

Role of an S4-S5 Linker in Sodium Channel Inactivation Probed by Mutagenesis and a Peptide Blocker

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ABSTRACT A pair of conserved methionine residues, located on the cytoplasmic linker between segments S4 and S5 in the fourth domain of human heart Na channels (hH1), plays a role in the kinetics and voltage dependence of inactivation. Substitution of these residues by either glutamine (M¹⁶⁵¹M¹⁶⁵²/QQ) or alanine (MM/AA) increases the inactivation time constant (τ_h) at depolarized voltages, shifts steady-state inactivation (h_∞) in a depolarized direction, and decreases the time constant for recovery from inactivation. The data indicate that the mutations affect the rate constants for both binding and unbinding of a hypothetical inactivation particle from its binding site. Cytoplasmic application of the pentapeptide KIFMK in Na channels mutated to remove inactivation produces current decays resembling inactivation (Eaholtz, G., T. Scheuer, and W.A. Catterall. 1994. *Neuron*. 12: 1041–1048.). KIFMK produces a concentration-dependent, voltage-independent increase in the decay rate of MM/QQ and MM/AA currents at positive membrane potentials ($K_i \sim 30 \mu\text{M}$), while producing only a small increase in the decay rate of wild-type currents at a concentration of 200 μM . Although MM/QQ inactivates ~ 2.5 -fold faster than MM/AA in the absence of peptide, the estimated rate constants for peptide block and unblock do not differ in these mutants. External Na⁺ ions antagonize the block by cytoplasmic KIFMK of MM/AA channels, but not the inactivation kinetics of this mutant in the absence of peptide. The effect of external [Na⁺] is interpreted as a voltage-dependent knock-off mechanism. The data provide evidence that KIFMK can only block channels when they are open and that peptide block does not mimic the inactivation process. Key words: cardiac muscle • complementary DNA • expression • mutagenesis

INTRODUCTION

The upstroke of the action potential in excitable cells (e.g., nerve and striated muscles) is caused by sodium influx via voltage-sensitive Na channels. In these cells depolarization causes the activation (opening) of the Na channels, which allows them to conduct ions. Shortly after opening, the channels spontaneously inactivate, a process that stops ion conduction. With membrane repolarization, the Na channels return to resting closed states from which they can be activated once again. The precise control of the activation and inactivation processes is critical for the action potential, so that it can be triggered reliably, that the depolarization is transient, and that the channels will recover appropriately from inactivation so that the membrane is again excitable.

Cytoplasmic amino acids are known to be involved in the inactivation of both Na and *Shaker* K channels. For

example, the cytoplasmic application of a variety of protein reagents, notably proteases, slows or abolishes inactivation (Rojas and Armstrong, 1971; Eaton et al., 1978; Oxford et al., 1978; Hoshi et al., 1990). In Na channels it has been proposed that the 54 amino acid cytoplasmic linker between domains 3 and 4, the D3-D4 linker, acts as the inactivation gate. Evidence in support of this hypothesis is (a) site-directed antibodies directed against this linker inhibit inactivation (Vassilev et al., 1988; Vassilev et al., 1989), (b) mutagenesis in the linker affects inactivation (Stühmer et al., 1989; Moorman et al., 1990; Patton et al., 1992; Hartmann et al., 1994; Yang et al., 1994; O'Leary et al., 1995), and (c) phosphorylation by C-kinase of a specific serine residue in this linker slows inactivation (West et al., 1991). The most important residues for inactivation in the D3-D4 linker are a triplet of three consecutive amino acids (IFM: isoleucine-phenylalanine-methionine). Mutation of all three hydrophobic residues to glutamine (IFM/QQQ) abolishes fast inactivation (West et al., 1992). Most of this effect in neuronal Na channels is caused by the F/Q substitution (West et al., 1992). Cytoplasmic application of a synthetic pentapeptide, KIFMK, partially modeled on these residues, restores a fast decay, resembling inactivation, to Na currents of the IFM/QQQ mutant (Eaholtz et al., 1994). By contrast, a syn-

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¹Abbreviations used in this paper: IFM, isoleucine-phenylalanine-methionine; WT, wild-type.

thetic pentapeptide with a glutamine substitution at the critical phenylalanine site (KIQMK) fails to affect the kinetics of the inactivation-impaired Na channel. These results suggest that the native IFM residues act as a critical portion of an inactivation particle, or gate, and that the binding of this particle to its receptor results in inactivation by occluding the transmembrane pore of the Na channel.

If the D3-D4 linker containing the IFM residues constitutes the inactivation gate, it raises the question of the identity of the receptor. Because the inactivation process appears to be highly voltage dependent, the affinity of the putative inactivation particle for its receptor must be coupled in some way to a voltage sensor. A preponderance of evidence suggests that the inactivation process derives most of its voltage dependence from being coupled to activation via conformational changes induced by voltage sensors in the activation pathway (for reviews see Armstrong, 1981; Bezanilla, 1985; Patlak, 1991; Keynes, 1994). Therefore the receptor for the inactivation gate could be closely associated with activation voltage sensors. The S4 transmembrane segments of each domain in Na channels, and in each subunit of voltage-dependent K channels, possess a positively charged amino acid, either arginine or lysine, every third residue, and it is these S4 segments that are thought to function as activation voltage sensors, moving outward in response to a depolarization (Yang and Horn, 1995; Larsson et al., 1996; Mannuzzu et al., 1996; Yang et al., 1996). Recent evidence in Na channels indicates a unique linkage between S4/D4 and inactivation. Point mutations in S4/D4 have a profound influence on the inactivation process with little effect on activation (Chahine et al., 1994; Bennett et al., 1995). Furthermore, the results from an extensive set of point mutations in S4 segments of all four domains (D1-D4) clearly indicate that S4/D4 mutations, but not S4 substitutions in D1-D3, slow inactivation kinetics and reduce their voltage dependence (Chen et al., 1995).

A cytoplasmic region of Na channels that may contribute directly to inactivation is, therefore, the S4-S5 loop in D4. This 12 amino acid loop is likely to undergo a conformational change when depolarization causes an outward movement of S4/D4 (Yang and Horn, 1995; Yang et al., 1996), and this movement could increase the affinity of the postulated inactivation particle for its receptor, especially if the S4-S5 loop of D4 forms part of the receptor. The S4-S5 loop of *Shaker* K channels has also been postulated to form part of the receptor for a cytoplasmic inactivation particle (Isacoff et al., 1991). In a study to be published elsewhere, we scanned the S4-S5 loop in D4 of the human heart Na channel (hH1) by site-directed mutations of adjacent pairs of residues and found a major effect on inactivation kinetics for substitutions of a pair of conserved me-

thionine residues (M¹⁶⁵¹M¹⁶⁵²; L. Tang and R.G. Kallen, manuscript in preparation). We show here that substitutions of these methionine residues destabilize an inactivated state, and evaluate the possibility that they constitute part of the binding site for a cytoplasmic inactivation gate. We also show that cytoplasmic application of the pentapeptide KIFMK increases the decay rate of inactivating currents in both wild-type and mutant Na channels but that this effect is distinctly different from native inactivation.

METHODS

Mutagenesis and Expression in tsA201 Cells

Site-directed mutagenesis was carried out using the Altered Sites-II in vitro mutagenesis system (Promega Corp., Madison, WI). Briefly, single-stranded template was prepared from JM109 cells with R408 helper phage. The synthetic antisense mutagenic oligonucleotide containing the desired mutations (shown underlined in bold) were as follows:

M1651Q/M1652Q:5'-GAGGGCAGGCAGGGACT**GCTG**GAGGGC-AAAGAGCAG-3'
M1651A/M1652A:5'-GAGGGCAGGCAGGGAT**GCTG**CGAGGGC-AAAGAGCAG-3'

The oligonucleotide was annealed to the single-stranded DNA template, converted to double-strand, closed, circular DNA, and the reaction mixture was transformed into ES1301 *mutS* cells. The plasmid DNA was isolated from antibiotic-selected ES1301 *mutS* cells and then transformed into JM109 cells. The colonies were screened and mutations verified by dideoxynucleotide sequencing. pCDNA-1 vector (Invitrogen Corp., San Diego, CA) was used to drive the expression of wild-type (WT) and mutant hH1 Na channels in the tsA201 cell line.

Na channels were transiently transfected into tsA201 cells, a transformed HEK293 cell line, by standard calcium phosphate methods. Briefly, cells were cotransfected with 10 μ g each of cDNAs encoding Na channels, either WT or mutant, and a surface marker, CD8 (Margolskee et al., 1993). Cells were used for recording 2-3 d after transfection. Transfected cells were selected by use of beads coated with an antibody against CD8 (Jurman et al., 1994). Greater than 90% of the bead-decorated cells expressed Na currents.

Electrophysiology and Data Analysis

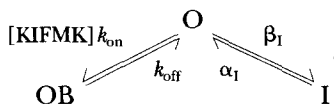
Whole cell voltage clamp recordings were carried out as described previously (O'Leary and Horn, 1994). Sylgard-coated (Dow Corning Corp., Midland, MI), fire-polished pipets of Corning 8161 glass were used. Currents were recorded and filtered at 5 kHz with an Axopatch 200A patch clamp amplifier (Axon Instruments, Inc., Burlingame, CA). Data were acquired using pCLAMP 6 and the Digidata 1200 interface (Axon Instruments). Cells were dialyzed at least 10 min before recording data. The series resistance was <1.6 M Ω after 80% compensation. Standard recording solutions were as follows. Bath solution (mM): 150 NaCl, 2 KCl, 1.5 CaCl₂, 1 MgCl₂, and 10 Na-HEPES, pH 7.4. Pipet solution (mM): 35 NaCl, 105 CsF, 10 EGTA, and 10 Cs-HEPES, pH 7.4. In some experiments we used a reversed gradient of [Na⁺]. The reversed-gradient bath solution was (mM): 140 choline-Cl, 10 NaCl, 2 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 Na-HEPES, pH 7.4; the reversed-gradient pipet solution was (mM): 100 NaF, 30 CsF, 10 CsCl, 5 EGTA, and 10 Cs-HEPES, pH 7.4. Corrections were

made for liquid junction potentials. The standard and reversed-gradient bath solutions listed above were exchanged on single cells (see Figs. 10 and 11) by use of a sigmacoted (Sigma Chemical Co., St. Louis, MO) macropipet (Yang et al., 1996).

