Published in final edited form as:

J Mol Cell Cardiol. 2013 August ; 61: 102–110. doi:10.1016/j.yjmcc.2013.03.013.

Caveolin-3 suppresses late sodium current by inhibiting nNOS-dependent S-nitrosylation of SCN5A

Jianding Cheng^{a,b,1}, Carmen R. Valdivia^{a,1}, Ravi Vaidyanathan^a, Ravi C. Balijepalli^a, Michael J. Ackerman^c, and Jonathan C. Makielski^{a,*}

^a Division of Cardiovascular Medicine, Department of Medicine, University of Wisconsin, Madison, WI 53792, USA

^b Department of Forensic Pathology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, China

^c Divisions of Cardiovascular Diseases and Pediatric Cardiology, Departments of Medicine, Pediatrics, and Molecular Pharmacology & Experimental Therapeutics, Mayo Clinic, Rochester, MN 55905, USA

Keywords

long QT syndrome; LQT9; caveolin-3; nitric oxide synthase; sodium current; sudden infant death syndrome

1. Introduction

Caveolae, a subset of membrane structures called lipid rafts, are 50-100 nm microdomains with an invaginated flask-shaped structure, abundantly present in most cell types including muscle cells [1]. Caveolae have been shown to play an important role in vesicular trafficking, protein targeting, second messenger signaling, and cholesterol homeostasis [1;2]. Several cardiac ion channels such as the pacemaker channels, L-type Ca^{2+} channels, K^{+} channels, cardiac Na^{+} channels, the $\text{Na}^{+}/\text{K}^{2+}$ ATPase, and the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger have been localized to caveolae and this subcellular localization to caveolae may allow the integration of these channels into specific macromolecular signaling complexes that provides for their precise regulation [2].

© 2013 Elsevier Ltd. All rights reserved.

* Corresponding author. Jonathan C. Makielski Department of Medicine, University of Wisconsin 600 Highland Ave H6/349, Madison, WI 53792, USA phone: +1 (608) 263-9648 fax: +1 (608) 263-0405 jcm@medicine.wisc.edu.

¹ These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Present address for CRV is the Center for Arrhythmia Research, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA

Supplementary material

Supplement material is available at *Journal of Molecular and Cellular Cardiology* online.

Conflict of interest: Dr. Ackerman is a consultant for PGxHealth and chairs their FAMILION Medical/Scientific Advisory Board (approved by Mayo Clinic's Medical-Industry Relations Office and Conflict of Interests Review Board). In addition, "cardiac channel gene screen" and "know-how relating to long QT genetic testing" license agreements, resulting in consideration and royalty payments, were established between Genaissance Pharmaceuticals (now PGxHealth) and Mayo Medical Ventures (now Mayo Clinic Health Solutions) in 2004.

Caveolins are the major proteins of caveolae and the 6 subtypes of caveolins (caveolin-1 α , -1 β , -2 α , -2 β , -2 γ , and caveolin-3) are encoded by separate genes (*CAV1*, *CAV2*, and *CAV3*) [3]. *CAV3*-encoded Caveolin-3 (Cav3) specifically expressed in the myocytes has been identified as critical for the formation of caveolae [2;4]. We previously implicated *CAV3* mutations for both type 9 long QT syndrome (LQT9) [5] and sudden infant death syndrome (SIDS) [6] by observing increased late sodium current (I_{Na}) when these mutations were coexpressed with *SCN5A*-encoded cardiac sodium channels (SCN5A, also termed Nav1.5). However, the mechanism by which Cav3 regulates caveolar SCN5A channels and how mutant Cav3 affects its function was unclear.

Neural nitric oxide synthase (nNOS)—mediates nitric oxide (NO) synthesis and excessive NO release in cardiomyocytes was shown to increase late I_{Na} [7]. Based on the identification of a macromolecular complex involving SCN5A- α 1-syntrophin (*SNTA1*)- nNOS-PMCA4b (cardiac isoform of the plasma membrane Ca-ATPase) in mouse heart and in transiently transfected HEK-293 cells [8], we implicated *SNTA1* as a novel LQTS [8] and SIDS [9] susceptibility gene based on the observations that *SNTA1* mutations disrupted binding with PMCA4b, releasing inhibition of nNOS, and accentuated both peak and late I_{Na} via S-nitrosylation of SCN5A. These findings strongly suggested the important role of nNOS complex in modulating late I_{Na} .

A number of caveolin-associated signaling molecules such as G-protein-coupled receptors, heterotrimeric G-proteins, steroid hormone receptors, receptor tyrosine kinases, nonreceptor tyrosine kinases, protein kinase A and C, phosphatidylinositol-3 –kinase/protein kinase B, MAP kinase (p42/44 MAPK), and NOS are concentrated within caveolae by caveolins and are shown to interact directly with caveolins [2]. The direct binding of Cav3 to nNOS suppresses the catalytic activity of nNOS [10]. The transgenic mice expressing mutant Cav3 (P104L) show deficiency of Cav3 in the sarcolemma and increased nNOS activity in skeletal muscle [11]. Based on these reports, we hypothesized that Cav3 may regulate cardiac I_{Na} through the nNOS complex and aimed to address the mechanism by which the LQT9-causative mutations, Cav3-F97C, increased late I_{Na} in a heterologous expression system as well as in native cardiomyocytes.

