The response of Na\textsubscript{v}1.3 sodium channels to ramp stimuli: multiple components and mechanisms

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Abstract

Nav1.3 voltage-gated sodium channels have been shown to be expressed at increased levels within axotomized dorsal root ganglion (DRG) neurons and within injured axons within neuromas and have been implicated in neuropathic pain. Like a number of other sodium channel isoforms, Nav1.3 channels produce a robust response to slow ramplike stimuli. Here we show that the response of Nav1.3 to ramp stimuli consists of two components: an early component, dependent upon ramp rate, that corresponds to a window current that is dependent upon closed-state inactivation, and a second component at more depolarized potentials that is correlated with persistent current which is detected for many tens of milliseconds after the start of a depolarizing pulse. We also assessed the K354Q Nav1.3 epilepsy-associated mutant channel, which is known to display an enhanced persistent current, and demonstrate a strong correlation with the second component of the ramp response. Our results show that a single sodium channel isoform can produce a ramp response with multiple components, reflecting multiple mechanisms, and suggest that the upregulated expression of Nav1.3 in axotomized DRG neurons and enhanced ramp current in K354Q mutant channels can contribute in several ways to hyperexcitability and abnormal spontaneous firing that contribute to hyperexcitability disorders such as epilepsy and neuropathic pain.
Nav1.3 sodium channels are upregulated within DRG neurons after axotomy (Black et al. 1999; Waxman et al. 1994) and other forms of peripheral nerve injury (Dib-Hajj et al. 1999) and accumulate within the injured tips of axons within experimental and human neuromas (Black et al. 1999; Black et al. 2008) where ectopic impulses underlying neuropathic pain are generated. Like a number of other sodium channel isoforms Nav1.7: (Cummins et al. 1998); Nav1.6: (Herzog et al. 2003), Nav1.3 channels produce a robust depolarizing response to slow ramp-like stimuli (Cummins et al. 2001; Cummins and Waxman 1997). Interestingly, spontaneously firing DRG neurons exhibit slow ramp-like depolarizations during the interspike interval (Estacion et al. 2011; Faber et al. 2012). Nav1.3 channels have also been noted to produce a persistent current which is detectable for many tens of milliseconds after the onset of a depolarizing pulse (Chen et al. 2000; Lampert et al. 2006; Sun et al. 2007).

The tetrodotoxin (TTX)-sensitive current evoked by slow ramp stimuli has alternatively been termed “persistent” or “ramp current” (Fleidervish and Gutnick 1996; Khaliq and Bean 2010; Pennartz et al. 1997; Taddese and Bean 2002). While in many cases the ramp current is tetrodotoxin (TTX)-sensitive and thus attributable to sodium channels, its underlying basis remains incompletely understood. Multiple mechanisms including a window current due to the overlap between activation and steady-state inactivation, a slowly-inactivating or persistent mode of channel gating, or generation by a separate channel isoform have all been suggested (Crill 1996; Kiss 2008). In many studies, ramp currents have been elicited and studied in intact neurons, so a contribution from multiple isoforms of sodium channels cannot be excluded.

In this study, we examined the properties of the currents evoked by ramp stimuli and the persistent currents recorded at the end of depolarizing pulses from a single isoform of heterologously expressed sodium channel, Nav1.3-WT channels. Using ramps of varying depolarization rates (1.2mV/msec to 0.2mV/msec), we could separate the ramp-evoked inward current produced by this single
sodium channel isoform into two peaks. We show that the two components of the ramp responses of Nav1.3 channels are produced by distinct mechanisms. In comparison, we assess the ramp response of the K354Q Nav1.3 mutant channel which was identified in a patient with cryptogenic pediatric partial epilepsy (Holland et al. 2008) and shown by voltage-clamp to increase persistent current (Estacion et al. 2010; Holland et al. 2008). Our results show that a single sodium channel isoform can produce a ramp response with multiple components, reflecting multiple mechanisms, and suggest that the upregulated expression of Nav1.3 in axotomized DRG neurons and the enhanced ramp response of K354Q can contribute in many ways to hyperexcitability and abnormal spontaneous firing that contribute to neuropathic pain.
Methods

Plasmid and transient transfection:

