Enhanced myocyte contractility and Ca\(^{2+}\) handling in a calcineurin transgenic model of heart failure

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Abstract

**Objective:** Impaired myocyte Ca\(^{2+}\) handling is a common characteristic of failing hearts and increases in calcineurin activity, a Ca\(^{2+}\)-sensitive phosphatase, have been implicated in heart failure phenotype. Transgenic mice with cardiac-specific expression of an active form of calcineurin display depressed function, hypertrophy and heart failure. We examined whether defects in cardiomyocyte Ca\(^{2+}\) handling properties contribute to the impaired cardiac function in calcineurin transgenic mice.

**Methods:** The levels of SR Ca handling proteins, SR Ca\(^{2+}\) transport function and cardiomyocyte mechanics, as well as Ca\(^{2+}\) kinetics were examined in mice overexpressing a constitutively active form of calcineurin.

**Results:** Transgenic expression of activated calcineurin catalytic subunit resulted in significant protein increases (66\%) in SERCA2 and decreases (35\%) in phospholamban, as well as enhanced (\(\geq 80\%\)) phospholamban phosphorylation. These alterations in the SR Ca\(^{2+}\)-transport proteins resulted in increased \(V_{\text{max}}\) and Ca\(^{2+}\)-affinity of SERCA2. The myofibrillar Mg-ATPase activity was also significantly increased at \(p\text{Ca}>6.0\). The enhanced SR Ca\(^{2+}\) handling and Mg-ATPase activity reflected significant elevation in myocyte contractile parameters (3-fold), Ca\(^{2+}\) transient amplitude (1.5-fold) and the rate of Ca\(^{2+}\) signal decay (2-fold). In contrast, in vivo cardiac function assessed by echocardiography, indicated severely depressed contractility in calcineurin hearts. The apparent disparity in contractile properties between the cellular and multicellular preparations may be partially due to tissue remodeling, including interstitial fibrosis and a marked reduction (45\%) dephosphorylation (81\%) and redistribution of the gap junctional protein connexin-43, which could compromise intercellular communication.

**Conclusion:** Despite enhanced SR Ca\(^{2+}\) handling and contractility in myocytes, pathological remodeling and defects in intercellular coupling may underlie contractile dysfunction of the calcineurin hearts.

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1. Introduction

Cardiac hypertrophy is an adaptive response to various congenital or acquired stimuli to balance the increased stress by reducing wall tension. In response to hypertrophic signals, a fundamental reprogramming occurs in adult cardiac myocytes, which is associated with the expression of genes encoding fetal protein isoforms [1]. Several studies have implicated Ca\(^{2+}\) as a primary inducer of hypertrophic growth through pathways involving angiotensin II, endothelin-1, adrenergic and Ca\(^{2+}\) channel agonists [2–7]. These stimuli result in activation of second messenger systems, which in turn elevate intracellular Ca\(^{2+}\) concentrations and inotropy. At the onset of hypertrophy, the amplitude of the intracellular Ca\(^{2+}\) transient...
increases and alterations in key Ca\(^{2+}\) handling proteins and signaling pathways occur to accommodate the altered Ca\(^{2+}\) homeostasis [8]. One of these signaling pathways involves calcineurin, the type 2B Ca\(^{2+}\)/calmodulin-dependent phosphatase [9]. Activated calcineurin dephosphorylates the NFAT transcription factors, allowing their translocation into the nucleus and induction of fetal cardiac gene expression. The role of calcineurin in the development of cardiac hypertrophy was originally described by Molkentin et al. [10], through transgenic expression of the active form of the enzyme in mouse hearts. Hypertrophy in the calcineurin transgenic mouse occurs as early as 18 days of age, and it progresses to dilated cardiomyopathy within 8–12 weeks with profound myocardial fibrosis, collagen deposition and depressed cardiac function [9,10]. Targeted inhibition of calcineurin has been shown to attenuate cardiac hypertrophy associated with pressure overload or hypertrophic agonists, such as angiotensin II and phenylephrine [9,11–14], suggesting a strong link between intracellular Ca\(^{2+}\)-calcineurin signaling and reprogramming of cardiac muscle gene expression.

