Interaction between the Pore and a Fast Gate of the Cardiac Sodium Channel

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ABSTRACT Permeant ions affect a fast gating process observed in human cardiac sodium channels (Townsend, C., H.A. Hartmann, and R. Horn. 1997. *J. Gen. Physiol.* 110:11–21). Removal of extracellular permeant ions causes a reduction of open probability at positive membrane potentials. These results suggest an intimate relationship between the ion-conducting pore and the gates of the channel. We tested this hypothesis by three sets of manipulations designed to affect the binding of cations within the pore: application of intracellular pore blockers, mutagenesis of residues known to contribute to permeation, and chemical modification of a native cysteine residue (C373) near the extracellular mouth of the pore. The coupling between extracellular permeant ions and this fast gating process is abolished both by pore blockers and by a mutation that severely affects selectivity. A more superficial pore mutation or chemical modification of C373 reduces single channel conductance while preserving both selectivity of the pore and the modulatory effects of extracellular cations. Our results demonstrate a modulatory gating role for a region deep within the pore and suggest that the structure of the permeation pathway is largely preserved when a channel is closed.

KEY WORDS: permeation • gating • block • site-directed mutagenesis

INTRODUCTION

Functional studies of ion channels typically concentrate on either of two processes, gating or permeation. Loosely speaking, gating is the mechanism that opens and closes a pore, and permeation is the process of ion transport through the open pore. The conceptual division of channel function into these two categories is supported by a variety of pharmacological and mutagenic experiments. For example, several biochemical reagents profoundly alter inactivation gating of sodium channels without affecting either the single channel conductance or the selectivity of the channels (e.g., *N*-bromoacetamide [Patlak and Horn, 1982], trypsin [Vandenberg and Horn, 1984], and tetramethrin [Yamamoto et al., 1984]). Moreover, mutations that affect either activation or inactivation gating rarely affect permeation (e.g., Moorman et al., 1990; Chahine et al., 1994; O'Leary et al., 1995).

At some level, this neat segregation of gates and pores must break down, however, because the gates need to communicate with the pore in some way during opening and closing. At minimum they must control the movement of ions into and out of the "business district" of the pore (Miller, 1982). The gating process, in fact, might completely collapse the permeation pathway during the closing of the activation gate (Liu et al., 1997). This appears unlikely in the case of potassium channels inactivated by a cytoplasmic "inactivation ball" because extracellular potassium, but not sodium, ions are capable of enhancing the dissociation of the inactivation ball from the inner mouth of the pore (Demo and Yellen, 1991).

It is reasonable to expect that some amino acid residues will play important roles in the coupling between the gates and the pore. Accordingly, mutations of certain residues can affect both permeation and gating. For example, the mutation of a putative pore residue in the outer mouth of a sodium channel reduces single channel conductance and simultaneously causes a marked acceleration of activation kinetics (Tomaselli et al., 1995). Another indication of a functional interaction between the pore and the gates is that permeant ions can have effects on gating. This is especially evident in voltage-gated potassium channels, where an increase of extracellular potassium concentration tends to increase the probability of a channel being open, as if a potassium ion bound selectively in the pore inhibits the closing of both activation and inactivation gates (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; Baukrowitz and Yellen, 1995; Levy and Deutsch, 1996a,b). Furthermore, the selectivity of a *Shaker* potassium channel changes as the channel

Portions of this work were previously published in abstract form (Townsend, C., H.A. Hartmann, and R. Horn. 1998. *Biophys. J.* 74: A401).

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passes through subconductance states in the activation pathway (Zheng and Sigworth, 1997). An enhancement of open probability is also observed in chloride-selective channels when chloride concentration is increased (Pusch et al., 1995; Chen and Miller, 1998). Finally, the dwell time in the open state of a calcium channel depends on the permeant ion species carrying the current (Nelson et al., 1984).

We recently reported an experiment that implicates an interaction between permeation and gating in cardiac sodium channels. Increasing the driving force for outward current at depolarized voltages, by reducing extracellular permeant ion concentration, causes a decrease in the current (Townsend et al., 1997). Single channel experiments revealed that this anomalous behavior was due to an effect of permeant ions on a fast voltage-dependent gating process that appeared to be distinct from activation. As for potassium and chloride channels, decreasing permeant ion concentration decreases open probability in these sodium channels. The molecular details of the cation-dependent modulation of open probability were not clear from our initial study. It is possible, for example, that the fast gating process was due to pore block by an unknown, perhaps tethered, endogenous molecule. This is an issue that we did not address directly, but is worth keeping in mind. We also had no evidence whether extracellular cations were reacting with the ion-selective pore itself or with another ion binding site on the channel protein.

Here we show not only that the critical binding site is deep within the pore, but also that the selectivity region of a channel with a closed activation gate is accessible to extracellular cations. The results are consistent with pore models having the activation gate located on the cytoplasmic end of the pore (reviewed in Armstrong, 1992; Hille, 1992).

METHODS

Mutagenesis

The mutants K1418C:F1485Q and D1422C:F1485Q were constructed using a three-step modified restriction site PCR strategy in the human heart sodium channel isoform hH1a (Hartmann et al., 1994). For each mutant, one reverse oligonucleotide primer was designed to create the desired mutation (K1418C or D1422C). This mutagenic primer and a forward primer corresponding to a sequence located upstream from the natural KpnI-restriction site (nucleotide 4224, primer A) were used in the first PCR step. A second set of amplification reactions was performed with a forward primer incorporating a mutation of the KpnI site and a reverse primer corresponding to a sequence located downstream from the natural BstEII restriction site (nucleotide 4624, primer B). In both steps, F1485Q hH1a-pGEM3 was used as template (Hartmann et al., 1994) and PCR products were purified by spin column chromatography (QIAGEN Inc.). The third PCR step involved primers A and B and a mixture of the purified products obtained in the first two PCR reactions as template. The final PCR products were digested with KpnI and BstEII, and the resulting 400-bp fragment ligated into the corresponding sites in the plasmid hH1a-pGEM3. The entire amplified region was then sequenced to verify the mutations. For expression into mammalian cells, the hH1a mutants were subcloned into the pcDNA3 vector (Invitrogen Corp.). Mutant hH1a-pGEM3 constructs were first digested with HindIII and VspI and the resulting 5′ overhangs flanking the hH1a insert were filled in with T4 DNA polymerase. The blunt-end products were then ligated into the EcoRV site of the pcDNA3 vector. All enzymes were purchased either from Boehringer Mannheim Biochemicals or from Promega Corp.

