Neuronal Na\(^+\) channel blockade suppresses arrhythmogenic diastolic Ca\(^{2+}\) release

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Aims

Sudden death resulting from cardiac arrhythmias is the most common consequence of cardiac disease. Certain arrhythmias caused by abnormal impulse formation including catecholaminergic polymorphic ventricular tachycardia (CPVT) are associated with delayed afterdepolarizations resulting from diastolic Ca\(^{2+}\) release (DCR) from the sarcoplasmic reticulum (SR). Despite high response of CPVT to agents directly affecting Ca\(^{2+}\) cycling, the incidence of refractory cases is still significant. Surprisingly, these patients often respond to treatment with Na\(^+\) channel blockers. However, the relationship between Na\(^+\) influx and disturbances in Ca\(^{2+}\) handling immediately preceding arrhythmias in CPVT remains poorly understood and is the object of this study.

Methods and results

We performed optical Ca\(^{2+}\) and membrane potential imaging in ventricular myocytes and intact cardiac muscles as well as surface ECGs on a CPVT mouse model with a mutation in cardiac calsequestrin. We demonstrate that a subpopulation of Na\(^+\) channels (neuronal Na\(^+\) channels; nNav) colocalize with ryanodine receptor Ca\(^{2+}\) release channels (RyR2). Disruption of the crosstalk between nNa\(^+\) and RyR2 by nNav blockade with riluzole reduced and also desynchronized DCR in isolated cardiomyocytes and in intact cardiac tissue. Such desynchronization of DCR on cellular and tissue level translated into decreased arrhythmias in CPVT mice.

Conclusions

Thus, our study offers the first evidence that nNa\(^+\) contribute to arrhythmogenic DCR, thereby providing a conceptual basis for mechanism-based antiarrhythmic therapy.

Keywords

Ventricular arrhythmias • Neuronal Na\(^+\) channels • Diastolic Ca\(^{2+}\) release

1. Introduction

Sudden arrhythmic death is a leading cause of mortality in the USA.\(^1\) Arrhythmias caused by abnormal impulse generation occur both in patients with structural heart disease,\(^2\) as well as in seemingly healthy individuals.\(^3\) In the latter, fatal arrhythmias are often associated with aberrant diastolic Ca\(^{2+}\) release (DCR) during exercise or stress-induced catecholamine surge.\(^1\) These are called Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT).\(^1\) Recent evidence suggests that CPVT is related to mutations in proteins comprising the ryanodine receptor Ca\(^{2+}\) release channel (RyR2) complex, including the RyR2 itself and the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-binding protein calsequestrin (CASQ2).\(^4\) These mutations appear to alter the ability of RyR2 to become refractory following systolic Ca\(^{2+}\) release, allowing it to remain open during diastole and leak SR Ca\(^{2+}\).\(^7\)–\(^10\) This can result in DCR, which in turn can be translated into membrane depolarization via electrogenic Na\(^+\)/Ca\(^{2+}\) exchange (NCX) resulting in triggered ventricular arrhythmia.\(^8\)\(^,\)\(^10\)–\(^12\)

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Despite high response of CPVT to combination of Ca\(^{2+}\) channel- and β-blockers, the incidence of refractory cases is still significant.\(^3\) Surprisingly, based on the Ca\(^{2+}\)-dependent mechanisms, such patients often respond to treatment with Na\(^{+}\) channel blockade.\(^3\) However, the precise contribution of Na\(^{+}\) influx to aberrant DCR and arrhythmogenesis is unclear. Data presented herein strongly suggest that a subpopulation of Na\(^{+}\) channels, neuronal Na\(^{+}\) channels (nNav), colocalize with RyR2. The electrogenic NCX couples Na\(^{+}\) fluxes in these compartments to Ca\(^{2+}\) influx and efflux as well as membrane potential. Disruption of coupling between nNav and RyR2 by nNav blockade reduced and desynchronized DCR in isolated cardiomyocytes as well as in tissue and decreased arrhythmias in CPVT mice. Thus, our study offers the first support for the concept that nNav contribute to the genesis and synchronization of arrhythmogenic DCR and identifies nNav, as a target for mechanism-based antiarrhythmic therapy.

2. 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).

