Polymorphic ventricular tachycardia and abnormal Ca\textsuperscript{2+} handling in very-long-chain acyl-CoA dehydrogenase null mice

Andreas A. Werdich,\textsuperscript{1} Franz Baudenbacher,\textsuperscript{1} Igor Dzhura,\textsuperscript{1} Loïce H. Jeyakumar,\textsuperscript{2} Prince J. Kannankeril,\textsuperscript{3} Sidney Fleischer,\textsuperscript{4} Alison LeGrone,\textsuperscript{3} Dejan Milatovic,\textsuperscript{3} Michael Aschner,\textsuperscript{3} Arnold W. Strauss,\textsuperscript{3} Mark E. Anderson,\textsuperscript{5} and Vernat J. Exil\textsuperscript{3}

\textsuperscript{1}Division of Cardiology, Department of Pediatrics, Vanderbilt University School of Medicine, Nashville; \textsuperscript{2}Division of Gastroenterology, Vanderbilt University Medical Center, Nashville; Departments of \textsuperscript{3}Biomedical Engineering and \textsuperscript{4}Biological Sciences, Vanderbilt University, Nashville, Tennessee; and \textsuperscript{5}Division of Cardiology, Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa

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DEFICIENCIES IN MITOCHONDRIAL fatty acid β-oxidation (FAO) enzymes have been implicated in cardiomyopathy, arrhythmias, sudden death, nonketotic hyperglycemia, heart and liver lipidoses, encephalopathy, and skeletal myopathy (21, 41, 46). Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is a FAO defect that usually presents with episodes of metabolic crisis and death in children (15) and causes ventricular tachycardia (VT) in almost 50% of the presenting cases (34). Most cases of sudden death occur in the first year of life (15). There has been little research on the mechanisms leading to cardiac dysfunction and ventricular arrhythmias in VLCAD deficiency. We developed a mouse model of VLCAD deficiency and characterized the cardiac phenotype in these mice, revealing lipid accumulation and mitochondrial proliferation in myocytes and inducible polymorphic VT, which is exacerbated by adrenergic stimulation (14).

We have hypothesized that altered intracellular Ca\textsuperscript{2+} homeostasis resulting from dysregulation of Ca\textsuperscript{2+}-regulated proteins may be at the basis of the VT in VLCAD deficiency. Our hypothesis originated from partial cDNA microarray data in which we found that levels of several of the Ca\textsuperscript{2+}-related proteins were quantitatively different in mouse hearts deficient in VLCAD at birth and 2 mo after birth. At a cellular level and in several metabolic pathways, Ca\textsuperscript{2+} is an important messenger that serves a vital role in cell signaling. In muscle, Ca\textsuperscript{2+} influx though sarcoplasmic ion channels triggers the mobilization of Ca\textsuperscript{2+} from intracellular stores (Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release), thereby leading to the contraction of the heart (systole; see Refs. 6 and 7). Abnormalities of intracellular Ca\textsuperscript{2+} handling are hypothesized to induce diastolic depolarization that can trigger ventricular arrhythmias (29, 30, 32, 33). Abnormalities in Ca\textsuperscript{2+} handling are also found in patients with familial catecholaminergic polymorphic ventricular tachycardia (CPVT), a genetic arrhythmia syndrome characterized by polymorphic VT associated with adrenergic stimulation. Intracellular Ca\textsuperscript{2+} homeostasis is modulated by intracellular Ca\textsuperscript{2+} release channels (ryanodine receptors, RyRs), which are widely distributed in tissues: RyR1 in skeletal muscle, RyR2 in cardiac muscle, and RyR3 in a variety of tissues but in minuscule amounts. Mutations in the cardiac ryanodine receptor (RyR2) gene are known to cause both CPVT, also termed familial polymorphic ventricular tachycardia (FPVT) or bidirectional VT (28, 29, 39, 48), and arrhythmogenic right ventricular dysplasia (40, 49). These mutations cause a “gain-of-function” or increased Ca\textsuperscript{2+} leak from the sarcoplasmic reticulum (SR; see Ref. 33).

Given that VLCAD-deficient babies present with severe forms of ventricular dysfunction and arrhythmias (34), and that our mouse model of VLCAD deficiency has easily inducible ventricular arrhythmias, we investigated whether defective cardiomyocyte Ca\textsuperscript{2+} handling plays a role in the development...
of cardiomyopathy in the VLCAD-deficient mouse. In this study, we used our mouse model of VLCAD deficiency (VLCAD−/−; see Ref. 14) to show a novel role for mitochondrial FAO defects in the regulation of Ca2+ signaling in heart. We assessed functional changes of intracellular Ca2+ homeostasis and dynamics in hearts from VLCAD−/+ and VLCAD−/− mice. Our results provide new insights into Ca2+ handling in the settings of VLCAD deficiency in mice and perhaps in humans.

