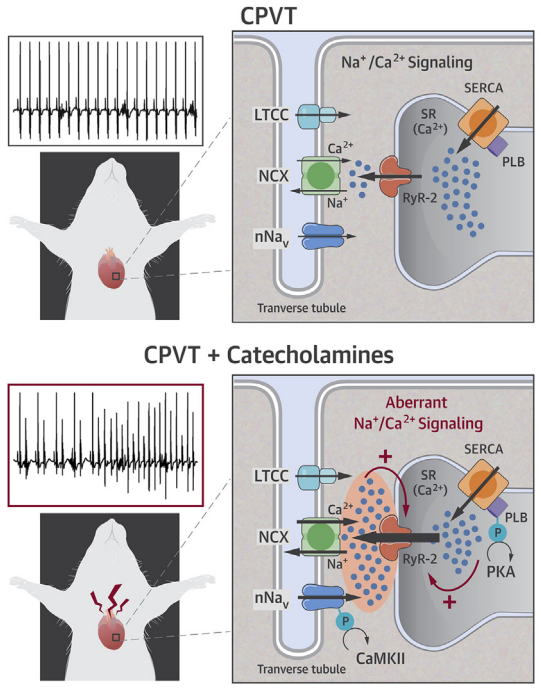
JACC: BASIC TO TRANSLATIONAL SCIENCE
VOL. 1, NO. 4, 2016
ISSN 2452-302X
http://dx.doi.org/10.1016/j.jacbts.2016.04.004

PRE-CLINICAL RESEARCH

Neuronal Na^+ Channels Are Integral Components of Pro-Arrhythmic Na^+/Ca^{2+} Signaling Nanodomain That Promotes Cardiac Arrhythmias During β-Adrenergic Stimulation

Przemysław B. Radwański, PharmD, PhD,[a,b,c] Hsiang-Ting Ho, PhD,[a,b] Rengasayee Veeraraghavan, PhD,[d] Lucia Brunello, PhD,[a,b] Bin Liu, PhD,[a,b] Andriy E. Belevych, PhD,[a,b] Sathya D. Unudurthi, PhD,[a,e] Michael A. Makara, PhD,[a,b] Silvia G. Priori, MD, PhD,[f] Pompeo Volpe, PhD,[g] Antonis A. Armoundas, PhD,[h] Wolfgang H. Dillmann, MD,[i] Bjorn C. Knollmann, MD, PhD,[j] Peter J. Mohler, PhD,[a,b] Thomas J. Hund, PhD,[a,d] Sándor Györke, PhD[a,b]

VISUAL ABSTRACT



Radwański, P.B. et al. J Am Coll Cardiol Basic Trans Science. 2016;1(4):251-66.

HIGHLIGHTS

- **CPVT is often caused by increased levels of circulating catecholamines; however, the mechanistic link between β-AR stimulation and the subcellular/molecular arrhythmogenic trigger(s) is unclear.**
- **In both CPVT and wild type mice, a subpopulation of Na^+ channels (nNa_v) colocalize with RyR2 and NCX.**
- **Augmented Na^+ entry via nNa_v and enhanced SR Ca^{2+}-ATPase (SERCA)-mediated SR Ca^{2+} refill are both essential and necessary for CPVT.**
- **Augmentation of Na^+ entry involves β-AR-mediated activation of Ca^{2+}/CAMKII.**
- **Selective pharmacological blockade as well as silencing of $Na_v1.6$ inhibit myocyte arrhythmic potential and prevent arrhythmias in vivo.**

From the [a]Dorothy M. Davis Heart and Lung Research Institute, College of Medicine, The Ohio State University Wexner Medical Center, Columbus, Ohio; [b]Department of Physiology and Cell Biology, College of Medicine, The Ohio State University, Columbus, Ohio; [c]Division of Pharmacy Practice and Sciences, College of Pharmacy, The Ohio State University, Columbus, Ohio; [d]Center for Heart and Regenerative Medicine Research, Virginia Tech Carilion Research Institute, Virginia Polytechnic University, Roanoke, Virginia; [e]Department of Biomedical Engineering, College of Engineering, The Ohio State University, Columbus, Ohio; [f]Division of

ABBREVIATIONS AND ACRONYMS

β-PMTX = β-pompilidotoxin

β-AR = β-adrenergic receptor

CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II

CASQ2 = calsequestrin

CPVT = catecholaminergic polymorphic ventricular tachycardia

DCR = diastolic Ca^{2+} release

I_{Na} = Na^+ current

ISO = isoproterenol

nNa_v = neuronal Na^+ channels

NCX = Na^+/Ca^{2+} exchange

PLB = phospholamban

Ril = riluzole

RyR2 = ryanodine receptor 2

SR = sarcoplasmic reticulum

SERCA2a = sarcoplasmic reticulum Ca^{2+}-ATPase 2a

TTX = tetrodotoxin

VT = ventricular tachycardia

WT = wild type

SUMMARY

Although triggered arrhythmias including catecholaminergic polymorphic ventricular tachycardia (CPVT) are often caused by increased levels of circulating catecholamines, the mechanistic link between β-adrenergic receptor (AR) stimulation and the subcellular/molecular arrhythmogenic trigger(s) is unclear. Here, we systematically investigated the subcellular and molecular consequences of β-AR stimulation in the promotion of catecholamine-induced cardiac arrhythmias. Using mouse models of cardiac calsequestrin-associated CPVT, we demonstrate that a subpopulation of Na^+ channels, mainly the neuronal Na^+ channels (nNa_v), colocalize with ryanodine receptor 2 (RyR2) and Na^+/Ca^{2+} exchanger (NCX) and 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 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 $Na_v1.6$ inhibit myocyte arrhythmic potential and prevent arrhythmias in vivo. Taken together, these data suggest that the arrhythmogenic alteration in Na^+/Ca^{2+} handling evidenced ruing β-AR stimulation results, at least in part, from enhanced Na^+ influx through nNa_v. Therefore, selective inhibition of these channels and of $Na_v1.6$ in particular can serve as a potential antiarrhythmic therapy. (J Am Coll Cardiol Basic Trans Science 2016;1:251–66) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Cardiac arrhythmias are a leading cause of death in the United States (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 1 of the regulatory proteins associated with the channel (i.e., calmodulin, calsequestrin [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 (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 $Ca_v1.2$, phospholamban (PLB), and RyR2, may be involved in the arrhythmogenic effects of β-AR stimulation (15). For instance, protein kinase A phosphorylation of PLB will

Cardiology and Molecular Cardiology, Maugeri Foundation-University of Pavia, Pavia, Italy; [g]Department of Biomedical Sciences, University of Padova, Padova, Italy; [h]Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts; [i]Department of Medicine, University of California San Diego, La Jolla, California; and the [j]Division of Clinical Pharmacology, Vanderbilt University Medical School, Nashville, Tennessee. This work was supported by National Institutes of Health grants R01-HL074045 and R01-HL063043 (to Dr. Györke); K99-HL127299 (to Dr. Radwański); R01-HL084583, R01-HL083422, and R01- HL075649 (to Dr. Mohler); and R01-HL114893 (to Dr. Hund). Additionally funding was provided by the Russian Science Foundation (N15-15-20008), which was used to support the image analysis. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Manuscript received February 9, 2016; revised manuscript received April 18, 2016, accepted April 24, 2016.

accelerate SR Ca^{2+}-ATPase 2a (SERCA2a)-mediated Ca^{2+} refilling of the SR, thereby providing adequate substrate for aberrant DCR (18). However, 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 cross talk. Furthermore, because 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. Because, 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 of Na^+/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).

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 U.S. National Institutes of Health (NIH Publication No. 85-23, revised 2011).

GENETICALLY-ENGINEERED MOUSE MODELS. All 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: 1) mice conditionally overexpressing SERCA2a in a doxycycline-dependent manner (on FVB/N background) (35); or 2) 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 [35], tetracycline response element-SERCA2a (35), and RyR2 S2814A mutation) using tail deoxyribonucleic acid. To induce the overexpression of SERCA2a, animals received doxycycline diet (Harlan TD 09295 1000 ppm Doxycycline Diet 2018, Harlan, Indianapolis, Indiana) for 14 to 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 Na^+/Ca^{2+} exchange (NCX) in aberrant Na^+/Ca^{2+} signaling (26).