Although evidence has been accumulating on the role of calcineurin and its substrates in transcriptional cascades, there is currently little information on the functional significance of this enzyme in modulating Ca\(^{2+}\) handling proteins, which may alter contractile parameters in cardiac muscle. In yeast, calcineurin was shown to induce the expression of genes encoding Ca\(^{2+}\) or Na\(^{+}\)-pumping ATPases [15–17] and to regulate SERCA activity [18]. In mammalian hearts, SERCA2 is the primary mediator of sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake, and its activity is under reversible regulation by phospholamban (PLB). Dephosphorylated PLB decreases SERCA2’s Ca\(^{2+}\) affinity, whereas phosphorylation of PLB relieves its inhibition by dissociating PLB from SERCA2. Calcineurin transgenic hearts have been reported to express decreased SERCA transcript levels [10] and this may result in depressed SR Ca\(^{2+}\) uptake and contractility. Calcineurin may also directly dephosphorylate PLB [19] or promote its dephosphorylation by activating a type-1 phosphatase [20] in the SR, leading to decreased Ca\(^{2+}\) affinity for SERCA. Thus, the depressed contractile function in calcineurin hearts may be partially due to depressed SR Ca\(^{2+}\) uptake activity, reflecting overall attenuated cell Ca\(^{2+}\) cycling. To determine the role of calcineurin on SR function and myocyte Ca\(^{2+}\) homeostasis, we examined the levels of SR Ca\(^{2+}\) handling proteins, SR Ca\(^{2+}\) transport rates and cardiac myocyte mechanics, as well as Ca\(^{2+}\) kinetics properties in calcineurin hearts. Surprisingly, our findings indicate alterations in the protein levels of SERCA and PLB, as well as the degree of PLB phosphorylation, contributing to enhanced SR Ca\(^{2+}\) handling and myocyte contractility in hearts, which expressed an activated form of calcineurin. However, alterations in connexin-43 (Cx43) expression and its phosphorylation may compromise intercellular communication, leading to depressed function in vivo.

2. Methods

2.1. Transgenic mice

Transgenic mice expressing activated calcineurin in the heart were previously described [10]. Calcineurin transgenic mice (line 37) and their wild type (WT) littermates between 4 and 6 weeks of age were used in the present study. The investigation conforms 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), and the Responsible Care and Use of Animals on Research and Education manual published by the University of Cincinnati.

2.2. Quantitative immunoblotting

Mouse hearts were homogenized, subjected to 13% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and blotted onto nitrocellulose or PVDF membranes, as described previously [21]. The membranes were then reacted with: either mouse monoclonal antibodies to PLB and ryanodine receptor (Affinity Bioreagents Inc., Golden, CO); or rabbit polyclonal antibodies to SR Ca\(^{2+}\)-ATPase, and calsequestrin (a gift from Dr Larry Jones, Indiana University). Membranes were processed [21] and the primary antibody binding was detected by ECL (Amer sham Pharmacia Biotech). To determine the total Cx43 levels, cardiac homogenates were subjected to 10% SDS–PAGE, blotted and probed with a polyclonal antibody to Cx43 (C6219; Sigma, St. Louis, MO and Zymed) and calsequestrin (a gift from Dr Larry Jones, Indiana University). Membranes were processed [21] and the primary antibody binding was detected by ECL (Amer sham Pharmacia Biotech). To determine the total Cx43 levels, cardiac homogenates were subjected to 10% SDS–PAGE, blotted and probed with a polyclonal antibody to Cx43 (C6219; Sigma, St. Louis, MO and Zymed) and processed as previously described [22]. For assessment of Cx43 phosphorylation, blots were probed with a monoclonal antibody (P2C4 raised to the last 23 amino acids of Cx43), which preferentially recognizes non-phosphorylated Cx43. The relative Cx43 phosphorylation levels were determined by subtracting the signal for the dephosphorylated Cx43 from the total Cx43.

2.3. SR Ca\(^{2+}\) uptake assays

Oxalate-supported initial rates of SR Ca\(^{2+}\) uptake were determined using 75 μg cardiac homogenate/assay, as described [23]. The reaction was initiated by the addition of ATP (final concentration, 5 mmol/l). The initial rates of Ca\(^{2+}\) uptake were calculated by least-squares linear regression analysis of Ca\(^{2+}\) uptake at 30, 60, and 90 s. Data were analyzed by nonlinear regression (Origin software).

