Impaired contractile function and calcium handling in hearts of cardiac-specific calcineurin b1-deficient mice

Paul J. Schaeffer,1,2 Jaime DeSantiago,5 John Yang,1,2 Thomas P. Flagg,3 Attila Kovacs,1,2 Carla J. Weinheimer,1,2 Michael Courtois,1,2 Teresa C. Leone,1,2 Colin G. Nichols,3 Donald M. Bers,5 and Daniel P. Kelly1,2,3,4

1Center for Cardiovascular Research, and Departments of 2Medicine, 3Molecular Biology and Pharmacology, and 4Pediatrics, Washington University School of Medicine, St. Louis, Missouri; and 5Department of Pharmacology, University of California Davis, Davis, California

Submitted 13 February 2009; accepted in final form 17 August 2009

Schaeffer PJ, DeSantiago J, Yang J, Flagg TP, Kovacs A, Weinheimer CJ, Courtois M, Leone TC, Nichols CG, Bers DM, Kelly DP. Impaired contractile function and calcium handling in hearts of cardiac-specific calcineurin b1-deficient mice. Am J Physiol Heart Circ Physiol 297: H1263–H1273, 2009. First published August 21, 2009; doi:10.1152/ajpheart.00152.2009.—To define the necessity of calcineurin (Cn) signaling for cardiac maturation and function, the postnatal phenotype of mice with cardiac-specific targeted ablation of the Cn B1 regulatory subunit (Ppp3r1) gene (csCnb1−/− mice) was characterized. csCnb1−/− mice develop a lethal cardiomyopathy, characterized by impaired postnatal growth of the heart and combined systolic and diastolic relaxation abnormalities, despite a lack of structural derangements. Notably, the csCnb1−/− hearts did not exhibit diastolic dilatation, despite the severe functional phenotype. Myocytes isolated from the mutant mice exhibited reduced rates of contraction/relaxation and abnormalities in calcium transients, consistent with altered sarcoplasmic reticulum loading. Levels of sarco(endo)plasmic reticulum Ca-ATPase 2a (Atp2a2) and phospholamban were normal, but phospholamban phosphorylation was markedly reduced at Ser16 and Thr17. In addition, levels of the Na/Ca exchanger (Slc8a1) were modestly attenuated following inhibition of Cn, whether pharmacologically (28) or by overexpression of Cn inhibiting proteins (10, 36). As a downstream effector of Ca2+ signaling, Cn also acts to regulate the activity of proteins involved in excitation-contraction coupling. Cn has been reported to modulate the activity of sarco(endo)plasmic reticulum Ca-ATPase (SERCA2a) (29), the ryanodine receptor (7), and phospholamban (PLN) (22).

The role of Cn in normal physiological processes in the heart and its downstream targets has not been fully elucidated (27, 42). Loss-of-function studies are necessary to delineate the biological roles of Cn signaling in vivo. Global Cn loss of function, via deletion of the CnB regulatory subunit, led to embryonic lethality due to failure of vascular patterning in the mouse embryo (13). To avoid this problem, Cn loss-of-func-tion mouse models, in which either the CnAα or the CnAβ subunit has been deleted, have been generated (6, 45). The CnA subunit has three isoforms, α, β, and γ, of which α and β are ubiquitously expressed. Deletion of CnAα had no reported cardiac phenotype (34, 45). CnAβ−/− mice also did not exhibit an overt cardiac phenotype at baseline (6). Thus, in both cases, the presence of the other CnA isoform was sufficient to prevent the embryonic lethality seen in the global CnB deletion. However, the CnAβ−/− mice were unable to mount a response to pathological hypertrophic stimuli (6). Additionally, when crossed with a muscular dystrophy mouse model, CnAβ−/− mice restored cardiac function, providing additional support for a role of Cn signaling in pathological cardiac remodeling (32). To further define the biological function of Cn signaling in the postnatal heart, we sought to develop mice with cardiac-specific Cn deficiency. To this end, we developed an independent line of mice with cardiac-restricted deletion of the Cnb1 gene using Cre recombinase driven by the α-myosin heavy chain gene promoter [csCnb1−/− mice (40)]. This strategy targets the Cnb1 subunit, which does not have functionally redundant isoform relatives in heart. Gene expression studies in these mice conducted in the early postnatal period revealed that genes involved in myocyte fatty acid oxidation were downregulated, consistent with the known role of Cn as an activator of the transcriptional coactivator peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), a master regulator of cellular energy metabolism (40). In this study, we have conducted rigorous cardiac phenotyping studies of csCnb1−/−