MYOCYTE ISOLATION, CONFOCAL CA^{2+} IMAGING, AND NA^+ CURRENT RECORDINGS. Ventricular myocytes were obtained by enzymatic isolation from 3- to 9-month-old mice of both sexes. Mice were anesthetized with isoflurane, and after a deep level of anesthesia was reached, the heart was rapidly removed and perfused via a Langendorff as previously described (14,26). Peak I_{Na} was recorded using an internal solution that contained (in mmol/l): 10 NaCl, 20 tetraethylammonium chloride, 123 CsCl, 1 $MgCl_2$, 0.1 Tris guanosine-5'-triphosphate, 5 Mg adenosine triphosphate, 10 HEPES, and 10 BAPTA (pH 7.2, CsOH). For persistent I_{Na} recordings, we substituted BAPTA with 1 mmol/l EGTA and maintained free Ca^{2+} 100 nmol/l with $CaCl_2$. The extracellular bathing solution for peak I_{Na} contained (in mmol/l): 10 NaCl, 130 tetraethylammonium chloride, 4 CsCl, 0.4 $CaCl_2$, 2 $MgCl_2$, 0.05 $CdCl_2$, 10 HEPES, and 10 glucose. The extracellular bathing solution for persistent I_{Na} recordings contained (in mmol/l): 140 NaCl, 4 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 0.05 $CdCl_2$, 10 HEPES, 10 glucose, 0.03 niflumic acid, 0.004 strophanthidin, and 0.2 $NiCl_2$. The 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 10 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 mmol/l): 140 NaCl, 5.4 KCl, 1.0 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, and 5.6 glucose (pH 7.4, NaOH). To assess the SR Ca^{2+} load, 20 mmol/l caffeine was applied at the end of the experiments. Intracellular Ca^{2+} cycling was monitored by a Nikon A1 laser scanning confocal microscope (Nikon Instruments Inc., Melville, New York). For intact myocytes, we used the cytosolic Ca^{2+}-sensitive indicators Fluo-3 AM. For more reliable measurements of SR Ca^{2+} release from inside the SR in control and isoproterenol-treated CPVT cardiomyocytes, we performed experiments in **Figure 1** using a low-affinity Ca^{2+} indicator Fluo-4FF-AM. The fluorescent probes were excited with the 488-nm line of an argon laser, and emission was collected at 500 to 600 nm. Fluo-3/Fluo-4FF fluorescence was recorded in the line scan mode of the confocal microscope. For Ca^{2+} wave recordings, myocytes were paced at 0.3 Hz using extracellular platinum electrodes 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 Ca^{2+} 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).

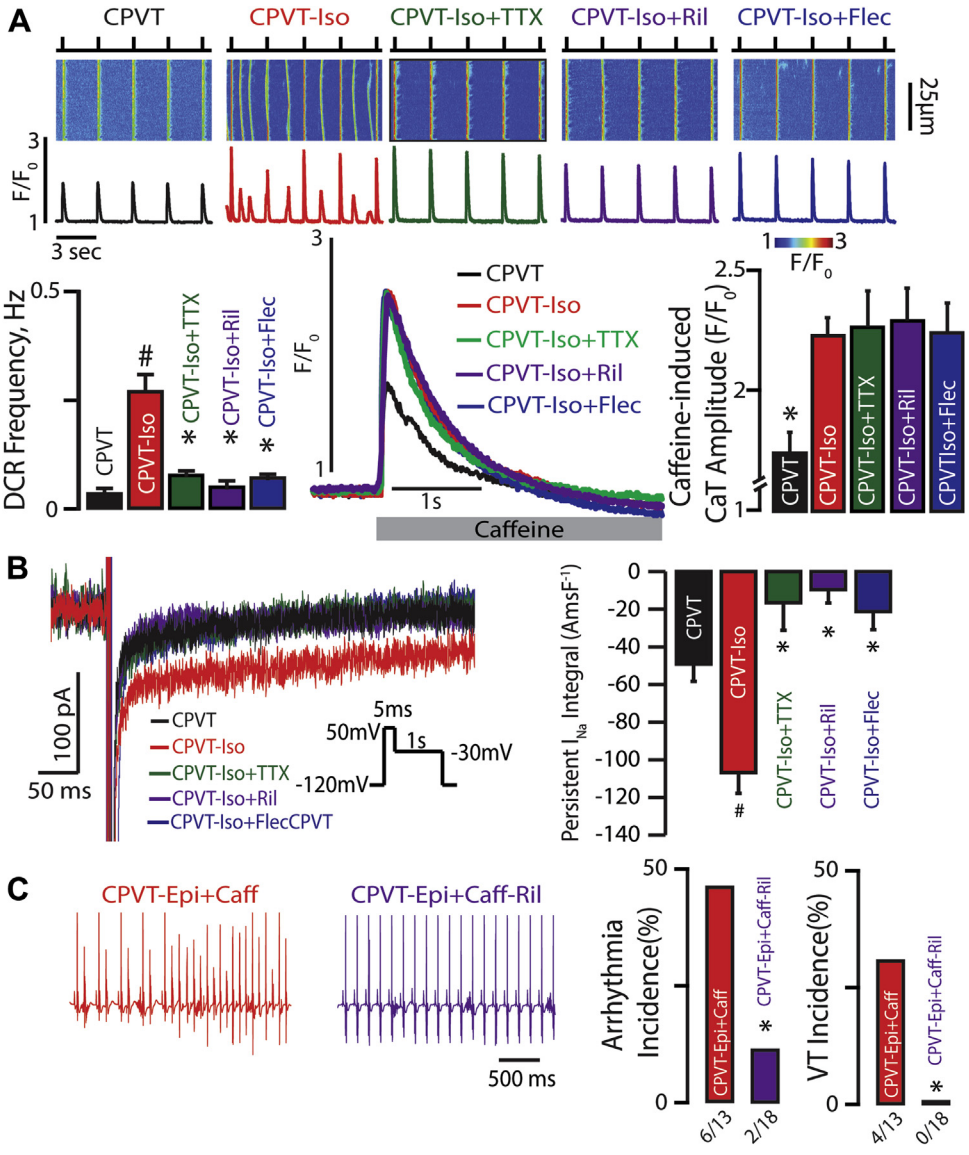
CONFOCAL MICROSCOPY OF IMMUNOLABELED MYOCYTES. Isolated ventricular myocytes were prepared for immunofluorescence as well as proximity ligation assay (PLA) as described previously (26). PLA is a histochemical/cytochemical and confocal microscopy technique for determining when specific proteins are colocalized 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, California) 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, for nNavs, Alomone, Jerusalem, Israel; $Na_v1.5$ was a generous gift from Dr. Peter Mohler; and RyR2 1:100 and NCX 1:50 were from Pierce Antibodies, Rockford, Illinois) overnight at 4°C. After washing for immunofluorescence, goat secondary antibodies (antimouse and antirabbit) conjugated to Alexa Fluor (Life Technologies, Grand Island, New York) were added for 1 h, whereas the PLA reactions were carried out using appropriate Duolink secondary antibodies (Sigma, St. Louis, Missouri) according to the manufacturer's instructions. The sensitivity of PLA was assessed by staining for $Na_v1.5$ (1:50, a generous gift from Dr. Peter Mohler) and Connexin 43 (1:100, Millipore, Billerica, Massachusetts) (Supplemental Figure 1), which were previously demonstrated to colocalize at the intercalated disc (37).

SILENCING RIBONUCLEIC ACID. Targeting silencing ribonucleic acid (siRNA) was purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Dallas, Texas). We used a previously validated approach of intraperitoneal injection (1.5 mg/kg) mixed with an equal volume of siPORT amine (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts,) in the live animal (38). We administered the siRNA every 24 h for 2 days. Silencing efficacy was evaluated 72 h after initiation of therapy by quantitative real-time (qRT) PCR as well as protein analysis.

QUANTITATIVE REAL-TIME PCR. Hearts were collected 72 h after initiations of siRNA therapy (n = 3 per each group). Total ribonucleic acid (RNA) was prepared from cells using an RNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) in accordance with the manufacturer's instructions. Total RNA was subjected to qRT PCR. RNA levels were analyzed using the TaqMan Gene Expression Assays in accordance with the 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, Waltham, Massachusetts). 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 the bicinchoninic acid (BCA) assay (Pierce), were loaded into 4% to 15% pre-cast TGX gels (Bio-Rad) and transferred to nitrocellulose membranes. Membranes were blocked for >1 h at room temperature in 3% bovine serum albumin and incubated in primary antibody overnight at 4°C. Primary antibodies included: $Na_v1.6$ (1:500, Alomone) and glyceraldehyde 3-phosphate dehydrogenase (1:5,000, Fitzgerald, Acton, Massachusetts). Secondary antibodies used were donkey antimouse-horseradish peroxidase (HRP) and donkey antirabbit-HRP (Jackson Laboratory, Farmington, Connecticut). Densitometric analysis was performed using Image Lab software (Bio-Rad Laboratories, Hercules, California), and all data was normalized to glyceraldehyde 3-phosphate dehydrogenase.