The rat Na\textsubscript{v}1.3 insert was cloned into a modified mammalian expression vector and converted to tetrodotoxin-resistant form (TTX-R, Na\textsubscript{v}1.3\textsubscript{R}) by the Y384S substitution which is useful for isolating the current when expressed in neuronal cells (Cummins et al. 2001). The K354Q mutation was introduced into rNa\textsubscript{v}1.3\textsubscript{R} using QuickChange XL II site-directed mutagenesis (Stratagene, La Jolla, CA). HEK293 cells, grown under standard culture conditions (5% CO\textsubscript{2}, 37°C) in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, were transiently-transfected with plasmids containing β1-IRES-CD4 and β2-IRES-GFP (Lossin et al. 2002) and either rNav1.3\textsubscript{R} (referred to as WT hereinafter) or the mutant channel rNav1.3\textsubscript{R}/K354Q (referred to as K345Q hereinafter) using Lipofectamine2000 (Invitrogen, Carlsbad, CA) with a stoichiometry of plasmids of 1:1:5 by mass.

Electrophysiology

Whole-cell voltage-clamp recordings were obtained as previously described (Estacion et al. 2010) using the following solutions. The extracellular solution contained (in mM): 140 NaCl, 3 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, and 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm with dextrose). The pipette solution contained (in mM): 135 Cs-Aspartate, 10 NaCl, 2 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{2}, 1.1 EGTA (pCa=8), 10 HEPES, pH 7.2 with CsOH (adjusted to 310 mOsm with dextrose). Patch-pipettes had a resistance of 1-3 MΩ when filled with pipette solution. The junction potential of 16 mV (calculated by JPcalc, CLAMP software) was compensated by setting holding potential during the seal test period to -16 mV. Once the seal had formed, these two solutions were no longer in contact and the applied potential was correct. Upon achieving whole-cell recording configuration, pipette and cell capacitance were manually minimized.
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using Axopatch 200B (Molecular Devices, Union City, CA) compensation circuitry. To reduce voltage
errors, 80-90% series resistance and prediction compensation were applied. Cells were excluded from
analysis if the predicted voltage error exceeded 3 mV. Recorded currents were digitized using pCLAMP
software (version 10) and a Digidata 1440A interface (Molecular Devices) at 50 kHz after passing
through a low-pass Bessel filter setting of 10 kHz. Linear leak and residual capacitance artifacts were
subtracted out using the P/N method (Clampex software). Sodium current recordings were initiated after
a 5 minute equilibration period once whole-cell configuration was achieved.

Data analysis was performed using Clampfit (Molecular Devices) or Origin (Microcal Software,
Northampton, MA). To generate activation curves, cells were held at -100 mV and stepped to -80 to
+50 mV in 5 mV increments for 100 msec. Peak inward currents from activation protocols were
converted to conductance values using the equation, \( G = \frac{I}{(V_m - E_{Na})} \), where \( G \) is conductance, \( I \) is peak
inward current, \( V_m \) is the membrane potential step used to elicit the response and \( E_{Na} \) is the sodium
reversal potential (determined for each cell using the x-axis intercept of a linear fit of peak inward current
responses). Conductance data were normalized by maximum conductance and fit with a Boltzmann
equation of the form \( G = G_{min} + (G_{max} - G_{min})/(1 + \exp[(V_{1/2} - V_m)/k]) \), where \( V_{1/2} \) is the activation midpoint
and \( k \) is slope factor. To generate steady-state fast-inactivation curves, cells were conditioned with 500
msec prepulses of -170 to -25 mV in 5 mV increments followed by a 50 msec test pulse to -20 mV. Peak
inward currents obtained from steady-state fast inactivation protocol were normalized by maximum
current amplitude and fit with a Boltzmann equation of the form \( I = I_{min} + (I_{max} - I_{min})/(1 + \exp[(V_m - V_{1/2})/k]) \), where \( V_m \) represents the inactivating pre-pulse membrane potential and \( V_{1/2} \) represents the
midpoint of inactivation. Persistent current was measured as the average current remaining during the last
10 msec of the 100msec activation pulses. The ramp stimulus protocol smoothly increases the command
potential starting at -100 mV and ending at +20mV over 600 msec (0.2mV/msec) and is repeated with
incrementally shorter durations to give the indicated ramp rates. The ramp-evoked current traces are post
acquisition filtered from 10 kHz down to 200 Hz to improve data display. Furthermore, nonspecific leak
currents indicated by a slope of the ramp traces over the range of -100mV to -70mV are removed by performing a linear leak subtraction based on the slope of this region. The amplitude and voltage of the peak inward ramp-evoked currents are identified by a 5-point averaging peak finding algorithm between pairs of user-defined cursors placed to bracket the two peaks observed in the ramp traces. Data are expressed as means ± standard error (SEM). Statistical significance was determined by Student’s t-test.
Results