derangements in cardiac myocyte calcium (Ca2+) homeostasis are linked to the development of heart failure (4). In addition to its role in excitation-contraction coupling, Ca2+ serves important functions in cellular signaling and metabolism. The serine-threonine phosphatase, calcineurin (Cn), is an important transducer of Ca2+ to cellular signaling events. The active Cn holoenzyme consists of a complex, including calmodulin and the Cn A and B subunits. This holoenzyme is activated by sustained elevation of intracellular Ca2+ levels, such as occurs in working striated muscle (37), and acts to regulate transcription of genes via activation of the nuclear factor of activated T cells family of transcription factors (9, 16). Cn has been implicated in the programs controlling pathological cardiac hypertrophic growth and remodeling (14, 42), skeletal muscle fiber-type determination (31), myocyte excitation-contraction coupling (7), and mitochondrial energy metabolism (40).
mice from neonatal to adult stages. We found that Cn signaling is critical for normal postnatal cardiac growth and function, as well as survival. Specifically, loss of Cn signaling results in reduced postnatal heart growth and a severe cardiomyopathy, with some features characteristic of restrictive physiology. The contractile phenotype of the Cn-deficient mice is associated with derangements in excitation-contraction coupling related to altered activity and expression of several key cellular Ca\(^{2+}\) pumps. These results demonstrate the importance of Cn signaling in postnatal heart maturation and in the regulation of myocyte Ca\(^{2+}\) homeostasis. The csCnb1\(^{-/-}\) mice should prove a useful model of Cn signaling in the adult myocardium.

**MATERIALS AND METHODS**

**Transgenic mice.** Transgenic mice expressing Cre-recombinase under control of the α-myosin heavy chain promoter (1) were crossed with mice in which exons 3–5 of the gene encoding the regulatory subunit of Cn (Ppp3r1l) are flanked by loxP sites (30) to generate cardiac-specific Cn bl null (csCnb1\(^{-/-}\)) mice. The final breeding resulted in the experimental mice, which all possessed the floxed allele (homozygous), but only 50% possessed Cre. Control mice possessed the floxed (Ppp3r1l) gene (homozygous), but lacked the Cre transgene. Mice possessing the Cre transgene alone were also included in initial studies to ensure that expression of Cre did not confer a cardiac phenotype. Mice were euthanized by carbon dioxide inhalation, and the hearts were harvested for subsequent analysis. All animal experiments and euthanasia protocols were conducted in accordance with the National Institutes of Health guidelines for humane treatment of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine.

**mRNA analysis.** Total RNA was isolated from left ventricular (LV) tissue of 3-mo-old male mice by the RNAzol method (Tel-Test, Friendswood, TX), as described previously (18). RNA was quantified spectrophotometrically, reverse transcription was performed, and quantitative PCR was carried out on a 7500 Sequence Detector (Applied Biosystems, Foster City, CA) using the mouse-specific primer-probe sets shown in Supplemental Table 1. (The online version of this article contains supplemental data.)

**Protein analysis.** Protein extracts from cardiac tissue of 3-mo-old male mice were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed using antibodies against SERCA2 (Affinity Bioreagents, Golden, CO), Na/Ca exchanger (Swant, Bellinzona, Switzerland), PLN (Zymed, Carlsbad, CA), phospho-PLN (Cyclacel, Dundee, UK), and actin (Sigma Chemicals, St. Louis, MO). Detection was performed by measuring chemiluminescent signal, as assayed by enhanced chemiluminescence (Amersham, Princeton, NJ). Mitral annulus velocity measurement was performed from the same four-chamber view by placing the TDI sample volume on the lateral portion of the mitral annulus. Digitally acquired echocardiographic images were analyzed offline by two observers blinded to the genotype of the animals.