FIGURE 1 β-AR Simulation Increases Propensity for CPVT by Augmenting TTX-Sensitive nNa_v-Mediated Late I_{Na}



(A) Effect of β-adrenergic receptor (β-AR) stimulation on neuronal Na^+ channel (nNa_v) blockade and Ca^{2+} handling. **(Top)** Representative examples of the line-scan images and corresponding Ca^{2+} transients (CaT) recorded in catecholaminergic polymorphic ventricular tachycardia (CPVT) ventricular cardiomyocytes loaded with Ca^{2+} indicator, Fluo-4FF AM, and paced at 0.3 Hz. Cells were treated with isoproterenol (Iso) (100 nmol/l) and tetrodotoxin (TTX) (100 nmol/l), riluzole (Ril) (10 μmol/l), or flecainide (Flec) (2.5 μmol/l). β-AR stimulation with Iso promotes diastolic Ca^{2+} release (DCR) events in the form of Ca^{2+} waves relative to untreated CPVT cardiomyocytes ($n = 166$ and $n = 34$ 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, respectively; $p < 0.001$ 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 (Caff)-induced (20 mmol/l) CaT. ISO significantly increased caffeine-induced CaT relative to untreated CPVT cardiomyocytes ($n = 13$ and $n = 11$ 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 of persistent Na^+ current (I_{Na}). Representative traces of persistent I_{Na} elicited using the protocol are shown in the **inset**. Iso enhanced persistent I_{Na} in CPVT cardiomyocytes ($n = 18$ and $n = 21$ 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 group vs. ISO). Summary data presented as persistent I_{Na} integral amp-ms/F ($AmsF^{-1}$). **(C)** Effect of β-AR stimulation on nNa_v-mediated ventricular arrhythmias in vivo. Representative electrocardiography (ECG) recordings of CPVT mice after catecholamine challenge with intraperitoneal injection of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg; **red** ECGs). A subset of mice was pre-treated 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. $n = 18$ CPVT-Epi+Caff vs. CPVT-Epi+Caff-Ril treated mice. *$p = 0.043$ and *$p = 0.023$ Fisher exact test for arrhythmia and VT incidence, respectively).

ELECTROCARDIOGRAPHIC RECORDINGS. Continuous electrocardiographic (ECG) recordings (PL3504 PowerLab 4/35, ADInstruments, Sydney, Australia) were obtained from mice anesthetized with isoflurane (1.0% to 1.5%) as previously described (26). Briefly, after baseline recording (5 min), a subset of animals received either riluzole (15 mg/kg) or β-pompilidotoxin (β-PMTX) (30 mg/kg). After 5 min, those animals that were pre-treated with β-PMTX received vehicle, riluzole, or flecainide (20 mg/kg). After an additional 5 to 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 min. We also obtained continuous ECG recordings from CPVT-SERCA mice pre- and post-doxycycline induction. After baseline recording (5 min), each CPVT-SERCA mouse received only β-PMTX (30 mg/kg) intraperitoneally, and ECG recording continued for 10 min. ECG recordings were analyzed using the LabChart 7.3 program (ADInstruments). Arrhythmia was defined as bigeminy or frequent ectopic ventricular activity, whereas ventricular tachycardia (VT) was defined as 3 or more premature ectopies.

REAGENTS. Unless otherwise stated, all chemicals were purchased from Sigma, Torcis (Bristol, United Kingdom), Focus Biomolecules (Plymouth Meeting, Pennsylvania), Cusabio (Wuhan, China), Medchemexpress LLC (Monmouth Junction, New Jersey), Millipore, or Alomone. Fluorescent dyes were purchased from Molecular Probes (Eugene, Oregon).

DATA ANALYSIS. I_{Na} analysis was performed using pCLAMP9 software (Molecular Devices, Sunnyvale, California). Line scanning images of Ca^{2+} were normalized for baseline fluorescence (14). The Ca^{2+} imaging data were processed using ImageJ (NIH, Bethesda, Maryland) and Origin 7.0 software OriginLab Corporation, Northampton, Massachusetts. 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 (per μm^2). Statistical analysis of the data was performed using a Wilcoxon signed rank test and Wilcoxon rank sum test for paired and nonpaired continuous data, respectively, or a Kruskal-Wallis test. The Šidák correction was applied to adjust for multiple comparisons. Fisher exact or McNemar tests were used to test differences in VT incidence. On the basis of our previous observations of mice with high incidence of VT (≥70%) (26), 4 CASQ2-R33Q or other high-VT incidence mice/group were required to have an 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. However, 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 analyses were performed using Origin 7.0 or R (R Foundation for Statistical Computing, Vienna, Austria). All values are reported as mean ± SEM unless otherwise noted. A p value <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 DCR in the absence of isoproterenol (ISO) (Sigma) (**Figure 1A**, black trace and bars). Addition of ISO (100 nmol/l) markedly increased the frequency of arrhythmogenic DCRs (**Figure 1A**, red traces and bars). This effect of ISO was accompanied by a significant increase in the SR Ca^{2+} content (**Figure 1A**, red trace and bar). The ISO-dependent increase in the frequency of arrhythmogenic DCRs could, therefore, be attributed to: 1) increase in the SR Ca^{2+} content (via phosphorylation of PLB and/or $Ca_v1.2$); 2) altered RyR2 function (via phosphorylation of RyR2 at S2814); or 3) augmented nNa_v-dependent local Na^+/Ca^{2+} signaling (26).

β-AR SIMULATION INCREASES PROPENSITY FOR CPVT BY AUGMENTING TTX-SENSITIVE nNa_V-MEDIATED PERSISTENT I_{Na}. In addition to the predominant TTX-resistant cardiac Na^+ channels ($Na_v1.5$) 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). The nNa_v blockade with 100 nmol TTX (Tocris Bioscience, Avonmouth, United Kingdom) significantly decreased the frequency of ISO-promoted DCRs (**Figure 1A**, green traces and bars). Recently, Na^+ channel inhibitors flecainide and riluzole emerged as effective therapies in CPVT models (23,26). Interestingly, riluzole (10 μmol/l, Sigma) and flecainide (2.5 μmol/l, Sigma) both also reduced DCR frequency (**Figure 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 (**Figure 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 I_{Na} in CPVT and WT cardiomyocytes. Exposure to ISO (100 nmol/l) elicited persistent I_{Na} both in CPVT and WT myocytes (**Figure 1B**, Supplemental Figure 2, respectively, red traces and bars). Notably, this current was sensitive to 100 nmol/l TTX (**Figure 1B**, Supplemental Figure 2, green traces and bars), riluzole, as well as flecainide (**Figure 1B**, purple and blue traces and bars, respectively), despite the 2 former agents exhibiting only a fraction of flecainide's total peak I_{Na} blocking potential (Supplemental Figure 3).

Next, we examined the effect 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 (**Figure 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 (**Figure 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.