2.1 Myocyte isolation, confocal Ca\(^{2+}\) imaging, Na\(^{+}\) current, and membrane potential recordings

Ventricular myocytes were obtained by enzymatic isolation from 3- to 5-month-old R33Q (in C57BL/6 background) and age-matched C57BL/6 WT (Jackson Lab) male mice. Mice were anaesthetized with isoflurane, and once a deep level of anaesthesia was reached, confirmed by lack of response to noxious stimuli, the heart was rapidly removed and perfused via a Langendorff as previously described. Single ventricular myocytes were isolated using Liberase Blendzymes (Roche, Applied Science, IN, USA). Whole-cell patch clamp recordings of action potentials were performed with an Axopatch 200b amplifier using external solution that contained (in mM): 140 NaCl, 5.4 KCl, 2.0 CaCl\(_2\), 0.5 MgCl\(_2\), 10 HEPES, and 5.6 glucose (pH 7.4). Patch pipettes were filled with a solution that contained (in mM): 90 K-aspartate, 50 KCl, 5 MgATP, 5 NaCl, 1 MgCl\(_2\), 0.1 Tris GTP, 10 HEPES, and 0.1 Fluo-4 K-salt (Molecular Probes, Eugene, OR, USA) (pH 7.2). Sodium currents (I\(_Na\)) were recorded using internal solution containing (in mM): 10 NaCl, 20 TEACl, 123 CsCl, 1 MgCl\(_2\), 0.1 Tris GTP, 5 MgATP, 10 HEPES, and 10 BAPTA (pH 7.2). The extracellular bathing solution contained (in mM): 10 NaCl, 130 TEACl, 4 CsCl, 0.4 CaCl\(_2\), 2 MgCl\(_2\), 0.05 CdCl\(_2\), 10 HEPES, and 10 glucose; pH was maintained at 7.4 with CsOH. Whole-cell capacitance and series resistance compensation (>60%) was applied along with leak subtraction. Electrical field stimulation experiments were performed using the following external solution (in mM): 140 NaCl, 5.4 KCl, 2.0 CaCl\(_2\), 0.5 MgCl\(_2\), 10 HEPES, and 5.6 glucose (pH 7.4). For Ca\(^{2+}\) spark recordings in permeabilized myocytes, the cardiac myocytes were permeabilized with saponin (0.01% for 20–30 s). The internal solution contained (in mM): 120 K-aspartate, 20 KCl, 0.81 MgCl\(_2\), 1 K\(_2\)HPO\(_4\), 0.5 EGTA (free [Ca\(^{2+}\)] \(\sim\) 50 nm), 3 MgATP, 10 phosphocreatine, 0.03 Fluo-4 K-salt, 20 HEPES (pH 7.2), and 5 U/mL creatine phosphokinase. To assess the SR Ca\(^{2+}\) load, 20 mM caffeine was applied at the end of the experiments. Intracellular Ca\(^{2+}\) cycling was monitored by a Nikon A1 laser scanning confocal microscope equipped with a ×63/1.4 numerical aperture oil objective (Leica, Buffalo Grove, IL, USA). Custom software written in Matlab (Mathworks, Natick, MA) was used to analyse images for colocalization and cross-correlation as well as to perform fast Fourier transform (FFT) morphological analysis. Briefly, colocalization was assessed from plots of red vs. green intensity as previously described.\(^4\) Additionally, the cross-correlation coefficient between the intensities of red and green immunofluorescent signals was calculated both row-wise as well as for the averaged intensity profile over a whole myocyte. Further morphological analysis was performed by calculating the two-dimensional (2D) FFT for the red and green channels of each image. The amplitude of the 2D FFT for the red and green channels, each itself in the form of a 2D image with the same dimensions as the original image, was then overlaid. Periodicity of immunofluorescent signal was assessed from the location and amplitude of peaks on the FFTs and the relative periodicities of the red and green immunofluorescent signals assessed from the overlaid FFTs of the respective channels. The antibodies used were rabbit anti-Na\(_v\), 1.1, 1.3, 1.6 (Alomone, Jerusalem, Israel), 1.5 (generous gift from Dr Peter Mohler), and mouse monoclonal anti-RyR2 (Pierce Antibodies, Rockford, IL, USA).