We examined block by cytoplasmic application of the cationic pentapeptide α -N-acetyl-Lys-Iso-Phe-Met-Lys-amide (KIFMK; Eaholtz et al., 1994). This pentapeptide was made by solid phase synthesis and purified by preparative HPLC in the Protein Chemistry Facility of the Jefferson Cancer Institute at Thomas Jefferson University. The chloride salt of KIFMK was dissolved in the "reversed-gradient" pipet solution at a concentration of 1 mM as a stock solution and diluted as necessary. Kinetically stable currents were achieved within ~ 4 min after the whole cell configuration was established, and recordings were made after 10 min. To measure the rapid decay kinetics accurately at positive voltages, especially after exposure to KIFMK, we usually cooled the bath to 14.7°C with a TC-10 controller (Dagan, Minneapolis, MN) connected to Peltier devices. Temperature was monitored by a thermistor at a distance < 100 μm from the cell. Other experiments were performed at room temperature, 19–21°C. Temperatures of all experiments are indicated in the figure legends.

Whole cell data were analyzed and plotted by a combination of pCLAMP programs, Microsoft Excel®, Origin (MicroCal, Northampton, MA), and our own FORTRAN programs. Data are expressed as mean \pm SEM. When quantifying the voltage sensitivity of various parameters (e.g., Boltzmann functions), we denote the steepness of the voltage dependence by the number of equivalent electronic charges (e_0) moving across the membrane electric field.

For kinetic modelling of the block by cytoplasmic application of KIFMK, we examined Na currents at positive voltages with a reversed $[\text{Na}^+]$ gradient. The rationale for focusing on positive potentials was to avoid a contribution by activation gates during the decaying phase of the current (O'Leary et al., 1994). The reversed $[\text{Na}^+]$ gradient allowed us to measure substantial currents at these voltages. We used the following kinetic model to describe the decay phase of Na currents in the presence of KIFMK:



In this model the channels are predominantly in the open state (O) at the current peak, and decay is due to entry into either of two nonconducting states, an inactivated state (I) or a peptide-blocked state (OB). This model explicitly assumes that the peptide has no effect on the rate constants for inactivation, α_1 and β_1 , and that block and inactivation are mutually exclusive. We further assume that closed channels preceding a depolarization are not blocked by KIFMK, and therefore the peptide must dissociate from its site before the channel achieves a resting closed state in the recovery period between depolarizations. The principle evidence for this assumption is that the amplitude of peak current does not decline during the first few minutes of whole cell recording as KIFMK diffuses from the patch pipet into the cell, whereas the decay rate of the Na current increases over this period (data not shown). The dissociation of KIFMK from the channel after a depolarization must be rapid, since 20-ms depolarizations to +40 mV presented at 4 Hz show no use-dependent decrease in current amplitude for WT channels or either methionine mutant at peptide concentrations up to 1 mM (data not shown).

We estimated parameters for the above kinetic scheme as follows. In the absence of peptide α_1 and β_1 were estimated from the inactivation time constant $\tau_h = (\alpha_1 + \beta_1)^{-1}$ and the steady-state outward current after a depolarization to voltages $\geq +20$ mV. The ratio of steady state to peak current is an estimate of $\alpha_1/(\alpha_1 + \beta_1)$, assuming that the peak open probability approaches unity at

these depolarized voltages (Sigworth, 1980). This assumption of the peak open probability had little effect on the estimates of k_{on} and k_{off} ; reducing the assumed peak open probability to 0.5 caused a change of $< 2\%$ in estimates of k_{on} and k_{off} . In the presence of peptide the current decay is expected to be a mixture of two exponential relaxations. However we typically observed only 1 exponential component, whose time constant was smaller than in the absence of peptide and depended strongly on $[\text{KIFMK}]$ (see Figs. 6–8). In some cases we observed two time constants, heavily weighted by the faster relaxation. Therefore we assume that the single relaxation time constant in the presence of peptide corresponds to the fast relaxation. For the above kinetic model the fast and slow time constants, τ_{fast} and τ_{slow} are given as follows:

$$1/\tau_{\text{fast}} = \{ S + \sqrt{S^2 - 4(\beta_1 k_{\text{off}} + \alpha_1 \{ [\text{KIFMK}] k_{\text{on}} + k_{\text{off}} \})} \} / 2,$$

$$1/\tau_{\text{slow}} = \{ S - \sqrt{S^2 - 4(\beta_1 k_{\text{off}} + \alpha_1 \{ [\text{KIFMK}] k_{\text{on}} + k_{\text{off}} \})} \} / 2,$$

$$\text{where } S = \alpha_1 + \beta_1 + [\text{KIFMK}] k_{\text{on}} + k_{\text{off}}. \quad (1)$$

At each membrane potential the rate constants k_{on} and k_{off} and their standard errors were estimated by a nonlinear least squares fit of the measured decay rates as a function of $[\text{KIFMK}]$ (e.g., see Fig. 8) using a variable metric algorithm (Powell, 1978). The equilibrium dissociation constant for peptide block, K_i , is the ratio of $k_{\text{off}}/k_{\text{on}}$. Its standard error was estimated from those of k_{on} and k_{off} by propagation of errors.

RESULTS

Fig. 1 shows families of Na currents of WT hH1 and the two double mutants, expressed transiently in tsA201 cells. The mutants exchange either glutamine ($\text{M}^{1651}\text{M}^{1652}/\text{QQ}$) or alanine (MM/AA) for two adjacent methionine residues in the cytoplasmic S4-S5 loop of domain 4 (D4). Both mutants cause a slowing of the current decay, with a more pronounced effect for the MM/AA mutant. Normalized peak current-voltage relationships (Fig. 2 A) show that MM/QQ and WT are indistinguishable, while MM/AA appears to activate at more negative voltages. The peak currents were converted to normalized conductances and plotted as conductance-voltage (G-V) relationships, as shown in Fig. 2 B. These data, fit by single Boltzmann relationships (solid lines), indicate that channels open (i.e., activate) over a voltage range of -80 to 0 mV. The G-V curve of MM/AA shows a hyperpolarizing shift of 9.4 mV, compared with WT, without a large change in the steepness of the voltage dependence (equivalent to $4.5 \pm 0.3 e_0$ and $3.8 \pm 0.2 e_0$ for MM/AA and WT, respectively; $P \cong 0.04$). Although the shift in the G-V curve suggests that MM/AA channels open at more negative voltages than WT channels, it is also possible that the slowed inactivation in this mutant reveals currents that would normally be masked by inactivation in WT channels (see arguments in Chahine et al., 1994).

The decay of Na current after a depolarization (Fig. 1) was usually well-fit by a single exponential relaxation at all voltages in both WT and mutant channels, al-

