The efficacy of Ranolazine on E1784K is altered by temperature and calcium

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E1784K is the most common mixed syndrome SCN5a mutation underpinning both Brugada syndrome type 1 (BrS1) and Long-QT syndrome type 3 (LQT3). The charge reversal mutant enhances the late sodium current (I_{Na}) passed by the cardiac voltage-gated sodium channel (Na\textsubscript{v}1.5), delaying cardiac repolarization. Exercise-induced triggers, like elevated temperature and cytosolic calcium, exacerbate E1784K late I_{Na}. In this study, we tested the effects of Ranolazine, the late I_{Na} blocker, on voltage-dependent and kinetic properties of E1784K at elevated temperature and cytosolic calcium. We used whole-cell patch clamp to measure I_{Na} from wild type and E1784K channels expressed in HEK293 cells. At elevated temperature, Ranolazine attenuated gain-of-function in E1784K by decreasing late I_{Na}, hyperpolarizing steady-state fast inactivation, and increasing use-dependent inactivation. Both elevated temperature and cytosolic calcium hampered the capacity of Ranolazine to suppress E1784K late I_{Na}. In-silico action potential (AP) simulations were done using a modified O’Hara Rudy (ORd) cardiac model. Simulations showed that Ranolazine failed to shorten AP duration, an effect augmented at febrile temperatures. The drug-channel interaction is clearly affected by external triggers, as reported previously with ischemia. Determining drug efficacy under various physiological states in SCN5a cohorts is crucial for accurate management of arrhythmias.

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We hypothesize that Ranolazine, which preferentially blocks late I_{Na}, is suitable for ameliorating the thermal and calcium-induced defects in E1784K. Although prescribed as an anti-anginal drug for diastolic dysfunction treatment, Ranolazine has anti-arrhythmic efficacy proven to be useful in treating SCN5a inherited conditions. Ranolazine efficacy is enhanced with SCN5a mutations or channel triggers, such as acidosis, which augment late I_{Na}. We predicted that the channel mutation-trigger interaction may alter drug efficacy. Our goal is to study the effects of Ranolazine on E1784K under conditions of elevated temperature and cytosolic calcium levels.

**Results**

**Ranolazine binds to NaV1.5 inner vestibule.** The NaV1.5 homology model based on NaV1.5-NaV1.5 Pas is shown in Fig. 2. The side view of the channel shows the four domains and their putative voltage and pore-forming segments (including the p-helices, extracellular and intracellular linkers). NaV1.5 Pas shares about 32% sequence identity with NaV1.5. The aligned sequences for DIII-DIV linker and CTD are shown in Fig. 2. Ranolazine was auto-docked against NaV1.5-NaV1.5 Pas using AutoDock4. The highest affinity (−7.7 kcal/mol) binding mode is enlarged in Fig. 2. The compound formed polar and van der Waals interaction with various residues located in all four domains: S401, V405, C896, N927, F1418, S1458, L1462, N1463, I1466, F1760, V1764, I1768. The aromatic residue, F1760, is outlined in Fig. 2 as it is a key putative binding site for many anti-arrhythmics, local anesthetics, and anticonvulsants. F1760 orientation with respect to Ranolazine supports its critical role in drug binding.

**Ranolazine does not affect conductance.** Raw current traces in Fig. 3 show the effects of 0 µM and 100 µM Ranolazine on WT and E1784K at 0 nM and 2500 nM cytosolic calcium (only 34 °C shown). E1784K reduced (p < 0.0001) the peak current and conductance density compared to WT. Elevated temperature (34 °C) increased (p < 0.0001) peak current and conductance density in WT but not in E1784K. Ranolazine had no effect (p > 0.05) on peak current or conductance density (Table 1 and Fig. 3B). Figure 3A shows normalized conductance plotted against the test potential at 34 °C. E1784K (p < 0.0001) and elevated temperature (p = 0.0003) depolarized the conductance midpoint (GV-V_{1/2}). The conductance slope (GV-z) was reduced (p < 0.0001) in E1784K compared to WT and increased (p < 0.0001) when temperature was elevated in both channel variants. The interaction between channel variant and temperature had no effect on GV-V_{1/2} and GV-z. Normalized conductance was unchanged in all conditions with Ranolazine (Table 2).

**E1784K availability is decreased in Ranolazine.** Normalized current is plotted against membrane potential in Fig. 4. E1784K hyperpolarized (p < 0.0001) the SSFI midpoint (SSFI-V_{1/2}) compared to WT. Elevated temperature depolarized (p < 0.0001) SSFI-V_{1/2} in both WT and E1784K. At 34 °C and 0 nM cytosolic calcium, SSFI-V_{1/2} in E1784K was hyperpolarized (p < 0.0001) in 100 µM Ranolazine compared to WT (Fig. 4C and Table 2). This effect was not significant at 2500 nM cytosolic calcium. Analogous to the shifts on GV-z, SSFI-z was decreased in E1784K and increased with elevated temperature (p < 0.0001). The slope was reduced in all conditions when Ranolazine was increased from 10 µM to 100 µM (p < 0.05, Table 2).
Fast inactivation onset kinetics are not altered with Ranolazine. Fast inactivation onset kinetics at depolarized potentials (>−50 mV) were measured from $\tau_{on}$ of the mono-exponential fits. E1784K fast inactivation onset kinetics were accelerated regardless of temperature (Fig. 5A,B). Onset kinetics were accelerated ($\tau_{on}$ decreased) with elevated temperature in WT compared to E1784K ($p < 0.01$). WT and E1784K onset kinetics were decelerated ($\tau_{on}$ increased, $p < 0.05$) in Ranolazine as a function of voltage and cytosolic calcium at 22 °C (values reported in Table 3). These drug effects on $\tau_{on}$ were not significant at elevated temperature.

Figure 5C shows the Q10 values at 0 $\mu$M and 10 $\mu$M Ranolazine for all conditions. We observed high variability in the temperature coefficient at −50 mV compared to other voltages. At −50 mV, both Ranolazine and cytosolic calcium mutually affect thermosensitivity in WT: Q10 decreased at 0 nM cytosolic calcium and increased at 2500 nM cytosolic calcium in 10 $\mu$M Ranolazine. At more depolarized voltages than −50 mV, subtle alterations occurred in Q10 (Fig. 5C). E1784K Q10 was not sensitive to Ranolazine. However, E1784K thermosensitivity was dampened in cytosolic calcium at −50 mV compared to other voltages and to WT.

Ranolazine does not suppress thermosensitive late INa in E1784K with elevated cytosolic calcium. Representative normalized late INa current traces are shown in Fig. 6A,B at 0 $\mu$M and 100 $\mu$M Ranolazine (only 34 °C shown). Late INa percent and density are shown in Fig. 6C,D as bar graphs. Late INa percent and density in E1784K increased ($p < 0.01$) by 11-fold and 7-fold, respectively, with elevated temperature at 0 nM cytosolic calcium and increased at 2500 nM cytosolic calcium in 10 $\mu$M Ranolazine. At more depolarized voltages than −50 mV, subtle alterations occurred in Q10 (Fig. 5C). E1784K Q10 was not sensitive to Ranolazine. However, E1784K thermosensitivity was dampened in cytosolic calcium at −50 mV compared to other voltages and to WT.

