The RyR2-P2328S mutation downregulates Nav1.5 producing arrhythmic substrate in murine ventricles

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Abstract Catecholaminergic polymorphic ventricular tachycardia (CPVT) predisposes to ventricular arrhythmia due to altered Ca2+ homeostasis and can arise from ryanodine receptor (RyR2) mutations including RyR2-P2328S. Previous reports established that homozygotic murine RyR2-P2328S (RyR2S/S) hearts show an atrial arrhythmic phenotype associated with reduced action potential (AP) conduction velocity and sodium channel (Na,v1.5) expression. We now relate ventricular arrhythmogenicity and slowed AP conduction in RyR2S/S hearts to connexin-43 (Cx43) and Na,v1.5 expression and Na+ current (INa). Stimulation protocols applying extrasystolic S2 stimulation following 8 Hz S1 pacing at progressively decremented S1S2 intervals confirmed an arrhythmic tendency despite unchanged ventricular effective refractory periods (VERPs) in Langendorff-perfused RyR2S/S hearts. Dynamic pacing imposing S1 stimuli then demonstrated that progressive reductions of basic cycle lengths (BCLs) produced greater reductions in conduction velocity at equivalent BCLs and diastolic intervals in RyR2S/S than WT, but comparable changes in AP durations (APD90) and their alternans. Western blot analyses demonstrated that Cx43 protein expression in whole ventricles was similar, but Na,v1.5 expression in both whole tissue and membrane fractions were significantly reduced in RyR2S/S compared to wild-type (WT). Loose patch-clamp studies similarly demonstrated reduced INa in RyR2S/S ventricles. We thus attribute arrhythmogenesis in RyR2S/S ventricles resulting from arrhythmic substrate produced by reduced conduction velocity to downregulated Na,v1.5 reducing INa, despite normal determinants of repolarization and passive conduction. The measured changes were quantitatively compatible with earlier predictions of linear relationships between conduction velocity and the peak INa of the AP but nonlinear relationships between peak INa and maximum Na+ permeability.

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Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmic syndrome characterized by episodic syncpe and/or sudden cardiac arrest, typically triggered by adrenergic stimulation as occurs during strenuous exercise or emotional stress [1, 34, 46]. CPVT has been associated with mutations in various Ca²⁺ handling proteins, which lead to abnormalities in Ca²⁺ homeostasis; most notably, these mutations are found in the cardiac ryanodine receptor-Ca²⁺ release channel (RyR2) [25, 35] and the sarcoplasmic reticulum (SR) Ca²⁺-binding protein calsequestrin 2 (CASQ2) [13, 19]. Ca²⁺-dependent calmodulin missense mutations also occur in a small number of cases [18]. Some RyR2 mutations also predispose to atrial arrhythmias [4, 34, 39]. For example, the RyR2-P2328S mutation is associated with high incidences of both CPVT and atrial tachycardia (AT) [25, 37, 40]. This RyR2 variant has been associated with a normal luminal SR Ca²⁺ release sensitivity but an increased sensitivity to cytosolic Ca²⁺ [31], giving rise to lower cytosolic Ca²⁺ thresholds leading to Ca²⁺ release. If reached during increased heart rates, these could be sufficient to elicit a ‘leak’ of SR Ca²⁺ consequently triggering arrhythmia.