2. Methods

Please see the online supplement for additional details of methods

2.1. Plasmid construction

The cDNA for SCN5A (hNav1.5, Genbank accession no. AB158469), SNTA1 (Genbank accession no. NM_003098), nNOS (GenBank accession no. NM_052799), and PMCA4b (GenBank accession no. AY560895), and Cav3 (GenBank accession no. NM_033337) were obtained and prepared as previously described [5;8]. Wild type (WT) human Cav3 was subcloned into pcDNA3 plasmid vector (Invitrogen, Carlsbad, California). The Cav3-F97C mutation was incorporated into Cav3-WT using a site-directed mutagenesis method as previously reported [5]. The cDNA of human SNTA1 was subcloned into pIRES2EGFP plasmid vector (Clontech Laboratories, Palo Alto, California). For transduction into primary cardiac myocytes, we generated the bicistronic adenovirus shuttle vector of Cav3 and GFP. Cav3 cDNA and the mutant Cav3-F97C were subcloned into an entry vector of pENTR1A-IRES2EGFP, which was created by incorporation of the multicloning sites, an internal ribosome entry site, and EGFP coding region from pIRES2EGFP into pENTR1A (Invitrogen). A recombination reaction between pAd/CMV/V5-DEST (Invitrogen) generated pAdCav3-IRES2EGFP. All clones were sequenced to confirm integrity.

2.2. Chemical reagent and antibodies

The NOS inhibitor, NG-monomethyl-L-arginine (L-NMMA), was obtained from Cayman chemical company (Ann Arbor, Michigan). The L-NMMA was diluted in PBS buffer (pH 7.2) 10 min before use. Rabbit anti-cardiac -Nav1.5 antibody (Millipore), rabbit anti-Syntrophin antibody (Sigma-Aldrich, St Louis, MO), mouse anti-nNOS antibody (Invitrogen, Carlsbad, CA), mouse anti-Cav3 antibody (BD Biosciences), goat HRP-conjugated anti-mouse IgG antibody (Charlottesville, VA), and goat HRP-conjugated anti-rabbit IgG (H+L) antibody (Biorad laboratories Hercules, CA) were obtained commercially.

2.3. Transfection into HEK-293 cells

The Cav3-WT or -F97C or empty vector was transiently cotransfected with expression vectors containing SCN5A, nNOS, and SNTA1 at a ratio of 1:1:1:0.2 respectively into HEK-293 cells with FuGENE6 reagent (Roche Diagnostics, Indianapolis, Indiana) according to manufacturer's instructions. Two days after transfection, GFP-positive HEK-293 cells were selected for functional study.

2.4. Adult rat ventricular myocytes isolation

Animal handling practices used in this study were approved by animal use committee at the University of Wisconsin and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Single ventricular myocytes were digested enzymatically from adult male Sprague-Dawley that were anesthetized with the inhalant anesthetic isoflurane administered by placing a soaked gauze pad into an airtight container with the animal which was then sacrificed by removal of the heart while under deep and continuous anesthesia. The heart was mounted on a Langendorff apparatus then perfused retrograde for 10 min at 37°C with oxygenated 1 mmol/L Ca^{2+} Ringers solution, followed by a 5 mmol/L Taurine of Ca^{2+} free Ringers solution for 5 min, and a 10 min perfusion with continuous recirculation of the same solution containing 0.5 mg/ml collagenase (type II, Worthington), and 0.35 mg/ml hyaluronidase (Type I-S, Sigma). Ca^{2+} was added back to bring Ca^{2+} concentrations to 0.5 mmol/L. After isolation the myocytes were maintained in 1 mmol/L Ca^{2+} Ringers solution.

2.5. Culture and adenovirus infection of adult rat ventricular myocytes

Isolated myocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 50 U/ml penicillin and 0.03 mmol/L streptomycin and (in mmol/L) 1 CaCl_2 , 5 taurine, 5 L-carnitine, and 5 creatine. The cells were plated on laminin-coated coverslips and incubated at 37°C, 5% CO_2 -95% room air for 2 hours. Then the culture medium was replaced with serum-free DMEM. Then the cells were transduced with adenovirus of either Cav3-WT or Cav3-F97C and incubated for 24 hours and the serum-free DMEM was changed and replenished daily. After 2-3 days beating, GFP-positive cardiomyocytes were selected for investigation.

2.6. Electrophysiological measurements

Macroscopic voltage-gated I_{Na} was measured with the standard whole-cell patch clamp method at 21°C to 23°C in the HEK-293 cells or cardiomyocytes as described previously [5;8;12]. Microelectrodes were manufactured from borosilicate glass using a puller (P-87, Sutter Instrument Co, Novato, California) and were heat polished with a microforge (MF-83, Narishige, Tokyo, Japan) and had resistances from 1.0 to 2.0 M Ω . Voltage clamp data were generated with pClamp software 10.2 and an Axopatch 200B amplifier (Axon Instruments, Foster City, California) with series-resistance compensation. Membrane current data were digitalized at 100 kHz, low-pass filtered at 5 kHz, and then normalized to membrane capacitance. Cells were continuously perfused with bath (extracellular) solution containing

140 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2 , 0.75 mM MgCl_2 , and 5 mM HEPES (pH 7.4 set with NaOH). The pipette (intracellular) solution contained 120 mM CsF, 20 mM CsCl_2 , 5 mM EGTA, and 5 mM HEPES (pH 7.4 set with CsOH). Late I_{Na} was normalized to peak I_{Na} after leak subtraction (in HEK293 cells) or after STX subtraction (in cardiomyocytes). Late I_{Na} was measured as the mean current between 600 and 700 ms after the initiation of the depolarization from -140 mV to -20 mV for 750 ms (see protocol inset) after passive leak subtraction. Additional details are in the online supplement.

2.7. Immunoprecipitation of SCN5A-nNOS complex from rat heart homogenates

Adult rat heart was dissected and homogenized by 10 strikes with a polytron probe in ice-cold RIPA buffer containing 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1% deoxycholate, and complete protease inhibitor tablet (Roche). The homogenate was transferred to a 1.5 ml tube and rotated for 1 hour at 4 °C, then centrifuged for 10 min at 10,000 RPM at 4 °C and the supernatant was transferred to a new tube for protein quantification and immunoprecipitation. Homogenate (1.0 mg/reaction) was mixed with anti-nNOS antibody (5 µg) and immunoprecipitated using the protein G-Dynabeads (GE Healthcare Life Sciences) at 4 °C overnight. The immunoprecipitated sample was analyzed by Western blotting by probing with anti-Nav1.5 (Millipore, 1:200), anti-nNOS (Invitrogen, 1:1000), anti-SNTA (Sigma, 1:250), and anti-Cav3 (BD transduction laboratories, 1:500) antibodies, followed by incubation with secondary antibody to (1:10,000).

