A Unique Role for the S4 Segment of Domain 4 in the Inactivation of Sodium Channels

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ABSTRACT Sodium channels have four homologous domains (D1-D4) each with six putative transmembrane segments (S1-S6). The highly charged S4 segments in each domain are postulated voltage sensors for gating. We made 15 charge-neutralizing or -reversing substitutions in the first or third basic residues (arginine or lysine) by replacement with histidine, glutamine, or glutamate in S4 segments of each domain of the human heart Na^+ channel. Nine of the mutations cause shifts in the conductance-voltage (G-V) midpoints, and all but two significantly decrease the voltage dependence of peak Na^+ current, consistent with a role of S4 segments in activation. The decreases in voltage dependence of activation were equivalent to a decrease in apparent gating charge of 0.5–2.1 elementary charges (e_0) per channel for single charge-neutralizing mutations. Three charge-reversing mutations gave decreases of 1.2–1.9 e_0 per channel in voltage dependence of activation. The steady-state inactivation (h_∞) curves were fit by single-component Boltzmann functions and show significant decreases in slope for 9 of the 15 mutants and shifts of midpoints in 9 mutants. The voltage dependence of inactivation time constants is markedly decreased by mutations only in S4D4, providing further evidence that this segment plays a unique role in activation-inactivation coupling.

KEY WORDS: sodium channels • gating • inactivation • S4 segments

INTRODUCTION

Sodium channels are responsible for the upstroke of the action potential in a variety of excitable cells, including neurons, skeletal muscle cells, and cardiac myocytes. Two voltage-dependent processes are observed in the gating of Na^+ channels: activation and inactivation. After a depolarization from the resting potential, channels initially respond by opening (activation). If the depolarization is maintained, the channels close to an inactivated state. Under voltage clamp conditions both the activation and the inactivation of Na^+ currents are voltage dependent (for reviews, see Armstrong, 1981; Bezanilla, 1985; Patlak, 1991; Keynes, 1994; Sigworth, 1994). Activation derives its voltage dependence from the ability of buried charges in the channel protein to move in response to changes in membrane potential (Sigworth, 1994). It is generally believed that inactivation derives most of its voltage dependence from being coupled to activation. In this coupling mechanism depolarization causes voltage-dependent activation gates to open, and the rate of inactivation increases as a consequence of these conformational changes. The molecular nature of this coupling is only poorly understood.

The predominant functional unit of the Na^+ channel is the α-subunit, a tandem arrangement of four homologous domains (D1-D4) in the primary sequence, each with six putative membrane spanning segments (S1-S6). Only one type of transmembrane segment, S4, is highly charged, containing 4, 5, 6, and 8 basic residues in D1-4, respectively. Every third amino acid in an S4 segment is positively charged (either arginine or lysine), and the intervening residues are hydrophobic. A plethora of experimental data indicate that the S4 segments of voltage-gated ion channels, including Na, K, and Ca channels with different numbers of positive charges on S4 segments, function as voltage sensors underlying activation (e.g., Stühmer et al., 1989; Auld et al., 1990; Liman et al., 1991; Lopez et al., 1991; McCormack et al., 1991; Papazian et al., 1991; Logothetis et al., 1992; Schoppa et al., 1992; Logothetis et al., 1993; Chahine et al., 1994; Fleig et al., 1994; Perozo et al., 1994; Papazian et al., 1995; Yang and Horn, 1995; Larsson et al., 1996; Mannuzzu et al., 1996; Yang et al., 1996).

Consistent with a coupling mechanism, there is a strong positive correlation between effects of S4 mutations on activation and inactivation (Stühmer et al., 1989; Papazian et al., 1991). This correlation has been observed for both the steepness of voltage dependence (i.e., the slopes of steady-state activation and inactivation) and the range over which the gates respond to changes of membrane potential (i.e., the midpoints of steady state activation and inactivation).

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One striking exception to this correlation was reported for naturally occurring mutations associated with the disease paramyotonia congenita (PC) (Chahine et al., 1994). In one PC mutant, the outermost basic residue of S4D4 of human skeletal muscle Na^+ channels (hSkM1), an arginine, is replaced by a cysteine (R1448C). This mutation has minor effects on activation, but profound effects on inactivation, slowing its rate at positive voltages, increasing the rate of recovery from inactivation, and abolishing the voltage dependence of the inactivation time constant, τ_h, at voltages between −40 and 0 mV. This is exactly the voltage range over which activation gates open and close, suggesting that S4D4 plays an important role in the coupling between activation and inactivation (Chahine et al., 1994). To test whether S4D4 differs from S4 segments in other domains, and whether other S4D4 residues play a similar role, we made a series of charge-neutralizing or -reversing substitutions for basic residues in the S4 segments of each domain of the human heart Na^+ channel (hH1; Gellens et al., 1992),[1] and examined their consequences on the gating of the channels. Our results show that S4D4 does play a unique role in both the kinetics and voltage dependence of the inactivation process in human heart sodium channels.

