Ranolazine block of human Na\textsubscript{1.4} sodium channels and paramyotonia congenita mutants

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Abbreviations: IC\textsubscript{50}, drug concentration that causes 50% block; hNa\textsubscript{1.4}, the human voltage-gated Na\textsuperscript{+} channel subtype 1.4; HEK, human embryonic kidney

The antianginal drug ranolazine exerts voltage- and use-dependent block (UDB) of several Na\textsuperscript{+} channel isoforms, including Na\textsubscript{1.4}. We hypothesized that ranolazine will similarly inhibit the paramyotonia congenita Na\textsubscript{1.4} gain-of-function mutations, R1448C, R1448H and R1448P that are associated with repetitive action potential firing. Whole-cell Na\textsuperscript{+} current (I\textsubscript{Na}) was recorded from HEK293 cells expressing the hNa\textsubscript{1.4} WT or R1448 mutants. At a holding potential (HP) of -140 mV, ranolazine exerted UDB (10 Hz) of WT and R1448 mutations (IC\textsubscript{50} = 59–71 \mu M). The potency for ranolazine UDB increased when the frequency of stimulation was raised to 30 Hz (IC\textsubscript{50} = 20–27 \mu M). When the HP was changed to -70 mV to mimic the resting potential of an injured skeletal muscle fiber, the potency of ranolazine to block I\textsubscript{Na} further increased; values of ranolazine IC\textsubscript{50} for block of WT, R1448C, R1448H and R1448P were 3.8, 0.9, 6.3 and 0.9 \mu M, respectively. Ranolazine (30 \mu M) also caused a hyperpolarizing shift in the voltage-dependence of inactivation of WT and R1448 mutations. The effects of ranolazine (30 \mu M) to reduce I\textsubscript{Na} were similar (~35% I\textsubscript{Na} inhibition) when different conditioning pulse durations (2–20 msec) were used. Ranolazine (10 \mu M) suppressed the abnormal I\textsubscript{Na} induced by slow voltage ramps for R1448C channels. In computer simulations, 3 \mu M ranolazine inhibited the sustained and excessive firing of skeletal muscle action potentials that are characteristic of myotonia. Taken together, the data indicate that ranolazine interacts with the open state and stabilizes the inactivated state(s) of Na\textsubscript{1.4} channels, causes voltage- and use-dependent block of I\textsubscript{Na} and suppresses persistent I\textsubscript{Na}. These data further suggest that ranolazine might be useful to reduce the sustained action potential firing seen in paramyotonia congenita.

Introduction

Gain-of-function mutations in SCN4A, the gene encoding the pore-forming alpha (\alpha) subunit of the human skeletal muscle voltage-gated Na\textsuperscript{+} channel type IV (hNa\textsubscript{1.4}) have been associated with non-dystrophic skeletal muscle pathologies including paramyotonia congenita,\textsuperscript{1} hyperkalaemic periodic paralysis,\textsuperscript{2} and potassium-aggravated myotonia\textsuperscript{3} (reviewed in refs. 4–6). Missense mutations in the outermost arginine (R1448C, R1448H, R1448P, R1448S and R1448L) of the domain IV voltage sensor (DIVS4) of hNa\textsubscript{1.4} have been identified in patients with paramyotonia congenita. Mutation of this positively charged arginine results in the general set of biophysical defects associated with paramyotonia congenita mutants, including slowed whole-cell current inactivation, reduced voltage-dependence of inactivation, enhanced rate of the recovery from inactivation and increased tetrodotoxin (TTX)-sensitive persistent (late) I\textsubscript{Na}.\textsuperscript{7,12} These features lead to enhanced Na\textsuperscript{+} entry into the cell causing depolarization of the resting membrane potential and sustained action potential firing which results in hyperexcitability (myotonia) or inexcitability (paralysis).\textsuperscript{10,13,14} Current therapies for patients with paramyotonia congenita include the Na\textsuperscript{+} channel blocking agents flecainide and mexiletine, but use of these drugs is limited by their side-effects.\textsuperscript{15}

Ranolazine is an anti-anginal drug that blocks cardiac (Na\textsubscript{1.5}) late I\textsubscript{Na} within its therapeutic concentration range (2–10 \mu M).\textsuperscript{16,17} Ranolazine exerts a state- and use-dependent block (UDB) of peak I\textsubscript{Na} in skeletal muscle (Na\textsubscript{1.4}), cardiac (Na\textsubscript{1.5}) and neuronal (Na\textsubscript{1.1}, Na\textsubscript{1.7} and Na\textsubscript{1.8}) Na\textsuperscript{+} channel isoforms.\textsuperscript{18–21} Furthermore, ranolazine exerts UDB of peak and late I\textsubscript{Na} of the long QT syndrome type 3 (LQT3) SCN5A (Na\textsubscript{1.5}) gain-of-function mutation, R1623Q. Similarly, ranolazine blocks late I\textsubscript{Na} evoked by Na\textsubscript{1.1} mutations associated with genetic epilepsy syndromes. Gene sequence alignment shows that the paramyotonia congenita SCN4A gain-of-function mutations R1448C, R1448H and R1448P correspond to the SCN5A gain-of-function mutation, R1623Q within the voltage-sensing DIVS4. These observations lead us to hypothesize that ranolazine, in addition to blocking late I\textsubscript{Na}, also exerts a state- and use-dependent block of the SCN4A gain-of-function R1448 mutant channel current. Using HEK293 cells transiently expressing either hNa\textsubscript{1.4} WT or R1448 mutant channels, we demonstrated...
that ranolazine (1) stabilizes the inactivated state(s) of Na\textsubscript{v1.4} channels, (2) exerts state- and use-dependent block of I\textsubscript{Na}, (3) causes a hyperpolarizing shift in the voltage-dependence of inactivation, (4) accelerates the rate of I\textsubscript{Na} whole-cell current decay, (4) slows the recovery from inactivation of I\textsubscript{Na}, and (5) suppresses I\textsubscript{Na} generated during slow voltage ramps. Computer simulations of myotonic skeletal muscle electrical activity using a generalized Markov model of Mutant-Nav1.4 and selective binding of ranolazine to the inactivated state of the channel support the experimental findings. The data suggest that ranolazine may decrease rapid firing of action potentials characteristic of skeletal muscle fibers expressing gain-of-function R1448 mutant Na\textsuperscript{+} channels.

