A negatively charged residue in the outer mouth of rat sodium channel determines the gating kinetics of the channel

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Ion channels are pore-forming proteins that underlie the excitability of nerves and muscles. Na+ channels are responsible for the conduction of electrical impulses throughout excitable tissues. Recently, direct information from crystal structures with regards to the pore and activation of K+ channels have been obtained (7, 12). As a complement to this new information, site-directed mutagenesis experiments continued to provide crucial functional tests utilizing data from crystal structures as a guide. However, this technique also makes certain basic assumptions that critically influence data interpretation; that is, the single amino acid substitutions do not significantly change the global structure of the proteins. Indeed, the good agreement between the reported crystal structure of KcsA K+ channel (7) and mutagenic work further confirmed the usefulness of such techniques in the study of ion channel structures.

Previous studies using the combined techniques of site-directed mutagenesis and electrophysiology have provided insights regarding the specific regions of Na+ channel, which are involved in the permeation and gating of the channels. Furthermore, there is evidence showing significant overlaps in the regions that are important for these two fundamental properties of the channels (18). We have previously shown that one pore-lining residue in S5-S6 region (P loop) in domain I of the μ1 skeletal muscle Na+ channel, was important in the gating of the channel. Here, we determined the role of an adjacent pore-lining negatively charged residue (E403) in channel gating. Charge neutralization or substitution with positively charged side chain at this position resulted in a marked delay in the rate of recovery from slow inactivation. Indeed, the fast inactivation process appeared intact. Restoration of the negatively charged side chain with a sulfhydryl modifier, MTS-ethylsulfonate, resulted in a reactivation profile from a slow-inactivated state, which was indistinguishable from that of the wild-type channels. We propose an additional functional role for the negatively charged residue. Assuming no major changes in the pore structure induced by the mutations, the negatively charged residue E403 may work in concert with other pore regions during recovery from slow inactivation of the channel. Our data represent the first report indicating the role of negative charge in the slow inactivation of the voltage-gated Na+ channel.

METHODS

Transient transfection of the μ1 skeletal muscle Na+ channels. The wild-type (WT) and mutant μ1 Na+ channel cDNA (19) and the rat brain β1 subunit (11) were transiently expressed in the human embryonic kidney cell line (HEK 293; American Type Culture Collection, Manassas, VA) using the calcium phosphate precipitation method for 6–12 h as previously described (3). A plasmid containing the green fluorescent protein (GFP; Invitrogen, Carlsbad, CA) was used as a transfection reporter.

Whole cell current recordings. All experiments were performed at room temperature (22–23°C). Macroscopic Na+...
currents \( (I_{Na}) \) were recorded from transfected HEK 293 cells using whole cell patch-clamp techniques \( (8) \) in bath solution containing \( (\text{in mM}) \) 140 NaCl, 5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 glucose, and 10 HEPES \((\text{pH} 7.4 \text{ with NaOH})\). Pipette solution contained \( (\text{in mM}) \) 35 NaCl, 105 CsF, 1 MgCl\(_2\), 10 HEPES, and 10 EGTA \((\text{pH} 7.2 \text{ with CsOH})\). The cell capacitance and series resistance were compensated electronically. In all experiments, a series resistance compensation of \( \approx 90\% \) was obtained. Current records were filtered at 10 kHz using a 4-pole Bessel filter and digitized at a sampling frequency of 50 kHz. A methanethiosulfonate (MTS)-based sulfhydryl-modifying reagent, MTS-ethylsulfonate (MTSES, 10 mM; Toronto Research Chemicals, ON, Canada), was used. Statistical significance was determined by using Student’s \( t \)-test or ANOVA with \( P < 0.05 \) representing significance (Systat, SYSTAT Software).