Electrophysiology and Data Analysis

Standard whole cell and single channel methods were used as described previously (Townsend et al., 1997; Townsend and Horn, 1997). In brief, whole cell recording was performed in transiently transfected tsA201 cells, a transformed mammalian cell line, and single channel recording was done with outside-out patches of *Xenopus* oocytes injected with cRNA encoding the identical sodium channel mutants. Oocytes were used for single channel recording because of the superior signal/noise ratio. Typically, however, they did not have high enough expression for macropatches. Our previous experience with the hH1a isoform shows that the biophysical properties of these sodium channels are similar, if not identical, in mammalian and oocyte expression. For whole cell recording, cotransfection with the surface protein CD8 was used to select positive transfectants with antibody-coated beads (Jurman et al., 1994).

Whole cell recordings were obtained with an Axopatch 200B amplifier (Axon Instruments) and were acquired with pCLAMP6 (Axon Instruments). Series resistance before 80% compensation was <2 MV. Cells were dialyzed at least 10 min before recording data. The pipette solution consisted of (mM) 140 NaF, 10 NaCl, 5 EGTA, and 10 HEPES, titrated to pH 7.4 with NaOH. In some experiments, some of the NaF was replaced by NaCl, tetraethylammonium (TEA)[1] chloride, diethylamine (DEA) hydrochloride, or sucrose, as indicated in the figure legends. The 150-mM Na^+ bath solution contained (mM) 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, titrated to pH 7.4 with NaOH. In the 150-mM Cs^+ solution, all Na^+ was replaced by Cs^+. All solution components were purchased from Sigma Chemical Co. Bath solutions were exchanged by application of a desired solution to single cells with a macropipette (Yang et al., 1997). Single channel currents were recorded from outside-out oocyte patches. Pipette and bath solutions were identical to those used in whole cell recordings from tsA201 cells. Single channel currents were filtered at 5 kHz, sampled at 20 kHz, and digitally filtered again at 5 kHz. Unless otherwise specified, all experiments were performed at room temperature (19–21°C).

Methanethiosulfonate-ethylsulfonate (MTSES; Toronto Research Chemicals Inc.) was kept at 4°C as a 250-mM stock solution in water and diluted in the appropriate bath solution immediately before use. The reagent (6.5 mM) was applied to outside-out patches with a macropipette for ~5 min. For whole cell recordings, 6.5 mM MTSES was present in the bath during the entire duration of the experiments. At the end of single channel and whole cell experiments, MTSES modification was verified by the absence of response to exposure to 1 mM MTS-ethyltrimethylammonium (MTSET). Application of MTSET to unmodified F1485Q hH1a

[1]*Abbreviations used in this paper:* DEA, diethylamine; I–V, current–voltage; MTSES, methanethiosulfonate-ethylsulfonate; NMG, *N*-methylglucamine; TEA, tetraethylammonium.

channels causes an ~80% reduction in current (Chen et al., 1997).

Whole cell and single channel data were analyzed with a combination of pCLAMP programs, Excel (Microsoft Corp.), and Origin (Microcal Software, Inc.). 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. Normalized open probability (P_{open}) was calculated from whole cell and single channel recordings with the following equation (Townsend et al., 1997):

$$P_{open} = \frac{I}{n \times i},$$

where I is the peak whole cell current in response to a depolarization, n is the number of channels, and i the single channel current. Because external cations affect whole cell currents in a reversible manner, n is assumed constant during the course of a given experiment, and channel open probabilities were normalized to the maximum P_{open} measured for the 150-mM Na^+ bath solution. Because of this normalization procedure, the estimates may in some cases be greater than 1.0 (e.g., see Fig. 6 D). This shows that P_{open} is an underestimate of the true open probability. To estimate P_{open} in the presence of internal TEA, whole cell currents were recorded with 17.5 mM TEA in the pipette. Single channel currents were recorded from outside-out patches with either 5 or 17.5 mM TEA in the pipette, depending upon the composition of the bath solution. Because TEA is a fast, voltage-dependent Na^+ channel blocker, single channel currents are reduced especially at positive voltages in the presence of internal TEA. In addition, replacing external Na^+ by Cs^+ enhanced single-channel block by TEA. Therefore, the effect of TEA on single channel currents with 150 mM Cs^+ in the bath was estimated with a reduced $[TEA]_i$ of 5 mM. The single channel data were fitted with the following equation (Woodhull, 1973):

$$(1 - F_u)/F_u = \frac{[TEA]}{K_0 \times \exp(-\delta\, VF/RT)},$$

where F_u represents the fraction of current unblocked, [TEA] is the concentration of TEA applied internally, K_0 is the inhibition constant at 0 mV, δ is the electrical distance for TEA block, V is the applied membrane voltage, and $RT/F = 25$ mV. For 17.5 mM TEA_i and 150 mM Na^+_o, K_0 was 15.4 mM and δ was ~0.36. With 5 mM TEA_i and 150 mM Cs^+ in the bath, K_0 was 6.15 mM and δ was ~0.29. These values and the above equation were used to estimate single channel currents in the presence of 17.5 mM TEA_i and 150 mM Cs^+_o. Channel open probability was then calculated, as described earlier, from measured whole cell currents and the estimated (Cs^+_o) or measured (Na^+_o) single channel currents. To estimate standard errors for P_{open}, we propagated uncertainties of all determinations (Bevington, 1969) including, in the case of TEA, standard errors of estimates of the blocking parameters K_0 and δ. Unless otherwise specified, data are expressed as mean ± SEM.

