

# Anomalous Effect of Permeant Ion Concentration on Peak Open Probability of Cardiac Na<sup>+</sup> Channels

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**ABSTRACT** Human heart Na<sup>+</sup> channels were expressed transiently in both mammalian cells and *Xenopus* oocytes, and Na<sup>+</sup> currents measured using 150 mM intracellular Na<sup>+</sup>. Decreasing extracellular permeant ion concentration decreases outward Na<sup>+</sup> current at positive voltages while increasing the driving force for the current. This anomalous effect of permeant ion concentration, especially obvious in a mutant (F1485Q) in which fast inactivation is partially abolished, is due to an alteration of open probability. The effect is only observed when a highly permeant cation (Na<sup>+</sup>, Li<sup>+</sup>, or hydrazinium) is substituted for a relatively impermeant cation (K<sup>+</sup>, Rb<sup>+</sup>, Cs<sup>+</sup>, *N*-methylglucamine, Tris, choline, or tetramethylammonium). With high concentrations of extracellular permeant cations, the peak open probability of Na<sup>+</sup> channels increases with depolarization and then saturates at positive voltages. By contrast, with low concentrations of permeant ions, the open probability reaches a maximum at approximately 0 mV and then decreases with further depolarization. There is little effect of permeant ion concentration on activation kinetics at depolarized voltages. Furthermore, the lowered open probability caused by a brief depolarization to +60 mV recovers within 5 ms upon repolarization to -140 mV, indicative of a gating process with rapid kinetics. Tail currents at reduced temperatures reveal the rapid onset of this gating process during a large depolarization. A large depolarization may drive a permeant cation out of a site within the extracellular mouth of the pore, reducing the efficiency with which the channel opens.

**KEY WORDS:** sodium channels • gating • single channel recording • kinetics

## INTRODUCTION

The selective pores of ion channels are said to be opened and closed by “gates,” which may be directly regulated by a variety of factors including the concentrations of agonists and antagonists (Hille, 1992). The gating of voltage-dependent ion channels (e.g., the Na<sup>+</sup>, Ca<sup>2+</sup>, and K<sup>+</sup> channels of nerve and striated muscle) is primarily controlled by transmembrane potential. The conformational transitions underlying voltage-dependent gating are also influenced by the concentration and identity of the permeant ion, sometimes in profound ways. For example, some Ca<sup>2+</sup> channels inactivate strongly when Ca<sup>2+</sup> ions carry the inward current but not when Ba<sup>2+</sup> is the permeant ion (Brehm and Eckert, 1978; Tillotson, 1979). The voltage-dependent gating of K<sup>+</sup> channels is also affected by K<sup>+</sup> concentration. In general an increase in extracellular K<sup>+</sup> concentration increases open probability at any membrane potential, inhibits inactivation, and increases the rate of recovery from inactivation (Swenson and Armstrong, 1981; Clay, 1986; Matteson and Swenson, 1986; Demo and Yellen, 1991; Pardo et al., 1992; Lopez-Barneo et al., 1993; Gomez-Lagunas and Armstrong, 1994; Bau-

rowitz and Yellen, 1995; Levy and Deutsch, 1996*a, b*). These effects are usually interpreted as a consequence of the binding of K<sup>+</sup> ions in or near the ion-conducting pore. For example, K<sup>+</sup> ions may bind near the extracellular mouth of the pore and interfere with the closing of either an activation or an inactivation gate. Alternatively the binding of K<sup>+</sup> ions inside the pore may enhance the opening of an inactivation gate. The open probability of a class of Cl<sup>-</sup> channels is also enhanced by increasing extracellular Cl<sup>-</sup> concentration (Pusch et al., 1995; Chen and Miller, 1996).

Although intracellular Na<sup>+</sup> concentration affects the rates and steady-state levels of inactivation of Na<sup>+</sup> channels (e.g., Chandler and Meves, 1965; 1970; Oxford and Yeh, 1985), changes of extracellular Na<sup>+</sup> concentration between 15 mM and 4 M have relatively small effects on either the kinetics or voltage dependence of Na<sup>+</sup> currents of squid axon (Armstrong and Bezanilla, 1974; Oxford and Yeh, 1985; Correa and Bezanilla, 1994; Bezanilla and Correa, 1995; A.M. Correa and F. Bezanilla, personal communication). Our own examination of currents from cardiac Na<sup>+</sup> channels also shows little effect of external Na<sup>+</sup> concentration on fast inactivation kinetics (O’Leary et al., 1994; Tang et al., 1996).

We report here an anomalous effect of changing extracellular permeant ion concentration, namely a decrease in Na<sup>+</sup> current in response to an increase of

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driving force. This effect is only observed when using impermeant substitutes for Na<sup>+</sup> ions and is especially obvious in a mutant in which fast inactivation is partially abolished. With low concentrations of extracellular permeant cations, a fraction of Na<sup>+</sup> channels do not open in response to large depolarizations. This concentration-dependent fraction is determined very rapidly after a depolarization and has little effect on the activation kinetics of the channels which open in response to the depolarization.

## METHODS

### Molecular Biology

The isolation of the hH1a Na<sup>+</sup> channel and the generation of the F1485Q mutant have been described previously (Hartmann et al., 1994). The cardiac clones hH1 and hH1a differ by 9 amino acid residues in regions that are poorly conserved among other isoforms of Na<sup>+</sup> channels (Hartmann et al., 1994). No functional differences are known between these two clones. In vitro transcription of wild-type (WT)<sup>1</sup> and F1485Q cDNA was performed as in Hartmann et al. (1994).

### Channel Expression in tsA201 Cells

Na<sup>+</sup> channels were transiently transfected into tsA201 cells, a transformed human kidney (HEK293) cell line, by standard calcium phosphate methods (Chahine et al., 1994). Cells were cotransfected with 10 µg each of cDNA encoding either WT or F1485Q hH1a channels and a surface marker, CD8 (Margolskee et al., 1993). Cells were maintained in standard growth medium at 37°C with 10% CO<sub>2</sub> until use. Whole cell Na<sup>+</sup> currents were recorded from cells 2–3 d after transfection. Transfected cells were selected using beads coated with an antibody against CD8 (Dynabeads M-450 CD8; Dynal, Lake Success, NY; Jurman et al., 1994). More than 90% of the bead-decorated cells expressed Na<sup>+</sup> currents.

### Channel Expression in Oocytes

Stage V or VI oocytes were defolliculated enzymatically and injected with WT or F1485Q cRNA (0.5–4 ng per oocyte). Injected oocytes were maintained at 18°C in a 50% diluted solution of Leibovitz's L-15 medium (Gibco BRL, Grand Island, NY) containing 15 mM HEPES (pH 7.6), 1 mM glutamine, and 100 µg/ml gentamycin. Single-channel recordings were obtained 1–3 d after injection.