Steady-state activation of \( I_{Na} \) was determined from \( I_{Na} \) conductance \( (\gamma_{Na}): \gamma_{Na} = I_{Na}(V_{m}-E_{rev}) \), where \( V_{m} \) is the membrane potential and \( E_{rev} \) is the zero current voltage. Steady-state inactivation \( (h_{Na}) \) of \( I_{Na} \) was measured using a conventional two-pulse voltage-clamp protocol. Conditioning pulses were 500 ms or 10 s in duration, followed by a test pulse to \(-10 \text{ mV} \) for 25 ms. Voltage dependence of inactivation was assessed as the relative current over a range of conditioning voltages. This was calculated by normalizing peak \( I_{Na} \) elicited by test depolarizations to \(-10 \text{ mV} \), to the value obtained after holding at \(-130 \text{ mV} \) conditioning voltage. Development of slow inactivation was assessed at \(-20 \text{ mV} \) using a two-pulse voltage-clamp protocol \((\text{inset, } \text{Fig. } 4E)\). The reduction in \( I_{Na} \) magnitude during the test pulse \( (P_2) \) relative to the first pulse \( (P_1) \) pulse is lengthened reflects occupancy of the slow inactivated state. The 50-ms recovery interval between \( P_1 \) and \( P_2 \) allows full recovery from fast inactivation but also allows some recovery from slow inactivation; hence, the magnitude of slow inactivation estimated by the analysis represents the lower limit \((17)\). Recovery from inactivation was assessed using a two-pulse voltage-clamp protocol with \( P_1 \) and \( P_2 \) at \(-20 \text{ mV} \), where \( P_1 \) was the first pulse with pulse duration fixed at four different values \((40 \text{ ms}, 100 \text{ ms}, 2.5 \text{ s}, \text{ and } 10 \text{ s}) \) and \( P_2 \) was the test pulse. The recovery interval at \(-120 \text{ mV} \) between \( P_1 \) and \( P_2 \) was varied. Deactivation kinetics of the currents was studied using standard tail current protocol \((\text{Fig. } 1B, \text{inset})\). Tail currents were elicited using voltage steps, which were varied between \(-100 \text{ mV} \)
to 60 mV in 10-mV increments after a prepulse to 0 mV for 1 ms to activate the channel.

RESULTS

Whole cell $I_{Na}$ were recorded from WT and E403C mutant channels to directly examine the activation, deactivation, inactivation, and reactivation kinetics of the channels. Experiments were performed in parallel using HEK 293 cells transfected with mutant or WT channels. Figure 1A shows examples of families of $I_{Na}$ records from WT and E403C mutant channels. As we have previously shown, cysteine substitution in this region of the channel is well tolerated (4, 5, 15, 16).

Figure 1B shows examples of tail currents used to assess the deactivation kinetics. Deactivation time constants were obtained by using single exponential fits to the tail currents. There were no significant changes in the deactivation time constants comparing WT or E403C mutant channels (Fig. 1C). However, close examination of the fast inactivation kinetics revealed that the activating prepulse used (1 ms) is longer than the time constant for fast inactivation at 0 mV ($\tau \sim 0.5$ ms, see Fig. 2A). This implies that a significant degree of fast inactivation will occur during this prepulse and that only a fraction of the channels examined in this experiment is undergoing deactivation during the tail current. Indeed, one may need to employ a cooling method to retard fast inactivation to properly examine the deactivation kinetics.

In addition, there were no significant changes in steady-state activation (Fig. 1D). However, there was a hyperpolarization shift in the steady-state availability
of the E403C mutant channel by ~10 mV compared with the WT channels (Fig. 1E). This shift in steady-state availability occurred without a significant change in the \( I_{Nax} \) decay kinetics assessed by using a pulse duration of 20 ms (Fig. 2, A and B). Assessment of recovery from inactivation, on the other hand, yielded important differences between the mutant and WT channels. While the recovery from inactivation of the mutant and WT channels was almost superimposed when a prepulse duration of 40 or 100 ms was used (Fig. 2, C and D), there was a significant delay in the recovery from slow inactivation in the E403C mutant channel when assessed using a prepulse duration of 2.5 or 10 s (Fig. 2, E and F). Therefore, this delay in the recovery from slow inactivation appeared to occur in the presence of intact fast inactivation. Furthermore, the mutation also resulted in a pronounced effect on the fraction of current recovering slowly from inactivation. The fraction of the current recovered from slow inactivation after a recovery interval of 500 ms (\( y_0 \)) was significantly reduced in the E403C mutant channel compared with the WT channel when prepulses of 2.5 and 10 s were used (Fig. 2, E and F). The data are quantified and summarized in Table 1.

