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Functional role of the C-terminus of voltage-gated sodium channel $Na_v1.8$

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Abstract Sodium channel $Na_v1.8$ requires stronger depolarization than other sodium channels for activation and inactivation. The contribution of $Na_v1.8$ C-terminus to this property was investigated by producing $Na_v1.8$ and $Na_v1.4$ chimeras and expressing them in ND7/23 cells. Current densities of the chimeras were significantly different than in parental channels, and the voltage-dependence of activation was depolarized in $Na_v1.4/1.8C$ compared to $Na_v1.4$. Analysis of steady-state inactivation showed that only $Na_v1.8$ and $Na_v1.4/1.8C$ currents demonstrate a non-inactivated fraction. Thus, the C-terminus of $Na_v1.8$ contributes to regulation of channel density at the cell surface, modulates channel gating, and regulates the generation of sustained current.

Keywords: Sodium channel; $Na_v1.8$; Voltage-gated sodium channel

1. Introduction

The voltage-gated sodium channel $Na_v1.8$ produces a slow-inactivating TTX-R current [1–3], and plays a key role in inflammatory pain [2,4–8]. $Na_v1.8$ requires depolarized voltages to activate and inactivate despite the conservation of the positively charged residues in the S4 segments and the IFMT peptide in L3 [1,3], and has been difficult to study because recombinant channels produce very small currents in mammalian expression systems, for example HEK 293 cell lines [9,10]. Thus, it is likely that sequence elements other than the S4 segments and the tetrapeptide IFMT, and/or channel partners, regulate the channel density at the cell surface and the kinetics and voltage-dependence of activation and inactivation of this channel.

The C-terminal polypeptides of several sodium channels have been shown to regulate the current density by enhancing trafficking of the channels to, or by removal from, the cell surface [11–13]. All channels, except $Na_v1.4$, contain the a.a. motif PXY which binds to members of the Nedd4 ubiquitin-ligase family and mediates channel removal from the cell surface [14]. However, most sodium channels which contain the PXY motif, e.g. $Na_v1.3$, produce robust currents in HEK 293 cells [15].

The C-termini of sodium channels contain sequence elements which regulate kinetic properties of inactivation (see for review [16]). Fibroblast growth factor homologous factor-1B (FHF1B) binds to the C-terminus of $Na_v1.9$ and $Na_v1.5$ and causes a hyperpolarizing shift in the voltage-dependence of inactivation of $Na_v1.5$ [17,18]. Calmodulin interacts with the IQ motif in the C-terminus of several sodium channels and influences the properties of $Na_v1.4$, $Na_v1.5$ and $Na_v1.6$ currents [19–21]. Exchanging the C-termini between $Na_v1.4$ and $Na_v1.5$ [22], and $Na_v1.2$ and $Na_v1.5$ [23] conferred parental inactivation properties, but not activation properties, on the chimera channels.

To investigate the role of $Na_v1.8$ C-terminus, we exchanged the C-termini of $Na_v1.8$ and $Na_v1.4$, and analyzed the sodium currents in the DRG-derived cell line ND7/23 [24]. Recombinant $Na_v1.8$ has been successfully expressed in ND7/23 cells [9,25]. Our data show that the C-terminus of $Na_v1.8$ plays a significant role in regulating channel trafficking and modulation of channel gating, including voltage-dependence of activation, and in the appearance of a sustained current.

2. Material and methods

2.1. Plasmids

The plasmid pBRG-$Na_v1.4_R$, which produces a TTX-R version of $Na_v1.4$, has been previously described [21]; pRK-$Na_v1.8$, which encodes $Na_v1.8$ was a gift from Dr. John Wood, University College London. The peptide sequence ILEN of S6 of domain 4 is invariant among sodium channels. We introduced a silent mutation to the L1591 codon of $rNa_v1.4$ to create a unique *Xho*I site (pBRG-$Na_v1.4_{RX}$). Plasmid pRK-$Na_v1.8$ contains *Xho*I sites in the 5′ polylinker, in the sequence encoding L2, and in the 3′ polylinker. The site in the 5′ polylinker was destroyed by digesting with the enzymes *Cla*I and *Hin*dIII, and repairing and re-ligating the ends. The *Xho*I site in L2 was destroyed by a silent, site-directed mutagenesis. A *Xho*I site was introduced by a silent substitution in the L1723 codon, which is analogous to the $Na_v1.4$-L1591 (pRK-$Na_v1.8_X$). A fragment encoding the C-terminus of $Na_v1.4$ was amplified and used to replace the *Xho*I–*Kpn*I fragment of the pRK-$Na_v1.8_X$ to produce the $Na_v1.8/1.4C$ chimera. Similarly, the C-terminus of $Na_v1.8$ was amplified and used to replace the *Xho*I–*Cla*I fragment of pBRG-$Na_v1.4_{RX}$ to produce the chimera $Na_v1.4_R/1.8C$. Identities of inserts were confirmed by sequencing.