For cardiac catheterization, 3-mo-old male mice were anesthetized with a mixture of xylazine (16 mg/kg) and ketamine (80 mg/kg). Closed-chest cardiac catheterization was performed by identifying and cannulating the right carotid artery and advancing a 1.4F Millar catheter into the ascending aorta, where it was secured. Hemodynamic measurements were then recorded. After acquisition of baseline data, dobutamine was serially infused at rates of 2, 4, 8, 16, and 32 ng·g\(^{-1}\)·min\(^{-1}\). Echocardiography and catheterization were performed by the Mouse Cardiac Phenotyping Core in the Center for Cardiovascular Research at Washington University School of Medicine.

**Mitochondrial respiration.** Mitochondria were isolated from both ventricles of 3-mo-old mice using a trypsin digestion procedure, as previously described (38). Briefly, tissue was minced, washed, and suspended in isolation medium (300 mM sucrose, 0.2 mM EDTA, 10 mM HEPES, pH = 7.4). Following digestion, tissues were gently homogenized with a Teflon-glass homogenizer (Eberbach, Ann Arbor, MI). Following centrifugation and washes, isolated mitochondria were resuspended in suspension buffer (230 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 20 mM Tris·HCl, 5 mM K$_2$HPO$_4$, pH = 7.4). Total protein was quantified by a bicinchoninic acid assay (Pierce, Rockford, IL), mitochondria (300 μg protein/ml) were placed in respiration media (125 mM KCl, 20 mM HEPES, 3 mM Mg- acetate, 0.4 mM EGTA, 0.3 mM dithiothreitol, 5 mM KH$_2$PO$_4$, 0.2% BSA, pH = 7.4) containing malate (5 mM) and either palmitoylcarnitine or pyruvate (10 mM), and respiration was determined as previously described (35) at 25°C using an optical oxygen sensor (FOXY Probe, Ocean Optics, Dunedin, FL). Following measurement of basal respiration, maximal (ADP-stimulated) respiration was determined by adding 1 mM ADP. Uncoupled respiration was evaluated following addition of oligomycin (1 μg/ml). The solubility of oxygen in the respiration buffer at 25°C was taken as 246.87 mM O$_2$/ml. Respiration rates were expressed as nanomoles of O$_2$ per minute per milligram of protein.

**Measurement of myocyte contractility and Ca\(^{2+}\) transients.** Ventricular myocytes were isolated from 3-mo-old female mice, and single-cell contraction and Ca\(^{2+}\) transients were measured as described (12, 19). Briefly, unloaded cells were maintained with normal Tyrode solution and stimulated with bipolar stimulating electrodes. A video edge detection system (Cresent Electronics, Sandy, UT) was used to measure myocyte length, and data were collected and analyzed using Axon Instruments (Molecular Devices, Sunnyvale, CA). Isolated myocytes were incubated 20 min at room temperature (24 ± 2°C) with 10 μM Fluo-LOJO (a leakage-resistant version of Fluo-4 acetoxyethyl ester, TefLabs, Austin, TX). Cells were superfused with 0.9 mM Ca\(^{2+}\) normal Tyrode solution for 20 min to wash out excess indicator and allow deesterification. Cells were held stimulated at 0.5 Hz until steady state, followed by a rapid application of 10 mM caffeine to release sarcoplasmic reticulum (SR) Ca\(^{2+}\) and to measure Ca\(^{2+}\) extrusion via Na/Ca exchanger (3). Fluo-LOJO was excited at 490 ± 3 nm and emitted fluorescence measured at 530 ± 20 nm. Background fluorescence (F) was subtracted before F/F0 was calculated (where F$_0$ is diastolic F under control conditions). Intracellular Ca concentration ([Ca\(^{2+}\)]c) decline is based on single-exponential (τ) curve fits.

**Statistical analysis.** The data are presented as means ± SE. One-way analysis of variance was used to evaluate heart mass and isolated cell mechanics. Individual mean differences were assessed using the Student-Newman-Keuls method. Unpaired t-tests were used for evaluating the effect of Cre-recombinase on gene expression, echocardiographic analysis, or mitochondrial respiration. Analysis of covariance was used to evaluate the effect of genotype and dobutamine infusion.
for cardiac catheterization studies. An α-level of 0.05 was used to indicate statistical significance.