2.4. Myofibrillar Mg\(^{2+}\)-ATPase activity

Myofibrillar Mg\(^{2+}\)-ATPase activity was determined in the myofibrillar preparations using the following incubation medium: 66 mM KCl, 60 mM imidazole, 6 mM MgCl\(_2\), 5 mM EGTA, and 5.33 mM ATP at pH 7.0. The reaction was initiated with the addition of the sample and stopped with 15% trichloroacetic acid after 5 min. The
reaction mixture was centrifuged and inorganic phosphate that was produced by the ATP hydrolysis was measured in the supernatant fraction by standard methods [24,25].

2.5. Myocyte studies

Single left ventricular myocytes were isolated from calcineurin and WT hearts [26,27]. For both mechanical and Ca$^{2+}$ transient measurements (Fura-2 loaded cells), isolated myocytes were perfused with Tyrode solution at room temperature (20–22 °C) and stimulated at 0.5 Hz, as previously described [28].

2.6. Echocardiography

Echocardiographic measurements were performed on 4–6-week-old WT and CLN mice, as described [9].

2.7. Immunohistochemistry

Frozen sections of hearts were prepared [29] and immunohistochemistry performed as previously detailed [30].

2.8. Electrocardiography

Unipolar needle electrodes were placed subcutaneously in the right and left axillary areas and the right and left lower abdomen of anesthetized mice. ECG leads I and III were recorded directly using two ECG amplifiers connected in series to a data acquisition unit. ECG leads II, aVR, aVL, and aVF were calculated and all six leads were recorded and displayed using data acquisition software (AccuKnowledge, Biopac, Santa Barbara, CA). Two 30-s ECG strips were recorded (2000 data points per second). The QRS duration was directly measured using an on-line caliper. The corrected QT interval (QTc) was calculated as the QT interval divided by the square root of the preceding R–R interval.

2.9. Statistics

Data are presented as mean±S.E.M. The number (n) of mice used is indicated. Statistical analysis was performed by Student’s t-test for comparisons between WT and calcineurin mice. Values were also tested by ANOVA using SUPER ANOVA software from Abacus, where applicable.

3. Results

3.1. Levels of phosphorylated phospholamban and SR Ca$^{2+}$-cycling proteins

To determine whether activation of calcineurin is associated with decreased PLB phosphorylation, which would reduce SR Ca$^{2+}$ uptake and may impair cardiac contractility, calcineurin transgenic mice (line 37) at 4–6 weeks of age were characterized in parallel with their isogenic wild-types. Surprisingly, quantitative immunoblotting revealed increases in pSer-16 (1.8-fold) in PLB (Fig. 1A and B), while there were no significant alterations in pThr-17–PLB, as revealed by the phosphorylation site-specific

![Fig. 1. Quantitative immunoblotting of SR Ca$^{2+}$-cycling proteins in wild-type (WT) and calcineurin (CLN) hearts. (A) Western blots of phospholamban (PLB), PLB with serine-16 phosphorylated (pSer-16), and SR Ca$^{2+}$-ATPase (SERCA). Various concentrations of pooled cardiac WT homogenates were used as standard for PLB (12, 8, 4, 2 μg), or for PLB pSer-16 and SERCA (20, 15, 10, 5 μg). (B) Quantitation of PLB and phosphorylation of Ser-16 in PLB, SERCA, CSQ and RyR expression levels in CLN (n=4) relative to WT (n=4) hearts. The solubilized samples were boiled for 5 min to fully dissociate the pentameric form of PLB into monomers. (C) Ratio of PLB/SR Ca$^{2+}$-ATPase in CLN relative to WT hearts. * P<0.05 vs. WT.](image-url)
antibodies. Similar results were obtained using $^{32}$P-labeled myocytes (data not shown). Furthermore, the total PLB protein levels decreased by $35\pm3\%$ in calcineurin hearts compared to WTs (100\%) (Fig. 1A and B), indicating a 4-fold increase in the relative PLB phosphorylation level, which should diminish its inhibitory effects on SERCA. In the same hearts, the SERCA protein levels were increased by $66\pm5\%$, compared to WTs. Thus, the ratio of PLB to SERCA was 0.4 in calcineurin compared to WT (1.0) hearts (Fig. 1C). Taken together, these findings on increased PLB phosphorylation and decreased PLB/SERCA ratio indicate that a larger portion of the SR Ca$^{2+}$-pumps were in the ‘uninhibited state’ relative to WTs. The alterations in the SR Ca$^{2+}$-uptake complex were not associated with any changes in the levels of the Ca$^{2+}$ storage protein, calsequestrin, or the Ca$^{2+}$-release channel, ryanodine receptor, in calcineurin hearts (Fig. 1B).