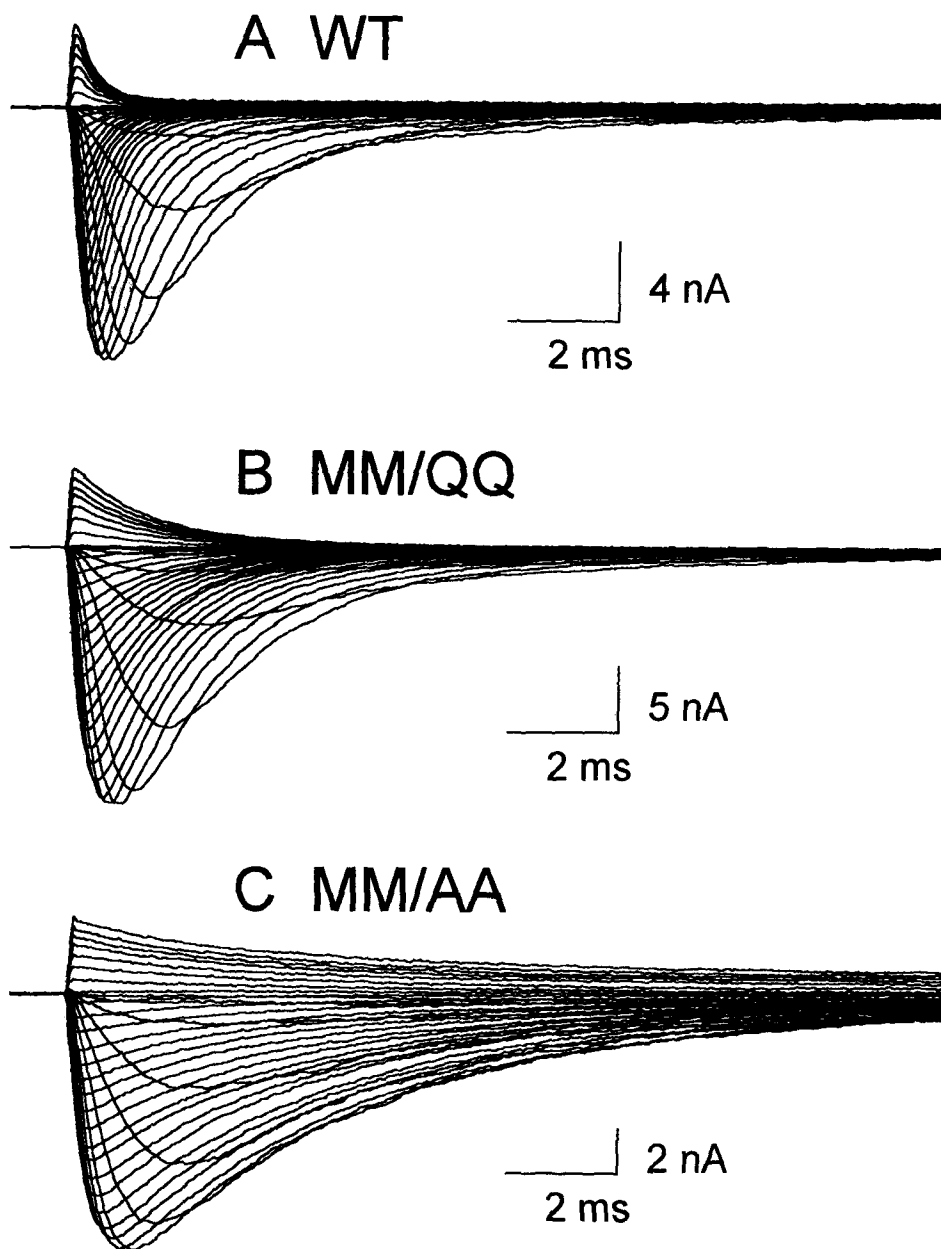


FIGURE 1. Effects of mutations of M^{1651}/M^{1652} on hH1 Na channel currents. Currents activated by depolarization in 5 mV increments between -80 mV and $+75$ mV from a holding potential of -120 mV (standard bath and pipet solutions, room temperature).

though in a few cells a second slower time constant, weighted $<10\%$, was observed. We restricted the following measurements to cells in which a single exponential relaxation produced a good fit to the current decay with a time constant τ_h (Fig. 3 A). Inactivation is slower in both mutants at voltages more positive than -60 mV. At 0 mV τ_h is 3.4- and 9.4-fold greater for MM/QQ and MM/AA, respectively, than for WT channels. Inactivation induced by 500-ms prepulses is shifted in a depolarizing direction in both mutants, 13.8 mV for MM/QQ and 10.5 mV for MM/AA (see Boltzmann fits to data of Fig. 3 B). In addition, the steepness of the h_∞ curve is decreased for MM/AA (Fig. 3 B).

The larger values of τ_h in the mutants and the right-

shifted h_∞ curves show that an inactivated state is somehow destabilized by these methionine mutations. Further indication of the destabilization of an inactivated conformation is that the rate of recovery from inactivation is increased in the mutants (Fig. 3, C and D). Inactivation was induced by a 15-ms prepulse to $+70$ mV, and recovery was measured after a variable-duration hyperpolarizing recovery pulse, as described previously (Chahine et al., 1994). The time course of recovery was fit by an exponential relaxation (Fig. 3 C) with a time constant of τ_{rec} at each recovery voltage. These recovery time constants are smaller for both mutants than for WT channels over a voltage range of -140 to -90 mV (Fig. 3 D). Note that the WT τ_{rec} has a maximum at

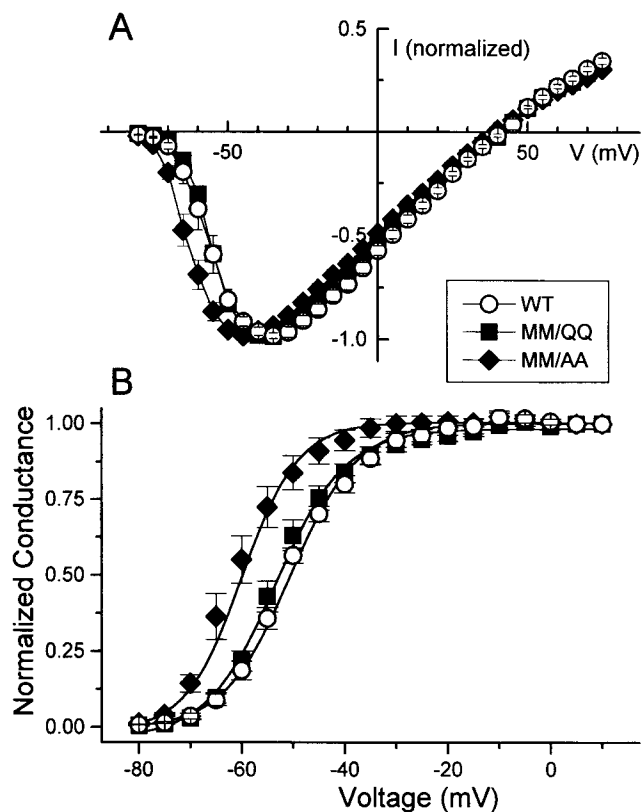


FIGURE 2. Effects of methionine mutations on peak currents. (A) Normalized peak current-voltage (I-V) relationship for wild-type (WT, $n = 5$), MM/QQ ($n = 9$), and MM/AA ($n = 6$). (B) Normalized conductance-voltage (G-V) curves from I-Vs. The G-V data were fit with a Boltzmann relationship (solid lines). The mean (\pm sem) midpoint and slope for WT are -50.5 ± 0.4 mV and $3.83 \pm 0.17 e_0$; for MM/QQ are -51.5 ± 0.7 mV and $4.34 \pm 0.25 e_0$; and for MM/AA are -59.9 ± 0.6 mV and $4.54 \pm 0.31 e_0$ (room temperature).

-100 mV, near the midpoint of the h_∞ curve, as we observed previously (O'Leary et al., 1995).

Inactivation is a complex process that is likely to involve the movement of activation voltage sensors as well as the actual opening and closing of the inactivation gate. However the single exponential kinetics and single Boltzmann function for steady-state inactivation suggest a predominant rate-limiting step in this process, and the destabilization of inactivation by the methionine mutations can be thought of as a decrease in affinity of the inactivation gate for its receptor. Formally, therefore, our data at negative voltages may be used to estimate an apparent association and dissociation rate constant for the hypothetical inactivation particle. The association rate constant, which involves the closing of the inactivation gate, is defined as β_1 , and the dissociation rate constant is α_1 . For this model the h_∞ curve represents the steady state probability of not being inactivated, $\alpha_1/(\alpha_1 + \beta_1)$, and $\tau_{rec} = (\alpha_1 + \beta_1)^{-1}$.

Therefore, from measured values of h_∞ and τ_{rec} we can estimate both α_1 and β_1 . Both of these estimated rate constants are voltage dependent (Fig. 4), β_1 increasing, and α_1 decreasing with depolarization. Note that for each clone α_1 is significantly larger than β_1 in Fig. 4 (plotted on different ordinates). This is expected, because except for the WT at -90 mV, α_1 and β_1 were estimated at voltages more hyperpolarized than the midpoint of the h_∞ curve. These two rate constants depend exponentially on membrane potential (theory curves in Fig. 4), with β_1 2–3 times more voltage dependent than α_1 for each clone (legend of Fig. 4). Fig. 4 also shows that the dissociation rate constants are increased in the mutants and that the association rate constants, β_1 , are also affected, especially in the MM/QQ mutant. If the paired methionine residues form part of the receptor for a hypothetical inactivation gate, these results suggest that they contribute to both the initial association between the gate and its receptor and the stabilization of the gate after it is bound. It is worth emphasizing, however, that the rate limiting step of this inactivation process may not be the actual binding and unbinding of an inactivation particle, but rather the voltage-dependent conformational changes that affect the receptor (O'Leary et al., 1995).

Effect of Methionine Mutations on Block by a Cytoplasmic Peptide

The native inactivation gate of the Na channel, like the so-called N-gate of *Shaker* K channels, is commonly believed to act like a cytoplasmic pore blocker of the channel. It has been proposed that a pentapeptide, KIFMK, when applied cytoplasmically, can serve the same function as the inactivation gate in Na channels mutated to remove inactivation (Eaholtz et al., 1994; Eaholtz et al., 1995; McPhee et al., 1995). This suggests that KIFMK can be used to probe the receptor for the native inactivation gate (McPhee et al., 1995). Because the methionine mutations appear to reduce the affinity of the inactivation gate for its receptor, as shown above, these mutations might also be expected to reduce the blocking potency of KIFMK. Based on the relative effects of the two methionine mutations on inactivation, the potency of peptide block is expected to be in the order WT > MM/QQ > MM/AA. We tested these hypotheses by examination of peptide block at voltages $\geq +20$ mV, where τ_h approximates the time constant for closing of the inactivation gate with little contribution from activation (Vandenberg and Horn, 1984; Scanley et al., 1990; Vandenberg and Bezanilla, 1991). To better resolve the currents at these positive potentials, we used a reversed gradient of Na^+ concentration in which internal $[Na^+]$ was 100 mM and external $[Na^+]$ was ~ 15 mM (see METHODS).