Ranolazine does not enhance UDI in E1784K with elevated cytosolic calcium. Sustained or repetitive depolarizations induce slow inactivation in NaV1.5, which was indirectly measured by the use-dependent inactivation (UDI) protocols described in the methods. We were unable to measure fast inactivation recovery kinetics in E1784K which may be decelerated by Ranolazine and contribute to UDI54. Use-dependence was measured at 1 Hz and 3 Hz, mimicking resting heart rate (60 bpm) and tachycardia (180 bpm), respectively. Normalized current plotted against time for UDI measured at 1 Hz and 3 Hz are shown in Fig. 7 (only 34 °C shown).

UDI plateau ($y_0$) was greater ($p = 0.0430$) at elevated temperature at both 1 Hz and 3 Hz, but the shift was larger in E1784K at 1 Hz (Table 5). $y_0$ decreased to different levels in Ranolazine (reported in Table 5). At high UDI frequencies, E1784K $y_0$ decreased in Ranolazine at 34 °C compared to WT (Fig. 7 shows only 34 °C, Table 5). Our statistical results suggest that the drug effects on UDI (3 Hz) in E1784K are limited in elevated cytosolic calcium (Table 5).
UDI onset kinetics were accelerated in E1784K and elevated temperature (p < 0.05), measured by τ_1, at 1 Hz and 3 Hz. Onset kinetics decelerated in elevated cytosolic calcium (p < 0.0001) at 1 Hz predominately in WT compared to E1784K. WT and E1784K τ_1 was decreased (p < 0.0001) in Ranolazine at 2500 nM cytosolic calcium and 34 °C (Table 5). τ_1 at 3 Hz was unaffected in Ranolazine. τ_2 was unaffected at 1 Hz and 3 Hz in all experimental conditions.

E1784K-induced alternans is exacerbated with Ranolazine. Biophysical data were extrapolated to physiological (37 °C) and febrile (41 °C) temperatures using a Q_{10} coefficient (Equation 6). Extrapolations were independently supported using Arrhenius relationships (Equation 7 and Table 6). Action potential (AP) traces are shown in Fig. 8 for simulations conducted in endocardial cells at three frequencies: 0.5 Hz (bradycardia), 1.5 Hz (sinus rhythm), and 2.5 Hz (tachycardia). At febrile temperature, simulated APs show accelerated depolarizations and repolarizations in WT compared to E1784K.

Electrical restitution curves (ERCs) at 90% repolarization were constructed from plotting APD_{90} against the diastolic interval as shown in Fig. 9A, B in endocardial cells. The last two beats were included in the ERCs to exemplify bifurcation and alternans-induction at critical diastolic intervals. The APD_{90} for WT follows a similar trend to previously published ERCs, typifying a relatively stable APD rate dependence. E1784K has a higher APD_{90} in all cardiac cells, especially the mid-myocardium, compared to WT (not shown in Fig. 9). At 37 °C, the mutant causes bifurcation in APD_{90}, mainly in epicardial cells, indicative of alternans (not shown); however, endocardial cells also experience alternans at febrile temperature in addition to epicardial cells (Fig. 9B). Upon drug perfusion, bifurcations were observed at higher diastolic intervals in E1784K (Fig. 9). The drug-induced bifurcations in ERCs were augmented at febrile temperature in all cardiac cells.

A linear relationship is established between the last two AP beats at each frequency step (shown in Fig. 9C), with no alternans. Divergence from linearity is indicative of alternans occurrence. At both 37 °C and 41 °C, WT cells had no alternans, even upon 10 μM Ranolazine perfusion, showing linearity with a slope = 1 (Fig. 9C).
At elevated stimulation frequencies and temperature, therapeutic Ranolazine decreased channel availability by the combination of both elevated temperature and cytosolic calcium. At 34 °C, E1784K late I Na percent was increasing channel use-dependence in E1784K. At 34 °C, Ranolazine did not attenuate gain-of-function in SCN5a drug-binding at high BCLs (low frequencies), but deviated from linearity beginning at intermediate BCLs; distortion in linearity is observed at lower frequencies in epicardial cells (Fig. 9C). This relationship in E1784K is augmented with febrile temperature. The prolonged APD∞ in E1784K were shortened with Ranolazine at very low frequencies, and alternans were quickly induced even during bradycardia, an effect exacerbated by febrile temperature (Fig. 9C).

### Discussion

Our goal was to determine whether Ranolazine reduced channel dysfunction in E1784K under the triggering conditions of elevated temperature and cytosolic calcium. Ranolazine did not attenuate gain-of-function in E1784K when temperature and cytosolic calcium were elevated. Ranolazine has minimal effects on conductance in NaV1.5. The drug follows the modulated receptor hypothesis, targeting the open/inactivated states at depolarized potentials, thereby suppressing late I Na. Physiological events, such as acidemia, enhance Ranolazine antiarrhythmic effect by augmenting late I Na, thus providing the drug with a larger open-state channel substrate to target. In addition to physiological modulators, SCN5a mutations often alter voltage-dependence of the channel, which modify drug effects on NaV1.5. To date, Ranolazine has been screened against only ∆KPQ, Y1767C, R1623Q, and D1790G. Our study is the first to show the combined external triggers and SCN5a mutation effects on Ranolazine.

Ranolazine efficacy was enhanced at elevated temperature. Similar to acidosis effects, elevated temperature increases the late open probability in E1784K. Late I Na percent and density increased by 11- and 7-fold, respectively, with elevated temperature. We previously reported a 3.54-fold increase in late I Na percent when temperature was elevated from 22 °C to 34 °C; however, we used CHO-1 cells to study E1784K thermosensitivity. The temperature coefficient (Q10) partly depends on lipid-channel interactions in the membrane, which differ between heterologous expression systems. The HEK293 lipid bilayer is less viscous than CHO1 cells as observed in our whole-cell recordings, which may justify the heightened Q10 in the late I Na measurements. At elevated stimulation frequencies and temperature, therapeutic Ranolazine decreased channel availability by increasing channel use-dependence in E1784K.

Although Ranolazine efficacy appears to be increased by temperature, its efficacy appears to be dampened by the combination of both elevated temperature and cytosolic calcium. At 34 °C, E1784K late I Na percent was depressed with elevated cytosolic calcium, but there was no effect on late I Na density. These opposing changes in late I Na percent and density may be attributed to the increased peak I Na density with elevated cytosolic calcium at 34 °C. Although not significant, the shift contributes to late I Na percent calculation (Table 1). Late I Na in E1784K was not attenuated by Ranolazine at elevated temperature and cytosolic calcium.

### Drug Binding in Mutant-Trigger Context

Ranolazine action on NaV1.5 is commonly associated with the modulated receptor hypothesis. Sokolov et al. argued that the drug follows a modified form of binding, as...
Activation and Steady-State Fast Inactivation.
were unavailable for drug binding in auto-docking due to their constricted sizes. It would be interesting to
the interaction between the channel and the fast inactivation particle. Fast inactivation onset is correlated with DIVS4 activation, whereas channel recovery is rate limited by charge immobilization of DIVS4. The charge reversal mutant, E1784K, may enhance the transition of DIVS4 between closed and open states, as suggested by the Peters-Ruben model. We postulate that this effect may be due to an electrostatic repulsion between the CTD mutant and conserved positive residues in DIVS4, given their close proximity. This repulsion could make the DIVS4 in E1784K more mobile, which might explain the accelerated fast inactivation onset and recovery kinetics. Fast inactivation kinetics in E1784K are not enhanced by temperature, so it does not seem justified to attribute the thermosensitive late \( I_{\text{IS}} \) in E1784K to increased recovery kinetics. Rather a rearrangement may occur in DIVS4, conforming the voltage sensor to a state in which conductance in E1784K is higher.

