Outward stabilization of the voltage sensor in domain II but not domain I speeds inactivation of voltage-gated sodium channels

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Sheets MF, Chen T, Hanck DA. Outward stabilization of the voltage sensor in domain II but not domain I speeds inactivation of voltage-gated sodium channels. Am J Physiol Heart Circ Physiol 305: H1213–H1221, 2013. First published July 26, 2013; doi:10.1152/ajpheart.00225.2013.—To determine the roles of the individual S4 segments in domains I and II to activation and inactivation kinetics of sodium current (I\textsubscript{Na}) in Na\textsubscript{v}1.5, we used a tethered biotin and avidin approach after a site-directed cysteine substitution was made in the second outermost Arg in each S4 (DI-R2C and DII-R2C). We first determined the fraction of gating charge contributed by the individual S4’s to maximal gating current (Q\textsubscript{max}), and found that the outermost Arg residue in each S4 contributed ~19% to Q\textsubscript{max} with minimal contributions by other arginines. Stabilization of the S4’s in DI-R2C and DII-R2C was confirmed by measuring the expected reduction in Q\textsubscript{max}. In DI-R2C, stabilization resulted in a decrease in peak I\textsubscript{Na} of ~45%, while its peak current-voltage (I-V) and voltage-dependent Na channel availability (SSI) curves were nearly unchanged from wild type (WT). In contrast, stabilization of the DII-R2C enhanced activation with a negative shift in the peak I-V relationship by ~7 mV and a larger ~17 mV shift in the voltage-dependent SSI curve. Furthermore, its I\textsubscript{Na} decay time constants and time-to-peak I\textsubscript{Na} became more rapid than WT. An explanation for these results is that the depolarized conformation of DII-S4, but not DI-S4, affects the receptor for the inactivation particle formed by the interdomain linker between DIII and IV. In addition, the leftward shifts of both activation and inactivation and the decrease in G\textsubscript{max} after stabilization of the DII-S4 support previous studies that showed β-scorpion toxins trap the voltage sensor of DII in an activated conformation. Sodium channels; gating charge; inactivation; voltage sensor; stabilization; β-scorpion toxin

Because voltage-gated sodium channels are intrinsically involved in the conduction of action potentials in the heart and the nervous system, they have become targets of both therapeutic medications and for nature’s toxins. One family of Na channel toxins, β-scorpion toxin (a site-4 toxin), has been shown to target the voltage sensor (S4) in the second domain (DII) of the Na channel (for review see 26). Voltage-gated Na channels contain a single α subunit of ~2,000 amino acids organized into four different domains, in contrast to the four separate, homologous domains of most voltage-gated potassium (K) channels that are thought to contribute to channel activation (2). Because all of the four domains are different from one another in the Na channel, each domain may make a unique contribution to channel kinetic transitions. For example, the outward movement of the fourth transmembrane segment in the fourth domain (DIV-S4) of the Na channel during a step depolarization has been associated with the Na channel kinetic transition from an open to a fast-inactivated state (8, 11, 21, 22, 43, 44). Furthermore, the movement of the DIV-S4 during a depolarization is slower than the other S4’s (10, 37), and it contributes a large component (nearly 32%) to the maximal gating charge (Q\textsubscript{max}) of the Na channel (10, 39) with the outermost extracellular arginine (DIV-R1) estimated to contribute the most charge, ~20%, to Q\textsubscript{max}, while DIV-R2 and R3 contribute the remaining 12% (39).

In contrast, a fluorescence-labeling study has shown that the DIII-S4 translocates very rapidly during a step depolarization consistent with a role in the activation process leading to channel opening (10). DIII-S4 also appears to have a role in closed-state inactivation as reflected by changes in the voltage-dependent steady-state Na channel availability curve after stabilization of the DIII-S4 by biotinylation of its third outermost charged residue (DIII-R3C) with MTSEA-biotin (36). Somewhat surprising, outward stabilization of the DIII-S4 produced only minimal changes in the peak I\textsubscript{Na}-voltage (I-V) relationship other than a decrease in maximal conductance, G\textsubscript{max} (36). Similar to the DIV-S4, the gating charge contributed by the DIII-S4 to Q\textsubscript{max} was ~30% with the outermost Arg of its S4 making the greatest contribution (35). Additional studies on the DII-S4 have implicated it in controlling recovery from fast inactivation (38). Together these data support a dual role for DIII-S4 in both activation and fast inactivation of Na channels.