METHODS

Mutagenesis

Mutations were introduced into pSelect-1 (pS1) containing the hH1 cDNA template using high efficiency mutagenesis protocols (Promega Corp., Madison, WI) (Chen et al., 1992). All clones contained the point mutation C373Y (see text for mutation nomenclature). The synthetic antisense oligonucleotides used were as follows:

WT	(C373Y)	5′-GCGCTCCCAGTAGTCCTGCGT-3′
D1:R1Q	(R219Q)	5′-GACTCGGAAGGTCTGTAAGGCT-GAGA-3′
D1:R1H	(R219H)	5′-TCGGAAGGTGTGTAAGGCTGA-3′
D1:R3Q	(R225Q)	5′-TTTCAGGGCCTGGAGGACTCG-3′
D1:R3E	(R225E)	5′-TTTCAGGGCCTCGAGGACTCGG-3′
D2:R1Q	(R808Q)	5′-AGCCGGAAGGACTGCAGCACC-GAC-3′
D2:R1H	(R808H)	5′-GCGGAAGGAGTGCAGCACCGA-3′
D2:R3Q	(R814Q)	5′-CTTGAAGACCTGCAGCAGGCG-3′
D2:R3E	(R814E)	5′-AGCTTGAAGACCTCCAGCAGGCG-GAA-3′
D3:K1Q	(R1300Q)	5′-CGCAGTGACTGGATGGGGCCC-3′
D3:K1H	(R1300H)	5′-GCGTCCGCAGTGAATGGATGGGGC-CCATCT-3′
D3:R3Q	(R1306Q)	5′-GGACGGAGTGCTTGCAGCGTC-CGCA-3′
D3:R3E	(R1306E)	5′-CAGAGGACGGAGTGCTTC-CAGCGTCCGCAGTGA-3′
D4:R1Q	(R1623Q)	5′-CAGGCGGATGACTTGGAA-GAGCGTCGGG-3′
D4:R1H	(R1623H)	5′-CCAGGCGGATGACATGGAA-GAGCGTCGG-3′
D4:R3Q	(R1629Q)	5′-GCGGCCTATTTGGGCCAGGCG-3′
D4:R3E	(R1629E)	5′-GAGGATGCGGCCTATTTCGGCCAG-GCGGATGAC-3′

The mismatched nucleotides are indicated by underlining. All mutations were confirmed by dideoxynucleotide sequencing. The Qiagen column-purified DNA containing mutations was linearized by PvuI restriction enzyme digestion, and cRNA was synthesized using T7 RNA polymerase (Promega Corp.). All mutations were functional except D1:R3E for which we tested five independent clones.

Electrophysiology and Data Analysis

Stage V or VI oocytes were injected with synthetic cRNA encoding mutants of hH1. The oocytes were maintained at 19°C in a 50% diluted solution of Leibovitz's L-15 medium (Gibco BRL, Grand Island, NY) enriched with 15 mM HEPES, pH 7.6, 1 mM glutamine, and 50 mg/ml gentamycin. Oocytes were used for experiments after 2–8 d.

The Na^+ currents from RNA-injected oocytes were measured by 2-microelectrode voltage clamp at room temperature. The cells were impaled with <1 MΩ electrodes containing 3 M KCl and were voltage-clamped with a TEC-01C oocyte clamp (npi Electronics, Tamm, Germany). The bath solution for microelectrode experiments contained (in mM): 116 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES, pH 7.6. In some experiments we used an extracellular KCl concentration of 20 mM. This had little effect on the Na^+ currents. The holding potential was −100 mV. To partially remove the capacity transients from the records we subtracted currents obtained when choline was used as a substitute for the sodium in the bath solution. Data were acquired and analyzed using pCLAMP (Axon Instruments, Foster City, CA). Further analysis and plotting used Origin (MicroCal Software, Northampton, MA). At least three measurements were made for each value presented as mean ± SEM. The normalized conductance-voltage relationship for peak current and steady-state inactivation for each oocyte were fit to a standard Boltzmann function with two free parameters, a midpoint and a slope. The slope is expressed in Table I in units of elementary charge (e_0) expected for the gating charge of a first-order transition.