Fig. 5 shows that the methionine mutations produce

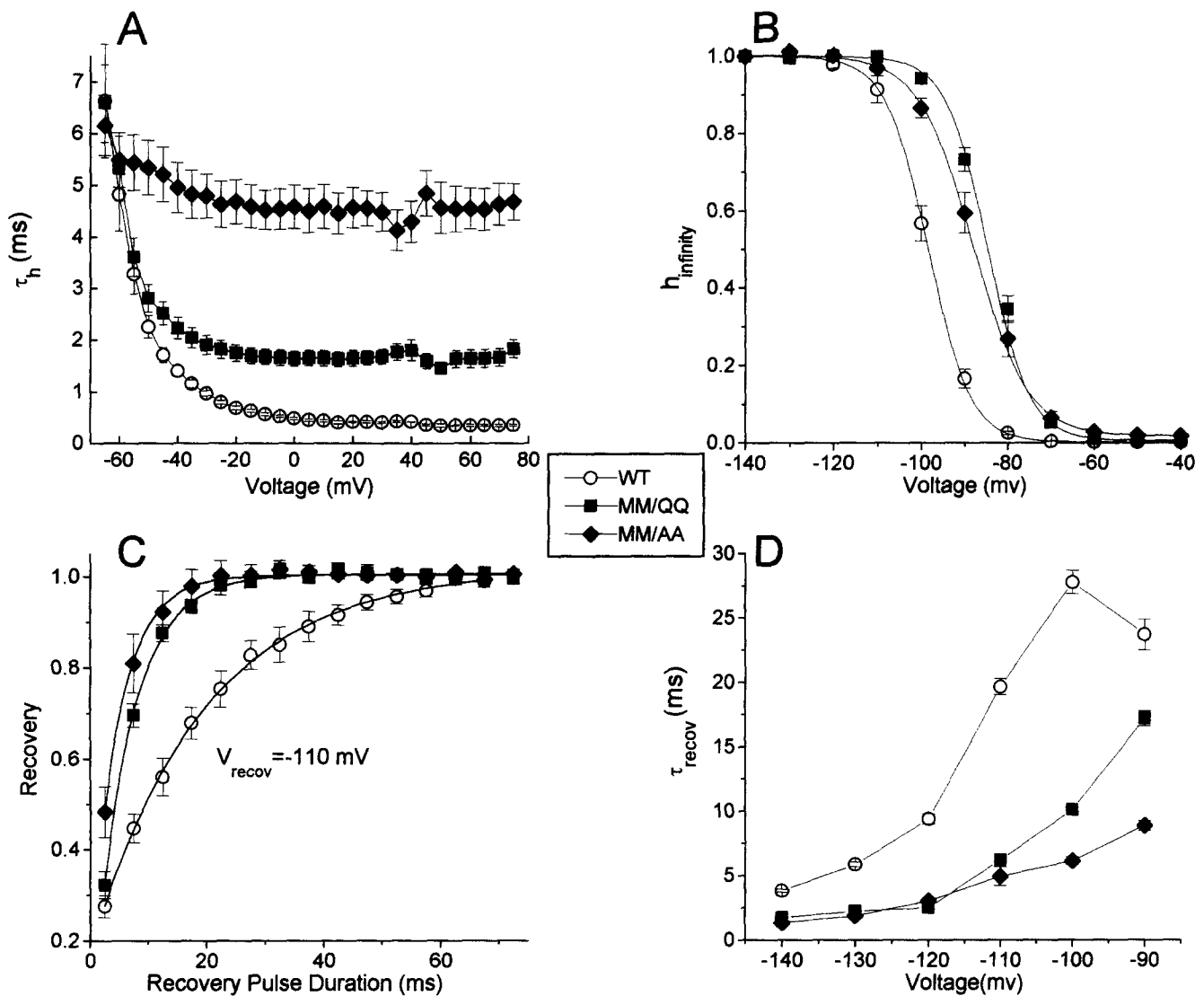


FIGURE 3. Effect of methionine mutations on inactivation. (A) Time constant (τ_h) of current decay as a function of pulse voltage during step depolarizations. The decay of current following peak was fit by a single exponential relaxation. (B) Steady-state inactivation (h_{∞}) curves. Currents were measured at a test potential of -30 mV after 500-ms conditioning pulses at indicated voltages from a holding potential of -90 mV. Each test peak current was normalized by maximal peak current and fit by the Boltzmann equation. The midpoint and slope for WT is -98.5 ± 0.5 mV and $5.16 \pm 0.19 e_0$; for MM/QQ is -84.7 ± 0.6 mV and $5.17 \pm 0.25 e_0$; and for MM/AA is -88.0 ± 0.7 mV and $3.95 \pm 0.20 e_0$. (C) Recovery at -110 mV from inactivation. Channels were inactivated by a 15-ms pulse to $+70$ mV from a holding potential of -120 mV. The membrane was repolarized to -110 mV for various durations followed by a test pulse at -30 mV. The data were fit (solid lines) by a single exponential relaxation with a time constant, τ_{rec} , of 19.7, 6.2, and 4.9 ms, for WT, MM/QQ, and MM/AA channels, respectively. (D) Recovery time constants from inactivation at indicated potentials derived from data in experiments as shown in C (room temperature).

similar effects on inactivation for reversed-gradient solutions as we described previously, namely an increase in τ_h which is greater for MM/AA than for MM/QQ. The mutations also decrease the steady state probability of a channel being inactivated, as indicated by the increased level of plateau currents at the end of the depolarization (Fig. 5). Because of the slow inactivation kinetics for MM/AA, we had to use 60-ms depolarizations to measure this steady state level (data not shown).

Fig. 6 shows that 200 μ M KIFMK causes an $\sim 40\%$ in-

crease in the decay rate of WT Na current, whereas lower concentrations produce a pronounced dose-dependent increase in the decay rate of Na current after its peak for both MM/QQ and MM/AA. The data shown are scaled, superimposed records for depolarizations to $+40$ mV, obtained from cells internally dialyzed with the indicated concentration of peptide. As in all of our whole cell measurements, recordings were obtained after at least 10 min of dialysis to allow for full diffusion of peptide into the cell. The kinetics and voltage de-

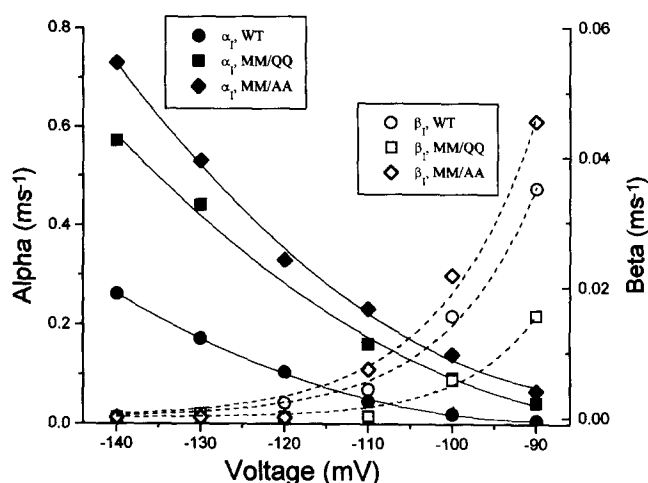


FIGURE 4. The association rate constant (α_1 , filled symbols) and dissociation rate constant (β_1 , open symbols) of inactivation plotted against voltage. These rate constants were calculated from the equations: $\tau_{\text{rec}} = 1/(\alpha_1 + \beta_1)$; $P_{\text{dosed}} = \alpha_1/(\alpha_1 + \beta_1)$, with τ_{rec} obtained from Fig. 3 D, and P_{dosed} obtained from steady-state inactivation (Fig. 3 B). The data for each rate constant were fit by a single exponential function (solid and dashed lines). Note the different absolute scales for α_1 (left axis) and β_1 (right axis). For β_1 the effective charge moved for gate closing was 2.3, 2.8, and 2.3 e_0 , for WT, MM/QQ, and MM/AA, respectively. For α_1 the effective charge moved for dissociation of the gate was 1.3, 1.1, and 1.0 e_0 , for WT, MM/QQ, and MM/AA, respectively (room temperature).

pendence of the Na currents were stable during the subsequent 5–10 min of recording required for each cell. This method of application of peptide was necessary, because the level of expression was not high enough to measure macroscopic currents in inside-out patches.

We were unable to characterize in detail the effects of KIFMK on WT channels, because we observed a slow “rundown” of WT currents for $[\text{KIFMK}] \geq 100 \mu\text{M}$. This effect was not observed for the methionine mutants or in WT channels when we used a normal, instead of a reversed, Na^+ gradient (data not shown). Because of this rundown in WT channels, we restricted the remaining experiments and analysis to a comparison of the two methionine mutants.

For simple presentation of KIFMK effects on the decay kinetics of Na currents, we used the following model: We assumed that the peptide binds to some portion of the receptor for the native inactivation gate and that peptide binding is mutually exclusive to the binding of the inactivation gate. Since we restricted our attention to positive voltages, where activation is assumed to be very fast and to contribute little to the decay rate of macroscopic currents, the kinetic model describing the decay phase of Na currents in the presence of KIFMK has three states: open, inactivated, and open-blocked (see METHODS). We estimated the second order blocking rate constant k_{on} and the dissociation rate constant k_{off} for such a model, as described in METHODS.

The aforementioned 3-state model produces, in general, double exponential kinetics. Our data, however, are usually well fit by a single decaying component. In some cases we observed a slower, second time constant as well, but it always had a small weight (i.e., <10%). Therefore the time constant for current decay is assumed to be the fast time constant, τ_{fast} , for this model. This time constant, unlike the slower time constant, is expected to decrease strongly with increasing $[\text{KIFMK}]$, consistent with our data (Figs. 6–8). The theoretical relationship between τ_{fast} and $[\text{KIFMK}]$ is given by Eq. 1 in METHODS.

We used the concentration dependence of KIFMK on decay kinetics to estimate the rate constants for its association and dissociation with open channels for each methionine mutant. To avoid the possibility of interference with activation kinetics, we restricted this analysis to KIFMK concentrations $\leq 100 \mu\text{M}$. Fig. 7 shows the effect of KIFMK on decay time constants for both mutants over a range of 0 to +60 mV. In this voltage range the WT τ_{h} is voltage independent for these reversed-gradient solutions (data not shown). There is little voltage dependence for the effect of KIFMK at any concentration, indicating that the binding site for the peptide is not located deeply within the membrane electric field. Although these data indicate a superficial binding site for KIFMK, below we will show that external Na^+ ions can induce a voltage dependence to the decay rate of the mutant MM/AA in the presence of the peptide. We will interpret this effect as a voltage dependence of Na^+ binding.