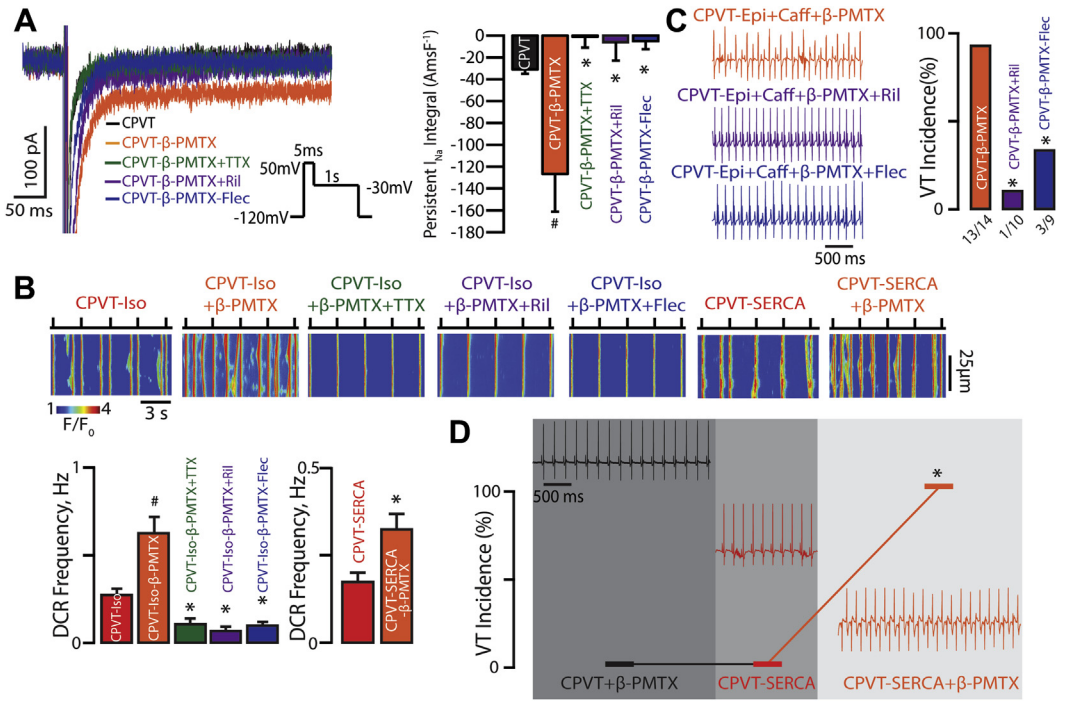
TTX-SENSITIVE nNa_v-MEDIATED PERSISTENT I_{Na} AUGMENTATION AND INCREASED SR CA^{2+} LOAD ARE NECESSARY AND SUFFICIENT FOR ARRHYTHMIAS IN CPVT. To determine whether augmentation of nNa_v-mediated Na^+ influx alone (independent of β-AR stimulation) is sufficient for inducing arrhythmogenic DCR, we induced persistent I_{Na} via nNa_v augmentation with β-PMTX (40) in CPVT cardiomyocytes (**Figure 2A**). Persistent I_{Na} induced by 40 μmol/l β-PMTX (Alomone) was completely reversed by TTX (100 nmol/l), riluzole (10 μmol/l) as well as flecainide (2.5 μmol/l) (**Figure 2A**). Stimulation of nNa_v channels by β-PMTX (30 mg/kg intraperitoneally) in the absence of catecholamine challenge, however, failed to induce VT in vivo (**Figure 2D**).

Of note, β-PMTX further promoted DCR in the presence of ISO on the cellular level (**Figure 2B**). This resulted in over 90% VT incidence in the CPVT mice undergoing concomitant β-PMTX treatment and catecholamine challenge (**Figure 2C**, orange ECG and bar). Confirming the involvement of nNa_v in this pro-arrhythmic process, Na^+-channel blockade—both selective and nonselective—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 (**Figures 2B and 2C**, Supplemental Figure 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 Figure 4A) and significantly more arrhythmic DCR events relative to ISO-naive CPVT myocytes (**Figures 1A and 2B**). However, this was insufficient to promote VT in vivo (**Figure 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 (**Figure 2B**) This, in turn, precipitated VT in all the CPVT-SERCA mice exposed to β-PMTX (**Figure 2D**, orange ECG and bar). Of note, in 2 instances when SERCA2a overexpression was reversed in CPVT-SERCA mice by stopping the 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.

PROARRYTHMIC 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 μmol/l, Sigma) prevented ISO-induced persistent

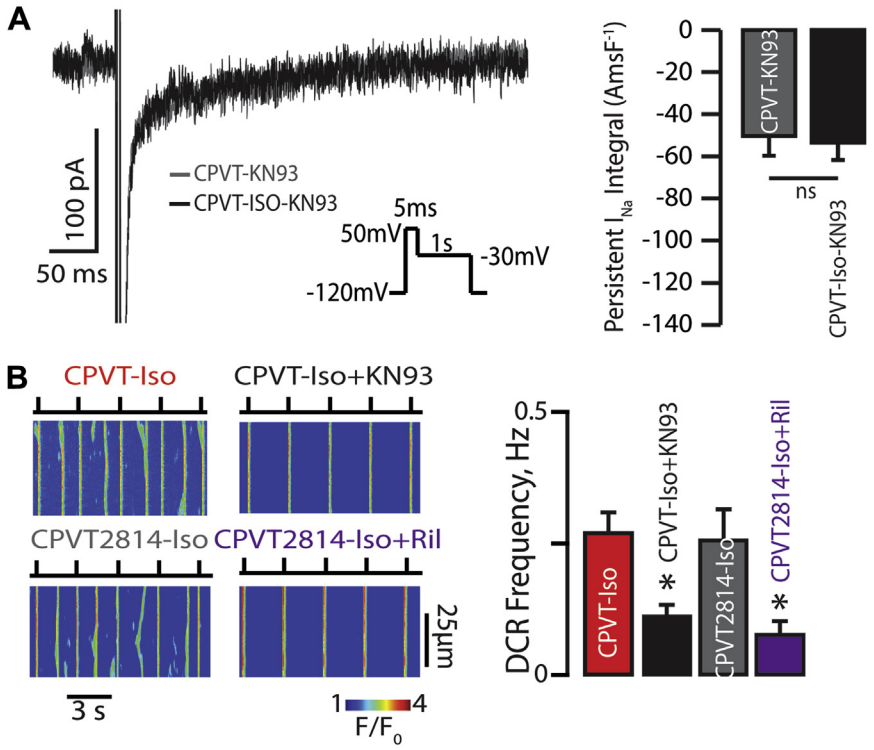
FIGURE 2 TTX-Sensitive nNa_v-Mediated Persistent I_{Na} Augmentation in Conjunction With Increased SR Ca^{2+} Load Contribute to CPVT



(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 μmol/l) in CPVT myocytes increased persistent I_{Na} relative to the control group ($n = 12$ and $n = 31$ cells, respectively; #$p = 0.004$ Wilcoxon rank sum test). β-PMTX-induced persistent I_{Na} was reduced by 100 nmol/l TTX, 10 μmol/l Ril, and 2.5 μmol/l 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 nmol/l) and β-PMTX (40 μmol/l) 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 intraperitoneal injection of epinephrine (1.5 mg/kg) and caffeine (120 mg/kg) and pre-treatment with β-PMTX (30 mg/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 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 (30 mg/kg; $n = 6$; *$p = 0.031$ McNemar 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 to 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. Abbreviations as in Figure 1.

I_{Na} (Figure 3A). Second, KN93 significantly reduced ISO-promoted DCR in CPVT myocytes (Figure 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 nonphosphorylatable 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 (Figure 3B, red and gray bars, respectively). Furthermore, the frequency of these aberrant DCRs was significantly reduced by Na^+ blockade with riluzole (Figure 3B, purple bar). Notably, none of the aforementioned interventions affected SR Ca^{2+} load (Supplemental Figure 4B). Thus CaMKII-mediated Na^+ influx