RESULTS

Abnormal postnatal cardiac growth and progressive cardiomyopathy in csCnb1−/− mice. Mice with cardiac-specific disruption of the gene encoding the Cn regulatory subunit B1 (csCnb1−/− mice) were generated by crossing mice possessing two floxed Ppp3r1 alleles with mice expressing Cre recombinase under control of the α-myosin heavy chain (MHC) promoter (40). Our initial analysis of the csCnb1−/− mice involved phenotypic characterization within the first 2 mo of life (40). By 3 wk of age, Cnb1 expression is reduced to ~15% of normal in whole hearts of the csCnb1−/− mice. At 2 mo of age, csCnb1−/− mice appeared grossly normal without evidence of neonatal lethality, based on Mendelian ratios (Ref. 40 and data not shown). Beginning at 2½ mo of age, the csCnb1−/− mice exhibited increased postnatal mortality compared with mice homozygous for the floxed allele, but lacking Cre (Cnb1+/− mice) or MHC-Cre control mice (Fig. 1). Approximately 50% of the csCnb1−/− mice died by 4 mo of age, with 100% mortality by age 7 mo (Fig. 1). Additionally, of 14 female csCnb1−/− breeders, none survived beyond 2 wk postpartum. In those few animals in which we observed mortality, death occurred following a period (~1 day) of lethargy. None of the MHC-Cre mice exhibited death or cardiac dysfunction by echocardiography (data not shown), and there was no increase in mortality during the study period (Fig. 1). Continuous telemetric electrocardiographic monitoring revealed no significant rhythm disturbances (data not shown).

Cardiac biventricular (BV) to BW ratios (BV/BW) were normal in 1-wk-old csCnb1−/− mice, consistent with the maintenance of Cnb1 expression during the first week of life. However, on loss of Cnb1 protein expression, and throughout the remainder of life, the mean BV/BW in csCnb1−/− mice was ~10% less than that of littermate csCnb1+/+ controls (Fig. 2A). Total BW was not different among the groups (data not shown). The gross appearance of hearts from 3- to 6-wk-old csCnb1−/− mice was normal, except that the ventricles of the mutant mice were slightly smaller (data not shown). However, beginning at 2–3 mo of age, the atria became massively dilated in the absence of ventricular dilatation (Fig. 2B). Although ventricular weights were less than normal, expression of the ANF and BNP genes was elevated in the ventricles of csCnb1−/− mice (Fig. 2C).

Echocardiographic analysis revealed marked reduction in systolic function and a decrease in relative wall thickness in csCnb1−/− mice as young as 1 mo of age (Fig. 3A and Supplemental Table 2). Strikingly, however, the ventricles of the mutant mice did not exhibit diastolic dilatation, even at 4 mo of age (Supplemental Table 2). Doppler recordings of transmural blood flow velocities and TDI of mitral annular velocities revealed characteristic patterns of restrictive physiology with elevated LV filling pressure, including decreased E wave deceleration times, diminished Ea velocity (peak velocity of early diastolic mitral annular flow), and an increased ratio of transmural E to mitral annular Ea (Fig. 3B and Supplemental Table 3).

To further investigate the cardiac phenotype of the csCnb1−/− mice, hemodynamics were obtained on 3-mo-old mice via closed-chest cardiac catheterization at baseline and following an infusion of the β-adrenergic agonist dobutamine. At baseline, LV end-diastolic pressure was significantly higher in the mutant mice compared with the controls. Following administration of dobutamine, heart rate and the rates of pressure development and relaxation failed to increase appropriately in the csCnb1−/− mice (ANOVA, P < 0.05). Tau, a separate measure of ventricular relaxation, decreased significantly less in csCnb1−/− mice than controls during the dobutamine infusion, while the elevated LV end-diastolic pressure was maintained. Additionally, the dobutamine-induced rise in maximal LV pressure was blunted in the csCnb1−/− mice during the dobutamine infusion (Fig. 4). Taken together with the morphological and echocardiographic data, these results indicate that the csCnb1−/− mice exhibit both systolic and diastolic ventricular dysfunction and features of severe restrictive physiology.