2.2. Transfection of ND7/23 cell line

The DRG-derived cell line ND7/23 [24] was used to express the parental and chimera channels. ND7/23 cells produce endogenous TTX-S, but not TTX-R currents [9,25]. However, the ND7/23 cell line has been used successfully to express recombinant $Na_v1.8$ channels [9,25]. ND7/23 cells were co-transfected with each channel

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DNA and pEGFP-N1 using Lipofectamine 2000 according to manufacturer's recommendations. Transfected cells were incubated at 37 °C for 24 h.

2.3. Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature with Axopatch 200B amplifiers (Axon Instrument, Foster City, CA) using the following solution: internal (mM), 140 CsF, 1 EGTA, 10 NaCl, and 10 HEPES, pH 7.3, adjusted to 310 mOsmol/L with sucrose; external (mM), 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 0.0003 TTX, 10 glucose, 20 HEPES, pH 7.3, adjusted to 320 mOsmol/L with sucrose. Recordings were started 5 min after establishing whole-cell configuration to allow currents to stabilize. To minimize the influence of time-dependent shifts in gating of $Na_v1.4$, all recordings were systematically made in the same order.

The currents were elicited from a holding potential of −120 mV, filtered at 5 kHz, and acquired at 50 kHz using pClamp 8.2. For current density measurements, membrane currents were normalized to membrane capacitance, and calculated as the integral of the transient current in response to a 5-ms hyperpolarizing pulse from the holding potential to −130 mV.

2.4. Experimental protocols and data analysis

Normalized curves of activation and steady-state inactivation were fitted using the Boltzmann distribution equation

$$Y = 1/\{1 + \exp[(V_{1/2} - V_m)/k]\},$$

where Y is the normalized conductance (G/G_{max}) or current (I/I_0), $V_{1/2}$ is the membrane potential at half-maximal conductance or current, and k is the slope factor.

Data analysis was performed using pClamp 8.2 and Origin 6.1. Statistical significance was determined using an unpaired t test. Results are presented as means ± S.E.M. and error bars represent standard errors.

3. Results

3.1. The effect of exchanging the C-terminus on current density

ND7/23 cells were transfected with parental and chimera channels and families of inward sodium currents were elicited in steps of 10 mV from a holding potential of −120 mV. All recordings were done in the presence of 300 nM tetrodotoxin (TTX), which was sufficient to block all endogenous TTX-S currents in ND7/23 cells. $Na_v1.4$ (Fig. 1A) produced a robust current (440.83 ± 68.18 pA/pF, $n = 20$) with fast inactivation properties, while $Na_v1.8$ (Fig. 1B) produced a small current (59.04 ± 11.44 pA/pF, $n = 25$) with slow inactivating properties as previously reported in DRG neurons and ND7/23 cells [1,9,25]. $Na_v1.4/1.8C$ chimera (Fig. 1C) current density (60.33 ± 8.95, $n = 20$) was significantly reduced ($P < 0.01$) to levels comparable to those of $Na_v1.8$. In contrast, the current density of $Na_v1.8/1.4C$ (Fig. 1D) was increased over 2-fold (155.29 ± 28.76, $n = 25$), compared to parental $Na_v1.8$ ($P < 0.01$).

3.2. The C-terminus regulates channel gating properties

The gating properties of channels were analyzed over a range of membrane potentials from a holding potential of −120 mV. Fig. 2A shows the mean normalized current–voltage (I–V) curve of parental and chimera channels. $Na_v1.4$ currents activate at potentials positive to −40 mV and peak near −10 mV. $Na_v1.8$ currents activate at potentials more positive to −30 mV and peak near +20 mV. Peak and threshold of activation voltages of $Na_v1.4/1.8C$ current were depolarized by ~10 and ~5 mV, respectively, compared to $Na_v1.4$. In contrast, the peak and threshold potentials of $Na_v1.8/1.4C$ were not changed, compared to $Na_v1.8$.

