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Multiple mechanisms underlie increased cardiac late sodium current

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Abstract

Background: We recently reported a quantitative relationship between the degree of functional perturbation reported in the literature for 356 variants in the cardiac sodium channel gene *SCN5A* and the penetrance of resulting arrhythmia phenotypes. In the course of that work, we identified multiple *SCN5A* variants, including R1193Q, that are common in populations but are reported in HEK cells to generate large late sodium current (I_{Na-L}).

Objective: To compare the functional properties of R1193Q to those of the well-studied LQT3 mutation KPQ.

Methods: We compared functional properties of *SCN5A* R1193Q to those of KPQ in CHO cells at baseline and following exposure to intracellular PIP₃, which inhibits I_{Na-L} generated by decreased PI3-kinase activity. We additionally used CRISPR/Cas9 editing to generate R1193Q in human induced pluripotent stem cells differentiated to cardiomyocytes (hiPSC-CMs).

Results: Both R1193Q and KPQ generated robust I_{Na-L} in CHO cells. PIP₃ abrogated the late current phenotype in R1193Q cells, but had no effect on KPQ. Homozygous R1193Q hiPSC-CMs displayed increased I_{Na-L} and long action potentials with frequent triggered beats, which were reversed with addition of PIP₃.

Conclusions: The consistency between the late current produced in HEK, CHO, and hiPSC-CMs suggests late current is a feature of the *SCN5A* R1193Q variant in human cardiomyocytes, but that the mechanism by which the late current is produced is distinct, and indirect, compared to the more highly penetrant KPQ. These data suggest observing a late current in an *in vitro* setting

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does not necessarily translate to highly pathogenic LQT3 phenotype but depends on underlying mechanism.

Keywords

SCN5A/Na_v1.5; human induced pluripotent stem cell cardiomyocytes hiPSC-CMs; Late/persistent current; LQT3; genetics

Introduction

Variants in *SCN5A* encoding the cardiac sodium channel Na_v1.5 are associated with several inherited arrhythmias, including type 3 long QT (LQT3) and Brugada syndromes, arrhythmia disorders that predispose individuals to sudden cardiac arrest. We have recently shown *SCN5A* variant functional perturbation is quantitatively associated with the degree of clinical penetrance.¹ However, we unexpectedly did not find that the magnitude of the late sodium current (I_{Na-L}) predicted penetrance in LQT3 and indeed some variants, notably R1193Q, are reported to generate large I_{Na-L} but are so common in certain populations that they cannot be invoked as causes of LQT3. Several studies initially implicated *SCN5A* R1193Q in sudden unexplained death, Brugada syndrome, and type 3 LQTS.^{2, 3} Functional assessment of R1193Q revealed a large late current,^{2, 4, 5} a feature previously associated with LQT3. However, aggregated exome sequencing results now estimate *SCN5A* R1193Q is present in 6.1% alleles in the East Asian population, with a 0.3% homozygous carrier rate (as reported in gnomAD, a large database of > 250,000 alleles from diverse ancestries).^{6, 7} Both homozygous and heterozygous carrier frequencies are much higher than the prevalence of LQT3, estimated at 0.005% in the general population (across all *SCN5A* mutations),⁸ suggesting little to no role of *SCN5A* R1193Q in disease presentation.⁹ These conflicting data—significant molecular phenotype, large late current, but high minor allele frequency—prompted us to test whether distinct mechanisms, some leading to high penetrance, are responsible for the ultimate expression of late current. In our previous assessment, the canonical KPQ variant (the originally-described LQT3 mutant in which three codons in *SCN5A* coding for residues 1505-1507 thought to be critical for fast inactivation are deleted)¹⁰⁻¹² displayed nearly 100% penetrance whereas penetrance was near zero for R1193Q.