Table 2. Activation and Steady-State Fast Inactivation. *p < 0.01 vs 0μM and 10μM Ran of same condition. 
*p < 0.05 vs 0μM and 10μM Ran of same condition.

| Condition | GV-V_{1/2} (mV) | GV-\( \alpha \) | N | SSFI-V_{1/2} (mV) | SSFI-\( \alpha \) | N |
|-----------|----------------|----------------|---|----------------|----------------|---|
| WT - 22 °C - 0 nM Ca\(^{2+}\) - 0μM Ran | −40.45 ± 1.24 | 3.76 ± 0.33 | 7 | −88.62 ± 1.99 | −3.93 ± 0.26 | 7 |
| WT - 22 °C - 0 nM Ca\(^{2+}\) - 10μM Ran | −45.59 ± 1.09 | 3.57 ± 0.29 | 6 | −86.51 ± 1.36 | −3.95 ± 0.16 | 6 |
| WT - 22 °C - 0 nM Ca\(^{2+}\) - 100μM Ran | −44.13 ± 0.67 | 4.84 ± 0.56 | 5 | −88.45 ± 3.18 | −2.87 ± 0.11 | 6 |
| WT - 22 °C - 2500 nM Ca\(^{2+}\) - 0μM Ran | −42.22 ± 1.16 | 4.08 ± 0.25 | 9 | −91.72 ± 1.72 | −3.68 ± 0.15 | 9 |
| WT - 22 °C - 2500 nM Ca\(^{2+}\) - 10μM Ran | −44.40 ± 2.22 | 3.87 ± 0.28 | 7 | −91.44 ± 1.63 | −3.93 ± 0.18 | 7 |
| WT - 22 °C - 2500 nM Ca\(^{2+}\) - 100μM Ran | −44.76 ± 2.09 | 4.38 ± 0.36 | 9 | −90.94 ± 1.64 | −2.20 ± 0.13 | 7 |
| WT - 34 °C - 0 nM Ca\(^{2+}\) - 0μM Ran | −43.44 ± 2.06 | 6.00 ± 0.45 | 6 | −80.31 ± 1.17 | −4.72 ± 0.18 | 6 |
| WT - 34 °C - 0 nM Ca\(^{2+}\) - 10μM Ran | −37.93 ± 1.87 | 4.95 ± 0.28 | 7 | −82.51 ± 2.26 | −6.00 ± 0.14 | 8 |
| WT - 34 °C - 0 nM Ca\(^{2+}\) - 100μM Ran | −37.58 ± 2.03 | 4.43 ± 0.33 | 7 | −87.30 ± 2.73 | −2.82 ± 0.16 | 6 |
| WT - 34 °C - 2500 nM Ca\(^{2+}\) - 0μM Ran | −40.62 ± 1.44 | 5.96 ± 0.35 | 6 | −80.25 ± 2.30 | −4.24 ± 0.08 | 7 |
| WT - 34 °C - 2500 nM Ca\(^{2+}\) - 10μM Ran | −38.03 ± 3.69 | 5.15 ± 0.40 | 6 | −83.18 ± 3.95 | −4.00 ± 0.13 | 6 |
| WT - 34 °C - 2500 nM Ca\(^{2+}\) - 100μM Ran | −43.69 ± 0.98 | 6.30 ± 0.48 | 6 | −90.43 ± 2.80 | −3.18 ± 0.23 | 6 |
| EK - 22 °C - 0 nM Ca\(^{2+}\) - 0μM Ran | −33.13 ± 1.70 | 3.99 ± 0.27 | 8 | −100.46 ± 1.51 | −2.97 ± 0.08 | 6 |
| EK - 22 °C - 0 nM Ca\(^{2+}\) - 10μM Ran | −35.83 ± 1.65 | 3.14 ± 0.27 | 10 | −99.37 ± 1.61 | −2.88 ± 0.08 | 10 |
| EK - 22 °C - 0 nM Ca\(^{2+}\) - 100μM Ran | −36.16 ± 1.87 | 3.95 ± 0.17 | 7 | −103.83 ± 2.42 | −1.77 ± 0.06 | 7 |
| EK - 22 °C - 2500 nM Ca\(^{2+}\) - 0μM Ran | −39.33 ± 2.02 | 3.09 ± 0.16 | 5 | −101.30 ± 3.14 | −3.07 ± 0.21 | 6 |
| EK - 22 °C - 2500 nM Ca\(^{2+}\) - 10μM Ran | −34.88 ± 1.57 | 2.83 ± 0.15 | 8 | −100.76 ± 1.83 | −3.11 ± 0.13 | 8 |
| EK - 22 °C - 2500 nM Ca\(^{2+}\) - 100μM Ran | −33.72 ± 1.52 | 3.02 ± 0.35 | 5 | −106.45 ± 2.29 | −2.12 ± 0.08 | 5 |
| EK - 34 °C - 0 nM Ca\(^{2+}\) - 0μM Ran | −30.22 ± 1.27 | 3.68 ± 0.28 | 9 | −91.02 ± 2.79 | −3.18 ± 0.14 | 9 |
| EK - 34 °C - 0 nM Ca\(^{2+}\) - 10μM Ran | −36.80 ± 1.20 | 4.31 ± 0.38 | 9 | −94.20 ± 3.73 | −3.14 ± 0.13 | 9 |
| EK - 34 °C - 0 nM Ca\(^{2+}\) - 100μM Ran | −35.53 ± 1.18 | 4.11 ± 0.22 | 8 | −105.48 ± 1.64 | −2.07 ± 0.07 | 8 |
| EK - 34 °C - 2500 nM Ca\(^{2+}\) - 0μM Ran | −34.74 ± 1.48 | 4.06 ± 0.41 | 8 | −96.78 ± 4.70 | −3.26 ± 0.12 | 8 |
| EK - 34 °C - 2500 nM Ca\(^{2+}\) - 10μM Ran | −31.25 ± 1.24 | 3.92 ± 0.35 | 7 | −95.01 ± 3.23 | −2.94 ± 0.15 | 7 |
| EK - 34 °C - 2500 nM Ca\(^{2+}\) - 100μM Ran | −33.22 ± 1.64 | 3.39 ± 0.15 | 9 | −105.73 ± 2.44 | −2.00 ± 0.06 | 10 |
(2) E1784K alters the structure of CTD by disrupting the native hydrophobic and electrostatic interactions holding the EF-like hand domain (α1–α4) tight with the IQ motif (α6)31. Calcium sensitivity is imparted in NaV 1.5 via CaM, which binds to the IQ motif (α6) via its C-lobe or N-lobe depending on cytosolic calcium levels71–73. During diastole or systole, CaM is calcified to different extents at its N-lobe 74. Calcified CaM has a lower affinity for the IQ motif and binds, via its C-lobe, to DIII-DIV linker, forming a tripartite complex. This interaction is thought to prevent the DIII-DIV linker fast inactivation particle from occluding the pore, increasing channel availability near resting potential71,73. With depolarized potentials, the CaM C-lobe stabilizes the fast inactivation particle, suppressing late INa, as in ∆KPQ and other mixed syndrome mutants37,40,44. Some studies refute the tripartite complex formation and favor a CaV 1.2-like regulation of inactivation in NaV 1.574,75. In those studies, the calcium-calmodulin complex is localized to CTD 76,77. The NaV Pas structure showed intermolecular interactions between DIII-DIV linker, CTD, and DIVS4 67. Motoike et al. reported CaM-independent interactions between the inter-linkers in NaV 1.536, suggesting that CaM acts as an auxiliary channel modifier during a calcium signal75. Calcium regulation in NaV 1.5 is mediated by CaM since the dual EF-like hand domains in CTD do not bind calcium 74,75. In light of these structural models, we speculate that E1784K decouples both the calcium-dependent and calcium–independent interactions between the DIII-DIV linker and the CTD. Thus, E1784K inhibits calcium–dependent facilitation in NaV 1.5. We propose that the decoupling in CTD caused by E1784K may create a high entropy, unstable structure. Upon a calcium signal, the calcified calmodulin has reduced affinity for the IQ motif, thus augmenting CTD entropy73.