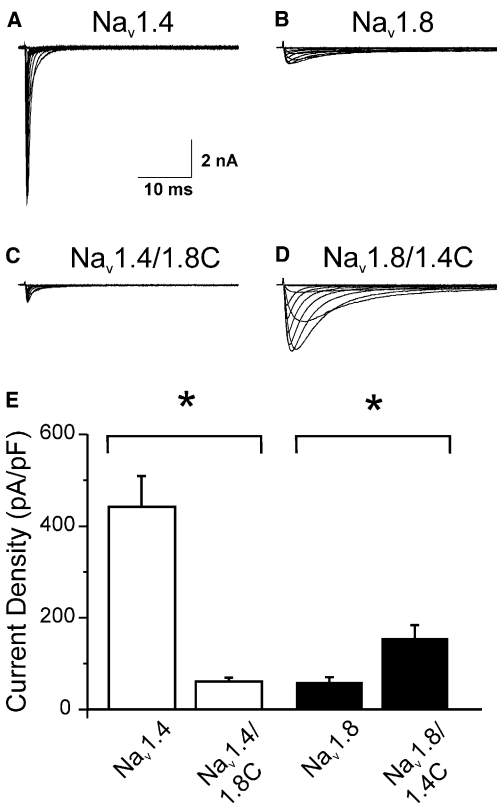

Fig. 1. Representative current traces and peak current densities for $Na_v1.4$, $Na_v1.8$ and chimera in ND7/23 cells. Whole-cell Na^+ currents elicited by 40 ms test pulses to potential between −45 and +45 mV for $Na_v1.4$ (A) and $Na_v1.4/1.8C$ (C) or −35 and +55 mV for $Na_v1.8$ (B) and $Na_v1.8/1.4C$ (D) in steps of 10 mV from a holding potential of −120 mV. Mean current densities of $Na_v1.4$, $Na_v1.4/1.8C$, $Na_v1.8$ and $Na_v1.8/1.4C$ were shown in (E). *$P < 0.01$ vs. parental channel.

Analysis of voltage-dependence of activation of parent and chimera channels confirms the role of the C-terminus on channel activation. Fig. 2B shows that the $V_{1/2}$ of activation of $Na_v1.8$ is 24 mV more positive than that of $Na_v1.4$. The $V_{1/2}$ of activation of $Na_v1.4/1.8C$ is 12 mV more depolarized compared to $Na_v1.4$, whereas the $V_{1/2}$ of activation of $Na_v1.8$ and Nav1.8/1.4C are similar.

Steady-state inactivation was studied by a conventional two-pulse protocol using 500 ms prepulse at various membrane potentials from a holding potential of −120 to −10 mV for $Na_v1.4$ and 0 mV for $Na_v1.4/1.8C$, and to +20 mV for $Na_v1.8$ and $Na_v1.8/1.4C$ (Fig. 3). The $V_{1/2}$ of steady-state inactivation of $Na_v1.4$ is 30 mV more hyperpolarized than the $V_{1/2}$ of $Na_v1.8$. The $V_{1/2}$ of $Na_v1.4/1.8C$ and $Na_v1.8/1.4C$ were similar to those of the parental channels. Unlike $Na_v1.8$ current in DRG neurons, inactivation of $Na_v1.8$ current in ND7/23 was incomplete and reached a plateau of 8.14 ± 1.36% ($n = 12$) of the peak current. The non-inactivated fraction of $Na_v1.8$ current increased with time as the recording progressed which caused an increase in the slope of the curve compared to the other channels. However, this phenomenon was not observed in the $Na_v1.8/1.4C$ current. Conversely, $Na_v1.4/1.8C$ shows a non-inactivated fraction (5.01 ± 1.05%, $n = 11$) similar to that observed for $Na_v1.8$.