We were interested to find out whether the loss of the negative charge was important for this change in the gating pattern. Thus we generated an E403R mutant channel. Figure 3A shows a family of current elicited from the E403R mutant channel. The charge reversal substitution was well tolerated. Compared with the WT channel, there were no significant changes in the fast or slow time constants for the current decay using step potentials with duration of 20 ms (Fig. 3B). In addition, the steady-state activation curve for the E403R mutant channel superimposed well with that of the WT channel (Fig. 3C). Similar to charge neutralization (E403C), charge reversal at this position resulted in a hyperpolarization shift in the steady-state inactivation but to a greater degree (~12 and 16 mV) with an associated increase in the slope factor when prepulses of 500 ms and 10 s, respectively, were used (Fig. 3, D and E).

Recovery from fast and slow inactivation, as well as the development of slow inactivation, was directly assessed in the E403R mutant channel compared with the WT channel. Experiments were performed in parallel by using HEK 293 cells transfected with mutant or WT channels. There were no significant changes in the recovery time course using short prepulses of 40 and 100 ms (Fig. 4, A and B). However, the mutant channel showed a significant delay in the recovery from slow inactivation when prepulse durations of 2.5 and 10 s were used (Fig. 4, C and D). Similar to the E403C mutant channel, the charge reversal at this position also resulted in a pronounced effect on the fraction of current recovering slowly from inactivation. The fraction of the current recovered from slow inactivation after a recovery interval of 500 ms (\( y_0 \)) was significantly reduced in the E403R mutant channel compared with the WT channel when prepulses of 2.5 and 10 s were used (Fig. 4, C and D). The data are summarized in Table 1.

The development of slow inactivation in the E403R mutant channel compared with the WT channel was assessed using a two-pulse voltage-clamp protocol (inset, Fig. 4E). The reduction in \( I_{Nax} \) magnitude during the second test pulse (\( P_2 \)) relative to the first pulse (\( P_1 \)) as the \( P_1 \) pulse is lengthened reflects occupancy of the slow inactivated state. The E403R mutant channel showed a significant increase in the fraction of the channel, which entered the slow inactivated state associated with a decrease in the time constant for the entry into the slow inactivated state (Fig. 4E). Taken together, these data suggest that the charge at E403 position is critical for the slow inactivation of the channel.

To crosscheck our interpretation of the significance of the negative charge at the 403 position, we exposed the E403C mutant channel to a permanently negatively charged sulfhydryl-specific modifier, MTSES, which would be expected to restore the negatively charged side chain to the cysteine residue (Fig. 5A). As previously published, \( \mu \) WT channels are not modifiable by MTS compounds (4, 5, 15, 16). In contrast, MTSES modification of E403C channels leads to a reduction in the current amplitude (4, 5). More importantly, MTSES restored the WT gating behavior; the modified channel showed an increase in the rate of

