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Article

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See related Commentary on pages 1223–1225.

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Introduction

Activation of cardiac muscle is mediated by the specialized electrical system of the heart. It consists of the sinoatrial (SA) and the atrioventricular (AV) nodes, the activation of which depends on an inward Ca^{2+} current, and of the bundle branches and Purkinje network, the activation of which depends on an inward Na^{+} current (1). Generally, congenital cardiac conduction anomalies are not well understood. In human fetuses and newborns, they are characterized by a progressive AV conduction delay resulting in severe bradycardia and cardiac dysfunction (2). Importantly, in the absence of electronic cardiac pacing, these disorders can be lethal. It is likely that some of these disorders result from abnormal development of the cardiac conductive system, which depends on the recruitment of cardiomyocytes (1, 3).

A number of mutations in muscle-specific genes, which affect cardiac development, have been reported (4). Recently, a calreticulin-deficient mouse was shown to be embryonic lethal (5, 6) because of a lesion in heart development (5). Given that calreticulin is a Ca^{2+}-binding chaperone located in the lumen of the endoplasmic reticulum (ER) (7), ER must play a significant role in cardiac development. Calreticulin is highly expressed in the developing heart but is downregulated in late embryonic stages and is maintained at very low levels in mature hearts (5). The physiological significance of this apparently tight control of calreticulin expression is not known, but it suggests that inappropriate levels of the protein could lead to cardiac pathology.

In this study, we produced transgenic mice that overexpress calreticulin in the heart and showed that postnatally elevated expression of calreticulin leads to severe cardiac pathology, including sinus bradycardia and AV node dysfunction with progressive prolongation of the P-R interval followed by complete cardiac block and sudden death. Cardiomyocytes overexpressing calreticulin have a decreased density of L-type Ca^{2+} channels and of the gap junction proteins, connexin43 and connexin40. It is interesting that the overexpression of calreticulin seems especially to result in pathological changes to the sinus and AV nodes, where an inward Ca^{2+} current is required for activation. Calreticulin may be part of one pathway of events involving the ER that can cause complete heart block.

Methods

Generation of transgenic mice. To generate DNA encoding HA tag the following oligodeoxynucleotides 5′-TCGAGTACCCATATGATGTTCCTGACTATGCTA-GACAGGCCAAGGACGAGCTGTAGT-3′ and 5′-CTA-

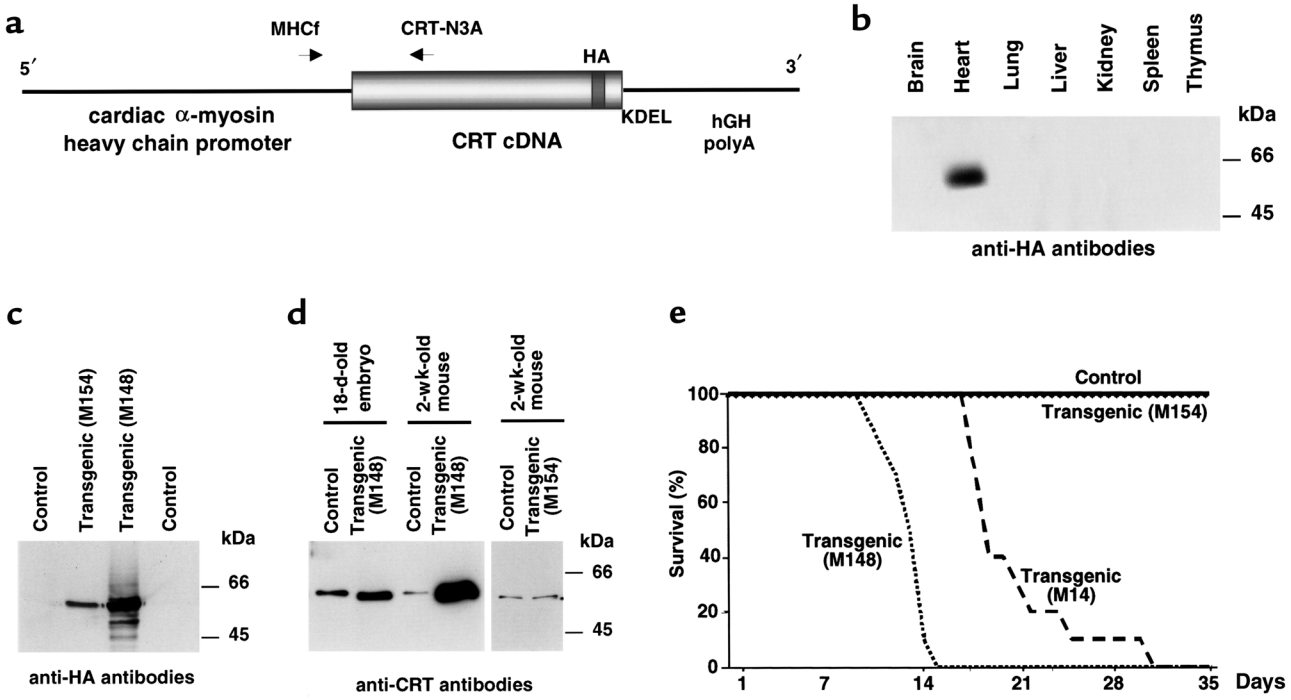

Figure 1

Generation of cardiac-specific calreticulin overexpresser transgenic mice. (**a**) Calreticulin cDNA encoding full-length protein including the NH_2-terminal signal sequence and the COOH-terminal KDEL ER retrieval signal was subcloned downstream from α-cardiac MHC promoter. The arrows indicate the PCR primers used for recognition of the transgene. (**b**–**d**) Western blot analysis of proteins extracted from control and transgenic mice. The blot was probed with the anti-HA antibodies (**b** and **c**) or with the rabbit anti-calreticulin antibodies (**d**). (**e**) Kaplan-Meier survival analysis of calreticulin transgenic mouse lines. hGH, human growth factor polyA sequences.