2.2 Cardiac muscles confocal Ca\(^{2+}\) and membrane potential imaging

Muscles for confocal Ca\(^{2+}\) and membrane potential imaging were prepared as previously described. Briefly, the hearts were removed and placed in a modified Krebs–Henseleit (KH) buffer, containing (in mM/L): 120 NaCl, 5 KCl, 2MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 20 NaHCO\(_3\), 0.25 CaCl\(_2\), and 10 glucose (pH 7.4), equilibrated with 95% O\(_2\)–5% CO\(_2\). Twenty millimolar 2,3-butanedione monoxyxime (BDM) was also added to the dissection buffer to minimize cutting injury. The recording chamber was placed on the stage of an Olympus Fluoview 1000 laser scanning confocal microscope, equipped with a 5 mW He–Ne laser and a UPLSAPO 60X water objective (NA 1.20, working distance 0.28 mm). Muscles were loaded with the cytosolic Ca\(^{2+}\) indicator Rhod-2 AM for 45 min and d-4-ANBQBS (Richard D. Berlin Center for Cell Analysis and Modeling, University of Connecticut Health Center, USA) for 10 min. The muscles were superfused with KH buffer containing 5 mM BDM and 10–20 μM blebbistatin to stop cell contraction. The Ca\(^{2+}\) concentration was also raised in the KH buffer to 1.5 mM. Line-scan images were acquired at the rate of 8 μs/pixel. 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.

2.3 Confocal microscopy of immunolabelled myocytes and image analysis

Isolated ventricular myocytes from R33Q hearts 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 overnight at 4 °C. After washing, goat secondary antibodies (anti-mouse and anti-rabbit) conjugated to Alexa Fluor (488, 594; Life Technologies, Grand Island, NY, USA) were added for 1 h. Coverslips were mounted to using ProLong Gold Anti-Fade Mounting Kit (Molecular Probes).

Immunolabelled myocytes were imaged on a TCS SP8 laser scanning confocal microscope equipped with a ×63/1.4 numerical aperture oil objective (Leica, Buffalo Grove, IL, USA). Custom software written in Matlab (Mathworks, Natick, MA) was used to analyse images for colocalization and cross-correlation as well as to perform fast Fourier transform (FFT) morphological analysis. Briefly, colocalization was assessed from plots of red vs. green intensity as previously described.\(^4\) Additionally, the cross-correlation coefficient between the intensities of red and green immunofluorescent signals was calculated both row-wise as well as for the averaged intensity profile over a whole myocyte. Further morphological analysis was performed by calculating the two-dimensional (2D) FFT for the red and green channels of each image. The amplitude of the 2D FFT for the red and green channels, each itself in the form of a 2D image with the same dimensions as the original image, was then overlaid. Periodicity of immunofluorescent signal was assessed from the location and amplitude of peaks on the FFTs and the relative periodicities of the red and green immunofluorescent signals assessed from the overlaid FFTs of the respective channels. The antibodies used were rabbit anti-Na\(_v\), 1.1, 1.3, 1.6 (Alomone, Jerusalem, Israel), 1.5 (generous gift from Dr Peter Mohler), and mouse monoclonal anti-RyR2 (Pierce Antibodies, Rockford, IL, USA).
2.4 ECG recordings

ECG recordings were performed before and after epinephrine and caffeine challenge. Continuous ECG recordings were obtained from mice anesthetized with isoflurane, at minimum effective concentration (1–1.5%). Subcutaneous needle electrodes were applied to the left, right upper limb, and right lower limb for ECG recording (PL3504 PowerLab 4/35, ADInstruments). After baseline recording (5 min.), each mouse received intraperitoneally vehicle (DMSO), riluzole (10 mg/kg) or SN-6 (40 mg/kg). After 5–10 min from the initial intervention, animals were exposed to an intraperitoneally epinephrine (Epi, 1.5 mg/kg) and caffeine (Caffe, 120 mg/kg) challenge, and ECG recording continued for 10 min. ECG recordings were analysed using the LabChart 7.3 program (ADInstruments). Ventricular tachycardia (VT) was defined as three or more premature ectopies, while arrhythmia was defined as frequent ectopies, bigeminies, and/or VT.

2.5 Reagents

Unless otherwise stated, all chemicals were purchased from Sigma (St Louis, MO, USA), Torcix (Bristol, UK), or Alomone. Fluorescent dyes were purchased from either Molecular Probes or Richard D. Berlin Center for Cell Analysis and Modeling (University of Connecticut Health Center, USA).

2.6 Data analysis

Membrane potential and I Na dependence analysis were performed using pCLAM9 software (Molecular Devices, Sunnyvale, CA, USA). Line scanning images of Ca2+ and membrane potential were normalized for baseline fluorescence.8 Ca2+ and membrane potential imaging data were processed using imagej and Origin software. Statistical analysis of the data was performed using a two-tailed Student’s t-test for paired and unpaired data or a single factor ANOVA. The Šidák correction was applied to adjust for multiple comparisons. A Fisher’s exact and a Mantel–Haenszel test were used to test differences in nominal data. All values are reported as means ± SEM unless otherwise noted. A value of P < 0.05 was considered statistically significant.