**Results**

**Ranolazine block of hNa\textsubscript{1.4} WT and R1448 mutant Na\textsuperscript{+} channels.** Normalized I\textsubscript{Na} recorded from HEK293 cells transiently expressing either hNa\textsubscript{1.4} WT, R1448C, R1448H or R1448P mutant Na\textsuperscript{+} channels as indicated along with the voltage-clamp protocol are shown in Figure 1A. As reported previously, fast inactivation for each R1448 mutant channel was impaired as evident by the significantly longer time constants for whole-cell current decay compared to WT. Values of \( \tau_{fast} \) and \( \tau_{slow} \) for WT were 0.43 ± 0.02 and 4.2 ± 0.6 msec (n = 10), respectively, whereas values of \( \tau_{fast} \) and \( \tau_{slow} \) for R1448C, R1448H and R1448P mutant channels as compared to WT. Data represent mean ± SEM. *indicates p < 0.05 vs. WT.

Ranolazine, at a concentration of 100 \( \mu \)M, caused minimal tonic block of peak I\textsubscript{Na} of WT (0.54 ± 0.35%; n = 8), R1448C (6.2 ± 5.4%; n = 11), R1448H (1.3 ± 1.3%; n = 8), but modest block of R1448P (21.4 ± 9.4%; n = 7). A high concentration of ranolazine (500 \( \mu \)M) caused block of WT and R1448C peak I\textsubscript{Na} by 19.6 ± 2.3% (n = 5) and 41.4 ± 4.4% (n = 7), respectively (Fig. 1C). A slowing of the decay rate of whole-cell I\textsubscript{Na} is a common feature of paramyotonia congenita mutations and we tested the effect of ranolazine on the decay rate of WT and R1448C. Ranolazine (500 \( \mu \)M) also significantly accelerated the whole-cell current decay rate of R1448C I\textsubscript{Na} for both fast (\( \tau_{fast} \)) and slow (\( \tau_{slow} \)) components (Fig. 1D, n = 7, p < 0.05 compared to control). Ranolazine had no effect on the decay rate of WT whole-cell I\textsubscript{Na} (n = 5).

Because paramyotonia congenita is characterized by repetitive action potential firing, we determined UDB by ranolazine using a series of voltage-step train depolarizations. First, steps to -20 mV from a holding potential (HP) of -140 mV were applied...
amplitude of WT and mutant channel $I_{Na}$ at both frequencies (at 10 Hz: 7.5–11.3%, $n = 4–8$; and at 30 Hz: 11.3–16.8%, $n = 5–7$). At 10 Hz, ranolazine (0.1–100 μM) caused a UDB of $I_{Na}$ with half-maximal inhibitory concentrations (IC$_{50}$ values) ranging from 59–71 μM for all tested channels (Table 1).

Increasing the stimulation frequency to 30 Hz produced IC$_{50}$ values for UDB of 20–27 μM (Table 1).

**Figure 2.** Block of hNa$_{1.4}$ WT and R1448 mutant channels by ranolazine. (A) Shows the $I_{Na}$ recordings from WT and R1448C in the absence (control) and presence of 30 μM of ranolazine (Ran, 30 μM) at either a holding potential of -140 mV (left) or -90 mV (right) stimulated at 10 Hz. For each condition, the peak current amplitude evoked by pulse 40 was normalized to the peak current evoked by pulse 1 and plotted as a function of ranolazine concentration. The reduction in the whole-cell currents represents UDB by ranolazine. (B) Shows the concentration-response relationships of block of WT and R1448 mutants by ranolazine at HPs of -140 (■), -90 mV (●) and -70 mV (▲). IC$_{50}$ values and Hill coefficients are given in Table 1. Data represent mean ± SEM.