GACTACAGCTCGTCCTTGGCCTGTCTAG-CATAGTCAGGAACATCATATGGGTAC-3′ were annealed to create double strand DNA fragment encoding NH_2-Y-P-Y-D-V-P-D-Y-A-R-Q-A-K-D-E-L-COOH amino acid sequence (hemagglutinin [HA] epitope is underlined followed by KDEL ER retrieval signal). cDNA encoding calreticulin was synthesized by a PCR-driven amplification (8) and cloned into SalI site of a plasmid containing the 5.5-kb mouse cardiac α-myosin heavy chain (α-MHC) (Figure 1a). Linearized pBS-α-MHC-CRT-HA was microinjected into the fertilized oocytes, which were transferred into the oviduct of pseudopregnant FVB/N mice. Transgenic mice were identified by PCR analysis of tail genomic DNA using a forward primer corresponding the 5′ end of the mouse the α-MHC promoter sequence (MHCf: 5′-TATCTCCCCCATAAGAGTTT-3′) and a reverse primer corresponding to the 5′ end of the calreticulin cDNA sequence (CRT-N3A: 5′-GTCAATCTTCACCTCAT-ACG-3′) (Figure 1a). Founder mice were identified, bred with wild-type FVB/N mice, and maintained in a pathogen-free environment.

SDS-PAGE and Western immunoblotting. Proteins from mouse tissues including heart, brain, lung, liver, kidney, and thymus were lysed, separated by SDS-PAGE followed by immunoblotting (9). Protein assays were carried out using DC Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, California, USA). Blots were probed with rabbit anti-HA antibodies, goat or rabbit anti-calreticulin antibodies (9, 10), rabbit anti-calnexin (Stress Gene, Victoria, British Columbia, Canada; 1:500 dilution), rabbit anti-BiP (1:2,000 dilution), rabbit anti-PDI (9) (1:500 dilution), rabbit anti-calsequestrin (10) (1:300 dilution), rabbit anti-Cx43 (1:20,000 dilution), or rabbit anti-SERCA2 antibodies (11) (1:1,000 dilution). Antibody binding was detected with appropriate peroxidase-conjugated secondary antibodies followed by a standard enhanced chemiluminescence development reaction.

Northern blot analysis. Northern blot analysis was carried out as described elsewhere (10). The following cDNA probes were used for hybridization: an approximately 600-bp ANP cDNA probe (PstI fragment), an approximately 400-bp BNP cDNA (HindIII/XbaI fragment), an approximately 1.5-kb Cx43 cDNA (NotI/SalI fragment), an approximately 1.2-kb Cx40 cDNA (EcoRI/XhoI fragment), and an approximately 800-bp GAPDH probe cDNA (XbaI fragment). The blots were analyzed using Phosphorimager BAS1000 (Fujix; Fuji Medical Systems, Stamford, Connecticut, USA).

Histological analysis and immunohistochemistry. We carried out histological analysis of mouse hearts as described previously (5). Sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome

stain or were probed with goat anti-calreticulin (1:25), rabbit anti-HA (1:50), and rabbit anti-calsequestrin (1:25) antibodies. Antibody binding was detected with FITC-conjugated donkey anti-goat IgG and goat anti-rabbit IgG antibodies followed by confocal microscopy.

M-mode echocardiography. Transgenic and control mice were anesthetized with methoxyflurane and maintained at 37°C to prevent hypothermia. For cardiac imaging, we used a 12-MHz phase array sector transducer with frequency fusion technology (SONOS 5500; Hewlett Packard, Andover, Massachusetts, USA). M-mode images were obtained in the parasternal short and long views at the papillary muscle level.

Electrocardiogram. ECG Leads I, II, III, AVR, and AVL of nonsedated transgenic and control mice from the age of 4 days until death were simultaneously recorded using E for M ECG amplifiers (PPG Biomedical Systems Inc.; Pleasantville, New York, USA). These recordings were filtered 1–100 Hz and sampled at 16 kHz using a Pentium computer containing National Instruments data acquisition board (National Instruments, Austin, Texas, USA) and custom software (Advanced Measurements, Calgary, Canada). ECGs of transgenic and wild-type mice were taken in the first (4–7 days), the second week of life (8–14 days), the third week of life (15–21 days). The ECG parameters compared included the sinus rate (P-P interval), the PR duration (onset of P wave to the onset of the QRS complex), the duration of atrial activation (P wave duration), and the duration of ventricular activation (QRS duration).