RESULTS

Uncoupling of Permeant Ion Modulation by Intracellular Pore Blockers

Reducing extracellular sodium concentration increases the electrochemical driving force for outward movement of sodium ions. In spite of this thermodynamic effect, macroscopic sodium currents at membrane potentials more positive than approximately +40 mV are reduced reversibly when extracellular sodium is replaced by the impermeant cation cesium (Townsend et al., 1997). This phenomenon is observed in both wild-type cardiac sodium channels and the mutant cardiac sodium channel we used in our experiments. The mutation (F1485Q) substitutes a glutamine for a phenylalanine in the cytoplasmic linker connecting the third and fourth homologous domains of the channel. This mutation strongly suppresses fast inactivation, facilitating both the ability of obtaining single channel data and the analysis of these data. In all of these experiments, we use a high concentration of intracellular sodium (150 mM) to maximize the amplitude of outward currents.

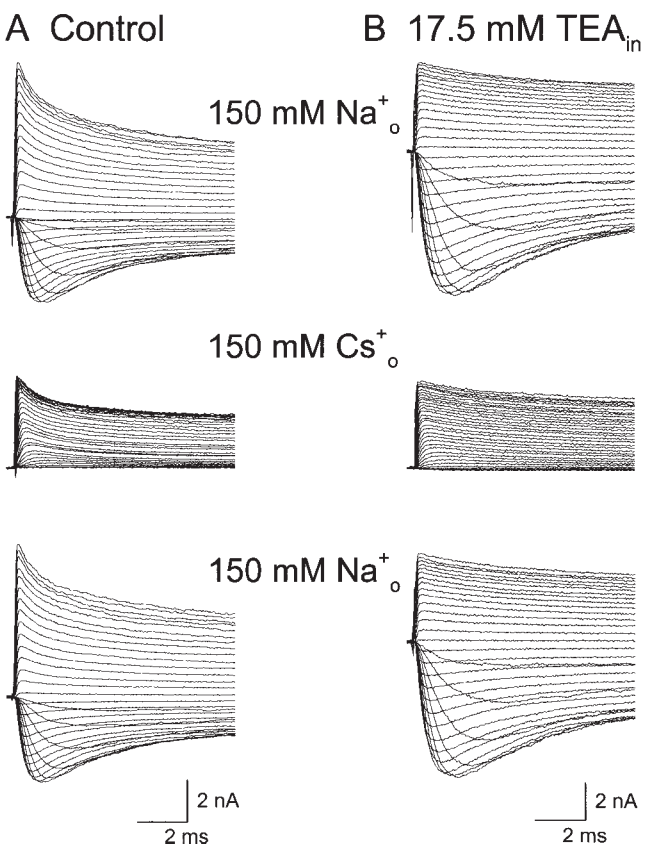

FIGURE 1. Effect of internal TEA on F1485Q hH1a whole cell currents. Na^+ currents were elicited by 9-ms depolarizations to voltages ranging from −85 to +70 mV in 5-mV increments (−140-mV holding potential). Recordings from two F1485Q-transfected cells successively bathed in 150 mM Na^+, 150 mM Cs^+, and 150 mM Na^+ solutions. The internal solution consisted of (mM): (A) 105 NaF, 45 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4; or (B) 105 NaF, 17.5 TEACl, 27.5 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4.

The anomalous effect of removing extracellular sodium on sodium current is apparent in both the raw currents (Fig. 1 A) and the peak current–voltage (I–V) relationships (Fig. 2, A and D). In the presence of so-

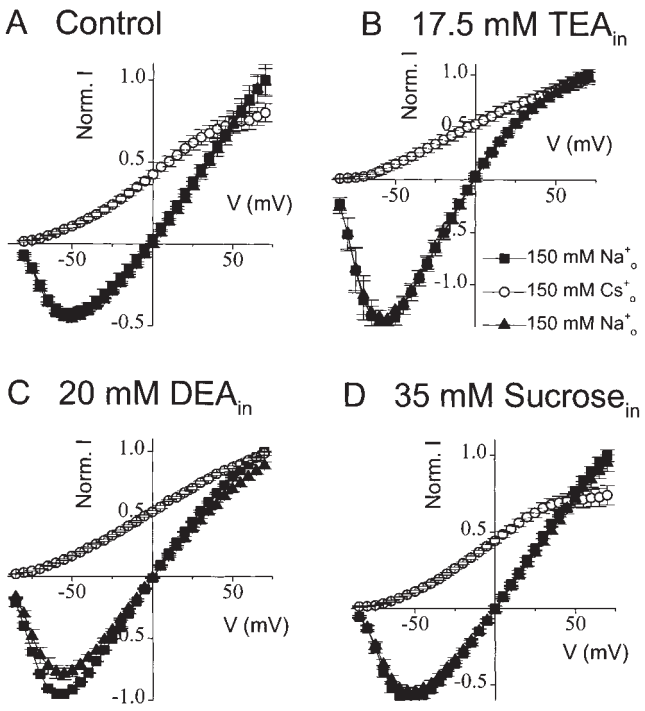

FIGURE 2. Current–voltage relations. Na^+ currents were elicited as described in Fig. 1. Peak currents are plotted versus test pulse voltage. F1485Q-transfected cells were sequentially bathed in 150 mM Na^+, 150 mM Cs^+, and 150 mM Na^+ solutions. Internal solutions consisted of (mM): 105 NaF, 45 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4 (A, $n = 3$); 105 NaF, 17.5 TEACl, 27.5 NaCl, 5 EGTA, 10 HEPES-NaOH pH 7.4 (B, $n = 3$); 105 NaF, 20 DEACl, 25 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4 (C, $n = 3$); and 105 NaF, 35 sucrose, 27.5 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4 (D, $n = 7$).

dium, the currents increase approximately linearly with depolarization at positive voltages. If extracellular sodium is replaced by the impermeant cation cesium, the I–V relationship saturates for voltages greater than approximately +20 mV. At voltages greater than approximately +40 mV, the outward currents are smaller in cesium than in sodium, leading to a crossing of the I–V relationships (Fig. 2, A and D).