FIGURE 3 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

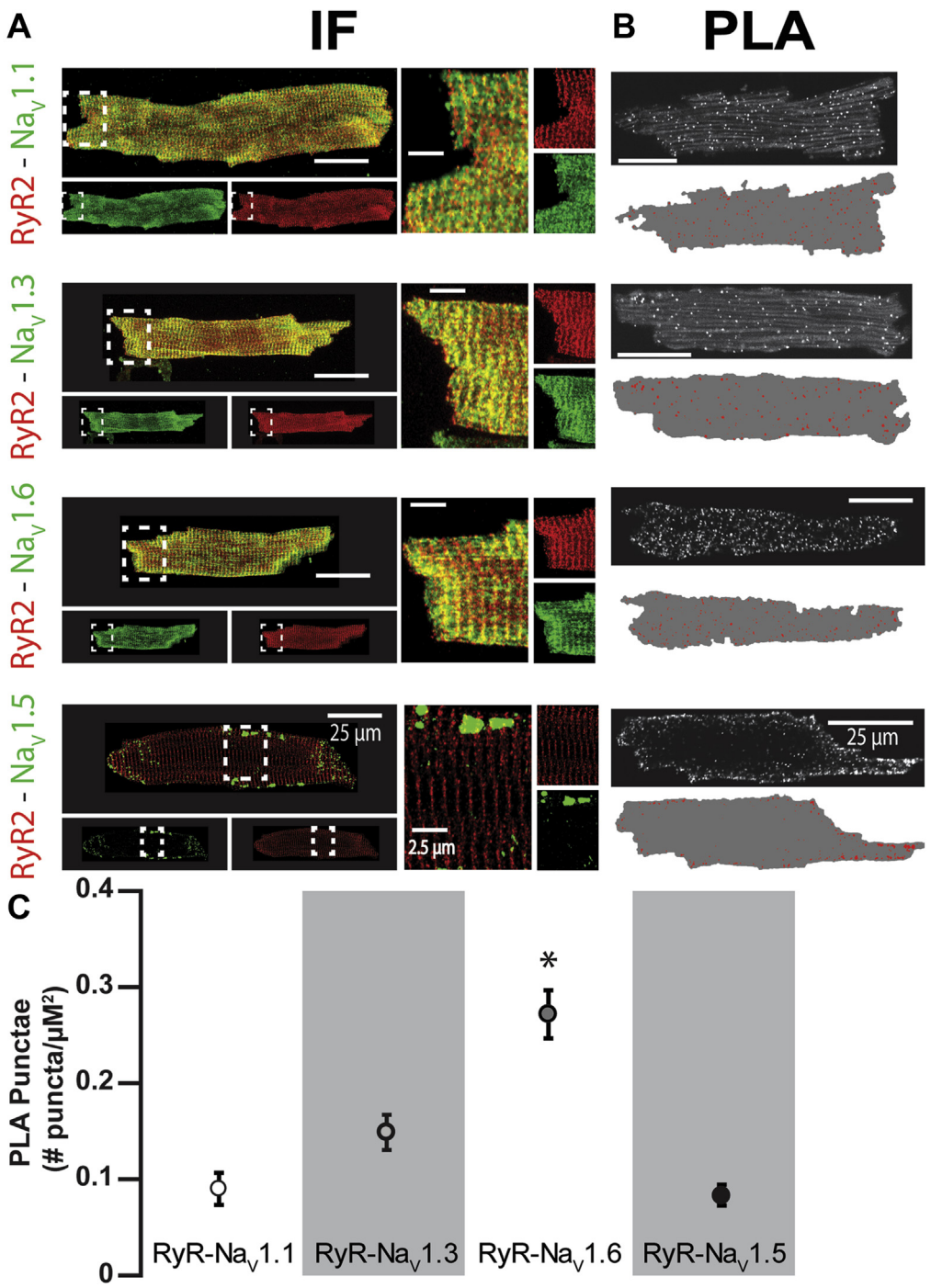


(A) The effect of β-AR stimulation on persistent I_{Na} is mediated through CaMKII. Representative traces of persistent I_{Na} recorded in CPVT cardiomyocytes exposed to CaMKII inhibitor KN93 (1 μmol/l) before and after treatment with 100 nmol/l Iso (n = 9 cells for both groups; p = 0.14 Wilcoxon signed 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 μmol/l KN93 reduced DCR frequency in Iso-treated CPVT cardiomyocytes (n = 166 and n = 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 μmol/l significantly reduced DCR frequency relative to Iso alone (n = 57 and n = 61 cells, respectively; *p = 0.003 Wilcoxon rank sum test). Abbreviations as in Figure 1.

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 (**Figure 4A**). To more precisely examine the localization of these channels with respect to RyR2 in CPVT, we performed a PLA (37). We found that all Na^+ channel isoforms were closely colocalized (within <40 nm [37]) with RyR2 (**Figure 4B**); however, $Na_v1.5$ appeared to be primarily colocalizing with RyR2 in the cell periphery, whereas the nNa_vs exhibited a more diffuse pattern of colocalization. Specifically, $Na_v1.6$ evidenced the highest degree of colocalization with RyR2 relative to the other nNa_v isoforms (**Figure 4C**). The pattern and degree of colocalization 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 Figure 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, a demyelinating disorder, through the blockade of $Na_v1.6$ (44). We therefore examined the functional role of $Na_v1.6$ in CPVT.

FIGURE 4 Neuronal Na^+ Channels and RyR2 Colocalize to the Same Discrete Subcellular Regions



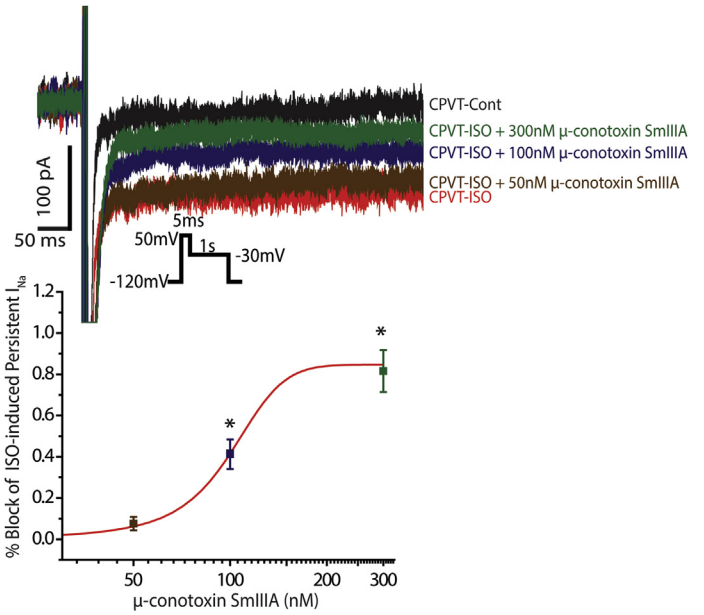
(A) Representative confocal micrographs of isolated CPVT ventricular myocytes labeled for RyR2 **(red)** with various Na_v isoforms (Na_v1.x, **green**) often resulted in an overlap between the immunofluorescent (IF) signals **(yellow)** when overlaid. **(Right)** Close-up views of regions highlighted 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 the results of digital segmentation, with the cell mask in **gray** and PLA signal in **red**. **(C)** Plot of average number of PLA punctae/μm^2 ($p < 0.001$ 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; $n = 1{,}231$, 1,223, 1,291, and 2,848 punctae from 7, 6, 12, and 7 cells for Na_v1.1, 1.3, 1.5, and 1.6, respectively). Abbreviations as in Figure 1.

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 nmol/l concentrations, μ-conotoxin SmIIIA inhibits $Na_v1.1$ and $Na_v1.3$ (45). Despite the putative inhibition of $Na_v1.1$ and $Na_v1.3$, 50 nmol/l μ-conotoxin SmIIIA (Cusabio, Wuhan, China) did not significantly alter ISO-induced persistent I_{Na} in CPVT myocytes (**Figure 5**). However, a concentration of μ-conotoxin SmIIIA near the IC_{50} for $Na_v1.6$ (100 nmol/l) (45) partially reduced ISO-induced persistent I_{Na}, whereas 300 nmol/l μ-conotoxin SmIIIA virtually abolished this ISO-induced phenomenon (**Figure 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 nmol/l 4,9-anhydro-TTX (**Figure 6A**), suggesting that this ISO-promoted persistent I_{Na} is for the most part carried by $Na_v1.6$.

Furthermore, the addition of 300 nmol/l 4,9-anhydro-TTX to CPVT myocytes reduced the frequency of ISO-promoted DCRs (**Figure 6B**). Similarly, pre-treatment of CPVT mice with 4,9-anhydro-TTX (750 μg/kg) markedly reduced VT vulnerability during catecholamine challenge (**Figure 6C**, blue ECG and bar). Notably, this intervention had no significant effect on SR Ca^{2+} load (Supplemental Figure 6). We further addressed the role of $Na_v1.6$ in CPVT by an siRNA approach to selectively target $Na_v1.6$ (Supplemental Figure 7). CPVT mice injected with siRNA against $Na_v1.6$ showed a marked decrease in arrhythmia episodes during catecholamine challenge (**Figure 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 NCX.

Last, to assess the potential role of NCX in the Na^+/Ca^{2+} signaling, we examined the structural correlation between NCX and nNa_vs as well as the functional effect of NCX inhibition on aberrant DCR. We found with the aid of PLA that NCX colocalizes with the TTX-sensitive nNa_v isoforms (Supplemental Figure 8). Furthermore, NCX inhibition with SEA0400 (48) (1 μmol/l, MedChem Express, Monmouth Junction, New Jersey) had a similar effect on DCR relative to that observed with 4,9-anhydro-TTX (**Figure 6B**). Therefore, these data suggest that NCX may be a component of the pro-arrhythmic interaction between nNa_vs and RyR2 that, in part, may be responsible for CPVT.