3. Results

3.1 Neuronal Na+ channel modulation regulates DCR

To determine whether nNa+, possess a unique action on intracellular Ca2+ handling that may serve as a substrate for triggered arrhythmias, we assessed the impact of nNa+, blockade on Ca2+ sparks and DCR in the form of Ca2+ waves in cardiomyocytes isolated from hearts expressing a CPVT-associated CASQ2 mutation (R33Q) and exposed to isoproterenol (Iso).15 In accord with previous observations,16 R33Q cardiomyocytes evidenced a high rate of SR Ca2+ leak that corresponded to high Ca2+ spark frequency and prolonged duration of release. Tetrodotoxin (TTX) at nanomolar concentration is known to selectively inhibit nNa+, without significantly affecting cardiac-type Na+ channels (Na+,1,5) or directly inhibiting RyR2.17–20 Line-scan confocal Ca2+ imaging showed that 100 nM TTX significantly decreased the frequency of iso-promoted Ca2+ sparks and DCR in resting and paced cardiomyocytes, respectively (Figure 1A–E). We also assessed DCR synchrony, a marker of Ca2+ release refractoriness.8,10 TTX significantly reduced DCR synchrony, as demonstrated by a broadened distribution of DCR first latencies and an increase in the S.D. of latency (Figure 1F). Notably, TTX’s antiarrhythmic effects were not accompanied by alterations in the SR Ca2+ content or Ca2+ transient amplitude (see Supplementary material online, Figure S1A–C), which is consistent with a previous report that utilized TTX concentrations of up to 5 μM.21 The sensitivity of DCR in R33Q ventricular cardiomyocytes to 100 nM TTX might have resulted from an adaptive up-regulation of nNa+. To address such possibility, we assessed peak Na+ current (I Na) in R33Q and WT myocytes. There was no significant difference in I Na density between the two study cohorts (Figure 2A and B).

Next, we investigated the antiarrhythmic effects of nNa+, inhibition using riluzole,22 an agent with an established clinical efficacy in management of amyotrophic lateral sclerosis (ALS).23 First, we assessed whether riluzole inhibits the TTX-sensitive component of I Na. To this end, we compared I Na reduction between 100 nM TTX and 10 μM riluzole. In WT cardiomyocytes, we observed no significant difference in the reduction of peak I Na (32.5 ± 7.7 vs. 35.1 ± 4.2%); between the two pharmacological interventions (Figure 2C). Furthermore, both riluzole and TTX facilitated I Na inactivation as evidenced by a reduction in the slow component of the decaying phase of I Na (Figure 2D and E). Importantly, effects of 10 μM riluzole on excitability (Supplementary material online, Figure S2A and B). Ca2+ release (Figure 1A–E, Supplementary material online, Figure S1A–C), and Ca2+ release synchrony (Figure 1E) were similar to those observed with TTX.20,24 Lastly, riluzole did not affect Ca2+ spark frequency in permeabilized cardiomyocytes (8.73 ± 0.49 vs. 7.69 ± 0.36 Sparks/100 μs, P = ns, n = 634–700 events), suggesting that riluzole lacks a direct effect on RyR2.

To assess whether modulation of nNa+, inactivation (Figure 2D and E) can in part contribute to the generation of DCR in quiescent (non-stimulated) myocytes, we facilitated as well as slowed nNa+, inactivation with riluzole (10 μM) and β-pompilidotoxin (β-PMTX, 50 μM), respectively.26 In catecholamine-free wild-type (WT) myocytes, we observed riluzole-mediated decrease in Ca2+ spark frequency (Figure 3A–C). On the other hand, β-PMTX increased frequency of Ca2+ sparks in WT myocytes despite the omission of catecholamines (Figure 3A–C). Furthermore, the enhanced Na+ flux at rest translated into increased rate of DCR frequency in stimulated, catecholamine-exposed WT cardiomyocytes (Figure 3D and E). Additionally, such rise in DCR was coupled to abbreviated latency to the first DCR and reduced SR Ca2+ content (Figure 3F; Supplementary material online, Figure S1E and F). This reduction in SR Ca2+ content can perhaps be ascribed to a large reverse mode NCX (Na+–out and Ca2+–in) in the junctional cleft prompted by enhanced Na+ influx via the incompletely inactivated nNa+. Of note, the effects of β-PMTX on Ca2+ cycling were reversed with riluzole (Figure 3A–E; Supplementary material online, Figure S1D–F). Importantly, the effects of nNa+, modulation were independent of global phosphorylation state of the cardiomyocyte. Neither Ca2+/calmodulin-dependent protein kinase II inhibition with KN-93 (5 μM) in the presence of Iso nor the complete omission of Iso from the superfusate prevented nNa+, modulation of aberrant Ca2+ release (Figure 3A–C; Supplementary material online, Figure S2C and D). Taken together these results strongly suggest that a specific subpopulation of Na+ channels (i.e. nNa+) exert a modulatory effect on DCR, which likely involves NCX.