### Electrophysiology

Whole-cell voltage clamp recordings were carried out as previously reported (O'Leary and Horn, 1994). Sylgard-coated (Dow-Corning Corp., Midland, MI), fire-polished pipettes of Corning 8161 glass were used. Currents were filtered at 5 kHz with an Axopatch 200A patch clamp amplifier (Axon Instruments Inc., Burlingame, CA). Data were acquired with pCLAMP6 and the Digidata 1200 interface (Axon Instruments). Cells were dialyzed at least 10 min before recording data. Series resistance was <2 MΩ

after 80% compensation. The pipette solution consisted of (in mM) 140 NaF, 10 NaCl, 5 EGTA, and 10 Cs-HEPES (pH 7.4). The 10 mM Na<sup>+</sup> bath solution contained (in mM) 10 NaCl, 140 NMG-OH (*N*-methyl-D-glucamine hydroxide), 2 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES (titrated to pH 7.4 with methanesulfonic acid). The 150 mM Na<sup>+</sup> bath solution contained (in mM) 150 NaCl, 2 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 HEPES (titrated to pH 7.4 with NaOH). In other solutions the NaCl was replaced by NMG-methanesulfonate, or by the chloride salts of Li<sup>+</sup>, K<sup>+</sup>, Rb<sup>+</sup>, Cs<sup>+</sup>, NMG, Tris(hydroxymethyl)-aminomethane, tetramethylammonium, choline, or hydrazinium. We found no difference between NMG-methanesulfonate and NMG-Cl in our experiments. The hydrazinium chloride solution was made by substituting 150 mM hydrazinium monochloride for 150 mM NaCl in the bath solution, and then titrating the solution to pH 7.4 with measured amounts of liquid hydrazine (Aldrich Chemical Co., Milwaukee, WI). Assuming a pK of 7.97 for the hydrazinium ion, we calculate the total concentration of cationic hydrazinium in our solution to be 138 mM. All solution components were purchased either from Aldrich or Sigma Chemical Co. (St. Louis, MO). Corrections were made for liquid junction potentials. After seal formation, cells were gently lifted off the bottom of the dish to allow better access to the entire cell surface. Bath solutions were exchanged by application of a desired solution to single cells with a Sigmacoted (Sigma) Corning Pyrex macropipette (Yang et al., 1996). Most experiments were performed at room temperature (19–21°C). The tail current experiments of Fig. 10 were done at a reduced temperature effected by Peltier devices regulated by a TC-10 Temperature Controller (Dagan Corp., Minneapolis, MN).

Single-channel currents were recorded from outside-out patches pulled from cRNA-injected *Xenopus* oocytes after removal of the vitelline membrane. Patch pipettes made of 1.5 mm O.D. quartz were coated with a mixture of Parafilm (American National Can, Neenah, WI) and heavy mineral oil. Under these conditions the recordings had a noise level typically <150 fA rms at a bandwidth of 5 kHz. Pipette and bath solutions were identical to those used in whole-cell recordings from tsA201 cells. Data acquisition was similar to that described for whole-cell recordings. Single-channel currents were filtered at 5 kHz, sampled at 20–100 kHz, and digitally filtered again at 5 kHz. The effective dead time for a detectable transition was ~50 µs.

### Data Analysis

Whole-cell and single-channel data were analyzed with a combination of pCLAMP programs, Microsoft Excel, Origin (Microcal, Northampton, MA), and our own Basic programs. Single-channel currents were examined with the pCLAMP program FETCHAN and the single-channel amplitudes were determined by fitting amplitude histograms to sums of Gaussian distributions with Origin. Unless otherwise specified, data are expressed as mean ± SEM.

Changes of the extracellular solution at any membrane potential can change the peak macroscopic Na<sup>+</sup> current  $\{I(V)\}$  by affecting the single channel current at that voltage  $\{i(V)\}$  and/or by affecting the open probability at that voltage  $\{P_{\text{open}}(V)\}$ . For a bath solution of interest, denoted by the superscript  $j$ , the peak open probability in a population of  $N$  channels is given by

$$P_{\text{open}}^j(V) = \frac{I^j(V)}{Ni^j(V)}. \quad (1)$$

Because we did not know the value of  $N$ , we arbitrarily normalized the maximum  $P_{\text{open}}$  in an extracellular solution of 150 mM Na<sup>+</sup> to unity in Fig. 5, and scaled the  $P_{\text{open}}$  values in other solutions to this value. To do this we obtained the ratio of single channel current amplitudes for any two extracellular solutions ( $j = a, b$ ) at each voltage. This ratio was used to estimate the rela-

<sup>1</sup>Abbreviations used in this paper: GHK, Goldman-Hodgkin-Katz; I-V, current-voltage; i-V, single-channel current-voltage;  $[Na^+]_o$ , external Na<sup>+</sup> concentration;  $P_{\text{open}}$ , peak open probability; P-V,  $P_{\text{open}}$ -voltage; NMG, *N*-methyl-D-glucamine; WT, wild type.

tive values of  $P_{\text{open}}$  for the two solutions from the relative macroscopic currents at each voltage, using the following equation:

$$\frac{P_{\text{open}}^a(V)}{P_{\text{open}}^b(V)} = \frac{i^b(V)}{i^a(V)} \frac{I^a(V)}{I^b(V)} \quad (2)$$

These probabilities are shown in Fig. 5, where solution  $b$  is 150 mM  $\text{Na}^+$ .

We obtained single channel records at positive voltages. To estimate a normalized  $P_{\text{open}}$ -voltage (P-V) relationship over the entire range of activation, we assumed that the single-channel current-voltage relationship had the form of the Goldman-Hodgkin-Katz (GHK) current equation (Hille, 1992, 341–345). Although the GHK model is insufficient to describe all details of permeation in  $\text{Na}^+$  channels (Hille, 1975), this model fit our data well over a range of positive voltages when the external cation was predominantly  $\text{Na}^+$  or NMG (see Fig. 3 C). In the case of symmetrical concentrations of  $\text{Na}^+$ , the GHK current-voltage relationship is linear, so that the P-V relationship is equivalent to a conductance-voltage relationship. We estimated the relative permeability of other cations to  $\text{Na}^+$  from biionic reversal potentials and calculated the current-voltage relationship for a single channel (i-V) obeying the GHK current equation. The normalized P-V relationship was then obtained by dividing the peak macroscopic I-V relationship by the GHK i-V relationship and scaling the maximum to unity (see Fig. 6).

## RESULTS

### *Anomalous Effect of $\text{Na}^+$ Driving Force on $\text{Na}^+$ Current Amplitude*

We examine here the effects of changing external  $\text{Na}^+$  concentration ( $[\text{Na}^+]_o$ ) on outward currents through both WT and mutant hH1a  $\text{Na}^+$  channels expressed transiently in tsA201 cells. In all cases we use a high concentration (150 mM) of intracellular  $\text{Na}^+$  to maximize the amplitude of outward currents. The F1485Q mutation used in most experiments substitutes a glutamine for a phenylalanine in the linker between domains 3 and 4, a cytoplasmic region postulated to act as the fast inactivation gate (Stühmer et al., 1989; Moorman et al., 1990; West et al., 1992). F1485Q channels inactivate much more slowly than WT channels (Hartmann et al., 1994).

Fig. 1 shows whole-cell  $\text{Na}^+$  currents of WT and F1485Q channels sequentially bathed in 10, 150, and 10 mM  $\text{Na}^+$  solutions. WT currents inactivate quickly and completely within a few milliseconds (Fig. 1 A), whereas the mutant channels inactivate more slowly and only partially (Fig. 1 B). Because of the 150 mM intracellular  $[\text{Na}^+]_i$  in all cases, we obtain only outward  $\text{Na}^+$  current for 10 mM external  $[\text{Na}^+]_o$  and both inward and outward current for 150 mM external  $[\text{Na}^+]_o$ , where the reversal potential is  $\sim 0$  mV. The peak outward currents for both WT and mutant channels at large positive voltages are reversibly increased by raising  $[\text{Na}^+]_o$ , in spite of the fact that the electrochemical driving force for outward current is decreased (Figs. 1 and 2, A and B). Based solely on the Nernst potential

