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Neuronal Na⁺ Channels Are Integral Components of Pro-arrhythmic Na⁺/Ca²⁺ Signaling Nanodomain That Promotes Cardiac Arrhythmias During β -adrenergic Stimulation

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Conflict of Interest

None

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Abstract

Background—Cardiac arrhythmias are a leading cause of death in the US. Vast majority of these arrhythmias including catecholaminergic polymorphic ventricular tachycardia (CPVT) are associated with increased levels of circulating catecholamines and involve abnormal impulse formation secondary to aberrant Ca^{2+} and Na^{+} handling. However, the mechanistic link between β -AR stimulation and the subcellular/molecular arrhythmogenic trigger(s) remains elusive.

Methods and Results—We performed functional and structural studies to assess Ca^{2+} and Na^{+} signaling in ventricular myocyte as well as surface electrocardiograms in mouse models of cardiac calsequestrin (CASQ2)-associated CPVT. We demonstrate that a subpopulation of Na^{+} channels (neuronal Na^{+} channels; nNa_v) that colocalize with RyR2 and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX) are a part of the β -AR-mediated arrhythmogenic process. Specifically, augmented Na^{+} entry via nNa_v in the settings of genetic defects within the RyR2 complex and enhanced sarcoplasmic reticulum (SR) Ca^{2+} -ATPase (SERCA)-mediated SR Ca^{2+} refill is both an essential and a necessary factor for the arrhythmogenesis. Furthermore, we show that augmentation of Na^{+} entry involves β -AR-mediated activation of CAMKII subsequently leading to nNa_v augmentation. Importantly, selective pharmacological inhibition as well as silencing of $\text{Na}_v1.6$ inhibit myocyte arrhythmic potential and prevent arrhythmias *in vivo*.

Conclusion—These data suggest that the arrhythmogenic alteration in $\text{Na}^{+}/\text{Ca}^{2+}$ handling evidenced during β -AR stimulation results, at least in part, from enhanced Na^{+} influx through nNa_v . Therefore, selective inhibition of these channels and $\text{Na}_v1.6$ in particular can serve as a potential antiarrhythmic therapy.

Keywords

β -adrenergic receptor; Ventricular arrhythmias; Neuronal Na^{+} channels; Diastolic Ca^{2+} release

Introduction

Cardiac arrhythmias are a leading cause of death in the US.¹ Arrhythmias caused by abnormal impulse generation are often associated with aberrant diastolic Ca^{2+} release (DCR) through dysregulated ryanodine receptor 2 (RyR2) Ca^{2+} release channels. This is especially evident when genetic defects in the RyR2 complex – either the RyR2 itself or one of the regulatory proteins associated with the channel (i.e., calmodulin, CASQ2, triadin and/or calstabin) – facilitate aberrant DCR.^{2–5} In particular, recent findings demonstrate that either dysfunction or loss of cardiac calsequestrin (CASQ2), an intra-sarcoplasmic reticulum (SR) Ca^{2+} -binding protein and a regulator of RyR2, impairs the ability of RyR2s to deactivate and become refractory following systolic Ca^{2+} release.^{6–12} This compromised refractoriness of Ca^{2+} release, in turn, permits the RyR2 channels to reopen during diastole, causing DCR to activate depolarizing membrane currents, resulting in pro-arrhythmic delayed afterdepolarizations (DADs).^{10,13–15} Independent of the underlying etiology, compromised RyR2 function is a hallmark of catecholaminergic polymorphic ventricular tachycardia (CPVT).

Episodes of cardiac arrhythmias in CPVT patients are precipitated by emotional stress or exercise, which are associated with increased levels of circulating catecholamines.^{2,11,16} In

accordance with the clinical presentation of a vast majority of these arrhythmias, β -blocker therapy is the mainstay of treatment for cardiac rhythm disorders.¹⁷ Recent years have witnessed research endeavors that have focused on alterations in Ca^{2+} handling and their roles in precipitating triggered arrhythmias; however, the precise mechanistic link between β -adrenergic receptor (β -AR) stimulation and arrhythmogenesis in Ca^{2+} -mediated arrhythmias remains elusive. Several targets for phosphorylation, including $\text{Ca}_v1.2$, phospholamban (PLB) and RyR2, may be involved in the arrhythmogenic effects of β -AR stimulation.¹⁵ For instance protein kinase A (PKA) phosphorylation of PLB will accelerate sarcoplasmic reticulum (SR) Ca^{2+} -ATPase 2a (SERCA2a)-mediated Ca^{2+} refilling of the SR thereby providing adequate substrate for aberrant DCR.¹⁸ On the other hand, it is unclear whether phosphorylation of RyR2 by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) plays a role in the pathogenesis of cardiac arrhythmias.¹⁹

Surprisingly, considering the Ca^{2+} -dependent nature of CPVT, these patients often respond to treatment with Na^+ channel blockers such as flecainide.^{20–22} It has been proposed that flecainide may exert its antiarrhythmic effect through a direct effect on RyR2,²³ however, this would not explain the effect of other Na^+ channel blockers on aberrant Ca^{2+} handling.^{24,25} Recently, we suggested that a subset of Na^+ channels, mainly the neuronal Na^+ channels (nNa_v), are present in the transverse (T)-tubule, near Ca^{2+} handling machinery.²⁶ These channels were initially described in neurons (hence their eponym) and are characterized by a high sensitivity to tetrodotoxin (TTX).^{24,27–29} However, little is known about the pro-arrhythmic interaction between nNa_v and aberrant Ca^{2+} handling during β -AR stimulation as well as the effects flecainide may have on this crosstalk. Furthermore, since there are multiple nNa_v isoforms expressed in cardiac myocytes^{30–32} their specific roles need to be characterized.

In this present study, we have systematically investigated the subcellular and molecular consequences of β -AR stimulation in the promotion of catecholamine-induced cardiac arrhythmias. Since, in certain variants of human CPVT, CASQ2 may be virtually absent or may exist at very low levels due to missense or other mutations; knocking out or mutating CASQ2 in a mouse realistically mimics the phenotype of human disease.^{2,33} Therefore, to investigate the role $\text{Na}^+/\text{Ca}^{2+}$ signaling we used well-established murine models of CVPT in which arrhythmogenic oscillation of intracellular Ca^{2+} and membrane potential are caused by depletion or dysfunction in CASQ2 (CASQ2 null and R33Q, respectively).^{6,14,26} We report that, in the setting of dysregulated RyR2 channels, catecholamines promote aberrant DCR by facilitating SR Ca^{2+} refilling, while enhancing nNa_v -mediated persistent Na^+ current (I_{Na}), respectively, forming the functional basis for catecholamine-induced polymorphic ventricular tachycardia (CPVT).

Materials and Methods

All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

Genetically-engineered mouse models

All the genetically-engineered mice used in our study were homozygous for their respective mutations and/or deletions. Cardiac calsequestrin (CASQ2) null mice (on mixed background)³⁴ were crossbred with a) mice conditionally overexpressing SERCA2a in a doxycycline dependent manner (on FVB/N background),³⁵ or b) with RyR2 S2814A mice (on C57BL/6 background; generous gift from Dr. Xander Wehrens).³⁶ The genotypes of the crossbred mice were confirmed by polymerase chain reactions (PCR; for CASQ2, reverse tetracycline transactivator driven by the cardiac specific α -myosin heavy chain promoter (MHC-rtTA),³⁵ tetracycline response element (Tet-RE)-SERCA2a,³⁵ and RyR2 S2814A mutation) using tail DNA. To induce the overexpression of SERCA2a, animals received doxycycline diet (Harlan TD 09295 1000 ppm Doxycycline Diet 2018) for 14-21 days. We also used cardiac CASQ2-R33Q as well as wild type (WT) mice (both on C57BL/6 background) to examine the role of Na_v1.6 and NCX in aberrant Na⁺/Ca²⁺ signaling.²⁶

Myocyte isolation, confocal Ca²⁺ imaging, Na⁺ current recordings

Ventricular myocytes were obtained by enzymatic isolation from 3-9 month old mice of both genders. Mice were anaesthetized with isoflurane and once a deep level of anaesthesia was reached the heart was rapidly removed and perfused via a Langendorff as previously described.^{14,26} Peak sodium currents (I_{Na}) were recorded using internal solution containing: 10 NaCl, 20 TEACl, 123 CsCl, 1 MgCl₂, 0.1 Tris GTP, 5 MgATP, 10 HEPES, 10 BAPTA (pH 7.2, CsOH). For persistent I_{Na} recordings we substituted BAPTA with 1 EGTA and maintained free Ca²⁺ 100 nmol/L with CaCl₂. The extracellular bathing solution for peak I_{Na} contained: 10 NaCl, 130 TEACl, 4 CsCl, 0.4 CaCl₂, 2 MgCl₂, 0.05 CdCl₂, 10 HEPES and 10 glucose. The extracellular bathing solution for persistent I_{Na} recordings contained: 140 NaCl, 4 CsCl, 1 CaCl₂, 2 MgCl₂, 0.05 CdCl₂, 10 HEPES, 10 glucose, 0.03 niflumic acid, 0.004 strophanthidin and 0.2 NiCl₂. pH was maintained at 7.4 with CsOH for both types of solutions. Whole-cell capacitance and series resistance compensation (60%) was applied along with leak subtraction. Signals were filtered with 2.9 kHz Bessel filter and I_{Na} was then normalized to membrane capacitance. Late I_{Na} was estimated by integrating I_{Na} between 50 and 450 ms.