The atrial and ventricular phenotypes are replicated by the RyR2-P2328S (RyR2S/S) murine model which shows potential arrhythmic triggers in the form of delayed afterdepolarizations [15, 22]. However, there remains a requirement for an electrophysiological tissue substrate in order to perpetuate and sustain arrhythmia, which has previously been typified by a reduced conduction velocity (θ) in systems showing reduced Na,1.5 expression [33], connexin 40 and/or 43 (atria) or connexin 43 (ventricle) expression [16, 23], or structural abnormalities, including fibrosis [44]. Interestingly, several reports have indicated the potential for Na,1.5 expression [6, 11, 42] and function [2, 41] to be modulated, both directly and indirectly, by alterations in cytosolic Ca²⁺. Rat cardiomyocytes showed reductions in Na⁺ channel activity following imposed increases of intracellular [Ca²⁺]. Additionally, the Ca²⁺ channel blocker verapamil and the Ca²⁺ ionophore calcimycin increased and decreased Na,1.5 mRNA and Na,1.5 protein expression respectively [11, 42]. In agreement with these findings, elevations and reductions of cytosolic [Ca²⁺], by chronic treatment with high extracellular [Ca²⁺] and [K⁺] or BAPTA-AM, decreased and increased Na⁺ current densities, respectively [6]. More recently, Casini et al. [5] demonstrated that acute increases in pipette [Ca²⁺] were capable of reducing both Na⁺ current density and (dV/dt)max.

Biochemical evidence accounting for the potential mechanisms of functional modulation of Na,1.5 by cytosolic [Ca²⁺] identifies both direct and indirect Ca²⁺ binding sites on Na,1.5. Direct Ca²⁺ binding to Na,1.5 is mediated at an EF hand motif resident at the carboxy-terminal region of Na,1.5 [47]. This binding results in a depolarizing shift of the voltage dependence of Na⁺ channel inactivation with a potential increase in Na⁺ channel activity [47]. Indirect mechanisms of Ca²⁺ binding have been attributed to both the presence of an additional binding site, the ‘IQ’ domain, within the C-terminal region of Na,1.5 for Ca²⁺/Calmodulin (Ca²⁺/CaM) and multiple phosphorylatable sites (including serines 516 and 571 and threonine 594) within the IDI-II linker region of Na,1.5 targeted by Ca²⁺/CaM Kinase II (CaMKII). These two mechanisms occur only subsequent to Ca²⁺ binding to the EF hand motifs of Ca²⁺/Calmodulin (Ca²⁺/CaM) or Ca²⁺/CaM binding at the IQ domain and CaMKII-dependent phosphorylation shifts Na⁺ current availability to a more depolarized membrane potential [2] and enhances slow inactivation of the Na⁺ current [41].

Recent reports have indeed implicated reduced Na,1.5 expression and Na⁺ channel function in the increased arrhythmogenicity in RyR2S/S atria [21, 22, 36]. They also demonstrated a reduced conduction velocity [22], resulting from a reduced Na⁺ current attributable either to a reduced Na,1.5 expression or the direct inhibitory effect on Na⁺ channel function of altered Ca²⁺ homeostasis outlined previously. Slowed conduction resulting from reduced Na,1.5 expression would potentially produce arrhythmogenic substrate, which would compound the arrhythmic effect of Ca²⁺-mediated triggered activity in the RyR2S/S [21, 22, 36].

The present study investigates for possible roles of Cx43 expression as well as Na,1.5 expression and function in RyR2S/S ventricular, as opposed to atrial, arrhythmogenicity. First, the arrhythmogenic properties of the RyR2S/S ventricle compared to the WT was confirmed in accordance with earlier reports [15] and correlated with measurements of action potential duration (APD), conduction velocity (θ) and their alternans, as well as ventricular effective refractory period (VERP). The stimulation protocols either interposed extrasystolic, S2, stimuli at progressively decremented S1S2 intervals within 8 Hz S1 pulse trains or applied steady stimulus frequencies at progressively decremented basic cycle lengths (BCLs). Second, to assess the underlying mechanism for the slowed conduction and arrhythmic phenotype, we assessed the ventricular expression of Cx43 and Na,1.5, the latter assessed in both the whole ventricle and the membrane fraction compared between WT and RyR2S/S hearts. Third, the corresponding functional evaluation of Na,1.5 was investigated through peak INa current recordings of WT and RyR2S/S ventricular tissue. These comparisons successfully correlated Na,1.5 expression and function, particularly within the functional Na,1.5 containing membrane fraction, with the incidence of ventricular arrhythmia, and the resulting conduction changes in RyR2S/S ventricles.
Materials and methods