Cardiac ventricles of csCnb1−/− mice do not exhibit histological abnormalities or evidence of cell death. Despite the dramatic cardiac functional phenotype, the histological appearance of the ventricles of csCnb1−/− mice was similar to that of control mice at both 2 and 4 mo of age. Specifically, there was no evidence of infiltrate, cellular disarray, or fibrosis (Fig. 5A). Moreover, staining for activated caspase-3 was remarkable for a lack of evidence for an apoptotic process (data not shown). These results strongly suggested that the cardiomyopathy of csCnb1−/− mice was not due to major alterations in the extracellular matrix or significant myocyte death. In striking contrast, atrial sections from 4-mo-old csCnb1−/− mice showed marked fibrosis and organized thrombosis, likely related to chronically elevated atrial pressure related to the noncompliant ventricles (Fig. 5B). Thus the ventricular dysfunction preceded any sign of atrial abnormality by at least 2 mo. Of note, there was evidence of pulmonary edema, but no parenchymal damage or pulmonary vascular changes in 3-mo-old mice (data not shown).

Mild impairment of fatty acid oxidation in mitochondria isolated from ventricles of csCnb1−/− mice. The lack of structural abnormalities in the cardiac ventricles of the csCnb1−/− mice suggested that the functional phenotype could be due to a primary metabolic abnormality. We and others have shown that the Cn signaling pathway activates the expression of the
nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α and its coactivator, PGC-1α, key transcriptional regulators of cardiac mitochondrial energy metabolism (40). To determine whether derangements in mitochondrial energy production caused the cardiomyopathic phenotype, respiratory function studies were performed on mitochondria isolated from ventricles of 3-mo-old control and csCnb1−/− mice and either pyruvate or palmitoylcarnitine as substrate. State 2 respiration rates of palmitoylcarnitine-driven respiration in the csCnb1−/− mice were normal, but state 3 rates were modestly but significantly diminished in the mitochondria isolated from the csCnb1−/− mice (Fig. 6). Uncoupled respiration was not different between the groups with either substrate (Fig. 6). These results were consistent with our previous observation that the expression of PGC-1α and its downstream targets involved in mitochondrial fatty acid oxidation are modestly but significantly reduced in csCnb1−/− mice (40). Whereas the impairment of maximal rates of palmitoylcarnitine-driven respiration in the csCnb1−/− cardiac mitochondria is consistent with the known role of Cn in the regulation of the PPARα/PGC-1α pathway, this relatively minimal impairment is highly unlikely to account for the observed mortality and severe cardiac phenotype. In support of this conclusion, mRNA expression of genes encoding citrate synthase, cytochrome oxidase IV, and ATP synthase-β is maintained in the hearts of the csCnb1−/− mice (data not shown).

Impaired contractility and excitation-contraction coupling in cardiac myocytes isolated from csCnb1−/− mice. Cardiac myocytes were isolated from 3-mo-old female csCnb1−/− mice and littermate csCnb1+/+ controls to further assess contractile function. csCnb1−/− myocytes exhibited a markedly reduced fractional shortening when stimulated at 0.5 Hz in the presence of 0.9 mM Ca2+ (near normal physiological level in the mouse; Fig. 7A). However, caffeine-induced contractures, indicative of SR Ca2+ content, were not significantly altered in csCnb1−/− myocytes (Fig. 7A). When extracellular [Ca2+] was increased from 0.9 to 1.8 mM (supra-physiological for mouse, and a [Ca2+] near which contraction is maximal), there was not a significant decrease in contraction amplitude when stimulated at 0.5–2.0 Hz (Fig. 7A). However, the rates of contraction and relaxation, based on changes in measured length per time during maximal contractions (measured at 1.8 mM [Ca2+]), were significantly lower in csCnb1−/− vs. control myocytes (Fig. 7B). Thus excitation-contraction coupling is defective in the csCnb1−/− myocytes, possibly due to altered Ca2+ handling.

We then measured myocyte Ca2+ transients. At 0.5 Hz, Ca2+ transient amplitude was lower in csCnb1−/− compared
with control, and this was also true for caffeine-induced Ca$^{2+}$ transients, indicating that SR Ca$^{2+}$ content is reduced in csCnb1$^{-/-}$ myocytes (Fig. 7C). The small decrease in SR Ca$^{2+}$ content could explain the larger reduction of twitch-induced Ca$^{2+}$ transients, because of the steep relationship between SR Ca$^{2+}$ content and fractional release (41). The rate of decline of twitch and caffeine-induced Ca$^{2+}$ transients provides information about SERCA and Na/Ca exchanger Ca$^{2+}$ transport rates, respectively (19). The results are consistent with the conclusion that both SERCA2a and Na/Ca exchanger transport Ca$^{2+}$ more slowly in csCnb1$^{-/-}$ myocytes. The large reduction in SR Ca$^{2+}$ uptake rate would be expected to reduce SR Ca$^{2+}$ content, but this may be partly mitigated by the reduced Na/Ca exchange function (which would slow Ca$^{2+}$ extrusion from the cell, allowing more SR Ca$^{2+}$ uptake).