RESULTS

All of our mutations in S4 segments are in a background in which a native cysteine residue in D1 is substituted by tyrosine (C373Y). This mutation in the extracellular linker between S5 and S6 increases the tetrodotoxin sensitivity of cardiac channels by ~60–100-fold (Chen, et al., 1992; Kirsch et al., 1994). We denote hH1 with this C373Y mutation as the wild-type (WT) channel. The S4 mutations are designated according to domain and the position of the substituted basic residue. For example, D2:R3Q is a glutamine substitution for the third basic residue (arginine) in S4 of the second domain, counting from the extracellular (amino) end of S4.

Families of Na^+ currents of hH1 obtained by two-microelectrode recordings from *Xenopus* oocytes show the effects of substituting a glutamine for the outermost basic residue of S4 in each domain (Fig. 1). The currents of the WT and 4 S4 mutants show a typical pattern of volt-

[1] *Abbreviations used in this paper:* hH1, human heart sodium channel subtype 1; WT, wild-type.

TABLE I

Midpoints and Slopes of S4 Mutants

	G-V		h_∞	
	Midpoint (mV)	Slope (e_0)	Midpoint (mV)	Slope (e_0)
WT	−31.9 ± 0.79	5.6 ± 0.41	−71.2 ± 1.08	4.7 ± 0.27
D1:R1Q	−28.6 ± 1.96	5.0 ± 0.49	−72.0 ± 1.54	4.5 ± 0.24
D1:R1H	−34.2 ± 1.21	3.5 ± 0.14*	−80.8 ± 1.13*	4.9 ± 0.28
D1:R3Q	−27.2 ± 1.21*	3.9 ± 0.37*	−77.0 ± 2.44	3.6 ± 0.18*
D2:R1Q	−18.6 ± 1.80*	4.4 ± 0.19*	−63.0 ± 0.72*	4.3 ± 0.17
D2:R1H	−25.6 ± 0.64*	4.1 ± 0.12*	−71.2 ± 0.97	3.3 ± 0.27*
D2:R3Q	−31.5 ± 0.92	4.0 ± 0.15*	−80.8 ± 0.60*	3.6 ± 0.05*
D2:R3E	−16.1 ± 2.15*	3.7 ± 0.13*	−73.6 ± 2.01	3.7 ± 0.28
D3:K1Q	−42.3 ± 1.50*	5.1 ± 0.42	−92.2 ± 2.36*	3.9 ± 0.09*
D3:K1H	−31.6 ± 1.23	3.9 ± 0.16*	−84.3 ± 0.53*	4.2 ± 0.11
D3:R3Q	−26.0 ± 1.49*	3.5 ± 0.25*	−81.8 ± 1.14*	4.1 ± 0.21
D3:R3E	−34.0 ± 0.79	3.9 ± 0.14*	−93.0 ± 1.46 *	3.3 ± 0.19*
D4:R1Q	−29.6 ± 0.79	4.0 ± 0.40*	−66.5 ± 0.75*	2.7 ± 0.05*
D4:R1H	−34.3 ± 0.42*	4.2 ± 0.13*	−72.1 ± 1.56	2.6 ± 0.06*
D4:R3Q	−23.3 ± 0.32*	4.0 ± 0.09*	−128.7 ± 0.07*	2.1 ± 0.07*
D4:R3E	−25.2 ± 0.99*	4.4 ± 0.14*	−74.9 ± 2.20	3.7 ± 0.17*

Each entry is the mean ± SEM obtained from individual fits of G-V and h_∞ data for each cell (n = 3–8). Starred entries are significantly different from the WT (t test, $P < 0.05$).

age-dependent activation and inactivation. The most noticeable effect of these mutations is the slowed inactivation of D2:R1Q and D4:R1Q.