We and others have recently shown that PI3-kinase inhibition generates late sodium current, which is reversed by the PI3K downstream effector phosphatidylinositol (3,4,5)-trisphosphate (PIP₃).^{13, 14} We show here that the late current generated by R1193Q is inhibited by PIP₃ whereas that generated by KPQ is not. These differences in sensitivity implicate distinct mechanisms in late current generation. There are many intracellular processes that modulate I_{Na-L}, such as Ca²⁺ dependent pathways, PIP₃ dependent pathways, etc.¹⁵ We therefore suggest structural variants directly inducing disruptions in inactivation and channel closure are more pathogenic in nature. The findings also demonstrate that screening novel genetic variants in *SCN5A* and other LQT-associated genes thought to be late current-inducing (such as CAV3 and syntactin) need to consider these diverse mechanisms.

Methods

Expression of *SCN5A* WT and variants in Chinese hamster ovary (CHO) cells.

Point mutations were introduced into *SCN5A* using the QuikChange Lightning kit (Agilent Technologies, Inc., Santa Clara, CA, USA). All recombinant cDNAs were sequenced to confirm the incorporation of R1193Q and KPQ variants. CHO cells were transfected with wildtype or mutant *SCN5A* pIRES2-GFP mammalian expression plasmids using the FuGENE transfection system. Cells were incubated at 37 °C for 48 hours in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, and 5% CO₂. Cells fluorescing green were selected for further electrophysiological studies. Ion currents in this study were recorded using the standard protocols as previously described.¹⁶ Additionally, 5 nM ATX^{17, 18} (Sigma-Aldrich, St. Louis, MO, USA) was used in the extracellular solution in some experiments where indicated.

Editing and differentiating human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs).

We introduced R1193Q into exon 19 of hiPSCs derived from a healthy individual. Guide RNA was designed using the Webserver <http://crispr.mit.edu/> and introduced into the Px459 vector coexpressing Cas9 and puromycin resistance. We used the following rescue template which includes the c.3578 G>A (bold) and the introduced silent variations, an NlaIII cut site and removal of the Protospacer-Adjacent Motif (PAM) site used in the guide RNA (both occupy the same site, underlined):

CCGGCGCTGTCCCTGCTGTGCGGTGGACACCACACAGGCCCCAGGGAAGGTCTG
GTGGC

aGTTCGCAAGACaTGCTACCACATCGTGGAGCACAGCTGGTTCGAGACATTCATC
ATCTT CATGATCCTACTCAGCAGTGGAGCGCTGGT. HiPSCs were grown to confluency and electroporated with the modified Px459 vector and recovery template using the Neon Transfection System (ThermoFisher Scientific, Waltham, MA). Resulting puromycin resistant colonies were isolated and assayed by restriction enzyme digestion and sanger sequenced for incorporation of the variant. For electrophysiologic characterization, cells homozygous for R1193Q were differentiated into cardiomyocytes by modulating Wnt signaling followed by glucose starvation to enrich the selection, as previously established.¹⁹ After 35 days or longer of differentiation, beating cells were dissociated from the Matrigel plate using TrypLE™ Select Enzyme (ThermoFisher Scientific, Waltham, MA) and passed through a 100 µm filter before electrophysiologic characterization.

Recordings of cardiac peak/late sodium currents and action potentials.

In whole-cell voltage clamp mode, we used previously described methods^{20, 21} to record peak and late sodium currents in *SCN5A*-transfected CHO cells and hiPSC-CMs by using an external K⁺- and Ca²⁺- free solution contained sodium concentration of 135 mM at room temperature (23°C). In current-clamp mode, we recorded action potentials (APs) from non-beating hiPSC-CMs by injection of a brief stimulus current (1-2 nA, 2-6 ms) at stimulation rates of 0.5 Hz and 0.1 Hz. Spontaneously-beating action potentials were also recorded without stimulation. For AP experiments, the bath (extracellular) solution was normal Tyrode's, containing (in mM): NaCl 135, KCl 4.0, CaCl₂ 1.8, and MgCl₂ 1.0, HEPES 5.0,

glucose 10, with a pH of 7.4. The pipette-filling (intracellular) solution contained (in mM): KCl 130, ATP-K₂ 5.0, MgCl₂ 1.0, CaCl₂ 1.0, BAPTA 0.1, and HEPES 5.0, with a pH of 7.3 (adjusted by KOH). In some experiments, 1 μ M PIP₃ was added into the intracellular solution to observe its effect on action potentials.