To assess the role of NCX in the interaction between nNa+ and RyR2, we inhibited reverse mode NCX (Na+–out and Ca2+–in) with SN6 (5 μM).27,28 Effects on DCR similar to those observed with nNa+, blockade were obtained with SN6 (Figures 1 and 3; Supplementary material online, Figure S1). Furthermore, no significant effect was observed on forward mode NCX (Ca2+–out and Na+–in) as assessed by the decay of the caffeine-induced Ca2+ transient (Supplementary material online, Figure S1C, inset). These data suggest that NCX can regulate Ca2+ release29 and is an integral part of the pro-arrhythmic interaction between nNa+ and RyR2 within R33Q cardiomyocytes.
3.2 Neuronal Na\(^+\) channels and RyR2 colocalize

Experiments in isolated myocytes suggest that localization of nNa\(_v\) in close proximity to SR Ca\(^{2+}\)-release machinery is consistent with their role in precipitating DCR. Therefore, we performed confocal immunolocalization experiments in ventricular myocytes isolated from R33Q hearts. Over 80% of the nNa\(_v\) (Nav1.1, 1.3, 1.6) immunofluorescent signal coincided with RyR2 labelling (Figure 4A–C and E–G) and exhibited a periodic pattern consistent with T-tubular localization (Supplementary material online, Figure S3A–C, E–G, S4, and S5A). On the other hand, very little spatial cross-correlation was observed between regions labelled with cardiac-type Na\(^+\) channels (Nav1.5) and RyR2 (Figure 4D and H; Supplementary material online, Figure S3D, H, and S5B). These results strongly suggest that nNa\(_v\) colocalize with RyR2 in the same subcellular regions in the R33Q mouse ventricle and in part may explain nNa\(_v\) contribution to the regulation of DCR in a setting of leaky RyR2.

3.3 Neuronal Na\(^+\) channel blockade desynchronizes DCR in tissue

We examined the role of nNa\(_v\), in DCR and focal excitation in tissue. We hypothesized that in a setting of leaky RyR2, nNa\(_v\) could facilitate temporal alignment or synchronization of DCRs, and the resultant membrane depolarization in neighbouring cells accounting for the generation of ventricular extrasystole across the myocardium. Therefore, we performed simultaneous confocal imaging of membrane potential and...
cytosolic $\text{Ca}^{2+}$ in cardiac muscle preparations from R33Q mice during nNa\textsubscript{v} blockade. Both riluzole (10 $\mu$M) and TTX (100 nM) significantly reduced frequency and synchronicity of Iso-promoted DCR (Figure 5A–E). Correspondingly, both drugs reduced the frequency of extrasystolic action potentials (Figure 5E). Thus, nNa\textsubscript{v} blockade with riluzole can reduce DCR, its synchronicity, and the resultant triggered arrhythmias in cardiac muscle.

### 3.4 Effect of neuronal Na\textsuperscript{+} channel blockade on CPVT

We next examined the therapeutic efficacy of disrupting the crosstalk between nNa\textsubscript{v} and the leaky RyR2 on the cellular level that desynchronized DCR in intact tissue. To this end, we assessed arrhythmia inducibility in R33Q mice. A caffeine and epinephrine challenge induced frequent ventricular bigeminy (Figure 6A), which degenerated into polymorphic VT. After riluzole treatment (10 mg/kg), half of the R33Q animals remained in sinus rhythm (Figure 6B) despite the caffeine and epinephrine challenge. Overall, riluzole reduced by half ventricular arrhythmias, including frequent ventricular extrasystoles and bigeminy (Figure 6D), while completely suppressing VTs in all R33Q mice tested (Figure 6E). Consistent with observations in isolated cardiomyocytes, riluzole’s antiarrhythmic effect in R33Q mice was modulated by perturbations of NCX-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} signalling. NCX inhibition with SN-6 (40 mg/kg, Figure 6C–E) resulted in a reduced overall arrhythmia burden. Taken together, these data suggest that perturbing local crosstalk between nNa\textsubscript{v} and RyR2 via nNa\textsubscript{v} blockade exerts an antiarrhythmic effect in vivo by reducing and desynchronizing DCR.