Altered expression of Na/Ca exchanger and PLN in the hearts of csCnb1$^{-/-}$ mice. As an initial step toward identifying the mechanistic basis for the observed abnormality in myocyte Ca$^{2+}$ transients in the csCnb1$^{-/-}$ mice, we quantified the expression of proteins involved in Ca$^{2+}$ release and re-uptake. Western blot studies demonstrated that the levels of the Na/Ca exchanger were significantly lower in the csCnb1$^{-/-}$ hearts (Fig. 8A). Expression of SERCA2a was not different in the csCnb1$^{-/-}$ mice compared with control (Fig. 8A). Additionally, no difference was seen in the levels of PLN protein expression. However, the ability of PLN to inhibit SERCA2a depends on its phosphorylation state. Therefore, we also determined the amount of phospho-PLN using antibodies specific to sites Ser$^{16}$ and Thr$^{17}$. Both antibodies demonstrated that the phosphorylation state of PLN was markedly lower in the csCnb1$^{-/-}$
hearts (Fig. 8A). No difference was detected in the mRNA expression of any target Ca\(^{2+}\) handling genes, indicating that, although Cn is known to play a role in excitation-transcriptional coupling, expression of these genes is not downregulated by loss of Cn activity (Fig. 8B).

**DISCUSSION**

Previous studies using mouse systems to overexpress Cn* or Cn inhibitory proteins have revealed that Cn signaling plays a critical role in the transduction of cardiac myocyte Ca\(^{2+}\).
Cardiac Calcineurin Deficiency

signals to pathological hypertrophic growth and to regulation of normal energy metabolism (40, 42, 44). Targeted gene disruption strategies in mice have confirmed a role for Cn signaling in pathological cardiac remodeling in response to pathophysiological stress, such as pressure overload (32, 33). However, the minimal baseline phenotype of previous CnA gene loss-of-function lines (α or β) has not provided a complete understanding of the biological necessity of this signaling pathway in postnatal heart, presumably due to compensation by the other CnA isoform. Recently, we generated mice with cardiac-specific deficiency of the Cn B1 regulatory subunit (csCnb1−/−) and found that they survived following birth, despite modest alterations in the expression of genes involved in mitochondrial fatty acid oxidation due to derangements in PGC-1α/PPAR-α signaling (40). As described here, the severe adult phenotype of the csCnb1−/− mice provides new information about the importance of Cn signaling in postnatal cardiac growth, ventricular contractility, and myocyte excitation-contraction coupling.

CsCnb1−/− mice die of heart failure due to a cardiac phenotype that includes a postnatal growth defect and an unusual cardiomyopathy that exhibits systolic ventricular dysfunction, combined with diastolic abnormalities and restrictive features. It is possible, given the surprising lack of ventricular dilatation, that the cardiomyopathy of the csCnb1−/− mice reflects, in part, the observed growth defect. Cardiac mass is normal in 1-wk-old csCnb1−/− mice, but, thereafter, is significantly lower than that in wild-type controls. These results are consistent with the known role of Cn signaling in programs directing cardiac hypertrophy (10, 24, 28, 36) and indicates that Cn signaling is necessary for normal physiological growth of the postnatal heart, as well as pathological forms of hypertrophy. Given the known role of Cn in driving hypertrophy, and the fact that the phenotype is mainly manifest in the postnatal period, it is likely that the reduced heart mass is due to size reduction of individual cells, rather than a loss in cell number. This conclusion is further supported by the absence of markers of increased cell death. However, our results do not exclude the possibility that reduced cell number does not contribute to the smaller heart size. It should also be noted that, due to the limitations of Cre-recombinase targeting approaches, we cannot be sure that a small residual amount of CnA remains in the csCnb1−/− mice.