MATERIALS AND METHODS

VLCAD−/− mice. All animals in this study were cared for according to the Institutional Animal Care and Use Committee at Vanderbilt University. Mice were generated and genotyped as previously described (13, 14). Mice of 2–3 mo of age were included in these studies. Intracardiac electrophysiological studies were performed in 12 VLCAD−/− and 16 VLCAD−/− male mice. Subcellular fractions were performed in 24 VLCAD−/− mice (16 males, 8 females) and 12 VLCAD−/+ mice (8 males, 4 females). Upon death or at autopsy, whole heart ventricles and skeletal muscle (slow and fast twitch) were harvested for total protein analysis.

Preparation and characterization of subcellular fractions from mouse hearts. Subcellular fractionation was performed by the method described (42). Myocytes were field stimulated at 600 and 250 beats/min via the performance of the isolated cells at the high stimulation frequency. Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). buffer (in mM: 48 Tris, 39 glycine, and 1.3 SDS, pH 9.2) using a gel, and the protein profile was visualized with Coomassie blue staining. Fractions enriched in SR were analyzed by SDS-PAGE (6% resolving gel), and 1.4 NA oil immersion lens (Plan Apochromat; Carl Zeiss) was placed in the right atrium and right ventricle, guided by electrogam tracing to verify placement.

Bipolar electrogram recordings were obtained from the right atrium, right ventricle, and His positions. Signals were amplified and filtered between 40 and 400 Hz. Bipolar pacing was performed using a programmable stimulator (Medtronic 2350) modified by the manufacturer to deliver coupling intervals as short as 10 ms. Pacing threshold (in mA) was determined for each pacing site, and stimulation was performed for 1.0–2.0 ms. Atrial width at two times the diastolic capture threshold. Electrophysiological intervals (RR, PR, QRS, QT, AH, HV, and AV) were measured in standard fashion. Standard clinical electrophysiological pacing protocols were used to determine all basic electrophysiological parameters. The AV-His-Purkinje conduction properties were assessed through rapid atrial pacing at rates up to 1,000 beats/min. The Wenckebach cycle length was defined as the longest atrial paced cycle length that failed to conduct 1:1 to the ventricle, was determined. Programmed atrial stimulation was performed to determine AV node effective refractory period, defined as the longest prematurity stimulus that failed to conduct to the ventricle. Ventricular effective refractory period was determined at three drive cycle lengths. Single, double, and triple extra stimuli were delivered at three drive cycle lengths to determine inducibility of VT. The duration and cycle length of induced tachycardias were recorded. After baseline measurements were completed, isoproterenol was administered (100 µg ip), and the protocols were repeated to assess the effects on conduction and refractoriness.

Preparation and characterization of subcellular fractions from mouse hearts. Subcellular fractionation was performed by the method described by Chamberlain and Fleischner (10). Cardiac microsomal fractions enriched in SR were analyzed by SDS-PAGE (6% resolving gel), and the protein profile was visualized with Coomassie blue staining. For Western blot analysis of RyR2 after SDS-PAGE, proteins were transferred to an Immobilon-P membrane in blot transfer cell (Bio-Rad). The vacant binding sites on the membrane were blocked by incubating the membrane in wash buffer (10 mM Tris·Cl, pH 8.0, 0.55 M NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk protein for 1 h. The Immobilon-P membrane was probed with a RyR2-specific antibody (1:1,000) in blocking buffer for 1 h, washed three times with wash buffer, and then incubated with secondary antibody goat anti-rabbit IgG conjugated to peroxidase (Sigma) in blocking buffer. The membrane was again washed three times with wash buffer, and immune complexes were detected by the enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia BioTech). RyR1, -2, and -3 specific antibodies were obtained from the Fleisher laboratory, Vanderbilt University (26). With the use of SDS-PAGE (10% resolving gels), Western blots were performed for VLCAD (antibody from Strauss laboratory Vanderbilt University; see Ref. 13), calsequestrin, SR Ca2+-ATPase (SERCA) 2a, and phospholamban (antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Quantification of the different blots was done by densitometry (NIH software image).

Preparation of ventricular myocytes. Mice were anesthetized by intraperitoneal injection of Avertin solution (5 mg Avertin/10 g body wt, T84802; Sigma-Aldrich) containing heparin (3 mg/10 ml, H9399; Sigma-Aldrich). The heart was rapidly excised and placed in ice-cold Ca2+-free and glucose-free HEPES-buffered Tyrode solution. The Tyrode solution contained (in mM) 140 NaCl, 4.5 KCl, 0.5 MgCl2, 150 glucose, 10 HEPES, 25 µM CaCl2, and 60 nM [3H]ryanodine (15,000 counts/min 1-µl pmol−1, obtained from Amersham) at 1 h at room temperature. Nonspecific binding was measured in the presence of 20 µM cold ryanodine (Sigma). Free ligand was separated from the bound ligand by sedimenting the microsomes in a Beckman TL 100.1 rotor at 95,000 revolutions/min (15 min at 4°C). The supernatants were removed by aspiration, the pellets were rinsed twice and resuspended in 200 µl water, and the radioactivity was measured in 5 ml of Cytosint (ICN, Cleveland, OH) in a Beckman LS 5000 TD scintillation counter. Upon completion of the experiment, radioactivity was measured and expressed as picomoles per milligram of protein. Binding was calculated based on the formula (total cpm − nonspecific cpm)/cpm/pmol × mg protein (26).