Fig. 8 plots the decay rate (i.e., the inverse of the decay time constant) as a function of $[\text{KIFMK}]$ for each mutant at +60 mV. These decay rates were fit at each membrane potential by Eq. 1 to obtain estimates of k_{on} and k_{off} (note the solid lines in Fig. 8, which are a reasonable approximation to the data for each mutant). The estimates of k_{on} and k_{off} from such fits are plotted in Fig. 9. Neither rate constant is voltage dependent, and there is no significant difference between the two methionine mutants with respect to either rate constant, even when the estimates are compared across all voltages ($P > 0.2$, Z-test using estimated rate constants and their estimated standard errors). This result is in sharp contrast with the pronounced differences in inactivation kinetics between the mutants (Figs. 5–7). Fig. 9 C also shows that the dissociation constant ($k_{\text{off}}/k_{\text{on}}$) for peptide block of the channel is about $30 \mu\text{M}$ and does not differ significantly between the two methionine mutants ($P > 0.25$).

Effects of Extracellular Na^+ Concentration

If KIFMK is acting as a cytoplasmic open-channel blocker, the block might be antagonized by extracellular Na^+ ions, as we observed previously for the pore

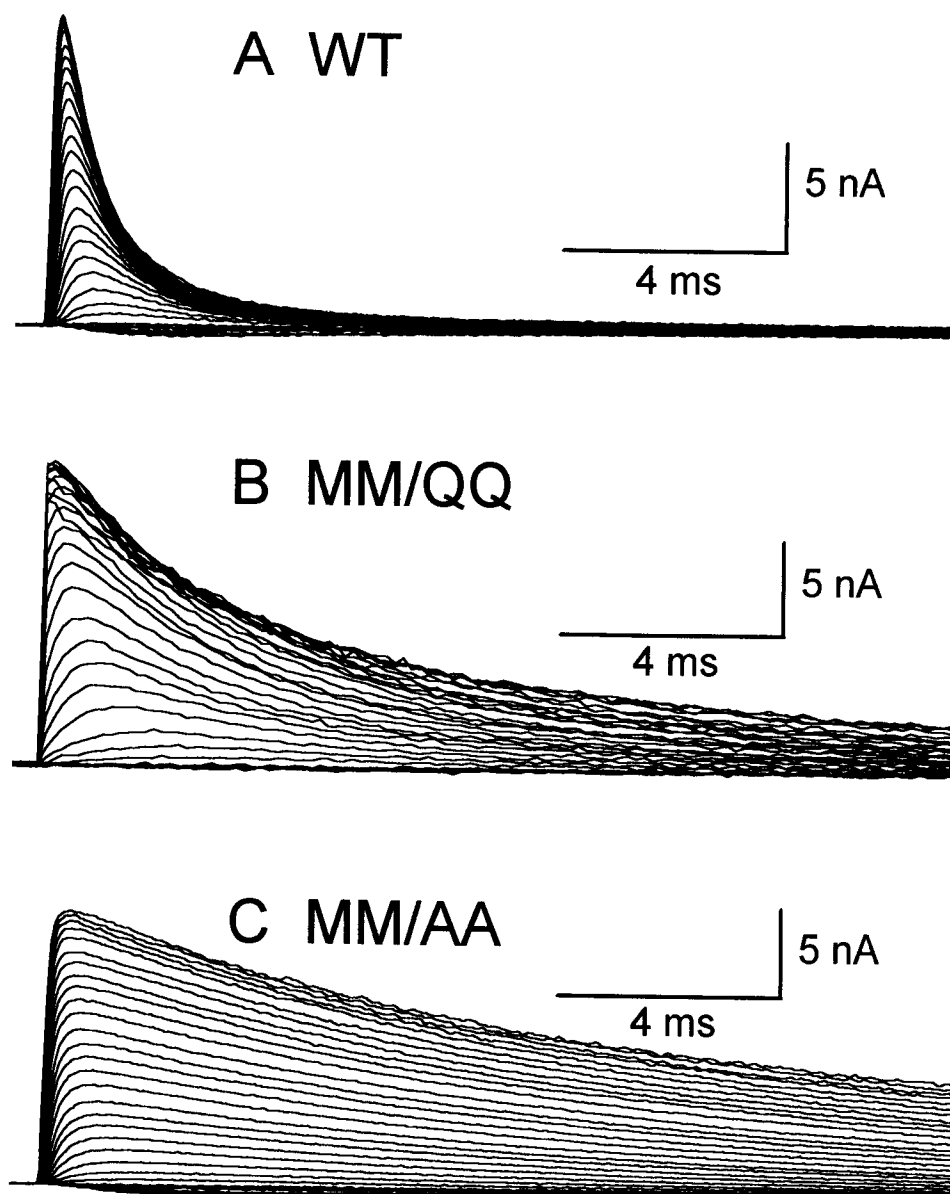


FIGURE 5. Effects of methionine mutations on outward currents, elicited in 5 mV steps between -20 and $+80$ mV from a holding potential of -120 mV. Reversed-gradient bath and pipet solutions; 14.7°C .

blocker tetrapentylammonium (O'Leary et al., 1994). An effect of permeant ion concentration on the opposite side of the membrane as the blocker is strong evidence that the blocker occludes the open pore (Armstrong, 1966; Yellen, 1984; MacKinnon and Miller, 1988; Demo and Yellen, 1991). To test this possibility we used the MM/AA mutant. When extracellular $[\text{Na}^+]$ was raised from 10 to 155 mM in the absence of peptide, we saw little effect on the inactivation kinetics of MM/AA between -35 and $+60$ mV (Fig. 10, A and B; Fig. 11 A, *open symbols*). In the presence of cytoplasmic $50 \mu\text{M}$ KIFMK, however, the higher extracellular $[\text{Na}^+]$ antagonizes the peptide block at more negative voltages (Fig. 10, C and D; Fig. 11 A, *filled symbols*). Notice that for high extracellular $[\text{Na}^+]$ the Na currents at the most negative voltage (-35 mV) decay at about the

same rate in the presence or absence of the peptide. The direction and extent of the voltage dependence ($\sim 0.7-1 e_0$) shown in Fig. 11 A suggest that extracellular Na^+ ions enter deeply into the channel to expel the blocker by increasing k_{off} (i.e., a knock-off effect). If k_{off} is sufficiently large, the kinetics of the current decay will be dominated by a slow component with a time constant (τ_{slow}) close to that of the channel in absence of blocker, according to the kinetic scheme in the METHODS. Therefore, the expected dwell time of the blocker in its site, normally 5–10 ms at very positive voltages (the inverse of k_{off} , Fig. 9 B), will be so abbreviated by the *trans* effect of high external $[\text{Na}^+]$, that the open channel will rarely contain the blocker, which therefore will be unable to impede the inactivation gate from closing at its normal rate.

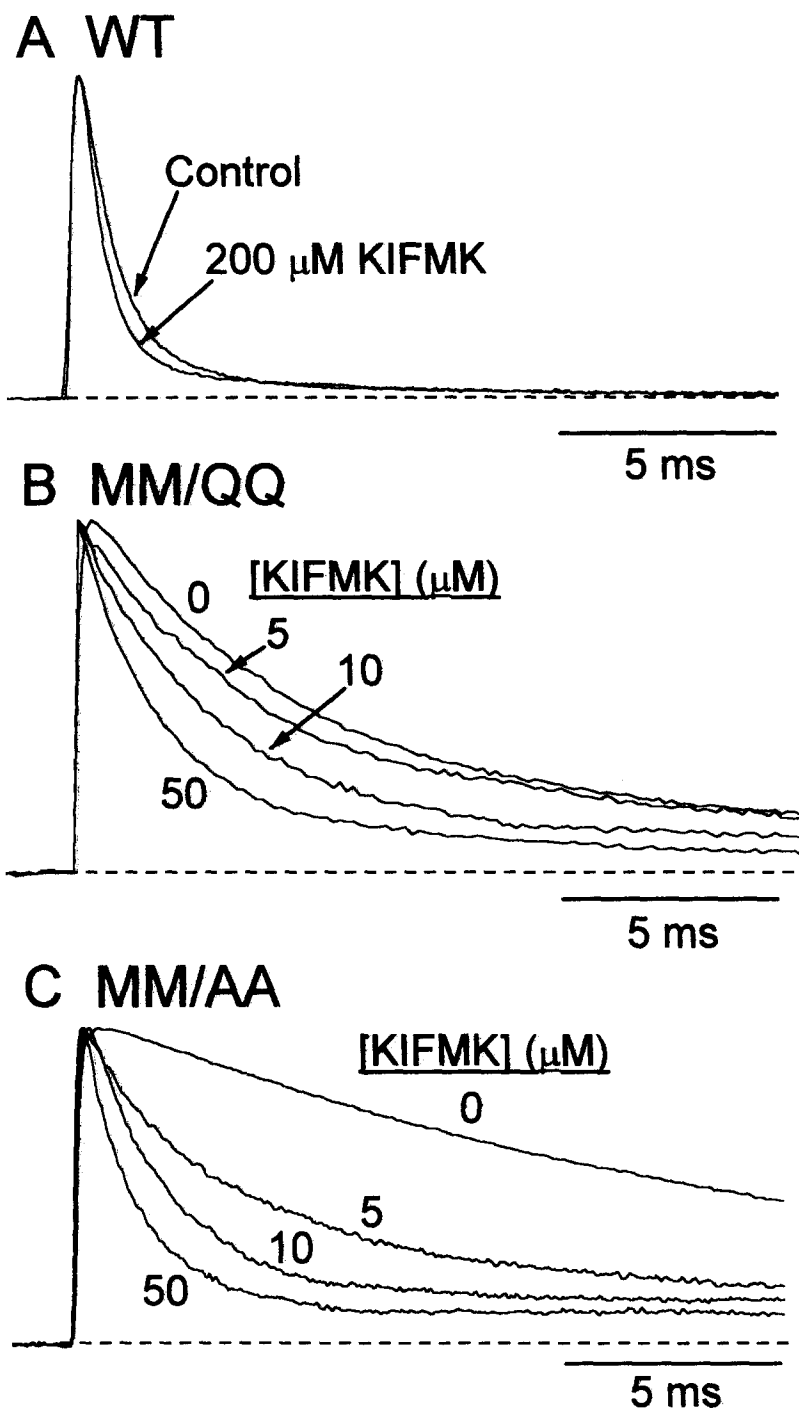


FIGURE 6. Effects of the pentapeptide KIFMK presented internally on Na currents at +40 mV using reversed-gradient solutions. Currents from different cells were normalized to the same peak current value. KIFMK was applied through the patch pipet at the indicated concentrations. Temperature, 14.7°C.