Patch clamp for L-type Ca^{2+} channel. Single ventricular myocytes from the 13- to 21-day neonatal mice were isolated as described previously (12). Ca^{2+} tolerant single myocytes with smooth surface and clear striation were used in these experiments. Whole cell currents were recorded at room temperature (~20°C) using conventional ruptured patch recording techniques. Borosilicate electrodes had resistances of 3–4 MΩ when they were filled with internal solution containing 100 mM Cs-Aspartate, 5 mM ATP-Na, 0.5 mM GTP-Na_2, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.2), 10 mM EGTA, 1 mM $CaCl_2$, and 10 mM CsCl. The external bath solution was HEPES-Tyrode solution containing extra 5 mM CsCl. A liquid junction potential of –10 mV was corrected with an axopatch 200B amplifier (Axon Instruments Inc., Foster City, California, USA). Data acquisition and analysis were carried out with pClamp software (Axon Instruments).

Results

Generation of transgenic mice overexpressing calreticulin in the heart. Cardiac-specific overexpression of calreticulin was driven by the cardiac MHC promoter. PCR analysis of genomic DNA, using the specific sets of primers depicted in Figure 1a, enabled identification of six

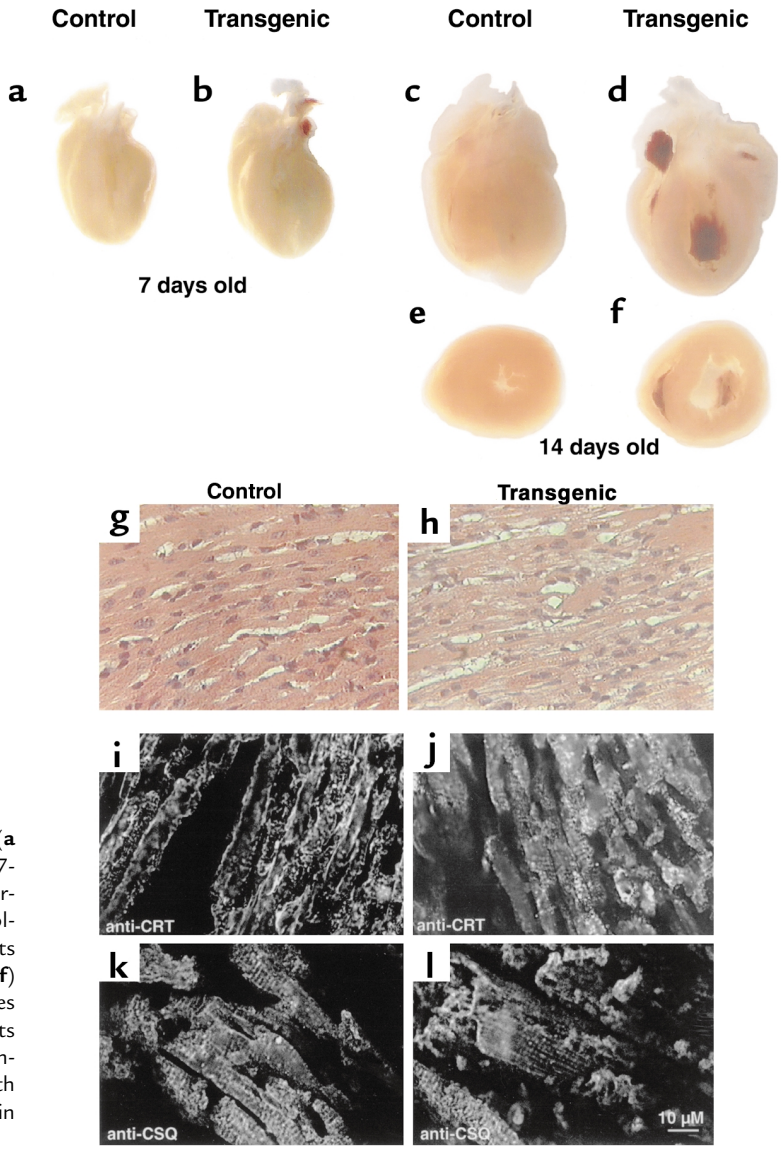

Figure 2
Pathological changes in calreticulin transgenic mice. (**a** and **b**) Gross morphology of control and transgenic 7-day-old mouse hearts, respectively. No significant morphological changes are observed. (**c–f**) Gross morphology of transgenic and control 14-day-old mouse hearts (line M148). (**c** and **d**) Transverse sections. (**e** and **f**) Sagittal sections. (**g** and **h**) High magnification images of control and transgenic 14-day-old mouse hearts stained with H&E. (**i–l**) Sections of transgenic and control (wild-type) cardiac tissue were immunostained with anti-calreticulin (anti-CRT) (**i** and **j**) or anti-calsequestrin (anti-CSQ) (**k** and **l**) antibodies.