### Table 1. Fitted parameters for recovery from inactivation

<table>
<thead>
<tr>
<th></th>
<th>( P_1 = 40 ) ms</th>
<th>( P_1 = 100 ) ms</th>
<th>( P_1 = 2.5 ) s</th>
<th>( P_1 = 10 ) s</th>
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<tr>
<td></td>
<td>( \tau_1 )</td>
<td>( \tau_2 )</td>
<td>( y_0 )</td>
<td>( A_1 )</td>
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<tr>
<td>WT</td>
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<td>3.2</td>
<td>1.0</td>
<td>0.3</td>
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<tr>
<td></td>
<td>( \pm 0.15 )</td>
<td>( \pm 0.53 )</td>
<td>( \pm 0.01 )</td>
<td>( \pm 0.05 )</td>
</tr>
<tr>
<td>E403C</td>
<td>0.9</td>
<td>5.6</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>( \pm 0.11 )</td>
<td>( \pm 0.11 )</td>
<td>( \pm 0.01 )</td>
<td>( \pm 0.01 )</td>
</tr>
<tr>
<td>E403C after MTSES</td>
<td>( \pm 0.5 )</td>
<td>( \pm 2.2 )</td>
<td>( \pm 0.01 )</td>
<td>( \pm 0.01 )</td>
</tr>
<tr>
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<td>1.9</td>
<td>40.9</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>( \pm 0.1 )</td>
<td>( \pm 2.7 )</td>
<td>( \pm 0.01 )</td>
<td>( \pm 0.01 )</td>
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* \( P < 0.05 \) compared with (WT) channel. MTSES, MTS-ethylenesulfonate; \( P_1 \) first pulse. Data were fitted using the following expression: \( y = y_0 + A_1(1 - \exp[-t/\tau_1]) + A_2(1 - \exp[-t/\tau_2]) \), where \( \tau_1 \) and \( \tau_2 \) are the fast and slow time constants, \( A_1 \) and \( A_2 \) are amplitude of the fast and slow components, and \( y_0 \) represents the fraction of current recovered after 500 ms.

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recovery from slow inactivation when a prepulse duration of 2.5 s was used (Fig. 5D). In addition, the modification resulted in an increase in the fraction of the current after a recovery interval of 500 ms from 0.75 ± 0.01 to 0.85 ± 0.01 (aP < 0.05, n = 7).

Finally, we performed experiments to further rule out the possibility that MTS reagent may unmask the normally unreactive native cysteine residue in the mutant channels. We directly tested the effects of MTSES on E403A mutant channel with a side chain size similar to that of cysteine, but it is expected to be unreactive to MTS compounds. Indeed, similar to the μ1 WT channel, the E403A mutant channel was not modifiable by MTSES. The peak current density when a step potential of −20 mV was used was 12.1 ± 1.2 and 12.0 ± 1.4 pA/pF in control and after treatment with 10 mM MTSES (n = 6, P = NS). In addition, there were no changes in the recovery kinetics when prepulse durations of 10 s were used after treatment of the E403A channel with MTSES. Time constants of recovery from inactivation are as follows: τ1 of 10 s, E403A (n = 3): τ1 = 13.2 ± 1.5 ms, τ2 = 78.7 ± 23.6 ms, after MTSES (n = 3): τ1 = 14.2 ± 1.0 ms, τ2 = 79.4 ± 12.0 ms (P = NS comparing τ before and after MTSES).

DISCUSSION

Functional roles of the negatively charged residue in the outer vestibule of Na⁺ channels. Since the first cloning of a voltage-gated Na⁺ channel (13), much information has been gained with regard to specific regions of the channel, which play crucial roles in specialized channel functions. Ample evidence suggested that the Na⁺ channel pore contains two rings of charge: an inner NH₂-terminal ring with one net negative charge (domains I–IV: D400, E755, K1237, and
Fig. 4. Comparison of \( I_{Na} \) decays and reactivation kinetics between E403R and wild-type channels. A–D: recovery from fast and slow inactivation using \( P_1 \) of 40 ms, 100 ms, 2.5 s, and 10 s. Solid lines indicate fits to 2 exponential functions as in Fig. 2. C–F. Note recovery from fast inactivation (A and B) are plotted as the log of recovery interval to expand the trajectory of data in the first 10 ms, similar to Fig. 2. Time constants of recovery from inactivation are summarized in Table 1 (* \( P < 0.05 \) comparing E403R and WT channels, \( n = 10 \) for each group). E: development of slow inactivation comparing E403R and WT channels using the pulse protocol as shown at right. Solid lines are fitted using a single exponential function: 
\[
y(t) = y_0 + A(1 - \exp(-t/\tau)),
\]
where \( t \) is the duration of \( P_1 \), and \( \tau \) is the time constant. Time constants of the development of slow inactivation are as follows: E403R (\( n = 8 \)): \( \tau = 2.8 \pm 0.2 \) s, WT (\( n = 8 \)): \( \tau = 3.8 \pm 0.3 \) s (* \( P < 0.05 \) comparing E403R and WT channels using ANOVA).