Electrical field stimulation experiments were performed using the following external solution (in mM): 140 NaCl, 5.4 KCl, 1.0 CaCl₂, 0.5 MgCl₂, 10 HEPES, and 5.6 glucose (pH 7.4, NaOH). To assess the SR Ca²⁺ load, 20 mM caffeine was applied at the end of the experiments. Intracellular Ca²⁺ cycling was monitored by a Nikon A1 laser scanning confocal microscope. For intact myocytes, we used the cytosolic Ca²⁺-sensitive indicators Fluo-3 AM. To more reliable measurements of SR Ca²⁺ release from inside the SR in control and isoproterenol-treated CPVT cardiomyocytes, we performed experiments in Fig. 1 using a low-affinity Ca²⁺ indicator Fluo-4FF-AM. The fluorescent probes were excited with the 488 nm line of an argon laser and emission was collected at 500–600 nm. Fluo-3/Fluo-4FF fluorescence was recorded in the line scan mode of the confocal microscope. For Ca²⁺ wave recordings myocytes were paced at 0.3 Hz using extracellular platinum electrodes in order to obtain DCR frequency. Any DCR event (i.e. wave, wavelet) that increased cell-wide fluorescence intensity above 10% of the signal generated by the preceding stimulated Ca²⁺ transient was included in the analysis. The fluorescence emitted

was expressed as F/F_0 , where F is the fluorescence at time t and F_0 represents the background signal. All experiments were performed at room temperature (26°C).

Confocal microscopy of immunolabeled myocytes

Isolated ventricular myocytes were prepared for immunofluorescence as well as proximity ligation assay (PLA) as described previously.²⁶ PLA is a histo/cyto-chemical and confocal microscopy technique for determining when specific proteins are co-localized within <40 nm.³⁷ Briefly, cells were plated on laminin-coated glass coverslips, fixed with 4% paraformaldehyde (5 min), permeabilized with 0.1% Triton X-100, and washed with PBS. Endogenous immunoglobulin was blocked using a mouse-on-mouse blocking reagent (M.O.M. kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature and subsequently incubated with primary antibodies ($Na_v1.1$, 1.3, 1.6, 1.5: 1:32, 1:32, 1:50, and 1:50, respectively; Alomone, Jerusalem, Israel for nNa_v s; while $Na_v1.5$ was a generous gift from Dr. Peter Mohler. RyR2 1:100 and NCX 1:50 Pierce Antibodies, Rockford, IL, USA.) overnight at 4°C. After washing, for immunofluorescence goat secondary antibodies (anti-mouse and anti-rabbit) conjugated to Alexa Fluor (488, 549; Life Technologies, Grand Island, NY, USA) were added for 1 h, while the PLA reactions were carried out using appropriate Duolink secondary antibodies (Sigma, St. Louise, MO, USA) according to the manufacturer's instructions. The sensitivity of PLA was assessed by staining for $Na_v1.5$ (1:50, generous gift from Dr. Peter Mohler) and Connexin 43 (Cx43, 1:100, Millipore; Supplemental Fig. 1), which were previously demonstrated to co-localize at the intercalated disc.³⁷

Silencing RNA

Targeting siRNA was purchased from Santa Cruz. We used a previously validated approach of intraperitoneal injection (1.5 mg/kg) mixed with an equal volume of siPORT amine (Ambion) in the live animal.³⁸ We administered the siRNA every 24 h for 2 days. Silencing efficacy was evaluated 72 hours after initiation of therapy by Quantitative Real-Time (qRT)-PCR as well protein analysis.

Quantitative Real-Time-PCR

Hearts were collected 72 h after initiations of siRNA therapy (n=3 per each group). Total RNA was prepared from cells using RNA Purification Kit (Norgen Biotek) in accordance with manufacturer's instructions. Total RNA was subjected to qRT-PCR. RNA levels were analyzed using the TaqMan Gene Expression Assays, in accordance with manufacturer's instructions (scn1a: Mm00450580_m1, scn3a: Mm00658167_m1, scn5a: Mm01342518_m1, and scn8a: Mm00488110_m1; Life Technologies). RNA concentrations were determined with a NanoDrop 20000 (Thermo Fisher Scientific, Inc.). Samples were normalized to OAZ1 for mRNAs (Life Technologies). Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Life Technologies). Comparative real-time PCR was performed in triplicate. Relative expression was calculated using the comparative Ct method.

Immunoblots

Heart tissue lysates, following quantitation by BCA assay (Pierce), were loaded into 4-15% precast TGX gels (BioRad) and transferred to nitrocellulose membranes. Membranes were blocked for >1hr at room temperature in 3% BSA and incubated in primary antibody overnight at 4°C. Primary antibodies included: Na_v1.6 (1:500, Alomone) and GAPDH (1:5000, Fitzgerald). Secondary antibodies used were donkey anti-mouse-HRP and donkey-anti-rabbit-HRP (Jackson Laboratories). Densitometric analysis was performed using Image Lab software and all data was normalized to GAPDH.

Electrocardiographic recordings

Continuous electrocardiographic (ECG) recordings (PL3504 PowerLab 4/35, ADInstruments) were obtained from mice anesthetized with isoflurane (1-1.5%) as previously described.²⁶ Briefly, after baseline recording (5 min.), a subset of animals received either riluzole (15 mg/kg) or β-PMTX (30mg/kg). After 5 min those animals that were pre-treated with β-PMTX received either vehicle, riluzole or flecainide (20mg/kg). After additional 5-10 min animals were exposed to an intraperitoneal epinephrine (1.5 mg/kg) and caffeine (120 mg/kg) challenge and ECG recording continued for 10 mins. We also obtained continuous ECG recordings from CPVT-SERCA mice pre- and post-doxycline induction. After baseline recording (5 min.), each CPVT-SERCA mouse received only β-PMTX (30mg/kg) intraperitoneally and ECG recording continued for 10 mins. ECG recordings were analyzed using the LabChart 7.3 program (ADInstruments). Arrhythmia was defined as bigeminy or frequent ectopic ventricular activity, while ventricular tachycardia (VT) was defined as three or more premature ectopies.

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma (St Louis, MO, USA), Torcis (Bristol, UK), Focus Biomolecules (Plymouth Meeting, PA, USA), Cusabio (Wuhan, China), Medchemexpress LLC (Monmouth Junction, NJ, USA), Millipore (Billerica, MA, USA) or Alomone (Jerusalem, Israel). Fluorescent dyes were purchased from Molecular Probes (Eugene, OR, USA).

Data analysis

I_{Na} analysis was performed using pCLAMP9 software (Molecular Devices, Sunnyvale, CA). Line scanning images of Ca²⁺ were normalized for baseline fluorescence.¹⁴ Ca²⁺ imaging data were processed using ImageJ and Origin software. Confocal micrographs of PLA signal were low pass filtered (Gaussian) and thresholded to generate a black and white mask of the whole myocyte. This was used to calculate myocyte area. The unfiltered image was then thresholded using Otsu's method, followed by nearest-neighbor cluster detection to segment the PLA punctae. The punctae within the whole cell mask areas were counted to determine the density of PLA punctae within the cell (#/μm²). Statistical analysis of the data was performed using a Wilcoxon signed rank test and Wilcoxon rank-sum test for paired and non-paired continuous data, respectively or a Kruskal-Wallis Test. The Šidák correction was applied to adjust for multiple comparisons. A Fisher's exact or McNemar's tests were used to test differences in VT incidence. Based on our previous observations of mice with high

incidence of VT (70%),²⁶ four CASQ2-R33Q or other high-VT incidence mice per group were required to have a 80% chance of detecting, as significant at the 5% level, a decrease in the VT incidence from 70% in the control group to 0% in the treatment group. On the other hand, due to a lower VT incidence in the CASQ null mice,³⁹ a total sample size of 30 mice in that group was needed. All statistical analysis was performed using Origin 7.0 or R. All values are reported as means \pm SEM unless otherwise noted. A $p < 0.05$ was considered statistically significant.

