoosmotic amount of the bigger impermeant cations choline$^+$ and Tris$^+$ (Hille, 1992). The results are shown in Fig. 7, *A* and

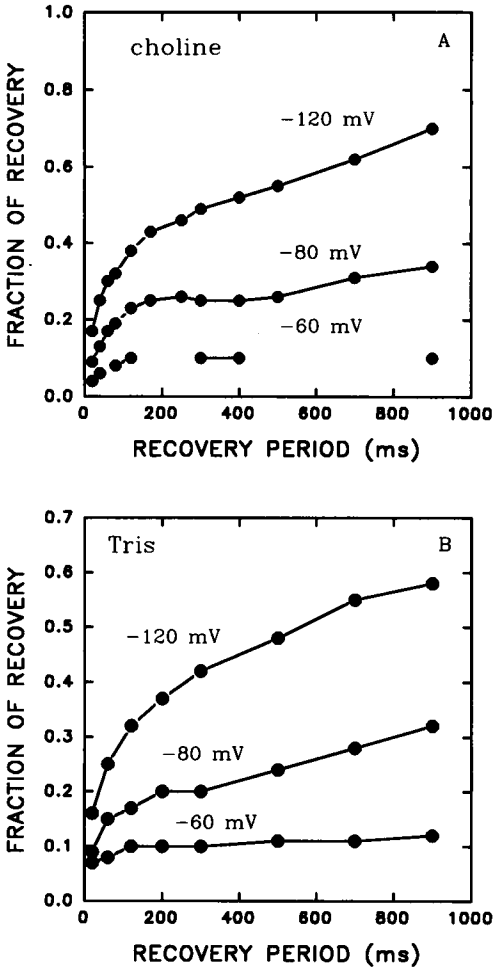

FIGURE 7 Voltage dependence of recovery from inactivation in choline$^+$ and Tris$^+$. (*A*) Recovery was measured with only the impermeant choline$^+$, by replacing all of the NaCl of the "normal" solution with an isoosmotic amount of choline chloride. (*B*) Recovery in the impermeant Tris$^+$. All of the Na$^+$ of the "normal" solution was replaced by an isosmotic amount of Trizma hydrochloride, pH 7.1, 2 mM $CaCl_2$. Experiments: mr123a & mr183a.

B, respectively, and it is clear that recovery is similar in all three of these impermeant ions. Both the fast and the slow phase of recovery show voltage dependence (see also Fig. 10).

A comparison of Figs. 6 and 7 also shows that recovery at pH 6.4 (Na$^+$ and choline$^+$) is not appreciably different from that at pH 7.0 (Tris$^+$). Also, it is worthwhile to note that the data in Fig. 6 and 7 were obtained with 2 mM Ca^{2+} in the external solution; neither Ca^{2+} in the range 1–40 mM (see above) nor Mg^{2+} up to 10 mM (not shown) has any effect on recovery from inactivation. Therefore, the voltage dependence seen in Figs. 6 and 7 cannot come from the effect of voltage on these ions.

Voltage dependence of recovery is stronger with ions that permeate or block

The preceding section demonstrates an intrinsic voltage dependence of recovery, but we find that the voltage dependence is stronger with ions that permeate or block. This is

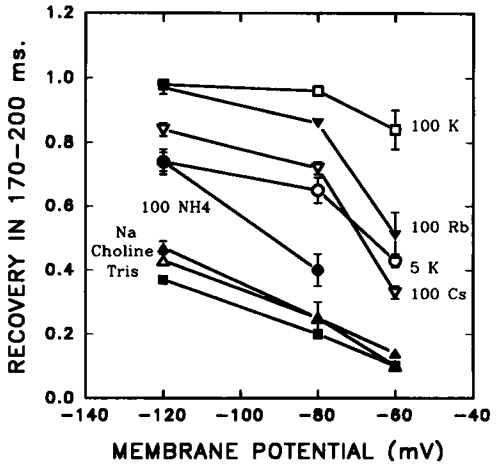

FIGURE 8 Voltage dependence of the extent of recovery from inactivation. The extent of recovery reached in 170–200 ms was measured as a function of the membrane potential during the interpulse. The points are the average of four experiments, with the exception of the points in choline$^+$ and Tris$^+$, which are from single representative experiments.

shown in Fig. 8, which plots the fractional recovery at 170 ms (the end of the fast phase) as a function of voltage, for several ionic conditions. Curves for the impermeant ions Na$^+$, choline$^+$, and Tris$^+$ are grouped together and show a small fractional recovery and a relatively weak voltage dependence. In 100 K$^+$ recovery is complete at all voltages, but