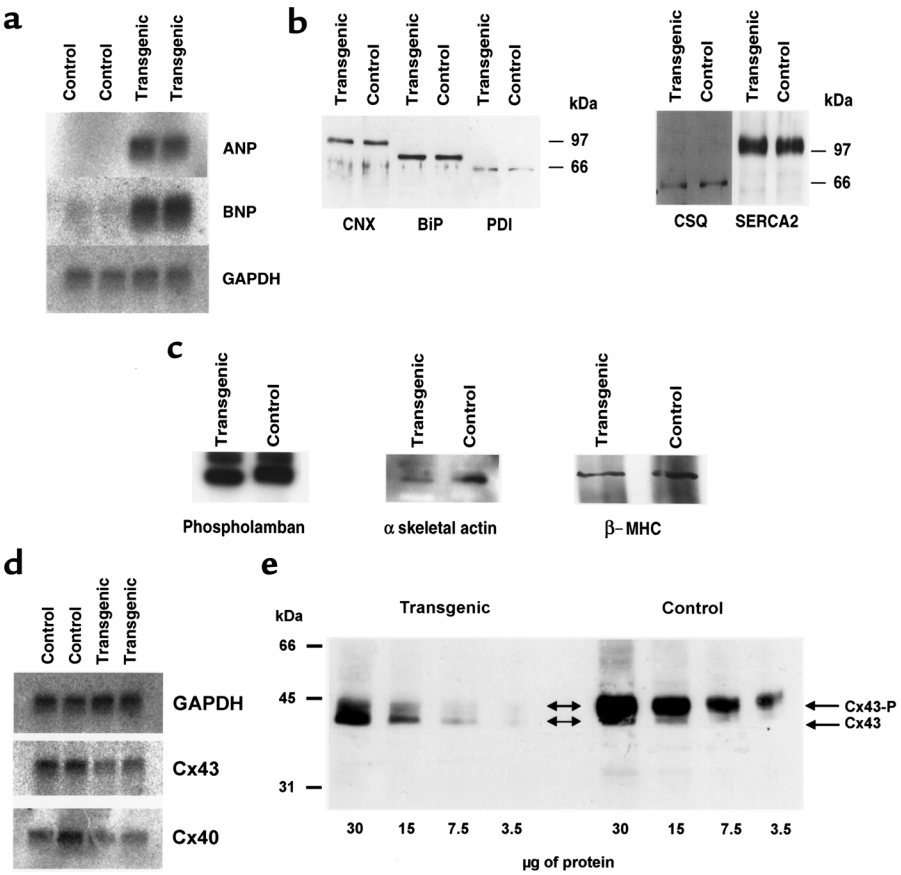

Figure 3
Northern and Western blot analysis of transgenic hearts. (**a**) Northern blot analysis of mRNA isolated from transgenic and control ventricle of 11-day-old calreticulin transgenic mice. The following cDNA probes were used: ANP, BNP, α-MHC, and GAPDH. (**b**) Western blot analysis of expression ER-resident chaperones and SR proteins in transgenic hearts. CNX, calnexin; BiP, immunoglobulin binding chaperone; PDI, protein disulfide isomerase; CSQ, calsequestrin. (**c**) Western blot analysis of expression of phospholamban, α skeletal actin, and β-MHC in transgenic and control mice. (**d**) Northern blot analysis of mRNA isolated from transgenic and control ventricle of 17-day-old transgenic mice. The Cx43, Cx40, and GAPDH cDNA probes were used. (**e**) Western blot analysis of expression of Cx43 in transgenic and control hearts. Cx43 and Cx43-P indicate the location of connexin43 and phosphorylated connexin43, respectively. The location of Bio-Rad Laboratories molecular markers is indicated.

transgenic founders, and four of them (M14, M148, M154 and M499) were chosen for further detailed analysis. With the exception of the line M154, they all exhibited an identical phenotype. To show that calreticulin in the transgenic mice was overexpressed specifically in the heart, we carried out Western blot analysis of different tissue homogenates. The influenza HA epitope was engineered into the COOH-terminus of the recombinant protein, making its identification easier (Figure 1a). This COOH-terminal HA tag has no effect on the function of the protein (13). Figure 1b shows that the recombinant calreticulin was expressed in the heart, but was not detected in brain, lung, liver, kidney, spleen, or thymus (Figure 1b). Further immunoblot analysis of cardiac homogenates from the transgenic mice (lines M154 and M148) revealed that line M148 had a significantly higher expression of recombinant calreticulin (Figure 1c). To determine the total levels of calreticulin (native plus recombinant) in the transgenic animals, cardiac homogenates were probed with rabbit anti-calreticulin antibodies (9). This antiserum recognizes both recombinant and endogenous forms of calreticulin. Figure 1d shows that the total amount of calreticulin in cardiac homogenates from transgenic line M154 was similar in homogenates from control and wild-type mice of the same age. In contrast, in line M148, the total cardiac expression of calreticulin was significantly elevated and changed with age (Figure 1d). Specifically, using the same rabbit anti-calreticulin antibodies, immunoblot analysis of cardiac homogenates from 18-day-old embryo and 2-week-old neonate revealed 1.9– and 20-fold increases in the level of calreticulin, respectively (Figure 1d). Total levels of cardiac calreticulin in transgenic lines M14 and M499 were also increased, 11- and 16-fold 2 weeks after birth, respectively (data not shown).

Phenotype of transgenic mice. During the first 1 or 2 weeks of life, the transgenic mice did not exhibit any obvious gross abnormalities, and they showed a normal increase in body weight. Subsequently, however, elevated expression of calreticulin in the heart (transgenic lines M148, M499, and M14) was associated with a loss of weight and with sudden death. For example, all the transgenic mice derived from line M148 died within 15 days of birth. Also, the majority (>80%) of transgenic mice from line M14 died 22 days after birth, and none survived beyond 5 weeks (Figure 1e). In contrast, transgenic animals derived from line M154, which expressed normal levels of total cardiac calreticulin, showed no abnormalities, and their phenotype was identical to the wild type, as expected (Figure 1e).