Experimental animals

Homozygous \(\text{RyR2}^\text{S/S}\) and WT mice (aged 4 to 6 months) with an inbred 129/Sv genetic background (supplied initially by Harlan, UK) were generated as described previously [15]. Mice were kept in plastic cages at room temperature in an animal facility, given free access to sterile rodent chow and water and subjected to 12 h light/dark cycles. Mice were killed by cervical dislocation for experimentation. All procedures conformed to the UK Animals (Scientific Procedures) Act 1986, approved by a university ethics review board. Hearts were rapidly excised and submerged in ice-cold Krebs-Henseleit (KH) buffer solution (containing, in mM, NaCl 119, NaHCO\(_3\) 25, KCl 4, KH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 1, CaCl\(_2\) 1.8, glucose 10 and Na-pyruvate 2, pH 7.4, 95% O\(_2\)/5% CO\(_2\); British Oxygen Company, Manchester, UK) for whole heart electrophysiological studies and Western blot analyses. All chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK), unless otherwise stated. Six WT and seven \(\text{RyR2}^\text{S/S}\) mice were used in whole heart electrophysiological investigations. Four WT and four \(\text{RyR2}^\text{S/S}\) hearts were used for Western blot studies of Cx43 expression. Seven WT and six \(\text{RyR2}^\text{S/S}\) mice were used for Western blot studies of Na\(_v\)1.5 expression in the whole tissue and membrane fraction. Four WT and three \(\text{RyR2}^\text{S/S}\) mice were used to give \(n=6\) and 12 patches, respectively, for loose patch-clamp studies of Na\(^+\) currents. Male and female mice were used in statistically equal numbers in each group.

Electrophysiological studies in whole heart

Excised hearts were cannulated and retrograde perfused on a Langendorff system as previously described [28, 29, 38, 48]. Prior to electrophysiological testing, hearts were perfused with KH solution for at least 5 min to achieve a steady state. Monophasic action potentials (MAPs) were recorded by microMAP electrodes (HugoSachs, Harvard Apparatus, UK) placed upon the epicardial surface. Recordings were amplified (Neurolog NL100 preamplifier; NL104 amplifier, Digitimer, Welwyn Garden City, UK), band-pass filtered (NL125/126 filter; 0.5 Hz to 1.0 kHz) and sampled at 5 kHz (micro1401 interface) for display using Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Hearts were paced at twice their excitation threshold voltage using a bipolar platinum-coated stimulating electrode placed on the ventricular septum connected to a DS2A-Mk.II stimulator (Digitimer). After pacing at 8 Hz for at least 5 min to attain and confirm a steady state, two types of pacing protocols were applied. A S1S2 protocol first stimulated hearts at frequencies of 10 Hz for 20 s; this was followed by cycles of drive trains of eight S1 beats delivered at 8 Hz followed by an S2 extra-stimulus, at S1-S2 coupling interval successively reduced by 1 ms with each subsequent cycle until either 2:1 block or sustained arrhythmia occurred. A dynamic pacing protocol [24, 28, 29] first assessed action potential (AP) properties at a BCLs of 134 ms duration for 100 stimulations. The BCL was then decremented by 5 ms, and the pacing sequence repeated until the hearts showed either entry into 2:1 block or sustained arrhythmia. Both stimulation protocols yielded incidences of arrhythmia. The S1S2 protocol additionally provided VERPs. The dynamic pacing protocol yielded APDs, and an indication of conduction velocity \(\theta\) (1/latency, which was measured as the time from the stimuli to the peak of the MAP) as a function of BCL, measured as the recovery time from AP peak to 90% full repolarization, APD\(_{90}\) (Fig. 1). The corresponding diastolic intervals (DIs) were calculated from the BCL and APD\(_{90}\) values using the relationship:

\[
\text{DI} = \text{BCL} - \text{APD}_{90}
\]

Protein extraction and Western blot analysis

The method of protein extraction was optimized for the protein of interest. For connexin proteins, which are primarily found in the surface membrane in hexameric clusters, a well-established high content sodium dodecyl sulphate (SDS) buffer [7, 12, 43] was used in order to fully solubilize the membrane and maximize release of the connexin proteins from the gap junction channels in the plasma membrane. For Na\(_v\)1.5 channels, we chose to use a milder buffer, followed by a centrifugation step and solubilization in order to separate out the membrane and non-membrane fractions based on the different distribution and abundance of Nav1.5 channels and their contribution to conduction.