Results

β -AR stimulation is necessary for aberrant DCR

In this study, we used mouse models of cardiac calsequestrin-associated CPVT. Consistent with the dependence of arrhythmia in CPVT patients on β -AR stimulation, CPVT murine myocytes presented only a few incidents of aberrant diastolic Ca^{2+} release (DCR) in the absence of isoproterenol (ISO; Sigma) (Fig. 1a, black trace and bars); Addition of ISO (100nM) markedly increased the frequency of arrhythmogenic DCRs (Fig. 1a, red traces and bars). This effect of ISO was accompanied by a significant increase in the SR Ca^{2+} content (Fig. 1a, red trace and bar). The ISO-dependent increase in the frequency of arrhythmogenic DCRs could therefore be attributed to either 1) increase in the SR Ca^{2+} content (via phosphorylation of PLB and/or $\text{Ca}_v1.2$); 2) altered RyR2 function (via phosphorylation of RyR2 at S2814); or 3) augmented neuronal Na^+ channel (nNa_v)-dependent local $\text{Na}^+/\text{Ca}^{2+}$ signaling.²⁶

β -AR stimulation increases propensity for CPVT by augmenting TTX-sensitive nNa_v -mediated persistent I_{Na}

In addition to the predominant TTX-resistant cardiac Na^+ channels ($\text{Na}_v1.5$) localized predominantly at the intercalated disc and lateral membrane,^{26,37} cardiac myocytes express several types of TTX-sensitive nNa_v localized in the cardiac T-tubule.^{26,30,31} nNa_v blockade with 100nM TTX (Tocris) significantly decreased the frequency of ISO-promoted DCRs (Fig. 1a, green traces and bars). Recently, Na^+ channel inhibitors flecainide and riluzole emerged as effective therapies in CPVT models.^{23,26} Interestingly, riluzole (10 μM ; Sigma) and flecainide (2.5 μM ; Sigma) both also reduced DCR frequency (Fig. 1a, purple and blue traces and bars). Notably, consistent with previous reports,^{25,26} none of the aforementioned interventions (i.e., TTX, riluzole or flecainide) was associated with alterations in the SR Ca^{2+} content (Fig. 1a). Taken together, these findings suggest that, in the setting of dysregulated RyR2 function, increased Na^+ influx through nNa_v during the post-systolic phase may contribute to the arrhythmogenesis evidenced in this model upon β -AR stimulation.

To examine the possibility of increased Na^+ flux through nNa_v during β -AR stimulation we assessed persistent Na^+ current (I_{Na}) in CPVT and WT cardiomyocytes. Exposure to ISO (100nM) elicited persistent I_{Na} both in CPVT and WT myocytes (Fig. 1b, Supplemental Fig. 2, respectively, red traces and bars). Notably, this current was sensitive to 100nM TTX (Fig. 1b, Supplemental Fig. 2, green traces and bars), riluzole as well as flecainide (Fig. 1b, purple

and blue traces and bars, respectively), despite the two former agents exhibiting only a fraction of flecainide's total peak I_{Na} blocking potential (Supplemental Fig. 3).

Next, we examined the impact of nNa_v -mediated persistent I_{Na} on CPVT *in vivo*. A catecholamine challenge composed of caffeine (Sigma) and epinephrine (Sigma) induced frequent ventricular arrhythmias, which degenerated into polymorphic VT (Fig. 1c, red ECG and bars). Consistent with the notion of β -AR-mediated TTX-sensitive persistent I_{Na} contributing to pro-arrhythmic DCR, the vast majority of CPVT animals tested remained in sinus rhythm when pre-treated with riluzole (Fig. 1c, purple ECG and bars). Therefore, augmentation of Na^+ influx through nNa_v by catecholamines appears to be necessary for the pro-arrhythmic aberrant Na^+/Ca^{2+} signaling in CPVT.

TTX-sensitive nNa_v -mediated persistent I_{Na} augmentation and increased SR Ca^{2+} load are necessary and sufficient for arrhythmias in CPVT

To determine whether augmentation of nNa_v -mediated Na^+ influx alone (independent of β -AR stimulation) is sufficient for inducing arrhythmogenic DCR, we induced persistent I_{Na} via nNa_v augmentation with β -Pompilidotoxin (β -PMTX)⁴⁰ in CPVT cardiomyocytes (Fig. 2a). Persistent I_{Na} induced by $40\mu M$ β -PMTX (Alomone) was completely reversed by TTX (100 nM), riluzole (10 μM) as well as flecainide (2.5 μM ; Fig. 2a). Stimulation of nNa_v channels by β -PMTX (30 mg/kg I.P.) in the absence of catecholamine challenge, however, failed to induce VT *in vivo* (Fig. 2d). Of note, β -PMTX further promoted DCR in the presence of ISO on the cellular level (Fig. 2b). This resulted in over 90% VT incidence in the CPVT mice undergoing concomitant β -PMTX treatment and catecholamine challenge (Fig. 2c, orange ECG and bar). Confirming the involvement of nNa_v in this pro-arrhythmic process, Na^+ channel blockade – both selective and non-selective – significantly reduced DCR and VT incidence in β -PMTX exposed, catecholamine challenged myocytes and animals, respectively which was independent of changes in SR Ca^{2+} load (Fig. 2b, 2c and Supplemental Fig. 4a, green, purple and blue bars and ECGs). Thus, stimulation of nNa_v alone, although necessary, is not sufficient to reproduce the proarrhythmic action of catecholamines in CPVT.

To test whether increased SR Ca^{2+} content is another necessary condition for arrhythmogenesis in CPVT we performed experiments in CPVT mice that conditionally overexpress SERCA2a (CPVT-SERCA).³⁵ Even without β -AR stimulation, CPVT-SERCA myocytes evidenced comparable SR Ca^{2+} load to ISO-exposed CPVT myocytes (Supplemental Fig. 4a) and significantly more arrhythmic DCR events relative to ISO-naive CPVT myocytes (Fig. 1a and 2b). However, this was insufficient to promote VT *in vivo* (Fig. 2d, red ECG and bar). Importantly, augmentation of Na^+ flux through nNa_v with β -PMTX in ISO-naive CPVT-SERCA myocytes was sufficient to significantly increase aberrant DCR on the cellular level, relative to untreated CPVT-SERCA myocytes. (Fig. 2b) This, in turn, precipitated VT in all the CPVT-SERCA mice exposed to β -PMTX (Fig. 2d, orange ECG and bar). Of note, in two instances when SERCA2a overexpression was reversed in CPVT-SERCA mice by stopping doxycycline-rich diet for 14 days, exposure to β -PMTX failed to induce VT. Taken together, these results suggest that nNa_v -mediated

persistent I_{Na} , combined with genetically impaired RyR2 function and enhanced SR Ca^{2+} refill, are necessary and sufficient for the arrhythmogenic phenotype responsible for CPVT.

Proarrhythmic effect of β -AR stimulation on TTX-sensitive persistent I_{Na} augmentation involves CaMKII phosphorylation of nNa_v and is independent of RyR2 phosphorylation

The aforementioned finding that β -AR stimulation promotes Na^+ influx through nNa_v suggests that catecholamines may modulate nNa_v function through phosphorylation. Recently, Na^+ channels have been shown to be subject to phosphorylation by CaMKII.^{41,42} To investigate the role of CaMKII-mediated modulation of Na^+/Ca^{2+} signaling in CPVT, we pharmacologically or genetically perturbed CaMKII signaling in CPVT cardiomyocytes. First, we observed that pharmacological blockade of CaMKII with KN93 (1 μ M; Sigma) prevented ISO-induced persistent I_{Na} (Fig. 3a). Secondly, KN93 significantly reduced ISO-promoted DCR in CPVT myocytes (Fig. 3b, black and red bars, respectively). These results suggested that CaMKII promotes aberrant Na^+/Ca^{2+} signaling by augmenting Na^+ influx through nNa_v . To examine the potential direct effects of CaMKII phosphorylation on RyR2 function in CPVT, we used CPVT-S2814A mice in which RyR2 is rendered non-phosphorylatable by CaMKII at S2814.³⁶ Cardiomyocytes isolated from CPVT-S2814A mice evidenced similar frequency of ISO-promoted DCR relative to those isolated from CPVT mice (Fig. 3b red and gray bars, respectively). Furthermore, the frequency of these aberrant DCRs was significantly reduced by Na^+ blockade with riluzole (Fig. 3b, purple bar). Notably, none of the aforementioned interventions affected SR Ca^{2+} load (Supplemental Fig. 4b). Thus CaMKII-mediated Na^+ influx through nNa_v can modulate DCR independently of RyR2 phosphorylation.