2.8. Immunocytochemistry

Isolated cardiomyocytes from adult rat hearts were cultured on laminin-coated coverslips, immediately after plating, the cells were transduced with the adenoviral constructs carrying the Cav3-WT or Cav3-F97C for 48 hours. Then cells were fixed with 4% paraformaldehyde in PBS (10 min), and permeabilized with 0.1% Triton X-100 in PBS (10 min). After blocking with 5% normal goat serum made in 0.1% Triton X-100 in PBS, the cells were incubated with specific primary antibodies (rabbit anti-Nav1.5 (Millipore, 1:200 dilution) and mouse anti-Cav3 (BD transduction laboratories, 1:500 dilution)) overnight at 4 °C according to manufacturer specifications. After washing with PBS-T (PBS with 0.1% Tween 20) 3 times five min each, coverslips were incubated in secondary antibodies, anti-rabbit Alexa 660 and anti-mouse Alexa 568 (Invitrogen) for 60 min. Cells were then washed in PBS-T three times 5 min each and mounted onto glass slides using ProLong Gold anti-fade mounting kit (Invitrogen). Fluorescence was analyzed, and images were acquired on a Nikon A1 confocal microscope. High resolution images were converted to 8-bit TIFF files. Figures were made using the Adobe suite of programs. Co-localization was determined by measuring Pearson's correlation coefficient using ImageJ software.

2.9. NADPH Diaphorase activity assay

The transfected HEK-293 cells plated on the coverslips were fixed with 4% paraformaldehyde in PBS buffer for 10 min at room temperature. The cells were then permeabilized with 0.2% Triton X-100 in PBS buffer for 20 min at 37 °C after a brief washing with PBS buffer. The cytochemical staining was performed for 1 hour in dark at 37 °C in 0.2% Triton X-100, 0.2 mmol/L NADPH (Sigma-Aldrich) and 0.2 mmol/L nitro blue tetrazolium (Sigma-Aldrich). The reaction was quenched by rinsing with PBS buffer.

2.10. S-Nitrosylated SCN5A protein detection

The S-nitrosylated SCN5A protein was detected using a detection assay kit (Cayman, Ann Arbor MI). In brief, the total proteins in HEK-293 cells transiently expressing Cav3-SCN5A-SNTA1-nNOS were isolated, the free thiols were blocked, then S-NO bonds were cleaved. The proteins were labeled with biotin by biotinylation of the newly formed SH

groups. The labeled proteins were then analyzed by SDS/PAGE (4–15% gradient gels; Bio-Rad) and transferred to PVDF membranes (Invitrogen) for Western blot.

2.11. Statistical analysis

Data points are reported as the mean value and the standard error of the mean (SEM). Determinations of statistical significance were performed using a Student *t*-test for comparisons of two means or using analysis of variance (ANOVA) for comparisons of multiple groups. Statistical significance was determined by a value of $P < 0.05$.

3. Results

3.1. Increased late I_{Na} caused by Cav3-F97C in HEK-293 cells was reversed by NOS inhibitor L-NMMA

Electrophysiological characterization of Cav3-F97C was first performed in HEK-293 cells with transiently expressed SCN5A and with either Cav3-WT, or Cav3-F97C, or pcDNA3 empty vector. There were no significant differences in peak I_{Na} , activation, inactivation (Table 1), or recovery from inactivation (data not shown) among the three groups. Late I_{Na} , as a percentage of peak I_{Na} , showed a significant 2-fold increase for Cav3-F97C compared with Cav3-WT while the empty vector showed no effect (Table 1 and Figure 1A-D).

To address the mechanism by which Cav3-F97C increased late I_{Na} , 100 μ M L-NMMA was introduced into the HEK-293 cell culture medium 12 hours prior to testing. The increased late I_{Na} observed with Cav3-F97C was reversed by L-NMMA without alteration of kinetic parameters (Table 1 and Figure 1A-D). These results suggested that Cav3-F97C increased late I_{Na} through a NOS-associated mechanism in HEK-293 cells.

3.2. Endogenous nNOS identified in HEK-293 cells

Based on previous studies in which interaction of nNOS with SCN5A was identified [5;8;12], we hypothesized that there might be endogenous nNOS activity in HEK-293 cells which might underlie the increased late I_{Na} for Cav3-F97C. We performed RT-PCR assay using nNOS-specific primers (forward: 5' acgtcttcctcatgtctaagttca; reverse: 5' ctgtgacaactcccgcctaca) to detect the nNOS mRNA in the cell lysates. HEK-293 cells transfected with nNOS showed a strong band while non-transfected HEK-293 cells showed faint but definite band, whereas no band was detected in Hela cells (Figure 2A). Based on these findings, we measured nNOS activity using a NADPH diaphorase (NDP) activity assay in HEK-293 cells expressing either Cav3-WT, or Cav3-F97C, or empty vector, and also in cells transfected with nNOS as a positive control. The positive control (Figure 2E) showed strong NDP-positive blue staining while the other three groups showed weaker staining (Figure 2B-D). Compared to Cav3-WT, both Cav3-F97C and empty vector showed significantly larger color densities (Figure 2F). When nitrites were measured directly the mutant showed significantly increased nitrites compared to WT, with vector only being intermediate (Supplemental Figure 1). These data suggest that traces of endogenous nNOS in HEK-293 cells may have been capable of nitrosylating SCN5A to produce late I_{Na} .