3.2. SR Ca$^{2+}$-uptake function

To evaluate whether the alterations in SR proteins were accompanied by enhanced SR Ca$^{2+}$-function in calcineurin hearts, Ca$^{2+}$-uptake assays were performed in calcineurin and WT cardiac homogenates, under conditions that restrict Ca$^{2+}$-uptake to SR vesicles [31]. The initial rates of Ca$^{2+}$-uptake indicated an increased $V_{\text{max}}$ in calcineurin hearts ($64\pm3.1$ nmol/mg/min, $n=3$), compared to WT ($44.8\pm3.0$ nmol/mg/min, $n=3$, $P<0.05$) hearts (Fig. 1C). Taken together, these findings on increased PLB phosphorylation and decreased PLB/SERCA ratio indicate that a larger portion of the SR Ca$^{2+}$-pumps were in the ‘uninhibited state’ relative to WTs. The system was decreased in calcineurin hearts (0.14$\pm$0.02 μM) versus WT (0.23$\pm$0.01 μM, $P<0.05$) hearts, reflecting the decreased PLB/SERCA ratio and the increased PLB phosphorylation. The enhanced SR Ca$^{2+}$-uptake function was also associated with increased SR Ca$^{2+}$ loading in

Fig. 2. SR Ca$^{2+}$-uptake rates. (A) Initial rates of SR Ca$^{2+}$-uptake in CLN ($n=3$) and WT ($n=3$) cardiac homogenates as a function of various pCa. (B) Time-course of SR Ca$^{2+}$-uptake at pCa 5.5. Data represents mean$\pm$S.E.M.
calcineurin (746±37 nmol/mg) compared to WT (251±17 nmol/mg, P<0.05) hearts (Fig. 2B). Thus, the SR function was significantly enhanced in hearts overexpressing calcineurin.

3.3. Myofibrillar Ca$^{2+}$-stimulated Mg-ATPase activity

The increased SR Ca$^{2+}$-uptake rates prompted us to examine whether there were any alterations at the level of the contractile apparatus in calcineurin hearts. Thus, myofibrillar preparations were isolated from calcineurin and WT hearts and the Mg-ATPase activity was measured over a wide range of Ca$^{2+}$ concentrations. The myofibrils from calcineurin hearts exhibited a leftward shift in the pCa–force relationship, compared to WTs (Fig. 3). Moreover, the maximal Ca$^{2+}$-stimulated Mg-ATPase activity was significantly higher in calcineurin, compared to WT fibers. Thus, the Ca$^{2+}$-sensitivity and the maximal velocity of the myofibrillar Mg-ATPase were significantly enhanced by calcineurin overexpression.

3.4. Myocyte mechanics and Ca$^{2+}$ transients

Based on the augmented SR Ca$^{2+}$ handling properties and increased Mg-ATPase activity in calcineurin hearts, it was expected that cardiac myocyte Ca$^{2+}$ cycling and contractility would be enhanced. Thus, left ventricular myocytes were isolated from calcineurin and WT hearts and their mechanical and Ca$^{2+}$ kinetic parameters were assessed at room temperature and a stimulation frequency of 0.5 Hz. These conditions were chosen to favor stability of cells [32] and allow comparison of the present findings with several literature reports [28,33–35]. Calcineurin myocytes were significantly longer (227.4±4.9 pF, n=208) compared with WTs (118.9±2.2 pF, n=174, P<0.01), consistent with the hypertrophic phenotype of calcineurin mice [10]. Furthermore, calcineurin myocytes exhibited significantly increased fractional shortening (171%), rate of cell shortening (+dL/dt: 303%) and rate of cell relengthening (−dL/dt: 276%) compared to WTs (100%) (Fig. 4B,C and Table 1). Analysis of Ca$^{2+}$ transients (Fig. 4D) indicated that there was no alteration in baseline cytosolic Ca$^{2+}$ levels, but the amplitude of the Ca$^{2+}$ signal was significantly increased in calcineurin (165%), compared to WT (100%) myocytes. The rate of Ca$^{2+}$ transient decay (T$_{90}$) was also significantly faster (52%) in calcineurin myocytes (Table 1), reflecting the enhanced SR function in these cells.