Less information is known about the role of the DI and DII voltage sensors. Early data in Xenopus oocytes in which DI-S4 residues were neutralized supported a contribution of their basic residues to channel activation (42). In addition, fluorescence labeling has demonstrated that these sensors move rapidly upon step depolarizations consistent with a role in activation leading to channel opening (10). However, the complex actions of the site-4 toxin, β-scorpion toxin (1, 20), on Na channel kinetics (5, 6) suggest that DII may affect more than only channel activation. We hypothesized that stabilization of the individual S4’s in either DI or DII may contribute to both channel activation and inactivation. In our present study we used a tethered biotin and avidin approach to stabilize the S4’s in DI and II in an outward, depolarized position to better understand their contribution to Na channel gating. This technique was first used with colicin (27, 41), and then in KvAP channels (19), whereby a site-directed cysteine substitution was made in an S4 segment, and then biotinylated with a sulfhydryl-reactive agent followed by exposure to avidin (19). Because this technique has been previously successful in the study of the S4’s in DIII and DIV of Nav1.5 (36), we applied it to study of DI and DII.
We first determined the fraction of gating charge contributed by basic residues in the S4’s of DI and II using lidocaine and a methodology similar to that previously used for the study of DIII and IV (35, 39), and found that the outermost Arg in each S4 was responsible for most of the charge contributed by those domains (each ~20%). Stabilization of each individual S4 in DI and DII was accomplished by the extracellular application of either MTSEA-biotinacip (or MTSEA-biotin) with avidin in mutant channels in which the second outermost Arg residue in each S4 of the two domains was substituted by a Cys (DI-R2C or DII-R2C) where confirmation of stabilization was determined by an appropriate reduction in Qmax. After stabilization of DI-S4, the primary effect was a decrease in peak Isa of ~45% compared with DI-R2C in control. The kinetic parameters of DI-R2C after stabilization were nearly unchanged compared with wild-type (WT) controls. The Isa decay time course, the half-point (V1/2) of activation, and parameters of the steady-state voltage-dependent availability curve were unchanged from control. In contrast, stabilization of the DII-S4 enhanced activation with a negative shift in the $V_{1/2}$ of the peak I-V relationship by ~7 mV while the $V_{1/2}$ of voltage-dependent availability curve was shifted further leftward by ~17 mV compared with WT. In addition, its decay time constants and time-to-peak of Isa in response to step depolarizations were more rapid.

METHODS

Molecular constructs. The cDNA for Nav1.5 (hH1a) was kindly provided by H. Hartmann and A. Brown (16). Four-primer PCR (17, 18) or QuickChange (Agilent Technologies, Santa Clara, CA) was used to substitute a cysteine for the native residue at one or more sites in the channel as follows [using the numbering system of hH1 (25)].

1. The second (DI-R2) or the third (DI-R3) outermost arginine in the S4 of DI at positions 222 and 225, respectively, were mutated to a Cys. 2. The first (DII-R1), second (DII-R2), or third (DII-R3) outermost arginine at positions 808, 811, or 814, respectively, were mutated to a Cys. These five mutations were abbreviated DI-R2C, DI-R3C, DII-R1C, DII-R2C, and DII-R3C, respectively. The entire inserts containing the mutated sites were confirmed by sequencing. In addition, all constructs (including WT) contained a Tyr substituted for the Cys in the external vestibule of the pore at position 373 (C373Y) to increase the sensitivity to block by tetrodotoxin (TTX) or saxitoxin.

For determination of peak Na kinetics, all constructs (including WT) contained a Tyr substituted for the Cys in the external vestibule of the pore at position 373 (C373Y) to increase the sensitivity to block by tetrodotoxin (TTX) or saxitoxin.

Na kinetic parameters. This allowed for comparison of DI-R2C and DII-R2C channels, but in a preferable modification of the DI-R2C and DII-R2C channels, but in a shorter time period.

For determination of peak I-V relationships step potentials were from ~130 to 20 mV for 50 ms at 1 Hz. Data were capacity corrected using four to eight scaled current responses recorded from voltage steps between ~150 mV and ~180 mV, and leak resistance was calculated as the reciprocal of the linear conductance between ~180 mV and ~110 mV. Peak Isa was taken as the mean of four data samples clustered around the maximal value of data digitally filtered at 5 kHz and leak-corrected by the amount of the calculated time-independent linear leak. Peak Isa, as a function of potential was fit with a Boltzmann distribution:

$$I_{Na} = \frac{(V_i - V_{rev})}{1 + \exp \left( \frac{V_i - V_{rev}}{s} \right)}$$

where Isa is the peak current in response to a step depolarization, $V_i$ is the test potential and the fitted parameters were $V_{rev}$, the half-point of the relationship; $s$, the slope factor (in mV), and $V_{rev}$, the reversal potential which was set to 40 mV. For comparison between cells, data

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were normalized to the maximum peak conductance ($G_{\text{max}}$) for each cell.