**Table 1.** Half-maximal inhibitory concentrations (IC$_{50}$) of use- and state-dependent block of hNa$_{1.4}$ WT and R1448 mutant Na$^+$ channels by ranolazine

| HP at -140 mV | 10 Hz | 30 Hz | WT | R1448C | R1448H | R1448P |
|---------------|-------|-------|----|--------|--------|--------|
| 63.9 ± 4.6    | 63.7 ± 5.4 | 58.7 ± 6.5 | 70.8 ± 4.8 |
| [0.77 ± 0.05] | [0.64 ± 0.04] | [0.80 ± 0.08] | [0.83 ± 0.05] | (n = 3–5) | (n = 3–5) | (n = 4) | (n = 3–4) |
| HP at -90 mV | 10 Hz | 18.1 ± 2.0 | 10.8 ± 1.7 | 17.4 ± 4.4 | 14.1 ± 4.7 |
| 3.8 ± 0.4    | 0.96 ± 0.18 | 6.3 ± 0.3 | 0.94 ± 0.11 |
| [0.70 ± 0.06] | [0.64 ± 0.08] | [0.93 ± 0.04] | [0.40 ± 0.03] | (n = 4–6) | (n = 5–6) | (n = 3) | (n = 3–4) |
| HP at -70 mV | 0.1 Hz | 3.8 ± 0.4 | 0.96 ± 0.18 | 6.3 ± 0.3 | 0.94 ± 0.11 |
| 3.8 ± 0.4    | 0.96 ± 0.18 | 6.3 ± 0.3 | 0.94 ± 0.11 |
| [0.70 ± 0.06] | [0.64 ± 0.08] | [0.93 ± 0.04] | [0.40 ± 0.03] | (n = 3–4) | (n = 3–4) | (n = 3–4) | (n = 6–8) |

HP, holding potential. The Hill coefficients are indicated in brackets. n is the number of cells per concentration tested.

either at 10 Hz (40 pulses of 40 msec duration) or 30 Hz (100 pulses of 20 msec duration) for WT, R1448C, R1448H and R1448P channels in the absence and presence of increasing concentrations of ranolazine (0.1–100 μM). Figure 2A shows representative currents recorded for WT and R1448C illustrating the UDB by 30 μM ranolazine at 10 Hz. Under control conditions (in absence of drug) there was minimal reduction in the amplitude of WT and mutant channel $I_{Na}$ at both frequencies (at 10 Hz: 7.5–11.3%, $n = 4–8$; and at 30 Hz: 11.3–16.8%, $n = 5–7$). At 10 Hz, ranolazine (0.1–100 μM) caused a UDB of $I_{Na}$ with half-maximal inhibitory concentrations (IC$_{50}$ values) ranging from 59–71 μM for all tested channels (Fig. 2B; Table 1). Increasing the stimulation frequency to 30 Hz produced IC$_{50}$ values for UDB of 20–27 μM (Table 1).
To mimic the resting membrane potential of a normal skeletal muscle fiber (~-84 mV), UDB experiments were repeated with a HP of -90 mV. A series of 40 depolarizing pulses to -20 mV at a frequency of 10 Hz was applied in the absence and presence of increasing concentrations of ranolazine (3–100 μM). Changing the HP to -90 mV decreased the IC50 values for ranolazine block to 10.8–18.1 μM (Fig. 2B and Table 1). To mimic the resting membrane potential of an injured skeletal muscle fiber (~-60 to -70 mV), a HP of -70 mV was used and the effect of ranolazine was tested at 0.1 Hz (Fig. 2B). Changing the HP to -70 mV further increased the potency of INa block by ranolazine with IC50 values between 1 and 6 μM (Fig. 2B and Table 1). These values are within the reported therapeutic concentration range of ranolazine in treatment of chronic angina (i.e., 2–10 μM).

Effects of ranolazine on the steady-state fast, intermediate and slow inactivation. Ranolazine caused a significant

Figure 3. Ranolazine enhances steady-state fast, intermediate and slow inactivation of hNaV1.4 WT and R1448C. Voltage-dependence of steady-state inactivation was assessed for WT (left) and R1448C (right) using inactivating prepulses of increasing duration for fast (100 msec), intermediate (1 sec) or slow (10 sec) inactivation processes (A–C, respectively). Steady-state inactivation was assessed in the absence of drug (■; control) or following the application of 30 μM ranolazine (□; Ran). Normalized peak INa for each condition was fit with a Boltzmann equation and fit parameters are provided in Table 2. Data represent mean ± SEM and insets represent voltage-clamp protocols.
hyperpolarizing shift in the $V_{1/2}$ of steady-state fast (Fig. 3A) and intermediate inactivation (Fig. 3B) of WT, R1448C, R1448H and R1448P without affecting slope factors (Table 2). The slope factors (k) obtained from Boltzmann fits of the steady-state inactivation curves highlight the decreased voltage dependence of inactivation gating for the mutant channels (slope factors of 8–11 mV/e-fold) compared to WT channels (slope factor of 6 mV/e-fold). Application of ranolazine did not significantly decrease the voltage dependence of inactivation gating for any of the constructs tested (Table 2).

To study the voltage dependence of steady-state slow inactivation, a 10 sec conditioning pre-pulse was applied (see Methods for details). Ranolazine (30 µM) caused a hyperpolarizing shift of the $V_{1/2}$ of steady-state slow inactivation of WT, R1448C, R1448H and R1448P channels (Fig. 3C and Table 2).

**Effects of ranolazine on the recovery from inactivation.** The time course of the recovery from inactivation for WT and the R1448 mutant channels was measured using a standard two-pulse protocol (see Methods for details). Data were fitted using a double exponential decay yielding two time constants $\tau_{fast}$ and $\tau_{slow}$ and respective amplitudes $A_{fast}$ and $A_{slow}$. Figure 4 illustrates the voltage-clamp protocol along with the recovery profiles of all channels in the absence and presence of ranolazine (30 µM). Ranolazine (30 µM) significantly ($p < 0.05$) slowed the recovery from inactivation for WT (increased $\tau_{slow}$), R1448C (increased $\tau_{fast}$ and $\tau_{slow}$) and R1448P (increased $\tau_{slow}$) channels. However, ranolazine had minimal effect on the recovery time course of R1448H channels (Table 3).