Measurement of intracellular Ca2+ transients. Dissociated cardiomyocytes were loaded for 15 min with the acetoxymethyl ester forms of the Ca2+-sensitive fluorescent indicators indo 1 (20 µM 1203; Invitrogen) or fura 2 (5 µM, F1221; Invitrogen). Cells were then centrifuged for 10 min at 27 g and resuspended in Tyrode solution containing 1.0 mM Ca2+. Ca2+ transients were measured in Tyrode solution containing 1.0 mM Ca2+ and 10 µM palmitate at 37°C, supplemented with an insulin-transferrin-selenium mixture (100X medium supplement, 41400, consisting of 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite; Gibco-Invitrogen) to improve the performance of the isolated cells at the high stimulation frequencies (42). Myocytes were field stimulated at 600 and 250 beats/min via two parallel platinum wires. Fluorescence measurements were carried out using an inverted microscope (Axiovert 200; Carl Zeiss) equipped with a ¥63, 1.4 NA oil immersion lens (Plan Apochromat; Carl Zeiss). For the fura 1 measurements, the excitation wavelength was rapidly switched between 340 ± 10 and 380 ± 10 nm at a rate of

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~200 ratios/s by using a monochromator (Optoscan; Cairn Research). For the indo 1 measurements, the excitation wavelength was fixed at 365 ± 15 nm. The excitation light was reflected on the cells by a dichroic mirror (415DCLP for fura 1 and 390DRLP for indo 1; Omega Optical). Fura 2 fluorescence emission was recorded at a wavelength of 510 nm.

With the use of a bandpass emission filter (510WB40; Omega Optical), indo 1 fluorescence was split into two components by a second dichroic mirror (450DCLP; Omega Optical), and each component passed through a 495-nm (495DF20; Omega Optical) or a 405-nm bandpass filter (405DF43; Omega Optical). The light was collected by 1-mm-diameter optical fibers mounted behind the emission filters imaging a ~16-μm-diameter spot on the cell. Fluorescence emission was registered by photomultiplier modules (H6780; Hamamatsu) and amplified by a custom-built low-noise direct current-coupled amplifier. The signals were digitized at a sampling rate of 20 kHz by an analog/digital (A/D) converter board (PCI-6071E; National Instruments) in a conventional personal computer (PC).

Fluorescence calibration. Fluorescence emission ratios were converted to intracellular Ca2+ concentrations ([Ca2+]i) according to the equation (20) 

\[
[Ca^{2+}]_i = K_0 \times (R_{max}/R) - R,
\]

published in vivo dissociation constants for fura 1 and indo 1 of 371 (23) and 844 (3) nM, respectively. R values in the previous equation were calculated as ratios of 405/495 nm fluorescence emission (indo 1) and 340/380 nm excitation at 510 nm emission (fura 2). β Values were calculated as ratios of Ca2+-free/saturated indicator at excitation/emission wavelengths of 365/495 nm (indo 1) and 380/510 nm (fura 2). Rmin and Rmax were determined in vivo. Cells were loaded with Ca2+ dye and exposed to 10 mM caffeine two times to empty the SR. For Rmin determination, cells were superfused with Ca2+-free Tyrode solution containing 5 mM EGTA and 10 μM of the nonfluorescent ionophore bromo-A-23187 (B7272; Sigma-Aldrich). Measurements were taken after the fluorescence reached stable values at both wavelengths. For Rmax determination, extracellular Ca2+ concentration was gradually increased to 10 mM in the presence of the metabolic inhibitor cyanide p-(trifluoromethoxy)-phenylhydrazone (3 μM, C2920; Sigma-Aldrich) to avoid hypercontracture. The Ca2+ ionophore was present throughout the calibration procedure.

Sarcoplasmyr contraction. Sarcoplasmic contraction was measured in single cardiac myocytes using a commercial contractility measurement system (Ionoptix) consisting of a 240-frame A CCD camera (MYO100; Ionoptix) connected to a side port of the microscope, a frame grabber FC card (FRGRAB; Ionoptix) and an A/D converter (DSI200; Ionoptix) to record the stimulus. Data were acquired using the IonWizard software. Cells were held stimulated at 240 beats/min to obtain 60 data points for each contraction at the maximum acquisition rate of 240 frames/s.

Electrophysiology. L-type Ca2+ current (Ic,a) was recorded in the whole cell mode voltage-clamp configuration according to previously published methods (22). Briefly, pipettes (2–3 MΩ) contained (in mM) 120 Cs+, 3 Ca2+, 126 Cl−, 1 MgATP, 2 NaGTP, 5 phosphocreatine, 10 HEPES, and 10 EGTA (1). The pH was adjusted to 7.2 with CsOH. The bath solution contained Tyrode solution as described above with 10 μM palmitic acid and 1.8 mM Ca2+. The holding potential was −90 mV. Ic,a was measured during 500-ms test pulses at potentials ranging from −40 to +40 mV following a 50-ms pulse at −50 mV to inactivate Na+ current (12, 31).