3.3. Cav3-F97C increased peak and late I_{Na} by nNOS-dependent S-nitrosylation of SCN5A through the SCN5A-SNTA1-nNOS-Cav3 complex

SCN5A, SNTA1, nNOS, and either the Cav3-WT, or Cav3-F97C, or empty vector were transiently expressed in HEK-293 cells. Compared with Cav3-WT, both Cav3-F97C and empty vector had significantly larger peak I_{Na} amplitudes and late I_{Na} by 3.3 and 3.5 fold, respectively (Table 1 and Figure 3A-D). Note that we report late I_{Na} as a percentage of peak, so the increase in late I_{Na} was out of proportion to the increase in peak I_{Na} . The inactivation for both Cav3-F97C and empty vector showed a subtle but statistically significant

depolarizing shift (*Table 1*). When treated with L-NMMA, the increase in late I_{Na} for Cav3-F97C and empty vector was abolished and the corresponding increase in peak I_{Na} was also reversed (*Table 1 and Figure 3A-D*). To demonstrate interactions between SCN5A with SNTA1, nNOS and Cav3, co-IP experiments in rat heart homogenates were performed using nNOS antibody to immunoprecipitate the complex and specific antibodies to detect SCN5A, SNTA1, and Cav3 from the complex (*Figure 3E*). When the Cav3.1 antibody was used to recognize Cav3, only half of the Cav3 was associated with the SCN5A complex as the other half of Cav3 was released in the 1st supernatant suggesting that only part of Cav3 associated with this complex (or pool of channels). We also performed immunoprecipitation with FLAG antibody using a FLAG-tagged SCN5A co-expressed with nNOS, SNTA1, and Cav3 (WT, F97C, or vector) in HEK cells and found that the complex remained intact with the mutant Cav3 (*Supplemental Figure 2*). Taken together, these results demonstrate that nNOS, SCN5A and SNTA1 form a complex with Cav3 in the heart.

To determine if direct S-nitrosylation of SCN5A was involved as the mechanism for increased peak and late I_{Na} by Cav3-F97C, we measured S-nitrosylated SCN5A protein using a biotin switch assay in HEK-293 cells transiently expressing SCN5A, SNTA1, nNOS, and either Cav3-WT or Cav3-F97C. S-nitrosylation of SCN5A was clearly increased with Cav3-F97C, while S-nitrosylation was less for Cav3-WT, Cav3-WT treated with L-NMMA, and Cav3-F97C treated with L-NMMA (*Figure 3F*). These results support the hypothesis that the nNOS-dependent S-nitrosylation of SCN5A is a mechanism for the Cav3-F97C induced gain of function for SCN5A.

3.4. Cell membrane expression of Cav3-F97C in both HEK-293 cells and adult rat cardiomyocytes

We investigated cell surface expression of SCN5A and Cav3-WT and Cav3-F97C using immunofluorescent staining. In HEK-293 cells transiently expressing SCN5A along with either Cav3-WT or Cav3-F97C, the mutant showed robust membrane expression levels similar to those seen with WT (data not shown). In adult rat cardiomyocytes overexpressing either Cav3-WT or Cav3-F97C, no apparent differences in SCN5A, Cav3-WT or Cav-F97C cell surface expression were observed (*Figure 4*). These results suggest that Cav3-F97C trafficks to the surface membrane normally. Quantification of the co-localization showed significant overlap between the fluorescent staining patterns (*Figure 4 plot inserts*) and localization of SCN5A and Cav3 at the subcellular areas (*Figure 4 arrows*). The combined results indicate that Cav3-F97C remains associated with SCN5A so that dissociation of Cav3 is not the likely mechanism for the apparent loss of nNOS inhibition. The detailed mechanism for this loss remains unknown.

3.5. Electrophysiology of adult rat cardiomyocytes overexpressing Cav3-F97C

To characterize Cav3-F97C in a native myocardial cell environment, we studied the biophysical properties of adult rat cardiomyocytes transduced for 2 days with adenovirus containing either Cav3-WT or Cav3-F97C. Cav3-F97C caused a significant increase in STX-sensitive late I_{Na} compared with Cav3-WT and the accentuation was reversed by L-NMMA (*Figure 5A-D*). Action potentials recorded in transduced cardiomyocytes showed significant prolongation in 90% of action potential duration (APD₉₀) for Cav3-F97C compared to Cav3-WT, and this prolongation was abolished by L-NMMA (*Figure 5E-F*). These results suggest that the pathogenic biophysical phenotype for LQTS9-causing Cav3-F97C is also NOS dependent in native cardiomyocytes.

4. Discussion

4.1. Cav3 identified as a regulator of cardiac late I_{Na} through suppression of nitrosylation

In both heterologous HEK-293 cells in which we established SCN5A-SNTA1-nNOS-Cav3 macromolecular complex and in native adult rat ventricular myocytes, we demonstrated a role for Cav3 as an endogenous negative regulator of late I_{Na} , and implicated a pathogenic molecular mechanism for Cav3-mediated LQT9. In the proposed mechanism, Cav3-F97C caused a loss of the normal inhibitory effect of Cav3-WT on nNOS, accentuated local NO, caused increased late I_{Na} via direct S-nitrosylation of SCN5A, and significantly prolonged the action potential duration which underlies the LQTS clinical electrophysiological phenotype.