Voltage-clamp recordings from HEK 293 cells transiently expressing rNaV1.3-WT channels

Whole-cell voltage-clamp recordings were performed on HEK 293 cells transiently expressing wild-type rNaV1.3R channels. The current traces elicited in response to the activation protocol of a representative cell are shown in Figure 1A. The currents elicited from the same cell in response to the fast-inactivation protocol are shown in Figure 1B. Both sets of traces indicate the presence of persistent current as defined by inward current remaining at the end of the stimulation pulse (100 msec for activation, 50 msec for fast-inactivation); an expansion of selected traces illustrating the persistent current is shown as an inset to panel A. The peak inward currents elicited by each stimulus are plotted as a function of the stimulus pulse voltage to generate an activation current versus voltage (I-V) curve (Figure 1C) and a fast-inactivation I-V curve (Figure 1D). The voltage-dependence of activation and fast-inactivation were examined by transforming the peak I-V curves into conductance versus voltage (G-V) curves as described in Methods. The activation and fast-inactivation G-V curves for this cell are normalized to Gmax and plotted together in Figure 1E. The persistent current analyzed from this cell is normalized to peak inward current and also plotted in red in Figure 1E. The shape and voltage-dependence of the persistent current in comparison to the activation and fast-inactivation curves are shown re-plotted with an expanded scale in Figure 1F. Notably, while there is a minor shoulder in the persistent current (arrow) within the voltage range of the window of overlap between activation and steady-state inactivation, the bulk of the persistent current falls outside of the voltage domain of the activation-inactivation window.

Ramp stimulus responses

Most of the Nav1.3-WT expressing cells exhibited a distinctive double inward peak in response to slow depolarizing ramp protocols although 3 of the 9 cells displayed only a small first peak which
Nav1.3 ramp currents

appeared as a shoulder rather than a well-defined peak. The response of the same cell illustrated in
Figure 1 to ramp stimuli with varying ramp rates is illustrated in Figure 2A. The ramp-evoked currents
clearly show two peaks. The first peak, which is elicited at more hyperpolarized potentials compared to
the second peak, is strongly reduced in size by slowing the ramp rate. The shape and voltage at peak for
the ramp-evoked current are compared to the persistent current measured for this cell in Figure 2B. Both
the ramp current and the persistent current were normalized to the maximal peak inward current and
plotted together for comparison (Figure 2B). The shape and voltage range for the persistent current do
not overlap with the first peak, but correspond more closely to the second peak of the ramp-evoked
current.

In the experiment shown in figure 2C, the current remaining at the end of the ramp protocol (-100
mV to +20 mV, 600 msec) was exposed to a mirror image slow hyperpolarization or “reverse ramp” (+20
mV to -100 mV, 600 msec) to determine whether either component could recover from inactivation and
also to assess whether either component of current deactivates. The reverse ramp current shows that none
of peak 1 current remains, suggesting that the peak 1 current does not recover from inactivation during the
reverse ramp, while a partial fraction of peak 2 current remains and exhibits deactivation.

Ramp response compared with persistent currents

The relationships between the two peaks of ramp-evoked current are examined in Figure 3. The
values of normalized peak amplitude and ramp voltage for the two peaks derived from the slow (0.2
mV/msec) ramp for a number of individual cells are plotted with a line linking the values corresponding
to each individual cell. In Figure 3A, the distribution of values derived from Nav1.3-WT expressing cells
is plotted. The average amplitude for peak 1 is $2.4 \pm 0.4\%$ occurring at $-37.3 \pm 1.2$ mV and the average
amplitude for peak 2 is $5.7 \pm 0.9\%$ occurring at $-17.4 \pm 1.0$ mV (n=9). The relationship between the
normalized amplitude of the second peak of the slow ramp evoked current and normalized persistent
current as measured at the end of sustained depolarizing pulses was evaluated for each of the cells
expressing Nav1.3-WT and shown in Figure 3B. The distribution of points was well described by a straight line indicating that these two parameters are highly correlated (adjusted R-square = 0.98).