Fig. 1 Two typical successive monophasic action potential (MAP) recordings at the LV epicardium of a WT heart obtained during dynamic pacing to highlight the derivation of the various parameters used for analysis; BCL, latency, APD\(_{90}\), and DI. BCL was the time interval between the adjacent stimuli, thus the pacing rate. Latency was measured as the time elapsed from the stimuli to the peak of MAP, APD\(_{90}\) is the time course over which 90% repolarization of the MAPs obtained. DI was measured as BCL-APD\(_{90}\), thus comprising the final 10% of MAP repolarization and up to the start of the next stimuli.
Ventricular tissues of the excised hearts were snap-frozen and crushed into powder by a clamp pre-cooled with liquid N2. The powdered tissue was homogenized in either SB20 (20 % SDS, 2 mM EDTA, 150 mM Tris) [7, 12, 43] and diluted to an appropriate gel loading concentration in sample loading buffer (90 % SB20, 5 % 2-mercaptoethanol, 5 % w/v bromophenol blue) for connexin 43 analysis, or the resuspension buffer (containing, in mM, Tris–HCl 50, NaCl 10, Sucrose 320, EDTA 5, EGTA 2.5 and Protease inhibitors (1 tablet/20 ml; Roche, West Sussex, UK), pH 7.4) and lysis buffer (containing, in mM, Tris–HCl 20, EDTA 2, NaCl 137 and Triton X-100 1 %, glycerol 10 %, pH 7.4) and then centrifuged for 15 min at 13,000 g and 4 °C for Na,1.5 analysis. The supernatant was divided into two parts. One was stored at −80 °C as the whole tissue fraction and the rest was centrifuged at 100,000 g and 4 °C for Na,v1.5 analysis. The occurrence of ventricular tachycardia (VT) was defined as an occurrence of two or more sequential spontaneous APs, as in previous work [36] in the course of programmed electrical stimulation. Figure 2 illustrates representative left ventricular epicardial traces from WT (A, B) and RyR2S/S (C-F) hearts, displaying regular activity (A), ectopic (B) and VT (C-E) and ventricular fibrillation (VF) (F) episodes during the S1S2 protocol. Six WT and seven RyR2S/S hearts were subject to the S1S2 protocol described in methods. None of the WT hearts showed VT, although one heart showed three separate ectopic events (at S1S2 intervals of 33, 31 and 30 ms). In contrast, the RyR2S/S hearts showed 30 episodes of arrhythmia, taking the form of either polymorphic or monomorphic VT in three of the hearts. Of these hearts, the first showed 7 episodes of VT, lasting approximately 2.8 s, with an additional VT episode that degenerated into VF lasting approximately 22.5 s. The second heart showed one episode of VT lasting approximately 11.2 s. The third heart showed 21 episodes of VT accounting for a total time of approximately 14 s.

Ventricular effective refractory periods (VERPs) were defined as the time period during which the myocardium is incapable of re-excitation in response to the twice-threshold stimulus employed in the S1S2 protocols [10, 32]. It was thus the S1S2 interval at which loss of S2 capture first occurred in an absence of VT. WT and RyR2S/S hearts typically became refractory at similar S1S2 pacing intervals (VERP: WT: 38.2±1.55 ms (n=6); RyR2S/S: 37.5±5.04 ms (n=5); P=0.9057). VERP could not be ascertained from all the mice studied due to arrhythmogenesis; sustained arrhythmias occurred in two of the seven RyR2S/S hearts during the S1S2 protocol.