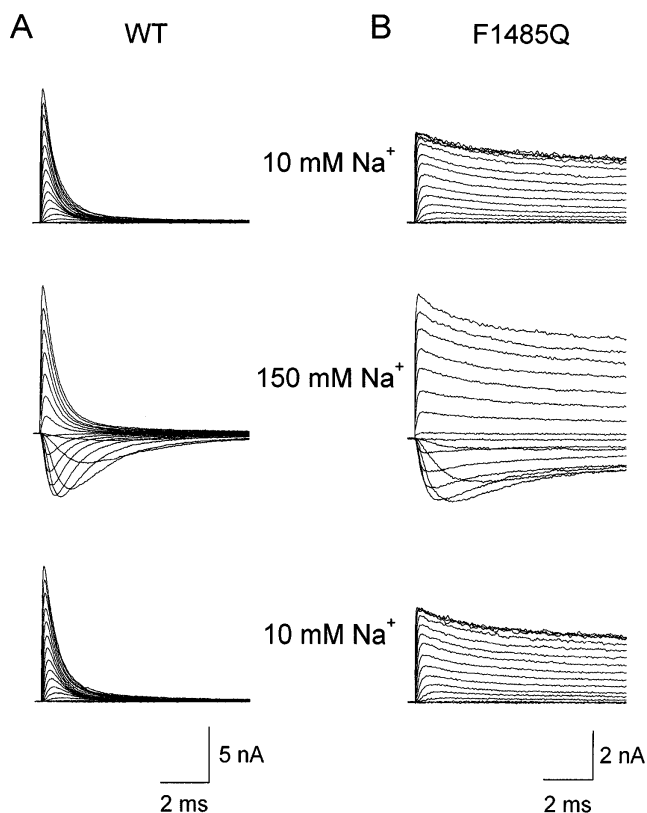


FIGURE 1. WT and F1485Q hH1a  $\text{Na}^+$  channel currents in 10 and 150 mM  $[\text{Na}^+]_o$ . Currents were elicited by 9-ms depolarizations to voltages ranging from  $-80$  to  $+70$  mV in 10-mV increments from a holding potential of  $-140$  mV and at a frequency of 0.5 Hz. Each panel shows families of  $\text{Na}^+$  currents obtained for one cell transfected with either WT (A) or F1485Q (B)  $\text{Na}^+$  channels and successively bathed in 10, 150, and 10 mM external  $\text{Na}^+$ . Intracellular  $[\text{Na}^+]_i$  was 150 mM.

for  $\text{Na}^+$  ions, the driving force is decreased by 68 mV when  $[\text{Na}^+]_o$  is raised from 10 to 150 mM, whereas the peak outward current at a test potential of  $+70$  mV increases by 12 and 46% in WT and F1485Q, respectively (Fig. 2, A and B). Because this anomalous effect of  $[\text{Na}^+]_o$  is more pronounced in the F1485Q mutant, we restricted the following experiments to this mutant.

In the experiments described above, extracellular NaCl was replaced with NMG-methanesulfonate, raising the possibility that the effects observed were a result of changing either the chloride or the NMG concentration. To test this we compared the effects of external NMG-methanesulfonate with those of  $\text{CsCl}$ .<sup>2</sup>  $\text{CsCl}$  has qualitatively the same effect as NMG-methanesulfonate (compare Fig. 2, B and C), although the magnitude of the effect of changing  $[\text{Na}^+]_o$  is less when  $\text{Cs}^+$  is used as a  $\text{Na}^+$  substitute (20 versus 46% at  $+70$  mV). The quantitative difference between NMG and  $\text{Cs}^+$  is shown

<sup>2</sup>Note that all substitutes for  $\text{Na}^+$  in this paper are predominantly monovalent cations at neutral pH.

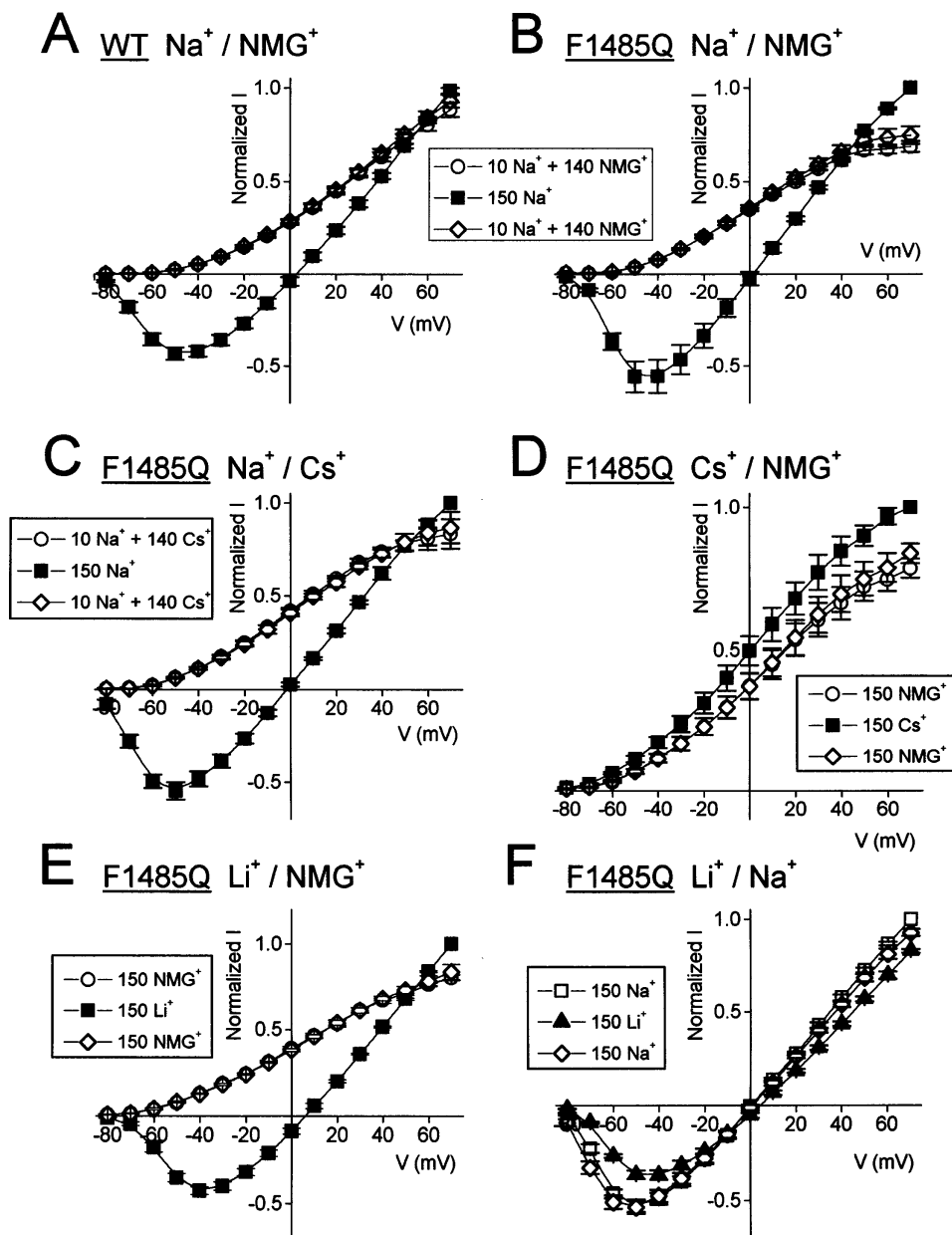


FIGURE 2. Effects of external cations on peak  $\text{Na}^+$  current-voltage relations. Currents were activated as described in the legend of Fig. 1. Normalized peak currents for WT- (A,  $n = 5$ ) and F1485Q- (B,  $n = 3$ ) transfected cells sequentially bathed in 10, 150, and 10 mM  $\text{Na}^+$ . (C, D, and E) Current-voltage relations for F1485Q-transfected cells successively bathed in (in mM): 10  $\text{Na}^+$  (140  $\text{Cs}^+$ ), 150  $\text{Na}^+$ , and 10  $\text{Na}^+$  (140  $\text{Cs}^+$ ) (C,  $n = 3$ ); 150 NMG, 150  $\text{Cs}^+$ , and 150 NMG (D,  $n = 5$ ); 150 NMG, 150  $\text{Li}^+$ , and 150 NMG (E,  $n = 4$ ); or 150  $\text{Na}^+$ , 150  $\text{Li}^+$ , and 150  $\text{Na}^+$  (F,  $n = 3$ ). Intracellular  $[\text{Na}^+]_i$  was 150 mM in all cases.

more clearly in Fig. 2 D, where complete replacement of 150 mM NMG by  $\text{Cs}^+$  reversibly increases outward  $\text{Na}^+$  current. We will show below that these impermeant cations differ primarily in their effects on the amplitudes of single channel currents and that  $[\text{Na}^+]_o$  influences both the amplitude of single channel current and the peak open probability ( $P_{\text{open}}$ ).