Steady-state voltage-dependent Na channel availability was evaluated from measurements of peak $I_{\text{Na}}$ during voltage steps to 0 mV after conditioning for 500 ms over a range of potentials from −150 to −40 mV at a cycle frequency of 0.4 Hz. Peak sodium currents as a function of conditioning potential were fitted with a Boltzmann distribution:

$$I_{\text{Na}}/I_{\text{max}} = 1/[1 + \exp (V_c - V_{1/2}/s)]$$

(2)

where $I_{\text{Na}}$ is the peak current after the conditioning pulse, $I_{\text{max}}$ is the maximal $I_{\text{Na}}$, $V_c$ is the conditioning potential, $V_{1/2}$ is the half-point of the relationship, and $s$ is the slope factor (in mV).

Decays of $I_{\text{Na}}$ traces recorded during peak $I-V$ relationships were trimmed from the front of the trace until its decay was clearly present, and then fit with a single-exponential equation:

$$I_t = \text{Amplitude of } I_{\text{Na}} \exp (-t/\tau) + \text{baseline}$$

(3)

where $I_t$ is the magnitude of current at time $t$, $\tau$ is the fitted single-exponential time constant, amplitude of $I_{\text{Na}}$ is the initial value of $I_{\text{Na}}$, and baseline is a fitted constant.

$I_t$ voltage-clamp protocols contained four repetitions at each test potential for 25 ms at 1 Hz. All $I_t$ were leak corrected by the mean of 2–4 ms of data usually beginning 8 ms after the change in test potential and capacity corrected using 4–8 scaled current responses to steps between the $V_{\text{tp}}$ and −170 or −180 mV taken immediately before and after the four test steps. $Q-V$ relationships were fit with a simple Boltzmann distribution:

$$Q = Q_{\text{max}}/[1 + \exp (V_{1/2} - V)/s]$$

(4)

where $Q$ is the charge during depolarizing step, $V_{1/2}$ is the test potential, the fitted parameters are as follows: $Q_{\text{max}}$ is the maximum charge, $V_{1/2}$ is the half-point of the relationship, and $s$ is the slope factor (in mV). Fractional $Q$ was calculated as $Q/Q_{\text{max}}$.

Data were analyzed and graphed using Matlab (The Mathworks, Natick, MA) and Origin (OriginLab, Northampton, MA). Unless otherwise specified summary statistics are expressed as means ± SD. Figures 1–4 show means ± standard error of the mean (SE). Paired $t$-tests and ANOVA (Tukey) were used to determine statistical significance at the $P < 0.05$ level.

RESULTS

Gating charge contributions of the individual S4’s in DI and DII to $Q_{\text{max}}$ in NaV1.5. Before designing experimental protocols for stabilization of the individual S4’s in DI and DII, we first determined the contribution of the individual S4’s in the two domains to the total gating charge using a general method previously published for investigation of the S4’s in DIII and IV (35, 39) where individual arginine residues were substituted with cysteines, expressed in tsA201 cells, and the maximum gating charge in the presence and absence of the local anesthetic, lidocaine, was measured. In WT NaV1.5 lidocaine reduces $Q_{\text{max}}$ by 38% by stabilization of the S4’s in domains III and IV (34). Thus, if a basic residue in the DI-S4 or DII-S4 were to make a large contribution to the overall $Q_{\text{max}}$ and that extracellular MTSEA-biotin could stabilize the S4’s in domains III and IV would be greater than for WT. Consequently, exposure to lidocaine would reduce charge by more than the 38% found for WT. Conversely, if an individual basic residue made no or little contribution to $Q_{\text{max}}$, then neutralization of that charge would have no effect on the magnitude of $Q_{\text{max}}$, and lidocaine would reduce $Q_{\text{max}}$ by the same amount found for WT. Beginning with the most straightforward assumption that overall $Q_{\text{max}}$ equals the sum of the individual contributions from each charged residue, we used the following equation to describe the relationship between the relative contribution from a specific residue in either DI or II to total gating charge and the fractional reduction in $Q_{\text{max}}$ of the mutant in lidocaine:

$$\text{relative contribution of a residue to } Q_{\text{max}}$$

$$= 1 - (0.38/\text{fractional reduction of } Q_{\text{max}} \text{ in mutant by lidocaine})$$

where 0.38 represents the decrease in $Q_{\text{max}}$ by lidocaine in WT NaV1.5.