SCN5A was shown previously to be colocalized with Cav3 at the sarcolemma in rat ventricular myocytes [13] and we confirmed the colocalization of SCN5A and Cav3 at the plasma membrane of human cardiomyocytes [5]. Cav3 has been shown to interact with nNOS and negatively regulate its activity [10]. In a study of LQTS- and SIDS-associated SNTA1 mutations, we provided evidence that SNTA1 linked nNOS to SCN5A to create a postulated NO micro-environment for regulating SCN5A [8;9]. The present study extends these observations to include Cav3 in a macromolecular complex SCN5A-SNTA1-nNOS-Cav3 that modifies late I_{Na} . Together with previously identified SCN5A-SNTA1-nNOS-PMCA4b complex [8;9], the complex of SCN5A-SNTA1-nNOS-Cav3 implicated in this study supports the idea that the adjustment of local concentration of NO through nNOS complex plays a role in regulating cardiac late I_{Na} . For the SCN5A-SNTA1-nNOS-PMCA4b complex it was shown that the SNTA1 mutation disrupted the nNOS suppressor PMCA4b from the complex [8;9]; in contrast, Cav3-F97C remains in the complex, but apparently has lost the ability to suppress nNOS. The nNOS inhibitory residues on CAV3 are 65 to 84 and 109 to 130 [10], flanking the F97C mutation suggesting perhaps an allosteric effect. The detailed mechanism for the loss of nNOS suppressing function remains to be determined. Notably, the recently reported influences of common variation involving the nNOS adaptor protein (NOS1AP, a nNOS regulator) on QT interval duration [14-17] and observed association of NOS1AP genetic variants with sudden cardiac death [18] SIDS [19], and drug-associated QT prolongation and arrhythmia [20] have also confirmed the key role of nNOS complex in LQTS-related disorders. Thus, the association of nNOS complex-related proteins with both SCN5A and other cardiac ion channels deserves further study.

4.2. An ongoing story: Cav3 mutations in caveolar cardiac ion channels and arrhythmias

The intricate signaling pathways in caveolar SCN5A complexes and their association with arrhythmia are not well understood. β -adrenergic receptor regulation was reported to increase current densities of caveolar SCN5A through both protein kinase A (PKA)-dependent phosphorylation of sodium channels [21] and direct $G\alpha_s$ interaction with Cav3 which promotes the presentation of SCN5A containing caveolae to the surface membrane [22]. Whether LQT9-associated Cav3 mutations affecting these two signaling pathways or other signaling macromolecular complexes linked with Cav3 also contribute to LQTS phenotype remains unknown.

Moreover, the localization of Cav3 to various cardiac ion channels such as pacemaker channels, L-type Ca^{2+} channels, K^+ channels, and Na^+/Ca^{2+} exchanger [2] also provides the possibility that Cav3 mutations may affect the function of these ion channels through specific signaling complexes [23-25]. We identified that hyper polarization-activated cyclic nucleotide-gated channel 4 (HCN4) associates with Cav3 to form a HCN4 macromolecular complex and that the disruption of caveolae using P104L alters HCN4 function and could cause a reduction of cardiac pacemaker activity [26]. Garg et al. demonstrated that Cav3

negatively regulates ATP-sensitive potassium channel (Kir 6.2/SUR2A) function in HEK-293T cells [27]. We also found that Cav3 mutants can cause loss of function of Kir2.1 channels in HEK-293 cells (data not shown) and that Cav3 has a clear inhibitory effect on the function of T type Ca^{2+} channel $\text{Ca}_v3.2$ [28]. Overall, the mechanism by which Cav3 mutants in caveolar cardiac ion channels contribute to both inherited arrhythmia syndromes and acquired arrhythmias in conditions are only beginning to be addressed.

4.3. A wider role in cardiac pathophysiology for Cav3 modulation of SCN5A through nNOS

The cardiomyocyte-specific overexpression of Cav3 enhanced the formation of caveolae and augmented the potential to protect hearts exposed to ischemia/reperfusion injury via phosphoinositide 3-kinase pathway in vivo [29]. Delayed cardiac protection induced by anesthetic preconditioning is also Cav3-dependent with increased amounts of Cav3 protein, increased incorporation of Cav3 and glucose transporter-4 to caveolae, and augmented delayed protection in the myocardium with ischemia-reperfusion injury [30]. Sodium overload through late I_{Na} [31] may cause calcium loading through decreased or reversed Na-Ca exchange. Furthermore, the Ca^{2+} -Calmodulin complex is known to activate nNOS. In view of the present findings that late I_{Na} was suppressed by Cav3 through the nNOS complex, it is interesting to speculate that the protective role of Cav3 may be to suppress NOS-mediated late I_{Na} associated cardiac disorders such as arrhythmias and heart failure.

4.4. Summary, limitations and implications

The biophysical data as well as biochemical findings presented here identified Cav3 as an important negative regulator for cardiac late I_{Na} via nNOS-dependent direct S-nitrosylation of SCN5A, and elucidated a possible molecular mechanism by which Cav3-F97C increased late I_{Na} to yield its LQTS biophysical and cellular phenotype. Due to the complexity of signaling network in caveolar cardiac ion channels, we cannot exclude the possibilities that Cav3-F97C may disturb other signaling molecular complexes associated with Cav3 to have additional effects on SCN5A, or alter biophysical properties of other caveolar cardiac ion channels through specific signaling pathways to affect cellular electrophysiology. In fact, as mentioned above, we expect effects of Cav3 on cellular cardiac electrophysiology beyond the effects on SCN5A.

We demonstrated the effects of Cav3 on I_{Na} in a “minimalist” heterologous expression system in which we over-expressed just those proteins that we hypothesized to be the key members of the complex for this effect. Although it is important and reassuring that the main findings were reproduced in native myocytes, the SCN5A complex in native tissue has many more components such as β subunits which we did not co-express in the heterologous cell model, and these and other proteins found in native heart could have key modulatory roles. Conversely, heterologous cells have many endogenous proteins (as we demonstrated in this study for nNOS) so we cannot claim that we have identified all of the key members of the complex necessary for this phenotype. L-NMMA is non-specific inhibitor and we have not performed experiments with specific NOS inhibitors. Other questions regarding the complicated association and interaction of each component in the SCN5A-SNTA1-nNOS-Cav3 macromolecular complex and the detailed mechanism by which Cav3-F97C causes LQT9 remain unknown. Also, for example does Cav3 connect directly nNOS to SCN5A in the caveolar sodium channel complex? Does the Cav3-F97C mutation cause a loss of inhibitory function on nNOS by dissociation of direct binding of Cav3-F97C to nNOS? How does nitrosylation of SCN5A cause increased late I_{Na} ?