The peak currents for all of the S4 mutants are plotted in the form of normalized conductance-voltage (G-V) curves (Fig. 2). The data points for each mutant were fit to a Boltzmann relationship with parameters given in Table I. S4 mutations of basic residues have two main effects on G-V curves, shifts in the midpoints and decreases in slope. 2 out of the 15 S4 mutations, one in D3 and one in D4, cause hyperpolarizing shifts of the G-V curves, 7 others cause depolarizing shifts and the remaining 6 mutants have no significant alteration of the G-V midpoint. All but 2 mutations decrease the voltage dependence of peak currents, as given by the G-V slopes. Although limiting slope factors better reflect the true gating charge, we have estimated apparent gating charge from the slopes of Boltzmann fits. Using this method, the decreases in voltage dependence are equivalent to a decrease in apparent gating charge of 0.5 to 2.1 elementary charges (e_0) per channel for the mutants that changed a single basic residue (lysine or arginine) to a neutral glutamine. We also made 3 mutations that changed a basic residue to negatively charged glutamate (D1:R3E was nonfunctional). D2:R3E, D3:R3E, and D4:R3E cause decreases in G-V slopes equivalent to decreases of apparent gating charge of 1.9, 1.7, and 1.2 e_0 per channel, respectively. These data strongly suggest that the S4 segments of all 4 domains play a role in the voltage-dependent activation of the channels, consistent with their postulated roles as voltage sensors.

Besides effects on steady-state activation, these S4 mutations also influence steady-state inactivation, obtained by measuring the decrease in peak current caused by 500-ms prepulses. The h_∞ curves show alterations in the best fit parameters for both slope and midpoint without a significant effect on the ability to fit the

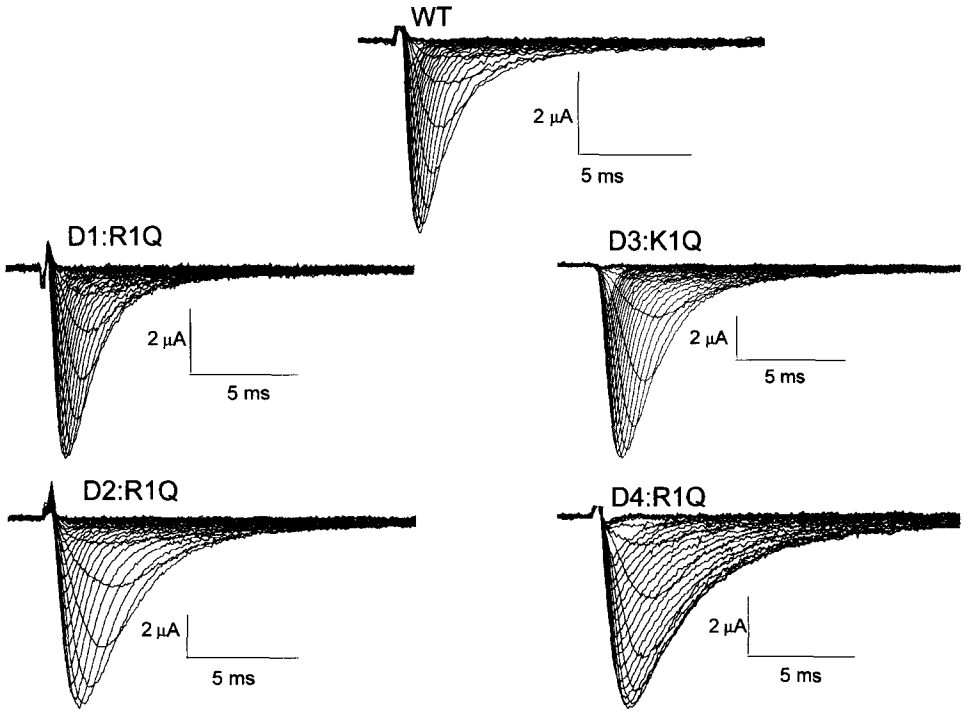

FIGURE 1. Effect of S4 mutations on Na^+ currents. Na^+ currents of hH1 activated by depolarizations in 5-mV increments from −80 to +50 mV. Currents obtained by two-microelectrode recording. Capacity transients partially removed by subtraction of records obtained with choline-Ringer.