Figure 1 and Table 1 show the gating charge data for mutant channels in which arginines in DI (R2C, R3C) and DII (R1C, R2C, R3C) were substituted with Cys. Previously published results have shown that the outermost Arg in DI (DI-R1C) contributed ~19% to $Q_{\text{max}}$ (34). The outermost Arg in DI (DI-R1C) also makes a large contribution (16%) to $Q_{\text{max}}$ while the second-outermost Arg in DII (DII-R2C) contributes only 3% to overall $Q_{\text{max}}$. No contribution could be detected for DI-R2C, DI-R3C, or DII-R3C. It is possible that charge rearrangement occurred in the mutated channels; however, the sum of the charge contributions from each of the four domains approximated a value of 1 (DI ~19%, DII ~19%, DIII ~30% and DIV ~31%) (35, 39), and support the assumption of adding the contributions of individual charged residues. In the remaining experiments DI-R2C and DII-R2C channels were used because those residues make little or no contribution to $Q_{\text{max}}$, thereby permitting confirmation of stabilization of each S4 by measuring a relatively large decrease in gating charge after biotin (or biotincap) and avidin.

Stabilization of the individual S4’s in DI and II. We used a variation of the tethered biotin and avidin approach (19, 41), whereby a site-directed cysteine substitution was made in the S4 segment in either DI or II, and the cell depolarized to allow biotinylation with a sulphydryl-reactive agent followed by exposure to avidin. Binding of the large protein, avidin, should prevent resetting of the voltage sensor, i.e., stabilize it in an outward position upon repolarization. Previously, we found that extracellular MTSEA-biotin could stabilize the S4’s in DIII and IV (as measured by the predicted reduction in $Q_{\text{max}}$ without the application of avidin (36). We tried the same approach to stabilization of the DI-S4 using DI-R2C. Because we found that the second outermost arginine did not make a measurable contribution to gating charge, stabilization of the S4 in DI-R2C would be expected to decrease $Q_{\text{max}}$ by nearly 20%.

Initially, we modified DI-R2C with 1 mM extracellular MTSEA-biotin during trains of step-depolarizations to 0 mV at 1 Hz until the decrease in $I_{\text{Na}}$ stabilized (typically after 10–15 min). However, $Q_{\text{max}}$ was decreased by only 11 ± 4% ($n = 4$ cells, data not shown). Moreover, exposure to extracellular avidin, which would be predicted to increase the size of the stabilizer, did not further decrease gating charge suggesting that the biotin moiety may not access the avidin binding site. To test whether the length of the tethered biotin was too short to allow a reaction with avidin, DI-R2C was exposed to extracellular MTSEA-biotincap, a reagent with a tether about 8 Å longer than MTSEA-biotin (12, 19). Similar to the effects of
Fig. 1. Effect of lidocaine on the gating charge-voltage (Q-V) relationships of DI-R2C (A), DI-R3C (B), DII-R1C (C), DII-R2C (D), DII-R3C (E) and wild type (WT) (F). The WT data were previously published in a study by Sheets et al. (34). Data plotted in A–E are means ± SE for cells in control (●) and after lidocaine (○), whereas the symbols are reversed in F. The solid lines represent the mean of the best fits to the data are given in Table 1.

MTSEA-biotin, Q_max was decreased by only 11% by 1 mM extracellular MTSEA-biotin cap (Fig. 2A). However, exposure of the biotinylated Cys to extracellular avidin at 40 μg/ml further reduced Q_max by an additional 11% for a total decrease of 22%, a value similar to ~19% estimated for the contribution of DI-S4 to Q_max. Both the slope factor and the V_50 [after accounting for the spontaneous shift in gating observed under these experimental conditions of ~0.13 mV/min (33)] of the Q-V relationship were not much different from control cells.