Morphological analysis of transgenic myocardium. There was no obvious increase in the size (dilation) of the cardiac chambers in 7-day-old transgenic animals (Figure 2, a and b). However, several gross pathological changes were apparent in the hearts of 2-week-old animals, including

dilation with marked atrial and ventricular enlargement (Figure 2, c–f). The lungs appeared normal, with no pathological changes suggestive of pulmonary edema or infarction (data not shown). There was no significant difference in cardiac weight between transgenic (54.5 ± 8.6 mg; mean ± SE; n = 5) and control (49.8 ± 6.8 mg; mean ± SE; n = 5) animals. Thinning of the ventricular walls indicated that the significant enlargement of the heart was not accomplished by a hypertrophic response (Figure 2, c and e). Comparison of sections of control and transgenic hearts (Figure 2, g and h) showed a mild hypertrophy with degenerative changes, including intracellular vacuolization. However, there was no evidence of myocyte disarray, necrosis, or myocarditis. Sections stained with Masson's trichrome indicated that fibrosis did not occur in response to the overexpression of calreticulin (data not shown).

We next compared the subcellular localization of calreticulin in transgenic and control cardiomyocytes, by immunostaining and confocal microscopy. The immunolocalization of calsequestrin was also assessed in these hearts. Calsequestrin is an SR luminal Ca^{2+}-binding/storage protein similar to calreticulin that is found primarily in cardiac and skeletal muscle (14). The localization of both calreticulin and calsequestrin was unchanged in transgenic cells overexpressing calreticulin (Figure 2, i–l). In a further experiment, we used TUNEL staining to determine whether apoptosis was involved in the observed cardiac pathology. We found no TUNEL-positive cells in either transgenic or control hearts (data not shown).

Molecular assessment of cardiomyocyte in the transgenic mice. Normally, increased volume overload induces a myocardial response including the transcriptional activation of several embryonic genes. For example, the synthesis and secretion of both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) from the ventricle increases in proportion to the severity of ventricle dysfunction (15). To determine whether the expression of ANP and BNP was changed in hearts of the transgenic mice, we carried out a Northern blot analysis. GAPDH cDNA probes were used to normalize for RNA loading. Figure 3 shows that the 11-day-old transgenic mice expressed higher levels of mRNA for ANP and BNP than did the wild-type, control animals (Figure 3a). We also looked for possible changes in the expression of proteins associated with the ER and SR. Figure 3b shows that the expression of calnexin, BiP (immunoglobulin binding chaperone, Grp78), protein disulfide isomerase (PDI), calsequestrin, and SERCA2 was unchanged in the hearts of transgenic mice. Transgenic hearts showed no significant change in expression of phospholamban and a slight decrease in α skeletal actin and β-MHC proteins (Figure 3c).

We also investigated whether transgenic mice that overexpress calreticulin have altered expression of Cx43 and Cx40 in the heart. First, we isolated cardiac RNA from 17-day-old transgenic and control mice and carried out a Northern blot analysis. Figure 3d shows that

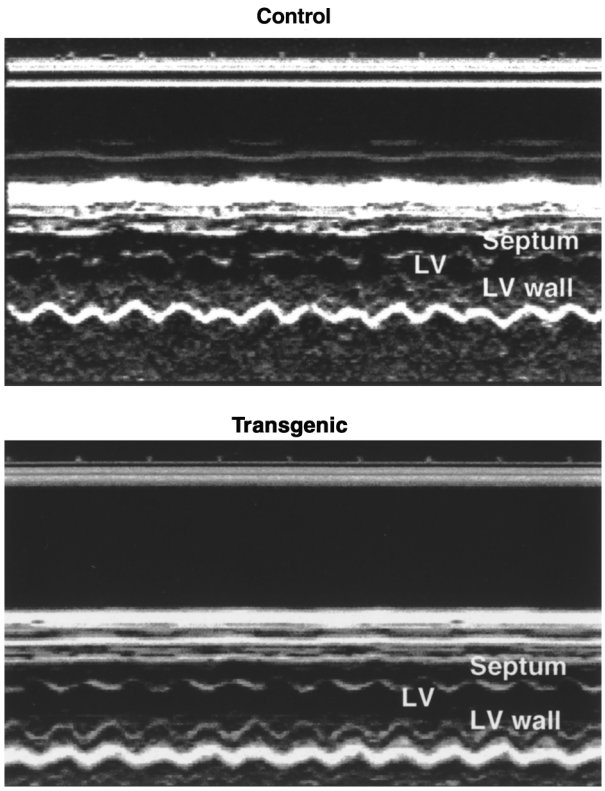

Figure 4
M-mode echoes from 17-day-old transgenic and control mice. LV, left ventricle.

the level of mRNA encoding Cx43 and Cx40 was significantly reduced in the transgenic hearts. Western blot analysis with anti-Cx43 antibodies showed that the level of Cx43 protein was also dramatically reduced (Figure 3e). The anti-Cx43 antibodies recognized two protein bands of 43-kDa and 41-kDa, respectively. Alkaline phosphatase treatment indicated that these two bands correspond to phosphorylated and unphosphorylated forms of Cx43, respectively (data not shown). Phosphorylated Cx43 accounted for more than 90% of the signal in the control heart (Figure 3e, control). Figure 3e (transgenic) shows that in hearts from 17-day-old transgenic mice (M148), there was a greater than 80% reduction in the level Cx43. Importantly, in hearts overexpressing calreticulin, there were negligible levels of the phosphorylated form of Cx43 (Figure 3e).