Assessment of structural context of SCN5A KPQ, R1193Q, and other I_{Na-L}-inducing variants.

We used the recently released cryo-EM structure of the electric eel Na_V1.4 (67% sequence identity to human Na_V1.5) structure (PDBID: 5XSY)²² to assess the spatial relationship between variants in the structurally modellable region of Na_V1.5 with high reported late current and high or low LQT3 penetrance. All variants assessed were previously reported to have late current at least 200% of WT and have been observed in at least five individuals. The low penetrance subset of this group had a penetrance < 20% (A185T, S216L, T353I, and R1193Q); the high penetrance subset had a penetrance > 20% (T1304M, N1325S, KPQ, R1644H, V1763M, Y1767C, V1777M, and E1784K). We visualized the overall shape and construction of the channel molecules using the PyMOL molecular graphics suite.²³ We then mapped onto this structure variants mentioned above to visualize which parts of the protein might be affected by these substitutions.

Results

Mechanism of late sodium current is distinct between SCN5A R1193Q and KPQ.

In CHO cells, both *SCN5A* R1193Q and KPQ generated large late currents: 0.3 ± 0.01 % (WT), 1.24 ± 0.09 % (R1193Q) and 2.0 ± 0.8 % (KPQ) of peak sodium current (Figures S1, 1 and 2) similar to what had been observed previously.^{2,4, 5,10-12} In addition, intracellular addition of 1 μ M PIP₃ significantly reduced *SCN5A* R1193Q late current from 1.5 ± 0.3 % to 0.4 ± 0.13 % ($p < 0.05$, Figure 1) without altering peak current, while PIP₃ addition had no effect on KPQ late current: 2.0 ± 0.7 % vs 2.0 ± 0.8 % (control, $p > 0.05$, Figure 2). These results suggested distinct mechanisms involved in late current generation between the two variants. Additionally, 5 nM ATX^{17, 18} generated late current recalcitrant to modulation by PIP₃ (Figure S2). These data further suggest the mechanisms resulting in late current in the genetic context of the *SCN5A* KPQ variant (or by the addition of ATX, which directly disrupts normal inactivation²⁴) is distinct from R1193Q.

hiPSC-CMs recapitulate late current phenotype and yield expected arrhythmogenic action potentials which are reversed by addition of PIP₃.

To further probe the late current phenotype of *SCN5A* R1193Q, we introduced the variant in human induced pluripotent stem cells. Five separate lines successfully incorporated the variant, three homozygous and two heterozygous (Figure S3). Endogenous late sodium current (I_{Na-L}) in cardiac cells is very small (~0.5% of peak sodium current) with a physiologic concentration of extracellular sodium.^{25, 26} Differentiated hiPS-CMs homozygous for the R1193Q edit also produced the large late current phenotype: 2.93 ± 0.4 % of peak I_{Na} vs WT 0.17 ± 0.01 % ($p < 0.01$, Figure 3). Measured action potentials had anomalous features of unusual prolongation and triggered activity (Figure 4): APD₉₀ at 0.5 Hz was 370 ± 50 ms (WT) vs 920 ± 110 ms (R1193Q, $p < 0.01$). Such long APs in

R1193Q cells were corrected by adding intracellular PIP₃ (1 μ M), to 380 ± 100 ms, which is indistinguishable from that seen in WT cells. R1193Q cells also displayed irregular spontaneous beating associated with delayed afterdepolarizations (DADs).