SR Ca2+ content. SR Ca2+ content was estimated by integrating current through the Na+/Ca2+ exchanger (NCX) as described by Diaz et al. (11). Briefly, Na+ and K+ currents were eliminated in the bath solution by adding Cs+ and tetraethylammonium chloride. Pipettes contained (in mM) 120 Cs+, 10 HEPES, 10 TEA, 5 phosphocreatine, 1 MgATP, and 1 NaGTP; pH was adjusted to 7.2 with CsOH. The extracellular bath solution contained (in mM) 137 N-methyl-D-glucamine (NMDG), 25 Cs+, 10 HEPES, 10 glucose, 1.8 Ca2+, and 0.5 Mg2+; pH was adjusted to 7.4 with HCl. Cells were rapidly perfused with a spritz of modified bath solution containing 20 mM caffeine and equimolar substitution of NaCl for NMDG. The resulting inward current was integrated using PCLAMP 9.2 (Molecular Devices) and normalized for total membrane capacitance vs. cell surface area.

Data analysis. Data were expressed as means ± SE. Statistical analysis for the Western blots was performed using the Mann-Whitney Test. Data analysis for the Ca2+ fluorescence was accomplished with Matlab (R14; The MathWorks). Ca2+ transient upstroke and decay were fit to mathematical functions for calculating numerical time derivatives (51). Because derivatives of high-bandwidth fluorescence recordings are extremely susceptible to noise, we used these empirical models to obtain smooth derivatives of the signal. Ca2+ transient upstroke was best fit by 

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[Ca^{2+}]_t = [Ca^{2+}]_{max} \times [P/(t + 1 - P)],
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t is time, [Ca2+]max is the amplitude of Ca2+ transient, with P(t) = 0.5(1 − e−t/τp). The parameters τ, n, and m were used as fit parameters and were not further analyzed. Transient decay was best fit by a sum of two exponentials, [Ca2+]t = Ae−t/τ1 + Be−t/τ2 with fit parameters A, B, τ1, and τ2 that were not further analyzed. The null hypothesis was rejected for P < 0.05.

RESULTS

Polymorphic and bidirectional VT in the VLCAD−/− mice. VLCAD−/− mice, although viable, demonstrated easily inducible VT in the absence of physiological stress. There was no difference in heart rate, PR interval, Wenczekbach cycle length (defined as the longest atrial paced cycle length with failure of 1:1 conduction to the ventricle), or ventricular effective refractory period (defined as the longest coupling interval that fails to capture the ventricle) between VLCAD−/− and VLCAD+/− mice. With the use of programmed ventricular stimulation, VT could be induced in 6/12 (50%) of VLCAD−/− mice compared with 2/16 (12%) wild-type mice. VT in the VLCAD−/− mice was consistently polymorphic. VT inducibility was increased in the VLCAD−/− mice to 10/12 (83%) when isoproterenol was used. VT with the typical bidirectional morphology with isoproterenol infusion was observed in 1 out of 10 mice (Fig. 1). Isoproterenol did not increase arrhythmia inducibility in wild-type mice.

Increased expression of RyR2 isoform in mouse hearts and augmented ryanodine binding in microsomes of VLCAD−/− mice. Given our microarray results suggesting changes in Ca2+-related genes and other reports showing a possible link between polymorphic/bidirectional VT and excessive RyR2 activity (32, 33, 39), we first tested whether RyRs were differentially expressed in the VLCAD−/− mice. This was accomplished with a sum of two exponentials, 

\[
\frac{[Ca^{2+}]}{t} = \frac{1}{\tau_1} \exp(-t/\tau_1) + \frac{1}{\tau_2} \exp(-t/\tau_2),
\]

where \(\tau_1, \tau_2, C_1, \) and \(C_2\) are fit parameters for the Ca2+ transient. The null hypothesis was rejected for P < 0.05.