We showed previously that the exchange of extracellular cations has little effect on the relationship between peak open probability (P_{open}) and voltage at negative voltages (Townsend et al., 1997). Therefore, we focussed in this study on positive voltages, where we can readily measure single channel current amplitudes. In this voltage range, P_{open}, calculated by dividing the peak whole cell currents by the amplitude of the single channel currents (see METHODS for details), is nearly independent of the membrane potential in the presence of extracellular sodium.

Fig. 3 A shows that the anomalous effect of removing extracellular sodium on peak sodium current (○) is due to a voltage-dependent reduction of peak open probability (P_{open}) at positive voltages. The effect on P_{open} is large enough to mask the opposing effect of sodium concentration on driving force for current flowing through open channels (Townsend et al., 1997).

The cation-dependent modulation of P_{open} is due to a fast-gating process that is apparently distinct from activation. Some evidence for this is that external cations decrease the probability of a single channel opening without affecting the average latency to the first opening after a depolarization (Townsend and Horn, 1997). This indicates that strong depolarization causes the fast gate to close rapidly without regard to the state of the activation gate. Furthermore, there is no indication of time-dependent changes in the amplitude of single channel currents during a depolarization (Townsend et al., 1997). Finally, macroscopic activation kinetics are not affected by external cations (see below). We have no direct information on the nature of the fast gate, but it is worth keeping in mind the possibility that it is an endogenous, cytoplasmic pore blocker that dif-

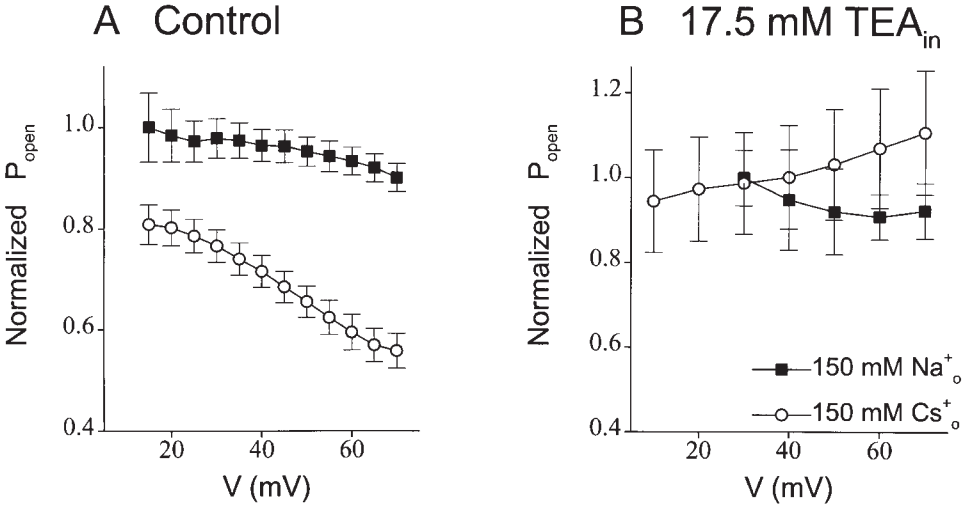

FIGURE 3. Channel open probability. F1485Q normalized open probability versus voltage relations for (A) control internal solution contained (mM): 105 NaF, 45 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4. (B) 17.5 mM TEA internal solution contained (mM): 17.5 TEACl, 27.5 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4. Normalized channel open probability was calculated from single channel and whole cell currents as described in METHODS.

fuses very slowly out of cells during whole cell recording (see DISCUSSION).

The modulatory effect of extracellular cations depends precisely on whether they are highly permeant or not. If sodium is replaced with lithium or hydrazinium, both permeant in sodium channels, there is no effect on P_{open} at depolarized voltages; however, replacement by any poorly permeant cation (e.g., potassium, cesium, choline, or *N*-methyl-glucamine [NMG]) causes the reduction of P_{open} seen in Fig. 3 A (Townsend et al., 1997). These results suggest that a binding site for extracellular cations within the permeation pathway is critical for our modulatory phenomenon. To test this, we attempted to disrupt cation binding within the pore by a *trans* effect of cationic pore blockers. We used either TEA or DEA, both of which block at an electrical distance nearly halfway through the membrane electric field when applied from the cytoplasmic face of the channel (O'Leary and Horn, 1994; Zamponi and French, 1993). The kinetics of these blockers are so fast that they cause an apparent reduction in the amplitude of single channel currents (O'Leary and Horn, 1994; Zamponi and French, 1993). Figs. 1 and 2 show the effect of 17.5 mM internal TEA on macroscopic currents. The voltage dependence of the block distorts the shape of the peak I–V relationship in the expected direction, causing a voltage-dependent decrease in both inward and outward current with increasing depolarization. The magnitude of this shape change is exactly predicted by a model in which TEA blocks open channels completely at a single site within the membrane electric field (Woodhull, 1973; O'Leary and Horn, 1994). The effect on P_{open} of replacement of extracellular sodium by cesium is abolished by internal TEA (Figs. 2 B and 3 B). Qualitatively similar results were obtained with 20 mM DEA (Fig. 2 C), a higher concentration (35 mM) of intracellular TEA, and in experiments in which extracellular sodium was substituted by NMG rather than cesium (data not shown). Because TEA was substituted for internal sodium, we tested whether the reduction of internal sodium itself could produce a similar effect as TEA. To do this, we substituted internal sodium by either cesium or sucrose. Neither substitution reduced the modulatory effect of extracellular sodium (e.g., Fig. 2 D).