Arrhythmogenesis in CPVT depends on $Na_v1.6$ -mediated persistent I_{Na}

As we have previously demonstrated,²⁶ cardiac myocytes contain several types of Na^+ channels, including TTX-sensitive nNa_v ($Na_v1.1$, $Na_v1.3$ and $Na_v1.6$) as well as the TTX-resistant $Na_v1.5$. The former are located in the vicinity of RyR2 in the junctional microdomain, and the latter, in the lateral membrane and the intercalated discs (Fig.4a). To more precisely examine the localization of these channels with respect to RyR2 in CPVT we performed a proximity ligation assay (PLA).³⁷ We found that all Na^+ channel isoforms were closely co-localized (within 40 nm³⁷) with RyR2 (Fig.4b); however, $Na_v1.5$ appeared to be primarily co-localizing with RyR2 in the cell periphery, while the nNa_v s exhibited a more diffuse pattern of co-localization. Specifically, $Na_v1.6$ evidenced the highest degree of co-localization with RyR2 relative to the other nNa_v isoforms (Fig.4c). The pattern and degree of co-localization of $Na_v1.6$ with RyR2 were similar between myocytes isolated from WT and CPVT hearts whereas this was not the case for $Na_v1.1$ and 1.3 (Supplemental Fig.5). These data, in the context of recent work suggesting a role for $Na_v1.6$ in progression of demyelinating disease,⁴³ led us to hypothesize a mechanistic role for $Na_v1.6$ in CPVT. Further, the nNa_v inhibitor riluzole may exert a therapeutic effect in Amyotrophic Lateral Sclerosis (ALS), a demyelinating disorder, through the blockade of $Na_v1.6$.⁴⁴ We therefore examined the functional role of $Na_v1.6$ in CPVT. To test this, first we conducted a dose response experiment with μ -conotoxin SmIIIa, which can discriminate between TTX-sensitive Na^+ channel isoforms.⁴⁵ Specifically, at very low nM concentrations μ -conotoxin SmIIIa inhibits $Na_v1.1$ and $Na_v1.3$.⁴⁵ Despite the putative inhibition of $Na_v1.1$ and $Na_v1.3$,

50nM μ -conotoxin SmIIIa (Cusabio Biotech) did not significantly alter ISO-induced persistent I_{Na} in CPVT myocytes (Fig.5). On the other hand, a concentration of μ -conotoxin SmIIIa near the IC_{50} for $Na_v1.6$ (100nM),⁴⁵ partially reduced ISO-induced persistent I_{Na} , while 300nM μ -conotoxin SmIIIa virtually abolished this ISO-induced phenomenon (Fig.5). These data suggest that $Na_v1.6$ can potentially contribute to the ISO-induced persistent I_{Na} and arrhythmias in CPVT. To examine this possibility further in both cardiac myocytes and *in vivo* we used a selective $Na_v1.6$ inhibitor, 4,9-anhydro-TTX (Focus Biomolecules).^{46,47} Notably, ISO-induced persistent I_{Na} in CPVT cardiomyocytes was sensitive to 300 nM 4,9-anhydro-TTX (Fig.6a), suggesting that this ISO-promoted persistent I_{Na} is for the most part carried by $Na_v1.6$. Furthermore, addition of 300 nM 4,9-anhydro-TTX to CPVT myocytes reduced the frequency of ISO-promoted DCRs (Fig.6b). Similarly, pretreatment of CPVT mice with 4,9-anhydro-TTX (750 μ g/kg) reduced markedly VT vulnerability during catecholamine challenge (Fig.6c, blue ECG and bar). Notably, this intervention had no significant effect on SR Ca^{2+} load (Supplemental Fig.6). We further addressed the role $Na_v1.6$ in CPVT by siRNA approach to selectively target $Na_v1.6$ (Supplemental Fig.7). CPVT mice injected with siRNA against $Na_v1.6$ showed a marked decrease in arrhythmia episodes during catecholamine challenge (Fig.6c, purple ECG and bar). Taken together, these results suggest that $Na_v1.6$ may be in part involved in CPVT-related arrhythmogenesis, which likely involves Na^+ - Ca^{2+} exchange (NCX).

Lastly, to assess the potential role of NCX in the Na^+ / Ca^{2+} signaling, we examined structural correlation between NCX and nNa_v s as well as functional effect of NCX inhibition on aberrant DCR. We found with the aid of PLA that NCX co-localizes with the TTX-sensitive nNa_v isoforms (Supplemental Fig.8). Furthermore, NCX inhibition with SEA0400⁴⁸ (1 μ M; Medchemexpress LLC) had a similar effect on DCR relative to that observed with 4,9-anhydro-TTX (Fig. 6b). Therefore, these data suggest that NCX may be a component of the pro-arrhythmic interaction between nNa_v s and RyR2 that in part may be responsible for CPVT.

Discussion

Cardiac arrhythmias are often precipitated by catecholamine release during physical or emotional stress. The role of β -AR stimulation is particularly evident in inherited forms of cardiac arrhythmia such as CPVT, where genetic defects in the RyR2 complex (i.e., RyR2, CaM, CASQ2, TRD and/or calstabin) alter RyR2 channel function and facilitate arrhythmogenic, aberrant DCR.²⁻⁵ Specifically, in the normal heart after each systolic Ca^{2+} release RyR2s become refractory via a process that involves a decrease in the SR luminal Ca^{2+} .¹⁰ An intra-SR Ca^{2+} buffering protein, CASQ2, has been implicated in this process, acting as a Ca^{2+} buffer and a luminal Ca^{2+} sensor that regulates RyR2 gating.^{7-9,11,12,15} Therefore, CPVT-associated mutations in CASQ2 impair the ability of the RyR2 channel to deactivate during the diastolic phase, thereby making RyR2s prone to premature activation that result in DCR.^{10,13-15} This defective RyR2 gating and the resulting DCR, which are evidenced in CPVT, are enhanced by β -AR stimulation. Despite the critical role of β -AR stimulation as an arrhythmia trigger, the precise mechanisms that link β -AR signaling to arrhythmogenesis remain elusive. Here we demonstrate that augmented Na^+ entry via nNa_v in the settings of the genetically compromised RyR2s and enhanced SR Ca^{2+} refill are

essential and necessary for the arrhythmogenesis during β -AR stimulation in CPVT. Furthermore, we show that augmentation of Na^+ entry involves β -AR-mediated activation of CAMKII subsequently leading to nNa_v augmentation. Importantly, selective inhibition of $\text{Na}_v1.6$ effectively prevents arrhythmia *in vivo*, thus potentially presenting a clinically useful antiarrhythmic approach.

Recently we and others have suggested that nNa_v may facilitate excitation-contraction coupling and contribute to aberrant local $\text{Na}^+/\text{Ca}^{2+}$ signaling, that, in part, may contribute to cardiac arrhythmias.^{24,26,49–52} Based on these studies, we set out to determine whether β -AR stimulation augments nNa_v -mediated Na^+ entry and thereby facilitate Ca^{2+} influx via the $\text{Na}^+-\text{Ca}^{2+}$ exchanger (NCX) that in turn may stimulate arrhythmogenic DCR through RyR2s. Here we show that Na^+ influx via nNa_v is not merely a compounding factor but rather that augmentation of this Na^+ influx plays a key role in mediating the proarrhythmic effect of β -AR stimulation in CPVT. Specifically, our findings highlight a distinct nanodomain where nNa_v are in close proximity (less than 40nm) to RyR2s (Fig. 4) and NCX (Supplemental Fig. 8), where β -AR-augmented Na^+ entry enhances aberrant $\text{Na}^+/\text{Ca}^{2+}$ signaling, including DCR, thus resulting in catecholamine-dependent arrhythmias (CPVT). Of note, the amplitude of the nNa_v -mediated persistent I_{Na} was similar between WT and CPVT myocytes both at baseline and in the presence of ISO (Fig. 1, 6 and Supplemental Fig. 2). Thus, putative “physiological” β -AR augmentation of nNa_v activity can become arrhythmogenic in a setting of genetically compromised RyR2 in CPVT.

Stimulation of β -AR has been previously shown to affect intracellular Na^+ influx both early and late after a depolarizing stimulus.^{53,54} In the case of peak I_{Na} , Yarbrough et al.⁵³ suggested that this phenomenon is coordinated by caveolin-3. Recently, caveolin-3 has been demonstrated to coordinate local nanodomain β_2 -AR-mediated regulation of L-type Ca^{2+} channels in the T-tubules.^{55,56} However, future studies will need to address the role of caveolin-3 compartmentation on regulation of β -AR-mediated signaling of subpopulations of Na^+ channels in various cellular compartments.