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H2204 ELECTROPHYSIOLOGICAL IMPLICATIONS OF VLCAD DEFICIENCY
Enhanced diastolic Ca\(^{2+}\) concentration, Ca\(^{2+}\) transients, and SR Ca\(^{2+}\) load in VLCAD\(^{-/-}\) cardiomyocytes. We tested whether the observed RyR2 upregulation and increased [\(^{3}\)H]ryanodine binding were associated with enhanced Ca\(^{2+}\) transients in intact cardiac myocytes from VLCAD\(^{-/-}\) hearts. We isolated ventricular cardiac myocytes from VLCAD\(^{+/+}\) and VLCAD\(^{-/-}\) mice and measured Ca\(^{2+}\) transients using Ca\(^{2+}\)-sensitive ratiometric dyes. We found that both diastolic fluorescence and amplitude of Ca\(^{2+}\) transient were increased in VLCAD\(^{-/-}\) cardiac myocytes as shown in Fig. 3. We confirmed our results in two series of independent experiments using two different fluorescence dyes with different Ca\(^{2+}\)-binding affinities, and at two different pacing rates. Because the physiological heart rate in a mouse is on the order of 600–700 beats/min, we initially measured Ca\(^{2+}\) transients at 10 Hz (Fig. 3A). Under these conditions, Ca\(^{2+}\) transients were significantly larger in VLCAD\(^{-/-}\) cardiac myocytes compared with VLCAD\(^{+/+}\) control cells. However, rapid pacing itself increased diastolic Ca\(^{2+}\) concentration in all cells (Fig 3C). The increased binding of Ca\(^{2+}\) to intracellular ligands that occurs when diastolic Ca\(^{2+}\) concentration is high may by itself increase free Ca\(^{2+}\) concentration and the amplitude of the Ca\(^{2+}\) transient. Therefore, we performed measurements at 4 Hz to achieve a lower baseline Ca\(^{2+}\) concentration and to reduce mitochondrial Ca\(^{2+}\) loading. Although diastolic fluorescence was significantly lower in VLCAD\(^{+/+}\) control myocytes at 4 Hz, i.e., 159 ± 40 nM (Fig. 3B), it was still threefold higher in VLCAD\(^{-/-}\) cardiac myocytes (Fig. 3, B and C). These data were consistent with the fluorescence data at 10 Hz. The amplitude of Ca\(^{2+}\) transient was significantly increased in VLCAD\(^{-/-}\) cells at 4 Hz (Fig. 3, B and D), and this increase was even more pronounced at the lower pacing rate (Fig. 3D).

To further characterize Ca\(^{2+}\) transients in VLCAD\(^{-/-}\) cardiomyocytes, we subsequently calculated the maximum time derivatives of Ca\(^{2+}\) transient upstroke and decay. We found that maximum slopes of the transients were significantly increased in VLCAD\(^{-/-}\) myocytes (Fig. 4A), although times of fluorescence rise and fall were about the same (Fig. 4B). One of our hypotheses was that the increase in Ca\(^{2+}\) transient amplitude was the result of an increase in SR Ca\(^{2+}\) content. We tested this hypothesis by measuring the integrated NCX current after fast application of caffeine (Fig. 4C). The integrated normalized NCX current was 3.38 ± 0.69 pC/pF in the VLCAD\(^{-/-}\) cardiomyocytes compared with 2.28 ± 0.55 pC/pF in wild-type control cells. This result indicated an increased SR Ca\(^{2+}\) load in VLCAD\(^{-/-}\) cardiomyocytes compared with WT control cells.
Fig. 3. Ca2+ transients in cardiac myocytes isolated from VLCAD-/- mice. A: mean Ca2+ release measured in single cardiac myocytes paced at the physiological rate of 10 Hz from VLCAD-/- (n = 20) and VLCAD+/+ (n = 18) mice. B: mean fura 2 Ca2+ transients measured in single cardiac myocytes paced at 4 Hz from VLCAD-/- (n = 10) and from VLCAD+/+ (n = 10) mice. C: diastolic Ca2+ concentrations in VLCAD-/- and VLCAD+/+ cells paced at 4 and 10 Hz as calculated from the measurements represented in A and B. D: systolic Ca2+ concentrations in VLCAD-/- and VLCAD+/+ cells paced at 4 and 10 Hz as calculated from measurements represented by A and B. Data were taken from 3 VLCAD-/- and 3 VLCAD+/+ mice. *, †, ‡, and §P < 0.005, VLCAD+/+ vs. VLCAD-/-.

Enhanced sarcomere contraction velocity in VLCAD-/- cardiomyocytes. We also measured sarcomere contraction and relaxation to test whether changes in time course and amplitude of Ca2+ transients had any impact on cardiomyocyte contractility. We found that sarcomere contraction was significantly hastened in VLCAD-/- cardiomyocytes compared with VLCAD+/+ control cells (Fig. 5A). Duration of contraction transient (as measured at 50% of the maximum contraction) was shortened by ~30% in VLCAD-/- cells (Fig. 5B), which was consistent with an increase in contraction velocity. Maximum contraction velocity was increased by 28% (Fig. 5D), and time-to-maximum was decreased by 25% (Fig. 5E) in VLCAD-/- cardiomyocytes. There was, however, no difference in sarcomere relaxation (Fig. 5, D and E). Interestingly, amplitude of sarcomere contraction was the same in VLCAD-/- (85 ± 15 nm) and VLCAD+/+ (96 ± 10 nm) cardiomyocytes (Fig. 5C). There was no difference in diastolic sarcomere lengths in VLCAD-/- (1.749 ± 0.003 μm) compared with VLCAD+/+ (1.744 ± 0.007 μm) cells (Fig. 5A).