Intracellular TEA also slows the kinetics of the inactivation that remains in the F1485Q mutant (Fig. 1). Although we did not characterize this kinetic effect in detail, the result contrasts with the lack of effect of intracellular TEA on inactivation of wild-type cardiac sodium channels (O'Leary and Horn, 1994). One interpretation of these data is that TEA is capable of interfering with the closing of a cytoplasmic inactivation gate, if the closing is partially impaired by the F1485Q mutation. The effect of TEA on the mutant channel is reminiscent of the inhibitory effects of internal TEA on the rate of inactivation caused by the cytoplasmic inactivation gate in the *Shaker* family of potassium channels (Choi et al., 1991), lending credence to the possibility of a similar inactivation mechanism in sodium channels.

The substitution of cesium for extracellular sodium has no effect on the kinetics of either activation or inactivation in the F1485Q mutant (Townsend et al., 1997). This observation holds in the presence of intracellular TEA. Fig. 4 shows that currents at both +20 and +70 mV are superimposable when cesium replaces sodium. Because of the decreased driving force for outward current in extracellular sodium, the currents at +20 mV were scaled up 1.49-fold. This result shows that the effects of TEA cannot be explained by an alteration of activation gating when external cations are exchanged.

The reduction of P_{open} and its voltage-dependent decrease in the absence of sodium (Fig. 3 A) are abolished in the presence of 17.5 mM intracellular TEA (Fig. 3 B). This concentration produces a fractional open-channel block >0.5 at positive voltages. The fractional block is greater when cesium is substituted for extracellular sodium (0.73 vs. 0.53 at 0 mV), suggesting a repulsive interaction between a sodium ion within the pore and a blocking cation on the cytoplasmic side of the selectivity filter. Previous data show that internal TEA has no effect on activation at negative voltages (O'Leary and Horn, 1994), consistent with the possibility that it can be trapped within its blocking site in a closed channel without an effect on the activation gate.

The effect of external cations on P_{open} is very fast, occurring in the submillisecond interval between the onset of a large depolarization and the time when the current reaches its peak value (Townsend et al., 1997). To observe the rapid kinetics of this process, we examined

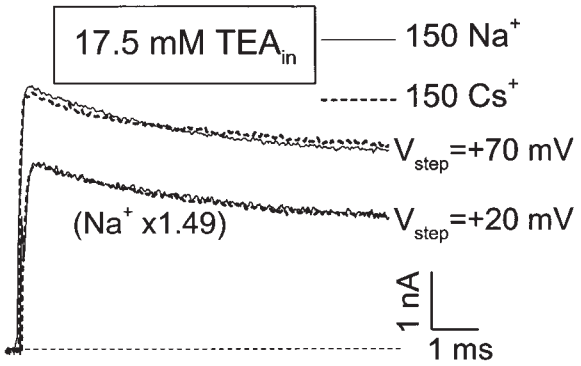

FIGURE 4. Lack of effect of external cations on kinetics. Superimposed Na^+ currents at +20 and +70 mV in Na^+ or Cs^+ bath. The internal solution contained 17.5 mM TEA. The current at +20 mV in the Na^+ bath was scaled up 1.49-fold. The currents at +70 mV were not scaled. The dashed line represents zero current. All currents from data shown in Fig. 1 B.

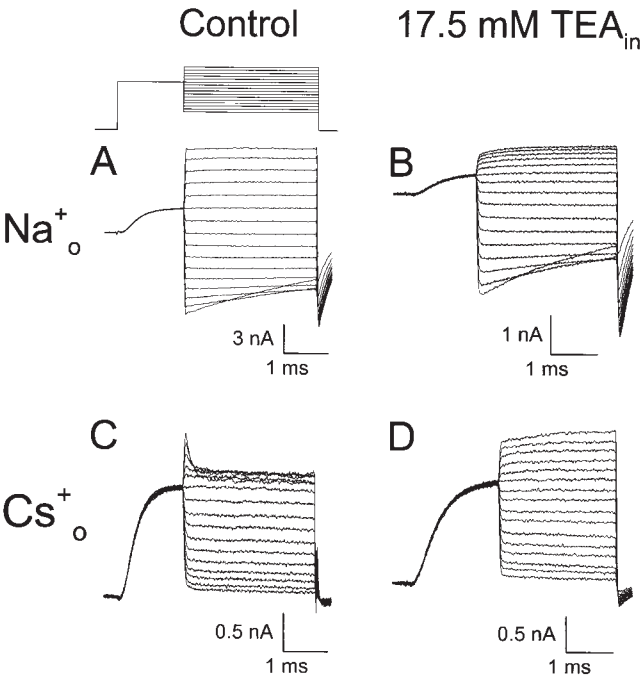

FIGURE 5. Effect of internal TEA on tail currents. Recordings from four F1485Q hH1a–transfected cells held at −140 mV and depolarized to +20 mV for 1.5–2 ms before 3–3.5-ms voltage steps ranging from −80 to +70 mV in 10-mV increments (inset). External bath solution was 150 mM Na^+ (A and B) or 150 mM Cs^+ (C and D). Internal solution was control (containing [mM]: 105 NaF, 45 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4; A and C) or 17.5 mM TEA (containing [mM]: 17.5 TEACl, 27.5 NaCl, 5 EGTA, 10 HEPES-NaOH, pH 7.4; B and D). Filter, 10 kHz; temperature, 9.6°C.