For experiments on stabilization of DII-S4, we again chose to substitute a Cys for the second outermost Arg (DII-R2C). Modification of I_Na by 1 mM MTSEA-biotin was rapid during

<p>| Table 1. Comparison of Boltzmann parameters from fits to Q-V relationships before and after lidocaine |
|----------------------------------|------------|------------|------------|------------|------------|------------|</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>DI-R2C (n = 4)</th>
<th>DI-R3C (n = 4)</th>
<th>DI-R1C (n = 4)</th>
<th>DI-R2C (n = 3)</th>
<th>DI-R3C (n = 4)</th>
<th>WT (n = 2)</th>
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</thead>
<tbody>
<tr>
<td>V/2, mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-68 ± 4</td>
<td>-60 ± 2</td>
<td>-65 ± 3</td>
<td>-57 ± 5</td>
<td>-60 ± 2</td>
<td>-56 ± 6</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>-80 ± 3*</td>
<td>-79 ± 2*</td>
<td>-83 ± 2*</td>
<td>-74 ± 8*</td>
<td>-76 ± 3*</td>
<td>-65 ± 1</td>
</tr>
<tr>
<td>s, mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 1*</td>
<td>10 ± 1</td>
<td>15 ± 1</td>
<td>15 ± 3</td>
<td>11 ± 2</td>
<td>-11 ± 1</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>17 ± 1*</td>
<td>16 ± 1*</td>
<td>23 ± 2*</td>
<td>25 ± 3*</td>
<td>18 ± 1*</td>
<td>-18 ± 1</td>
</tr>
<tr>
<td>Reduction in Q_max in lidocaine</td>
<td>0.38 ± 0.01*</td>
<td>0.36 ± 0.02*</td>
<td>0.45 ± 0.06*</td>
<td>0.39 ± 0.02*</td>
<td>0.36 ± 0.02*</td>
<td>38 ± 2</td>
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<tr>
<td>Fractional contribution to Q_max</td>
<td>0</td>
<td>0</td>
<td>0.16</td>
<td>0.03</td>
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</tr>
</tbody>
</table>

Values are means ± SD. Q-V, gating charge-voltage relationship. *Wild type (WT) coexpressed with B1, previously published in Sheets et al. (34). *For values <0 the fractional contribution was set to 0. *Significant P < 0.05 for paired t-test for each channel in control compared with lidocaine. See text for definitions of parameters.
The Qmax for each cell was normalized to the fitted Qmax value in control (Eq. 4). In control the slope factor was 13 ± 2 mV and the V½ was −68 ± 6 mV, while after only MTSEA-biotin the Qmax decreased to 0.92 ± 0.05 (P < 0.03 compared with control), the slope was 15.4 ± 1 mV, and the V½ was −70 ± 7 mV. After both MTSEA-biotin and avidin, the Qmax decreased to 0.78 ± 0.05 (P < 0.05 compared with control), the slope was 15 ± 1 mV, and the V½ was −75 ± 6 mV (P < 0.05 compared with control). However, the V½ was nearly unchanged after accounting for the spontaneous leftward shift in Na channel kinetics as a function of time of internal perfusion (estimated to be about −9 mV due to nearly 70 min between data collection in control and after MTSEA-biotin with avidin; see text). In four WT cells (data not shown) the value of the fitted, normalized Qmax after a comparable time (67 ± 12 min) was 1.01 ± 0.03 compared with their first determination.

A: the Q-V relationships for five cells expressing DI-R2C are plotted (means ± SE) in control (●), after exposure to only N-biotinylcaproylaminoethyl methanethiosulfonate (MTSEA-biotin) (○), and after exposure to both MTSEA-biotin and avidin (■). The Qmax for each cell was normalized to the fitted Qmax value in control (Eq. 4). In control the slope factor was 14 ± 2 mV and the V½ was −66 ± 4 mV, while after only MTSEA-biotin the Qmax decreased to 0.92 ± 0.03 (P < 0.05 compared with their control), the slope was 15.7 ± 1 mV, and the V½ was −68 ± 5 mV. After both MTSEA-biotin and avidin, the Qmax decreased to 0.80 ± 0.04 (P < 0.05), the slope was 17 ± 1 mV, and the V½ was −74 ± 6 mV (P < 0.05 compared with their control). As for DI-R2C, the difference (−8 mV) between the V½’s was nearly unchanged after accounting for the nearly 80 min between data collection in control and after MTSEA-biotin with avidin.

B: the Q-V relationships for cells expressing DII-R2C are plotted (means ± SE) in control (●, n = 5 cells), after exposure to only N-biotinylcaproylaminoethyl methanethiosulfonate (MTSEA-biotin) (○, n = 3 cells), and after exposure to both MTSEA-biotin and avidin (■, n = 4 cells). The Qmax for each cell was normalized to the fitted Qmax value in control (Eq. 4). In control the slope factor was 13 ± 2 mV and the V½ was −68 ± 6 mV, while after only MTSEA-biotin the Qmax decreased to 0.89 ± 0.02 (P < 0.05), the slope was 15.4 ± 1 mV, and the V½ was −70 ± 7 mV. After both MTSEA-biotin and avidin, the Qmax decreased to 0.78 ± 0.05 (P < 0.05 compared with control), the slope was 15 ± 1 mV, and the V½ was −75 ± 6 mV (P < 0.05 compared with control). However, the V½ was nearly unchanged after accounting for the spontaneous leftward shift in Na channel kinetics as a function of time of internal perfusion (estimated to be about −9 mV due to nearly 70 min between data collection in control and after MTSEA-biotin with avidin; see text). In four WT cells (data not shown) the value of the fitted, normalized Qmax after a comparable time (67 ± 12 min) was 1.01 ± 0.03 compared with their first determination.