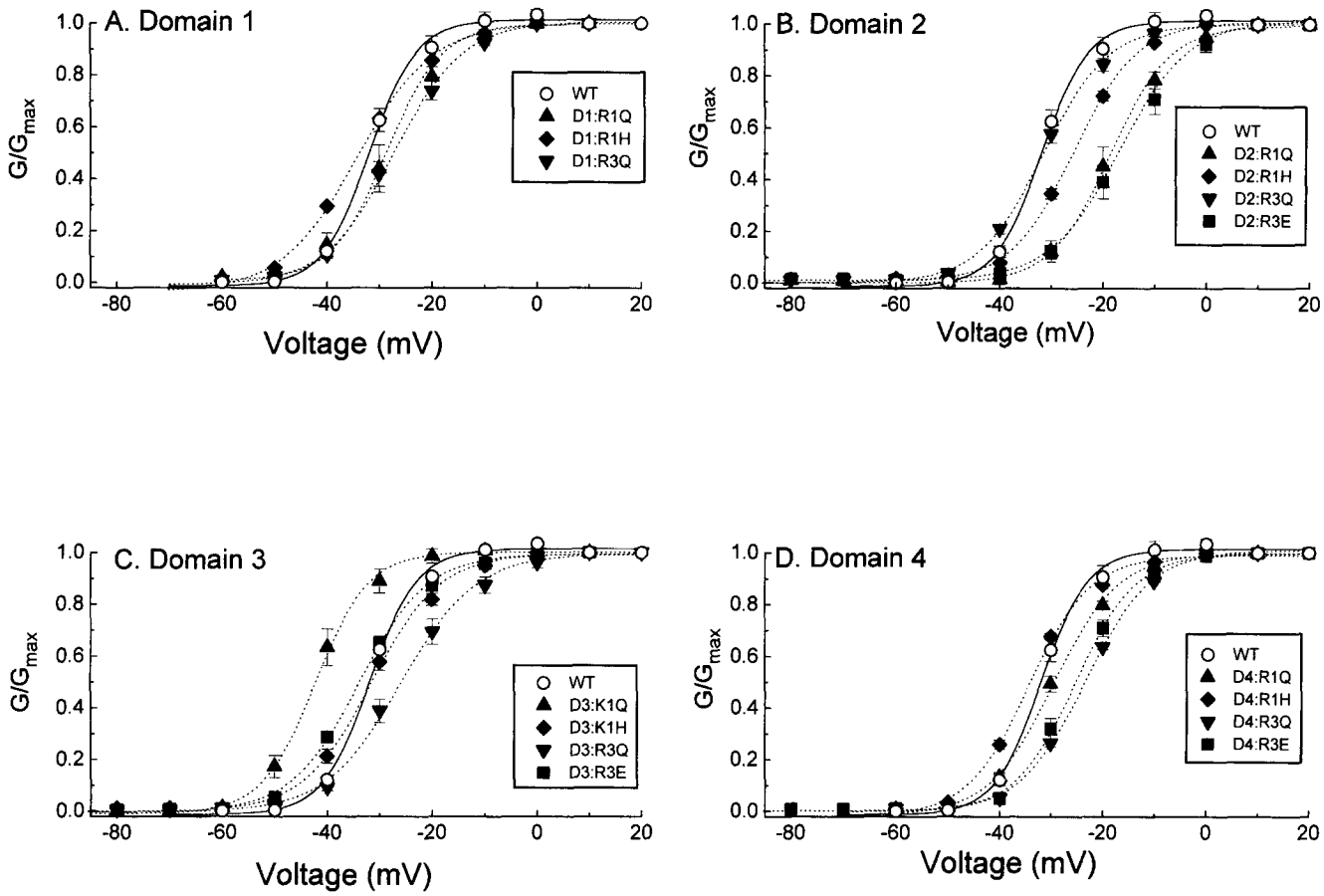

FIGURE 2. Effects of S4 mutations on peak activation. Normalized G-V curves fit by standard Boltzmann equation. Parameters for fits in Table I.

curves to a single-component Boltzmann function (Fig. 3 and Table I). Whereas S4 mutations primarily cause depolarizing shifts of G-V curves, they generally produce hyperpolarizing shifts of h_∞ curves. Only two mutants, D2:R1Q and D4:R1Q, show a depolarizing shift. The shifts of h_∞ curves are especially obvious in D3 and D4, most notably D4:R3Q whose h_∞ midpoint is 57.5 mV more hyperpolarized than that of WT (Fig. 3). Significant decreases in slope are also obtained in 9 of 15 mutants. The incidence and magnitude of slope changes are greatest for S4 mutants in D4 (Table I). Surprisingly, the D4 mutant with the biggest decrease in positive charge, D4:R3E, has the steepest h_∞ slope.