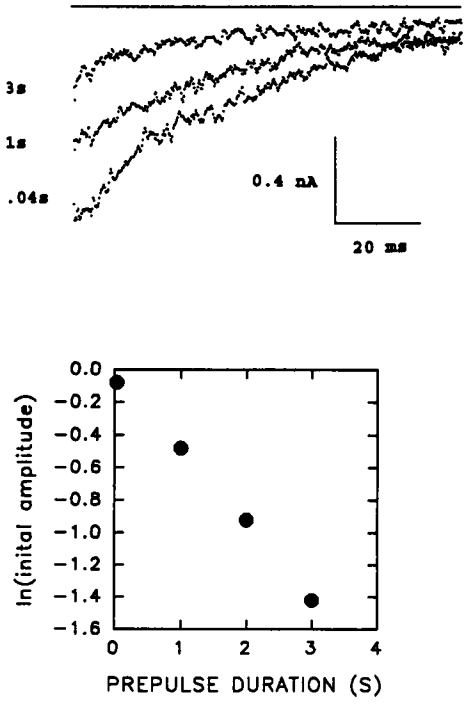

FIGURE 9 The initial amplitude of the slow tails decays exponentially with a time constant of 2.5 s. Slow tails were recorded at −80 mV after +60 mV depolarizations of variable length (indicated). The external solution contained 100 mM K$^+$. The lower panel is a plot of the logarithm of the absolute value of the initial amplitude of the current, measured by fitting an exponential to the slow tails. Experiment ar173a.

in 5 K^+ there is evidence for a strong voltage dependence, particularly between −60 and −80 mV. The same is true for Rb^+ and Cs^+ at 100 mM. Ammonium ion, which is intermediate in permeability, is also intermediate with respect to fractional recovery and voltage dependence. Thus, ions that can be driven through (K^+, Rb^+, $(NH_4)^+$) or into (Cs^+) the membrane have a stronger voltage dependence than the impermeant ions Na^+, choline$^+$, and Tris$^+$.

A hypothesis: there are at least two inactivated states associated with N-type inactivation

An inspection of the time course of recovery in Na^+, choline$^+$, and Tris$^+$ in Figs. 6 and 7 (also Fig. 2) shows that at potentials positive to −120 mV, the fast phase of recovery is followed by a slow phase. Thus, after about 300 ms, it seems that the inactivated channels have passed into a state from which recovery is much slower. This behavior is strikingly similar to the recovery from block by internal TEA derivatives in squid K channels (Armstrong, 1971). The phenomenon in that case was interpreted as trapping of the blocker in the channel by closing of the activation gate. This raises the possibility that the slow phase of recovery is related to N-type inactivation: perhaps the inactivation ball gets trapped in the channel in some way. Or, it is possible that the slow phase is related to another form of inactivation.

In addition to N-type (I_N), *Sh*B has slow or C-type inactivation (I_C) (Iverson and Rudy, 1990) that can be reached from either the open O or the fast (N-type) inactivated state (Hoshi et al., 1991). To our knowledge, only the rate constant for inactivation from the open state has been measured precisely, in channels devoid of fast inactivation. In the mutant *Sh*B 6-46, C-type inactivation develops with a time constant of hundreds of milliseconds to seconds depending on the ionic composition of the external solution (Labarca and MacKinnon, 1992; López-Barneo et al., 1993) and does not depend on voltage in the range of −25 to +50 mV (Hoshi et al., 1991).

At the end of the +60 mV/40 ms pulse, used to inactivate the channels, nearly all of them are in state I_N, which develops with a time constant of only 2 ms. Their presence in I_N is also consistent with the fact that the current recovers with the time course of a single exponential in high external K^+. To get information about the state from which the channels recover slowly in Figs. 6 and 7, we first tried to determine how fast the step is from I_N to I_C. We used the initial amplitude of the slow tails after +60 mV depolarizations of variable length as a measure of the rate of the step from I_N to I_C. The above procedure is based on the following assumptions: (a) after the +60 mV/40 ms pulse, keeping the membrane at +60 mV leads the channels from I_N to I_C; and (b) the initial amplitude of the slow tails is proportional to the fraction of the channels in I_N.