Effects of stabilization of the individual S4’s in DI and II on Na current kinetics. Having established that DI and DII S4 segments can be stabilized outward, we next determined how that affected peak I-V relationships and steady-state Na channel availability curves. Because Na channel kinetic parameters spontaneously shift leftward during the lifetime of an internally perfused cell (15, 33), and due to the length of time required for the modification of the mutant channels with both the MTSEA reagent and avidin (see METHODS), we used WT cells recorded at comparable cell lifetimes for comparison. Figure 3 shows families of Na currents after S4 stabilization of DI-R2C (Fig. 3A), DII-R2C (Fig. 3B), and WT (Fig. 3C) at a corresponding time. The most obvious difference is a more rapid time course of INa decay in DII-R2C after stabilization compared with DI-R2C and WT. Figure 3D shows the normalized peak I-V relationships for each of the stabilized channels with a corresponding time-control for WT. Note the decrease in Gmax of ~50% (see inset) for both DI-R2C and DII-R2C after modification by MTSEA reagents with avidin compared with the minor decrease in WT as a function of cell lifetime. Although both stabilized channels showed a leftward shift in the V½ of activation, the shift was only large enough (~7 mV in DII-R2C) to reach statistical significance (Table 2).

In contrast to the modest changes in the peak I-V relationships after stabilization of S4’s in DI and DII, steady-state Na channel availability was markedly shifted leftward after DII-R2C was stabilized (~17 mV), but unchanged for DI-R2C (Fig. 3E). Sodium currents are shown normalized to a value of one to better illustrate the shift in the V½ of the relationships. It should also be noted that hyperpolarization of the holding membrane potential to as much as −180 mV did not restore the current magnitude for stabilized mutant channels (data not shown).

Although peak INa was reduced in both mutant channels after stabilization of their S4’s, the INa kinetics differed between the two channels. Figure 4A shows the time to peak INa from the same cells as above recorded during step depolarizations from the Vhp, while Fig. 4B shows their single-exponential decay time constants. Both the time to peak INa and INa decay time constants were not significantly different from WT for DI-R2C after modification by MTSEA-biotin and avidin, but were markedly shortened for DII-R2C after MTSEA-biotin and avidin.

DISCUSSION

We have shown that application of the tethered biotin and avidin technique (19, 27, 41) to the voltage-gated NaV1.5 channel can stabilize the individual S4’s in both DI and II. Using lidocaine to calculate the relative contribution of individual basic residues in DI and DII to overall gating charge, we estimate that each S4 contributes ~20% to the Qmax of Nav1.5
with the outermost Arg residue in each S4 responsible for almost all of the charge. These results are similar to those from previous reports showing that the outermost Arg residues in the S4’s in DIII and IV also contributed the majority of the gating charge even though those S4’s made an overall greater contribution to gating charge (~30% each) (35, 39).

$\Delta$Na decay time course, the voltage dependence of activation assessed by peak I-V relationships, and the steady-state Na channel availability curves were not significantly different from WT when the DI-S4 was stabilized in an outward position. The only significant effect of positioning DI-S4 into a presumed channel-activation conformation was a reduction in $G_{\text{max}}$ of 45%. In contrast, when the DII-S4 was held stabilized and held outward, in addition to a reduction in $G_{\text{max}}$, there was a prominent speeding-up of $I_{\text{Na}}$ decay and a faster time to peak $I_{\text{Na}}$ accompanied by a small leftward shift of $-7$ mV in the peak I-V relationship but with a large $-17$ mV leftward shift of the steady-state Na channel availability curve.

In voltage-gated K channels such as Shaker K channels, most if not all of the gating charge results from channel activation transitions leading to channel opening (3, 29, 30, 45) with little or no gating charge movement after channel opening. Although there are fewer studies on the discrete transitions in the activation processes of Na channels, the S4’s from domains I, II, and III are thought to be principally involved in channel activation (2) while inactivation has been associated with the S4 in DIV (8, 10, 11, 21, 22, 37, 43, 44). Studies of individual fluorescent-tagged voltage sensors in rat skeletal muscle Na channels (rSkM1, Nav1.4) showed that the S4’s in domains I–III all translocated rapidly during a step depolarization consistent with their role in the activation process (10).