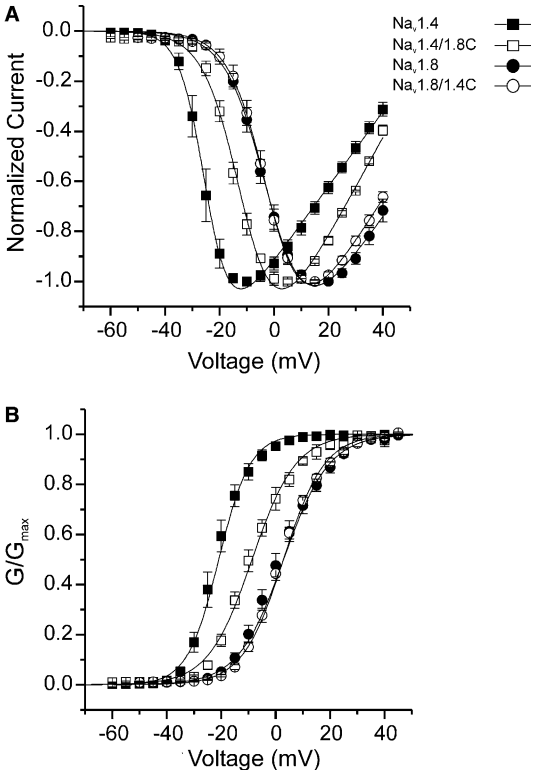

Fig. 2. Effect of channel's C-terminus on voltage-dependence of activation. Mean normalized I–V curves (A) for $Na_v1.4$, $Na_v1.4/1.8C$, $Na_v1.8$ and $Na_v1.8/1.4C$. The steady-state activation curves (B) were fitted by Boltzmann distribution equation: $Na_v1.4$, $V_{1/2} = -20.90 \pm 0.20$ mV, $k = 5.78 \pm 0.18$ mV ($n = 8$); $Na_v1.4/1.8C$, $V_{1/2} = -8.69 \pm 0.29$ mV, $k = 8.05 \pm 0.26$ mV ($n = 6$); $Na_v1.8$, $V_{1/2} = 2.90 \pm 0.69$ mV, $k = 8.38 \pm 0.26$ mV ($n = 5$); $Na_v1.8/1.4C$, $V_{1/2} = 2.83 \pm 0.64$ mV, $k = 7.55 \pm 0.27$ mV ($n = 10$). The $V_{1/2}$ of $Na_v1.4/1.8C$ was significantly different from those of the other channels ($P < 0.001$ vs. $Na_v1.4$ or $Na_v1.8$).

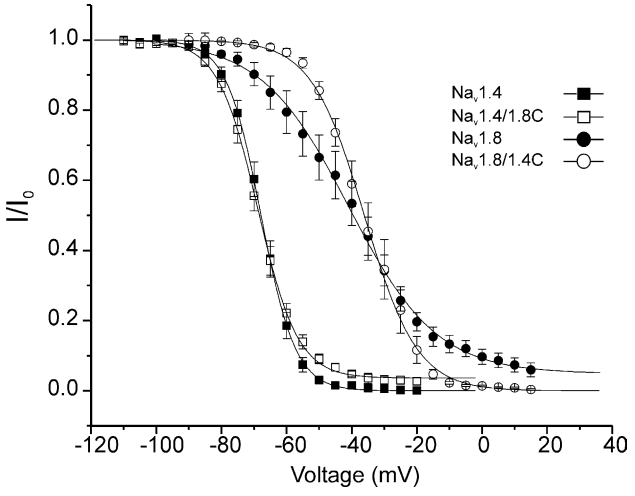

Fig. 3. Steady-state inactivation properties of parent and chimera channels. Best-fitted curves were generated by Bolzmann distribution equation: $Na_v1.4$ ($n = 9$), $V_{1/2} = -67.82 \pm 0.06$ mV, $k = 5.33 \pm 0.05$ mV; $Na_v1.4/1.8C$ ($n = 11$), $V_{1/2} = -68.78 \pm 0.13$ mV, $k = 6.15 \pm 0.11$ mV; $Na_v1.8$ ($n = 12$), $V_{1/2} = -40.90 \pm 0.45$ mV, $k = 13.45 \pm 0.37$ mV; $Na_v1.8/1.4C$ ($n = 9$), $V_{1/2} = -36.12 \pm 0.23$ mV, $k = 8.12 \pm 0.20$ mV. Note that $Na_v1.8$ and $Na_v1.4/1.8C$ show a non-inactivating current at strong depolarized potentials.

3.3. The C-terminus regulates kinetics of activation and inactivation of channels

Kinetic properties of activation and inactivation of parental and chimera channels were investigated for currents elicited by a strong depolarization from a holding potential of −120 to +30 mV (Fig. 4A). $Na_v1.8$ activates and inactivates much slower than $Na_v1.4$ (Fig. 4A). The time-to-peak of $Na_v1.4/1.8C$ is slightly delayed at all voltages compared to $Na_v1.4$ (Fig. 4B; 1.36 ± 0.17 and 1.72 ± 0.19 ms at −30 mV, 0.39 ± 0.01 and 0.46 ± 0.01 ms at +50 mV for $Na_v1.4$ and $Na_v1.4/1.8C$, respectively; $P < 0.05$). A ~10 mV depolarizing shift was apparent at all voltages. In contrast, the time-to-peak of $Na_v1.8/1.4C$ was not different from that of $Na_v1.8$.