The differing arrhythmic properties of WT and RyR2S/S were further confirmed in the dynamic pacing protocol.

Statistical analysis

Statistical analysis for differences between experimental groups was performed using Graphpad Prism software (La Jolla, CA 92037 USA), applying unpaired Student’s t tests. A value of P<0.05 was considered statistically significant. Data are presented as means±SEM.

Results

Comparison of ventricular arrhythmogenicity in S1S2 protocols and dynamic protocols

We initially confirmed the arrhythogenic phenotype of the RyR2S/S murine heart as previously reported [15]. An S1S2 stimulation protocol was used to determine the incidence, frequency and duration of ventricular arrhythmia in isolated Langendorff-perfused hearts.

The differing arrhythmic properties of WT and RyR2S/S were further confirmed in the dynamic pacing protocol,
which subjected hearts to systematically decreasing BCLs. Two of the six WT hearts showed VT at BCLs of 39 and 44 ms. However, these correspond to BCLs which are substantially lower, thus a much higher heart rate than those experienced during normal physiological maximal exercise [8]. RyR2S/S hearts not only demonstrated higher incidences of VT and VF but they occurred also at higher BCLs than WT (54, 64, 54 and 74 ms in four RyR2S/S hearts respectively; these necessitated termination of the protocol), suggesting a reduced capacity to tolerate increased heart rates as may be observed during emotional or physical stress such as exercise [8].

**Action potential properties and conduction at varying pacing rates**

AP propagation and recovery properties at different pacing rates were then investigated using the dynamic pacing protocol. Figure 3 illustrates typical APs thus obtained from the LV epicardium of WT (left column) and RyR2S/S hearts (right column). In both cases, AP amplitude decreased with increasing pacing rate in every heart, independent of genotype (Fig. 3a–e). At lower pacing rates, RyR2S/S hearts showed a higher incidence of alternans (Fig. 3a, right column) compared with WT hearts (Fig. 3c, left column). Around half of both the WT and the RyR2S/S hearts had shown either a loss of capture or arrhythmogenesis when the BCL reached 54 ms (Fig. 3e).

Figure 4 plots averaged (mean±SEM) APD90 (A, C) and $\theta'$ (=1/latency) (B, D) values in WT (filled symbols) and RyR2S/S (open symbols) hearts against BCL (A, B) and DI (C, D). At BCLs, where significant differences between readings at the two genotypes were obtained, this is indicated (*$P<0.05$; **$P<0.01$). Both WT and RyR2S/S showed similar ($P>0.05$) values of APD90 at each BCL and DI. These both declined with decreasing BCL and DI. RyR2S/S and WT hearts additionally showed 2:1 block at similar values of BCL (WT 56.5±5.95 ms, $n=4$; RyR2S/S: 54±2.5 ms, $n=3$; $P=0.751$). As with VERP for the S1S2 protocol, 2:1 block was not obtained from all the mice studied; this was due to arrhythmogenesis warranting termination of the dynamic pacing protocol in four of the seven RyR2S/S hearts and two of six WT hearts. In contrast to similar APD90 at equivalent BCLs, RyR2S/S hearts showed consistently lower $\theta'$ than WT hearts at equivalent BCLs. Indeed, the highest mean $\theta'$ showed by the RyR2S/S (0.043±0.003 m s$^{-1}$), which was observed at the highest BCL, was similar to the lowest $\theta'$ (0.042±0.006 m s$^{-1}$) shown by the WT, which was observed at the shortest BCLs. These findings were corroborated when the APD90 and $\theta'$ values were plotted against their preceding DIs reflecting recovery times from the preceding APs (C, D). The present findings...
demonstrate normal AP repolarization characteristics, but compromised AP conduction in the RyR2<sup>S/S</sup> arrhythmic phenotype, which could arise from abnormalities in gap junction channels and/or Nav1.5.