Our findings do add to the growing understanding of caveolar cardiac ion channel macromolecular complexes and their role in inherited cardiac arrhythmias involving mutations of Cav3. The modulatory mechanisms of late I_{Na} may yield new therapeutic

strategies to correct late I_{Na} , rescue normal repolarization, improve Ca^{2+} handling and contractility, and prevent arrhythmia of the failing or ischemic heart [32].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Amanda L. Vega, Evi Lim, Wei Guo (University of Wisconsin) for technical assistance in the isolation of cardiomyocytes and plasmid construction.

Funding

This work was supported by the University of Wisconsin Cellular and Molecular Arrhythmia Research Program (J.C.M.), grant HL71092 (J.C.M.) from the National Institutes of Health, USA, and grants 30973367 & 81172901 (J.C.) from the National Natural Science Foundation of China.

Reference List

1. Westermann M, Steiniger F, Richter W. Belt-like localisation of caveolin in deep caveolae and its re-distribution after cholesterol depletion. *Histochem Cell Biol.* 2005; 123:613–620. [PubMed: 15889267]
2. Balijepalli RC, Kamp TJ. Caveolae, ion channels and cardiac arrhythmias. *Prog Biophys Mol Biol.* 2008; 98:149–160. [PubMed: 19351512]
3. Parton RG, Simons K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol.* 2007; 8:185–194. [PubMed: 17318224]
4. Li S, Galbati F, Volonte D, Sargiacomo M, Engelman JA, Das K, Scherer PE, Lisanti MP. Mutational analysis of caveolin-induced vesicle formation. Expression of caveolin-1 recruits caveolin-2 to caveolae membranes. *FEBS Lett.* 1998; 434:127–134. [PubMed: 9738464]
5. Vatta M, Ackerman MJ, Ye B, Makielski JC, Ughanze EE, Taylor EW, Tester DJ, Balijepalli RC, Foell JD, Li Z, Kamp TJ, Towbin JA. Mutant caveolin-3 induces persistent late sodium current and is associated with long-QT syndrome. *Circulation.* 2006; 114:2104–2112. [PubMed: 17060380]
6. Cronk LB, Ye B, Kaku T, Tester DJ, Vatta M, Makielski JC, Ackerman MJ. Novel mechanism for sudden infant death syndrome: Persistent late sodium current secondary to mutations in caveolin-3. *Heart Rhythm.* 2007; 4:161–166. [PubMed: 17275750]
7. Ahern GP, Hsu SF, Klyachko VA, Jackson MB. Induction of persistent sodium current by exogenous and endogenous nitric oxide. *J Biol Chem.* 2000; 275:28810–28815. [PubMed: 10833522]
8. Ueda K, Valdivia C, Medeiros-Domingo A, Tester DJ, Vatta M, Farrugia G, Ackerman MJ, Makielski JC. Syntrophin mutation associated with long QT syndrome through activation of the nNOS-SCN5A macromolecular complex. *Proc Natl Acad Sci U S A.* 2008; 105:9355–9360. [PubMed: 18591664]
9. Cheng J, Van Norstrand DW, Medeiros-Domingo A, Valdivia C, Tan BH, Ye B, Kroboth S, Vatta M, Tester DJ, January CT, Makielski JC, Ackerman MJ. Alpha1-syntrophin mutations identified in sudden infant death syndrome cause an increase in late cardiac sodium current. *Circ Arrhythm Electrophysiol.* 2009; 2:667–676. [PubMed: 20009079]
10. Venema VJ, Ju H, Zou R, Venema RC. Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. *J Biol Chem.* 1997; 272:28187–28190. [PubMed: 9353265]
11. Sunada Y, Ohi H, Hase A, Ohi H, Hosono T, Arata S, Higuchi S, Matsumura K, Shimizu T. Transgenic mice expressing mutant caveolin-3 show severe myopathy associated with increased nNOS activity. *Hum Mol Genet.* 2001; 10:173–178. [PubMed: 11159934]
12. Tan BH, Pundi KN, Van Norstrand DW, Valdivia CR, Tester DJ, Medeiros-Domingo A, Makielski JC, Ackerman MJ. Sudden infant death syndrome-associated mutations in the sodium channel beta subunits. *Heart Rhythm.* 2010; 7:771–778. [PubMed: 20226894]