Altered Ca2+ release in VLCAD-/- mice was not the result of changes in the function of the L-type Ca2+ channel. We tested whether the observed increase in Ca2+ transient amplitude could be the result of an increase in transmembrane L-type ICa. An increase in Ca2+ transient amplitude could have been the result of an increase in Ca2+ influx through sarcolemmal ion channels. Ca2+ release from the SR is solely graded via Ca2+ influx through the L-type Ca2+ channel, given that contribution from NCX reverse mode is believed to be small (~7%) in mouse and rat ventricles (5, 6). We found that both amplitude and time course of ICa were not changed under voltage-clamp conditions in VLCAD-/- cardiac myocytes using a test potential of 0 mV (Fig. 6A). The slow and the fast time constants of ICa inactivation were the same, with 92 ± 6 and 12.0 ± 3 ms in VLCAD-/- and 92 ± 5 and 12 ± 3 ms in VLCAD+/+ cardiomyocytes as indicated by the recovery period of the current traces in Fig. 6A. We also measured the current-voltage relationship of mean peak ICa and found no differences between VLCAD-/- and VLCAD+/+ cells (Fig. 6B), supporting that the increase in Ca2+ transient amplitude in VLCAD-/- cells was not because of an increase in transmembrane ICa.

Ablation of the VLCAD gene in mice leads to alteration in other Ca2+-related proteins in heart. Given the changes in Ca2+ homeostasis and the observed increase in amplitude of Ca2+ transient in VLCAD-/- cardiomyocytes, we tested whether the expression of other Ca2+-related proteins was also changed in our model. Western blot analyses showed a sevenfold increase in calsequestrin levels (Fig. 7, A and B). Protein levels of SERCA2a were not changed (Fig. 7, A and D). The monomeric form of phospholamban was unchanged although the pentameric form of phospholamban was increased 6.0 ± 0.9-fold in the VLCAD-/- cells compared with control (Fig. 7D).

DISCUSSION

VLCAD deficiency in mice leads to polymorphic and bidirectional VT. Children with mitochondrial VLCAD deficiency present with cardiomyopathy, ventricular arrhythmias, and
Our results in VLCAD−/− cardiac myocytes demonstrate that VLCAD deficiency in mice leads to catecholamine-sensitive Ca2+ transient amplitude and SR Ca2+ load in isolated cardiomyocytes. One major finding in this paper is that, in VLCAD−/− cardiac myocytes, the Ca2+ transient is rapidly attenuated with a variety of Ca2+ buffers (4–6). These buffers are Ca2+-binding sites that are unoccupied in diastole, but they bind part of the Ca2+ released during systole, thus attenuating the rise in [Ca2+]i. Convervatively, the increase in baseline Ca2+ concentration was comparable to the total buffer capacity of the cytosol equal to 162 μM. Under these assumptions, we found that the increase in diastolic Ca2+ concentration (i.e., the increase in Ca2+ buffering capacity) in VLCAD−/− cardiac myocytes that could be expected from the increase in diastolic Ca2+ concentration alone and compared it with our measured values. Based on the findings of Berlin et al. (4), we used the traditional Michaelis-Menten relationship to estimate total [Ca2+]i and a total buffer capacity of the cytosol equal to 162 μM. These buffers (4–6) were used to study the contribution of mitochondrial Ca2+ buffering capacity (i.e., the increase in Ca2+ buffering capacity) in VLCAD−/− cardiomyocytes. We estimated the increase in free Ca2+ concentration alone and compared it with our measured values. Based on the findings of Berlin et al. (4), we used the traditional Michaelis-Menten relationship to estimate total [Ca2+]i and a total buffer capacity of the cytosol equal to 162 μM. Under these assumptions, we found that the increase in diastolic Ca2+ concentration (i.e., the increase in Ca2+ buffering capacity) in VLCAD−/− cardiac myocytes could account for nearly 80% of the increase in diastolic Ca2+ concentration in these cells when stimulated at 10 Hz (Fig. 3A).

The interpretation of our high baseline Ca2+ fluorescence levels in the VLCAD−/− cardiomyocytes needs to also take into account the contribution of mitochondrial Ca2+ loading. Mitochondrial Ca2+ has been shown to be significant when resting Ca2+ concentration exceeds 300–500 nM, as demonstrated by Zhou et al. (53). Mitochondrial dye loading cannot be avoided using the ester form of indo 1. This is why we subsequently performed Ca2+ fluorescence measurements at a lower stimulation frequency of 4 Hz using fura 2 (Fig. 3B). The ester form of fura 2 accumulates less in intracellular compartments (9). The lower pacing rate using fura 2 significantly reduced diastolic Ca2+ concentration in wild-type control cardiac myocytes to a value of <200 nM (Fig. 3, B and C), which compares well with literature data (8, 18). However, under the slow pacing condition, amplitude of Ca2+ transients was significantly increased compared with wild-type control cells. Under the same pacing conditions, diastolic [Ca2+]i was further increased to ~700 nM in the VLCAD−/− cells (Fig. 3C). These high diastolic Ca2+ levels were most likely from SR inability to sequester sufficient Ca2+ at such high stimulation rate as suggested by Field et al. (16).