### 4. Discussion

Here we studied CPVT-associated arrhythmogenesis resulting from aberrant DCR.\textsuperscript{15,35} Previous studies showed that CPVT-linked mutations disrupt RyR2 gating, thereby precipitating synchronous DCR and triggered arrhythmias.\textsuperscript{5,8,36} In recent years, Na\textsuperscript{+} channel blockade has emerged as a potential antiarrhythmic strategy in genetic and acquired models of Ca\textsuperscript{2+}-mediated arrhythmias.\textsuperscript{13,19,21} Previous studies have suggested two mechanisms for the antiarrhythmic effect of Na\textsuperscript{+} channel blockers such as flecainide: (i) direct inhibition of the RyR2\textsuperscript{37} or (ii) decreased membrane excitability due to cardiac-type Na\textsuperscript{+} channel (Nav1.5) blockade uncoupling DCR from membrane depolarization.\textsuperscript{38} Our results demonstrate a novel mechanism for Ca\textsuperscript{2+}-mediated arrhythmogenesis which involves Na\textsuperscript{+}/Ca\textsuperscript{2+} signalling independent of direct RyR2 block and reduced excitability. Importantly, disruption of the Na\textsuperscript{+}/Ca\textsuperscript{2+} signalling via inhibition of neuronal Na\textsuperscript{+} channels (nNa\textsubscript{v}) effectively suppressed arrhythmias in CPVT mice, thus suggesting a new therapeutic approach in management of CPVT.

Initially, the antiarrhythmic effect of Na\textsuperscript{+} channel blockers, such as flecainide, was attributed to the direct inhibition of the RyR2.\textsuperscript{37} Yet, riluzole did not reduce spark frequency in permeabilized myocytes in the present study arguing against its interacting directly with RyR2. Subsequent studies have suggested that decreased membrane excitability as...
a result of Na⁺ channel blockade uncouples DCR from membrane depolarization. However, neither 10 μM riluzole in our study nor 100 nM TTX in previous ones altered membrane excitability. Importantly, our findings point to a distinct microdomain where nNav are localized near RyR2s (Figure 4), forming the functional unit underlying arrhythmogenic DCR in CPVT (Figure 6F). This is in accord with previous studies that have demonstrated the presence of nNa⁺ in the T-tubule. We further demonstrate using pharmacological modulation of nNa⁺ (i.e. inhibition with TTX and riluzole and augmentation with β-PMTX) as well as NCX activity (SN6) that the unique interplay between Na⁺ and Ca²⁺ handling within this domain is key to the pro-arrhythmic process. More precisely, in CPVT where cardiomyocytes exhibit increased baseline pro-arrhythmic RyR2 Ca²⁺ leak, nNav, and NCX blockade reduced DCR and suppressed arrhythmic activity on cellular level (Figures 1 and 3) and in tissue isolated from CPVT hearts (Figure 5). The results of these functional experiments taken together with colocalization of nNav (but not cardiac isoform of Na⁺ channel) with RyR2 make a very compelling case for the involvement of nNav in the modulation of the arrhythmic DCR. However, this does not preclude the cardiac isoform of the Na⁺ channel from participating in the arrhythmogenic