Figure 4. Ranolazine effects on steady-state fast inactivation. Panels A, B (22 °C) and C (34 °C) shows steady-state fast inactivation as normalized current plotted against the prepulse potential (pulse protocol shown in C inset).

Figure 5. Ranolazine effects on fast inactivation onset time constants. Panels A, B show the single-exponential time constants plotted against voltage. The pulse protocol is identical to that used to measure channel conductance (refer to Methods). Panel C includes Q10 coefficient values for all conditions between –50 mV to +10 mV. Cytosolic calcium seems to modulate Ranolazine effects on WT Q10 as elevated cytosolic calcium heightened thermosensitivity. Cytosolic calcium made –50 mV fast inactivation onset in E1784K less thermosensitive, consistent with decoupling between CTD and Domain III-IV linker mechanism, explained in the discussion.
or calcium-induced reduction in late I_{Na} especially in the right epicardium, results in complete action potential failure. E1784K channels are less available as myocardial ischemia or infarction, and heart failure. The majority of patients with this expressivity are finely tuned by channel switches, like temperature and cytosolic calcium.

Both mechanisms (1) and (2) may occur simultaneously in E1784K. The calcium effects in Na_{v}1.5 are localized to CTD. No reports have shown direct interaction between calcium-calmodulin and DIVS4, so if mechanism (2) occurs, it may be via an indirect effect on DIVS4.

In light of the discussed structural insights, we speculate that Ranolazine can easily access the inner vestibule with non-
calciﬁed calmodulin, since the molecule binds tightly to the IQ motif. Ranolazine efficacy, however, is hampered by cytosolic calcium, suggesting an interaction between the drug and the channel at CTD. The high entropy CTD in calcified calmodulin seems to physically hinder Ranolazine from entering into the inner vestibule.

### Physiological and Medical Implications

Elevated temperature and cytosolic calcium are two of many other physiological triggers that occur during exercise and are common to other pathophysiological states, such as myocardial ischemia or infarction, and heart failure. The majority patients with SCN5a mutations show ameliorated LQT3 phenotype during exercise. Functional studies have correlated this to a stimulation frequency or calcium-induced reduction in late I_{Na}. However, it is clear from our study, focusing on E1784K, that the SCN5a mutant response to triggers can be unique. Thus, it is necessary to study antiarrhythmics in SCN5a cohorts during different physiological states as the mutant-trigger effect may determine drug efficacy.

Our AP simulations clearly show pro-arrhythmic effects of Ranolazine, which are exacerbated by febrile temperatures. Electrical restitution curves clearly show a critical diastolic interval at which alternans are triggered. Our AP simulations provide evidence of Ranolazine’s arrhythmogenicity, as it does not shorten APD_{90} in E1784K at high heart rates. At low heart rates and at body core temperature, the drug shortens APD_{90} in cardiac cells. However, with normal and elevated heart rates, the drug induces alternans, an effect exacerbated at febrile temperature. The critical diastolic intervals at which alternans are caused by the drug appear earlier (at higher BCLs) at febrile temperatures.

E1784K induces alternans with higher prevalence in epicardial cells at low heart rates. This result coincides with the phase 2 re-entry phenomenon constituting the repolarization hypothesis in BrS. The high I_{Na} density, especially in the right epicardium, results in complete action potential failure. E1784K channels are less available for activation due to the hyperpolarized SSFI-V_{10}. This seems to be the main mechanism behind the decrease in AP upstroke velocity in cardiac cells, especially the epicardial cells, despite the mutant and triggers-exacerbated increased late I_{Na}. Thus, E1784K expresses both gain- and loss-of-function at the electrical level in cardiac cells. However, this expressivity is finely tuned by channel switches, like temperature and cytosolic calcium.

Our previous and current data suggest exercise, and its accompanying physiological triggers, differentially affect mixed syndrome mutations, especially E1784K. The action of different antiarrhythmics appear to differ depending on physiological state.
Conclusions

Appropriate management of cardiac arrhythmias in SCN5a patients requires careful investigation of antiarrhythmic drug efficacy under various physiological states. Our results suggest that Ranolazine may increase the susceptibility for arrhythmia development in E1784K carriers at sinus rhythm and tachycardia. The risk is augmented under febrile conditions. Although exercise is commonly associated with high heart rates, other pathophysiological states share common triggers, as in heart failure or myocardial ischemia and infarction. Other antiarrhythmics should also be screened against E1784K and other channel mutants under various physiological conditions.

Methods

Homology Modelling and Auto-Docking. Homology modeling was performed using the Swiss-Model server (https://swissmodel.expasy.org). The newly cryo-EM solved American cockroach voltage-gated sodium channel (NaV Pas) structure (3.8-Å resolution) was used as a template against the NaV 1.5 sequence. Modeling was done according to the protocol established by Bordoli et al. Sequence alignment was performed using Uniprot Align (http://www.uniprot.org/align/) for SCN5A_HUMAN (NaV 1.5) and SCNA1_PERAM (NaV Pas).

Ranolazine was virtually docked using AutoDock4 against the NaV 1.5 homology model built on NaV Pas (NaV 1.5-NaV Pas). PyMOL-pdb viewer was used for optimization and visualization of the auto-docking results.

Ethical approval. The research was approved by Biohazards review 251–2012 issued by the office of the Environmental Health and Safety at Simon Fraser University, Burnaby, BC, Canada.

Cell Culture. HEK293 cells were grown at pH 7.4 in a DMEM (1x) nutrient medium (Life Technologies, NY, USA), supplemented with 10% FBS and maintained in a humidified environment at 37 °C with 5% CO₂. The α subunits (WT or E1784K) were co-transfected with the β1 subunit and green fluorescent protein, eGFP (1.50 μg: 0.75 μg: 1.50 μg, respectively). The cDNA mixture was then allowed to incubate with the HEK293 cells before plating on coverslips. The HEK293 cells were selected for this study since they contain a relatively elevated [CaM] free level compared to other cell lines, thereby controlling for calcium-calmodulin effects on NaV 1.5.
**Table 4.** Late I$_{Na}$ Current Density and Percentage. *p < 0.01 vs 10 μM and 100 μM Ran of same condition.