**Open-state block of WT and R1448 mutant Na⁺ channels by ranolazine.** To test whether ranolazine has an effect on the open state of the channel, a train of repetitive depolarizing pulses (10 Hz) to -20 mV with variable step durations (2, 5 or 20 msec) was used. If ranolazine binds to the open state, then we would expect similar peak $I_{Na}$ reduction to occur at the end of each of train of pulses, regardless of pulse duration. Figure 5 shows the peak $I_{Na}$ amplitude of each pulse normalized to that of the first pulse recorded in the absence (control, filled symbols) or presence of 30 µM ranolazine (Ran, open symbols) for WT, R1448C, R1448H and R1448P channels (Fig. 5A–D). In the absence of drug, there was minimal reduction of peak $I_{Na}$, suggesting minimal loss of channel availability at 10 Hz (<9%, n = 4–6) (data not shown). At a pulse duration of 2 msec, ranolazine (30 µM) reduced peak $I_{Na}$ of WT, R1448C, R1448H and R1448P channels by 24.5 ± 0.8%, 33.9 ± 2.3%, 30.3 ± 3.5% and 32.1 ± 3.3% (n = 4–6), respectively. Channel block by ranolazine was minimally affected by changing the pulse duration from 2 to 5 to 20 msec (Fig. 5). These findings confirm previously published results that ranolazine binds to the open states of rNa v1.4, hNa v1.7 and rNa v1.8 channels.20,21

**Suppression of R1448C ramp current by ranolazine.** Paramyotonia congenita muscle fibers have been shown to have increased persistent $I_{Na}$ that is responsible for increased Na⁺ influx and intracellular Na⁺ concentration, resulting in a depolarized resting membrane potential causing muscle weakness.10,14,22 We used slow voltage ramps to evoke $I_{Na}$, as an indirect measure of persistent current. Compared to cells expressing WT Na⁺ channels, cells expressing R1448C exhibited an increased $I_{Na}$ in response to the slow ramp protocol in the absence of drug (R1448C-control, Fig. 6A). To account for a potential difference in transfection efficiency between WT and mutant channels, the TTX-subtracted ramp current was normalized to peak $I_{Na}$, measured during a step depolarization to -20 mV (Fig. 6B). Normalized values of ramp $I_{Na}$ were: WT, 19.7 ± 2.7 pC/nA (n = 6); R1448C channels in the absence of drug, 44.2 ± 5.8 pC/nA (n = 11), p < 0.05 compared to WT-control; 3 µM, 31.4 ± 6.4 pC/nA (n = 6); p > 0.05 compared to WT-control and 10 µM, 25.7 ± 3.1 pC/nA (n = 6); respectively, p > 0.05 compared to WT-control. Ranolazine at a concentration of 10 µM (R1448C-Ran) blocked R1448C ramp $I_{Na}$ back to WT levels.

**Ranolazine block of simulated myotonic action potentials.** The persistent current observed in paramyotonia congenita muscle fibers significantly impacts the ability of these fibers to sense and respond to incoming stimuli. Computational models of WT-Na v1.4 and Mutant-Na v1.4 were constructed to investigate the effect of ranolazine to inhibit the abnormal myotonic action potential generation in skeletal muscle. Figure 7A shows
Figure 4. Time course recovery from inactivation of hNav1.4 WT and R1448 mutant channels. Recovery from inactivation was assessed using a two pulse protocol consisting of an inactivation prepulse followed by a variable length interpulse recovery period. (A–D) show normalized INa plotted as a function of recovery time (rt) in the absence (■, control) or presence of 30 µM ranolazine (□, Ran) for WT (A), R1448C (B), R1448H (C) and R1448P (D). Insets represent voltage-clamp protocols, data represent mean ± SEM and the fit parameters are provided in Table 3.

Table 3. Parameters of the time course recovery from inactivation of hNav1.4 WT and R1448 mutant Na+ channels in the absence and presence of ranolazine

|          | WT Control | (Ran 30 µM) n | R1448C Control | (Ran 30 µM) n | R1448H Control | (Ran 30 µM) n | R1448P Control | (Ran 30 µM) n |
|----------|------------|---------------|----------------|---------------|----------------|---------------|----------------|---------------|
| τfast (msec) | 1.4 ± 0.1  | 2.3 ± 0.7     | 18.6 ± 4.3†   | 66.8 ± 5.8*   | 3.6 ± 0.5†     | 2.6 ± 0.7     | 1.9 ± 0.4      | 4.3 ± 0.9      |
| τslow (msec) | 98.4 ± 57.0 | 430.2 ± 107.1* | 303.8 ± 45.4  | 2688.1 ± 700.0* | 324.8 ± 79.7   | 243.0 ± 52.0  | 125.8 ± 22.4   | 535.8 ± 93.9*  |
| Afast (%)    | 79.1 ± 7.0  | 69.7 ± 9.7    | 51.0 ± 3.3†   | 55.4 ± 2.0    | 61.6 ± 5.8     | 72.0 ± 4.9    | 64.8 ± 2.8     | 65.7 ± 2.2     |
| Aslow (%)    | 20.9 ± 7.0  | 30.3 ± 9.7    | 49.0 ± 3.3†   | 44.6 ± 2.0    | 38.4 ± 5.8     | 28.0 ± 4.9    | 35.2 ± 2.8     | 34.3 ± 2.2     |