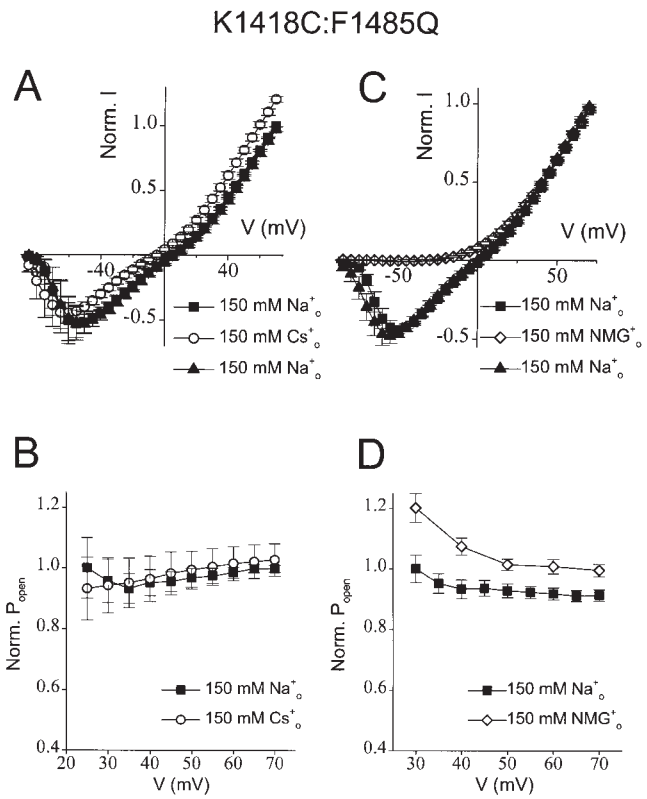

FIGURE 6. K1418C:F1485Q hH1a current– and open probability–voltage relations. (A and C) Peak current versus voltage relations for K1418C:F1485Q hH1a–transfected cells successively bathed in either Na^+, Cs^+, or Na^+ bath solutions (A, $n = 3$), or Na^+, NMG^+, and Na^+ bath solutions (C, $n = 5$). (B and D) Corresponding normalized open probability versus voltage relations (see METHODS, $n = 3$ single-channel patches for all bath solutions except 150 mM Na^+, where $n = 2$ patches).

tail currents at a reduced temperature. Fig. 5 C shows a rapid relaxation ($\tau \cong 0.13$ ms at +60 mV) after a depolarization from +20 mV in the presence of extracellular Cs^+. This relaxation, representing a rapid decrease of P_{open} at voltages more positive than approximately +30 mV, is not observed when sodium is the predominant extracellular cation (Fig. 5 A). Note that after ~200 ms, the outward currents saturate only when cesium is the external cation, as in Fig. 1 A. Internal 17.5 mM TEA abolishes the relaxation (Fig. 5, B and D), consistent with its effects on P_{open} (Fig. 3). The rapid transient in Fig. 5 C is the kinetic counterpart to the voltage-dependent decrease in P_{open}. Like the fast inactivation of potassium channels, this transient is suggestive of a pore block that is competitively inhibited by an even faster blocker, namely TEA (see DISCUSSION).

Effect of Pore Mutations on Cationic Modulation of Gating

The selective effect of permeant versus impermeant cations on gating is evidence that the selectivity filter plays a direct role in the modulatory effects of external cations. To test this, we made mutations of two residues in the putative pore region of the channel. One residue, a lysine in domain 3 (K1418), is known to be critical for selectivity of the sodium channel among cations (Heinemann et al., 1992; Favre et al., 1996). The other residue, an aspartate situated more superficially in the same pore region (D1422), is believed to affect the local concentration of cations in the extracellular mouth of the channel, while playing little role in selectivity (Chiamvimonvat et al., 1996a,b; Chen et al., 1997; Tsushima et al., 1997). We mutated each of these residues individually to cysteine in the background of F1485Q, and tested whether modulation of P_{open} was affected by the mutations.

The K1418C mutation had profound effects on selectivity. Fig. 6 A shows that the mutation nearly abolishes the ability of the channel to discriminate between sodium and cesium ions ($P_{Cs}/P_{Na} = 0.67 \pm 0.03$; $n = 3$ cells). Because both sodium and cesium are permeant, we expected that replacement of sodium by cesium would have no effect on P_{open}, a prediction verified by the data in Fig. 6. The I–V relationships are linear and do not cross for voltages greater than +30 mV (Fig. 6 A), and P_{open} remains high at depolarized voltages after

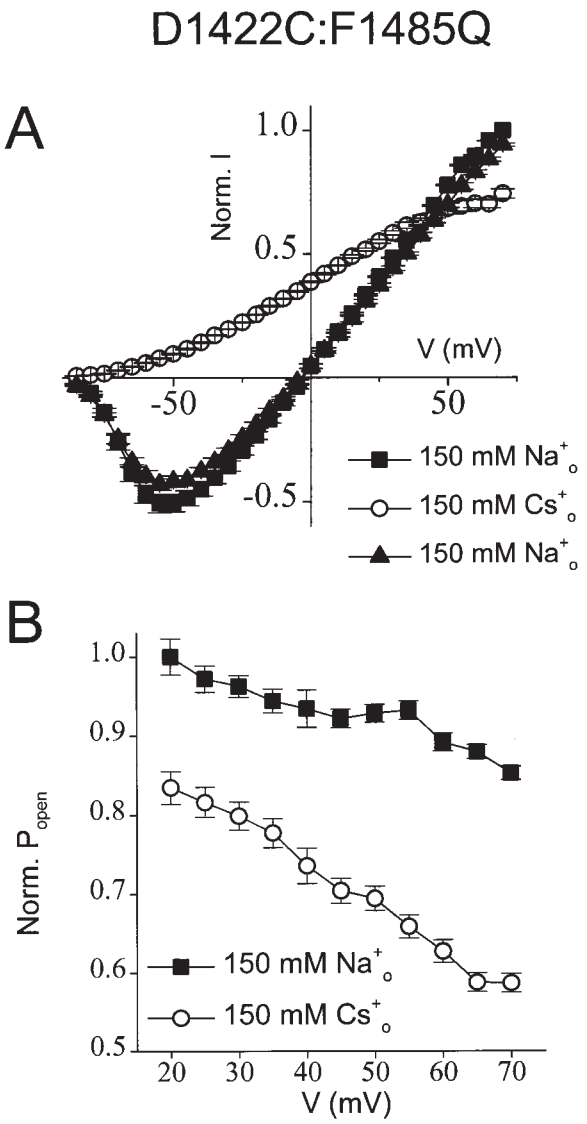

FIGURE 7. D1422C:F1485Q hH1a current– and open probability–voltage relations. (A) Peak current–voltage relations for D1422C:F1485Q hH1a–transfected cells successively bathed in either Na^+, Cs^+, or Na^+ bath solutions ($n = 3$). (B) Corresponding normalized open probability versus voltage relations (see METHODS, $n = 3$ single-channel patches for each bath solution).