3.5. In vivo cardiac function

The enhanced Ca$^{2+}$ cycling and contractile properties observed in calcineurin myocytes isolated from 4–6-week-old mice were in contrast to previous in vivo studies in 8–12-week-old transgenics, which indicated depressed cardiac function [9]. To determine whether calcineurin mice exhibit depressed cardiac function at this earlier age or whether the previously reported phenotype was altered through propagation of the transgenic line, we re-assessed cardiac function and histology in calcineurin mice at 4–6 week of age. Calcineurin mice exhibited depressed left ventricular function (Fig. 5A) and left ventricular dilation (Fig. 5B), as determined by echocardiography. Furthermore, there was myocyte disarray (Fig. 5D) and interstitial fibrosis (Fig. 5F) in calcineurin transgenic hearts, which was absent in WT hearts (Fig. 5C,E).

3.6. Connexin 43 expression, phosphorylation and distribution

The discord between the contractile function assessed in isolated myocytes and intact hearts, suggested that intercellular coupling between the calcineurin myocytes may be compromised, and thus, unable to efficiently transduce the mechanical forces generated at the cellular level to multicellular preparations. This finding prompted us to examine the expression patterns of Cx43, which is the predominant component of gap junctions in cardiomyocytes and is important in intercellular communication [36]. Immunohistochemical analysis of frozen sections for Cx43 revealed a marked reduction in antibody reactivity in calcineurin ventricles (Fig. 6A) compared to WTs (Fig. 6B). The decrease in Cx43 staining was not localized to the lesioned areas, but occurred throughout the cal-
Fig. 4. Cardiac myocyte contraction and Ca^{2+} kinetics in WT and CLN cardiomyocytes. Single cardiomyocytes isolated from sex- and age-matched CLN and WT mice (A). Bar=50 μm. Representative recordings of myocyte cell shortening (B), rates of shortening and relengthening (C) and Ca^{2+} transients (D) from WT and CLN hearts.

calcineurin hearts. Furthermore, both the pattern and overall distribution of the signal were disarrayed in longitudinal (Fig. 6A,B) and cross sections (data not shown). Whereas Cx43 staining in WT hearts was predominantly localized to the intercalated discs, the staining in the transgenics hearts was distributed along the entire surface of the long axis of the cells in a punctuate pattern (Fig. 6). The intensity of staining was also weaker in the calcineurin hearts than WTs, suggesting both qualitative and quantitative changes in Cx43 expression. Consistent with these findings, quantitative immunoblotting revealed a 56±4% decrease in Cx43 protein levels in calcineurin hearts compared to WTs (Fig. 6C). In addition, Cx43 was mainly (2.5-fold) present in the nonphosphorylated form in calcineurin hearts com-
weeks of age [10]. Surprisingly, our data demonstrated mouse model with cardiac-specific expression of a con-However, alterations in the phosphorylation status of special emphasis on SR Ca-transport function in a Ca storage and/or release in the transgenic hearts.

examine the alterations in myocyte Ca-kinetics, with receptor were not altered, suggesting no alterations in SR Ca-homeostasis [38], the precise mechanisms respon-
sible for the modulations observed in cardiac hypertrophy longed rates of Ca transient decay [41,42]. Importantly, of systems have been identified in the regulation of cellular studies in human and animal models of end-stage heart

trophy is associated with alterations in intracellular Ca2+-transport function, leading to increased rates of relaxation and increased force of contraction, respectively [21,33,39]. Furthermore, the phosphorylation status of PLB was increased in calcineurin hearts, contributing to the reduced inhibition of SERCA by PLB. The finding on increased PLB phosphorylation was surprising since calcineurin has been previously shown to activate protein phosphatase-1 in hippocampal neurons, leading to dephosphorylation of the transcription factor CREB (cAMP response element-binding protein) [40]. Thus, calcineurin was also expected to activate the SR associated type-1 protein phosphatase and dephosphorylate PLB in the transgenic hearts. However, it is possible that the SR type-1 phosphatase may not be regulated by calcineurin in a similar manner as the enzyme, which dephosphorylates CREB in neuronal tissue. The increased SERCA2 and decreased PLB protein levels as well as enhanced phosphorylation of PLB may be due to either a direct consequence of calcineurin activation or an adaptive, early response to cardiac hypertrophy in this model. Since calcineurin was previously shown to act through the CRZ1/TCN1-encoded transcription factor and regulate the expression of genes encoding Ca2+- or Na+-pumping ATPases [15–17] as well as regulate SERCA activity in yeast [18], it is interesting to propose that calcineurin may be also capable of regulating SERCA at the transcriptional and/or translational levels in the mammalian heart. Nevertheless, these observed alterations in the SR Ca2+ transport ensemble and myocyte contractility highlight the uniqueness of the calcineurin model with respect to previous studies in human and animal models of end-stage heart failure, exhibiting decreases in SERCA2 levels and prolonged rates of Ca2+ transient decay [41,42]. Importantly, the protein levels of calsequestrin and the ryanodine receptor were not altered, suggesting no alterations in SR Ca2+ storage and/or release in the transgenic hearts. However, alterations in the phosphorylation status of