Alternans of electrophysiological parameters, reflecting temporal variability, often presages arrhythmic activity. Figure 5 assesses the average (mean±SEM) degree of alternans in AP amplitude (A, D) [30], APD<sub>90</sub> (B, E) and θ<sup>'</sup> (C, F) at different BCLs (A-C) and DIs (D-F) in WT (filled symbols) and RyR2<sup>S/S</sup> (open symbols) hearts. Alternans reflects system instability through the mean difference between alternating high and low values of a given parameter normalized to the mean value of the parameter. Both the RyR2<sup>S/S</sup> and the WT demonstrated similarly increasing AP amplitude instabilities with either decreasing BCL or decreasing DI. RyR2<sup>S/S</sup> and WT showed similar APD<sub>90</sub> and θ<sup>'</sup> instabilities which similarly varied with decreasing BCL or DI. θ<sup>'</sup> instabilities were relatively small in contrast to the large changes in their mean values described.

These findings implicate abnormal conduction as opposed to abnormal repolarization in the RyR2<sup>S/S</sup> ventricular arrhythmic phenotype. The underlying mechanism/cause of this abnormal conduction is thus investigated in the next sections.

**Cx43 expression is comparable between the ventricles of WT and RyR2<sup>S/S</sup> murine hearts**

Abnormal cardiac conduction can arise from one of three factors: abnormal connexin expression/gap junction formation, impaired Na channel function and/or structural abnormalities such as with fibrosis or hypertrophy. Due to the structural similarity of WT and RyR2<sup>S/S</sup> hearts [49], we pursued the remaining two factors.

We first assessed the expression of the ventricular gap junction protein, Cx43. Western blots of whole tissue ventricular lysates from WT and RyR2<sup>S/S</sup> hearts demonstrate that the overall expression of Cx43, normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was not significantly different between...
WT and RyR2S/S ventricles (0.59±0.07; n=4 and 0.79±0.1; n=4, respectively; P>0.05, Fig. 6). This suggests that a loss of Cx43 expression is not a contributory factor to the slowed ventricular conduction and increased arrhythmogenesis observed in the ventricles of RyR2S/S hearts, in parallel to findings in the atria of the same model [21].

Decreased Na,1.5 expression in the ventricles of RyR2S/S murine hearts

Western blots of WT and RyR2S/S ventricular homogenates (Fig. 7) illustrate a decreased Na,1.5 expression in the RyR2S/S relative to the WT in both the whole tissue fraction (Fig. 7a; left panel) (1.17±0.2; n=6, and 1.69±0.15; n=7, respectively; (n=7, open symbols) hearts. APD90 is virtually superimposable at all BCLs between WT and RyR2S/S hearts and thus shows no statistically significant variation. However, θ is consistently lower in RyR2S/S hearts as compared to WT hearts with significant differences between the genotypes denoted by asterisks; *P<0.05 and **P<0.01.

Fig. 4 Plots of APD90 and θ at different BCLs and DIs in WT and RyR2S/S hearts. a, b Mean (± SEM) values for APD90 and θ respectively at different BCLs (134, 129, 124, 119, 114, 109, 104, 99, 94, 89, 84, 79, 74 and 69 ms) for WT (n=6, filled symbols) and RyR2S/S (n=7, open symbols) hearts. c, d Mean (± SEM) values for APD90 and θ respectively, at different DIs for WT (n=6, filled symbols) and RyR2S/S (n=7, open symbols) hearts. APD90 is virtually superimposable at all BCLs between WT and RyR2S/S hearts and thus shows no statistically significant variation. However, θ is consistently lower in RyR2S/S hearts as compared to WT hearts with significant differences between the genotypes denoted by asterisks; *P<0.05 and **P<0.01.