There is a striking difference between the behavior of the time constants of inactivation, τ_h, obtained from the decay of current after a depolarization (Fig. 1), for the S4 mutants in D4 compared to those of D1-D3 (Fig. 4). The τ_h values for S4D4 mutants are larger than the τ_h^{WT} value for three out of the four S4 mutants in D4. Furthermore, the voltage dependence of τ_h is almost completely abolished for these three mutants, especially for voltages more negative than 0 mV where the τ_h^{WT} values have the steepest voltage dependence. A moderate slowing of τ_h and a slight decrease in its voltage dependence is also observed for the mutant D2:R1Q, but this may be explained in part as a depolarizing shift in the τ_h versus voltage relationship; D2:R1Q causes the largest depolarizing shift in inactivation of all the S4 mutants and the second largest depolarizing shift in activation (Table I). One S4D4 mutant that has τ_h values close to those of the WT is D4:R3Q. However, with respect to inactivation this mutation is exceptional in other ways. Besides having the lowest voltage dependence of steady-state inactivation of all the clones we examined, D4:R3Q causes large shifts in G-V and h_∞ curves in the opposite directions: an 8.6-mV depolarizing shift of G-V and a 57.5-mV hyperpolarizing shift of h_∞ (Table I). In the voltage range where we measured τ_h (−50 to +30 mV), these large shifts would tend to increase the rate of channel opening and the rate of inactivating from the open state. Both effects would contribute to a fast macroscopic rate of inactivation (Aldrich et al., 1983; Vandenberg and Horn, 1984).

DISCUSSION

S4 segments are found in all members of the superfamily that includes K^+, Ca^{2+}, and Na^+ channels. It is gen-