Prolonged P-R interval, bradycardia, and AV block in the transgenic mice. M-mode echo analysis revealed ventricle dilation in the transgenic mice was demonstrated in both the systolic and diastolic phase (Figure 4). The ejection fraction of the transgenic heart was decreased 15% (from 0.85 in control animals to 0.72 in transgenic animals) and was not sufficient to cause sudden death. During echocardiography of the transgenic heart, aortic Doppler echo showed that heart rates in the transgenic mice were slower than in control mice (data not shown). To measure differences in heart rate without sedation, we recorded ECGs in mice from 4 days after

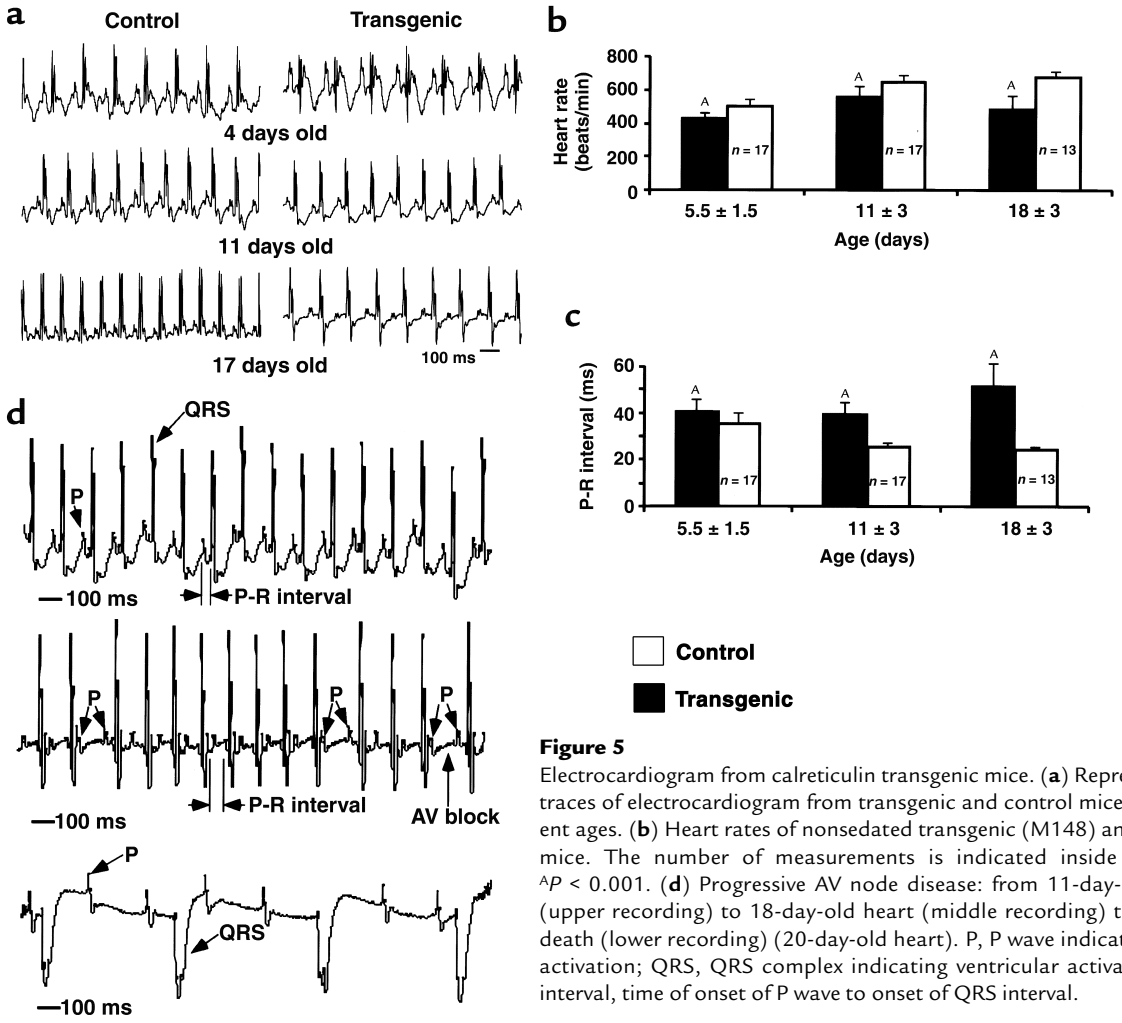

Figure 5

Electrocardiogram from calreticulin transgenic mice. (**a**) Representative traces of electrocardiogram from transgenic and control mice of different ages. (**b**) Heart rates of nonsedated transgenic (M148) and control mice. The number of measurements is indicated inside the bar. $^{A}P < 0.001$. (**d**) Progressive AV node disease: from 11-day-old heart (upper recording) to 18-day-old heart (middle recording) to sudden death (lower recording) (20-day-old heart). P, P wave indicating atrial activation; QRS, QRS complex indicating ventricular activation; P-R interval, time of onset of P wave to onset of QRS interval.

birth until the transgenic mice died (Figure 5a). Limb-leads ECG traces, I, II, III, AVL, and AVF, were monitored and recorded synchronously. At the earliest age (5.5 ± 1.5 days), the heart rate was significantly lower in transgenic mice than in wild-type controls (Figure 5b). With increasing age (11 ± 3 days), the heart rate increased in both transgenic mice and wild-type mice, but remained lower in the transgenic mice (Figure 5b). At 18 ± 3 days, the heart rate in the transgenic mice decreased, whereas in the wild-type control animals it reached adult levels (Figure 5b).