To test if the modulatory action of  $\text{Na}^+$  ions may be generalized to other permeant cations, we examined the effects of  $\text{Li}^+$  ions on peak outward  $\text{Na}^+$  currents.  $\text{Li}^+$  is more permeant through  $\text{Na}^+$  channels than  $\text{Na}^+$ , as indicated by the positively shifted reversal potential ( $P_{\text{Li}}/P_{\text{Na}} \cong 1.2$ ; Fig. 2, E and F), and is less conductive than  $\text{Na}^+$ , as indicated by the smaller amplitude of inward current at negative voltages (Fig. 2 F). In spite of

the fact that  $\text{Li}^+$  decreases the driving force for outward current more than  $\text{Na}^+$ , there is still a 25% increase in outward current at +70 mV when 150 mM NMG is replaced by 150 mM external  $\text{Li}^+$  (Fig. 2 E). The quantitative difference observed between  $\text{Na}^+$  and  $\text{Li}^+$  ions could arise from different permeation properties of these two cations, a possibility explored below.

Although lowering the extracellular concentration of permeant ions has the expected consequence of increasing the driving force for outward current, it decreases outward  $\text{Na}^+$  current. This anomalous effect on peak current amplitude is due either to an decrease in single-channel current at positive voltages or to a decreased  $P_{\text{open}}$ . To determine which of these is affected by  $[\text{Na}^+]_o$ , we examined the effects of extracellular ions

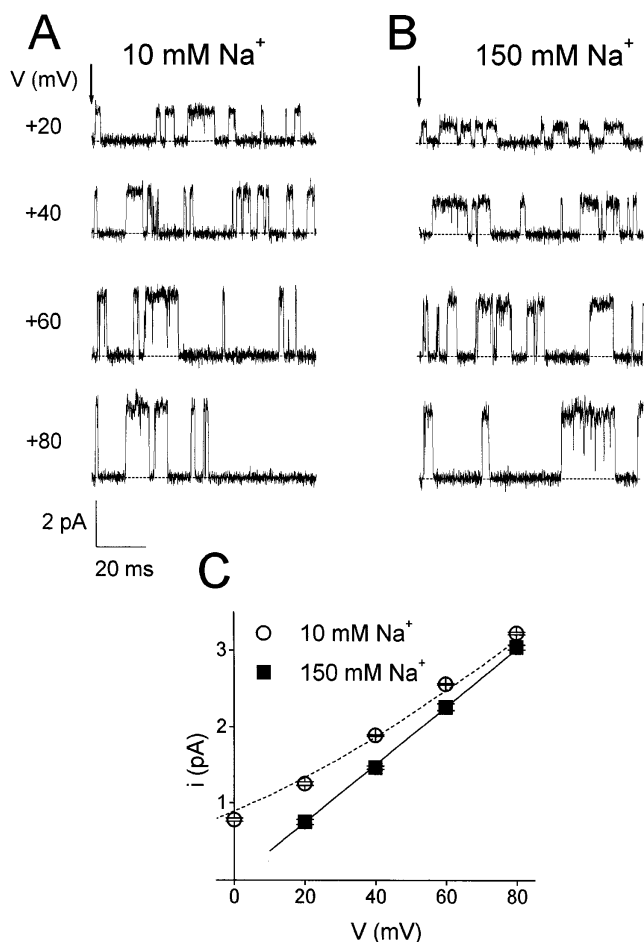


FIGURE 3. Effects of  $[Na^+]_o$  on single-F1485Q channel currents. Selected single-channel current recordings from outside-out patches bathed in 10 (A) and 150 mM  $Na^+$  (B). Internal  $[Na^+]_i$  was 150 mM. Currents were activated by 90-ms depolarizations (arrow) to voltages ranging from +20 to +80 mV (as indicated to the left of the traces) from a holding potential of  $-140$  mV. The dotted lines represent the closed level. (C) Single-channel current-voltage relations in 10 ( $n = 2$ ) and 150 mM ( $n = 4$ )  $Na^+_o$ . Data points for 150 mM  $Na^+$  were fit by linear regression, yielding an estimate of the GHK permeability (solid line). The dotted line represents single-channel currents for 10 mM  $Na^+_o$  as predicted by the GHK current equation.

on single-channel currents at positive voltages. Because membrane patches from *Xenopus* oocytes yield more stable and lower noise recordings than do patches from tsA201 cells, this experiment was performed with patches from oocytes injected with cRNA encoding F1485Q hH1a. Single channel recordings from outside-out patches bathed in 10 and 150 mM  $Na^+$  are shown in Fig. 3. NMG was used as a  $Na^+$  substitute. Single-channel current amplitudes at voltages ranging from +20 to +80 mV are larger in 10 mM than in 150 mM extracellular  $Na^+$ , as predicted by the GHK current equation (dashed line, Fig. 3 C). This is in contrast with the observation that lower  $[Na^+]_o$  causes a reduction in

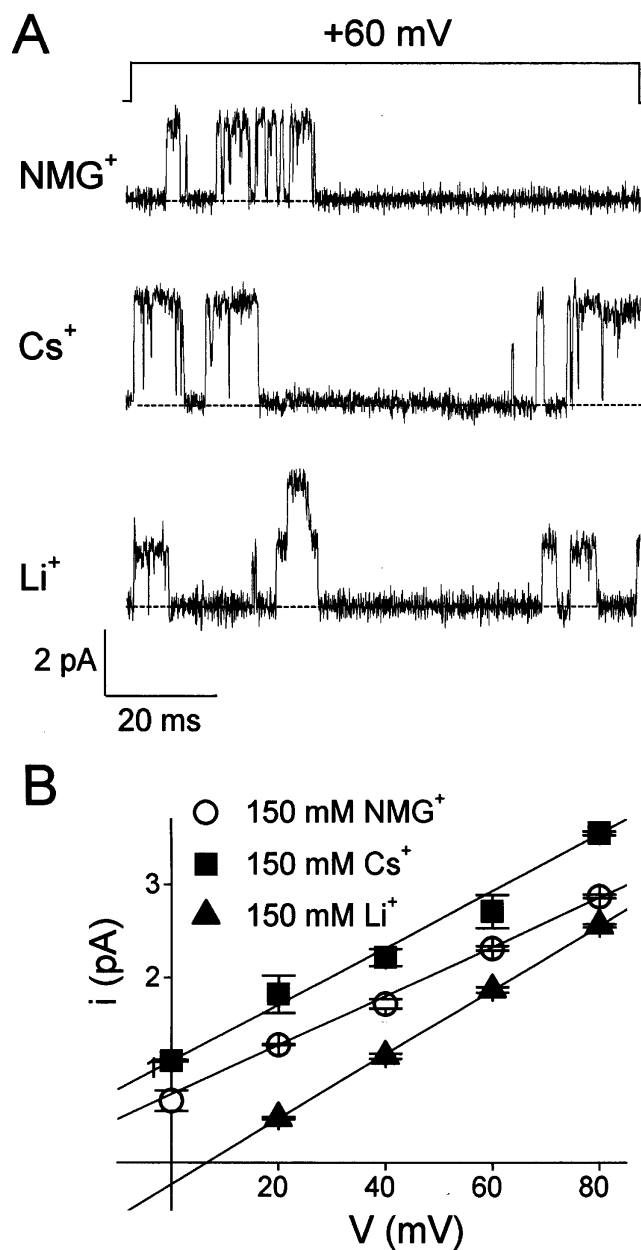


FIGURE 4. Effects of external cations on single-channel outward currents. Currents were evoked as described in the legend of Fig. 3. (A) Selected single-channel recordings obtained in the presence of 150 mM NMG, 150 mM  $Cs^+$ , and 150 mM  $Li^+$  in the bath solution ( $V = +60$  mV). (B) Current-voltage relations for 150 mM NMG $_o$  ( $n = 2$ ), 150 mM  $Cs^+_o$  ( $n = 3$ ), and 150 mM  $Li^+_o$  ( $n = 3$ ). Data points were fit to straight lines with slopes of 27, 31, and 35 pS for NMG,  $Cs^+$ , and  $Li^+$ , respectively.