DISCUSSION

Our data indicate a gating role for two methionine residues ($\text{M}^{1651}\text{M}^{1652}$) in the cytoplasmic S4-S5 linker in D4 of the human heart Na channel. Relative to WT hH1, mutations of these adjacent residues to either glutamine or alanine cause a decrease in the rate of inactivation at depolarized voltages (Figs. 1, 3 A, and 5), depolarizing shifts in steady-state inactivation (Fig. 3 B), and in-

creases in the rate of recovery from inactivation (Fig. 3, C and D). All of these effects are consistent with a destabilization of an inactivated state. The MM/AA mutant has a more severe phenotype than that of MM/QQ. For example, the inactivation time constant τ_h at depolarized voltages is ~ 2.5 -fold larger for MM/AA than for MM/QQ (Figs. 3, A and 7). We have not examined the individual methionine residues in this study. However,

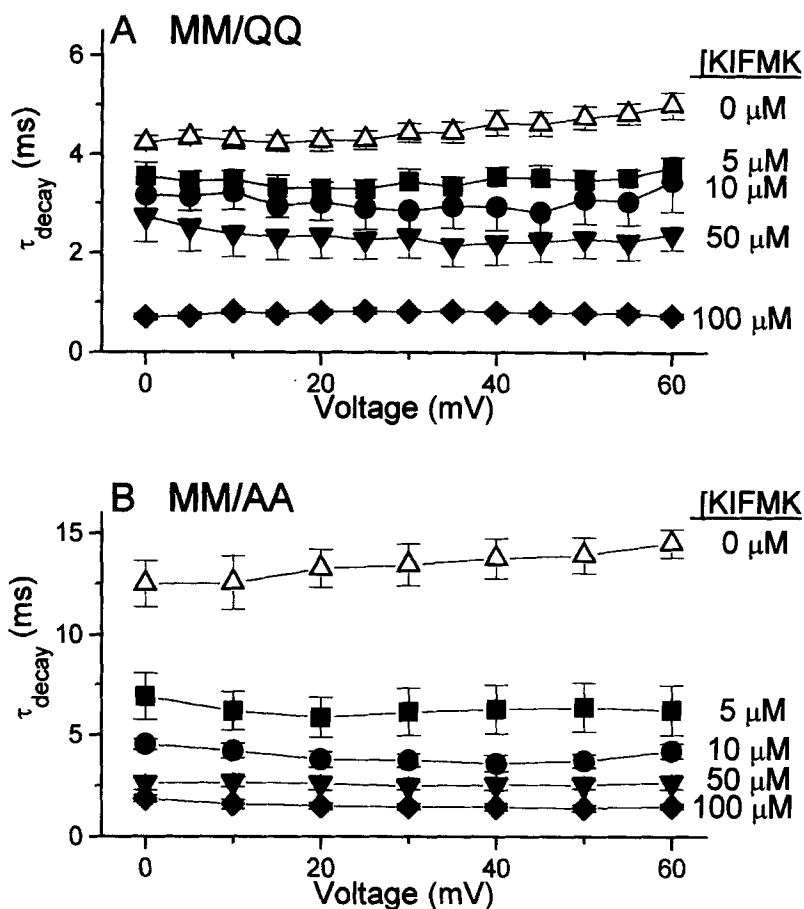


FIGURE 7. Effect of voltage and [KIFMK] on current decay. Time constants for current decay were fit to single exponential relaxation for both MM/QQ and MM/AA channels. Temperature, 14.7°C.

single mutations of the homologous residues in the rat skeletal muscle channel rSkM1 also affect τ_h (R.L. Barchi, personal communication), suggesting that both methionine residues contribute to inactivation of Na

channels. We can only speculate why alanine substitution has a greater effect on inactivation than glutamine substitution, but glutamine is more similar to the WT methionine with respect to its volume and surface area (Creighton, 1993), suggesting that alanine is too small to fulfill the roles played by the native methionine. In spite of the differences between MM/QQ and MM/AA in our study, the block by a pentapeptide postulated to mimic the inactivation process (Eaholtz et al., 1994) does not differ between the mutants. Furthermore, the peptide has a smaller effect on WT channels at a concentration that has a very large effect on the mutants. We will use these data to explore a gating model in which inactivation is caused by the binding of the cytoplasmic D3-D4 linker to a receptor that includes the S4-S5 loops.

In its most simplistic terms the inactivation model we are considering is analogous to the ball-and-chain mechanism of inactivation originally proposed for Na currents of squid axon (Armstrong and Bezanilla, 1977; Bezanilla and Armstrong, 1977). In this model, depolarization causes a movement of activation voltage sensors, and this conformational change increases the affinity of a binding site for the cytoplasmically tethered inactivation ball. Inactivation is then caused by the binding of the inactivation ball to its site, in or near the

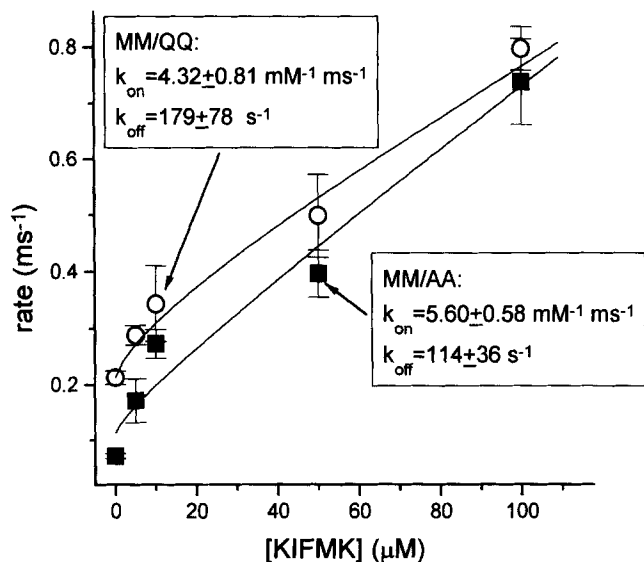


FIGURE 8. Decay rate of Na currents at +60 mV for MM/QQ and MM/AA, plotted against [KIFMK]. Decay rates are the inverse of the decay time constants in Fig. 7. The theoretical curves are best fits of Eq. 1 (METHODS) with the indicated parameters; 14.7°C.

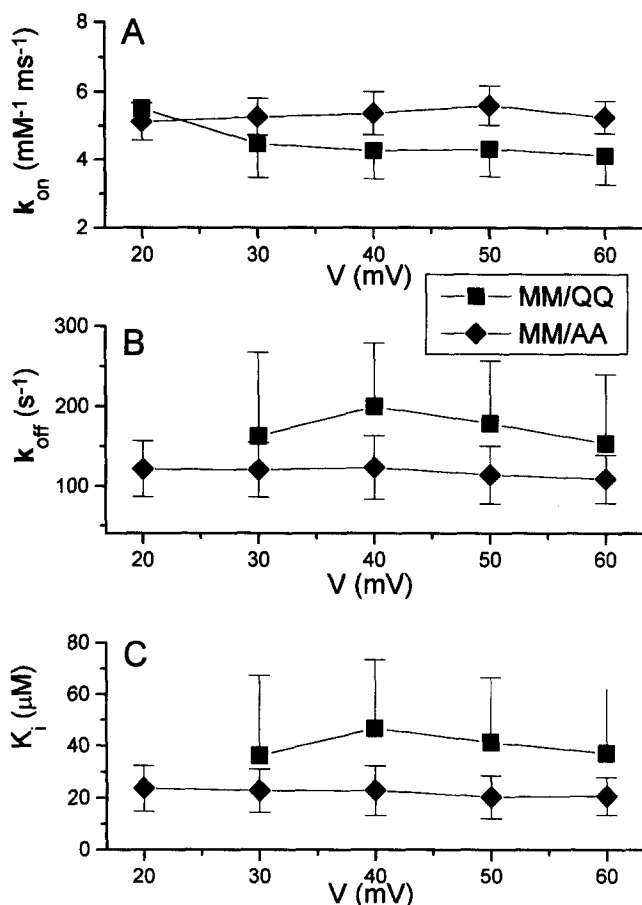


FIGURE 9. (A) Association rate constant for peptide block (k_{on}), obtained by fits to Eq. 1 (see Fig. 8). (B) Dissociation rate constant for peptide block (k_{off}) (C) Inhibition constant (K_i) calculated from k_{off}/k_{on} , plotted against test potential. 14.7°C.