High diastolic Ca2+ levels also alter cytosolic Ca2+ buffering efficiency, thereby complicating the interpretation of Ca2+ transients in humans (17). Approximately 99% of the total Ca2+ that enters the cytosol during a Ca2+ transient is rapidly complexed with a variety of Ca2+ buffers (4–6). These buffers are Ca2+-binding sites that are unoccupied in diastole, but they bind part of the Ca2+ released during systole, thus attenuating the rise in [Ca2+]i. Conversely, in the setting of our high diastolic Ca2+ levels, there would be fewer binding sites available to buffer the rise in [Ca2+]i following a release. As a consequence, we tested whether the increase in transient amplitude was the result of less cytosolic Ca2+ buffering in the VLCAD−/− cardiomyocytes. We estimated the increase in free Ca2+ concentration alone and compared it with our measured values. Based on the findings of Berlin et al. (4), we used the traditional Michaelis-Menten relationship to estimate total [Ca2+]i, using a lumped Ka of 0.63 μM for all cytosolic Ca2+ buffers and a total buffer capacity of the cytosol equal to 162 μM. Under these assumptions, we found that the increase in diastolic Ca2+ concentration (i.e., the increase in Ca2+ buffering capacity) in VLCAD−/− cardiac myocytes could account for nearly 80% of the increase in diastolic Ca2+ concentration in these cells when stimulated at 10 Hz (Fig. 3A).

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diastolic Ca\(^{2+}\) concentrations at the lower pacing rate. Ca\(^{2+}\) buffering in VLCAD\(^{-/-}\) myocytes is expected to be higher at the slow compared with the fast pacing rate because more intracellular Ca\(^{2+}\) buffer binding sites should be available when diastolic Ca\(^{2+}\) concentration is lower. We estimated that, at the slower pacing rate of 4 Hz, the change in Ca\(^{2+}\) buffering may account for ~60% of the increase in transient amplitude. The observed increase in transient amplitude was even higher at the lower pacing rate. Likely, at these lower diastolic Ca\(^{2+}\) levels, the effect of VLCAD deficiency on the amplitude of Ca\(^{2+}\) transient was more pronounced because of the larger Ca\(^{2+}\) concentration gradient between the SR and the cytosol (negative staircase). Interestingly, the normalized integrated NCX current was increased by 48% after rapid caffeine application in VLCAD\(^{-/-}\) cells (Fig. 4C). The increased caffeine-induced Ca\(^{2+}\) release together with the increased expression of SR Ca\(^{2+}\)-binding protein calsalequestrin (Fig. 7, A and B) indicate that the SR Ca\(^{2+}\) content was increased in VLCAD\(^{-/-}\) cardiac myocytes.

**VLCAD deficiency in mice accelerates sarcomere shortening.**

Biochemical and physiological changes in Ca\(^{2+}\) homeostasis led to changes in sarcomere shortening velocity in the VLCAD\(^{-/-}\) cardiomyocytes. Sarcomere contraction was significantly hastened in VLCAD\(^{-/-}\) myocytes compared with control cells (Figs. 5, A and D), which was consistent with an enhanced Ca\(^{2+}\) transient in these cells (Figs. 3 and 4). Faster contraction was evident in a shorter contraction transient (Fig. 5B), a larger time derivative of the upstroke (Fig. 5D), and a shorter time-to-minimum sarcomere length (Fig. 5E). Surprisingly, the mean contraction amplitude was not changed in the VLCAD\(^{-/-}\) cardiomyocytes (Fig. 5C; \(P = 0.53\)). The observed increase in diastolic Ca\(^{2+}\) in VLCAD\(^{-/-}\) cardiac myocytes did not translate into a decreased diastolic sarcomere length (Fig. 5A). It might be speculated that myofilament response to cytosolic Ca\(^{2+}\) was altered in VLCAD\(^{-/-}\) cardiac myocytes, but a direct measurement of myofilament Ca\(^{2+}\) sensitivity in the VLCAD cardiomyocytes would be required to test this hypothesis.

**VLCAD deficiency alters expression of Ca\(^{2+}\) regulatory proteins.**

We have previously shown that VLCAD\(^{-/-}\) mice have a number of biochemical changes suggestive of complex alteration in lipid metabolism and lipid transport that are present in the heart at birth, whereas ultrastructural abnormalities develop postnatally (14). Although these changes may be part of the compensatory molecular events that occur in the absence of VLCAD, it is also conceivable that the biochemical changes that we presently report are directly related to the disease phenotype. Here, we report changes in the expression of several Ca\(^{2+}\) regulatory proteins in heart. These findings have not been previously reported in VLCAD deficiency or in other models of mitochondrial FAO defects. We found that all RyR isoforms (i.e., RyR1 in skeletal muscle, RyR2 in heart, and RyR3 in brain) were upregulated. \([\text{H}]\)ryanodine binding was also increased in VLCAD\(^{-/-}\) microsomes compared with microsomes from control mouse hearts. In our model, we also found that the pentameric form of phospholamban was upregulated in VLCAD\(^{-/-}\) myocytes (Fig. 7D). Although several studies show that phospholamban asserts its inhibitory function by binding to SERCA in its monomeric form (2, 24, 25) there is some evidence that the pentameric form of phospholamban may directly interact with SERCA2a, causing a decrease in the affinity of the pump for Ca\(^{2+}\) (45, 52). However, the mechanism of this interaction is not fully understood. Pentameric phospholamban has been proposed to form an ion pore in SR
membranes (1a, 27, 43, 44) that is selectively permeable to Ca\textsuperscript{2+}/H\textsubscript{10001} with spontaneous opening and closing properties (27).