Comparison to Nav1.3-K354Q mutation

As a comparison to Nav1.3-WT, we studied the K354Q Nav1.3 mutation, described in a patient with epilepsy (Holland et al. 2008) and shown by voltage-clamp to increase persistent current to 8.1% compared to 4.4% in WT Nav1.3 channels (Estacion et al. 2010). We examined the relationship between the activation and fast-inactivation conductances, and persistent current by plotting them together (Figure 4A) for a selected cell (similar as for a WT cell, Figure 1E). Similar to Nav1.3-WT cells, the persistent current of K354Q-expressing cells shows only a small shoulder over the voltage range for predicted window current (Figure 4B, shaded area and arrow) and is much larger at more depolarized membrane voltages. The ramp response of this cell to ramp stimuli with different ramp rates is shown in Figure 5A and it too shows two peaks. The first peak again shows a strong decrease as the ramp rate is slowed. The persistent current measured from this cell is plotted against the slow ramp (0.2 mV/msec) response in Figure 5B. Note the good correspondence of the persistent current and the second peak of the ramp response. As for wild-type Nav1.3, a reverse ramp evoked peak 2 current but no discernible peak 1 current from Nav1.3-K354Q expressing cells (Figure 5C).

In Figure 6A, the ramp-evoked peak values derived from Nav1.3-K354Q expressing cells are plotted. The average amplitude for peak 1 of the current evoked by the slow ramp is 2.6 ± 0.4% occurring at -38.5 ± 1.4 mV, and the average amplitude for peak 2 is 10.1 ± 2.1%, occurring at -17.2 ± 0.8 mV (n=12). Consistent with a substantial contribution of persistent current to the second peak of the ramp current, the average normalized peak 2 ramp current of K354Q channels (10.1 ± 2.1 %, n=12) was increased compared to WT expressing cells (5.7 ± 0.9 %, n=9, p=0.09). The distribution of points from cells expressing Nav1.3-K354Q mutant also shows a strong correlation between the second peak of the slow ramp-evoked response and the persistent current (Figure 6B).
Further support for the correlation of the second peak of the slow ramp evoked current to persistent current is seen when the ramp evoked currents from both WT and K354Q expressing cells are normalized and then averaged (Figure 7). Although on average the first peak smooths into a shoulder on the slow ramp evoked response, the response of WT and K354Q cells overlay each other during the voltage range of -60 mV through -35 mV. Over the voltage range for the second peak, however, the K354Q ramp response is approximately double that of the ramp response for WT Nav1.3.
In this study, we examined more closely the properties of the current evoked by slow ramp stimuli from a single sodium channel isoform, Nav1.3. Using ramps of varying depolarization rates (1.2 mV/msec to 0.2 mV/msec) to study Nav1.3-WT channels, we could separate the slow inward currents into two peaks. The first peak showed clear dependence on ramp rate. Since ramp rate determines whether the rate of depolarization is fast enough to activate current before inactivation develops due to depolarization in earlier parts of the ramp, it is likely that the first component of the ramp response is strongly modulated by closed-state inactivation (Cummins et al. 1998). Consistent with this interpretation, the first peak more closely corresponded to the window current predicted by the overlap of the voltage-dependence of activation and fast-inactivation. The second peak occurred at more depolarized potentials and appeared to be relatively insensitive to closed-state inactivation. Lampert et al. (2006) demonstrated that Nav1.3 channels can produce a persistent current that is seen as late as tens of msec in activation protocols. Sun et al. (2007) reported that Nav1.3 persistent current is both larger and shifted to more depolarized voltages than the predicted window current. The current density of this second inward ramp current was poorly correlated to transient inward peak. However, consistent with a major contribution of persistent current to the second component of the ramp response, the amplitude of the second ramp component was very well correlated with the current density of persistent current as measured at the end of long depolarization steps. Also suggesting a major contribution of persistent current to the second ramp component, this component of the ramp response was enhanced in the K354Q mutant Nav1.3 channel, which is known (Estacion et al. 2010) to display an enhanced persistent current.

In the literature, reversed ramps have been used to evaluate the persistence and voltage-dependence of deactivation of subthreshold inward currents (Astman et al. 2006; Dai and Jordan 2011; Kononenko et al. 2004; Theiss et al. 2007). If the response to the reverse ramp shows hysteresis, that also indicates continuing slow-inactivation for the subthreshold inward current. The response of Nav1.3 to the reverse ramp indicates that the second ramp component exhibits continued voltage-dependent inactivation.
since the magnitude of the second peak is smaller in the reverse direction. The reverse ramp response also indicates a clear voltage-dependent deactivation of the second peak and reveals a clear loss of current in the voltage range of the first peak.