Importantly, the P-R interval in transgenic mice was already prolonged at 5.5 ± 1.5 days after birth (Figure 5c). With increasing age (18 ± 3 days), it became even more prolonged (Figure 5c). Arrhythmias were recorded in nine (53%) of the transgenic mice during ECG acquisition of 10–15 minutes. Second- or third-degree heart block was recorded in six of the animals, and atrial arrhythmias, including sinus pauses, were recorded in three mice. For example, Figure 5d shows electrocardiograms from a transgenic mouse at various stages of development. At 11 days of age (Figure 5d, upper recording) there is one-to-one conduction through the AV node, despite the P-R interval being prolonged compared with that of a normal mouse. By day 18 (Figure 5d, middle recording), there was marked prolongation of the P-R interval, and second-degree AV block was noted three times in an interval of 1.8 seconds. By day 20 (Figure 5d, lower recording), there was a complete heart block followed by sudden cardiac death.

We next investigated whether increasing the heart rate in transgenic animals would help to overcome the cardiac pathology that results from the overexpression of calreticulin. Transgenic mice were given atropine (80 ng/g of body weight) three times daily by intraperitoneal injection. The administration of atropine significantly extended the life span of the transgenic mice. (Thirty percent of these animals survived up to 6 days longer than did the untreated transgenic mice.) However, it did not prevent sudden cardiac death (data not shown).

A previous study has suggested that complete heart block may be associated with production of an autoantibody against calreticulin (16, 17). Therefore, we investigated the possibility that overexpression of calreticulin in the transgenic mice might lead to secretion of the protein and/or production of anti-calreticulin antibod-

ies. However, we detected neither calreticulin nor anti-calreticulin antibodies in serum collected from transgenic and wild-type control mice (data not shown).

L-Type Ca^{2+} channels in transgenic mice. A Ca^{2+} current through the L-type Ca^{2+} channel supports excitation of the SA and AV nodes, and conduction down the AV node (18, 19). Ca^{2+} influx through L-type Ca^{2+} channels is also a prerequisite for the initiation of myocardial contraction (20). Thus, inhibition of L-type Ca^{2+} channels may interfere with the generation of electrical impulses and conduction and myocardial contractility. We investigated the properties of L-type Ca^{2+} channels in cardiomyocytes from the transgenic animals. In a whole cell, voltage-clamp configuration, L-type Ca^{2+} current ($I_{Ca,L}$) was induced, at different potentials, by 300-ms depolarization pulses. This was carried out from a holding potential of –80 mV to +40 mV by 10 mV step voltage increases. Sodium and T-type calcium currents were inactivated by application of a 100-ms conditional pulse. The potassium conductance of the cell was completely blocked by Cs^{+} in the internal and external solutions. Figure 6 shows two representatives of $I_{Ca,L}$ in control (Figure 6a) and transgenic cardiomyocytes (Figure 6b). At every voltage, the transient inward $I_{Ca,L}$ in transgenic cardiomyocytes was significantly less than in the control cells (Figure 6c). The current density of peak inward $I_{Ca,L}$ elicited at 0 mV in transgenic cardiomyocytes was 4.0 ± 0.9 pA/pF (n = 5), significantly smaller than the 13.1 ± 2.8 pA/pF measured in control cardiomyocytes under the same conditions (n = 5).

The inactivation of $I_{Ca,L}$ in both groups closely fitted a bi-exponential decay. At 0 mV, the taus of fast and slow components were 16.8 ± 3.2 and 78.3 ± 9.2 ms in control (n = 5) and 28.9 ± 8.9 and 92.6 ± 10.0 ms in transgenic cardiomyocytes (n = 5), respectively, but these changes did not reach statistical significance. Both time constants were slower in the transgenic cardiomyocytes than in the control, but no statistically significant difference was found between inactivation time constants in control and transgenic cardiomyocytes.

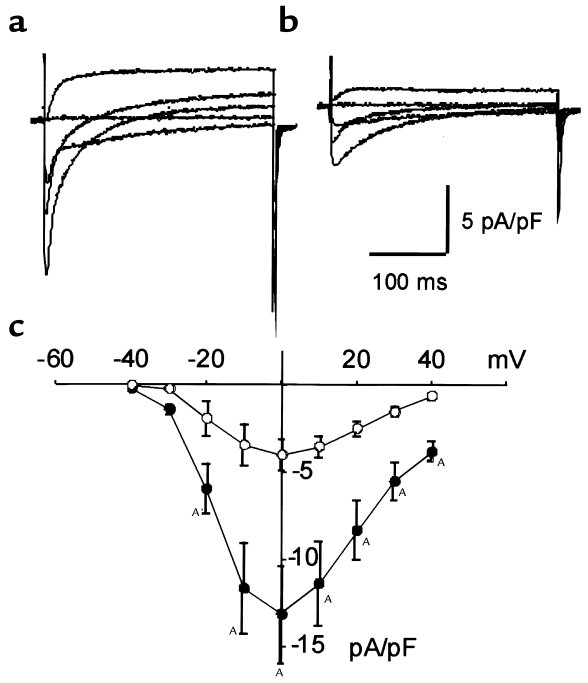

Figure 6
Patch clamp for L-type Ca^{2+} channel. The density of $I_{Ca,L}$ in the ventricular myocytes in control and transgenic mice. The representative current trace families of $I_{Ca,L}$ in control (**a**) and transgenic (**b**) mice. (**c**) I-V relationships of peak in control (filled circles) and transgenic (open circles) mice. Data are presented as mean ± SEM. $^{A}P < 0.05$.