*p < 0.05 vs. Control, †p < 0.05 vs. WT, n is the number of cells. Ran, ranolazine.

the whole-cell currents generated by the Markov models for WT-Na1.4 (top left) and Mutant-Na1.4 (top right) channels in response to step depolarizations to between -80 and 20 mV from a holding potential of -120 mV. The whole-cell currents replicated accurately current traces recorded from heterologously expressed Na1.4 channels (Fig. 7A, bottom). The rate constants of model Mutant-Na1.4 channels were tuned to qualitatively reproduce the general biophysical defects typically reported for paramyotonia congenita SCN4A mutations. These defects include a negative shift in the voltage-dependence of activation (V1/2 of -23.1 mV and k of 8.3 mV/e-fold) and steady-state fast inactivation (V1/2 of -68.8 mV and k of 9.0 mV/e-fold) compared to the WT-Na1.4
channel (activation $V_{1/2}$ of -20.0 mV and $k$ of 8.5 mV/e-fold, steady-state fast inactivation $V_{1/2}$ of -61.3 mV and $k$ of 7.7 mV/e-fold). Similar shifts in the activation and steady-state fast inactivation of R1448C were also observed in the current study (Fig. 7B, bottom). Note that the Mutant-Nav1.4 model is designed to reproduce effects caused by SCN4A paramyotonia congenita mutations, but not specific gating defects associated with R1448C. The mutant-Nav1.4 model channel also exhibited a delayed recovery from fast inactivation ($\tau_{fast} = 4.74$ msec, 88%; $\tau_{slow} = 35.0$ msec, 11%) compared to the model WT-Nav1.4 ($\tau_{fast} = 2.12$ msec, 82%; $\tau_{slow} = 25.7$ msec, 18%). Mutant-Nav1.4 generated a significantly increased persistent current (2.3% of peak current) compared to the model WT-Nav1.4 (0.3% of peak current) in response to a 200 msec step depolarization from -120 mV to -10 mV.

We next determined the effect of ranolazine to inhibit the abnormal excitability of myotonic muscle using a previously published mathematical model of skeletal muscle fiber action potentials. We replaced the original Na channel model with our Markov models, either WT-Nav1.4, alone or in heterozygous combination with Mutant-Nav1.4 (Fig. 7C and D, respectively). Although the total sodium conductance was held constant, the resting membrane potential of the heterozygous mutant fiber was depolarized 3 mV compared to homozygous WT-Nav1.4 fiber (-80.3 mV compared to -83.3 mV, respectively). Using the model, a train of action potentials in response to a stimulus (24 μA/cm²) was seen in fibers expressing Mutant-Nav1.4 channels (Fig. 7C, left inset).

Simulated binding of ranolazine to the primary fast-inactivated state of model WT-Nav1.4 did not alter the action potential train. In contrast, heterozygous expression of WT-Nav1.4 and Mutant-Nav1.4 produced a train of action potentials during the stimulation followed by after depolarization induced action potentials (Fig. 7D, left) resembling myotonia. In the drug free simulation, the myotonic action potentials continued for 2,500 msec following the onset of the stimulation. Simulated application of ranolazine inhibited the myotonic action potentials. These data suggest that fast inactivation state binding of ranolazine to mutant Nav1.4 channels is sufficient to block simulated action potential generation in myotonic muscle fibers.

**Discussion**

Our findings show that ranolazine (1) was effective in blocking hNav1.4 WT and paramyotonia congenita hNa v1.4 mutant channels R1448C, R1448H and R1448P $I_{na}$ in a voltage- and use-dependent manner, (2) interacts with the open state and stabilizes inactivated state(s) of Na+ channels, (3) accelerated $I_{na}$ decay rates, (4) caused a hyperpolarizing shift of the voltage-dependence of inactivation, (5) slowed the rate of recovery from inactivation and (6) blocked the TTX-sensitive R1448C ramp $I_{na}$. Furthermore, a low concentration of ranolazine inhibited myotonic action potentials and restored normal excitability in a computer simulation of a muscle fiber response to stimulation. Lehmann-Horn and colleagues demonstrated that the resting membrane potentials recorded from isolated fiber segments of normal subjects and patients with myotonia were -84 mV and -60 to -70 mV, respectively. This depolarized membrane
potential resulted in excessive and sustained action potential firing (myotonic activity). It was also shown that the late (persistent) openings of Na+ channels were more frequent (0.75% of peak I\textsubscript{Na}) in the myotonic fiber segments than in the fiber segments from normal subjects (0.11% of peak I\textsubscript{Na}). These increased late openings of Na+ channels were TTX sensitive. In our experiments with hNa\textsubscript{1.4} WT and paramyotonia congenita mutant channels, the potencies for ranolazine to cause UDB at hyperpolarized membrane potentials (-140 mV) were low (IC\textsubscript{50} values of 58.7–70.8 μM, Fig. 2B and Table 1). In contrast, higher affinity block (IC\textsubscript{50} of 10.8–18.1 μM, Fig. 2B and Table 1) by ranolazine of both WT and R1448 mutant channels was observed when the HP was changed to -90 mV, which is close to the resting membrane potential of a normal muscle fiber. Interestingly, when the HP of HEK293 cells was changed to -70 mV to mimic the resting membrane potential of an injured skeletal muscle fiber, the potency of ranolazine increased further (IC\textsubscript{50} of 0.94–6.3 μM, Table 1). In summary, the UDB of peak I\textsubscript{Na} by ranolazine increased with the stimulating frequency (10 to 30 Hz) and with depolarization of the resting membrane potential (from -140 mV to -90 mV at 10 Hz). The increased potency of ranolazine in UDB could be due to incomplete recovery of drug-bound channels or to increased access of ranolazine to an open state binding site during frequent stimulation. Data suggests that concentrations of ranolazine achieved therapeutically may be expected to reduce repetitive firing of depolarized skeletal muscle fibers in patients with paramyotonia congenita.