| Condition | Late I$_{Na}$ Density (pA/pF) | N | Late Percent (%) | N |
|-----------|-----------------------------|---|------------------|---|
| WT - 22 °C - 0 nM Ca$^{2+}$ - 0 μM Ran | 1.95 ± 0.34 | 9 | 1.71 ± 0.54 | 9 |
| WT - 22 °C - 0 nM Ca$^{2+}$ - 10 μM Ran | 1.35 ± 0.44 | 8 | 0.35 ± 0.12 | 8 |
| WT - 22 °C - 0 nM Ca$^{2+}$ - 100 μM Ran | 1.55 ± 0.79 | 6 | 0.45 ± 0.20 | 6 |
| WT - 22 °C - 2500 nM Ca$^{2+}$ - 0 μM Ran | 8.83 ± 1.33 | 8 | 2.41 ± 0.72 | 6 |
| WT - 22 °C - 2500 nM Ca$^{2+}$ - 10 μM Ran | 3.78 ± 1.64 | 8 | 0.80 ± 0.48 | 7 |
| WT - 22 °C - 2500 nM Ca$^{2+}$ - 100 μM Ran | 1.01 ± 0.38 | 7 | 0.30 ± 0.12 | 6 |
| WT - 34 °C - 0 nM Ca$^{2+}$ - 0 μM Ran | 5.04 ± 1.42 | 9 | 1.57 ± 0.52 | 9 |
| WT - 34 °C - 0 nM Ca$^{2+}$ - 100 μM Ran | 7.21 ± 1.61 | 8 | 1.54 ± 0.54 | 8 |
| WT - 34 °C - 0 nM Ca$^{2+}$ - 100 μM Ran | 7.66 ± 2.47 | 9 | 1.67 ± 0.42 | 9 |
| WT - 34 °C - 2500 nM Ca$^{2+}$ - 0 μM Ran | 10.57 ± 3.2 | 7 | 2.71 ± 0.90 | 9 |
| WT - 34 °C - 2500 nM Ca$^{2+}$ - 10 μM Ran | 6.95 ± 1.97 | 8 | 1.09 ± 0.26 | 8 |
| WT - 34 °C - 2500 nM Ca$^{2+}$ - 100 μM Ran | 5.11 ± 1.67 | 4 | 0.98 ± 0.25 | 5 |
| EK - 22 °C - 0 nM Ca$^{2+}$ - 0 μM Ran | 2.51 ± 0.63 | 9 | 0.85 ± 0.20 | 9 |
| EK - 22 °C - 0 nM Ca$^{2+}$ - 10 μM Ran | 2.86 ± 0.88 | 9 | 0.68 ± 0.21 | 8 |
| EK - 22 °C - 0 nM Ca$^{2+}$ - 100 μM Ran | 3.01 ± 1.08 | 10 | 1.02 ± 0.39 | 8 |
| EK - 22 °C - 2500 nM Ca$^{2+}$ - 0 μM Ran | 9.02 ± 2.31 | 5 | 2.64 ± 0.81 | 4 |
| EK - 22 °C - 2500 nM Ca$^{2+}$ - 10 μM Ran | 3.68 ± 0.51 | 9 | 1.65 ± 0.30 | 9 |
| EK - 22 °C - 2500 nM Ca$^{2+}$ - 100 μM Ran | 1.78 ± 0.65 | 8 | 0.61 ± 0.30 | 6 |
| EK - 34 °C - 0 nM Ca$^{2+}$ - 0 μM Ran | 17.47 ± 1.54 | 6 | 8.97 ± 1.02 | 6 |
| EK - 34 °C - 0 nM Ca$^{2+}$ - 10 μM Ran | 3.84 ± 1.39 | 4 | 0.86 ± 0.04 | 4 |
| EK - 34 °C - 0 nM Ca$^{2+}$ - 100 μM Ran | 3.09 ± 0.96 | 6 | 0.89 ± 0.22 | 6 |
| EK - 34 °C - 2500 nM Ca$^{2+}$ - 0 μM Ran | 11.01 ± 2.12 | 7 | 2.00 ± 0.34 | 9 |
| EK - 34 °C - 2500 nM Ca$^{2+}$ - 10 μM Ran | 10.05 ± 3.08 | 5 | 3.28 ± 0.93 | 6 |
| EK - 34 °C - 2500 nM Ca$^{2+}$ - 100 μM Ran | 15.73 ± 6.24 | 5 | 3.76 ± 1.10 | 4 |

**Electrophysiology.** Whole-cell patch clamp recordings were performed in extracellular solution containing (mM): 96 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, and 10 HEPES (pH 7.4). Solutions were titrated with CsOH to pH 7.4. Pipettes were fabricated with a P-1000 puller using borosilicate glass (Sutter Instruments, CA, USA), dipped in dental wax to reduce capacitance, then thermostatically polished to a resistance of 1.0–1.5 MΩ. Low resistance electrodes were used to minimize series resistance between pipette and intracellular solution resulting in typical access resistances of 3.5 MΩ or less, thereby minimizing voltage measurement error. Pipettes were filled with intracellular solution. For minimal cytosolic calcium levels, pipettes contained (mM): 130 CsF, 9.6 NaCl, 10 HEPES, 10 EGTA titrated to pH 7.4. The intracellular pipette solution was manipulated to mimic peak systolic cytosolic calcium$^{85,86}$. To do so, we calculated, using the Ca-EGTA Calculator v1.3, the amount of CaCl$_2$ (in mM) added to bring cytosolic calcium to 2500 nM at both 22 °C and 34 °C. We extrapolated data to physiological temperatures using a Q$_{10}$ relationship, which was supported with Arrhenius calculations, (described below). After a giga ohm seal resistance was achieved, the whole-cell configuration was attained. The holding potential between protocols was −110 mV. We recorded I$_{Na}$ from cells that expressed currents no greater than −5 nA. The average voltage error calculated for all cells used in this study (n = 250) is 6.06 mV ± 0.40 mV obtained (Table 7). There are no differences between the voltage-errors in the different conditions (p > 0.05).

**Drug Preparation.** Ranolazine was obtained from Gilead Sciences (Foster City, CA) in powder form, diluted to 100 mM stock in 0.1 M HCl, aliquoted at 10 mM and stored at −20°C. Working concentrations of 10 μM (therapeutic concentration) or 100 μM (non-therapeutic) were freshly prepared in bath solution. pH was readjusted before performing electrophysiological experiments. Due to the large number of experimental conditions and the challenges of maintaining whole-cell recordings at elevated temperature, we performed unmatched pair experiments.

**Analysis and Statistics.** Analysis and graphing were done using FitMaster software (HEKA Elektronik, Lambrecht, Germany) and Igor Pro (WaveMetrics, Lake Oswego, OR, USA) with statistical information derived using JMP statistical software. Statistical significance was accepted at p < 0.05 using a four-factor completely randomized design (CRD) ANOVA test followed by a post-hoc Tukey test. Our statistical model was a full factorial in...
which all the factors were allowed to interact together yielding multiple effect tests: Ranolazine, Channel Variant, Ranolazine × Channel Variant, Temperature, Ranolazine × Temperature, Channel Variant × Temperature, Ranolazine × Channel Variant × Temperature, Calcium, Ranolazine × Calcium, Channel Variant × Calcium, Ranolazine × Channel Variant × Calcium, Temperature × Calcium, Ranolazine × Temperature × Calcium, Channel Variant × Temperature × Calcium, Ranolazine × Channel Variant × Temperature × Calcium. All values reported in the results sections are given as means ± standard error of means.

Voltage Protocols.  

Current Density.  We measured current density from the ratio of current amplitude to the cell membrane capacitance (pA/pF).

Conductance Density.  Channel conductance was calculated from peak INa using Ohm’s law at 0 mV.

\[
G_{Na} = \frac{I_{Na}}{V - E_{rev}}
\]

(1)

where \(G_{Na}\) is sodium channel conductance, \(I_{Na}\) is peak sodium current in response to the command potential \(V = 0\) mV, and \(E_{rev}\) is the reversal potential. We measured conductance density from the ratio of conductance to the cell membrane capacitance (nS/pF).