Our structural and functional studies make a very compelling case for the involvement of nNa_v in the arrhythmogenic process. However, this does not preclude the cardiac isoform of the Na^+ channels ($\text{Na}_v1.5$) from contributing to arrhythmogenesis. In fact, early reports have described late I_{Na} as a component of the cardiac I_{Na} that can be inhibited by ranolazine.⁵⁴ This late I_{Na} was presumably carried by $\text{Na}_v1.5$ and is a reflection of cell-wide sarcolemmal $\text{Na}_v1.5$ activity. Here we show that $\text{Na}_v1.5$ is present in the core-compartment of cardiomyocytes presumably in the T-tubules, albeit its presence in that compartment is very limited (Fig. 4). On the other hand, nNa_v , which include $\text{Na}_v1.6$, are the predominant isoforms present within these distinct nanodomains (Fig. 4) and are responsible for the persistent I_{Na} phenotype during β -AR stimulation (Fig. 1, 6 and Supplemental Fig. 2). In this vein, non-selective ($\text{Na}_v1.5$ and nNa_v) inhibition with flecainide,⁵⁷ despite having similar effect on persistent I_{Na} , more profoundly affected peak I_{Na} relative to 100 nM TTX (Supplemental Fig. 3), a concentration that completely blocks nNa_v while mostly sparing $\text{Na}_v1.5$.^{24,27–29} These data would further suggest that since 10 μM riluzole inhibits both peak and persistent I_{Na} to similar extent as 100nM TTX, that it perhaps may elicit its DCR-

stabilizing effect through blockade of $n\text{Na}_v$. However, future studies will need to determine the specific Na^+ channel isoforms blocked by this agent.

At least three isoforms of $n\text{Na}_v$ have been identified in the heart, $\text{Na}_v1.1$, 1.3 and 1.6 (Fig. 4).^{26,30–32,58} In order to examine whether a particular $n\text{Na}_v$ isoform is essential for both aberrant $\text{Na}^+/\text{Ca}^{2+}$ signaling and *in vivo* arrhythmia in CPVT, we employed structural and functional assays. PLA as well as pharmacological and silencing approaches (Fig. 4b, Supplemental Fig. 5 and Fig. 5 & 6, respectively) pointed to the involvement of $\text{Na}_v1.6$ in the arrhythmogenic process. Moreover, WT and CPVT myocytes exhibited a similar degree of $\text{Na}_v1.6$ and RyR2 colocalization (Fig. 4 and Supplemental Fig. 5), in contrast to changes in RyR2 colocalization observed with other $n\text{Na}_v$ isoforms: thus, the bulk of ISO-promoted late I_{Na} in WT and CPVT is likely carried by $\text{Na}_v1.6$. Taken together, these findings are consistent with the prevalence of this Na^+ channel isoform in cardiomyocytes,^{26,30–32,58} its substantial persistent current^{59,60} and localization in the T-tubules in the vicinity of the RyR2 (Fig. 4). Furthermore, the persistent I_{Na} that was generated by $\text{Na}_v1.6$ during application of ISO was modulated by CaMKII-dependent $n\text{Na}_v$ augmentation (Fig. 3). Although the exact CaMKII phosphorylation site(s) in $\text{Na}_v1.6$ or the other $n\text{Na}_v$ s are not known, there are consensus CaMKII phosphorylation sites in these channels which correspond to DI-II linker conforming to Arg/Lys-X-X-Ser/Thr.⁶¹ In particular, S571 in $\text{Na}_v1.5$ appears to be conserved in TTX-sensitive $n\text{Na}_v$ s, suggesting that this might be the putative CaMKII phosphorylation site; however, future studies will need to determine the particular phosphorylation site(s) responsible for catecholamine-mediated augmentation of persistent I_{Na} .

What other factors, apart from $n\text{Na}_v$ stimulation, are critical to arrhythmogenesis in CPVT? To address this question, we omitted exposure to catecholamines and selectively slowed $n\text{Na}_v$ inactivation with β -PMTX⁴⁰ to mimic β -AR-induced $n\text{Na}_v$ augmentation in CPVT mice with inducible SERCA2a overexpression. These studies suggested that enhanced SR Ca^{2+} refilling or phosphorylation of effector sites such as RyR2 may be necessary for arrhythmogenesis in cardiac CASQ2-associated CPVT. Further experiments where we inhibited $n\text{Na}_v$ activity in CPVT mice exposed to β -AR stimulation that were deficient in RyR2 CaMKII phosphorylation (S2814A) revealed that CaMKII phosphorylation of RyR2 does not play a pivotal role in CASQ2-associated CPVT. Taken together these data suggest a novel conceptual framework for β -AR-promoted arrhythmogenesis (Fig. 7): Mainly, the cross-talk between $n\text{Na}_v$, NCX and RyR2 may play a critical role in triggering aberrant DCR during β -AR stimulation in CPVT. Furthermore, it is likely that a similar mechanism may contribute to arrhythmogenesis in other genetic and acquired forms of catecholamine-dependent arrhythmias. Likewise, there is evidence to suggest that CPVT is associated with DCR in the atria as well.^{34,62} Further, aberrant Ca^{2+} release events are also observed in atria of patients with various forms of atrial fibrillation.⁶³ Thus, it is very likely that the mechanism described herein may apply to the atrium as well. However, future studies will need to address the involvement of such aberrant $\text{Na}^+/\text{Ca}^{2+}$ signaling in atrial as well as ventricular variants of genetic and acquired forms of catecholamine-dependent arrhythmias.

Although Na⁺ channel blockade, with flecainide in particular, has been shown to be effective in management of CPVT,^{21,23} the mechanism through which it alleviates arrhythmia remains to be clarified. Initially, the antiarrhythmic effect of flecainide was attributed to the direct inhibition of the RyR2.²³ Subsequent studies have suggested that it reduces the availability of cardiac-type Na⁺ channels (Na_v1.5), thus preventing the development of triggered activity.⁶⁴ Here we propose an additional and novel antiarrhythmic mechanism for flecainide in CPVT: Antagonizing catecholamine-dependent augmentation of Na⁺ influx via nNa_vs in general and, Na_v1.6 in particular. Considering that altered RyR2 function contributes to acquired arrhythmias of various etiologies, including ischemic and nonischemic cardiomyopathy,⁶⁵ inhibition of nNa_v can potentially be applied to treat these diverse conditions. Interestingly, while non-isoform-selective Na⁺ channel inhibition initially appeared beneficial in the management of Ca²⁺-mediated arrhythmias due to myocardial infarction,²⁰ it has proven to be pro-arrhythmic and enhance the risk of arrhythmic death in patients with structural heart disease evidently due to reduced electrical excitability of the myocardium.^{66,67} In this context, nNa_v appear particularly suitable antiarrhythmic target, where the antiarrhythmic effect of selective nNa_v blockade can be uncoupled from the proarrhythmic effect of reduced cellular excitability associated with Na_v1.5 inhibition. Taken together our study brings well established findings on the global plane regarding the efficacy of Na⁺ channel as well as β-AR blockers under one mechanistic umbrella. Specifically, the novel catecholamine-mediated arrhythmogenic mechanism described herein relies on the maintenance of enhanced SR Ca²⁺ load in the setting of genetically compromised RyR2 along with augmentation of nNa_v activity. The combination of these factors promotes aberrant Na⁺/Ca²⁺ signaling resulting in DCR and arrhythmias *in vivo*. Selective inhibition of nNa_vs in general, and Na_v1.6 in particular, may represent effective treatment for a wide range of arrhythmias associated with altered RyR2 function and sympathetic stimulation.

COMPETENCY IN MEDICAL KNOWLEDGE

In a mouse heart, catecholamines promote proarrhythmic aberrant diastolic Ca²⁺ release (DCR) by enhancing neuronal Na⁺ channel (nNa_v)-mediated persistent Na⁺ current (I_{Na}). Thus, these form the functional basis for catecholamine-induced polymorphic ventricular tachycardia (CPVT).