The reduction in the rate of I\textsubscript{Na} decay, and the enhanced rate of recovery from inactivation, exhibited by R1448 mutant channels are features which may increase excitability and contribute to myotonia. As shown in Figure 1, the rate of whole-cell I\textsubscript{Na} decay in R1448 mutant cells was slower than hNa\textsubscript{1.4} WT cells. However, the recovery from inactivation (τ\textsubscript{slow}) for the R1448 mutations was not enhanced in our study. Our data are in disagreement with some, but are in accordance with other reports. Differences in results could be due to the voltage-clamp protocol or the co-expression of the β1-subunit in our study. Importantly, ranolazine corrected the defective inactivation of R1448C by accelerating the whole-cell INa decay rate (both fast and slow components; see Fig. 1C and D). Ranolazine also prolonged the recovery from inactivation of hNa\textsubscript{1.4} WT, R1448C and R1448P that would further reduce sodium influx into the affected muscle fibers.

Extending the pulse duration from 2 to 5 and 20 msec did not alter the extent of UDB (10 Hz) of hNa\textsubscript{1.4} WT and R1448C, R1448H and R1448P I\textsubscript{Na} by ranolazine suggesting that the drug could rapidly bind to the open state of the hNa\textsubscript{1.4} channels. This is in agreement with a recent report by Wang and colleagues showing that ranolazine preferentially bound to the open but not to the rested or inactivated states of Na+ channels. However, our data suggest that ranolazine also stabilizes inactivated state(s) of the channel. Specifically, ranolazine caused greater UDB at 30 Hz than at 10 Hz and high affinity voltage-dependent block when the HP was changed from -140 to -90 mV (IC\textsubscript{50} of 10.8–18.1 μM) and -70 mV (IC\textsubscript{50} of 0.94–6.3 μM). The strongest evidence for inactivated state binding is that ranolazine caused a hyperpolarizing shift in the mid-points of fast, intermediate and slow-inactivation of WT and the paramyotonia congenita mutants (Fig. 3), similar to lidocaine.

An important characteristic of paramyotonia congenita mutant channels is increased persistent I\textsubscript{Na}, which produces muscle fiber inexcitability, followed by paralysis. In the present study, the presence of increased persistent I\textsubscript{Na} in cells expressing the paramyotonia congenita mutation, R1448C was observed using slow depolarizing ramps. At 3 μM, which is within the

Figure 6. Ranolazine blocks the increased ramp current carried by R1448C. (A) shows representative TTX-subtracted I\textsubscript{Na} recordings from WT (blue line) and R1448C (black line) in the absence of drug. Application of 10 μM ranolazine to R1448C (orange line) reduces the abnormal I\textsubscript{Na} elicited by the slow ramp depolarization. The dashed line represents zero current. (B) shows a plot of the normalized charge conducted between -50 and 0 mV for R1448C ramp I\textsubscript{Na} in absence (black bar) and presence of 3 (green bar) and 10 μM (orange bar) ranolazine compared to WT (blue bar). Data represent mean ± SEM. * and †represent p < 0.05 vs. WT-control and R1448C-control, respectively.
demonstrated that mutant channel gating induced myotonic action potentials. This abnormal excitability was not seen in simulations using the WT-Na\textsubscript{v}1.4 model and could be completely inhibited by simulated application of 3-μM ranolazine. It is worth noting that in these simulations ranolazine binding was limited to the main fast inactivated state within the model. Binding to open states was not required for the block of simulated myotonic action potentials.

The Na\textsuperscript{+} channel blockers carbamazepine, flecainide and mexiletine have been shown to be useful in treating myotonia. The beneficial effect of these drugs is due to their strong UDB of Na\textsuperscript{+} anti-anginal therapeutic plasma concentrations of 2–10 μM, ranolazine decreased the difference in R1448C persistent \(I_{\text{Na}}\) compared to WT by 52% whereas 10 μM reduced persistent \(I_{\text{Na}}\) to a level similar to that of WT (Fig. 6).