Discussion

Although there is a clear correlation between *SCN5A* variant perturbation measured by heterologous expression and clinical presentation,¹ there are variants where relatively high minor allele frequency (as reported in gnomAD) conflicts with the severity of functional perturbation, especially variants characterized by a large “late current” gain-of-function phenotype. *SCN5A* R1193Q was shown previously to generate large late currents, a well-established mechanism leading to type 3 LQTS, by heterologous expression in HEK cells.²⁷⁻³¹ However, the prevalence of this variant in ostensibly unaffected populations, 6.1% in East Asian alleles, argues strongly that it cannot play a significant role in disease presentation.⁹ This conflict prompted us to assess whether underlying mechanisms which induce late current may be identifiably distinct between high and low penetrant variants with similar late current phenotypes. We additionally examined the response in the more native like hiPS-CM context closer to the native context. Here we showed that the *SCN5A* R1193Q late current phenotype is produced in both CHO cells and hiPS-CMs and that the mechanism of this late current is distinct from the KPQ variant.

Given the differences in physical location, and without locations proposed common to both, we suspected these variants perturb channel function by distinct mechanisms (Figure 5). Residue 1193 is located on the “S0” helix which precedes the voltage-sensing, four-helix bundle of Domain III known to be partly responsible for activation of voltage-gated sodium channels,³² whereas KPQ is located on the DIII-DIV linker (residues 1481 to 1515). Residue 1193, as for residues 185 and 216, both with late current greater than 200% of WT and estimated LQT3 penetrance < 20%, lies peripherally on the Nav1.5 molecule and is likely indirectly involved in inactivation (Figure 5). The molecular mechanism of voltage-gated sodium channel inactivation or how late current is generated are unknown, however the DIII-DIV linker is a key intracellular component. In fact, substituting three key residues (I1488, F1489, and M1490) near the KPQ site (K1505-Q1507) with glutamines removes all inactivation.³³ Additionally, the DIII-DIV linker is dynamic along most its length³⁴ which may be necessary to exhibit the multiple conformations observed in recently determined structures of sodium channels;^{22, 35} this property is likely perturbed in the context of KPQ (Figure 5). As opposed to residue 1193, residues 1505-1507 are centrally located within Nav1.5 as are other highly penetrant, high late current variants (Figure 5), which we believe suggests a more direct role in perturbing inactivation of the channel.

Variants with high late current and high penetrance localize most often near the region of the Nav1.5 molecular responsible for inactivation. Of the higher penetrance variants modeled (Figure 5, light red), all but one lies on the intracellular side of the Nav1.5 channel. Furthermore, all higher penetrance variants lie toward the DIII-DIV half of the molecule that houses the inactivation machinery, including the DIII-DIV linker. The lower penetrance variants appear distributed more peripherally, suggesting distinct roles structurally between these classes of variants in *SCN5A*. In fact, of the 15 variants with low LQT3 penetrance

identified in our earlier work, 9 lie outside the modellable region (R458C, L619F, R689H, P1177L, E1901Q, S1904L, R1913H, F2004L, and P2006A), mostly in interdomain linkers and the C-terminus. However, none of the 11 higher penetrance variants lie outside the modellable region. These data imply a subset of variants with direct impact on inactivation and late current lead to a higher penetrance than other more peripherally located *SCN5A* variants which influence the channel more indirectly.

We tried a pharmacological approach to assess differences between the late current found in R1193Q compared to the more highly penetrant KPQ. PIP₃ contributes to regulation of several intracellular pathways including PDK1, SGK, and Akt. The mechanism by which Na_v1.5 is regulated by PIP₃ is not known; however, certain QT prolonging drugs that downregulate PI3K α , an upstream regulator of PIP₃, are known to modulate cardiac sodium late current, an effect rescued by addition of PIP₃ intracellularly.^{13, 21} We therefore tested the hypothesis of distinct mechanisms resulting in late current between R1193Q and KPQ by observing the response to intracellular PIP₃. The sensitivity of R1193Q to PIP₃, but not KPQ or the ATX-Na_v1.5 complex, suggests distinct mechanisms inducing late current have distinct pathologies which need to be considered when interpreting the severity of the excess late current functional defect in Na_v1.5. This distinction may in part explain the heterogeneity in LQT3 penetrance among variants with similarly large late currents. In addition, our data suggest a PIP₃-influenced compensatory pathway leading to susceptibility to QT prolongation in other contexts beyond lesions in *SCN5A* such as drug challenges, other genetic variants, electrolyte abnormalities or other processes known to modulate late current, Ca²⁺ concentration, β subunit interaction, oxidative stress, and phosphorylation.¹⁵