Table 1

<table>
<thead>
<tr>
<th>Mechanical parameters (n)</th>
<th>WT</th>
<th>TG</th>
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<tbody>
<tr>
<td>Resting cell length (µm)</td>
<td>110±4</td>
<td>198±15*</td>
</tr>
<tr>
<td>% Shortening</td>
<td>7±1</td>
<td>12±2*</td>
</tr>
<tr>
<td>+ dL/dt (µm/s)</td>
<td>166±22</td>
<td>503±23*</td>
</tr>
<tr>
<td>− dL/dt (µm/s)</td>
<td>136±20</td>
<td>375±14*</td>
</tr>
<tr>
<td>Ca2+ kinetics (n)</td>
<td>(4)</td>
<td>(7)</td>
</tr>
<tr>
<td>Ca2+ amplitude (340/380)</td>
<td>1.86±0.34</td>
<td>3.08±0.27*</td>
</tr>
<tr>
<td>T50 (s)</td>
<td>0.42±0.06</td>
<td>0.20±0.03*</td>
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+ dL/dt, rate of cell shortening; − dL/dt, rate of cell lengthening; T50, time for 80% decay of the Ca2+ signal. Data are presented as mean±S.E.M. (n=number of mice). Parameters were assessed for an average of 11 myocytes from each heart. A single heart was considered as a single sample. * Indicates that the value significantly differed (P<0.05) in CLN myocytes from WT myocytes.

pared to WTs, as revealed by probing the blots with a monoclonal antibody (P2C4), which preferentially binds nonphosphorylated connexin (Fig. 6D).

3.7. Electrocardiography

Alterations in Cx43 levels, distribution and phosphorylation have been linked to arrhythmogenesis in cardiac muscle [36,37]. Thus, we performed electrocardiography studies in calcineurin and WT mice. Fig. 7 shows the six-lead ECG from a WT and calcineurin mouse. The QRS duration was 1.8±0.1-fold longer in calcineurin (n=6) than WT (n=4) mice. The WT tracing (Fig. 7A) showed normal sinus rhythm with a heart rate of approximately 540 beats/min. The calcineurin mouse (Fig. 7B) showed no clear relationship between P waves and QRS complexes, with the P waves occurring more frequently than the QRS complexes, suggesting complete heart block in three of the six calcineurin mice. The other three calcineurin mice exhibited sinus bradycardia.

4. Discussion

Numerous studies have suggested that cardiac hypertrophy is associated with alterations in intracellular Ca2+-handling and myocardial contractility. Although a number of systems have been identified in the regulation of cellular Ca2+ homeostasis [38], the precise mechanisms responsible for the modulations observed in cardiac hypertrophy are not well known. The present study was designed to examine the alterations in myocyte Ca2+-kinetics, with special emphasis on SR Ca2+-transport function in a mouse model with cardiac-specific expression of a constitutively active calcineurin, which displays cardiac dysfunction and hypertrophy, but no overt failure at 4–6 weeks of age [10]. Surprisingly, our data demonstrated enhanced myocyte contractile and Ca2+-handling properties, which were associated with increases in SR Ca2+ cycling and function in calcineurin hearts.