cesium substitution (Fig. 6 B). The lack of an effect of cesium is not due, however, to the fact that it is permeant in this mutant. Even when sodium is replaced by the impermeant organic cation NMG, there is no effect on P_{open} (Fig. 6, C and D). We interpret these results as follows: the lysine mutation either abolishes the ability of the selectivity filter to interact with the gating mechanism under study, or else drastically alters the occupancy of permeant ions near the selectivity filter. Either interpretation supports a significant role for the selectivity filter in this modulatory process.

To further test for a role of the selectivity filter, we examined a more superficial pore mutant (D1422C) that affects single channel conductance, but not selectivity. This charge-neutralizing mutation reduced inward sodium conductance (25.5 vs. 34.6 pS), but not outward conductance (35.5 vs. 36.6 pS), consistent with a role of the wild-type aspartate in concentrating cations at the extracellular mouth of the pore. Fig. 7 shows that sodium replacement by cesium in the D1422C mutant has the hallmark effects observed in wild-type channels, namely a crossing of the I–V relationships and a voltage-dependent reduction of P_{open} at positive voltages in the absence of extracellular permeant cations. Therefore, modulation by external cations is preserved in this pore mutant.

Modification of a Native Cysteine Residue Near the Extracellular Pore Mouth

The cardiac sodium channel contains a cysteine residue (C373) in the putative pore region of domain 1. A variety of experiments show that the side chain of this residue lies within the permeation pathway. For example, pore block by extracellular cadmium ions is reduced if C373 is mutated to another amino acid (Backx et al., 1992; Satin et al., 1992). Furthermore, C373 is capable of forming disulfide links with cysteines introduced into the pore-lining regions of other domains (Tsushima et al., 1997; Bénitah et al., 1997). Finally, modification of C373 by hydrophilic cysteine reagents reduces the single channel conductance without significantly affecting selectivity (Chen et al., 1997). We made use of this strategy to test whether an alteration of the extracellular mouth of the channel, but not of the selectivity region, affects modulation of gating by extracellular cations.

Fig. 8, A and B, shows that covalent modification by the anionic reagent MTSES reduces the amplitudes of single channel currents. However, as in the case of the D1422C mutant, the modulation by extracellular cations is preserved (Fig. 8, C and D), again supporting a critical role for the selectivity region.

DISCUSSION

We previously reported that sodium channel gating is modulated by the concentration and nature of extracellular monovalent cations. Specifically, lowering the extracellular concentration of permeant ions decreases peak open probability (P_{open}) at large positive voltages (Townsend et al., 1997). The underlying gating process is apparently distinct from activation and is rapid, typically occurring faster than the channel opening induced by a large depolarization. The clear segregation between permeant and impermeant ions for the decrease of P_{open} led us to explore the permeation pathway for interaction sites between external cations and the channel protein.

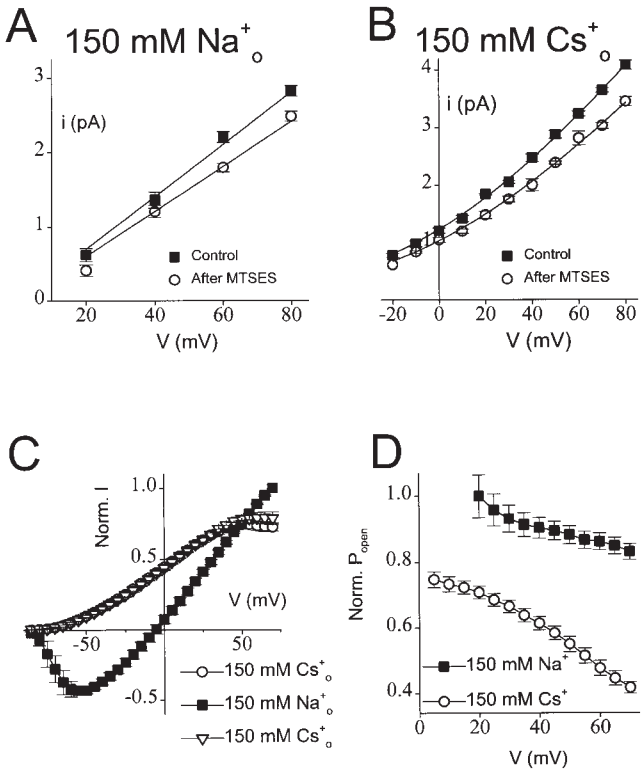

FIGURE 8. Effects of MTSES modification on F1485Q currents and open probability. (A and B) Single channel current–voltage relations obtained from outside-out patches bathed in either Na^+ (A, $n = 3$) or Cs^+ (B, $n = 3$) before and after exposure to 6.5 mM MTSES. Data points in A were fit by linear regression (solid lines). In B, the lines are fits to the Goldman-Hodgkin-Katz equation. (C) Current–voltage relations obtained from MTSES-treated cells ($n = 4$). (D) Normalized channel open probability versus voltage relations after MTSES modification (calculated from the data shown in A–C, as described in METHODS).