FIGURE 5 Dose Response of ISO-Induced Persistent I_{Na} in CPVT Cardiomyocytes to nNa_v Blockade With μ-Conotoxin SmIIIA

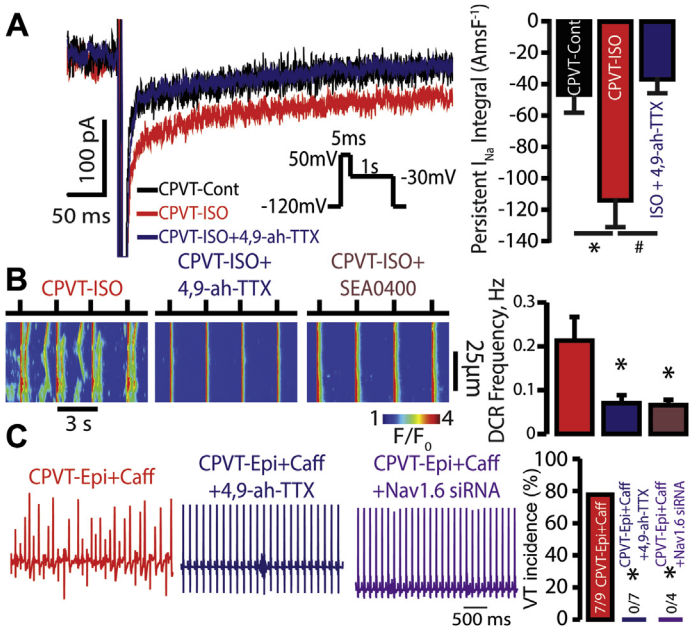


(Top) Representative traces of persistent I_{Na} recorded in CPVT cardiomyocytes exposed to ISO (100 nmol/l) and subsequent increasing concentrations of μ-conotoxin SmIIIA (50, 100, and 300 nmol/l). **(Bottom)** Summary of ISO-induced persistent I_{Na} dose response to μ-conotoxin SmIIIA. ISO-induced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

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

FIGURE 6 Arrhythmogenesis in CPVT Depends on $Na_v1.6$-Mediated Persistent I_{Na}



(A) The effect of $Na_v1.6$ blockade on ISO-induced persistent I_{Na}. Representative traces of persistent I_{Na} pre- and post-exposure to 100 nmol/l 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 300 nmol/l 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 Ca^{2+} indicator, Fluo-3 AM, and were paced at 0.3 Hz. $Na_v1.6$ inhibition with 300 nmol/l 4,9-ah-TTX as well as NCX inhibition with SEA0400 (1 μmol/l) 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 $Na_v1.6$. Pre-treatment with 4,9-ah-TTX (750 μg/kg intraperitoneally) 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 exact test for CPVT- Epi+Caff +4,9ah-TTX and CPVT-Epi+Caff+$Na_v1.6$ siRNA vs. CPVT-Epi+Caff, respectively). Abbreviations as in **Figure 1**.

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 $Na_v1.6$ 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 Na^+/Ca^{2+} signaling, that, in part, may contribute to cardiac arrhythmias (24,26,49–52). On the basis of these studies, we set out to determine whether β-AR stimulation augments nNa_v-mediated Na^+ entry and thereby facilitates Ca^{2+} influx via the 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 (<40 nm) to RyR2s (**Figure 4**) and NCX (Supplemental Figure 8), and where β-AR–augmented Na^+ entry enhances aberrant Na^+/Ca^{2+} signaling, including DCR, thus resulting in 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 (**Figures 1 and 6**, Supplemental Figure 2). Thus, putative "physiological" β-AR augmentation of nNa_v activity can become arrhythmogenic in a setting of genetically compromised RyR2 in CPVT.

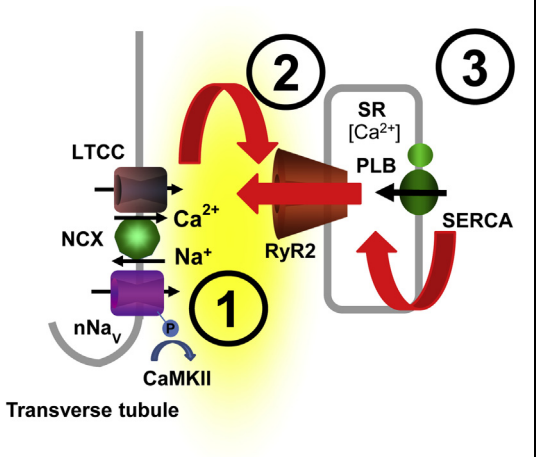
Stimulation of β-AR has been previously shown to affect intracellular Na^+ influx both early and late after a depolarizing stimulus (53,54). In the case of peak I_{Na}, Yarbrough et al. (53) suggested that this phenomenon is coordinated by caveolin-3. Recently, caveolin-3 has been demonstrated to coordinate 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 ($Na_v1.5$) 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 $Na_v1.5$ and is a reflection of cell-wide sarcolemmal $Na_v1.5$ activity. Here, we show that $Na_v1.5$ is present

in the core-compartment of cardiomyocytes, presumably in the T-tubules, albeit its presence in that compartment is very limited (**Figure 4**). However, nNa_v, which include $Na_v1.6$, are the predominant isoforms present within these distinct nanodomains (**Figure 4**) and are responsible for the persistent I_{Na} phenotype during β-AR stimulation (**Figures 1 and 6**, Supplemental Figure 2). In this vein, nonselective ($Na_v1.5$ and nNa_v) inhibition with flecainide (57), despite having similar effect on persistent I_{Na}, more profoundly affected peak I_{Na} relative to 100 nmol/l TTX (Supplemental Figure 3), a concentration that completely blocks nNa_v although mostly sparing $Na_v1.5$ (24,27–29). These data would further suggest that because 10 μmol/l riluzole inhibits both peak and persistent I_{Na} to a similar extent as 100 nmol/l TTX, it may perhaps elicit its DCR-stabilizing effect through blockade of nNa_v. However, future studies will need to determine the specific Na^+ channel isoforms blocked by this agent.

At least 3 isoforms of nNa_v have been identified in the heart as follows: $Na_v1.1$, $Na_v1.3$, and $Na_v1.6$ (**Figure 4**) (26,30–32,58). To examine whether a particular nNa_v isoform is essential for both aberrant Na^+/Ca^{2+} signaling and in vivo arrhythmia in CPVT, we used structural and functional assays. PLA as well as pharmacological and silencing approaches (**Figure 4B**, Supplemental Figure 5, and **Figures 5 and 6**, respectively) pointed to the involvement of $Na_v1.6$ in the arrhythmogenic process. Moreover, WT and CPVT myocytes exhibited a similar degree of $Na_v1.6$ and RyR2 colocalization (**Figure 4**, Supplemental Figure 5), in contrast to changes in RyR2 colocalization observed with other nNa_v isoforms; thus, the bulk of ISO-promoted late I_{Na} in WT and CPVT is likely carried by $Na_v1.6$. 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 its localization in the T-tubules in the vicinity of the RyR2 (**Figure 4**). Furthermore, the persistent I_{Na} that was generated by $Na_v1.6$ during application of ISO was modulated by CAMKII-dependent nNa_v augmentation (**Figure 3**). Although the exact CaMKII phosphorylation site(s) in $Na_v1.6$ or the other nNa_vs are not known, there are consensus CaMKII phosphorylation sites in these channels that correspond to DI-II linker conforming to Arg/Lys-X-X-Ser/Thr (61). In particular, S571 in $Na_v1.5$ appears to be conserved in TTX-sensitive nNa_vs, 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}.

FIGURE 7 Arrhythmogenic Targets of β-AR Stimulation Responsible for CPVT



During the initiation of excitation-contraction (EC) coupling, Ca^{2+} influx through the L-type Ca2+ channels (LTCC) results in activation of RyR2 and subsequent Ca^{2+} release from the SR (68). On the basis of 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 Ca^{2+} release (DCR) by enhancing Na^+-Ca^{2+} exchange (NCX)-dependent Ca^{2+} accumulation in the dyadic cleft (i.e., space between the sarcolemma and the RyR2); ② this nanodomain Ca^{2+} accumulation in turn promotes DCR via Ca^{2+}-induced Ca^{2+} release from the RyR2 that are sensitized due to genetic defects in the RyR2 complex (i.e., RyR2, CaM, CASQ2, TRD, and/or calstabin) (2–5); which along with ③ increased SR Ca^{2+} load play a critical role in triggering aberrant DCR during β-AR stimulation in CPVT. Abbreviations as in **Figure 1**.