Figure 7. Simulated myotonic action potentials are inhibited by ranolazine binding to Na\textsubscript{v} fast inactivated states. (A) Illustrates the strong concordance between the whole-cell \(I_{\text{Na}}\) generated by the Markov models for WT-Na\textsubscript{v}1.4 and Mutant-Na\textsubscript{v}1.4 (top) and \(I_{\text{Na}}\) recorded from heterologously expressed WT-Na\textsubscript{v}1.4 or R1448C (bottom). (B) The Mutant-Na\textsubscript{v}1.4 model accurately reproduces the negative shifts in activation and steady-state inactivation reported for several paramyotonia congenita mutations (top), including heterologously expressed R1448C (bottom). The model Mutant-Na\textsubscript{v}1.4 is proposed as a generic or proof of principle model for SCN4A associated paramyotonia congenita and therefore does not strictly follow the biophysical defects of R1448C. (C and D) Show sarcolemma action potentials using the homozygous expression of WT-Na\textsubscript{v}1.4 (C) or the heterozygous expression of WT-Na\textsubscript{v}1.4 and Mutant-Na\textsubscript{v}1.4 (D). In all experiments the total sodium conductance was held constant and action potentials were elicited by a 100 msec depolarizing current injection. Post-stimulation myotonic action potentials caused by after depolarizations were predicted by the model in cells expressing heterozygous channels. Myotonic action potentials were inhibited by simulated ranolazine application in a dose dependent manner (0 μM, 1 μM and 3 μM from left to right).
channels. In addition, mexiletine has been shown to preferentially stabilize the inactivated state of both WT and paramyotonia congenita mutant channels.32 Flecainide was shown to be useful in severe forms of myotonias where mexiletine was not effective.33 The clinical efficacy of mexiletine (therapeutic concentrations of 2.8–11 μM),34 has been attributed to its effect to either induce a UDB of peak hNa\textsubscript{1.4} \textsubscript{I\textsubscript{Na}}, or to block the persistent I\textsubscript{Na} (IC\textsubscript{50} of 7.5 μM for block of chloramine-T induced current).35 The clinical efficacy of flecainide (therapeutic concentrations of 0.4–2 μM),36 was attributed to its UDB of peak hNa\textsubscript{1.4} \textsubscript{I\textsubscript{Na}} at a HP of -90 mV at a frequency of 50 Hz (IC\textsubscript{50} of 3.1 μM).37 However, long-term management of myotonic episodes by either mexiletine or flecainide is limited due to their cardiovascular and CNS-related side-effects.3 It is important to note that flecainide has a very narrow therapeutic index and can exhibit toxicity at therapeutic concentrations. Ranolazine is approved for the treatment of chronic angina pectoris (therapeutic plasma level of 2–10 μM) and has been shown to have minimal side-effects in patients.38,39

In conclusion, although extrapolation from the HEK293 expression system to paramyotonia congenita patients should be done with caution, ranolazine may have the ability to block excessive and sustained firing of action potentials in these patients.

Materials and Methods

Generation of R1448 mutant constructs. The vector expressing the human wild-type (WT) skeletal muscle voltage-gated Na\textsuperscript{+} channel α-subunit, pRC/CMV-hSkM1,40 was used to construct the R1448C, R1448H and R1448P mutant channel vectors. Site-directed mutagenesis using the QuickChange II XL mutagenesis system (Stratagene, La Jolla, California) was used as per manufacturer’s instructions to create the single mutations at the position 1448 in segment 4 of domain IV of the hNav1.4 α-subunit substituting the positively charged arginine for the neutral amino acids cysteine (R1448C), histidine (R1448H) and proline (R1448P). Mutagenic oligonucleotides were as follows: R1448C, 5’-CCC ACC CTG TTT TCT GTG ATC CGC C-3’; R1448H, 5’-CCC ACC CTG TTT CAT GTG ATC CGC C-3’; and R1448P, 5’-CCC ACC CTG TTT CCT GTG ATC CGC C-3’. DNA sequencing was used to verify presence of desired mutations.

Heterologous expression of hNav1.4 WT and R1448 mutant constructs. Following mutagenesis, hSkM1 pRC/CMV constructs encoding the full-length human Na\textsubscript{1.4} WT and mutant α-subunits were introduced into HEK293 cells by transient transfection using Polyfect (Qiagen, Valencia, California) as per manufacturer’s instructions. Briefly, cells grown in T25 tissue culture flasks (Corning, Corning, New York) reaching 50–60% confluence were transfected using 0.75–1 μg of α-subunit of WT or mutant construct DNA with p1-subunit plasmid (SCI19540, OriGene, Rockville, Maryland) and green fluorescence protein plasmid (pEGFP-N1; Clontech Laboratories Inc., Mountain View, CA) in a respective mass ratio of 1:1:0.2 for 24–48 hours before being used for current recordings. Cells were routinely grown in Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12) (Gibco\textsuperscript{TM}, Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (Gibco\textsuperscript{TM}, Invitrogen) and 1% penicillin/streptomycin (CellGro, Mediatech Inc., Manassas, Virginia) and were incubated at 37°C in a humid environment supplemented with 5% CO\textsubscript{2}.

Electrophysiology and data analysis. Whole cell I\textsubscript{Na} recordings were performed as previously described in reference 41. Patch glass pipettes (World Precision Instruments, Sarasota, Florida) were pulled with a DMZ Universal puller (Dagan Corporation, Minneapolis, Minnesota) and were filled (resistance of 1–3 MΩ) with internal solution containing (in mM) 120 CsF, 20 CsCl, 2 EGTA and 5 HEPES (pH 7.4; adjusted with CsOH). Cells were continuously superfused with a bath external solution containing (in mM) 140 NaCl, 4 KCl, 1.8 CaCl\textsubscript{2}, 2H\textsubscript{2}O, 0.75 MgCl\textsubscript{2},6H\textsubscript{2}O and 5 HEPES (pH 7.4; adjusted with NaOH). Currents were recorded using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California) at room temperature (21 ± 1°C). After establishing a patch with whole-cell configuration, a period of 10–15 minutes was given for cells to stabilize before conducting experiments. Only currents ≤10 nA were considered for data collection. Whole-cell data were acquired using pCLAMP software (Molecular Devices) and were analyzed using pCLAMP, Microcal Origin (OriginLab Corporation, Northampton, Massachusetts) and GraphPad Prism (GraphPad Software, Inc., La Jolla, California) software programs.