Modulation and the Selectivity Filter

Three independent experiments support the idea that cation binding sites at or near the selectivity filter are critical for the modulatory effects of extracellular cations. (a) Application of the intracellular pore blockers TEA and DEA disrupts modulation by external cations and prevents the voltage-dependent decrease of P_{open}. This result further supports the idea that the activation gate is separate from the fast gating process we are investigating, because internal TEA has no effect on activation of sodium channels (O'Leary and Horn, 1994). (b) Mutation of a pore residue that significantly contributes to selectivity (lysine 1418) disrupts modulation, whereas a more superficial mutation (D1422C) that affects conductance, but not selectivity, retains the modulation. It is noteworthy that mutation of K1418 abolishes modulation not only when comparing external sodium and cesium, both of which are permeant in the K1418C mutant, but also when comparing sodium and the impermeant cation NMG. (c) Covalent modification of a native cysteine in the extracellular mouth of the pore region of domain 1 (C373), a manipulation that affects conductance but not selectivity, preserves the modulation of gating by external cations. The accessibility of C373 to cysteine reagents considerably larger than permeant ions indicates that this residue is in a region more superficial than the narrow selectivity filter. Moreover, mutation of this cysteine to tyrosine (C373Y) produces a similar biophysical profile as that of covalent modification, affecting conductance only while retaining modulation (data not shown).

These results demonstrate a critical role for a region deep within the permeation pathway in this modulatory phenomenon. They also suggest that the absence of a permeant ion in the pore causes a reduction of P_{open} at depolarized voltages.

Relationship between Pore Block and P_{open}

Our experiments indicate that closing of a fast gate is inhibited by the presence of cations in the pore. Two types of sites may be involved, both located deeply within the membrane electric field. The more cytoplasmic site, when occupied by a blocker, inhibits the closing of the fast gate. In addition, a more external site is accessible to permeant cations in the extracellular solution.

There is ample evidence that an internal blocker can decrease the occupancy of a selective site deep within the pore. For example, a *trans*-channel inhibition between internal and external cationic blockers has been observed in sodium channels (Cahalan and Almers, 1979b; French et al., 1996; Sierralta et al., 1998). The cause of the inhibition may be an electrostatic repulsion between external and internal cations, although an allosteric effect cannot be ruled out. The blockers we have used, TEA and DEA, appear to enter deeply (30–50%) into the membrane electric field from the inside (Zamponi and French, 1993; O'Leary and Horn, 1994). We do not know the location in the electric field of the binding site for external permeant cations;[2] however, it is likely to be deeper than ~30% from the outside. This follows from two observations. The first is that external pore residues that affect permeation, but not selectivity, contribute to a cadmium blocking site when replaced by cysteine; this site is located at an apparent distance of up to ~28% into the electric field (Chiamvimonvat et al., 1996b). The second is that highly selective effects of, for example, sodium versus cesium ions on gating suggest that permeant ions reach a site even deeper within the pore. This idea is supported both by the topology revealed by cysteine scan-

[2]The apparent location of a blocker in the electric field may be affected by ion–ion interactions within the pore, and therefore has to be interpreted with caution (Hille, 1992).

ning (Chiamvimonvat et al., 1996b; Yamagishi et al., 1997) and by the effects we report here on pore mutations. Therefore, the equivalent electrical distance between, for example, the TEA blocking site and the selectivity filter is likely to encompass <30% of the electric field. If the sodium channel pore is structurally similar to the pore of potassium channels, then the physical distance between these sites is on the order of 10 Å (Doyle et al., 1998), a distance close to the Debye length in free solution, further supporting the possibility of electrostatic repulsion between an internal blocker and a permeant cation situated within the selectivity filter. In addition, mutation of a positively charged pore residue, K1418, to a neutral residue, cysteine, increases the potency of TEA block (Townsend and Horn, unpublished observations). As noted above, residue K1418 contributes significantly to selectivity. Finally, we found that the internal TEA affinity was enhanced 2.5-fold when external sodium was replaced by cesium, further suggesting an intrapore repulsion between sodium and TEA. This is consistent with the idea that permeant extracellular cations have access to deeper sites than impermeant cations.

The above considerations argue strongly that the binding of TEA or DEA inhibits the binding of a permeant ion within the selectivity filter, perhaps by electrostatic repulsion within a multiply occupied pore. Because these blockers have a similar effect as that of extracellular permeant ions, we propose that the presence of either in deep regions of the permeation pathway prevents the closing of the fast gate. Although we do not know the molecular identity of this gate, it resembles in some ways a highly voltage-dependent pore blocker that is competitively inhibited by occupancy of the pore by cations. This raises the possibility that it is an exogenous polyvalent cation, such as spermine (Lopatin et al., 1994). It might also be a tethered blocker, like the inactivation ball of potassium channels (Hoshi et al., 1990). Finally, it may be intrinsic to the core of the protein, like the activation gate. We are currently investigating these alternatives.

Implications about the Selectivity Filter and the Activation Gate

Because the selective effect of external cations can occur before channels open in response to a depolarization (Townsend et al., 1997; Townsend and Horn, 1997), these cations must have access to the selectivity filter when the activation gate of the channels is closed. This has two general implications. (a) The selectivity region of a channel with a closed activation gate is not collapsed. This indicates that the activation gate is physically separate from the selectivity filter, whose properties are preserved during the opening and closing of the channels. Note that our experiments do not address the possible effects of inactivation on the selectivity filter. It has been suggested that selectivity is altered during C-type inactivation of potassium channels (Starkus et al., 1997; Kiss et al., 1998). (b) The activation gate must lie on the cytoplasmic side of the selectivity filter, a result consistent with previous results on both sodium channels (Strichartz, 1973; Yeh and Narahashi, 1977; Cahalan and Almers, 1979a; Eaholtz et al., 1994) and potassium channels (Armstrong, 1975; French and Shoukimas, 1981; Choi et al., 1993; Liu et al., 1997).

We thank Drs. Roger Sorensen and Hali A. Hartmann for generous advice and oligonucleotides used for mutagenesis and Dr. Robert French for insightful comments on the manuscript.

This study was supported by National Institutes of Health grant AR-49691 (R. Horn) and National Research Service Award fellowship NS-10439 (C. Townsend).

Original version received 18 August 1998 and accepted version received 8 December 1998.

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