Beginning at 2 mo of age, the atria of the csCnb1−/− mice become massively dilated in the absence of ventricular dilatation, also consistent with severe ventricular diastolic dysfunction. Doppler recordings of transmural blood flow velocities, TDI of mitral annular velocities, and invasive hemodynamic measurements confirmed characteristic patterns of diastolic ventricular dysfunction and restrictive physiology in the mutant mice (with the exception of structural abnormality). Two-dimensional echocardiographic analysis revealed marked reduction in systolic function and a decrease in relative wall thickness in csCnb1−/− mice, yet the ventricles did not exhibit diastolic dilatation.

Surprisingly, despite evidence of a severe cardiomyopathy, we did not find structural or cellular derangements, including fibrosis, cell death, or infiltration in the myocardium of the csCnb1−/− mice. These findings led us to explore a metabolic basis for the cardiomyopathy. Recently, we found reduced expression of transcriptional activators (PGC-1α, PPAR-α) and downstream target genes involved in mitochondrial energy metabolism in the hearts of young csCnb1−/− mice (40). However, other than a modest impairment in maximal rates of palmitoylcarnitine-driven respiration, mitochondrial function was normal in the hearts of the csCnb1−/− mice, making energy metabolic derangements as the primary cause of the cardiomyopathy unlikely. Therefore, we assessed cardiac myocyte excitation-contraction properties in the mutant mice. CsCnb1−/− myocytes exhibited a markedly reduced fractional shortening, which was improved by increasing the [Ca2+] in the media, yet rates of contraction and relaxation (changes in measured length per time) remained abnormal, despite improved Ca2+ transient amplitudes. These results suggested a defect in myocyte Ca2+ transients, which was confirmed. CsCnb1−/− myocyte Ca2+ transients exhibited reduced amplitude compared with control, and the SR Ca2+ content was diminished, as evidenced by reduced caffeine-induced Ca2+ transients, consistent with a reduced SR Ca2+ content. The rate of decline of twitch and caffeine-induced Ca2+ transients suggested functional impairment of both SERCA2a and Na/Ca exchanger in csCnb1−/− myocytes. Indeed, we found that the levels of the Na/Ca exchanger were reduced in the hearts of the mutant mice. In addition, although both SERCA2a and its negative modulator, PLN, were expressed at normal levels in the csCnb1−/− mice, the phosphorylation state of PLN was...
Fig. 7. Altered contractile parameters of isolated adult cardiac myocytes from csCnb1⁻/⁻ mice. A: mean contraction amplitudes as a percentage of total cell length of cardiac myocytes (n = 13 cells per group) isolated from csCnb1⁺/⁺ and csCnb1⁻/⁻ ventricles measured at 1 Hz and 0.9 mM Ca²⁺ (left) or 0.5–2 Hz and 1.8 mM Ca²⁺ (right). B: mean rates of contraction (left) and relaxation (right) of cardiac myocytes maintained in 1.8 mM Ca²⁺ and stimulated at 0.5–2 Hz. C: Ca²⁺ transients induced by electrical stimulation (0.5 Hz) or rapid exposure to 10 mM caffeine (0.9 mM Ca²⁺ concentration). Amplitudes (left) and time constants (τ) of intracellular Ca²⁺ (Cai) concentration decline (right) are shown. *P < 0.05 compared with controls at the same contraction frequency. WT, wild type; KO, knockout; F/F₀, ratio of background fluorescence to diastolic fluorescence under control conditions.
greatly diminished. PLN, an integral membrane protein of the SR, functions as a modulator of the SERCA2a Ca\(^{2+}\) pump. In its dephosphorylated state, PLN binds to SERCA2a and inhibits its pump function (22). By dephosphorylating PLN, Cn acts to enhance the kinetics of Ca\(^{2+}\) handling, as demonstrated in mice overexpressing activated Cn (8).