TRANSLATIONAL OUTLOOK

Na⁺ channel blockade has been shown to be effective in management of CPVT. Considering that altered RyR2 function contributes to both genetic and acquired arrhythmias of various etiologies, including ischemic and nonischemic cardiomyopathy, inhibition of nNa_v can potentially be applied to treat these diverse conditions. Interestingly, while non-isoform-selective Na⁺ channel inhibition initially appeared beneficial in the management of Ca²⁺-mediated arrhythmias due to myocardial infarction, it has proven to be pro-arrhythmic and enhance the risk of arrhythmic death in patients with structural heart disease evidently due to reduced availability of Na_v1.5 and the consequent loss of myocardial excitability. In this context, nNa_v appear particularly suitable antiarrhythmic target, where the antiarrhythmic effect of selective nNa_v blockade can be uncoupled from the proarrhythmic effect of reduced cellular excitability associated with Na_v1.5 inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Acronyms

ALS	amyotrophic lateral sclerosis
AmsF⁻¹	Amp.ms/F
β-PMTX	β-Pompilidotoxin
β-AR	β-adrenergic receptor
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CASQ2	calsequestrin
CPVT	Catecholaminergic Polymorphic Ventricular Tachycardia
DAD	delayed afterdepolarizations
DCR	diastolic Ca ²⁺ release
I_{Na}	Na ⁺ current
ISO	isoproterenol
nNa_v	neuronal Na ⁺ Channels
NCX	Na ⁺ /Ca ²⁺ exchange
PLB	phospholamban
PKA	protein kinase A
RyR2	ryanodine-receptor Ca ²⁺ release channels
SR	sarcoplasmic reticulum
SERCA2a	sarcoplasmic reticulum Ca ²⁺ -ATPase 2a
TTX	tetrodotoxin
VT	ventricular tachycardia
WT	wild type

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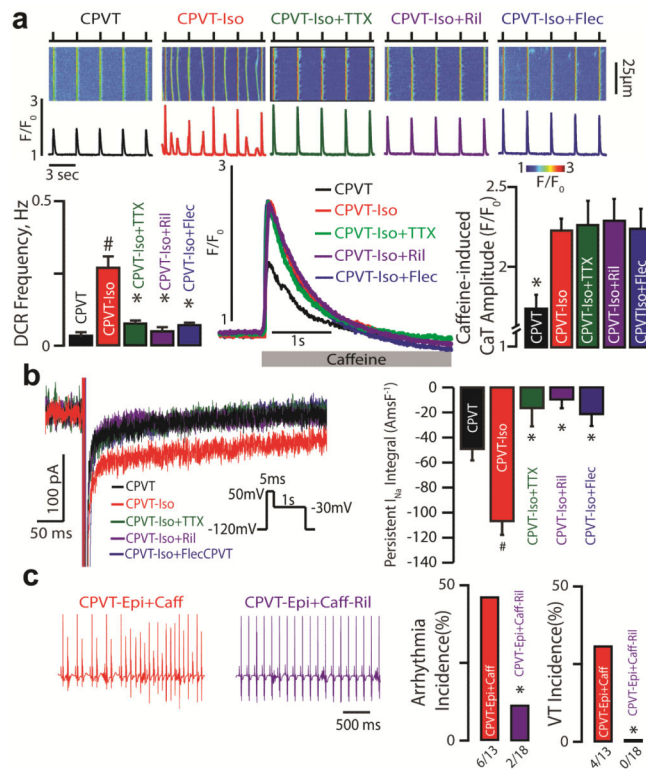


Fig. 1. β -AR stimulation increases propensity for CPVT by augmenting TTX-sensitive nNa_v -mediated late I_{Na}

(a) Effect of β -AR stimulation on nNa_v blockade and Ca^{2+} handling. (Top) Representative examples of the line-scan images and corresponding Ca^{2+} transients (CaT) recorded in CPVT ventricular cardiomyocytes loaded with Ca^{2+} indicator, Fluo-4FF AM and paced at 0.3 Hz. Cells were treated with isoproterenol (Iso, 100 nM) and tetrodotoxin (TTX, 100 nM), riluzole (Ril, 10 μ M) or flecainide (Flec, 2.5 μ M). β -AR stimulation with Iso promotes DCR events in the form of Ca^{2+} waves relative to untreated CPVT cardiomyocytes ($n=166$ and 34 cells, respectively; #, $p<0.001$ Wilcoxon rank-sum test). TTX, Ril and Flec significantly decreased DCR frequency in CPVT cardiomyocytes exposed to Iso ($n=109$, 48 and 66 cells, $p<0.001$ Kruskal-Wallis test; *, $p=0.003$, $p<0.001$ and $p=0.032$ Wilcoxon rank-sum test for TTX, Ril and Flec vs. ISO, respectively). (Bottom) Representative caffeine-induced (20 mM) CaT. ISO significantly increased caffeine-induced CaT relative to untreated CPVT cardiomyocytes ($n=13$ and 11 cells, respectively; *, $p=0.005$ Wilcoxon rank-sum test). This elevation in caffeine-induced CaT persisted despite concomitant treatment with TTX, Ril and Flec ($n=11$, 13 and 10 cells, respectively, $p=0.99$ Kruskal-Wallis test). (b) Effect of β -AR stimulation and subsequent nNa_v blockade on persistent I_{Na} . Representative traces of persistent I_{Na} elicited using the protocol shown in the inset. Iso enhanced persistent I_{Na} in CPVT cardiomyocytes ($n=18$ and 21 cells, respectively; #, $p=0.004$ Wilcoxon rank-sum test). This response to Iso was completely abolished upon addition of TTX, Ril or Flec ($n=9$, 7 and 9 cells, respectively, $p<0.001$ Kruskal-Wallis Test; *, $p<0.001$ Wilcoxon rank-sum test for each treatment groups vs. ISO). Summary data presented as persistent I_{Na} integral amp-msec/F ($AmsF^{-1}$). (c) Effect of β -AR stimulation on nNa_v -mediated ventricular arrhythmias *in vivo*. Representative ECG recordings of CPVT

mice after catecholamine challenge with intraperitoneal injection (i.p.) of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg; red ECGs). A subset of mice was pretreated with Ril (15 mg/kg; purple ECGs). Arrhythmia and ventricular tachycardia (VT) incidence (%) in CPVT mice exposed to catecholamine challenge during Na⁺ channel blockade with riluzole (n = 13 vs 18 CPVT-Epi+Caff vs CPVT-Epi+Caff-Ril treated mice. *, $p=0.043$ and *, $p=0.023$ Fisher's exact test for Arrhythmia and VT incidence, respectively).

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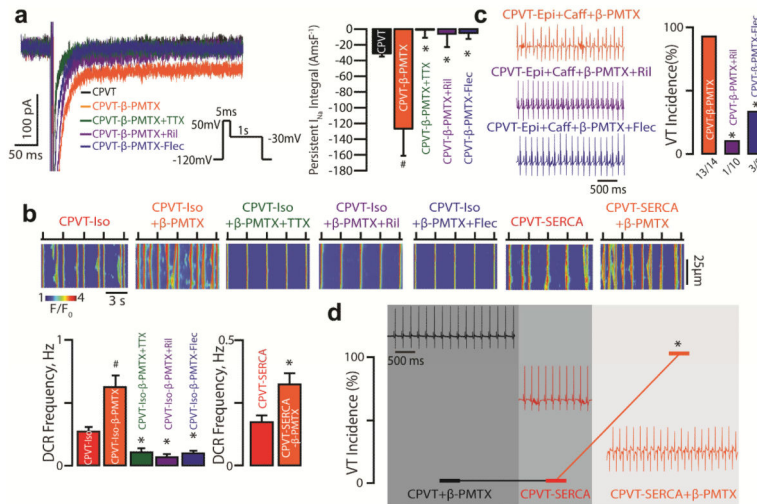


Fig. 2. TTX-sensitive nNa_v -mediated persistent I_{Na} augmentation in conjunction with increased SR Ca^{2+} load contribute to CPVT