13. Yarbrough TL, Lu T, Lee HC, Shibata EF. Localization of cardiac sodium channels in caveolin-rich membrane domains: regulation of sodium current amplitude. *Circ Res.* 2002; 90:443–449. [PubMed: 11884374]
14. Arking DE, Pfeufer A, Post W, Kao WH, Newton-Cheh C, Ikeda M, West K, Kashuk C, Akyol M, Perz S, Jalilzadeh S, Illig T, Gieger C, Guo CY, Larson MG, Wichmann HE, Marban E, O'Donnell CJ, Hirschhorn JN, Kaab S, Spooner PM, Meitinger T, Chakravarti A. A common genetic variant in the NOS1 regulator NOS1AP modulates cardiac repolarization. *Nat Genet.* 2006; 38:644–651. [PubMed: 16648850]
15. Aarnoudse AJ, Newton-Cheh C, de Bakker PI, Straus SM, Kors JA, Hofman A, Uitterlinden AG, Witteman JC, Stricker BH. Common NOS1AP variants are associated with a prolonged QTc interval in the Rotterdam Study. *Circulation.* 2007; 116:10–16. [PubMed: 17576865]
16. Lehtinen AB, Newton-Cheh C, Ziegler JT, Langefeld CD, Freedman BI, Daniel KR, Herrington DM, Bowden DW. Association of NOS1AP genetic variants with QT interval duration in families from the Diabetes Heart Study. *Diabetes.* 2008; 57:1108–1114. [PubMed: 18235038]
17. Chang KC, Barth AS, Sasano T, Kizana E, Kashiwakura Y, Zhang Y, Foster DB, Marban E. CAPON modulates cardiac repolarization via neuronal nitric oxide synthase signaling in the heart. *Proc Natl Acad Sci U S A.* 2008; 105:4477–4482. [PubMed: 18337493]
18. Kao WH, Arking DE, Post W, Rea TD, Sotoodehnia N, Prineas RJ, Bishe B, Doan BQ, Boerwinkle E, Psaty BM, Tomaselli GF, Coresh J, Siscovick DS, Marban E, Spooner PM, Burke GL, Chakravarti A. Genetic variations in nitric oxide synthase 1 adaptor protein are associated with sudden cardiac death in US white community-based populations. *Circulation.* 2009; 119:940–951. [PubMed: 19204306]
19. Osawa M, Kimura R, Hasegawa I, Mukasa N, Satoh F. SNP association and sequence analysis of the NOS1AP gene in SIDS. *Leg Med (Tokyo).* 2009; 11(Suppl 1):S307–S308. [PubMed: 19289301]
20. Jamshidi Y, Nolte IM, Dalageorgou C, Zheng D, Johnson T, Bastiaenen R, Ruddy S, Talbott D, Norris KJ, Snieder H, George AL, Marshall V, Shakir S, Kannankeril PJ, Munroe PB, Camm AJ, Jeffery S, Roden DM, Behr ER. Common Variation in the NOS1AP Gene Is Associated With Drug-Induced QT Prolongation and Ventricular Arrhythmia. *J Am Coll Cardiol.* 2012; 60:841–850. [PubMed: 22682551]
21. Frohnwieser B, Chen LQ, Schreibmayer W, Kallen RG. Modulation of the human cardiac sodium channel alpha-subunit by cAMP-dependent protein kinase and the responsible sequence domain. *J Physiol (Lond).* 1997; 498(Pt 2):309–318. [PubMed: 9032680]
22. Palygin OA, Pettus JM, Shibata EF. Regulation of caveolar cardiac sodium current by a single Gsalpha histidine residue. *Am J Physiol Heart Circ Physiol.* 2008; 294:H1693–H1699. [PubMed: 18281377]
23. Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Berretta RM, Rizzo V, Molkentin JD, Houser SR. A caveolae-targeted L-type Ca(2)+ channel antagonist inhibits hypertrophic signaling without reducing cardiac contractility. *Circ Res.* 2012; 110:669–674. [PubMed: 22302787]
24. Pflieger C, Ebeling G, Blasche R, Patton M, Patel HH, Kasper M, Barth K. Detection of caveolin-3/caveolin-1/P2X7R complexes in mice atrial cardiomyocytes in vivo and in vitro. *Histochem Cell Biol.* 2012; 138:231–241. [PubMed: 22585038]
25. Gazzzerro E, Bonetto A, Minetti C. Caveolinopathies: translational implications of caveolin-3 in skeletal and cardiac muscle disorders. *Handb Clin Neurol.* 2011; 101:135–142. [PubMed: 21496630]
26. Ye B, Balijepalli RC, Foell JD, Kroboth S, Ye Q, Luo YH, Shi NQ. Caveolin-3 associates with and affects the function of hyperpolarization-activated cyclic nucleotide-gated channel 4. *Biochemistry.* 2008; 47:12312–12318. [PubMed: 19238754]
27. Garg V, Sun W, Hu K. Caveolin-3 negatively regulates recombinant cardiac K(ATP) channels. *Biochem Biophys Res Commun.* 2009; 385:472–477. [PubMed: 19481058]
28. Markandeya YS, Fahey JM, Pluteanu F, Cribbs LL, Balijepalli RC. Caveolin-3 regulates protein kinase A modulation of the Ca(V)3.2 (alpha1H) T-type Ca2+ channels. *J Biol Chem.* 2011; 286:2433–2444. [PubMed: 21084288]

29. Tsutsumi YM, Horikawa YT, Jennings MM, Kidd MW, Niesman IR, Yokoyama U, Head BP, Hagiwara Y, Ishikawa Y, Miyanohara A, Patel PM, Insel PA, Patel HH, Roth DM. Cardiac-specific overexpression of caveolin-3 induces endogenous cardiac protection by mimicking ischemic preconditioning. *Circulation*. 2008; 118:1979–1988. [PubMed: 18936328]
30. Tsutsumi YM, Kawaraguchi Y, Horikawa YT, Niesman IR, Kidd MW, Chin-Lee B, Head BP, Patel PM, Roth DM, Patel HH. Role of caveolin-3 and glucose transporter-4 in isoflurane-induced delayed cardiac protection. *Anesthesiology*. 2010; 112:1136–1145. [PubMed: 20418694]
31. Makielski JC, Farley AL. Na(+) current in human ventricle: implications for sodium loading and homeostasis. *J Cardiovasc Electrophysiol*. 2006; 17(Suppl 1):S15–S20. [PubMed: 16686671]
32. Hale SL, Shryock JC, Belardinelli L, Sweeney M, Kloner RA. Late sodium current inhibition as a new cardioprotective approach. *J Mol Cell Cardiol*. 2008; 44:954–967. [PubMed: 18462746]

Highlights for review

The LQT9 mutation F97C in Caveolin3 increases late sodium current by direct nitrosylation of SCN5A.

The increased late I_{Na} can be reversed by blockers of nitrosylation.

HEK293 cells have native nitrosylation capability

The Caveolin3 SCN5A association is not disrupted by the mutation F97C in CAV3

Figure 1. NOS inhibitor L-NMMA suppressed the increased late I_{Na} caused by Cav3-F97C in HEK-293 cells

Representative traces showing late I_{Na} associated with (A) Cav3-WT, (B) Cav3-F97C, (C) Empty vector, and the corresponding traces after being treated with L-NMMA. (D) Summary data of late I_{Na} normalized to peak I_{Na} after leak subtraction. See Table 1 for numbers of experiments.