Magistretti and Alonso (1999) have commented on the definition of persistent sodium current as measured with voltage-ramps, and note that, depending on ramp speed, multiple kinetic components can be distinguished. Importantly, however, their studies were carried out on dissociated neurons, which likely express multiple isoforms of sodium channels. Whether these components derive from a single channel isoform or from different isoforms with different kinetics is not clear. In our experiments we were able to distinguish multiple components of the ramp response from a single sodium channel isoform, Nav1.3, expressed in a heterologous expression system.

The identity and properties of persistent inward current have been evaluated in many ways. Although persistent inward currents may also be carried by calcium channels (Khaliq and Bean 2010; Perez-Reyes 2003) or non-voltage-gated sodium permeable channels (Khaliq and Bean 2010), many studies in neurons have implicated voltage-gated sodium channels as contributing to persistent or ramp currents. Some studies e.g. (Del Negro et al. 2002) and (Agrawal et al. 2001) refer to ramp responses as persistent currents. Previous studies in intact neurons have demonstrated contributions of window current and persistent current to the ramp response. Consistent with our observations on the first component of the ramp response, Fleidervish and Gutnick (1996) presented evidence indicating that in neocortical neurons, the ramp-evoked inward current through voltage-gated sodium channels is modulated by closed-state inactivation. However, Magistretti and Alonso (1999), in a study on entorhinal cortex neurons, observed that the current evoked by ramp stimuli had an amplitude and voltage-dependence that could not be accounted for by the window current.

Our description of the two components of the ramp current is not without precedent. For example in a study on medullary neurons Rybak et al. (2003) demonstrated two components of ramp current.
Many recordings of ramp current from neurons, however, explore the voltage range between -80mV and about -30mV (Astman et al. 2006; Theiss et al. 2007) since more depolarized voltages evoke large outward currents as voltage-gated potassium channels activate, confounding direct observation of an additional peak of inward ramp current unless the potassium channels are inhibited (Rybak et al. 2003, Zeng et al. 2005). Our results, using heterologous expression into HEK cells which have little endogenous voltage-gated currents, allowed demonstration of two components of ramp current from a single sodium channel isoform, Nav1.3. Further studies will be needed to determine whether other sodium channel isoforms similarly produce ramp currents with multiple components.

It has been suggested (French et al. 1990) that persistent sodium current including persistent currents evoked by ramp stimuli (Magistretti and Alonso 1999) may be produced by a specialized sodium channel with biophysical characteristics including late channel (re) openings different from those of the fast transient channels. Our results indicate that a single sodium channel isoform, Nav1.3, can generate at least two mechanistically distinct components of ramp-evoked responses, as well as fast-transient current as demonstrated by (Cummins et al. 2001). It remains to be determined whether these functionally distinct currents are produced by differentially modulated Nav1.3 channels or by different gating modes of the channel. However, while kinetic gating properties of Nav1.3 are known to be modulated by cell type-specific factors (Chen et al. 2000; Cusdin et al. 2010), it is notable that the three currents can be discerned in a heterologous non-neuronal expression system. Possible mechanisms for the second persistent component of the ramp response include direct modulation of the channels by G-protein mediated pathways (Mantegazza et al. 2005; Pinet et al. 2008) or cytokine-induced phosphorylation (Binshtok et al. 2008) or by association with regulatory beta subunits (Cusdin et al. 2010). The formation of an enhanced population of persistent channels by the K354Q mutant, however suggests the possibility of some other mechanisms, since the mutation substitutes an amino acid within the extracellular linker between the S5 helix and the pore-loop motif of domain I. The absence of correlation between the fraction of true persistent current and peak inward current suggests that persistent current may not be due
to an altered gating scheme for all channels, but is more consistent with the presence of a subpopulation of channels that are able to maintain sustained openings. Parri and Crunelli (1998) modulated the fraction of persistent current by removing fast-inactivation with papain and showed that the voltage-dependence of the newly formed \( I_{\text{nap}} \) was identical to the \( I_{\text{nap}} \) found in their neurons.