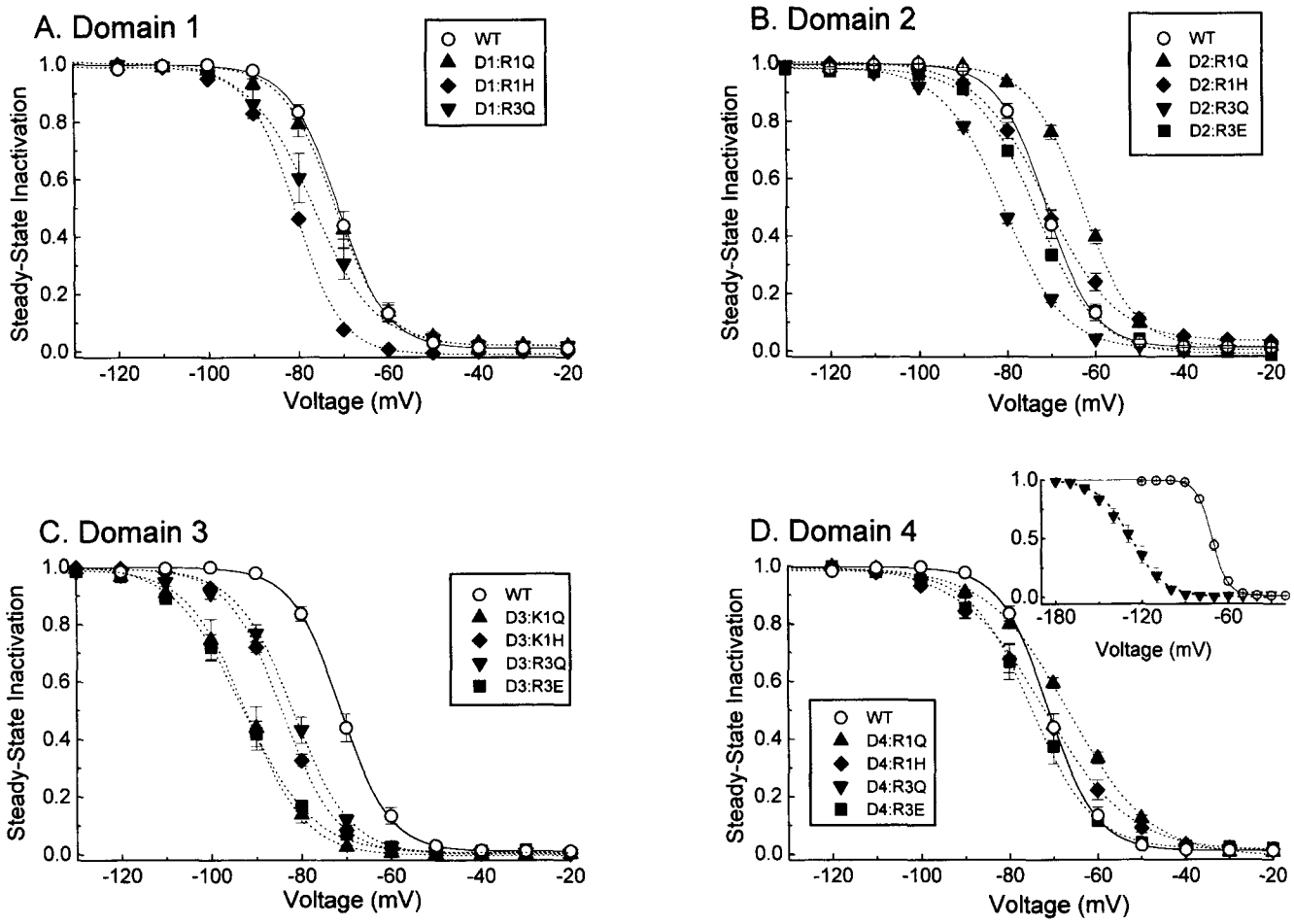

FIGURE 3. Effects of S4 mutations on steady-state inactivation. Steady-state inactivation assayed at −10 mV induced by 500-ms conditioning pulse at the indicated voltage followed by a 0.1-ms prepulse to −100 mV. Holding potential, −100 mV. Curves are fit to a standard Boltzmann equation (Table I). Note that the data for D4:R3Q are plotted in an inset to panel *D*.

erally believed that S4 segments act as voltage sensors for activation, and our data are consistent with this view. Each of the 15 S4 mutants in this study reduces the net positive charge of an S4 segment, and 13 of these mutants cause a significant decrease in the voltage dependence of activation, as estimated from decreases in the slopes of G-V curves. These results indicate effects on activation gating. In addition, most of the S4 mutants show alterations in the slopes and midpoints of steady-state inactivation. S4 mutations in all 4 domains have effects on both activation and inactivation. By contrast with the typically large effects of these mutations on steady-state inactivation, inactivation time constants, measured from the current decay during activating depolarizations, are not strongly affected by S4 mutations, except in D4. S4D4 mutations cause dramatic increases in τ_h, along with an abolition of its voltage dependence, especially in the voltage range where channels activate. This shows that S4D4 plays a predominant role in inactivation kinetics and in the coupling between activation and inactivation. Large effects of mutations of D4:R1 were previously observed for the skeletal muscle isoforms, hSkM1 and rSkM1 (Chahine et al., 1994). Our data suggest that S4D4 in hH1, which has much less primary sequence identity compared to the two previously studied skeletal muscle Na channels (Trimmer et al., 1989), plays a similar role in inactivation.

Activation

The first direct evidence for the possibility that S4 segments act as voltage sensors was a study in which the total reduction of positive charge in D1 and D2 of neuronal Na^+ channels correlated well with the reduction in the slope of G-V curves (Stühmer et al., 1989). This correlation was, in general, independent of the location of the mutated basic residues. Subsequent experiments on K^+ channels failed to show such a simple correlation (e.g., Liman et al., 1991; Papazian et al., 1991), calling into question whether the effects of S4 mutants in Na^+ channels were due entirely to the change of charge. Furthermore, charge-conserving or neutral mu-