Fig. 5 Plots of alternans at different BCLs and DIs. The mean (± SEM) alternans characteristics of AP amplitude (a, d), APD90 (b, e) and θ (c, f) for WT (filled symbols) and RyR2S/S (open symbols) hearts have been plotted as percentage variation between each beat as a function of BCL (a–c) and DI (d, e) AP magnitude displays an increasing degree of alternans with decreasing BCL and DI; however, this does not vary between genotypes. Similarly, a small degree of alternans is observed in the APD90 and less so the θ with decreasing BCL and DI, but these do not vary significantly between the WT and RyR2S/S hearts.
P<0.05) and within the membrane fraction (Fig. 7a; right panel) (2.06±0.33; n=4, and 0.91±0.13; n=4, respectively; P<0.01). Thus, Na\textsubscript{v1.5} expression in RyR\textsubscript{2}\textsuperscript{S/S} ventricles was approximately 69 % of that seen in the WT whole tissue fraction and down to only 44 % of WT in the membrane fraction (Fig. 7b). This significant reduction of Na\textsubscript{v1.5} expression in the ventricular membrane where the function of Na\textsubscript{v1.5} channels is crucial to cardiac excitability, and conduction would be expected to lead to a significant reduction in I\textsubscript{Na} in the RyR\textsubscript{2}\textsuperscript{S/S} heart compared to the WT.

**Decreased I\textsubscript{Na} in the ventricles of RyR\textsubscript{2}\textsuperscript{S/S} murine hearts**

To assess whether the reduced expression of Na\textsubscript{v1.5} in RyR\textsubscript{2}\textsuperscript{S/S} ventricles correlated with a functional alteration of Na\textsubscript{v1.5}, we measured I\textsubscript{Na} in both WT and RyR\textsubscript{2}\textsuperscript{S/S} ventricles using the loose patch clamp technique. Figure 8a illustrates representative currents elicited by WT and RyR\textsubscript{2}\textsuperscript{S/S} ventricles following a series (20–120 mV voltage excursions) of depolarizing test pulses. The peak current elicited at each voltage excursion and the overall peak current for both WT and RyR\textsubscript{2}\textsuperscript{S/S} ventricles are shown in Fig. 8b, c, respectively. Currents recorded from the WT ventricle were significantly larger than those recorded in the RyR\textsubscript{2}\textsuperscript{S/S} ventricle at depolarizing pulses of 60 mV or greater (P<0.01). The overall peak current in the RyR\textsubscript{2}\textsuperscript{S/S} was −14.45 nA±0.88 nA while in the WT it was −21.3 nA±1.87 nA (P<0.01); this equates to a 32 % reduction in peak I\textsubscript{Na} in the RyR\textsubscript{2}\textsuperscript{S/S}.

**Discussion**

The present experiments demonstrate that reduced Na\textsubscript{v1.5} expression and Na current is associated with the reduced conduction velocity and consequent arrhythmic substrate and ventricular arrhythmogenesis in homozygotic murine RyR\textsubscript{2}-P2328S (RyR\textsubscript{2}\textsuperscript{S/S}) hearts. The quantitative changes were compatible with earlier reports of linear relationships predicted between the conduction velocity and the peak I\textsubscript{Na} of the AP, but a nonlinear (logarithmic) relationship between peak I\textsubscript{Na} and maximum Na\textsuperscript{+} permeability [20]. Thus, increased arrhythmogenicity was associated with a reduced conduction velocity of ~22 % during steady 8 Hz pacing and in the region of a ~33 % reduction during dynamic pacing, which would correspond to comparable reductions in AP wavelength given an absence of significant changes in repolarization characteristics (VERP and APD\textsubscript{90}), and determinants of passive conduction reflected in Cx43 expression. These in turn
accompanied reductions in membrane Na,1.5 expression of ∼56 % and peak INa of ∼32 %.