(a) Slowed inactivation of nNa_v with β -PMTX results in TTX-sensitive persistent I_{Na} in CPVT mice. Representative traces of persistent I_{Na} recorded in CPVT cardiomyocytes. Direct augmentation of nNa_v -mediated persistent I_{Na} with β -PMTX (40 μ M) in CPVT myocytes increased persistent I_{Na} relative to control (n = 12 and 31 cells, respectively, # $p=0.004$ Wilcoxon rank-sum test). β -PMTX-induced persistent I_{Na} was reduced by 100 nM TTX, 10 μ M Ril and 2.5 μ M Flec (n = 8, 6 and 5 cells, $p < 0.001$ Kruskal-Wallis test; *, $p=0.002$, $p=0.039$, $p=0.003$ Wilcoxon rank-sum test vs β -PMTX alone, respectively). (b) Pharmacological augmentation of nNa_v -mediated persistent I_{Na} promotes DCR in CPVT. Representative line-scan images obtained from CPVT cardiomyocytes and those conditionally overexpressing SERCA2a (CPVT-SERCA) that were loaded with Ca^{2+} indicator, Fluo-3 AM and paced at 0.3 Hz. Concomitant application of Iso (100 nM) and β -PMTX (40 μ M) further promoted DCR frequency in CPVT cardiomyocytes relative to CPVT myocytes exposed to Iso alone (n = 79 and 166 cells, respectively, # $p=0.001$ Wilcoxon rank-sum test). Addition of TTX (n = 38), Ril (n = 61) or Flec (n = 70) significantly reduced Iso/ β -PMTX-promoted DCRs ($p < 0.001$ Kruskal-Wallis test; *, $p < 0.001$ Wilcoxon rank-sum test for each experimental group vs ISO+ β -PMTX). In the absence of catecholamines, CPVT-SERCA cardiomyocytes exposed to β -PMTX evidenced greater DCR frequency relative to the untreated ones (n = 80 and 83 cells, respectively;*, $p=0.01$ Wilcoxon rank-sum test). (c) Representative ECG recordings of CPVT mice after catecholamine challenge with i.p. injection of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg) and pretreatment with, β -PMTX (30mg/kg), β -PMTX+Ril (15 mg/kg) or β -PMTX +Flec (20 mg/kg). VT incidence in CPVT mice exposed to catecholamine challenge during various interventions (n = 14, 10 and 9 mice for CPVT- β -PMTX, CPVT- β -PMTX+Ril and CPVT- β -PMTX+Flec, respectively. * $p < 0.001$ and $p=0.005$ Fisher's exact test for CPVT- β -PMTX vs. CPVT- β -PMTX+Ril and CPVT- β -PMTX vs. CPVT- β -PMTX+Flec, respectively). (d) Representative ECG recordings and summary VT incidence of CPVT +SERCA mice before and after doxycycline-induced SERCA2a overexpression and in the presence or absence of β -PMTX (30mg/kg; n = 6, * $p = 0.031$ McNemar's test for CPVT-SERCA vs. CPVT-SERCA+ β -PMTX). All experiments in CPVT-SERCA mice were

conducted in the absence of epinephrine and caffeine. Before induction of SERCA2a overexpression all 6 mice were exposed to β -PMTX. After 2-3 weeks of doxycycline-supplemented diet, all 6 mice (horizontal line connecting the hash marks that represent VT incidence) were assessed for arrhythmias after which they were again exposed to β -PMTX.

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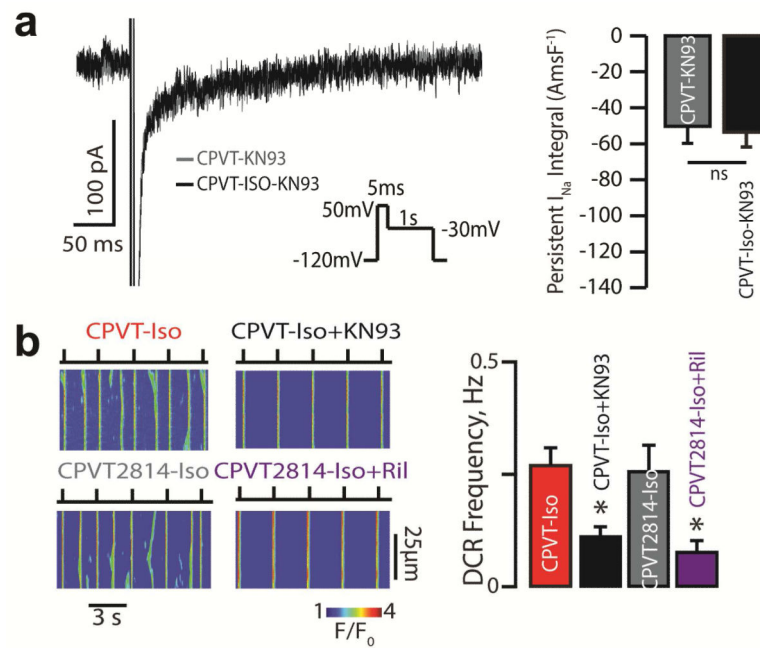


Fig. 3. Proarrhythmic effect of β -AR stimulation on TTX-sensitive persistent I_{Na} involves CaMKII phosphorylation of nNa_v and is independent of RyR2 phosphorylation

(a) Effect of β -AR stimulation on persistent I_{Na} is mediated through CaMKII.

Representative traces of persistent I_{Na} recorded in CPVT cardiomyocytes exposed to CaMKII inhibitor KN93 (1 μ M) before and after treatment with 100 nM Iso ($n = 9$ cells for both groups, $p=0.14$ Wilcoxon signed-rank test).

(b) CaMKII modulates DCR independent of RyR2 phosphorylation at S2814. Representative line-scan images recorded in CPVT ventricular cardiomyocytes as well as those expressing RyR2 that cannot be phosphorylated by CaMKII at site 2814 (S2814A). Myocytes were loaded with Ca^{2+} indicator, Fluo-3 AM and paced at 0.3 Hz. CaMKII inhibition with 1 μ M KN93 reduced DCR frequency in Iso treated CPVT cardiomyocytes ($n=166$ and 105 , respectively; *, $p = 0.001$ Wilcoxon rank-sum test). CPVT2814 cardiomyocytes did not evidence altered DCR frequency relative to Iso-treated CPVT myocytes; however, exposure of Iso-treated CPVT2814 cardiomyocytes to Ril 10 μ M significantly reduced DCR frequency relative to Iso alone ($n = 57$ and 61 cells, respectively; *, $p=0.003$ Wilcoxon rank-sum test).

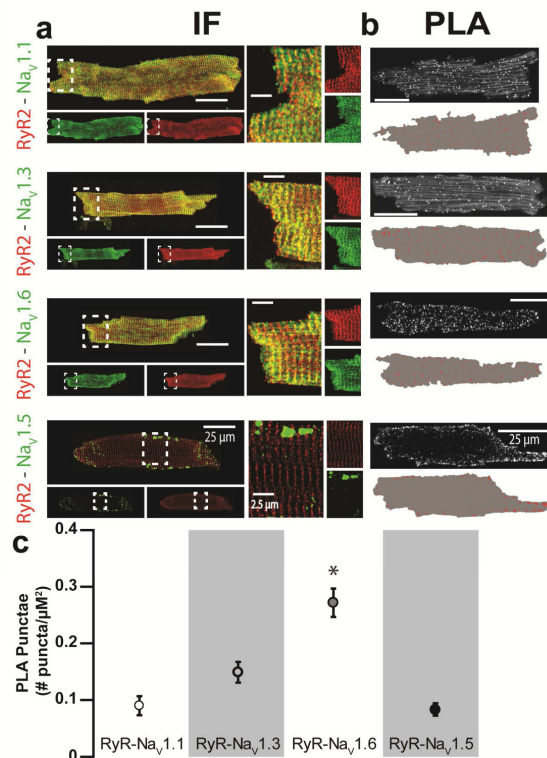


Fig. 4. Neuronal Na⁺ channels and RyR2 colocalize to the same discrete subcellular regions
(a) Representative confocal micrographs of isolated CPVT ventricular myocytes labeled for RyR2 (red) with various Na_v isoforms (Na_v1.x; green) often resulted in an overlap between the immunofluorescent (IF) signals (yellow) when overlaid. Panels on the right show close up views of regions highlighted by dashed white boxes. **(b)** Representative confocal micrographs of ventricular myocytes isolated from CPVT mice showing fluorescent proximity ligation assay (PLA) signal for RyR2 with different nNa_v isoforms (Na_v1.x). Below each image, are shown the results of digital segmentation with the cell mask in grey and PLA signal in red. **(c)** Plot of average number of PLA punctae per μm² ($p < 0.001$ Kruskal-Wallis test; *, $p=0.002$, $p=0.019$, $p<0.001$ Wilcoxon rank-sum test for Na_v1.6 vs. Na_v1.1, 1.3 and 1.5, respectively; $n = 1231$, 1223 , 1291 and 2848 punctae from 7 , 6 , 12 and 7 cells for Na_v1.1, 1.3, 1.5 and 1.6, respectively).