The exact role of RyR upregulation and elevated levels of calsequestrin and other Ca\textsuperscript{2+}/H\textsubscript{10001}-related proteins in VLCAD deficiency is not entirely clear. Future studies are needed to assess their biological and clinical implications for other models of mitochondrial fatty acid oxidation defects in mice and perhaps in humans.

Altogether, the present work supports the concept of a previously unrecognized connection between a clinically important metabolic defect, upregulation of SR Ca\textsuperscript{2+} stores and release proteins, enhanced sarcomere contraction, and increased susceptibility to adrenergically mediated arrhythmias. Our current hypothesis is that changes in the regulation of [Ca\textsuperscript{2+}], are likely directly related to the cardiac phenotype of VLCAD deficiency and may serve as an added substrate for VT in the VLCAD\textsuperscript{−/−} mouse. Enhanced SR Ca\textsuperscript{2+} load may increase spontaneous Ca\textsuperscript{2+} release, which is a hypothesized molecular mechanism of polymorphic and bidirectional VT. Whether changes in RyR, phospholamban, or calsequestrin were true contributors to the observed ventricular arrhythmias is not known. Our findings, however, support a mechanism in which VLCAD deficiency, perhaps through decreased ATP production, leads to alteration in intracellular Ca\textsuperscript{2+} homeostasis and elevated SR Ca\textsuperscript{2+} load. These changes have the potential to alter excitation-contraction coupling, leading to cardiac rhythm abnormalities. In the future, we will need to assess whether there are inherent or preexisting changes in RyR2, phospholamban, and/or calsequestrin that lead to abnormal Ca\textsuperscript{2+} handling or whether the accumulation of toxic metabolites resulting from VLCAD deficiency causes the observed changes in Ca\textsuperscript{2+} homeostasis. Given that these Ca\textsuperscript{2+} changes were consistently observed in the VLCAD\textsuperscript{−/−} mice, abnormalities in intracellular Ca\textsuperscript{2+} handling may represent a plausible cellular mechanism for ventricular arrhythmia in VLCAD deficiency and/or several of the FAO enzyme defects.

There are several limitations to our study. Ca\textsuperscript{2+} fluorescence amplitude may differ from SR Ca\textsuperscript{2+} release because sarcolem-
mal ion currents were not blocked in our experiments. Although in mouse ventricles most of the Ca$^{2+}$ that enters the cytosol during systole is released from the SR (92%, compared with $\sim 70\%$ in humans), other ion currents (such as the reverse-mode NCX) might have partly contributed to the increase in systolic free Ca$^{2+}$ concentration in VLCAD$^{-/-}$ myocytes (5, 6). These currents were not individually measured. Furthermore, mitochondrial Ca$^{2+}$ loading can be expected to occur in VLCAD$^{-/-}$ myocytes even when diastolic Ca$^{2+}$ concentration is low (19, 53). Because both indo 1 and fura 2 are known to accumulate in noncytoplasmic compartments (9), fluorescence originating from Ca$^{2+}$ trapped in the mitochondria may superimpose the cytosolic component of Ca$^{2+}$ transient. Therefore, it is important to note that quantitative changes in maximum time derivatives of total Ca$^{2+}$ fluorescence are only suggestive of changes in SR Ca$^{2+}$ release and uptake fluxes. Additional experiments would be required to determine the exact contribution of Ca$^{2+}$ fluxes resulting from noncytoplasmic compartments, the sarcolemma, and the mitochondria. Notwithstanding these limitations, our findings remain true: Ca$^{2+}$ homeostasis is altered in the VLCAD$^{-/-}$ mouse heart, which is a possible underlying mechanism for polymorphic VT in mice and perhaps in humans.

In summary, in this research report, we have provided evidence that VLCAD deficiency in mice is associated with polymorphic and bidirectional VT, elevated levels of critical Ca$^{2+}$ regulatory proteins with elevated diastolic and systolic Ca$^{2+}$, and elevated SR Ca$^{2+}$ stores. Findings in this mouse model raise the following critical questions. 1) Does VLCAD deficiency in humans contribute to a subset of cardiac arrhythmias that resembles defects in Ca$^{2+}$ release channels of the heart? 2) Do our findings represent a molecular mechanism for arrhythmias in other models of FAO deficiency? Long-term follow-up of patients with VLCAD deficiency has been limited by the fact that most of the cases present in childhood with sudden death as presenting sign. Patients who survive into adulthood may have no symptoms and structurally normal hearts (without cardiac hypertrophy or dilation). Our data, however, raise the possibility that these patients may still be at risk for VT specifically related to changes in Ca$^{2+}$ handling in the absence of cardiac hypertrophy or dilation. Our results also suggest that VLCAD deficiency in humans may contribute to a subset of cases with FPVT/CPVT and point to a molecular mechanism for arrhythmias in other cases of FAO deficiency.

REFERENCES