Activation (GV).  To determine the voltage dependence of activation, we measured the peak current amplitude at test pulse potentials ranging from \(-100\) mV to \(+80\) mV in increments of \(+10\) mV for 19 ms. Prior to the test pulse, channels were allowed to recover from fast inactivation at \(-130\) mV for 197 ms. Channel conductance was calculated from peak \(I_{Na}\) using Formula (1). Calculated values for conductance were normalized to the maximal conductance and fit with the Boltzmann function:

\[
\frac{G}{G_{max}} = \frac{1}{1 + \exp \left( -ze_{0}[V_m - V_{1/2}]/kT \right)}
\]

(2)

where \(G/G_{max}\) is the normalized conductance amplitude, \(V_m\) is the command potential, \(z\) is the apparent valence, \(e_0\) is the elementary charge, \(V_{1/2}\) is the midpoint voltage, \(k\) is the Boltzmann constant, and \(T\) is temperature in °K.

Steady-State Fast Inactivation (SSFI).  The voltage-dependence of SSFI was measured by preconditioning the channels to a hyperpolarizing potential of \(-130\) mV and then eliciting prepulses from \(-130\) or \(-150\) to \(+10\) mV in increments of \(10\) mV for 500 ms. Channel availability was assessed during a test pulse to 0 mV. Normalized current amplitude as a function of voltage was fit using the Boltzmann function:

\[
\frac{I}{I_{max}} = \frac{1}{1 + \exp \left( -ze_{0}(V_m - V_{1/2})/kT \right)}
\]

(3)

where \(I/I_{max}\) is the normalized current amplitude, \(z\) is apparent valence, \(e_0\) is the elementary charge, \(V_m\) is the prepulse potential, \(V_{1/2}\) is the midpoint voltage of SSFI, \(k\) is the Boltzmann constant, and \(T\) is temperature in °K.
Table 5. Use-Dependence (1 Hz and 3 Hz).  *p < 0.0001 vs 0 μM and 10 μM Ran of same condition. **p < 0.001 vs 0 μM Ran of same condition. ***p < 0.0001 vs 10 μM and 100 μM Ran of same condition. ****p < 0.001 vs 0 μM and 100 μM Ran of same condition.

**Fast Inactivation Onset.** Time constants for open-state fast inactivation were derived by fitting a single exponential function to the decay of current obtained from the activation protocol.

\[ I = I_{ss} + \alpha \exp(-(t - t_0)/\tau) \]  

where \( I \) is current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha \) is the amplitude at time 0 for time constant \( \tau \), and \( t \) is time.

**Late \( I_{ss} \) Current.** Late \( I_{ss} \) was measured between 40–50 ms during a 50 ms depolarizing pulse to 0 mV from a holding potential of –130 mV. An average of 10 pulses was used to increase the signal-to-noise ratio.

**Use-Dependent Inactivation (UDI, 1 Hz and 3 Hz).** Channels accumulated into a use-dependent inactivated state during either a series of 300 380 ms depolarizing pulses to 0 mV followed by a 615 ms–110 mV recovery pulse at a frequency 1 Hz, or 500 220 ms depolarizing pulses to 0 mV followed by a 110 ms–110 mV recovery pulse at a frequency 3 Hz. Normalized current amplitude as a function of time was fit with a double exponential.

\[ I = I_{ss} + \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \]  

where \( I \) is current amplitude, \( I_{ss} \) is the plateau amplitude, \( \alpha_1 \) and \( \alpha_2 \) are the amplitudes at time 0 for time constants \( \tau_1 \) and \( \tau_2 \), and \( t \) is time.

**Q_{10} Coefficients.** The temperature coefficient for kinetic and thermodynamic parameters plotted as a function temperature was calculated in Igor:

\[ Q_{10} = \left( \frac{R_b}{R_a} \right)^{10/(T_2 - T_1)} \]  

where \( R \) is the rate and \( T \) is temperature (1 and 2 are the two temperatures measured). Rate was calculated by the inverse of the \( \tau \) value. \( Q_{10} \) fits for steady-state midpoints and slopes were calculated by replacing the \( R_b \) with \( V_{1/2} \) and \( z \) values. Fits for \( V_b \) were calculated based of the 1/\( V_b \) to yield optimal \( Q_{10} \) values. The fit was extrapolated to physiological (37°C) and febrile (41°C) temperatures.

**Arrhenius Calculations.** The Arrhenius linear relationship for the natural exponent of kinetic or thermodynamic parameters as a function of inverse temperature was calculated in Igor:
### Condition 37°C (Q10) 41°C (Q10) 37°C (Arrhenius) 41°C (Arrhenius)

| Condition | Peak GV Density (nS/pF) | Late I\(_{Na}\) Density (pA/pf) | Late Percent (%) | GV-V\(_{1/2}\) (mV) | GV-z |
|-----------|-------------------------|----------------------------------|------------------|------------------|------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | 16179.18 | 1126.32 | 1.54 | -44.23 | 6.79 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | 22678.58 | 99329.99 | 117.26 | -36.12 | 4.84 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 27586.50 | 131656.89 | 521.76 | -40.20 | 6.59 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 15043.82 | 48489.81 | 521.76 | -36.50 | 5.55 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | 3575.20 | 3527.30 | 339.21 | -29.04 | 3.89 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | 12400.15 | 35482.80 | 339.21 | -29.04 | 4.68 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 14940.82 | 51358.73 | 430.22 | -23.06 | 4.36 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 2866.49 | 2528.37 | 430.22 | -23.06 | 4.28 |
| Peak I\(_{Na}\) Density (pA/pf) | 446.63 | 75.26 | 270.93 | 3575.20 | 114.21 |
| Late I\(_{Na}\) Density (pA/pf) | 564.39 | 706.75 | 3575.20 | 430.22 | 22.38 |
| Late Percent (%) | 623.14 | 754.26 | 3575.20 | 430.22 | 22.38 |
| GV-V\(_{1/2}\) (mV) | 521.76 | 633.36 | 3575.20 | 430.22 | 22.38 |
| GV-z | 27511.5 | 1550.0 | 22.38 | 10.61 | 2.80 |

Continued
| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | −78.24 | −75.70 | −77.94 | −75.59 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | −81.49 | −80.21 | −81.32 | −80.15 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | −77.46 | −74.07 | −77.02 | −73.92 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | −81.11 | −78.58 | −80.79 | −78.47 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | −88.67 | −85.79 | −88.30 | −85.67 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | −92.87 | −91.23 | −92.66 | −91.15 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | −95.62 | −94.16 | −95.43 | −94.10 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | −93.54 | −91.72 | −93.31 | −91.64 |

**SSFI z**

| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | −4.95 | −5.26 | −4.99 | −5.27 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | −4.02 | −4.04 | −4.02 | −4.04 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | −4.41 | −4.63 | −4.44 | −4.63 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | −4.02 | −4.05 | −4.03 | −4.05 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | −3.24 | −3.31 | −3.25 | −3.32 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | −3.21 | −3.30 | −3.22 | −3.31 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | −3.31 | −3.38 | −3.32 | −3.38 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | −2.90 | −2.85 | −2.89 | −2.84 |

**−50mV FI τ (ms)**

| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | 2.04 | 1.77 | 2.00 | 1.76 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | 2.71 | 2.58 | 2.69 | 2.57 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 2.19 | 1.92 | 2.15 | 1.91 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 1.27 | 1.01 | 1.24 | 1.00 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.80 | 0.69 | 0.78 | 0.69 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.77 | 0.65 | 0.75 | 0.64 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.60 | 0.57 | 0.59 | 0.57 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 1.04 | 0.99 | 1.03 | 0.99 |