Our own studies with inactivation-deficient channels also support this finding (32, 38).

However, prepositioning the DII-S4 outward produced a more complex set of changes compared with stabilization of the DI-S4. $I_{\text{Na}}$ decay in WT channels is critically dependent upon inactivation from the open state (8, 11, 14, 21, 22, 37, 43, 44); consequently the more rapid decay of $I_{\text{Na}}$ following stabilization of the DII-S4 suggests that inactivation has also become faster. Furthermore, the large negative shift in the $V_{\text{th}}$ of the steady-state Na channel availability curve after DII-S4 was stabilized also suggests it has a role in closed state inactivation, presumably by helping form or helping control

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**Table 2. Comparison of Boltzmann parameters from fits to peak I-V (IVP) and voltage-dependent Na channel availability (SSI) plots for DI-R2C, DII-R2C, and WT**

<table>
<thead>
<tr>
<th>Cells</th>
<th>$V_{\text{th}}$, mV</th>
<th>$\alpha$, mV</th>
<th>Time, min</th>
</tr>
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<tbody>
<tr>
<td>DI-R2C (n = 6)</td>
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</tr>
<tr>
<td>IVP</td>
<td>$-59 \pm 4$</td>
<td>$-9.1 \pm 0.4^*$</td>
<td>$44 \pm 2$</td>
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<tr>
<td>SSI</td>
<td>$-116 \pm 3$</td>
<td>$9.6 \pm 0.9$</td>
<td>$46 \pm 2$</td>
</tr>
<tr>
<td>DII-R2C (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVP</td>
<td>$-62 \pm 3^*$</td>
<td>$-7.4 \pm 0.6^*$</td>
<td>$47 \pm 3$</td>
</tr>
<tr>
<td>SSI</td>
<td>$-130 \pm 6^+$</td>
<td>$8.7 \pm 1$</td>
<td>$47 \pm 3$</td>
</tr>
<tr>
<td>WT (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVP</td>
<td>$-55 \pm 4$</td>
<td>$-7.0 \pm 0.9$</td>
<td>$44 \pm 2$</td>
</tr>
<tr>
<td>SSI</td>
<td>$-113 \pm 5$</td>
<td>$9.4 \pm 2$</td>
<td>$45 \pm 2$</td>
</tr>
</tbody>
</table>

Values are means ± SD. I-V, current-voltage relationship. *MTSEA-biotin-cap for DI-R2C, MTSEA-biotin for DII-R2C, and control solution for WT.

*Significant at $P < 0.05$ compared with WT using ANOVA (Tukey). †Significant at $P < 0.05$ compared with DI-R2C using ANOVA (Tukey).
formation of the binding site for the inactivation lid. The enhanced inactivation after stabilization of the DII-S4 would help explain the decrease in its $G_{\text{max}}$. Both the leftward shift in its steady-state Na channel availability curve combined with a more rapid decay of $I_{\text{Na}}$ during step depolarizations would be expected to decrease $G_{\text{max}}$. Not surprisingly, holding the membrane potential more negative than $-150 \text{ mV}$ after stabilization of DII-S4 did not result in an increase in $I_{\text{Na}}$ consistent with the large size of avidin prohibiting a more negative membrane potential to retract the stabilized DII-S4 into a more normal, inward resting position.

An explanation for the decrease in $G_{\text{max}}$ after stabilization of the DI-S4 is less easily understood. Its steady-state Na channel availability was not significantly shifted nor were its $I_{\text{Na}}$ decay time constants shorter than WT suggesting that inactivation was unchanged. Stabilization of the DI-S4 by itself would not be expected to alter single-channel conductance because the channel modification should have affected only the voltage-sensor domain and not the pore domain of the channel. It is possible that the presence of either the bound avidin and/or MTSEA-biotin might obstruct or electrostatically affect the external vestibule, thereby affecting single-channel conductance. Another possible explanation for the decrease in $G_{\text{max}}$ is that stabilization of the DI-S4 secondarily affected the position of the DIV-S4, a voltage sensor that has been shown to be critically involved in Na channel inactivation (8, 10, 11, 14, 21, 22, 37, 39, 43, 44). It has been reported that S4 movements in different domains of Na channels are cooperative, particularly between the S4’s in domains I and IV (9). Consequently, even though stabilization of the DI-S4 may have no direct effect on inactivation, it may have an indirect effect on inactivation via cooperativity between domains I and IV resulting in a decrease in peak $I_{\text{Na}}$ after stabilization of the DI-S4.