mouth of the pore. This mechanism of inactivation has been demonstrated rather convincingly for certain K channels in the *Shaker* subfamily (Hoshi et al., 1990; Zagotta et al., 1990; Demo and Yellen, 1991; MacKinnon et al., 1993). However differences between inactivation of Na and K channels suggest that the ball-and-chain model needs a careful evaluation in Na channels (O'Leary et al., 1995). One variant of this model, the hinged lid hypothesis (West et al., 1992), proposes that the critical residues of the inactivation particle (i.e., the ball) are three consecutive amino acids near the middle of the D3-D4 linker, namely IFM (isoleucine-phenylalanine-methionine). Mutation of all three of these residues to glutamine (IFM/QQQ) abolishes fast inactivation (West et al., 1992). Single mutations of each of these residues show that the phenylalanine residue is the most important in supporting rapid inactivation kinetics (West et al., 1992; Hartmann et al., 1994). In the hinged lid model depolarization exposes a binding site for the D3-D4 linker, much as depolarization appar-

ently creates a binding site for the inactivation ball in K channels. If this model is correct, the hydrophobic nature of the IFM residues suggests that part of the receptor for the inactivation gate is also hydrophobic and could include the methionine residues in the S4-S5 loop of D4. The voltage dependence of inactivation could be explained in part, therefore, by a voltage dependence of the conformation of the S4-S5 loops in each domain as the S4 segments move in response to changes in membrane potential (Yang and Horn, 1995; Larsson et al., 1996; Mannuzzu et al., 1996; Yang et al., 1996). For example, depolarization could expose these two methionines so they would be able to bind either the IFM residues or other portions of the D3-D4 linker. Alternatively, the methionine mutations may simply alter the tertiary structure of the S4-S5 loop without contributing directly to the receptor for the IFM residues. A final possibility is that the methionine mutations destabilize inactivation without directly affecting the binding of a cytoplasmic inactivation particle. It is not possible to discriminate clearly among these possibilities until the nature of Na channel inactivation is better understood. For example, it has not been demonstrated convincingly that inactivation is the result of the binding of a cytoplasmic inactivation particle to the mouth of the channel. Nevertheless, we will explore some of the expected consequences of the possibility that these two methionines directly contribute to the receptor for the D3-D4 linker.

Assuming that inactivation is a consequence of the binding of a hypothetical inactivation particle, we can estimate the change in the affinity of this particle caused by a double methionine mutation. We use MM/QQ for this calculation because, unlike MM/AA, this mutation does not change the steepness of the h_{∞} curve compared with WT channels (Fig. 3 B). At negative voltages the equilibrium probability of a channel being inactivated is given by $1 - h_{\infty}$. MM/QQ causes a 13.8 mV depolarizing shift (ΔV) of h_{∞} , indicating that additional work (W_{shift}) must be done to cause this mutant to be inactivated. This work depends both on ΔV and on the steepness of the h_{∞} curve ($q_h = 5.16 e_0$) as follows (French et al., 1996):

$$W_{shift} = q_h \cdot \Delta V \cdot N_{Avog} \cdot e = 6.87 \text{ kJ/mol},$$

where $N_{Avog} = 6.022 \times 10^{23}$ charges/mol and $e = 1.602 \times 10^{-19}$ coulomb. For a binding reaction, W_{shift} is equivalent to the change in free energy of binding. The increase of the effective dissociation constant caused by the mutation MM/QQ is $\exp(W_{shift}/RT) = 16.8$, where R is the universal gas constant and T is the temperature in degrees Kelvin. Thus, WT channels have a 16.8-fold higher affinity for the postulated inactivation particle with its receptor than MM/QQ channels. The data for MM/QQ in Fig. 4 show that this change in affinity is

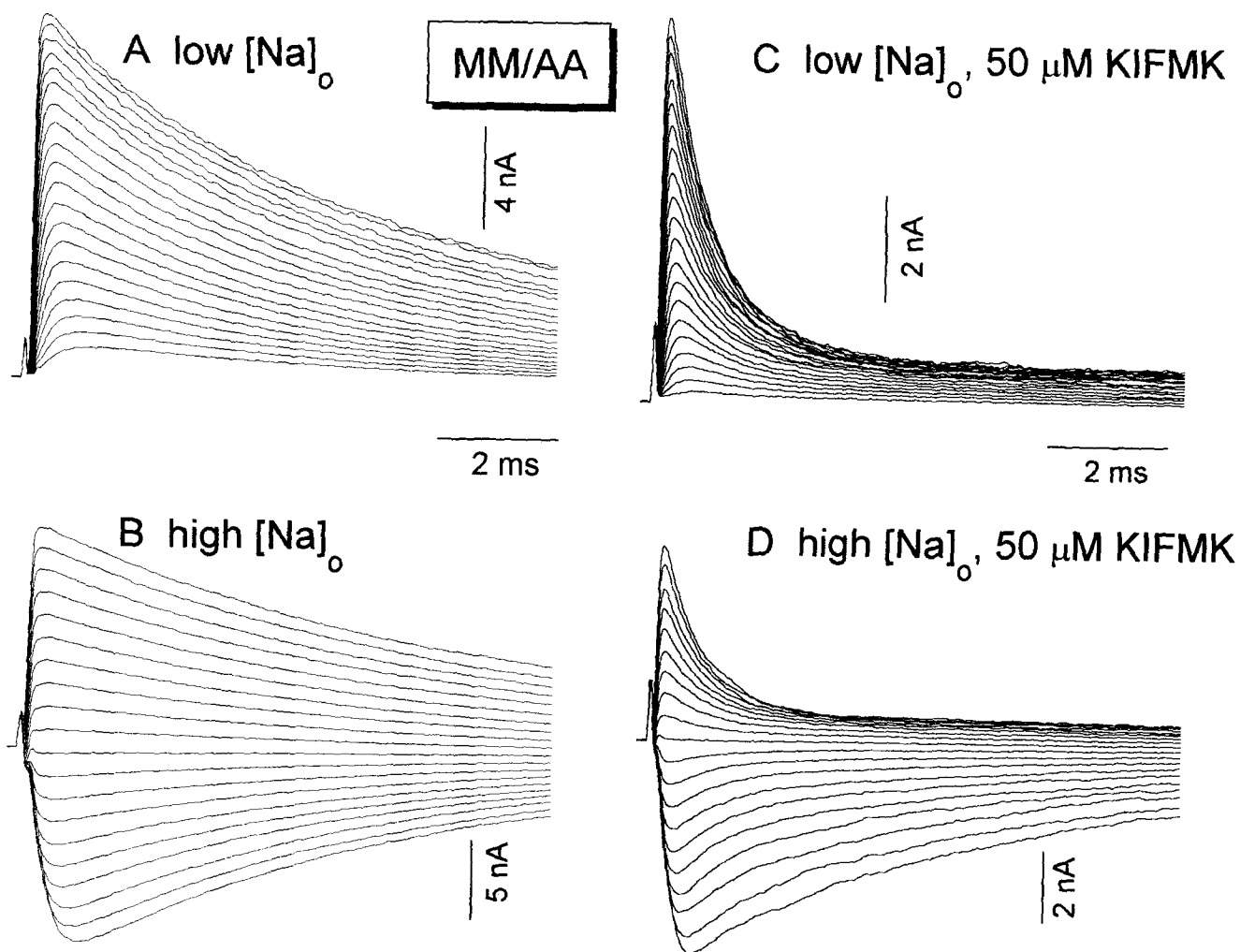


FIGURE 10. Effect of extracellular Na^+ concentration on currents of MM/AA, in the presence or absence of $50 \mu\text{M}$ KIFMK, applied through the patch pipet. The reversed-gradient pipet solution was used. The extracellular bath solution was exchanged from reversed-gradient (*top*) to standard solution (*bottom*) in each case. The pipet solution for the cell on the right contained $50 \mu\text{M}$ KIFMK. Depolarizations were from -35 to $+60$ mV in 5 mV increments; holding potential, -120 mV. Temperature, 21°C .

due both to an increase in the off rate constant for the binding reaction and a decrease in the rate constant for binding of the hypothetical inactivation particle.

Peptide Block and Inactivation Are Distinct Processes

It is therefore possible, formally, to describe the effects of these methionine mutations as a partial disruption of the binding site for the putative hinged lid. To explore this possibility in further detail, we used a hydrophilic peptide, KIFMK, postulated to mimic part of the natural inactivation gate and compete with it for its binding site (Eaholtz et al., 1994). If methionine mutations partially disrupt the binding site for the inactivation gate, then these mutants might also be expected to decrease the block by KIFMK. Although KIFMK, introduced cytoplasmically, appears to act as an open chan-

nel blocker, our data (Figs. 6–11) are not consistent with a model in which it competes with an endogenous inactivation particle for the same site, as discussed below.

KIFMK produces a concentration-dependent, voltage-independent increase in the rate of decay of both methionine mutants at positive membrane potentials, as if the peptide acts as an open channel blocker. This conclusion is supported by the concentration dependence of the decay rate (Fig. 8). The antagonistic effect of extracellular Na^+ also supports the idea that KIFMK is a pore blocker (Figs. 10 and 11). These data do not show, however, whether the peptide can occupy its pore-blocking site when the channel is closed. Some pore blockers can only occupy their binding sites when the channel is open (e.g., intracellular tetraethylammonium on squid axon K channels; Armstrong, 1966). We