The upper panel in Fig. 9 shows a family of slow tails at −80 mV after +60 mV pulses of variable length. As the duration of the pulse increases, the amplitude of the tails gets smaller; however, even after 3 s there is a noticeable tail, which indicates that the channels leave I_N very slowly. The time course of the slow tails is the same regardless of their initial amplitude, i.e., regardless of the duration of the +60 mV pulse (not shown). The lower panel shows that the initial amplitude of the slow tails decays exponentially with a time constant of about 2.5 s, with 100 K^+ in the external solution.

Even though it is known that the rate of C-type inactivation is slower in K^+ than in Na^+ (López-Barneo et al., 1993), the time course in Fig. 9 seems too slow to account for the steady state in recovery from fast inactivation, which in Na^+, Tris$^+$, and choline$^+$ is achieved in about 300 ms at −80 mV, and

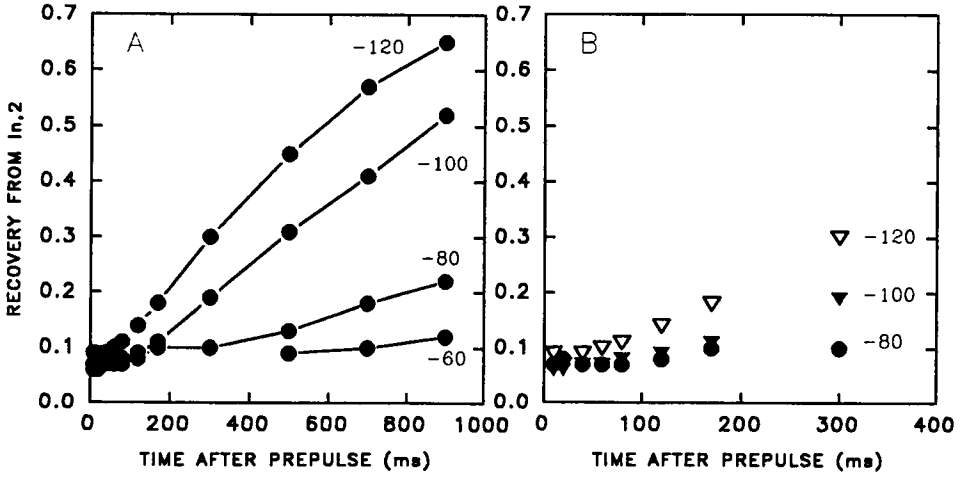

FIGURE 10 Recovery from I_{N2}. (*A*) A variation of the two-pulse protocol in the Na^+ "normal" solution was used to first put about 90% of the channels in I_{N2}, and then to study the recovery from that state: a first +60 mV/40 ms pulse was applied to inactivate the channels, then the membrane was repolarized to −60 mV for 300 ms (this brings about 90% of the channels to I_{N2}, see Figs. 6 and 7). After that time, the membrane potential was changed for a variable "interpulse" time to the indicated voltages. A second +60 mV/40 ms pulse was used to see the recovery during the "interpulse." The fraction of recovery was calculated as in Fig. 2. (*B*) The first 400 ms of recovery in *A* are shown in an expanded time scale. Notice that there is an initial lag that lasts for up to 100 milliseconds, and the lag is voltage-dependent. Experiment jy123a.

in about 150 ms at −60 mV (Figs. 6 and 7). More important, in *Shaker*B, the rate of C-type inactivation is the same in Na^+, Cs^+, and NH_4^+ (τ of 1.8, 2.5, and 1.2 s, respectively) and is much faster with large impermeant molecules like *N*-methyl glucamine (Fig. 1 *C* and Table 1 of López-Barneo et al., 1993). This contrasts markedly with the behavior seen in Figs. 2, 6, and 7, where the fraction of the channels that recover slowly is quite different in Cs^+, $(NH_4)^+$, and Na^+ (20, 50, and 80%, respectively) and is about the same with Na^+, choline$^+$, or Tris$^+$.

Therefore, it seems reasonable to conclude that the slow phase of recovery is unlikely to be the result of C-type inactivation. It is most reasonable to suppose that the slow phase is associated with N-type inactivation.

In accord with the above hypothesis, we will refer to the state from which the channels recover slowly as I_{N2} because at potentials that allow recovery, the conditions that make a substantial fraction of the channels go into state I_{N2} are those that make recovery from I_N slow: low K^+ (<5 mM) and a depolarized potential (≤−80 mV), as shown in Figs. 6, 7, and 8.