What other factors, apart from nNa_v stimulation, are critical to arrhythmogenesis in CPVT? To address this question, we omitted exposure to catecholamines and selectively slowed nNa_v inactivation with β-PMTX (40) to mimic β-AR-induced nNa_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 nNa_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 (**Figure 7**). Mainly, the cross talk among nNa_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 Na^+/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 as follows: 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, although non-isoform-selective Na^+ channel inhibition initially appeared to be beneficial in the management of Ca^{2+}-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 appears to be a 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 1 mechanistic umbrella. Specifically, the novel catecholamine-mediated arrhythmogenic mechanism described herein relies on the maintenance of enhanced SR Ca^{2+} load in the setting of genetically compromised RyR2 along with augmentation of nNa_v activity. The combination of these factors promotes aberrant Na^+/Ca^{2+} 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.

ACKNOWLEDGMENTS The authors would like to thank Ms. Megan Koleske for critical reading of the manuscript. Furthermore, they would like to thank Dr. Igor Kubasov and Dr. Dan Bobkov (Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences) for their valuable suggestions on the manuscript and help with quantitative image analysis.

REPRINT REQUESTS AND CORRESPONDENCE: Dr. Przemysław B. Radwański, Davis Heart and Lung Research Institute, The Ohio State University, 473 West 12th Avenue, Room 510, Columbus, Ohio 43210. E-mail: Przemyslaw.Radwanski@osumc.edu. OR Dr. Sándor Györke, Davis Heart and Lung Research Institute The Ohio State University, 473 West 12th Avenue, Room 507, Columbus, Ohio 43210. E-mail: Sandor.Gyorke@osumc.edu.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In a mouse heart, catecholamines promote pro-arrhythmic aberrant diastolic Ca^{2+} release (DCR) by enhancing neuronal Na^+ channel (nNa_v)-mediated persistent Na^+ current (I_{Na}) along with maintaining adequate SR Ca^{2+} load. Thus, these form the functional basis for 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, although non-isoform-selective Na^+ channel inhibition initially appeared beneficial in the management of Ca^{2+}-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 appears to be 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.

REFERENCES

1. Kong MH, Fonarow GC, Peterson ED, et al. Systematic review of the incidence of sudden cardiac death in the United States. J Am Coll Cardiol 2011;57:794–801.

2. Knollmann BC, Chopra N, Hlaing T, et al. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. J Clin Invest 2006;116:2510–20.

3. Priori SG, Napolitano C, Tiso N, et al. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. Circulation 2001;103:196–200.

4. Iyer V, Hajjar RJ, Armoundas AA. Mechanisms of abnormal calcium homeostasis in mutations responsible for catecholaminergic polymorphic ventricular tachycardia. Circ Res 2007;100:e22–31.

5. Marx SO, Marks AR. Dysfunctional ryanodine receptors in the heart: new insights into complex cardiovascular diseases. J Mol Cell Cardiol 2013;58:225–31.

6. Stevens SCW, Terentyev D, Kalyanasundaram A, Periasamy M, Györke S. Intra-sarcoplasmic reticulum Ca2+ oscillations are driven by dynamic regulation of ryanodine receptor function by luminal Ca2+ in cardiomyocytes. J Physiol 2009;587:4863–72.

7. Qin J, Valle G, Nani A, et al. Ryanodine receptor luminal Ca2+ regulation: swapping calsequestrin and channel isoforms. Biophys J 2009;97:1961–70.

8. Beard NA, Casarotto MG, Wei L, Varsányi M, Laver DR, Dulhunty AF. Regulation of ryanodine receptors by calsequestrin: effect of high luminal Ca2+ and phosphorylation. Biophys J 2005;88:3444–54.

9. Qin J, Valle G, Nani A, et al. Luminal Ca2+ regulation of single cardiac ryanodine receptors: insights provided by calsequestrin and its mutants. J Gen Physiol 2008;131:325–34.

10. Radwański PB, Belevych AE, Brunello L, Carnes CA, Györke S. Store-dependent deactivation: cooling the chain-reaction of myocardial calcium signaling. J Mol Cell Cardiol 2013;58:77–83.

11. Terentyev D, Nori A, Santoro M, et al. Abnormal interactions of calsequestrin with the ryanodine receptor calcium release channel complex linked to exercise-induced sudden cardiac death. Circ Res 2006;98:1151–8.

12. Knollmann BC. New roles of calsequestrin and triadin in cardiac muscle. J Physiol 2009;587:3081–7.

13. Kornyeyev D, Petrosky AD, Zepeda B, Ferreiro M, Knollmann B, Escobar AL. Calsequestrin 2 deletion shortens the refractoriness of Ca^{2+} release and reduces rate-dependent Ca^{2+}-alternans in intact mouse hearts. J Mol Cell Cardiol 2012;52:21–31.

14. Brunello L, Slabaugh JL, Radwanski PB, et al. Decreased RyR2 refractoriness determines myocardial synchronization of aberrant Ca2+ release in a genetic model of arrhythmia. Proc Natl Acad Sci U S A 2013;110:10312–7.

15. Györke S. Molecular basis of catecholaminergic polymorphic ventricular tachycardia. Heart Rhythm 2009;6:123–9.

16. Di Barletta MR, Viatchenko-Karpinski S, Nori A, et al. Clinical phenotype and functional characterization of CASQ2 mutations associated with catecholaminergic polymorphic ventricular tachycardia. Circulation 2006;114:1012–9.

17. Priori SG, Aliot E, Blomstrom-Lundqvist C, et al., for the European Society of Cardiology. Update of the guidelines on sudden cardiac death of the European Society of Cardiology. Eur Heart J 2003;24:13–5.

18. Domeier TL, Maxwell JT, Blatter LA. β-adrenergic stimulation increases the intra-sarcoplasmic reticulum Ca2+ threshold for Ca2+ wave generation. J Physiol 2012;590:6093–108.

19. Dobrev D, Wehrens XHT. Role of RyR2 phosphorylation in heart failure and arrhythmias: controversies around ryanodine receptor phosphorylation in cardiac disease. Circ Res 2014;114:1311–9, discussion 1319.

20. The CAPS Investigators. The Cardiac Arrhythmia Pilot Study. Am J Cardiol 1986;57:91–5.

21. Van der Werf C, Kannankeril PJ, Sacher F, et al. Flecainide therapy reduces exercise-induced ventricular arrhythmias in patients with catecholaminergic polymorphic ventricular tachycardia. J Am Coll Cardiol 2011;57:2244–54.

22. Knollmann BC. Power and pitfalls of using transgenic mice to optimize therapy for CPVT—a need for prospective placebo-controlled clinical trials in genetic arrhythmia disorders. Heart Rhythm 2010;7:1683–5.

23. Watanabe H, Chopra N, Laver D, et al. Flecainide prevents catecholaminergic polymorphic ventricular tachycardia in mice and humans. Nat Med 2009;15:380–3.

24. Radwański PB, Greer-Short A, Poelzing S. Inhibition of Na+ channels ameliorates arrhythmias in a drug-induced model of Andersen-Tawil syndrome. Heart Rhythm 2013;10:255–63.

25. Sikkel MB, Collins TP, Rowlands C, et al. Flecainide reduces Ca(2+) spark and wave frequency via inhibition of the sarcolemmal sodium current. Cardiovasc Res 2013;98:286–96.

26. Radwański PB, Brunello L, Veeraraghavan R, et al. Neuronal Na+ channel blockade suppresses arrhythmogenic diastolic Ca2+ release. Cardiovasc Res 2015;106:143–52.

27. Ritchie JM, Rogart RB. The binding of saxitoxin and tetrodotoxin to excitable tissue. Rev Physiol Biochem Pharmacol 1977;79:1–50.

28. Renaud JF, Kazazoglou T, Lombet A, et al. The Na+ channel in mammalian cardiac cells. Two kinds of tetrodotoxin receptors in rat heart membranes. J Biol Chem 1983;258:8799–805.

29. Satin J, Kyle JW, Chen M, et al. A mutant of TTX-resistant cardiac sodium channels with TTX-sensitive properties. Science 1992;256:1202–5.

30. Maier SKG, Westenbroek RE, McCormick KA, Curtis R, Scheuer T, Catterall WA. Distinct subcellular localization of different sodium channel alpha and beta subunits in single ventricular myocytes from mouse heart. Circulation 2004;109:1421–7.

31. Westenbroek RE, Bischoff S, Fu Y, Maier SKG, Catterall WA, Scheuer T. Localization of sodium channel subtypes in mouse ventricular myocytes using quantitative immunocytochemistry. J Mol Cell Cardiol 2013;64:69–78.