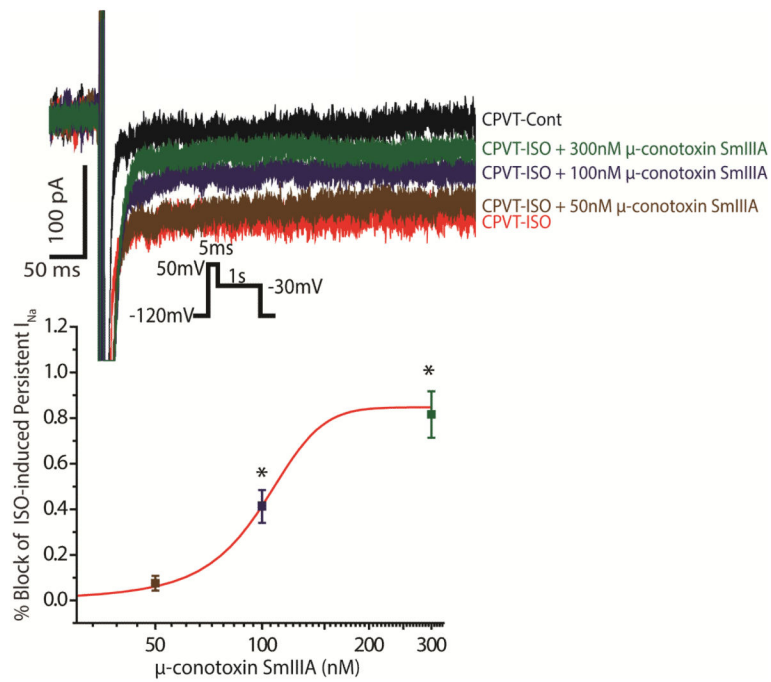


Fig. 5. Dose response of ISO-induced persistent I_{Na} in CPVT cardiomyocytes to nNa_v blockade with μ-conotoxin SmIIIA

(Top) Representative traces of persistent I_{Na} recorded in CPVT cardiomyocytes exposed to ISO (100 nM and subsequent increasing concentrations of μ-conotoxin SmIIIA (50, 100 and 300 nM). (Bottom) Summary of ISO-induced persistent I_{Na} dose response to μ-conotoxin SmIIIA. ISO-induced persistent I_{Na} was not significantly affected by 50 nM μ-conotoxin SmIIIA (n = 10 for both CPVT-ISO and CPVT-ISO+50nM μ-conotoxin SmIIIA, *p* = 1 Wilcoxon rank-sum test), partially inhibited by 100 nM (n = 9; *, *p* = 0.048 Wilcoxon rank-sum test vs. CPVT-ISO) and almost completely blocked by 300 nM (n = 6; *, *p* = 0.003 Wilcoxon rank-sum test vs. CPVT-ISO).

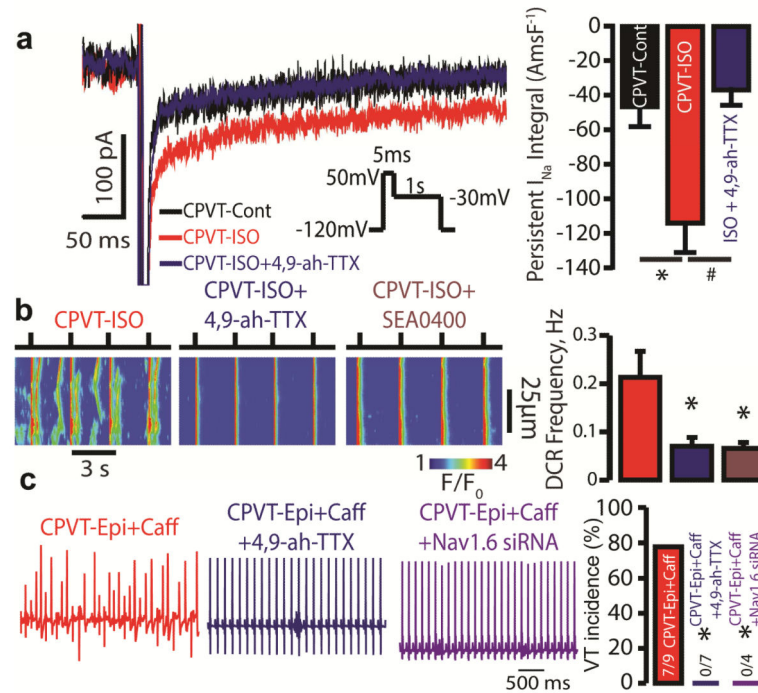


Fig. 6. Arrhythmogenesis in CPVT depends on $Na_v1.6$ -mediated persistent I_{Na}
(a) Effect of $Na_v1.6$ blockade on ISO-induced persistent I_{Na} . Representative traces of persistent I_{Na} pre- and post-exposure to 100nM ISO in CPVT cardiomyocytes ($p = 0.007$ Kruskal-Wallis test, $n = 6$ for both; *, $p = 0.035$ Wilcoxon rank-sum test vs. CPVT-control). The persistent I_{Na} response to ISO was abolished by 300nM 4,9-anhydro-TTX (4,9-ah-TTX; $n = 5$; #, $p = 0.016$ Wilcoxon rank-sum test vs. CPVT-ISO). **(b)** $Na_v1.6$ and NCX blockade reduce DCR. Representative line-scan images recorded in CPVT ventricular cardiomyocytes that were loaded with Ca²⁺ indicator, Fluo-3 AM and were paced at 0.3 Hz. $Na_v1.6$ inhibition with 300nM 4,9-ah-TTX as well as NCX inhibition with SEA0400 (1 μ M) reduced DCR frequency in ISO treated CPVT cardiomyocytes ($n = 99$, 123 and 74 cells for ISO-4,9-ah-TTX, ISO-SEA0400 and ISO treated cells, respectively). $p = 0.026$ Kruskal-Wallis test; *, $p = 0.031$ and *, $p = 0.032$ Wilcoxon rank-sum test for ISO-4,9-ah-TTX and ISO-SEA0400 vs. ISO, respectively). **(c)** Representative ECG recordings and summary VT incidence of CPVT mice exposed to catecholamine challenge (epinephrine and caffeine) as well as those with pharmacological or genetic inactivation of $Na_v1.6$. Pretreatment with 4,9-ah-TTX (750 μ g/kg I.P.) or administration of siRNA selectively targeting $Na_v1.6$ prevented VT during catecholamine challenge ($n = 9$, 7 and 4 mice for CPVT-Epi+Caff, CPVT-Epi+Caff +4,9ah-TTX and CPVT-Epi+Caff+ $Na_v1.6$ siRNA, respectively). *, $p = 0.003$ and *, $p = 0.021$ Fisher's exact test for CPVT-Epi+Caff +4,9ah-TTX and CPVT-Epi+Caff+ $Na_v1.6$ siRNA vs. CPVT-Epi+Caff, respectively).

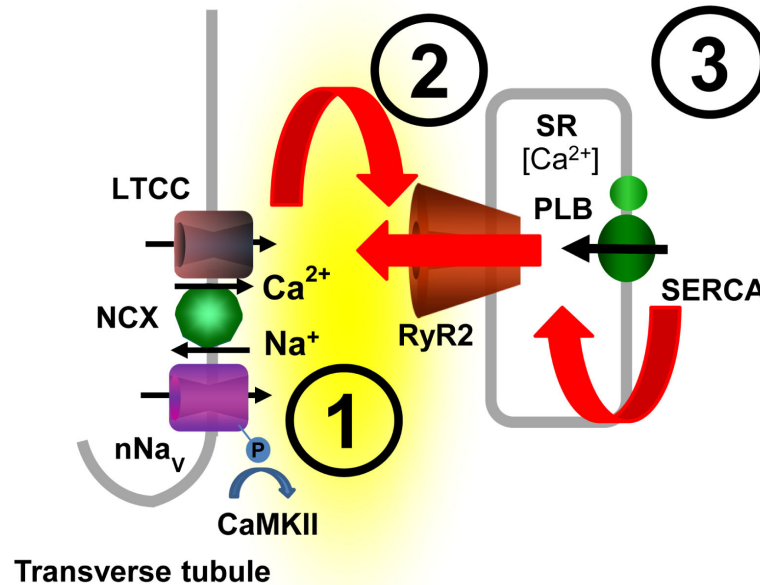


Fig.7. Arrhythmogenic targets of β-AR stimulation responsible for CPVT

During the initiation of excitation-contraction (EC) coupling, Ca²⁺ influx through the L-type Ca²⁺ channels (LTCC) results in activation of RyR2 and subsequent Ca²⁺ release from the SR.⁶⁸ Based on the present study, in the presence of β-adrenergic receptor (β-AR) agonist isoproterenol, **1**) CaMKII-dependent augmentation of Na⁺ influx during the post-systolic phase (i.e., persistent Na⁺ current) may facilitate diastolic Ca²⁺ release (DCR) by enhancing Na⁺-Ca²⁺ exchange (NCX)-dependent Ca²⁺ accumulation in the dyadic cleft (i.e., space between the sarcolemma and the RyR2). **2**) This nanodomain Ca²⁺ accumulation in turn promotes DCR via Ca²⁺-induced Ca²⁺ release from the RyR2 that are sensitized due genetic defects in the RyR2 complex (i.e., RyR2, CaM, CASQ2, TRD and/or calstabin),²⁻⁵ These along with **3**) increased SR Ca²⁺ load play a critical role in triggering aberrant DCR during β-AR stimulation in CPVT.