Conclusion

We propose that late current is a functional perturbation that arises from heterogeneous mechanisms of Na_v1.5 dysfunction, one direct and structural (KPQ) and another indirect (R1193Q). The consistency between the *SCN5A* R1193Q late current produced in CHO and hiPS-CM cells suggest late current is a feature of this variant in human cardiomyocytes, but that the mechanism by which the late current is produced is distinct compared to KPQ. We believe this mechanistic distinction gives rise to differences in penetrance, near 0% for R1193Q and near 100% for KPQ, and suggest certain mechanisms resulting in additional late current can be compensated for in the human myocardium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Internal addition of PIP₃ reversed R1193Q variant-expressed late current in CHO cells. A) Control peak and late current traces recorded with the protocol shown as an insert. B) Peak and late current traces recorded with internally-added PIP₃. C) Summary of control and peak I_{Na} recorded after two minutes with internal PIP₃. D) Summary of control and late I_{Na} recorded after two minutes with internal PIP₃.

Figure 2:

No effect of internal addition of PIP_3 on KPQ-expressed peak and late I_{Na} in CHO cells.

A) control peak and late current traces recorded with the protocol shown as an insert. B) Peak and late current traces recorded with internally-added PIP_3 . C) Summary of control and peak I_{Na} recorded after two minutes with internal PIP_3 . D) Summary of control and late I_{Na} recorded after two minutes with internal PIP_3 . Note the observed late current is not modulated by addition of PIP_3 , in contrast to R1193Q.

Figure 3: *SCN5A* WT- and R1193Q-expressed late I_{Na} from CRISPR/Cas9 edited hiPS-CMs. A-B) Normalized late I_{Na} traces from WT (A) and R1193Q cells with a single-pulse voltage protocol shown as an insert. C) Comparison of average late I_{Na} between WT and R1193Q.

Figure 4:

Action potentials of *SCN5A* WT and R1193Q in hiPS-CMs. A-B) Action potentials generated by stimulating hiPS-CMs derived from an unaffected individual at 0.5 and 0.1 Hz. C) Action potentials from *SCN5A* R1193Q edited otherwise isogenic control stimulated at 0.5 Hz as in A. Action potentials are much longer for R1193Q than the unedited control line. D) Cells could not be stimulated at 0.1 Hz due to spontaneous beats. Arrhythmogenic DADs were also observed in the absence of AP stimulation. E) Long action potential phenotype observed in R1193Q hiPS-CMs can be rescued by addition of PIP₃, a known late current modulator.

Figure 5:

Cartoon representation of a model of Nav_v1.5 highlighting the membrane spanning segments as well as R1193 and residues 1505-1507 (KPQ, removed in KPQ). Rightmost image is from the perspective of inside the cell looking out through the channel and membrane.

R1193 is in the amphipathic helix preceding the transmembrane domain of domain III while KPQ is in the domain III-IV linker, the segment of *SCN5A* known to influence inactivation.³³ The model is based on electric eel Nav_v1.4 (67% sequence identity to human Nav_v1.5) structure (PDBID: 5XSY).²² The four helix voltage-sensing modules of each domain (I-IV) are noted by black circles. Residues where variants with at least five carriers and late current greater than 200% that of WT are known are shown as spheres. Light blue spheres are variants where the predicted penetrance is < 20%; light red spheres are variants where predicted penetrance is > 20%. The high-penetrance variants are spatially proximal to the inactivation machinery, in contrast with low-penetrance variants.