**−30mV FI τ (ms)**

| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.54 | 0.43 | 0.53 | 0.43 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.61 | 0.52 | 0.60 | 0.52 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.36 | 0.26 | 0.35 | 0.25 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 0.51 | 0.42 | 0.50 | 0.42 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.30 | 0.25 | 0.29 | 0.25 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.26 | 0.21 | 0.25 | 0.21 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.23 | 0.20 | 0.23 | 0.20 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 0.31 | 0.27 | 0.31 | 0.27 |

**−10mV FI τ (ms)**

| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.28 | 0.22 | 0.27 | 0.21 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.30 | 0.25 | 0.29 | 0.25 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.21 | 0.16 | 0.20 | 0.15 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 0.24 | 0.19 | 0.23 | 0.19 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.20 | 0.16 | 0.19 | 0.16 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.18 | 0.15 | 0.18 | 0.15 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.15 | 0.13 | 0.15 | 0.13 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 0.19 | 0.16 | 0.18 | 0.16 |

**+10 mV FI τ (ms)**

| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.19 | 0.14 | 0.18 | 0.14 |
| WT - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.20 | 0.16 | 0.19 | 0.16 |
| WT - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.16 | 0.12 | 0.16 | 0.12 |
| WT - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 0.16 | 0.13 | 0.16 | 0.12 |
| EK - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.16 | 0.13 | 0.15 | 0.13 |
| EK - 0 nM Ca\(^{2+}\) - 10 µM Ran | 0.15 | 0.12 | 0.14 | 0.12 |
| EK - 2500 nM Ca\(^{2+}\) - 0 µM Ran | 0.12 | 0.10 | 0.12 | 0.10 |
| EK - 2500 nM Ca\(^{2+}\) - 10 µM Ran | 0.15 | 0.12 | 0.14 | 0.12 |

**1 Hz y6**

| Condition | 37°C (Q10) | 41°C (Q10) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-----------|-----------|-----------|-----------------|-----------------|
| WT - 0 nM Ca\(^{2+}\) - 0 µM Ran | 0.94 | 0.97 | 0.94 | 0.97 |

Continued
| Condition                      | 37°C (Q_{10}) | 41°C (Q_{10}) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-------------------------------|---------------|---------------|-----------------|-----------------|
| WT - 0 nM Ca^{2+} - 0 µM Ran | 0.76          | 0.76          | 0.76            | 0.76            |
| WT - 2500 nM Ca^{2+} - 0 µM Ran | 0.90        | 0.90          | 0.93            | 0.93            |
| WT - 2500 nM Ca^{2+} - 10 µM Ran | 0.83      | 0.85          | 0.84            | 0.85            |
| EK - 0 nM Ca^{2+} - 0 µM Ran | 0.86          | 0.92          | 0.87            | 0.92            |
| EK - 0 nM Ca^{2+} - 10 µM Ran | 0.83          | 0.91          | 0.84            | 0.92            |
| EK - 2500 nM Ca^{2+} - 0 µM Ran | 0.92        | 0.97          | 0.92            | 0.97            |
| EK - 2500 nM Ca^{2+} - 10 µM Ran | 0.83      | 0.90          | 0.84            | 0.91            |

1 Hz - τ
| Condition                      | 37°C (Q_{10}) | 41°C (Q_{10}) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-------------------------------|---------------|---------------|-----------------|-----------------|
| WT - 0 nM Ca^{2+} - 0 µM Ran | 5.34          | 6.15          | 5.43            | 6.18            |
| WT - 0 nM Ca^{2+} - 10 µM Ran | 6.34          | 7.76          | 6.51            | 7.83            |
| WT - 2500 nM Ca^{2+} - 0 µM Ran | 42.49      | 65.65         | 44.88           | 66.93           |
| WT - 2500 nM Ca^{2+} - 10 µM Ran | 2.23        | 1.65          | 2.15            | 1.63            |
| EK - 0 nM Ca^{2+} - 0 µM Ran | 8.14          | 10.28         | 8.38            | 10.38           |
| EK - 0 nM Ca^{2+} - 10 µM Ran | 2.22          | 2.01          | 2.19            | 2.00            |
| EK - 2500 nM Ca^{2+} - 0 µM Ran | 23.75       | 50.72         | 28.04           | 52.25           |
| EK - 2500 nM Ca^{2+} - 10 µM Ran | 1.22        | 0.77          | 1.15            | 0.75            |

3 Hz - y₀
| Condition                      | 37°C (Q_{10}) | 41°C (Q_{10}) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-------------------------------|---------------|---------------|-----------------|-----------------|
| WT - 0 nM Ca^{2+} - 0 µM Ran | 0.90          | 0.99          | 0.91            | 1.00            |
| WT - 0 nM Ca^{2+} - 10 µM Ran | 0.69          | 0.75          | 0.70            | 0.75            |
| WT - 2500 nM Ca^{2+} - 0 µM Ran | 0.83        | 0.88          | 0.84            | 0.88            |
| WT - 2500 nM Ca^{2+} - 10 µM Ran | 0.63        | 0.65          | 0.63            | 0.66            |
| EK - 0 nM Ca^{2+} - 0 µM Ran | 0.79          | 0.82          | 0.79            | 0.83            |
| EK - 0 nM Ca^{2+} - 10 µM Ran | 0.57          | 0.60          | 0.57            | 0.60            |
| EK - 2500 nM Ca^{2+} - 0 µM Ran | 0.76        | 0.78          | 0.76            | 0.78            |
| EK - 2500 nM Ca^{2+} - 10 µM Ran | 0.68        | 0.76          | 0.69            | 0.76            |

3 Hz - τ
| Condition                      | 37°C (Q_{10}) | 41°C (Q_{10}) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-------------------------------|---------------|---------------|-----------------|-----------------|
| WT - 0 nM Ca^{2+} - 0 µM Ran | 2.97          | 3.29          | 3.01            | 3.30            |
| WT - 0 nM Ca^{2+} - 10 µM Ran | 0.70          | 0.46          | 0.67            | 0.46            |
| WT - 2500 nM Ca^{2+} - 0 µM Ran | 1.38        | 1.28          | 1.36            | 1.27            |
| WT - 2500 nM Ca^{2+} - 10 µM Ran | 0.98        | 0.93          | 0.97            | 0.92            |
| EK - 0 nM Ca^{2+} - 0 µM Ran | 3.07          | 3.69          | 3.14            | 3.72            |
| EK - 0 nM Ca^{2+} - 10 µM Ran | 1.47          | 1.29          | 1.45            | 1.28            |
| EK - 2500 nM Ca^{2+} - 0 µM Ran | 2.27        | 2.01          | 2.24            | 2.00            |
| EK - 2500 nM Ca^{2+} - 10 µM Ran | 0.84        | 0.58          | 0.81             | 0.57            |

3 Hz - τ
| Condition                      | 37°C (Q_{10}) | 41°C (Q_{10}) | 37°C (Arrhenius) | 41°C (Arrhenius) |
|-------------------------------|---------------|---------------|-----------------|-----------------|
| WT - 0 nM Ca^{2+} - 0 µM Ran | 14.24         | 10.80         | 13.75           | 10.66           |
| WT - 0 nM Ca^{2+} - 10 µM Ran | 15.28         | 13.72         | 15.08           | 13.65           |
| WT - 2500 nM Ca^{2+} - 0 µM Ran | 39.52       | 40.39         | 39.63           | 40.43           |
| WT - 2500 nM Ca^{2+} - 10 µM Ran | 20.81       | 26.32         | 21.43           | 26.60           |
| EK - 0 nM Ca^{2+} - 0 µM Ran | 54.26         | 61.55         | 55.14           | 61.90           |
| EK - 0 nM Ca^{2+} - 10 µM Ran | 31.12         | 36.95         | 31.81           | 37.24           |
| EK - 2500 nM Ca^{2+} - 0 µM Ran | 43.81       | 68.26         | 46.41           | 69.75           |
| EK - 2500 nM Ca^{2+} - 10 µM Ran | 5.29        | 4.15          | 5.13            | 4.11            |

Table 6. Values at 37 °C and 41 °C based on Q_{10} and Arrhenius Calculations.
ln(k) = ln(A) − (Ea/R) × (1/T)  
(7)

where k is the rate constant, steady-state midpoint, or slope, A is the pre-exponential factor, Ea is the activation energy, R is the universal gas constant, and T is temperature in °K.