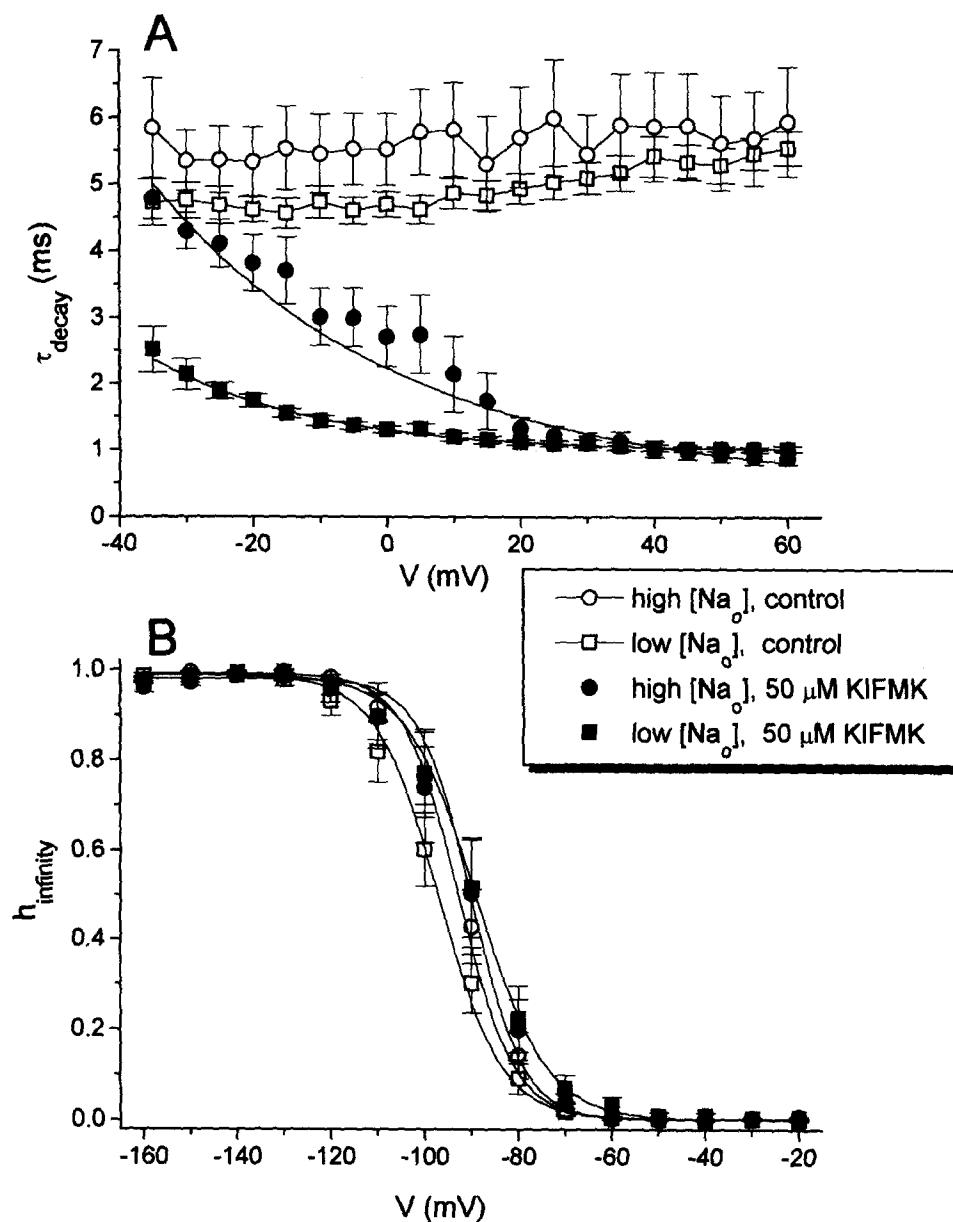


FIGURE 11. Effect of extracellular Na^+ concentration on decay kinetics and steady state inactivation of MM/AA, in the presence (filled symbols) or absence (open symbols) of 50 μM KIFMK. (A) The decay of currents, obtained as in Fig. 10, were fit to a single exponential relaxation with the indicated time constants. In the presence of 50 μM KIFMK (filled symbols) the data were fit by an exponential function (solid lines) indicating an effective charge of $1.05 \pm 0.05 e_0$ and $0.68 \pm 0.08 e_0$ for low and high $[\text{Na}]_o$, respectively. (B) Steady state inactivation induced by a 500-ms prepulse to the indicated voltage. Boltzmann fits to the data have (midpoints, slopes) of: $(-97.2 \pm 2.9 \text{ mV}, 3.93 \pm 0.16 e_0)$ for control cells in low $[\text{Na}]_o$; $(-91.8 \pm 2.4 \text{ mV}, 4.27 \pm 0.15 e_0)$ for control cells in high $[\text{Na}]_o$; $(-89.9 \pm 3.8 \text{ mV}, 3.84 \pm 0.27 e_0)$ for cells in low $[\text{Na}]_o$ with 50 μM KIFMK; $(-91.0 \pm 4.3 \text{ mV}, 4.19 \pm 0.15 e_0)$ for cells in high $[\text{Na}]_o$ with 50 μM KIFMK. Temperature, 21°C.

believe this to be the case for KIFMK in Na channels. First, unlike cytoplasmic tetraalkylammoniums (O'Leary and Horn, 1994), KIFMK does not reduce the peak Na current as it diffuses into the cell. Furthermore, KIFMK apparently has little effect on the equilibrium between closed channels that are either noninactivated or inactivated, because steady state inactivation of MM/AA channels is not influenced strongly by the peptide (Fig. 11 B). It is noteworthy that at potentials between -40 and $+60$ mV the inactivation kinetics of MM/AA channels are voltage independent, whereas the decay kinetics in the presence of 50 μM KIFMK are clearly voltage dependent between -40 and $+20$ mV (Fig. 11 A). Although most of this voltage dependence may be attributed to a *trans* effect of Na^+ ions, it is also possible that

the voltage dependence of channel opening plays a role at negative voltages, where the open probability is low. If a pore blocker can only block open channels, then a decrease in the fraction of open channels at more negative voltages would be expected to decrease the effect of the blocker on the decay of the current (Armstrong, 1966; Choi et al., 1993). Note that the inactivation gate, unlike the peptide, is capable of occluding both closed and open channels. For example, steady state inactivation is primarily a voltage-dependent reaction between closed-inactivated and closed-noninactivated conformations.

The nature of KIFMK block contrasts with results obtained with tetraalkylammoniums, which block closed and open channels indiscriminately, at least for WT hH1

(O'Leary and Horn, 1994). This discrepancy could be a consequence of the larger size of KIFMK, which is not able to access its binding site when the channel is closed. Another difference between KIFMK and other smaller cationic blockers (e.g., QX314: Gingrich et al., 1993; tetraalkylammoniums: O'Leary et al., 1994) is that the block by KIFMK has little voltage dependence at membrane potentials ≥ 0 mV (Figs. 7, 9, and 11 A) and therefore does not penetrate deeply into the pore. This lack of voltage dependence for KIFMK block is consistent with usual inactivation models having relatively voltage-independent rate constants between an open and inactivated state.

KIFMK is apparently a poor blocker of WT channels ($V_{\text{test}} = +40$ mV; Fig. 6 A). This contrasts with the expectations of a model in which the methionine mutations disrupt the binding site for both the natural inactivation gate and KIFMK. If this were the case, the peptide would block WT better than mutant channels. A conceivable explanation for the weak effect of KIFMK on WT channels is that the WT inactivation gate closes rapidly, perhaps before the peptide has a chance to bind to its site (e.g., see Eaholtz et al., 1994). To explore this idea, we can predict the expected effect of KIFMK assuming it has the same blocking kinetics on WT channels as estimated for both the methionine mutants. Fig. 9 gives approximate values of $5 \text{ mM}^{-1}\text{ms}^{-1}$ and 150 s^{-1} for k_{on} and k_{off} , respectively. After determining the rate constants underlying inactivation of WT channels at +40 mV in the absence of peptide (see METHODS), Eq. 1 can be used to determine the expected fast time constant for decay of WT current in the presence of $200 \text{ }\mu\text{M}$ KIFMK. For the WT data shown in Fig. 6 A the expected τ_{fast} is $458 \text{ }\mu\text{s}$, 42% smaller than the observed τ_{decay} of $796 \text{ }\mu\text{s}$. The calculation suggests, therefore, that the peptide blocking site is poorly accessible in WT channels. This conclusion may reflect a limitation of our simplistic kinetic model. For example, our model does not allow for concurrent peptide block and inactivation. However, it is possible that the peptide binding site is partially occluded in WT channels and that the methionine mutations cause

a conformational change that exposes it. For example, the methionine mutations may break the postulated link between the D3-D4 linker and the activation voltage sensor to which it is attached (O'Leary et al., 1995). If this link is broken in the methionine mutants, a decrease of voltage dependence of τ_{h} might also be expected, consistent with our data (Fig. 3 A).

Our data reveal several differences between peptide block and native inactivation. First, KIFMK block of MM/QQ and MM/AA is indistinguishable, in spite of the significant differences in inactivation kinetics observed between these two mutants. Second, the peptide blocks the methionine mutants better than WT channels, in spite of the fact that inactivation is considerably destabilized in the mutants. Third, peptide block is voltage dependent in a range where inactivation is not (Fig. 11 A). This could be due in part to the fact that the peptide, unlike the native inactivation gate, is apparently incapable of occluding closed channels. Finally, external Na^+ ions antagonize cytoplasmic peptide block, but have little effect on the inactivation of MM/AA in the absence of peptide. The accumulated results rule out the possibility that these methionine residues are the binding site for KIFMK. A similar conclusion was reached for mutations in S6/D4, which also affected inactivation without altering the binding of KIFMK (McPhee et al., 1995). The possibility remains, however, that the M¹⁶⁵¹M¹⁶⁵² residues contribute, either directly or indirectly, to the receptor for a cytoplasmic inactivation gate. It is likely, if the D3-D4 linker is the inactivation gate itself, that the receptor is extensive and consists of cytoplasmic regions of several or all four domains. It is known, for example, that a naturally occurring mutation in the S4-S5 loop of D3 (A1156T of the human skeletal muscle Na channel) is genetically linked to a myotonic disorder (McClatchey et al., 1992) and that this mutation destabilizes inactivation (Yang et al., 1994). It remains to be shown whether cationic pore blockers like KIFMK are useful probes of the inactivation receptor, or even if inactivation of Na channels occurs by a mechanism based on occlusion of the inner mouth of the ion-conducting pore by a tethered particle.

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