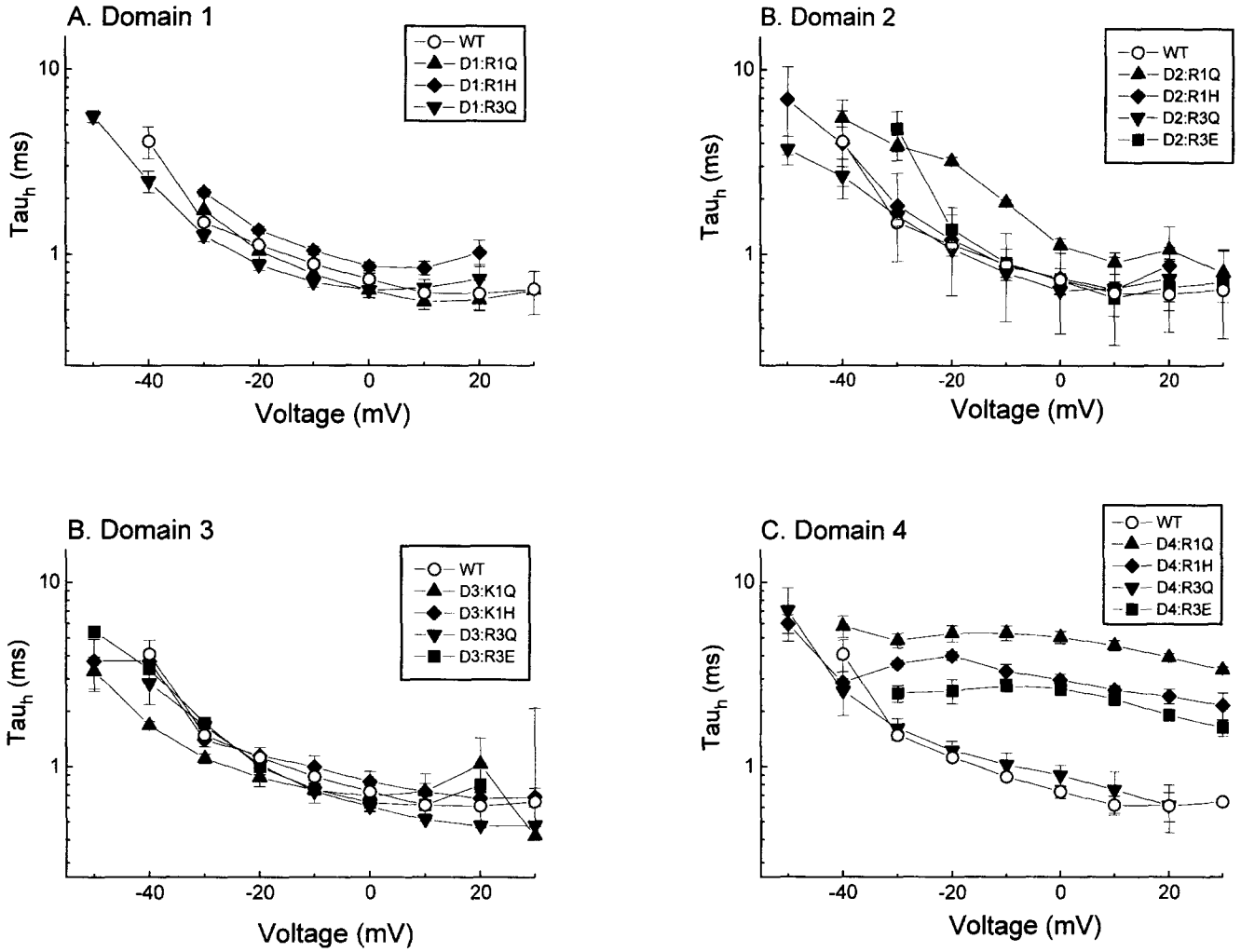

FIGURE 4. Effect of S4 mutants on τ_h. τ_h measured from single exponential relaxation of current after the peak.

tations in S4 and elsewhere can reduce the slope of G-V curves in both Na^+ and K^+ channels (Auld et al., 1990; Lopez et al., 1991; McCormack et al., 1991; Schoppa et al., 1992; Perozo et al., 1994). A more general interpretation of all these data is that S4 segments contribute to activation gating, and that S4 mutations can disrupt activation (Sigworth, 1994). This interpretation does not require a strict correlation between the total number of basic residues and the G-V slopes. Our data conform to this idea. For example, glutamate substitutions in D3 and D4 cause less of a reduction of G-V slope than glutamine substitutions, in spite of their greater reduction of net positive charge in the S4 segment (Table I). Furthermore, only a subset of the basic residues in S4 segments move through the electric field during changes of membrane potential (Larsson et al., 1996; Yang et al., 1996) showing that individual residues have unique contributions to voltage sensing.

Inactivation

S4 mutations in each domain have effects on inactivation, as evidenced by shifts and decreased slopes in h_∞ curves (Fig. 3, Table I). Many of these effects can be explained, at least in part, by a coupling of inactivation to activation. If S4 mutations primarily affect the conformations of states in the activation pathway, these conformations could have effects on the state of the inactivation gate. This explanation is in accordance with current hypotheses on coupling of inactivation to activation in Na^+ channels (Patlak, 1991; Keynes, 1994; Kuo and Bean, 1994; O'Leary et al., 1995).

The effects of S4D4 mutations on inactivation differ strikingly from those in other domains. First, they produce the largest reductions of the h_∞ slopes. Second, mutations of both D4:R1 and D4:R3 profoundly affect τ_h, increasing its value and decreasing its voltage de-

pendence. The decrease in voltage dependence is especially extreme in the voltage range where the G-V curves are steeply voltage dependent, evidence for uncoupling of activation and inactivation. The data show not only that S4D4 has a predominant role in inactivation gating, but also that this role pertains for residues other than the outermost arginine. In fact our preliminary data show inactivation effects of S4D4 mutations at several positions between D4:R1 and D4:R8 (unpublished data), suggesting that the entire transmembrane segment plays a role in the inactivation process. If the inactivation gate is cytoplasmic, as proposed previously (Stühmer et al., 1989; Moorman et al., 1990; Patton et al., 1992; West et al., 1992; Hartmann et al., 1994), the consequences of mutations near the extracellular part of S4D4 may be due to the altered movement of the entire S4 segment, the bottom of which could interact directly or indirectly with a cytoplasmic inactivation gate (Tang et al., 1996).

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