Targeted Inhibition of Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II in Cardiac Longitudinal Sarcoplasmic Reticulum Results in Decreased Phospholamban Phosphorylation at Threonine 17* 

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To investigate the role of Ca\(^{2+}\)/calmodulin-dependent kinase II in cardiac sarcoplasmic reticulum function, transgenic mice were designed and generated to target the expression of a Ca\(^{2+}\)/calmodulin-dependent kinase II inhibitory peptide in cardiac longitudinal sarcoplasmic reticulum using a truncated phospholamban transmembrane domain. The expressed inhibitory peptide was highly concentrated in cardiac sarcoplasmic reticulum. This resulted in a 59.7 and 73.8% decrease in phospholamban phosphorylation at threonine 17 under basal and β-adrenergic stimulated conditions without changing phospholamban phosphorylation at serine 16. Sarcoplasmic reticulum Ca\(^{2+}\) uptake assays showed that the V\(_{\text{max}}\) was decreased by ~30% although the apparent affinity for Ca\(^{2+}\) was unchanged in heterozygous hearts. The in vivo measurement of cardiac function showed no significant reductions in positive and negative dP/dt, but a moderate 18% decrease in dP/dt\(_{40}\) indicative of isovolumic contractility, and a 26.1% increase in the time constant of relaxation (τ) under basal conditions. The changes in these parameters indicate a moderate cardiac dysfunction in transgenic mice. Although the 3- and 4-month-old transgenic mice displayed no overt signs of cardiac disease, when stressed by gestation and parturition, the 7-month-old female mice develop dilated heart failure, suggesting the important role of Ca\(^{2+}\)/calmodulin-dependent kinase II pathway in the development of cardiac disease.

Calcium plays a central role in cardiac excitation-contraction coupling. The sarcoplasmic reticulum (SR) releases Ca\(^{2+}\) to trigger contraction and uptakes Ca\(^{2+}\) to initiate relaxation. Ca\(^{2+}\)-induced Ca\(^{2+}\) release occurs via ryanodine-sensitive SR Ca\(^{2+}\) release channels located mainly at junctional SR ("foot" structure), whereas Ca\(^{2+}\) uptake is mediated principally by the SR Ca\(^{2+}\)-ATPase (SERCA) which is located in the longitudinal SR. Both Ca\(^{2+}\) release and uptake are proposed to be regulated in a Ca\(^{2+}\)-dependent manner via Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) (1).

CaMKII is a member of a family of Ca\(^{2+}\)/calmodulin-regulated enzymes. Four CaMKII isoforms are derived from four closely related genes, α, β, γ, and δ (2). The δ and γ are the primary cardiac CaMKII isoforms expressed in the adult heart (3–5). CaMKII distributes in distinct compartments of the cardiomyocytes including sarcolemma, cytosol, SR, and nucleus (6), which may represent its functional relevance in the heart. The link between specific isoforms of CaMKII with particular regulatory properties, intracellular localization, and cellular substrates is not established.

The activity of SERCA is primarily regulated by an SR intrinsic protein, phospholamban (PLB). PLB physically interacts with SERCA to inhibit pump activity (7). The phosphorylation of PLB disrupts the interaction of PLB with the SERCA pump, relieving its inhibitory effect and resulting in an increase in the apparent affinity of SERCA for Ca\(^{2+}\) (7). CaMP-dependent protein kinase (PKA) mediates PLB phosphorylation at serine 16 (7, 8), whereas CaMKII mediates phosphorylation of PLB at threonine 17 (8, 9). In the intact heart, both PLB serine 16 and threonine 17 are phosphorylated by PKA and CaMKII, respectively, in response to β-adrenergic stimulation (10, 11). In vitro studies have indicated that SERCA is also phosphorylated by CaMKII at Ser\(^{38}\), which may enhance the maximal velocity (V\(_{\text{max}}\)) of calcium uptake (12–14). However, this pathway remains controversial (15, 16), due to a lack of in vivo evidence. In addition to Ca\(^{2+}\) uptake, CaMKII may also play a role in regulating Ca\(^{2+}\)-induced Ca\(^{2+}\) release by phosphorylation of serine 2809 of the cardiac SR calcium release channel (ryanodine receptor, RyR) which can also be a target for PKA (17, 18). However, whether the phosphorylation of RyR leads to opening or closing the Ca\(^{2+}\) release channel remains undefined (17–19). Therefore, the role of CaMKII in the regulation of RyR in intact heart is not clear.

Recently, several studies (20) have shown that the level of cardiac isoform CaMKII δ is significantly increased in dilated cardiomyopathy patients. Currie (21) and Kirchhefer et al. (22) have independently reported that the activity of CaMKII is significantly increased in hypertrophied animal models as well as in human heart failure. However, Netticadan et al. (23, 24) showed that the endogenous SR-associated CaMKII-mediated phosphorylation of SR Ca\(^{2+}\)-handling proteins is depressed in heart failure due to either ischemia-reperfusion or myocardial infarction. These data, taken together, suggest that SR CaMKII is associated with the abnormal Ca\(^{2+}\) handling of the

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‡ The abbreviations used are: SR, sarcoplasmic reticulum; CaMKII, Ca\(^{2+}\)/calmodulin-dependent kinase II; SERCA, SR Ca\(^{2+}\)-ATPase; TG, transgenic; NTG, non-transgenic; PLB, phospholamban; PKA, cAMP-dependent protein kinase; AIP, autocamtide inhibitory peptide; α-MIC, α-miosin heavy chain; RyR, ryanodine receptor; LV, left ventricle; LVEDP, LV end-diastolic pressure; PP, protein phosphatases.