Discussion

In this study, we showed that elevated expression of calreticulin in the hearts of newborn mice leads to severe cardiac pathology, including heart block and early postnatal death. Our results indicate that cardiomyocytes are sensitive to changes in the abundance of calreticulin and that it is physiologically important to downregulate the expression of calreticulin after birth, to prevent severe cardiac pathology. Calreticulin is a chaperone and Ca^{2+} storage protein resident in the ER (7). To our knowledge, this is the first report indicating a role in cardiac pathology for a protein that is resident in the ER.

The overexpression of calreticulin in cardiac tissue results in sinus node dysfunction, which is demonstrated by a decline in sinus node activity with age and by marked sinus pauses. It also results in AV node dysfunction, which manifests as prolongation of the P-R interval and heart block. Our patch clamp studies demonstrated a significant decrease in inward Ca^{2+} current in myocytes overexpressing calreticulin. This inward Ca^{2+} current is responsible for depolarization in both the sinus and AV nodes (18, 19). Furthermore, the L-type Ca^{2+} channel is essential for normal pacemaker activity in mouse heart (21). We also observed a significantly reduced expression of the gap junction proteins Cx43 and Cx40 in hearts that were overexpressing calreticulin. The molecular composition and spatial distribution of gap junctions are important determinants of the conduction properties of different cardiac tissues (22). Decreasing level of Cx43 has been found in the cardiac pathology including congestive heart failure, ischemia, and hypertrophy (23, 24). Cx43 is phosphorylated at multiple serine residues affecting channel function and the rates of channel assembly (22). The mechanisms responsible for calreticulin-dependent downregulation of Cx43 and Cx40 are presently not known. Calreticulin may play a role in folding of connexin and assembly of gap junction. Transgenic hearts overexpressing calreticulin have a negligible level of phosphorylated Cx43, indicating that calreticulin may also, via regulation of Ca^{2+} homeostasis, influence the function of protein kinases involved in phosphorylation of connexin.

In the ventricular chamber of the mouse, Cx40 is localized to the myocardial component of the conductive system, whereas Cx43 is expressed throughout the ventricular myocardium (1). Cx40 appears to be a

major conductor of intercellular current in atrial muscle (25). As seen here in mice overexpressing calreticulin, Cx40-deficient mice also have cardiac conductive abnormalities characteristic of first-degree AV block associated with an extended P-R interval (2). Furthermore, mice with cardiac-specific decreases in Cx43 develop spontaneous ventricular arrhythmias and sudden cardiac death (26). It seems likely, therefore, that the calreticulin-dependent bradyarrhythmias and cardiac block involve impaired function of both the L-type Ca^{2+} channel and of gap junction connexins. Calreticulin may exert these effects via action as a negative regulator of distinct Ca^{2+} channels, as observed in nonmuscle cells (27–30). Alternatively, in its role as a chaperone (7), calreticulin may affect the proper folding, post-translational modification, assembly and/or intracellular trafficking of channel proteins. It is also possible that downregulation of Cx43 and Cx40 is secondary to the dilation.

An important finding of this study is that calreticulin, an ER protein, is part of a "pathway" involved in pathophysiological development of the conductive system in the heart. A recent study indicates that there is a well-defined mechanism(s) for activation and repression of the calreticulin gene during cardiac development (5). Here we show that postnatal repression of the calreticulin gene is essential for proper development of the cardiac conductive system. The mechanisms controlling the expression of calreticulin in the heart are not known. During embryonic development, the gene may be activated by the transcription factor Nkx2.5, whereas COUP-TF1 may be involved in its postnatal repression (31).

Recently, Chien's group produced mice that are deficient in the transcription factor HF-1b (32). These animals exhibit sudden cardiac death and defects in the cardiac conduction system, including spontaneous ventricular tachycardia and a high incidence of AV block, suggesting that HF-1b plays an essential role in the development of the conductive system (32). Our work indicates that altered expression of calreticulin in the heart also plays a role the development and function of the cardiac conductive system. However, instead of the spontaneous ventricular tachycardia and AV block seen in HF-1b–deficient mice, calreticulin overexpressers develop bradycardia, prolonged P-R interval, complete cardiac block, and sudden cardiac death.

Several existing transgenic mouse models show elevated expression of cardiac muscle membrane proteins (33, 34). However, here we describe what is to our knowledge the first transgenic model overexpressing an ER protein, which is normally downregulated in postnatal and mature hearts (5). Interestingly, the pathophysiology of the heart block and heart failure seen in mice that overexpress calreticulin is distinctly different from that associated with the animal models that overexpress other membrane-associated proteins (33, 34). For example, the overexpression of SERCA2 is associated with increased rates of myocyte relengthening and shortening, which result from an increased maximum velocity of SR Ca^{2+} transport (35). The overexpression of phospholamban is associated with an inhibition of contractile parameters (36), and this is related to its role in the regulation of SERCA. Mouse hearts that overexpress the SR Ca^{2+}-binding protein calsequestrin develop severe cardiac hypertrophy, but they show no problems in development and function of the conductive system (37, 38).

Our transgenic calreticulin overexpressers may prove to be a mouse model of complete heart block that closely resembles the pathological, hemodynamic, and clinical features of the human disease. As such, they may enable us to identify and understand a novel causative pathway for complete heart block. They will also provide an excellent model for further investigation of developmental cardiac pathologies in general.

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