Recovery from state I_{N2} depends on V but not on external K^+

We explored the factors that speed the exit from I_{N2} beginning with voltage. We placed the cells in a Na^+ external solution and used a variation of the two-pulse protocol, to put most channels into I_{N2}, and then measured recovery from that state. A first pulse of +60 mV/40 ms to fast inactivate the channels was followed by a 300-ms repolarization to −60 mV (prepulse), at the end of which approximately 90% of the channels were in state I_{N2}. The potential was then changed to a series of values for a variable recovery period, and a second pulse of +60 mV/40 ms tested the recovery.

The results of a typical experiment are shown in Fig. 10, which shows that the exit from I_{N2}, i.e., recovery after the −60 mV/300 ms conditioning period, is voltage-dependent. The extent of recovery increases as the membrane potential becomes more negative. It is also seen that recovery at the new voltage begins after a lag of several milliseconds (>60 ms). The lag shortens as the potential is made more negative. This point is easier to see in Fig. 10 *B*, where only the first 400 ms of recovery are shown. We observed the same result in four other cells. The presence of a latency can be interpreted as an indication that I_{N2} is more than one state, but for simplicity we will refer to it as one.

Recovery in the fast phase, from state I_N, is influenced by permeant ions in the external medium. Is the same true for recovery from I_{N2}? In Fig. 11, we examined the dependence on external K^+. Most of the channels were put in I_{N2} as above, but 500 ms after the first pulse the external K^+ was raised to 100 mM. After 500 ms in K^+, a second pulse was applied to see the recovery. The second pulse had a duration of 2.5 ms, and an inward tail current at pulse-end made it clear that K^+ indeed surrounded the cell (Fig. 11 *B*). In evaluating this experiment, it is necessary to keep in mind that raising external K^+ causes only a small reduction in outward current at +60 mV, expected to be 8% using the Goldman Hodgkin Katz (GHK) equation. However, it is known that in some K channels the external K^+ modulates the magnitude of the outward current in a rather more complex way than that predicted by the GHK equation (Almers and Armstrong, 1980; Oxford and Wagoner, 1989; Pardo et al., 1992; López-Barneo et al., 1993), so we measured, in 12 other experiments, the actual reduction in the current brought about by just the addition of K^+; we found a reduction of 32 ± 6%.