whole-cell F1485Q channel currents at voltages  $> +40$  mV (Fig. 2 B). Therefore, decreasing  $[Na^+]_o$  must decrease  $P_{open}$ . The close prediction to the GHK equation further suggests that NMG is acting as an inert substitute for  $Na^+$ . To test the influence of  $Cs^+$  and  $Li^+$  on single-channel current amplitudes, we compared outward currents in 150 mM  $Cs^+_o$ , 150 mM  $Li^+_o$ , and 150

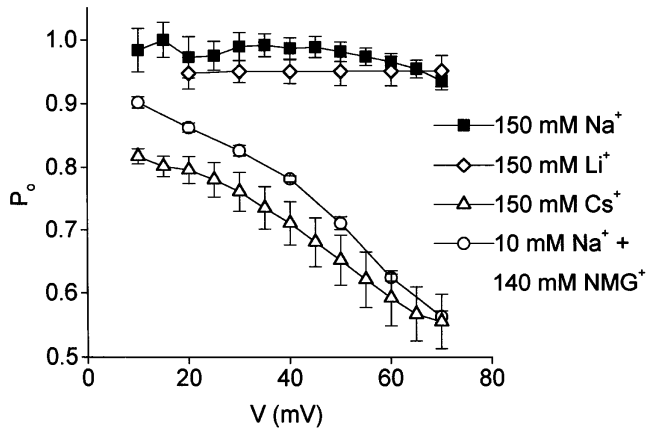


FIGURE 5. Effect of extracellular  $\text{Na}^+$ ,  $\text{Cs}^+$ , and  $\text{Li}^+$  on  $P_{\text{open}}$  of F1485Q  $\text{Na}^+$  channels.  $P_{\text{open}}$  ( $P_o$ ) was calculated from whole-cell peak currents (Fig. 2) and single-channel i-V relations (Figs. 3 and 4) as described in METHODS. NMG replaced  $\text{Na}^+$  in the 10 mM  $\text{Na}^+$  bath solution. Data are means  $\pm$  SEM normalized to the maximum  $P_o$  obtained in 150 mM  $\text{Na}^+$ .

mM  $\text{NMG}_o$ . Fig. 4 shows that between 0 and +80 mV the amplitude of single channel currents increases in the following order:  $\text{Li}^+ < \text{NMG} < \text{Cs}^+$ .

#### Voltage-dependent Effect of $[\text{Na}^+]_o$

When  $[\text{Na}^+]_o$  is  $\leq 10$  mM the peak amplitudes of outward currents saturate as the voltage increases (Figs. 1 B and 2), although the single-channel current-voltage relationship is nearly linear in this range (Figs. 3 and 4). The saturation of the peak current-voltage relationship is indicative in these experiments of the predominance of an impermeant cation (i.e.,  $\text{Cs}^+$  or NMG) in the bath. To quantify this effect of extracellular cations, we divided the peak macroscopic currents from whole cell recordings by the single channel current amplitudes at positive voltages to produce scaled estimates of  $P_{\text{open}}$  (Eqs. 1 and 2 in METHODS). Although  $P_{\text{open}}$  is relatively constant at positive voltages in high  $[\text{Na}^+]_o$  or  $[\text{Li}^+]_o$ , it decreases with depolarization in both  $\text{Cs}^+$  and NMG solutions (Fig. 5).

We tested the generality of this effect of impermeant cations by replacing all the  $\text{Na}^+$  in the bath solution with  $\text{Li}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$ ,  $\text{Cs}^+$ , NMG, Tris, choline, or tetramethylammonium, and estimating the  $P_{\text{open}}$ -voltage (P-V) relationship from whole cell currents over the entire voltage range (see METHODS). We obtained saturating current-voltage relationships only for impermeant cations,<sup>3</sup> and the resultant P-V curves, normalized to a maximum of 1.0, show a voltage-dependent decrease in a voltage range where the P-V curve is relatively constant in high  $[\text{Na}^+]_o$  (*dashed line* in each panel of Fig. 6). At negative

<sup>3</sup> $\text{K}^+$  was measurably permeant in these experiments, however, with a permeability ratio of 0.08 compared with  $\text{Na}^+$ .

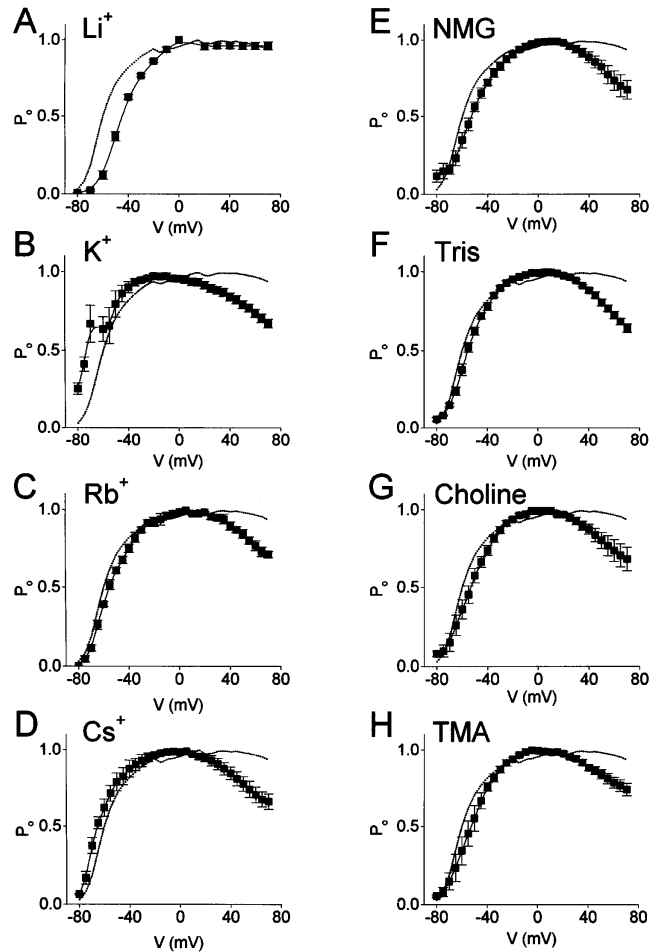


FIGURE 6. Effects of impermeant cations on normalized P-V relationships. Peak  $P_{\text{open}}$  ( $P_o$ ) was determined from whole-cell and single-channel current-voltage relations as described in METHODS. Data from F1485Q-transfected cells bathed in 150 mM of the indicated cations. In each panel, the dotted line corresponds to the P-V curve obtained with 150 mM  $\text{Na}^+$ . Data are means  $\pm$  SEM with maximums normalized to unity.