**Figure 3** Neuronal Na⁺ channel contribute to Na⁺/Ca²⁺ signalling and DCR. (A) Representative examples of the line-scan images of Ca²⁺ sparks recorded in WT ventricular cardiomyocytes. nNa⁺ blockade with Ril in WT myocytes reduced Ca²⁺ spark frequency, while augmentation of the current passed by nNa⁺ with β-PMTX increased Ca²⁺ spark frequency. (B) Ril decreased (n = 199 events from 49 cells isolated from two mice) while β-PMTX increased Ca²⁺ spark frequency (n = 2413 events from 159 cells isolated from six mice) relative to catecholamine-free control (n = 1363 events from 115 cells isolated from eight mice). (C) Ril (n = 27 cells isolated from two mice) decreased while β-PMTX (n = 36 cells isolated from six mice) increased SR Ca²⁺ load normalized spark-mediated SR Ca²⁺ leak relative to catecholamine-free control (n = 29 cells isolated from eight mice, *P < 0.05). Ril and SN6 significantly reversed the effects of β-PMTX on Ca²⁺ spark properties (n = 293 and 753 events from 51 and 65 cells isolated from three mice for Ca²⁺ spark, respectively, and n = 27 and 24 cells isolated from three mice for SR Ca²⁺ load normalized spark-mediated SR Ca²⁺ leak, respectively, #P < 0.05). (D) Representative examples of the line-scan images of Iso-stimulated WT ventricular cardiomyocytes paced at 0.3 Hz. (E) Augmentation of the current passed by nNa⁺ with β-PMTX in WT myocytes (n = 95 cells isolated from seven mice for β-PMTX and n = 38 cells isolated from five mice for Iso alone, *P < 0.05) increased DCR frequency and (F) shortened the latency to the first DCR relative to Iso alone (n = 309 events for PMTX and n = 121 for Iso alone, *P < 0.05). Ril and SN6 significantly reversed the effects of β-PMTX on DCR frequency (n = 30 and 86 cells isolated from three mice, respectively, #P < 0.05, *P < 0.05 vs. Iso alone) and latency (n = 97 and 343 events, respectively).
Neuronal Na\textsuperscript{+} channels and RyR2 colocalize to the same discrete subcellular regions. Confocal micrographs of myocytes from R33Q mutant mice with red labelling for RyR2s (top left) and green labelling (bottom left) for (A) Na\textsubscript{av}1.1, (B) Na\textsubscript{av}1.3, (C) Na\textsubscript{av}1.6, and (D) Na\textsubscript{av}1.5 and the overlay of the two signals (right). Scale bars represent 30 \textmu m. The dashed white box marks the region displayed in high magnification in the insets. Results of colocalization analysis for RyR2 (red) and (E) Na\textsubscript{av}1.1, (F) Na\textsubscript{av}1.3, (G) Na\textsubscript{av}1.6, and (H) Na\textsubscript{av}1.5. Images show colocalizing pixels in white, pixels with only RyR2 signal in red, and only Na\textsubscript{av}1.x signal in green. Inset plots show green (Na\textsubscript{av}1.x) signal intensity plotted against red (RyR2) signal intensity. Pixels with colocalizing signals are marked in yellow, only red signal in red, only green signal in green, and weak signal intensity (background fluorescence) in black.
process. Future studies will need to address the involvement of this particular subpopulation of Na$^+$ channels in aberrant Ca$^{2+}$ handling.

The concept of Na$^+$/Ca$^{2+}$ signalling in the vicinity of RyR2 that our data suggest is supported by previous observations that, reverse mode NCX (Na$^+$-out/Ca$^{2+}$-in) activated by the TTX-sensitive inward Na$^+$ current early during the action potential, can contribute to stimulated Ca$^{2+}$ release.\textsuperscript{17,44–47} These studies demonstrated that, during a normal heart beat, Na$^+$ channels modulate the efficiency of Ca$^{2+}$ release by supplying the necessary Na$^+$ for reverse NCX, which in turn can facilitate RyR2 function (i.e. priming RyR2). The present study extends this concept to elucidate the mechanism of Ca$^{2+}$-dependent arrhythmias by providing the first evidence that the crosstalk between nNav along with NCX and RyR2 is critical to aberrant DCR and triggered activity. Specifically, it has been previously demonstrated that nNav inactivate at relatively high potentials\textsuperscript{48} and carry a substantial residual Na$^+$ current during depolarization.\textsuperscript{24} nNav have also been shown to exhibit substantial activity at negative membrane potentials,\textsuperscript{25} where they are prone to activation and do not completely inactivate.\textsuperscript{48} Thus, nNav activity in both stimulated and quiescent cardiomyocytes could account for the nNav-dependent effects observed herein. Our results further suggest that Na$^+$ influx through the not fully inactivated nNav, (Figure 2D)\textsuperscript{48} in the wake of electrical stimuli (Figures 1D–F and 3D–F) or those that open at rest (Figures 1A–C and 3A–C) may facilitate DCR by enhancing NCX-dependent Ca$^{2+}$ accumulation in the space between the sarcolemma and the RyR2. This microdomain Ca$^{2+}$ accumulation in turn promotes Ca$^{2+}$-induced Ca$^{2+}$ release via RyR2 that are sensitized due to the presence of a pathogenic mutation in CASQ2 (Figures 1, 3, and 6F).\textsuperscript{7–10} While a direct measurement of microdomain Na$^+$ and Ca$^{2+}$ flux in a physiologically relevant setting might be presently not feasible, our functional and structural assays targeting the key components of the Na$^+$/Ca$^{2+}$ signalling provide strong evidence for its role in CPVT-associated arrhythmogenesis. Importantly, they reveal Na$^+$/Ca$^{2+}$ signalling and in particular nNa, as a therapeutic target. Future studies will have to, however, address the specific subtypes of nNa, involved in this pro-arrhythmic process.