The current decay for $Na_v1.8$ and $Na_v1.8/1.4C$ is well fitted by a single exponential function, while the current decay for $Na_v1.4$ and $Na_v1.4/1.8C$ is well fitted by a double exponential function. The time constant of inactivation of $Na_v1.8/1.4C$ was unaltered at all voltages compared to $Na_v1.8$ (Fig. 4C). However the late current of $Na_v1.8/1.4C$ at the end of the 40 ms pulse was much smaller than the parental channel. Compared to $Na_v1.4$, the rate of the slow component of $Na_v1.4/1.8C$ inactivation was significantly decreased at all voltages, but the rate of the fast component was unchanged (Fig. 4C and D). Interestingly $Na_v1.4/1.8C$ inactivated as quickly as $Na_v1.4$ but not completely. The existence of non-inactivated current of $Na_v1.8$ and $Na_v1.4/1.8C$ is consistent with the appearance of a non-inactivated fraction in the steady-state inactivation studies.

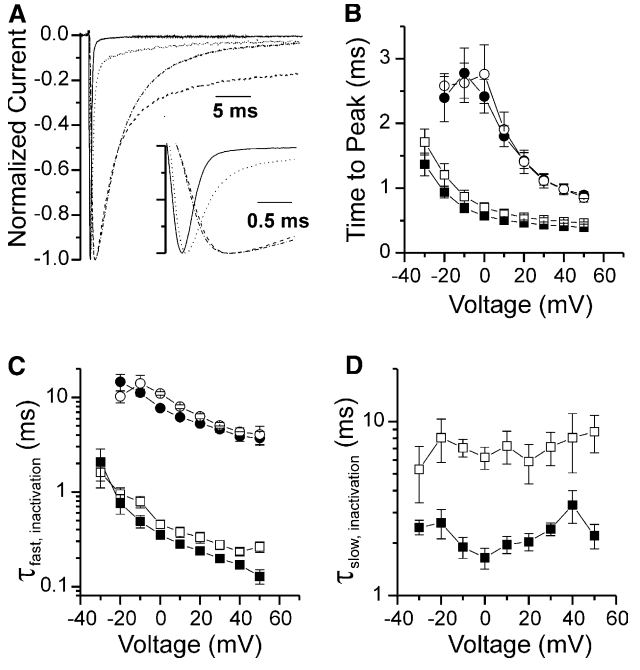

Fig. 4. Kinetics of activation and inactivation. (A) Mean currents of $Na_v1.4$ (—), $Na_v1.8$ (--), $Na_v1.4/1.8C$ (···) and $Na_v1.8/1.4C$ (-·-) recorded during a 40-ms step depolarization to +30 from −120 mV of holding potential. Inset in (A) represents re-scaled mean currents. (B) Time to peak as a function of test potentials for $Na_v1.4$ (■; $n = 6$), $Na_v1.4/1.8C$ (□; $n = 6$), $Na_v1.8$ (●; $n = 8$) and $Na_v1.8/1.4C$ (○; $n = 6$). The fast ($\tau_{fast,inactivation}$) and slow ($\tau_{slow,inactivation}$) components of inactivation decay as a function of test potentials are shown in (C) and (D), respectively.

4. Discussion

The C-terminal polypeptide of sodium channels contain multiple sequence motifs which play a role in channel trafficking [12–14,26]. The presence of the ubiquitin ligase binding motif PXY in the C-terminus of $Na_v1.8$ but not $Na_v1.4$ suggests a molecular mechanism for the rapid removal of $Na_v1.8$ and $Na_v1.4/1.8C$ from the cell surface which results in the small amplitude of their currents. Indeed, the $Na_v1.8$ current in *Xenopus* oocytes is reduced by the co-expression of Nedd4-2 [26], similar to the effect of Nedd4 on $Na_v1.5$ [14]. Thus, a putative Nedd4 variant which binds specifically to $Na_v1.8$ could account for the small current amplitude in the HEK 293 cells ([9,10] and our unpublished data). However, disruption of this motif in $Na_v1.8$ did not rescue a robust current in HEK 293 cells [10]. Thus Nedd4-mediated channel internalization is not sufficient to explain the small current amplitudes of $Na_v1.8$ in non-neuronal heterologous expression systems.