voltages there are also small effects of some of these  $\text{Na}^+$  substitutes on the shape of the P-V relationship. This may be due in part to the simplified assumption that the single-channel i-V relationship has the form of the GHK equation. However the foot of the P-V relationship is shifted to more depolarized voltages for  $\text{Li}^+$  than for other cations. By contrast with all the impermeant cations, the nonmetal monovalent cation hydrazinium ( $\text{H}_2\text{N-NH}_3^+$ ), which has appreciable permeability in  $\text{Na}^+$  channels (Hille, 1971), produces a peak current-voltage relationship that increases nearly linearly at positive voltages (Fig. 7 A) and a P-V relationship fairly constant at positive voltages (Fig. 7 B). Based on the reversal potential of  $-12.8$  mV and the cationic concentration of 138 mM (see METHODS), the permeability ratio of hydrazinium to  $\text{Na}^+$  is 0.65. Our data show, therefore, that permeant and impermeant cat-

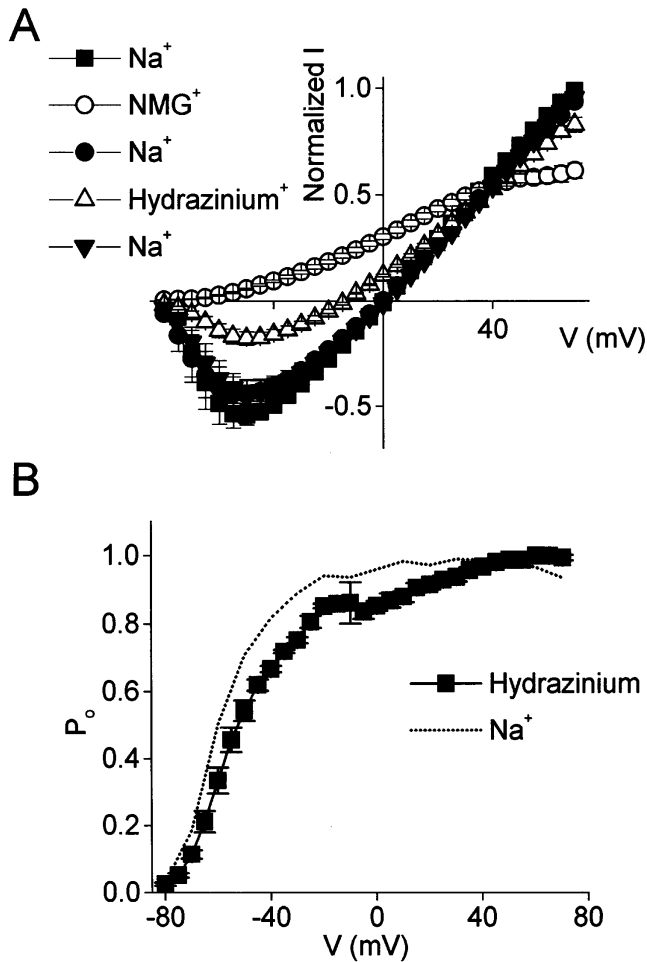


FIGURE 7. Effect of hydrazinium on the I-V relationship and channel open probability. Currents were evoked as described in the legend of Fig. 1 except that 5-mV increments were used between each pulse. (A) I-V relationships obtained from F1485Q-transfected cells were successively bathed in Na<sup>+</sup>, NMG, Na<sup>+</sup>, hydrazinium, and Na<sup>+</sup> (150 mM except [hydrazinium]<sub>o</sub> ~138 mM, see METHODS). Data are means  $\pm$  SEM from 3 cells. (B) Peak  $P_{open}$  ( $P_o$ ) versus voltage relations for cells bathed in hydrazinium (138 mM,  $n = 3$ , see METHODS). The dotted line corresponds to the P-V curve obtained with 150 mM Na<sup>+</sup>. Data are means  $\pm$  SEM with maximums normalized to unity.

ions represent separate classes of ions; impermeant cations are associated with a voltage-dependent decrease of  $P_{open}$  at positive voltages.

The progressive decrease in  $P_{open}$  for increasingly large depolarizations in low  $[Na^+]_o$  is not due to an accumulation of inactivation for the successive depolarizations used for the experiments of Figs. 1, 2, 5–7 (9-ms test pulses presented at 0.5 Hz from a holding potential of  $-140$  mV), because a similar saturation of the current-voltage relationship is obtained when test pulses are presented in the reverse order, from  $+70$  mV to  $-80$  mV (data not shown). The effect of low  $[Na^+]_o$  on  $P_{open}$  in Figs. 5 and 6 must therefore reflect a voltage-dependent process rapid enough to prevent channels from

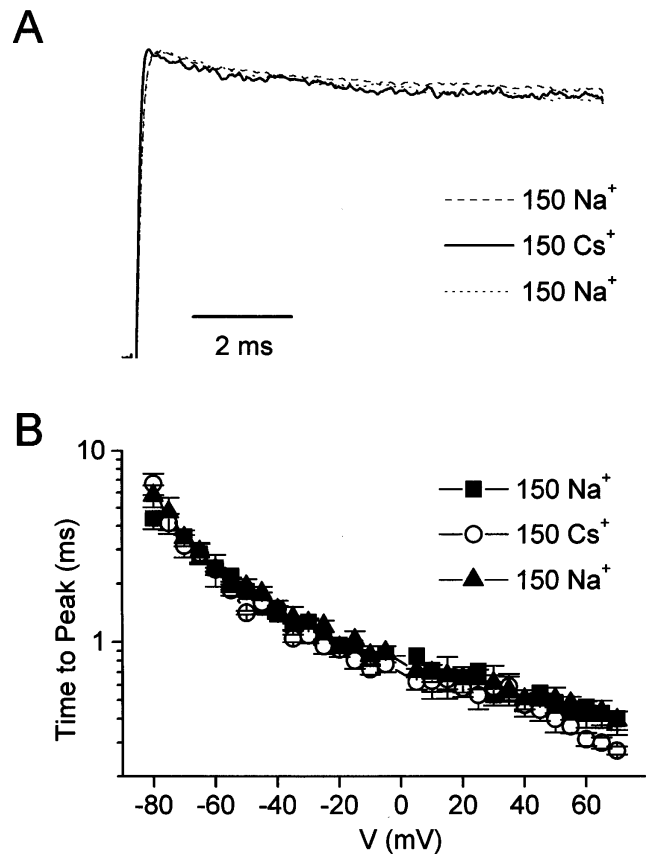


FIGURE 8. Lack of effect of  $[Na^+]_o$  on activation kinetics. (A) Scaled Na<sup>+</sup> currents from a cell bathed sequentially in Na<sup>+</sup>, Cs<sup>+</sup>, and Na<sup>+</sup> (150 mM).  $V = +60$  mV,  $-140$  mV holding potential. (B) Time to peak versus voltage for cells successively bathed in Na<sup>+</sup>, Cs<sup>+</sup>, and Na<sup>+</sup> (150 mM). Currents were evoked as described in the legend of Fig. 1 except that 5-mV increments were used. Data are means  $\pm$  SEM from three cells.

opening after a large depolarization. Scaled currents in high and low  $[Na^+]_o$  have comparable activation kinetics at  $+60$  mV (Fig. 8 A) and indeed over the whole activation range (Fig. 8 B), indicating that the effects of permeant ion concentration on  $P_{open}$  occur in the sub-millisecond interval between the depolarization and the opening of the channels. These data further suggest that high  $[Na^+]_o$  would reduce the number of blank records in single channel records, a prediction we have not tested.

Recovery from the decreased  $P_{open}$  after a large depolarization is also rapid. This is evident from the kinetics of recovery after a 0.8-ms depolarization to  $+60$  mV, using a 2-pulse voltage protocol with a variable interpulse interval to  $-140$  mV (Fig. 9 A). In both high and low  $[Na^+]_o$ , the current for a test pulse to  $+20$  mV recovers to full amplitude in less than 5 ms.<sup>4</sup> In fact the recovery

<sup>4</sup>Cs<sup>+</sup> was used as a Na<sup>+</sup> substitute in this experiment to avoid effects on slow inactivation (Townsend and Horn, 1997).