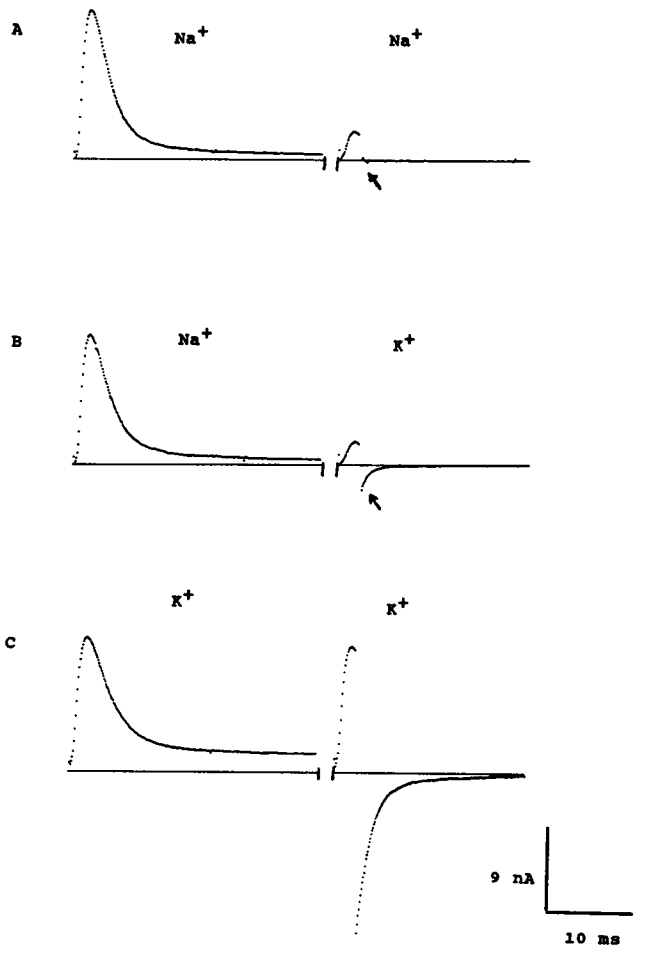

FIGURE 11 Effect of external K^+ on recovery from I_{N2}. A variation of the two-pulse protocol in the Na^+ normal solution was used, as in Fig. 10, to put about 90% of the channels in I_{N2}. (*A*) Control in Na^+. The second pulse (+70 mV/2.5 ms) was applied after 1000 ms at −60 mV. Notice that, as expected, there is no inward deactivation tail (*arrow*). Notice also the poor extent of recovery. (*B*) Effect of K^+. After the first pulse (+70 mV/40 ms), the membrane was repolarized to −60 mV/500 ms and then, while the voltage was kept at −60 mV, a pulse of 100 mM K^+/500 ms was applied to the cell, with a second pipette connected to a pressure ejection system (not sampled). Then a +70 mV/2.5 ms pulse was applied to see the recovery. Notice the inward deactivation tail (*arrow*), which demonstrates that there was K^+ outside the cell; however, the extent of recovery was the same as in Na^+. Thus, recovery from I_{N2} does not depend on the ionic composition of the external solution. (*C*) Control in K^+. After having obtained the traces in *A* and *B*, the same cell was put in a 100 mM K^+ external solution and the pulse protocol in *A* was applied. The trace shows that, as opposed to I_{N2}, the N-type inactivation of the channels was relieved by the external K^+, as expected. Experiment: ju043a.

A comparison of the traces in Figs. 11 *A*, with Na^+ surrounding the cell, with those in Fig. 11 *B*, where K^+ surrounded the cell for 500 ms, shows that external K^+ does not modify efficiently the recovery from I_{N2}. The peak current during the second pulse, in Fig. 11 *B*, is quite small; it has an 83% reduction relative to that in the first pulse. This is far more than expected by just the addition of K^+, and it is in marked contrast with the effectiveness of K^+ during recovery from I_N. For a control, Fig. 11 *C* shows the currents with the two pulse protocol when the same cell was continuously bathed in 100 mM K^+. We observed the same result in three other cells.

Fig. 11 shows that in state I_{N2} the inactivation balls are screened from the influence of external K^+. This suggests that the channels do not reflux through the open state while recovering from I_{N2}, because if that were the case K^+ should speed recovery as it does in the fast phase (recovery from I_N).

DISCUSSION

Two or more inactivated states

The time course of recovery shows two distinct phases and suggests that after 200–300 ms the channels that are still inactivated enter a new conformational state, I_{N2}, from which recovery is very slow. I_{N2} is quite different from the initial inactivated state (I_N) in that external K^+ no longer speeds recovery like it does during the fast phase (Fig. 11). Entry into this state is favored by the absence of permeant cations externally and by relatively positive V_m (−60 mV). Recovery from I_{N2} is speeded by very negative voltage, even when applied after a lag, as in Fig. 10.

The precise nature of state I_{N2} (which may be more than one state, Fig. 10 *B*) is not clear, but it seems likely that the conformational change from I_N to I_{N2} precludes further movement of ions into and out of the outer part of the pore, thus removing the influence of external ions on recovery. Further, it seems unlikely that channels reflux through the conducting state as they recover from I_{N2}, again because of the lack of influence of external ions. Thus, channels recovering from I_{N2} may bypass the conducting state, as is the case during recovery from inactivation in Na channels, which do not conduct during recovery from inactivation (Armstrong and Croop, 1982).

Ion permeation and recovery from inactivation

External cations influence recovery only during the fast phase, before channels enter state I_{N2}. It is convenient to divide the tested ions based on their permeability through K channels, and their tendency to block. The fast phase of recovery is smallest with the impermeant cations Na^+, choline$^+$, and Tris$^+$. In the presence of the permeant cations K^+ and Rb^+ at 100 mM, recovery is virtually complete during the fast phase. The sparingly permeant $(NH_4)^+$ ion has a less strong influence in speeding the fast phase of recovery.

Somewhat surprisingly, the impermeant Cs^+ ion speeds recovery more effectively than does the sparingly permeant $(NH_4)^+$ ion. Judging from the electrical distance, Cs^+ penetrates deeply into the pore, and presumably occupies a site near the inactivating ball. Ca^{2+} also blocks K channels (Gómez-Lagunas and Armstrong, 1993), but does penetrate as far into the channel, as judged by its electrical distance, and has no influence on the fast phase of recovery.

The voltage dependence of recovery

Our results show that recovery both from state I_N and from state I_{N2} increases with hyperpolarization. From both states, hyperpolarization speeds recovery, even in the absence of permeant cations, suggesting an intrinsic voltage dependence to the recovery process. The influence of hyperpolarization is much more pronounced, however, in K^+, Rb^+, or Cs^+. Driving these ions into the pore apparently adds to the intrinsic voltage dependence, making the overall effect of voltage much stronger.

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