The murine RyR2S/S heart has proven a useful experimental model for CPVT in reproducing a particular clinically observed human CPVT genotype [25, 40], RyR2S/S ventricular myocytes show features of altered Ca2+ homeostasis [15] thought to result from an increased RyR2-mediated Ca2+ leak reflecting an increased sensitivity of Ca2+ release to cytosolic though not to SR levels [Ca2+] [31]. The consequent increase in cytosolic [Ca2+] would result in increased sodium-calcium exchanger (NCX) activity whose electrogenic actions would result in triggering events including delayed afterdepolarizations leading to ectopic APs that could potentially initiate ventricular arrhythmia. However, these initial studies did not explore for the presence or otherwise for arrhythmic substrate that could sustain the resulting arrhythmia.

Genetic modifications in RyR2 are also associated with AF phenotypes [17, 31, 37]. This has also been modeled by the RyR2S/S system which demonstrates abnormal atrial Ca2+ homeostasis, delayed triggering events and atrial arrhythmia [22, 48]. However, they also demonstrated reductions in conduction velocity that could provide an arrhythmic substrate [22]. This was attributed to a reduced Na+ current which could be either attributed to a reduced Na,1.5 expression or a direct inhibitory effect on Na+ channel function of altered Ca2+ homeostasis [21]. This could arise from either increased leak of SR Ca2+ or the consequently elevated diastolic Ca2+. Indeed, recent evidence suggests that altered Ca2+ homeostasis can acutely affect cardiac excitability due to both direct [47] and indirect actions on the Na+ channel [2, 5, 41]. CaMKII has been shown to directly interact with Na,1.5, shifting Na+ current availability to a more depolarized membrane potential, thus enhancing the accumulation of Na+ channels into an intermediate inactivated state [2]. Increases in CaMKII activity additionally is known to phosphorylate RyR2 which itself increases SR Ca2+ leak [45]. Intracellular Ca2+ concentration can also acutely modulate Na+ current density in ventricular myocytes [5]. Atrial conduction slowing has also been observed in further models of RyR2-mediated Ca2+ leak including a CSQ2 mutant [14, 26].

These findings suggest that altered Ca2+ homeostasis following the chronic atrial alterations in SR Ca2+ release in the RyR2S/S system could compromise Na,1.5 expression or function as a result of the elevated diastolic Ca2+. The present study now demonstrates that RyR2S/S ventricles similarly displayed a reduced Na,1.5 expression and consequently reduced peak INa, that could explain similar reductions in their conduction velocities [49]. It further extends these findings in localizing this altered expression to the membrane, as well as the whole tissue, fraction (Fig. 7), leading to a reduced maximum rate of AP depolarization, which would be expected to reduce AP conduction velocity, thus creating an arrhythmic substrate. These findings accompanied a greater arrhythmogenicity of RyR2S/S murine ventricles, which showed arrhythmic events on extrasystolic (S2) stimulation unlike WT and more frequent arrhythmias that occurred at higher BCLs during dynamic stimulation. These findings took place despite indistinguishable AP recovery characteristics in WT and RyR2S/S ventricles, as reflected in VERP and APD90, readings, thereby excluding re-entrant mechanisms involving recovery phases of the AP [27]. In contrast, RyR2S/S showed reduced indices of conduction velocity, θ through all BCLs examined compared to WT, despite indistinguishable AP amplitude, APD90 and θ alternans and their variation with BCL or DI, particularly at low BCLs.

Our findings therefore suggest that the arrhythmic substrate results from reduced expression of Na,1.5 in the membrane, where a reduced INa leads to slowed AP conduction velocity, in the ventricles of RyR2S/S mice. This would be consistent with a situation in which abnormalities in cytosolic Ca2+ exert both short- and long-term effects. In the short term, ectopic activity can follow transient elevations in cytosolic [Ca2+]. In the long term, chronic elevations in cytosolic [Ca2+] can result in a downregulation of either Na,1.5 expression or activity, thereby reducing action potential conduction and resulting in arrhythmic substrate. In such a situation, short-term triggering events could potentially form a means for initiating electrical events then perpetuated by the pre-existing arrhythmic substrate. These findings may have broader implications for the mode of therapeutic intervention in a variety of Ca2+ dependent, and potentially some Na,1.5 dependent, arrhythmia.

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