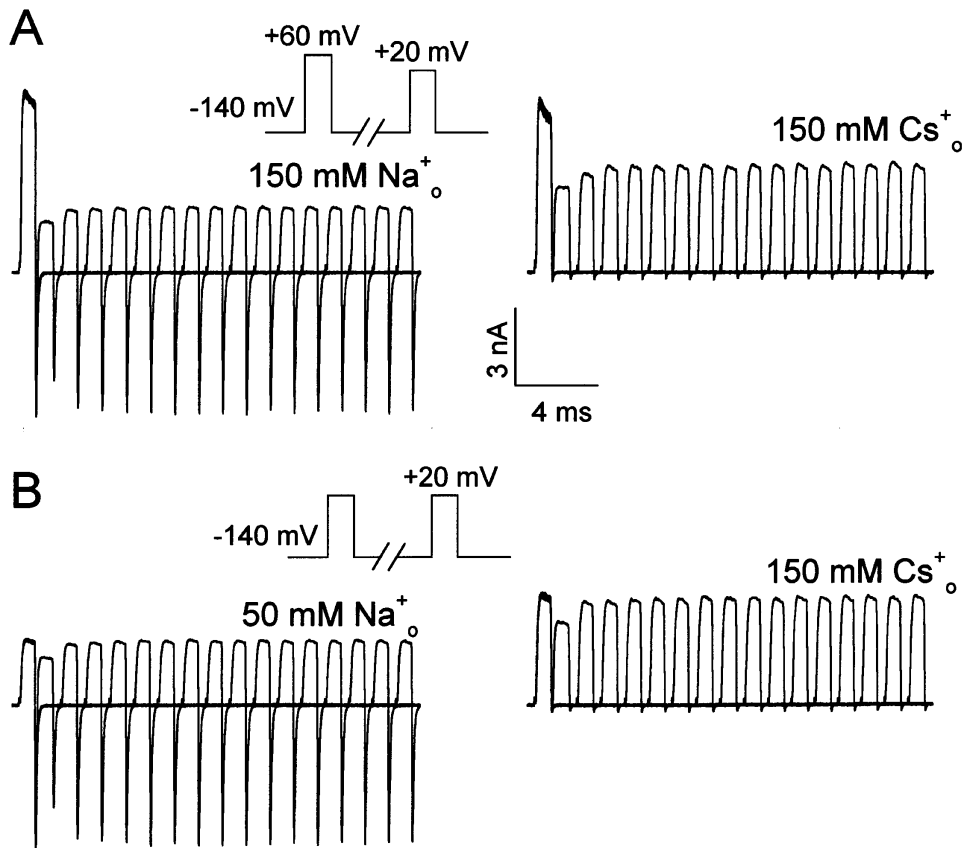


FIGURE 9. Rapid kinetics of recovery after alteration of  $P_{\text{open}}$ . Recovery at  $-140$  mV after a 0.8-ms prepulse to  $+60$  (A) or  $+20$  mV (B, see insets). 16 0.8-ms recovery test pulses to  $+20$  mV were given 0.1–18.1 ms after the prepulse (superimposed traces). Data are from one cell successively bathed in 150 mM  $\text{Na}^+$  and  $\text{Cs}^+$ .

has a similar time course after a prepulse to only  $+20$  mV (Fig. 9 B), where  $[\text{Na}^+]_o$  has less effect on  $P_{\text{open}}$  (Fig. 5). The data of Fig. 9 B reveal a use-dependent reduction of outward current for brief depolarizations to  $+20$  mV in both high and low  $[\text{Na}^+]_o$  (note that the peak current during the second depolarization is smaller than the current at the end of the first depolarization). This reduction is due either to closed channel inactivation or to a short-term disruption of activation. Either of these mechanisms may also contribute to the  $[\text{Na}^+]_o$ -dependent reduction of  $P_{\text{open}}$  (see DISCUSSION).

The data of Fig. 8 show that the reduction of  $P_{\text{open}}$  during a large depolarization is at least as rapid as activation. To reveal these kinetics, we examined tail currents at reduced temperatures for F1485Q currents bathed in either 150 mM  $\text{Na}^+$  or 150 mM  $\text{Cs}^+$ . Depolarizations up to  $+70$  mV show the typical pattern observed at room temperature (Fig. 10 A). However tail currents elicited after a 1.5-ms prepulse to 0 mV have a rapid ( $\tau < 200$   $\mu\text{s}$ ) relaxation in the absence of extracellular  $\text{Na}^+$  at potentials more positive than  $+30$  mV (Fig. 10 B). This is exactly the voltage range over which  $P_{\text{open}}$  decreases sharply (Figs. 5–7). Moreover the outward current amplitude in 150 mM  $\text{Cs}^+$  increases approximately linearly with voltage and then saturates after this relaxation. A similar rapid relaxation in low  $[\text{Na}^+]_o$  was also observed for WT channels (data not

shown). As expected these relaxations are faster than activation kinetics at these voltages (Fig. 10 A). These tail current experiments suggest that open channels can either close or inactivate rapidly in low  $[\text{Na}^+]_o$ . For depolarizations from a negative holding potential, however, it is likely that a fraction of channels never reach the open state in the absence of extracellular permeant cations.

In summary our data indicate that lowering extracellular permeant ion concentration causes a voltage-dependent reduction of  $P_{\text{open}}$ .

## DISCUSSION

Although the gating (opening and closing) of an ion channel is usually considered a separate process from the permeation of ions through its open pore, there are now several examples where the concentration or nature of the permeant ion appears to have effects on gating (see INTRODUCTION). In general these effects involve a modulation of either the kinetics of gating or the free energies of certain gating states. Such effects may be understood as an allosteric action of the permeant ion, when bound within the permeation pathway, on the various conformational transitions that underlie gating. Our data are consistent with such a mechanism. A permeant cation bound selectively within the



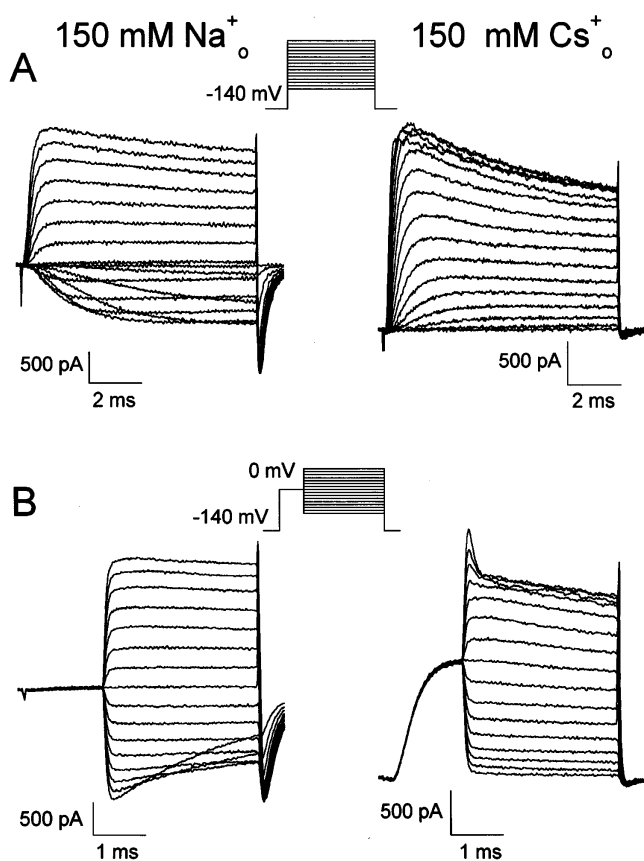


FIGURE 10. Effect of external  $[\text{Na}^+]_o$  on F1485Q tail currents. (A) Families of currents elicited by 9-ms depolarizations to voltages ranging from  $-80$  to  $+70$  mV. Holding potential,  $-140$  mV. Currents shown were recorded from two cells bathed in either  $150$  mM  $\text{Na}^+$  (left) or  $150$  mM  $\text{Cs}^+$  at  $8.0$  and  $7.1^\circ\text{C}$ , respectively. (B) Tail currents were recorded from  $-80$  to  $+70$  mV after a brief depolarization ( $1.5$  ms) to  $0$  mV from a holding potential of  $-140$  mV. Currents recorded from the same two cells shown in A at the same temperatures.

permeation pathway enhances the probability that a channel will open after a large depolarization. Although we have no direct evidence that the binding site is in the  $\text{Na}^+$  channel pore, this idea is supported by the fact that there is a clean segregation between the effects of three highly permeant and seven relatively impermeant cations in our experiments. Only the impermeant cations produce saturating current-voltage relationships, and therefore voltage-dependent reductions of  $P_{\text{open}}$  at positive membrane potentials.