The presence of cell-specific factors which regulate $Na_v1.8$ density at the neuronal cell membrane remains a distinct possibility. While the co-expression of $Na_v1.8$ and the auxiliary β1 or β3 subunits increased the current density in *Xenopus* oocytes [27,28], the co-expression of these subunits with $Na_v1.8$ in HEK 293 cells did not increase the current density [9,10]. Because mutations in the IQ motif in the C-terminus of $Na_v1.4$, which disrupt the binding of calmodulin, result in a total loss of the $Na_v1.4$ current [21], calmodulin is considered a candidate partner to deliver $Na_v1.8$ to the cell surface. However, we have recently shown in a biochemical pull-down assay that, in contrast to $Na_v1.4$, the $Na_v1.8$ C-terminus does not bind calmodulin [21]. While the sequence of the IQ motif in $Na_v1.8$ is conserved and is predicted to bind calmodulin, the presence of tryptophan (W1849) instead of arginine (R) just upstream of the IQ motif may disrupt calmodulin binding. W1849 of $Na_v1.8$ is analogous to the R1902C mutation in $Na_v1.2$ which significantly reduces the affinity of the interaction of the channel with Ca^+-bound calmodulin [29]. Surprisingly, the W1849R mutation of $Na_v1.8$ did not elevate the $Na_v1.8$ currents in HEK 293 cells [10]. Although auxiliary β-subunits, calmodulin and Nedd4 may contribute to $Na_v1.8$ trafficking, their role is not rate-limiting.

The LQT mutation D1790G in the membrane proximal, acidic-rich sequence of the C-terminus of $Na_v1.5$ causes a depolarizing shift in activation, providing evidence for a role of the C-terminus in regulating activation [18,30]. The $V_{1/2}$ of activation of the $Na_v1.4/1.8C$ chimera is depolarized by 12 mV compared to $Na_v1.4$, while the $V_{1/2}$ of inactivation was not affected, suggesting a role of the C-terminus of $Na_v1.8$ in the channel's activation. Surprisingly, the $Na_v1.8/1.4C$ chimera did not show a change in either activation or inactivation. Chimeras $Na_v1.2/1.5C$ and $Na_v1.5/1.2C$ [23], and $Na_v1.4/1.5C$ and $Na_v1.5/1.4C$ [22] show a difference in voltage-dependence of inactivation but not activation, compared to parent channels. While the D1790G substitution in the C-terminus of $Na_v1.5$ causes a depolarizing shift of activation [18,30], an aspartic acid (D) is present at the analogous position in $Na_v1.8$ C-terminus. Therefore, an alternative mechanism might be responsible for this effect in the $Na_v1.4/1.8C$ chimera.

$Na_v1.8$ produced a sustained current after a 40 ms depolarization of ND7/23 cells (Fig. 4A). The exchange of the C-terminus of $Na_v1.8$ with that of $Na_v1.4$ ($Na_v1.8/1.4C$) attenuated the sustained current, and exchange of $Na_v1.4$ C-terminus with that of $Na_v1.8$ produced a sustained current under similar recording conditions (Fig. 4A). This is reminiscent of the sustained current reported for a deletion mutation of $Na_v1.5$ where the channel is truncated just upstream of the IQ motif in the C-terminus [31]. The authors attributed the sustained current to the loss of an interaction between the C-terminus and the fast inactivation particle. It is possible that the proposed interaction of Nav1.8 C-terminus with the inactivation particle is weaker than that of $Na_v1.4$ which results in the sustained current in the ND7/23 cells.

We show here that exchanging the C-termini of $Na_v1.4$ and $Na_v1.8$ channels causes significant changes in current density and gating properties of the channels. While analysis of steady-state inactivation shows that $Na_v1.8$ and $Na_v1.4/1.8C$ but not $Na_v1.4$ produce a non-inactivated current in ND7/23 cells, $Na_v1.8$ does not produce a sustained current in native DRG neurons [2,4,11], suggesting the presence in DRG neurons of factor(s) that is not present in ND7/23 cells or DRG-specific post-translational modification of the channel. Thus, the C-terminus of $Na_v1.8$ appears to play a significant role in enhancing the current density and the modulation of channel gating, including voltage-dependence of activation, and in producing a non-inactivating current depending on the cell background.

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