Quantitative immunoblotting was used to examine the levels of SR Ca2+-transport, storage and release proteins. SERCA protein was significantly increased, although previous studies indicated decreases in SERCA mRNA in calcineurin hearts [10]. The apparent disparity between SERCA transcript and protein levels may be due to mRNA stability and/or protein turnover. The increased SERCA protein levels were consistent with findings in yeast, where activation of calcineurin induced the expression of PMC1 and PMR1, which encode Ca2+-ATPases in the vacuole and Golgi, respectively [15,18]. In addition, the decrease in PLB levels in transgenic hearts would result in increased rates of SR Ca2+ transport and higher SR Ca2+ load, leading to increased rates of relaxation and increased force of contraction, respectively [21,33,39]. Furthermore, the phosphorylation status of PLB was increased in calcineurin hearts, contributing to the reduced inhibition of SERCA by PLB. The finding on increased PLB phosphorylation was surprising since calcineurin has been previously shown to activate protein phosphatase-1 in hippocampal neurons, leading to dephosphorylation of the transcription factor CREB (cAMP response element-binding protein) [40]. Thus, calcineurin was also expected to activate the SR associated type-1 protein phosphatase and dephosphorylate PLB in the transgenic hearts. However, it is possible that the SR type-1 phosphatase may not be regulated by calcineurin in a similar manner as the enzyme, which dephosphorylates CREB in neuronal tissue. The increased SERCA2 and decreased PLB protein levels as well as enhanced phosphorylation of PLB may be due to either a direct consequence of calcineurin activation or an adaptive, early response to cardiac hypertrophy in this model. Since calcineurin was previously shown to act through the CRZ1/TCN1-encoded transcription factor and regulate the expression of genes encoding Ca2+ or Na+-pumping ATPases [15–17] as well as regulate SERCA activity in yeast [18], it is interesting to propose that calcineurin may be also capable of regulating SERCA at the transcriptional and/or translational levels in the mammalian heart. Nevertheless, these observed alterations in the SR Ca2+ transport ensemble and myocyte contractility highlight the uniqueness of the calcineurin model with respect to previous studies in human and animal models of end-stage heart failure, exhibiting decreases in SERCA2 levels and prolonged rates of Ca2+ transient decay [41,42]. Importantly, the protein levels of calsequestrin and the ryanodine receptor were not altered, suggesting no alterations in SR Ca2+ storage and/or release in the transgenic hearts. However, alterations in the phosphorylation status of
Fig. 5. In vivo cardiac function and histology of CLN hearts. Echocardiographic studies performed on 4–6-week-old CLN and WT mice revealed decreased fractional shortening (A) and increased left ventricular end-diastolic dimensions (B), indicating impaired function and cardiac remodeling in CLN mice. Hematoxylin/eosin-stained histologic sections of WT (C) and CLN (D) hearts at 200× magnification demonstrated hypertrophy and myocyte disarray in CLN hearts. Masson’s trichrome-stained sections from CLN hearts (F) revealed wide-spread interstitial fibrosis, which was absent in WT hearts (E). * P < 0.05 vs. WT.
Fig. 6. Connexin-43 protein expression, distribution and phosphorylation in WT and CLN hearts. Immunohistochemical staining of longitudinal sections from WT and CLN hearts show differences in the staining pattern with Cx43 antibodies. WT hearts exhibit a characteristic distribution of Cx43 in gap junctional complexes at the ends of myofibers. In CLN hearts, the pattern is weaker and more diffuse with small, punctuate, and less well-organized complexes suggesting a general disorganization of gap junctions. Bar = 50 μm. (C) Western blot using a Cx43 polyclonal antibody that recognizes total Cx43. (D) Immunoblots probed with a monoclonal antibody (P2C4) raised to the last 23 amino acids of Cx43, which preferentially binds non-phosphorylated Cx43. Cx43 in CLN hearts migrated with an apparent lower molecular weight than in WT hearts, consistent with a more dephosphorylated form of Cx43 in the transgenic. * P < 0.05 vs. WT.
Fig. 7. Six lead ECG in WT and CLN mice. P waves and QRS complexes are indicated on the tracings. (A) A QRS complex follows each P wave, indicating sinus rhythm in WT. (B) P waves and QRS complexes are not related to one another, suggesting complete heart block in CLN. The time and electrical scales are identical for each tracing. Each tracing represents approximately 2 s. P, P wave; QRS, QRS complex.

Ryanodine receptor cannot be excluded and may contribute to the phenotype of the transgenic hearts.