Ranolazine was synthesized by the Department of Chemistry, Gilead Sciences, Inc., (Palo Alto, California). A stock solution of 50 mM was prepared using 0.1 N HCl and further dilutions were made freshly on the day of experiment.

Values for each determination are expressed as mean ± SEM. Statistical significance (p < 0.05) was determined using the Student’s t-test. The number of cells used in each experiment is provided in the Tables and Results section.

Concentration-response relationships were fit using the Hill equation:

\[
\frac{I_{\text{drug}}}{I_{\text{control}}} = \frac{1}{1 + \left(D/IC_{50}\right)^{n_{H}}}
\]

where \(I_{\text{drug}}/I_{\text{control}}\) is fractional block, D is drug concentration, IC\textsubscript{50} is the drug concentration that causes 50% block and \(n_{H}\) is the Hill coefficient.

For assessing the voltage dependence of steady-state fast and intermediate inactivation, pre-pulses ranging from -150 to 0 mV were applied for a period of 100 and 1,000 msec, respectively, followed by a 40 msec depolarizing step to 0 mV. The peak currents (\(I_{\text{peak}}\)) were normalized to the maximal values (\(I_{\text{max}}\)) obtained at a HP of -140 mV and plotted against the conditioning pulse potential. Data were fit to a Boltzmann equation:

\[
I/I_{\text{max}} = \frac{1}{1 + \exp((V - V_{\text{0.5}})/k)}
\]

where V is the membrane potential during the pre-pulse, \(V_{0.5}\) is the potential at which the half-maximal channel inactivation occurs and \(k\) is the slope factor.

For assessing the voltage dependence of steady-state slow inactivation, pre-pulses ranging from -150 to 0 mV were applied for
a period of 10 sec, followed by a 100 msec hyperpolarizing step to -160 mV (to allow channels to recover from fast inactivation), then inactivation was assessed by a 40 msec depolarizing step to 0 mV. The peak currents (I) were normalized relative to the maximal values (I_{max}) obtained at a HP of -140 mV and plotted against the conditioning pulse potential. Data were fit to a modified Boltzmann equation:

\[ \frac{I}{I_{max}} = \frac{1}{(1 + \exp(-(V - V_c)/k))} \]

where V, V_c, and k were as above and I_{resid} is the residual (non-inactivating) fraction of the channel.

To derive current decay time constants (\(\tau_{decay}\)), I_{Na} whole-cell currents at a test potential of -20 mV were normalized and fitted to a double exponential equation.

The time course of recovery from inactivation was measured using a standard two-pulse protocol of 40 msec duration with an incremental time delay of 1 to 7,000 msec interpulse duration (holding potential of -140 mV; test potential of -20 mV). The peak I_{Na} elicited by the second pulse was normalized relative to the maximum current value and plotted against the time delay between the two pulses and fit to a double exponential function yielding two time constants, \(\tau_{fast}\) and \(\tau_{slow}\) and their relative amplitudes.

UDB of I_{Na} by ranolazine was assessed with repetitive voltage step (-20 mV, 10 Hz) using increasing step durations (2, 5 and 20 msec). The I_{Na} for each step was normalized to the current normalized to the corresponding peak INa evoked by a voltage step to -20 mV (pC/mA). Representative currents were low pass filtered at 10 Hz to reduce the noise and maintain the current tracing.

Computational modeling. Skeletal muscle fiber action potentials were simulated as previously described by Cannon, et al.\(^{30}\) Briefly, this model reproduces the sarcolemma using compartments for the surface membrane and the transverse tubule (T-tubule) membranes. Both membrane compartments contain active conductances (Na_ and K_ channels) as well as a passive leak conductance. This model tracks the concentration of T-tubule K_ concentration [K_]|_{Ttub} that is used to continuously update the reversal potential for the T-tubule K_ and leak conductances. Similar to the previously published model, the [K_]|_{Ttub} peaked at 5.7 mM in the wild-type model but was increased to 7.1 mM with the heterozygous expression of Mutant-Na_1.4. The original Hodgkin-Huxley Na_ channel model was replaced by our Markov models for Na_1.4. All implementation parameters were identical to the original report\(^{31}\) unless otherwise noted. All simulations were performed within the NEURON simulation environment (www.neuron.yale.edu) using the CVODE variable time step routine.\(^{44}\)

Markov models of WT-Na_1.4 and Mutant-Na_1.4 were constructed using our previously described model of Na_1.1 as a template.\(^{31}\) This approach was feasible as recombinant hNa_1.1 and hNa_1.4 channels share many aspects of channel function including rapid activation and inactivation kinetics (Figs. 1–4 of the current study and Figs. 1 and 3 of Chahine et al.). Altering rate constants to generate the Mutant-Na_1.4 produced a model that accurately reflects the biophysical defects associated with paramyotonia congenita SCN4A mutations (see results section). For action potential simulations, ranolazine binding was constrained to the final (absorbing) fast inactivated state of both Na_1.4 models using moderate K_ON and K_OFF rates (750 M^{-1}ms^{-1} and 5 x 10^-7 ms^{-1}, respectively).\(^{31,43-47}\)

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