Our results show that a single sodium channel isoform, Nav1.3 can contribute multiple components to the ramp response. Whether the two components of ramp-evoked inward current play different roles in regulating excitability of neurons is currently unknown. The expression of Nav1.3 within DRG neurons is increased after axonal injury (Black et al. 1999; Dib-Hajj et al. 1999; Waxman et al. 1994). Moreover, Nav1.3 is known to accumulate in the injured tips of axons within experimental (Black et al. 1999) and human neuromas (Black et al. 2008), where ectopic impulses are generated. The more hyperpolarized component of ramp current from Nav1.3 is more likely to be involved in altering threshold. The more depolarized second component of ramp current may, in contrast, play a role in interspike interval pacemaking when neurons or their axons are depolarized after injury. A pharmacological approach to address these hypotheses may be possible since Sun et al. (2007) report that the concentrations of topiramate required to block Nav1.3 persistent current is much lower than the concentrations required to reduce availability via a drug-induced shift of fast-inactivation voltage-dependence. Interestingly, spontaneously firing DRG neurons exhibit interspike interval waveforms which mimic ramp stimuli (Estacion et al. 2011; Faber et al. 2012). Moreover, hippocampal neurons expressing the K354Q Nav1.3 mutant channel, identified in a patient with epilepsy (Holland et al. 2008), display bursts of action potentials with interspike waveforms with a ramp-like trajectory (Estacion et al. 2010). We suggest that the current evoked by slow ramp-like stimuli in WT Nav1.3 channels in axotomized DRG neurons, and by K354Q mutant channels in central neurons, contribute to hyperexcitability, thus playing a role in both neuropathic pain and epilepsy.
References


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Figure Legends

**Figure 1** Voltage-clamp of Nav1.3-WT channels.

(A) Superimposed traces recorded from HEK293 cells expressing Nav1.3-WT channels in response to
the activation stimulation protocol (100 msec duration pulses). Persistent current is seen as the current
remaining at the end of the 100-msec duration stimulus. Selected traces are shown in an expanded view
(inset). (B) Superimposed traces illustrating the response to the fast-inactivation protocol. (C) The
peak currents for each trace in panel A were measured to obtain the activation current-voltage (I-V)
relation as described in the Methods. (D) The peak responses to the fast-inactivation protocol in panel B
were analyzed to obtain the voltage-dependence of fast-inactivation as described in the Methods. (E)
The G-V curves for both activation ($V_{1/2} = -24.3$ mV, slope = 7.6, black circles) and fast-inactivation ($V_{1/2} = -65.8$ mV, slope = 5.9, blue squares) were normalized to obtain the plotted data. In addition, the
normalized persistent current (red diamonds) is also plotted. (F) The data in panel E are re-plotted with
expanded scales to better visualize the relationship between the activation and fast-inactivation curves to
the persistent current measured at the end of a 100 msec depolarizing pulse. The grey area indicates the
voltage range and approximate shape of the predicted window current.

**Figure 2:** Nav1.3-WT channels exhibit two components of ramp response

(A) The currents evoked during a smoothly increasing voltage ramp from -100 mV to +20 mV over 600
msec (black line) are shown from the same Nav1.3-WT expressing cell analyzed in Figure 1. Ramp
response currents recorded using faster ramp rates (see stimulus protocol inset) display a first peak which
occurs at more hyperpolarized potentials and varies in size with ramp rate, decreasing as the ramp rate
slows. The second peak is relatively insensitive to ramp rate, consistent with a persistent current. (B)
Superimposition of the persistent current measured at the end of 100-msec stimulus pulses and the slow
ramp response (0.2 mV/msec) recorded from the same cell. Both the ramp data and the persistent current
data have been normalized to peak current. The filled symbols are the persistent current recorded from this cell (Figure 1F) using 100 msec stimulus pulses and show correspondence to the second slow ramp peak (grey line, from Panel A, 0.2 mV/msec). (C) Currents recorded in response to a ramp protocol that reverses direction as shown in the inset. The current recorded to the rising phase of the ramp stimulus is shown in black and the current recorded to the falling phase of the ramp stimulus is shown in red. These traces are from a different cell than shown in panel A and no leak subtraction was performed on the data traces.

Figure 3 Average peak amplitude and voltage at peak for Nav1.3-WT inward ramp responses

(A) The peak amplitude and the voltage at peak for the two components of the slow ramp (0.2 mV/msec) response are plotted for 9 cells expressing Nav1.3-WT currents. The normalized amplitude and membrane voltage of the two peaks are plotted and the relationship between the first peak (black circles) and the second peak (red circles) for each cell are indicated by the connecting lines. The average values are shown as colored diamonds with both x and y error bars (SEM). (B) There was a good correlation (adjusted R-square = 0.98) between the normalized amplitude of the second peak of the slow ramp response and the normalized persistent current measured for each Nav1.3-WT expressing cell.