In DI-R2C, modification of the Cys required use of MTSEA-biotincap rather than MTSEA-biotin consistent with the idea that the 8 Å longer tether in MTSEA-biotincap was required for the biotin moiety to access the pocket in avidin (see 19). This suggests that the second outermost Arg in DI during depolarization is further from the extracellular surface than the corresponding Arg residue in DII where avidin appears to bind to DII-R2C after modification by MTSEA-biotin.

Relationship to action of β-scorpion toxins. β-Scorpion toxin, a site-4 toxin (1, 20), has been shown to bind to the extracellular surface of Na channels with a critical interaction near the DII-S4 resulting in trapping the DII-S4 in an activated conformation (5, 6). There is also evidence that β-scorpion toxin binding to the pore loop of DII (24) suggesting that the binding site of β-scorpion toxin may involve a more complex interaction with the Na channel. Some of the effects of β-scorpion toxin appear to be isoform dependent because both Nav1.2 and Nav1.4 display enhanced activation (by about $-30 \text{ mV}$) accompanied by a leftward, negative shift in steady-state Na channel availability (ranging from $-12$ to $-21 \text{ mV}$) after modification, while Nav1.5 channels show little or no change in either the peak $I-V$ relationship or voltage-dependent steady-state availability other than an overall decrease in $G_{\text{max}}$ (4, 6, 23).

In contrast to the minimal effects of β-scorpion toxins on Nav1.5 other than a reduction in $G_{\text{max}}$ due to a decrease in open channel probability (23), we found that stabilization of the DII-S4 in Nav1.5 caused both a leftward ($-7 \text{ mV}$) shift in the half-point of the peak $I-V$ relationship and a larger leftward shift ($-17 \text{ mV}$) in the voltage-dependent steady-state availability curve. Previous studies have identified a specific Gly residue (Gly845 in Nav1.2) in the S3-S4 linker of DII that is critical for β-scorpion toxin enhancement of $I_{\text{Na}}$ activation (6); however, Nav1.5 has an Asn (Asn803) at the corresponding position in the S3-S4 linker in DII. As anticipated, mutation of the Asn to a Gly (N803G) in Nav1.5 caused $-\text{scorpion toxin}$ to enhance Na channel activation (23). The fact that we found leftward shifts in both activation and inactivation after stabilization of the DII-S4 in Nav1.5 even though it has an Asn at position 803 is not unexpected because the tethered biotin and avidin approach does not depend upon the presence of a specific interaction(s) site for β-scorpion toxin. In addition, we found that stabilization of the DII-S4 in Nav1.5 was also
accompanied by a decrease in peak $I_{Na}$ and a speeding of $I_{Na}$ decay similar to that reported for $\beta$-scorpion toxins (7, 13). Thus our results after stabilization of the DII-S4 are similar to those observed for Nav1.2 and Nav1.4 after $\beta$-scorpion toxin, and support the interpretation that site-4 toxins trap the voltage sensor in DII in activation conformation as proposed by Ces tele et al. (6).

In summary, we have shown that the S4’s in both DI and II of Nav1.5 each contribute $\sim 20\%$ to channel gating charge ($Q_{\text{max}}$) with the outermost Arg making most of the contribution in each case. Stabilization of the DI-S4 in the outward position produced only a reduction in size of $I_{Na}$ (by 45%); peak I-V relationships and voltage-dependent steady-state Na channel availability curves were not significantly different from WT. In contrast, stabilization of DII-S4 resulted in a small, significant ($-7$ mV) leftward shift of the peak I-V relationship, a larger ($-17$ mV) leftward shift in steady-state Na channel availability, a speeding of $I_{Na}$ decay and time to peak $I_{Na}$, and a decrease in $G_{\text{max}}$. The changes associated with the stabilization of DII-S4 in an outward position replicate those published for the site-4 toxin, $\beta$-scorpion toxin.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.F.S. and D.A.H. conception and design of research; M.F.S. and T.C. performed experiments; M.F.S. and T.C. analyzed data; M.F.S. and D.A.H. interpreted results of experiments; M.F.S. and D.A.H. prepared figures; M.F.S. drafted manuscript; M.F.S. and D.A.H. edited and revised manuscript; M.F.S., T.C., and D.A.H. approved final version of manuscript.

REFERENCES