Phosphorylation of PLN at Ser\(^{16}\) and Thr\(^{17}\) occurs in response to \(\beta\)-adrenergic signaling via PKA and Ca\(^{2+}\)-triggered events through CAMKII, respectively (11, 23). We found that both sites are equally and nearly completely dephosphorylated in the csCnb1 \(^{-/-}\) mice, indicating that Cn is necessary for the activity of these signaling pathways. In the absence of Cn signaling, SERCA2a is subject to inhibition by PLN, contributing to cardiac dysfunction. The specific function for phosphorylation at each site (2, 5, 26, 46) and their interaction is an area of active investigation (23). The lack of Cn, a phosphatase, cannot directly explain the lower phosphorylation, so the effect must be indirect. This indirect pathway must also differ from that in the failing heart, where Cn is activated and PLN phosphorylation reduced (29). Phosphatases other than Cn (especially PP1) are the primary determinants of the phosphorylation state of PLN. Direct links between Cn and the PKA and CAMKII circuits have not been fully defined. In fact, Cn has been shown to oppose both CAMKII (by dephosphorylation of Thr\(^{17}\)) and PKA (by opposing the inhibition of PP1 by PKA) (23). It is possible that, in the context of chronic ablation of Cn action, the actions of PP1 or other relevant phosphatases become dominant in this system. However, the hearts of mice overexpressing Cn\(^{+}\) exhibit increased phosphorylation of PLN (8), consistent with our results. In addition, others have suggested that interactions between Cn and MAPK signaling cascade (39) can modulate PLN phosphorylation. Further studies will be necessary to define the links between Cn signaling and PLN.

In addition to SERCA2a, Ca\(^{2+}\) is removed from the cytosol by the Na/Ca exchanger (\(\text{Slc8a1}\)), extruding Ca\(^{2+}\) across the plasma membrane. We found that the expression of the \(\text{Slc8a1}\) Na/Ca exchanger is reduced at the protein level in the csCnb1 \(^{-/-}\) mice, although mRNA levels are unchanged. Thus loss of Cn activity likely alters either translation and/or stability of this exchanger. Loss of Na/Ca exchanger activity is consistent with the slowed intracellular [Ca\(^{2+}\)] decline during caffeine exposure, as the SERCA pumps are inactivated and would tend to limit the decline in SR Ca\(^{2+}\) content (3, 19, 41).
However, cardiac-specific Na/Ca exchanger gene ablation in mice is associated with very limited impairment of contractility and no observed mortality (15). Thus the slightly reduced Na/Ca exchanger expression and function is unlikely to be causative of depressed cardiac function in the csCnb1−/− mice. Rather, the reduction of Na/Ca exchanger activity may be compensatory in these animals, as the dramatic reduction in SERCA2a pump activity would be expected to deplete SR Ca2+ stores with normal Na/Ca exchanger function.

Reduction in SERCA2a activity via PLN-mediated inhibition is a likely contribution to the impaired ventricular systolic and diastolic function in the csCnb1−/− mice. The impact of PLN on SERCA2a activity and cardiac function has been nicely demonstrated using gene ablation and overexpression strategies in mice. PLN loss of function results in decreased diastolic and systolic ventricular function (17), a phenotype similar to that of the csCnb1−/− mice. Transgenic mice overexpressing glycogen synthase kinase-3β, which opposes Cn action, exhibit diastolic dysfunction with impaired Ca2+ kinetics (25). The precise link between CnA signaling and PLN phosphorylation has not been established previously or by our results. It is possible that CnA acts within the PKA and CAMKII circuits. Alternatively, CnA could control a postnatal developmental maturation program that is required for proper control of PLN.

In summary, we conclude that loss of Cn signaling in the postnatal heart results in an impairment in postnatal growth and chronic PLN-mediated inhibition of SERCA2a, leading to a cardiomyopathy with features of impaired ventricular relaxation and systolic function, in the absence of ventricular remodeling.

ACKNOWLEDGMENTS

We thank Mary Wingate and Shonna Hyde for expert assistance in the preparation of the manuscript, Jefferson Gomes for assistance in the preparation of adult mouse cardiac myocytes, and Deanna Loseke for assistance with the Na/Ca exchanger and SERCA2a protein immunoblotting experiments. We also thank Dr. Gerald Crabtree for providing the Cnb1 “floxed” mice.

Present addresses: P. J. Schaeffer, Department of Zoology, Miami University, Oxford, OH 45056; T. C. Leone and D. P. Kelly, Burnham Institute for Medical Research, Orlando, FL 32827.

GRANTS

This work was supported by National Institutes of Health Grants F32 AR48758 and RO1 HL058427, the Digestive Diseases Research Core Center GRANTS Medical Research, Orlando, FL 32827.

REFERENCES