Figure 4 Voltage-clamp of Nav1.3-K354Q channels.

(A) The G-V curves for both activation ($V_{1/2} = -33.8$ mV, slope = 7.3, black circles) and fast-inactivation ($V_{1/2} = -74.8$ mV, slope = 6.9, blue squares) for a representative Nav1.3-K254Q expressing cell obtained by analyzing the activation and fast-inactivation data as described in Figure 1. In addition, the normalized persistent current (red diamonds) is also plotted. (B) The data in panel A are re-plotted with expanded
scales to better visualize the relationship between the activation and fast-inactivation curves and the
persistent current measured at the end of 100 msec depolarizing pulses.

**Figure 5** Nav1.3-K354Q channels exhibit two components of ramp response

(A) The currents evoked during a smoothly increasing voltage ramp from -100 mV to +20 mV over 600 msec (black line) are shown from the same Nav1.3-K354Q expressing cell analyzed in Figure 4. Ramp response currents recorded using faster ramp rates (see inset) indicate that the first peak, which occurs at more hyperpolarized potentials, varies in amplitude with ramp rate, decreasing as the ramp rate slows. The second peak is less sensitive to ramp rate, consistent with a persistent current. (B) The relationship between persistent current measured at the end of 100-msec stimulus pulses is plotted against the slow ramp response (0.2 mV/msec) recorded from the same cell. Both the ramp data and the persistent current data have been normalized to peak current. The filled symbols are the persistent current recorded from this cell (Figure 4B) using 100 msec stimulus pulses and shows correspondence to the second slow ramp peak (grey line, from Panel A, 0.2 mV/msec). (C) Currents recorded in response to a ramp protocol that reverses direction as shown in the inset to figure 2C. The current recorded to the rising phase of the ramp stimulus is shown in black and the current recorded to the falling phase of the ramp stimulus is shown in red. These traces are from a different cell than shown in panel A and no leak subtraction was performed on the data traces.

**Figure 6** Average peak amplitude and voltage at peak for K354Q inward ramp responses

(A) The peak and the voltage at peak for the two components of the slow ramp (0.2 mV/msec) response are plotted for 12 cells expressing Nav1.3-K354Q currents. The normalized amplitude and membrane voltage of the two peaks are plotted and the relationship between the first peak (black circles) and the
second peak (red circles) for each cell are indicated by connecting lines. Average values are shown as colored diamonds with both x and y error bars (SEM). (B) There was a good correlation (adjusted R-square = 0.88) between the normalized amplitude of the second peak of the slow ramp response and the normalized persistent current measured for each Nav1.3-K354Q expressing cell.

**Figure 7 Comparison of averaged WT and K354Q responses**

The currents evoked by the 0.2 mV/msec slow ramp stimulus for WT expressing cells and for K354Q expressing cells were normalized to peak current and then averaged together. The average response from WT expressing cells (n=9) is shown in black, while the average response from K354Q expressing cells is shown in red. In this analysis, the first peak appears as a shoulder compared to the second peak. The average normalized amplitudes of the first and second peaks are shown in the inset.
A

Fraction of peak current vs. ramp voltage (mV) for different current rates: 0.2 mV/msec, 0.24 mV/msec, 0.3 mV/msec, 0.4 mV/msec, 0.6 mV/msec, 1.2 mV/msec.

B

Fraction of peak current vs. voltage (mV) for HEK + rNav1.3r-WT and norm persistent.

C

Current (pA) vs. ramp voltage (mV) for forward and reverse ramps with 20 mV and 600 msec.
Slow ramp peak responses
Nav1.3-WT

Normalized current

-0.12 -0.10 -0.08 -0.06 -0.04 -0.02 0.00

0.00 0.02 0.04 0.06 0.08 0.10 0.12

mV

Ramp peak2

Linear Fit R-Square = 0.98

Ramp peak2 vs. persistent current

Linear Fit R-Square = 0.98
Slow ramp peak responses
Nav1.3-K354Q

A

B

Linear Fit R-Square = 0.88
Average normalized ramps

-120 -100 -80 -60 -40 -20 0 20 40

-0.12 -0.10 -0.08 -0.06 -0.04 -0.02 0.00 0.02

avg WT
avg K354Q

peak 1 peak 2