Our data provide a few clues to the identity of the gating mechanism affected by external cations. Because the kinetics of activation are relatively insensitive to  $[\text{Na}^+]_o$  (Fig. 8), the effects on  $P_{\text{open}}$  cannot be ascribed simply to alterations of rate constants in the activation pathway. In fact the reduction of  $P_{\text{open}}$  can occur after activation is complete (Fig. 10). We also see little effect of external cation ( $\text{Na}^+$  versus NMG) on the latency to first opening after a depolarization in single

channel experiments (Townsend and Horn, 1997). The external cation has a clear influence, however, on whether a channel opens or not. We show in the following paper that external cations also affect a much slower process, slow inactivation. Because of its kinetics, slow inactivation cannot, however, account for the effects of impermeant cations described here. Furthermore, a different set of external cations influences slow inactivation than those we report here (Townsend and Horn, 1997).

The appearance of a voltage-dependent reduction of  $P_{\text{open}}$  with low concentrations of extracellular permeant ions indicates either the introduction of a new voltage-dependent process or an increase in the magnitude (or voltage dependence) of a pre-existing gating reaction. We propose that in the absence of extracellular permeant ions large depolarizations drive  $\text{Na}^+$  ions out of a site within the external mouth of the pore. If the channel is closed, for example, a permeant ion trapped within it could sense the membrane electric field and be driven out by a large depolarization. If extracellular permeant ion concentration is low, the binding site would remain empty, and a closed channel with an empty site would have a lower probability of opening. The voltage dependence for such a process comes from the movement of the trapped permeant ion through the electric field. A mechanism of this type has some precedence. Movement of a  $\text{Cl}^-$  ion within a closed  $\text{Cl}^-$  channel, for example, acts as a voltage sensor for gating (Pusch et al., 1995; Chen and Miller, 1996). Our tail current experiments suggest, however, that open channels are also susceptible to modulation by  $[\text{Na}^+]_o$ , presumably due also to its effect on the probability of the occupancy of a pore site by a permeant ion.

The fact that  $\text{K}^+$  has a low, but finite, permeability in these  $\text{Na}^+$  channels ( $P_{\text{K}}/P_{\text{Na}} \cong 0.08$ ) deserves a few words of discussion. Measurable  $\text{K}^+$  currents indicate that  $\text{K}^+$  ions can bind very rapidly with an open channel. Yet  $\text{K}^+$  behaves like all impermeant cations in its effects on gating (Fig. 6). This could be explained by assuming that  $\text{K}^+$  ions have a very low affinity for the binding site, so that although the rate of binding to the site might be very high, the probability of occupancy of the site is very low (i.e., the off rate is much higher for  $\text{K}^+$  than for  $\text{Na}^+$ ) once a large depolarization has emptied it.

We propose, without direct evidence, one of the following two mechanisms to explain the effects of permeant ion concentration on  $P_{\text{open}}$ .

The first mechanism is that the reduction of  $P_{\text{open}}$  at large depolarizations is due to pore block by an intracellular blocker. The blocker could either be tethered to the channel or freely diffusible in the cytoplasm of the cells. If the blocker is diffusible, it must remain within the cells for at least the 10 min that we wait after obtain-

ing the whole cell configuration. By this mechanism, depolarization drives a blocker into the pore, and the affinity of the blocker is reduced by the binding of extracellular  $\text{Na}^+$  ions in the pore. The main argument we have against this possibility derives from our previous experiments studying the effects of  $[\text{Na}^+]_o$  on intracellular cationic blockers (O'Leary et al., 1994; Tang et al., 1996). These studies showed smaller effects of  $[\text{Na}^+]_o$  for large versus small depolarizations, the opposite of the trend that we report here.

A second possible mechanism is a decrease in the magnitude of a rate constant of inactivation when a permeant ion is bound within the pore. The reduction of  $P_{\text{open}}$  has such rapid kinetics (Fig. 10), that many channels would presumably inactivate before opening. In this mechanism the presence or absence of a bound permeant ion has an allosteric effect on a voltage-dependent reaction inherent within the  $\text{Na}^+$  channel protein. An allosteric effect of permeant ions is observed in *Shaker*  $\text{K}^+$  channels, where C-type inactivation is much slower when a site within the extracellular mouth of the pore contains a  $\text{K}^+$  ion (Baukrowitz and Yellen, 1995). Moreover, C-type inactivation may be so rapid in some  $\text{K}^+$  channels that in response to a large depolarization a very small fraction of channels open before inactivating (Smith et al., 1996).

Inactivation of closed channels is prevalent in WT  $\text{Na}^+$  channels, as shown in part by a substantial fraction of blank (null) traces in response to a series of depolarizations (Horn et al., 1981). This type of inactivation is almost completely abolished by the F1485Q mutation

observed with a normal  $[\text{Na}^+]_o$  gradient (Hartmann et al., 1994). This might explain why the mutant is more sensitive to  $[\text{Na}^+]_o$  than WT channels (Figs. 1 and 2). In WT channels a substantial fraction of channels do not open even in high  $[\text{Na}^+]_o$ , whereas closed-channel inactivation can be considerably "upregulated" in F1485Q channels by removing extracellular permeant cations.

Our results suggest that the charge movement underlying gating should be sensitive to  $[\text{Na}^+]_o$ , a prediction that has not been fulfilled in previous studies. This may be a consequence of the fact that the changes in gating current are either too small or too fast to be observed. However most  $\text{Na}^+$  channel gating current measurements involve the use of the extracellular pore blocker tetrodotoxin, which would exclude the exchange of  $\text{Na}^+$  ions between the extracellular solution and binding sites within the pore. Likewise, mutations of the pore that prevent conduction (e.g., Perozo et al., 1993) might also prevent  $\text{Na}^+$  entry into the pore, obviating their use for studies of the  $[\text{Na}^+]_o$ -dependent modulation of gating current.

Our results show that permeant ions enhance the efficiency with which a channel opens in response to a large depolarization, a process with rapid kinetics. In the following paper we show that  $[\text{Na}^+]_o$  also affects slow inactivation. Little is known about the molecular mechanisms for either gating process. We anticipate that the modulation of gating by  $[\text{Na}^+]_o$  will provide valuable insight into the relationship between the  $\text{Na}^+$ -selective pore and the gates with which it interacts.

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We thank Drs. Enrico Stefani for his "goop" recipe for coating pipette tips, Chris Miller for insightful discussions, Larry Goldman for suggesting the tail-current experiment of Fig. 10, Carol Deutsch for extensive comments on the manuscript, and Al Lefer who inspired the creation of the Institute of Hyperexcitability.

Supported by National Institutes of Health grant AR41691 (R. Horn).

*Original version received 12 February 1997 and accepted version received 28 April 1997.*

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