In addition to enhanced SR Ca\(^{2+}\) transport, the myofibrillar Mg-ATPase activity was increased in calcineurin hearts, possibly reflecting higher myofilament phosphorylation of troponin I [43] and/or the regulatory myosin light chains [44]. Collectively, the alterations in SR and myofibrils, together with the increased Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channel [44] may contribute to the enhanced Ca\(^{2+}\) handling and contractile performance of isolated calcineurin myocytes, observed in this study. The enhanced Ca\(^{2+}\) cycling properties of calcineurin cells, is consistent with recent observations in neonatal cardiac myocytes, which exhibited an increase in the Ca\(^{2+}\)-transient before the development of significant hypertrophy, upon calcineurin adenoviral infection [45]. Furthermore, recent studies have shown that calcineurin may regulate the transcription of Na\(^+\)/Ca\(^{2+}\) exchanger isoforms [46] and the activity of IP\(_3\) receptors [47] in neuronal tissues, which are also involved in the regulation of Ca\(^{2+}\) homeostasis. Thus, calcineurin may act as a Ca\(^{2+}\) sensor contributing to regulation of Ca\(^{2+}\)-dynamics in myocardial cells, and calcineurin activation in vivo may occur in specific subcellular compartments, exhibiting microdomain elevations in Ca\(^{2+}\) levels and spatially different sensing mechanisms. However, it is not clear whether activation of endogenous calcineurin by sustained increases in intracellular Ca\(^{2+}\) may result in effects similar to transgenic expression of activated calcineurin.

The enhanced Ca\(^{2+}\) cycling and contractility, observed in calcineurin myocytes, was totally unexpected and was in contrast to the decreased function of intact hearts at the same age. Thus, our hypothesis was that the dissonance in function between multicellular preparations and single isolated myocytes may result from compromised cell-to-cell interactions, such that the force of contracting myocytes could not be translated to the whole heart level. This may be due at least partially to cardiac remodeling in calcineurin mice, including myocyte hypertrophy, interstitial fibrosis and geometric alterations. Such remodeling may compromise intercellular architectural organization or even trigger re-organization of gap junctions. Interestingly, Cx43 was significantly down-regulated and mainly dephosphorylated in the calcineurin hearts, which may be associated with alterations in connexin trafficking, channel gating, gap junction assembly and turnover [48–50]. Furthermore, reduction in the expression levels and redistribution of Cx43, is associated with altered intercellular architectural organization or remodeling of gap junctions and diminished electrical coupling leading to arrhythmogenesis [36]. Indeed, ECG recording of CLN mice showed abnormal electrical rhythm including slower heart rates and prolonged QRS complex, indicating a decreased conduction velocity in transgenic hearts.

Our findings on downregulation of Cx43 are consistent with recent reports on Cx43 cardiac-restricted knockouts [51], as well as studies in regions bordering healing infarcts [37] and in human hibernating myocardium [52]. The dephosphorylation of Cx43 may be mediated directly by calcineurin, similar to recent observations in cultured astrocytes, where inhibition of calcineurin with cyclosporin A or FK506 reduced Cx43 dephosphorylation and junctional uncoupling observed after hypoxia [53]. Alternatively, calcineurin may dephosphorylate and modulate the binding of accessory proteins to Cx43, which participate in the regulation of gap junctional intercellular communica-
tion [54]. However, our findings indicate that phosphatase activity, either directly or indirectly, may affect normal Cx43 processing and protein turnover in the heart. Thus, cellular disorganization, myocyte loss and abnormal intercellular coupling in CLN hearts are expected to lead to depressed cardiac function, which may provide further stimulus to hypertrophy in the surviving myocytes. As a compensatory mechanism to the increases in cardiac work by the morphological changes, Ca\(^{2+}\) handling mechanisms are augmented at the cellular level.

In summary, chronic activation of calcineurin signaling was associated with increases in SERCA protein levels and concomitant decreases in PLB, which was highly phosphorylated. The enhanced SR Ca\(^{2+}\) transport activity coupled with increases in the myofilament Mg-ATPase activity resulted in augmented myocyte Ca\(^{2+}\) cycling and contractility. However, the cellular disorganization and abnormal intercellular coupling in CLN hearts may lead to depressed global cardiac function, which may provide a further stimulus to hypertrophy in the surviving myocytes. Future studies may be designed to determine whether the alterations in SERCA and PLB are mediated by activated calcineurin at the transcriptional level or occur as secondary responses to compensate for the depressed function in calcineurin hearts.

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