Fig. 5. Modification of the charge of the E403C mutant channel using methanethiosulfonate derivative, MTSES. The reaction of the MTSES with a cysteine residue adds \( -\text{SCH}_2\text{CH}_2\text{SO}_3^- \) to the thiol side chain via a disulfide bond. A: an example of whole cell \( I_{Na} \) elicited at a test potential of \(-20 \) mV from a holding potential of \(-120 \) mV before and after modification with 10 mM MTSES. B–D: recovery from inactivation using \( P_1 \) of 40, 100, and 2500 ms, respectively, to assess recovery from fast and slow inactivation. Note recovery from fast inactivation (B and C) are plotted as the log of recovery interval to expand the trajectory of data in the first 10 ms, similar to Figs. 2 and 4. Time constants of recovery from inactivation are summarized in Table 1. (* \( P < 0.05 \) comparing E403C before and after MTSES, \( n = 7 \)).
A1529 in μ1 Na⁺ channel) and an outer COOH-terminal ring with four negative charges (domains I–IV: E403, E758, D1241, and D1532 in μ1 Na⁺ channel; Ref. 13). Residues from both rings have been shown to play crucial roles in ionic selectivity, channel conductance, and tetrodotoxin (TTX) and saxitoxin (STX) binding (4, 5, 9, 15, 20).

Noda et al. (14) first discovered that the negatively charged residue in the external ring of domain I (E403 for μ1) was crucial in the binding of TTX and STX to the channel. Subsequent studies have unequivocally found that this acidic residue lines the external mouth of the channel to increase local concentration of permeant ions and to increase conductance of the channel. In the present study, we determined the effect of charge neutralization or reversal at the external ring in domain I on the gating kinetics. Charge neutralization or reversal at this position resulted in a marked delay in the rate of recovery from slow inactivation, whereas that of fast inactivation appeared to be preserved. Indeed, restoration of the negatively charged side chain with the sulphydryl modifier, MTSES, resulted in a reactivation profile that was indistinguishable from that of the WT channels.

**Slow inactivation.** Whereas fast inactivation in Na⁺ channels is mediated by cytoplasmic occlusion of the pore by III–IV linker residues, the structural features of slow inactivation are quite distinct. Slow inactivation appears to involve conformational changes in the external pore (1, 2, 6, 10, 21). We have previously shown that mutation of an adjacent residue from E403, i.e., W402C mutation in domain I, eliminated slow inactivation (2). Our present data suggest that the adjacent negatively charged residue is also critical for the slow inactivation. However, in contrast to W402 residue, neutralization of E403 resulted in marked delay in the recovery from slow inactivation. The finding that E403 replacement by cysteine has nearly the opposite effect of W402 replacement is rather unexpected. Nevertheless, the two adjacent residues are quite distinct. The native bulky tryptophan residue may serve to retard the conformation changes around the mouth of the channel required for the slow inactivation of the channel. On the other hand, the negative charge on E403 residue may interact with nearby positively charged residues, possibly positive charge(s) in the voltage sensor, during open conformation. Elimination of the negative charge on E403 may result in a channel that favors the entry into a slow-inactivated state and slows the recovery kinetics, as seen with E403C or E403R. In summary, our new data suggest an additional role of the negative charge on residue E403 located in the outer vestibule of the channels. Assuming no major changes in the pore structure were induced by the mutations, the negatively charged residue E403 may work in concert with other pore regions during slow inactivation of the channel. Our previous data have shown that cysteine mutagenesis in this region of the channel is well tolerated. The mutant channels remained selective to Na⁺ ions and retained the activation and inactivation gating kinetics similar to the WT channel (2, 4, 5, 15, 16, 18). Our data represent the first report indicating the role of negative charge in the slow inactivation of the voltage-gated Na⁺ channel.

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