**Myocardial Action Potential (AP) Modeling.** Simulations. Action potentials were simulated using a modified version of the O’Hara Rudy (ORD) model at 37 °C and 41 °C programmed in Matlab. The sodium data was extrapolated to physiological and febrile temperatures Q10 values for WT and E1784K at 0 µM and 10 µM. Ranolazine. The maximal GNa density was 150 mS/µF in all conditions simulated. We modified the gating INa parameters data in accordance with our biophysical data for the various conditions. The GV and SSFI midpoints and slopes were extrapolated to 37 °C and 41 °C and normalized to the original ORd parameters. The phospho-rylated steady-state fast inactivation midpoints in all channel variants were equally hyperpolarized by 6.2 mV. Late INa density was normalized to the original ORd value and multiplied by the percentage of late to peak INa calculated above.

Figure 8. Endocardial action potential simulations. AP simulations are plotted against time (inset is shown in bottom right corner) at 37 °C and 41 °C. The last two AP beats were plotted in E1784K to show alternans-induction. Simulations only included therapeutic concentrations of Ranolazine (10 µM).

Figure 9. Cardiac electrical restitution properties. Panel A,B shows the endocardial ERC curves at 37 °C and 41 °C. Panels C shows plots of the last two AP beats to determine alternans-induction in the three myocardial cells at 37 °C and 41 °C.
value, prior to the current stimulus pulse, by 0.9. The APD90 of the final two beats in the frequency step were
initial duration (APD) was measured at 90% of repolarization by multiplying the resting membrane potential (RMP)
Analysis.
gradually from 0.5 Hz to 2.5 Hz, with a 1000 beats per frequency step to ensure attainment of steady-state.

| Condition | Voltage Error (mV) | N  |
|-----------|-------------------|----|
| WT - 22°C - 0 nM Ca²⁺ - 0 µM Ran | 3.52 ± 0.97 | 9 |
| WT - 22°C - 0 nM Ca²⁺ - 10 µM Ran | 7.83 ± 1.47 | 8 |
| WT - 22°C - 0 nM Ca²⁺ - 100 µM Ran | 5.70 ± 2.05 | 6 |
| WT - 22°C - 2500 nM Ca²⁺ - 0µM Ran | 4.89 ± 1.29 | 9 |
| WT - 22°C - 2500 nM Ca²⁺ - 10 µM Ran | 6.54 ± 1.30 | 11 |
| WT - 22°C - 2500 nM Ca²⁺ - 100 µM Ran | 3.12 ± 0.88 | 10 |
| WT - 34°C - 0 nM Ca²⁺ - 0 µM Ran | 8.35 ± 1.56 | 11 |
| WT - 34°C - 0 nM Ca²⁺ - 10 µM Ran | 9.08 ± 1.32 | 9 |
| WT - 34°C - 0 nM Ca²⁺ - 100 µM Ran | 7.18 ± 1.16 | 19 |
| WT - 34°C - 2500 nM Ca²⁺ - 0 µM Ran | 8.64 ± 0.98 | 7 |
| WT - 34°C - 2500 nM Ca²⁺ - 10 µM Ran | 8.53 ± 1.79 | 9 |
| WT - 34°C - 2500 nM Ca²⁺ - 100 µM Ran | 8.14 ± 1.57 | 6 |
| EK - 22°C - 0 nM Ca²⁺ - 0 µM Ran | 4.41 ± 0.65 | 19 |
| EK - 22°C - 0 nM Ca²⁺ - 10 µM Ran | 5.99 ± 1.22 | 10 |
| EK - 22°C - 0 nM Ca²⁺ - 100 µM Ran | 4.28 ± 0.72 | 14 |
| EK - 22°C - 2500 nM Ca²⁺ - 0 µM Ran | 3.01 ± 0.71 | 6 |
| EK - 22°C - 2500 nM Ca²⁺ - 10 µM Ran | 4.49 ± 1.04 | 9 |
| EK - 22°C - 2500 nM Ca²⁺ - 100 µM Ran | 4.63 ± 1.21 | 10 |
| EK - 34°C - 0 nM Ca²⁺ - 0 µM Ran | 6.50 ± 1.09 | 19 |
| EK - 34°C - 0 nM Ca²⁺ - 10 µM Ran | 6.74 ± 1.73 | 10 |
| EK - 34°C - 0 nM Ca²⁺ - 100 µM Ran | 5.52 ± 0.98 | 12 |
| EK - 34°C - 2500 nM Ca²⁺ - 0 µM Ran | 5.63 ± 1.32 | 8 |
| EK - 34°C - 2500 nM Ca²⁺ - 10 µM Ran | 8.95 ± 1.97 | 8 |
| EK - 34°C - 2500 nM Ca²⁺ - 100 µM Ran | 3.67 ± 0.69 | 11 |

Table 7. Voltage Error.

To model the calcium-dependence of our late \( I_{Na} \) data, we fit the biophysical parameters extrapolated to 37°C and 41°C with a Hill equation:

\[
Z = Y_0 + \left( \frac{Y_M - Y_0}{1 + \left( X_{1/2}/X \right)^b} \right) \tag{8}
\]

where \( Z \) is the biophysical parameter of interest, \( Y_0 \) is the minimum value, \( Y_M \) is the maximum value, \( X_{1/2} \) is the midpoint of the curve, \( X \) is the intracellular cytosolic calcium, \( b \) is the rate.

Subspace calcium was not accounted for due to the lack of experimental data. Thus, the modified ORd model is a dynamic simulation of the calcium-induced shifts which are observed with increasing intracellular calcium levels as a function of pacing frequency, comprising the positive staircase phenomenon\(^{88,89}\).

Simulations at febrile temperature (41°C) included modifications to the major ionic currents, \( I_{Kt} \)\(^{94}\), in the ORd model based on previously published Q 10 values.

Simulations were run on endocardial, midmyocardial, and epicardial ventricular myocytes using a 0.5 ms stimulus pulse with an amplitude of −80 µA/µF. The stimulus protocol was designed to step up the frequency gradually from 0.5 Hz to 2.5 Hz, with a 1000 beats per frequency step to ensure attainment of steady-state.

Analysis. Analysis of APs only included those that fully recovered and were restored to baseline. Action potential duration (APD) was measured at 90% of repolarization by multiplying the resting membrane potential (RMP) value, prior to the current stimulus pulse, by 0.9. The APD₉₀ of the final two beats in the frequency step were plotted versus the diastolic interval (DI = BCL − APD₉₀), where BCL is the basic cycle length, creating electrical restitution curves.

Data Availability. All data generated or analysed during this study are included in this published article.

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**Author Contributions**

M.A. and co-author collected, assembled, analyzed, and interpreted the data, designed the experiments, and drafted the manuscript. M.R. collected, assembled, and analyzed data. P.C.R. conceived the experiments and revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript and qualify for authorship.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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