Figure 2. Identification and activity of nNOS in HEK-293 cells

The nNOS mRNA fragment was detected as a strong positive band in HEK-293 cells transfected with nNOS, a faint positive band in HEK-293 cells, and was absent in Hela cells using RT-PCR assay (A). The representative NADPH diaphorase (NDP) positive-staining (blue particles) cells showing nNOS activity in HEK-293 cells expressing either (B) Cav3-WT, or (C) Cav3-F97C, or (D) empty vector, or (E) nNOS (positive control). Three hundred HEK-293 cells were randomly picked up from the coverslips ($n = 3$) of each group to be analyzed by integrated color density under microscopy using ImageJ software (ImageJ 1.33 u National Institutes of Health, USA). (F) Summary data of integrated color densities from each group. * $P < 0.05$ versus Cav3-WT. The integrate color densities for positive control is $4 \times 10^7 \pm 4 \times 10^6$.

Figure 3. Cav3-F97C significantly increased late I_{Na} by nNOS-dependent S-nitrosylation of SCN5A through SCN5A-SNTA1-nNOS-Cav3 complex

Representative traces showing late I_{Na} associated with (A) Cav3-WT, (B) Cav3-F97C, (C) Empty vector, and the corresponding traces after being treated with L-NMMA. (D) Summary data of late I_{Na} normalized to peak I_{Na} after leak subtraction. See Table 1 for numbers of experiments. (E) CoIP using nNOS to precipitate the complex and specific antibody to pull down SCN5A, SNTA and Cav3. (F) Cav3-F97C was shown to cause increased S-nitrosylation of SCN5A compared to Cav3-WT and the S-nitrosylation was reversed by L-NMMA.

Figure 4. Cav3F97C does not change the pattern of localization of SCN5A in rat myocytes
 Panel A illustrates the localization of Nav1.5 (red), caveolin3 (green) in rat ventricular tissue sections. Insets on the right are higher magnification of the area identified by box in the merged panel. In these zoomed in panels, asterix denotes intercalated disc and the double arrow denotes sarcomeric pattern of staining. Isolated cardiomyocytes shown in panel B were infected with Cav3-WT and that in Panel C were infected with Cav3-F97C construct. Top panels of B and C, illustrates immunolocalization of Cav3 (green, panel B, Cav3-WT and panel C, Cav3-F97C) with Nav1.5 (red) in isolated adult rat ventricular myocytes. Insets on the right are higher magnification of the area identified by box in the merged panel. In these zoomed in panels the double arrow denotes sarcomeric pattern of staining. DAPI staining for nuclei are shown in blue for all merged panels. Scale bar denotes 20μm. Bottom left panels of B and C, are the distance-intensity plots of for Nav1.5 and caveolin-3. Co-localization was quantified by Pearson's correlation coefficient. Pearson's correlation coefficient is 0.78 (Panel A), 0.84 (Panel B) and 0.77 (Panel C) (>0.5 is considered significant) suggesting significant co-localization also apparent from the overlap of the peak signal intensity for caveolin3 and Nav1.5 in Panels B and C.

Figure 5. The biophysical characteristics of adult rat cardiomyocytes overexpressing Cav3 Representative traces showing STX-sensitive late I_{Na} associated with (A) Cav3-WT, (B) Cav3-F97C, (C) Cav3-F97C being treated with L-NMMA. (D) is summary data of late I_{Na} normalized to peak I_{Na} after STX subtraction. The number of tested cells is indicated above the bar. (E) Representative action potential recording from cardiomyocytes overexpressing Cav3 in a current-clamp mode showed Cav3-F97C prolonged the action potential duration (APD) compared to Cav3-WT and L-NMMA reversed the alteration. (F) Summary data of APD₉₀ (AP duration at 90% repolarization) for Cav3-WT and Cav3-F97C. The number of tested cells is indicated above the bar.

Table 1
Biophysical properties of SCN5A co-expressing either Cav3, or Cav3 and nNOS complex in HEK-293 cells

Samples	Peak I_{Na}		Activation		Inactivation		Late I_{Na}	
	pA/pF	n	V1/2(mV)	K	n	V1/2(mV)	K	n
Cav3-WT	-287 ± 31	29	-34.4 ± 0.5	4.5	27	-72.1 ± 0.5	4.6	29
Cav3-F97C	-297 ± 30	38	-34.9 ± 0.8	4.2	27	-72.2 ± 0.6	4.8	39
Empty vector	-320 ± 37	7	-33.1 ± 0.6	4.1	7	-69.7 ± 0.5	4.6	20
Cav3-WT +L-NMMA	-239 ± 42	20	-34.4 ± 1.0	4.2	16	-72.5 ± 0.6	4.7	17
Cav3-F97C +L-NMMA	-276 ± 540	6	-33.4 ± 0.6	4.1	6	-70.3 ± 1.0	4.7	20
Empty vector +L-NMMA	-279 ± 42	6	-34.5 ± 0.8	4.2	8	-71.5 ± 0.8	4.2	13
Cav3-WT +nNOS complex [‡]	-202 ± 21	36	-33.4 ± 0.6	4.3	32	-72.9 ± 0.8	4.7	32
Cav3-F97C +nNOS complex	-302 ± 21 [‡]	35	-33.92 ± 0.4	4.1	25	-70.3 ± 0.4 [‡]	4.5	39
Empty vector +nNOS complex	-309 ± 21 [‡]	39	-34.5 ± 0.7	4.2	25	-70.5 ± 0.5 [‡]	4.8	39
Cav3-WT +nNOS complex +L-NMMA	-236 ± 26	7	-34.8 ± 1.0	4.2	7	-72.2 ± 0.7	4.3	15
Cav3-F97C +nNOS complex +L-NMMA	-256 ± 40	7	-34.2 ± 0.9	34.1	7	-70.9 ± 0.8	4.9	16
Empty vector +nNOS complex +L-NMMA	-249 ± 38	7	-34.2 ± 0.8	4.3	7	-70.6 ± 0.7	4.2	16

* $P < 0.01$ versus Cav3-WT.

[‡] nNOS complex means SNTA1 + nNOS.

[‡] $P < 0.05$ versus Cav3-WT + nNOS complete