**Figure 5** Neuronal Na$^+$ channel blockade desynchronizes DCR in tissue. Representative Ca$^{2+}$ (top) and voltage (bottom) line-scan images of ISO-treated R33Q muscle preparations paced at 1 Hz before (A) and after (B) exposure to riluzole (Ril, 10 μM), along with the corresponding Rhod-2 fluorescence profiles from each myocyte and average di-4-ANBDQBS signal. Dashed white lines outline myocyte borders. Red asterisks indicate tissue-wide extrasystolic Ca$^{2+}$ release (DCR) that resulted in triggered activity (TA). (C) Temporal distribution of DCR in cardiac muscles (n = 264 events for Iso alone, n = 355 for Ril and n = 372 for 100 nM TTX). (D) Median latencies to first DCR (solid bars) and their S.D. (hashed bars) were increased by Ril (n = 355 events) and TTX (n = 372 events) relative to Iso alone (n = 264 events). (E) DCRs and TA frequency per second (Hz) before and after treatment with Ril (10 μM; n = 26 cells from four preparations isolated from three mice, *P < 0.05) or TTX (100 nM; n = 29 cells from three preparations isolated from three mice, *P < 0.05).
An additional unanswered question that our study attempts to tackle is how DCR can occur synchronously in sufficient amount of myocytes to elicit a ventricular extrasystole within the entire myocardium. An extrasystolic trigger must provide a depolarizing current source large enough to overcome the electrical load of connected neighbouring myocytes serving as current sinks. Our data suggest that in addition to facilitating arrhythmogenic DCR on the cellular level, Na+/Ca2+ signalling synchronizes aberrant DCR across the myocardium which results in triggered activity in intact cardiac tissue (Figure 5). The latter effect enables DCR-induced depolarization to overcome the source-sink mismatch, thereby generating ventricular extrasystole.

Interestingly, over 25 years ago Na+ channel blockade has emerged as a promising strategy in management of Ca2+-mediated arrhythmias due to heart failure. Our data suggest that in addition to facilitating arrhythmogenic DCR on the cellular level, Na+/Ca2+ signalling synchronizes aberrant DCR across the myocardium which results in triggered activity in intact cardiac tissue (Figure 5). The latter effect enables DCR-induced depolarization to overcome the source-sink mismatch, thereby generating ventricular extrasystole.

**Figure 6** Effect of neuronal Na+ channel blockade on CPVT. (A) Representative ECG recordings of R33Q mutant mice before (top) and after (bottom) injection (i.p.) of epinephrine (Epi, 1.5 mg/kg) and caffeine (Caff, 120 mg/kg). As illustrated in the ECG trace, Epi + Caff challenge resulted in numerous repetitive ventricular extrasystoles (arrows), bigeminy, and the induction of ventricular tachycardia (VT). (B) ECG of R33Q mice after administration of Ril (10 mg/kg), targeting plasma concentrations comparable to those achieved in experiments conducted in isolated cardiomyocytes, before (top) and after (bottom) Epi + Caff challenge. (C) ECG of SN-6 (40 mg/kg) treated mice 10 min before (top) and after (bottom) Epi + Caff challenge. (D) Arrhythmia and (E) ventricular tachycardia (VT) incidence (%) in R33Q mice exposed to Epi + Caff during various interventions (n = 6–11 mice, *P < 0.05). (F) Na+/Ca2+ signalling; colocalization to the same discrete subcellular region allows crosstalk between neuronal Na+ channels (nNa+) and leaky Ca2+ release channels (RyR2) through the NCX resulting in aberrant DCR that underlie ventricular extrasystoles, which in turn trigger VT.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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**References**

19. Radwan´ski PB, Greer-Short A, Poelzing S. Inhibition of Na
27. Niu C-F, Watanabe Y, Ono K, Iwamoto T, Yamashita K, Satoh H, Urushida T, Hayashi H,
24. Conforti L, Tohse N, Sperelakis N. Tetrodotoxin-sensitive sodium current in rat fetal
16. Liu N, Denegri M, Dun W, Boncompagni S, Lodola F, Protasi F, Napolitano C, Boyden PA,
11. Radwan´ski PB, Belevych AE, Brunello L, Carnes CA, Gyo¨rke S. Store-dependent deacti-
12. ter Keurs HEDJ, Boyden PA. Calcium and arrhythmogenesis.
47. Murphy E, Eisner DA. Regulation of intracellular and mitochondrial sodium in health and
160
11. Radwan´ski PB, Belevych AE, Brunello L, Carnes CA, Gyo¨rke S. Store-dependent deacti-
12. ter Keurs HEDJ, Boyden PA. Calcium and arrhythmogenesis.