32. Lin X, Liu N, Lu J, et al. Subcellular heterogeneity of sodium current properties in adult cardiac ventricular myocytes. Heart Rhythm 2011;8:1923–30.

33. Rizzi N, Liu N, Napolitano C, et al. Unexpected structural and functional consequences of the R33Q homozygous mutation in cardiac calsequestrin: a complex arrhythmogenic cascade in a knock in mouse model. Circ Res 2008;103:298–306.

34. Lou Q, Belevych AE, Radwański PB, et al. Alternating membrane potential/calcium interplay underlies repetitive focal activity in a genetic model of calcium-dependent atrial arrhythmias. J Physiol 2015;593:1443–58.

35. Suarez J, Gloss B, Belke DD, et al. Doxycycline inducible expression of SERCA2a improves calcium handling and reverts cardiac dysfunction in pressure overload-induced cardiac hypertrophy. Am J Physiol Heart Circ Physiol 2004;287:H2164–72.

36. Van Oort RJ, McCauley MD, Dixit SS, et al. Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. Circulation 2010;122:2669–79.

37. Rhett JM, Ongstad EL, Jourdan J, Gourdie RG. Cx43 associates with Na(v)1.5 in the cardiomyocyte perinexus. J Membr Biol 2012;245:411–22.

38. Mezzaroma E, Toldo S, Farkas D, et al. The inflammasome promotes adverse cardiac remodeling following acute myocardial infarction in the mouse. Proc Natl Acad Sci U S A 2011;108:19725–30.

39. Liu B, Ho H-T, Velez-Cortes F, et al. Genetic ablation of ryanodine receptor 2 phosphorylation at Ser-2808 aggravates Ca(2+)-dependent cardiomyopathy by exacerbating diastolic Ca2+ release. J Physiol 2014;592:1957–73.

40. Schiavon E, Stevens M, Zaharenko AJ, Konno K, Tytgat J, Wanke E. Voltage-gated sodium channel isoform-specific effects of pompilidotoxins. FEBS J 2010;277:918–30.

41. Hund TJ, Koval OM, Li J, et al. A β(IV)-spectrin/CaMKII signaling complex is essential for membrane excitability in mice. J Clin Invest 2010;120:3508–19.

42. Ashpole NM, Herren AW, Ginsburg KS, et al. Ca2+/calmodulin-dependent protein kinase II

(CaMKII) regulates cardiac sodium channel NaV1.5 gating by multiple phosphorylation sites. J Biol Chem 2012;287:19856–69.

43. Craner MJ, Newcombe J, Black JA, Hartle C, Cuzner ML, Waxman SG. Molecular changes in neurons in multiple sclerosis: altered axonal expression of Nav1.2 and Nav1.6 sodium channels and Na+/Ca2+ exchanger. Proc Natl Acad Sci U S A 2004;101:8168–73.

44. Sierra Bello O, Gonzalez J, Capani F, Barreto GE. In silico docking reveals possible Riluzole binding sites on Nav1.6 sodium channel: implications for amyotrophic lateral sclerosis therapy. J Theor Biol 2012;315:53–63.

45. Wilson MJ, Yoshikami D, Azam L, et al. μ-conotoxins that differentially block sodium channels NaV1.1 through 1.8 identify those responsible for action potentials in sciatic nerve. Proc Natl Acad Sci U S A 2011;108:10302–7.

46. Rosker C, Lohberger B, Hofer D, Steinecker B, Quasthoff S, Schreibmayer W. The TTX metabolite 4,9-anhydro-TTX is a highly specific blocker of the Na(v1.6) voltage-dependent sodium channel. Am J Physiol Cell Physiol 2007;293:C783–9.

47. Hargus NJ, Nigam A, Bertram EH 3rd, Patel MK. Evidence for a role of Nav1.6 in facilitating increases in neuronal hyperexcitability during epileptogenesis. J Neurophysiol 2013;110:1144–57.

48. Bourgonje VJA, Vos MA, Ozdemir S, et al. Combined Na(+)/Ca(2+) exchanger and L-type calcium channel block as a potential strategy to suppress arrhythmias and maintain ventricular function. Circ Arrhythm Electrophysiol 2013;6:371–9.

49. Lin X, O'Malley H, Chen C, et al. Scn1b deletion leads to increased tetrodotoxin-sensitive sodium current, altered intracellular calcium homeostasis and arrhythmias in murine hearts. J Physiol 2015;593:1389–407.

50. Mishra S, Reznikov V, Maltsev VA, Undrovinas NA, Sabbah HN, Undrovinas A. Contribution of sodium channel neuronal isoform Nav1.1 to late sodium current in ventricular myocytes from failing hearts. J Physiol 2015;593:1409–27.

51. Biet M, Morin N, Lessard-Beaudoin M, et al. Prolongation of action potential duration and QT interval during epilepsy linked to increased contribution of neuronal sodium channels to cardiac late Na+ current: a potential mechanism for sudden death in epilepsy. Circ Arrhythm Electrophysiol 2015;8:912–20.

52. Torres NS, Larbig R, Rock A, Goldhaber JI, Bridge JHB. Na+ currents are required for efficient excitation-contraction coupling in rabbit ventricular myocytes: a possible contribution of neuronal Na+ channels. J Physiol 2010;588:4249–60.

53. Yarbrough TL, Lu T, Lee H-C, Shibata EF. Localization of cardiac sodium channels in caveolin-rich membrane domains: regulation of sodium current amplitude. Circ Res 2002;90:443–9.

54. Dybkova N, Wagner S, Backs J, et al. Tubulin polymerization disrupts cardiac β-adrenergic regulation of late INa. Cardiovasc Res 2014;103:168–77.

55. Nikolaev VO, Moshkov A, Lyon AR, et al. Beta2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. Science 2010;327:1653–7.

56. Wright PT, Nikolaev VO, O'Hara T, et al. Caveolin-3 regulates compartmentation of cardiomyocyte beta2-adrenergic receptor-mediated cAMP signaling. J Mol Cell Cardiol 2014;67:38–48.

57. Ramos E, O'Leary ME. State-dependent trapping of flecainide in the cardiac sodium channel. J Physiol 2004;560:37–49.

58. Maier SKG, Westenbroek RE, Schenkman KA, Feigl EO, Scheuer T, Catterall WA. An unexpected role for brain-type sodium channels in coupling of cell surface depolarization to contraction in the heart. Proc Natl Acad Sci U S A 2002;99:4073–8.

59. Rush AM, Dib-Hajj SD, Waxman SG. Electrophysiological properties of two axonal sodium channels, Nav1.2 and Nav1.6, expressed in mouse spinal sensory neurones. J Physiol 2005;564:803–15.

60. Chen Y, Yu FH, Sharp EM, Beacham D, Scheuer T, Catterall WA. Functional properties and differential neuromodulation of Na(v)1.6 channels. Mol Cell Neurosci 2008;38:607–15.

61. Marionneau C, Lichti CF, Lindenbaum P, et al. Mass spectrometry-based identification of native cardiac Nav1.5 channel α subunit phosphorylation sites. J Proteome Res 2012;11:5994–6007.

62. Faggioni M, Savio-Galimberti E, Venkataraman R, et al. Suppression of spontaneous ca elevations prevents atrial fibrillation in calsequestrin 2-null hearts. Circ Arrhythm Electrophysiol 2014;7:313–20.

63. Wakili R, Voigt N, Kääb S, Dobrev D, Nattel S. Recent advances in the molecular pathophysiology of atrial fibrillation. J Clin Invest 2011;121:2955–68.

64. Liu N, Denegri M, Ruan Y, et al. Short communication: flecainide exerts an antiarrhythmic effect in a mouse model of catecholaminergic polymorphic ventricular tachycardia by increasing the threshold for triggered activity. Circ Res 2011;109:291–5.

65. Belevych AE, Radwański PB, Carnes CA, Györke S. "Ryanopathy": causes and manifestations of RyR2 dysfunction in heart failure. Cardiovasc Res 2013;98:240–7.

66. Starmer CF, Lastra AA, Nesterenko VV, Grant AO. Proarrhythmic response to sodium channel blockade. Theoretical model and numerical experiments. Circulation 1991;84:1364–77.

67. Echt DS, Liebson PR, Mitchell LB, et al. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The Cardiac Arrhythmia Suppression Trial. N Engl J Med 1991;324:781–8.

68. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol 1983;245:C1–14.

KEY WORDS β-adrenergic receptor, diastolic Ca^{2+} release, neuronal Na^+ channels, ventricular arrhythmias

APPENDIX For supplemental figures, please see the online version of this article.