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SR in cardiomyocytes. However, the role of CaMKII in the regulation of SR Ca$^{2+}$-handling proteins under pathophysiological conditions is not clear.

In order to investigate the role of SR-associated CaMKII in the regulation of SR Ca$^{2+}$ transport, as well as in regulating cardiac function, transgenic mice were generated to target specifically the expression of CaMKII inhibitory peptide to cardiac longitudinal SR. The SR targeting sequence is defined by the transmembrane portion of the PLB protein, encoding a double mutant that obviates PLB function. We demonstrate that targeted inhibition of SR CaMKII activity results in a significant decrease in PLB phosphorylation at threonine 17. When stressed, the transgenic mice develop dilated heart failure, suggesting the important role of CaMKII pathway in the development of cardiac disease.

**EXPERIMENTAL PROCEDURES**

*Generation of CaMKII-AIP$_4$ Transgenic Mice—* A synthetic gene expression unit was engineered to encode three functional domains using oligonucleotides produced by the DNA Core Facility at the University of Cincinnati. Starting at the amino terminus, the expression unit contains nucleotides that will encode a CaMKII inhibitor, a FLAG epitope, and an SR localization signal. The CaMKII inhibitor domain consisted of sequences encoding a tetramer of the CaMKII autocamtide inhibitory peptide (AIP$_4$), this 13-amino acid sequence (KKALRRQAEVDAL) is known to be a highly specific and potent inhibitor of CaMKII (25). Sequences encoding a FLAG epitope (amino acids DYKDDDDK) (Eastman Kodak Co.) were placed 3’ of the AIP tetramer. In addition, a synthetic sequence encoding a truncated PLB transmembrane domain (amino acids 23–52) was inserted to serve as SR localization signal with mutations at Leu31 (L31A) and Asn34 (N34A) (Genomatix, Ltd., patent pending). Previous studies have shown that mutants L31A and N34A result in loss of the PLB inhibition to SERCA activity (26). This expression unit was subsequently subcloned into a pBluescript-based vector between the 5.5-kb murine a-myosin heavy chain (a-MHC) promoter (a gift from J. Robbins, Children’s hospital, Cincinnati, OH) and an SV40 polyadenylation signal. The linear transgene fragments were released from SalI sites and were purified by gel purification kit (Qiagen). The transgene was injected into pronuclei of fertilized mouse oocytes by the Transgenic Core at University of Cincinnati. The resultant pups were screened for the presence of the transgene by PCR, using an a-MHC-specific primer (5’-GCCCACACCGGAGATGAGCA-3’) and an AIP-specific primer (5’-ACTCTGAGCAGGAGCTAGATA-3’). The founder mice were confirmed by Southern blot analyses. Founder mice were bred with FVB/N wild type mice. Heterozygous animals from at least the third generation were used for all studies, with their non-transgenic (NTG) littermates serving as controls. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

*Southern Blotting Analysis—* Southern blotting analysis was performed to determine the copy number. According to the formula, 1 copy of transgene = $6 \times 10^6$ bp (genomic DNA/copy) $\times$ µg of transgene DNA loaded/bp of transgene, different amounts of transgene DNA was loaded to estimate the different copy number that served as control. Ten µg of genomic DNA prepared from tail biopsies was digested with KpnI and electrophoresed. The blots were hybridized with the SalI to SalI fragment of the released transgene. The TG samples produce 5.8- and 1-kb bands. Intensities of the TG band were measured using a PhosphorImager (Amersham Biosciences) and were compared with the control bands to estimate copy number.

*Preparation of Cardiac Homogenates, SR-enriched Microsomes, and Cytosol Fraction—* Cardiac homogenates, SR-enriched membrane fractions, as well as cytosol fractions were prepared as described previously with a slight modification (27). In brief, an individual mouse heart was used for preparation of cardiac homogenates with 1 ml of ice-cold buffer A containing (mM) 10 imidazole, pH 7.0, 300 sucrose, 10 NaF, 1 EDTA, 0.3 phenylmethylsulfonyl fluoride, 0.5 dithiothreitol, and proteinase inhibitors. For SR isolation, 10–15 pooled mouse hearts were homogenized. Cardiac homogenates were centrifuged at 8,000 g for 20 min; the supernatant was collected, and the resulting pellet (first pellet) was homogenized in buffer A and re-centrifuged as above. The supernatant from two spins were pooled, mixed with buffer B (same as buffer A except that 300 mM sucrose was substituted by NaCl to obtain the final concentration of 600 mM), and centrifuged at 100,000 g for 60 min. The supernatant was concentrated through Centricon (Millipore) and was treated as a cytosol fraction. The resulting pellet (second pellet) was washed in buffer A and re-centrifuged at 100,000 x g for an additional 60 min. The final pellet was used as an SR membrane. Protein concentration was measured by Bradford assay (Bio-Rad).

*Quantitative Immunoblotting Analysis—* The antibodies used for immunoblotting were as follows: mouse anti-FLAG M2 and anti-PP1 α-subunit antibodies (Sigma), polyclonal anti-calsequestrin and monoclonal anti-PLB, anti-RyR antibodies (Affinity Bioreagents, Inc.), mouse anti-PP2A antibody and rabbit anti-CaMKII-α antibody (Calbiochem). The appropriate secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG, were obtained from Sigma. Enhanced chemiluminescence was performed using the SuperSignal Chemiluminescent Detection System (Amersham Biosciences). Calsequestrin protein was used as internal control. The data were analyzed using Image Pro 4.0 (Media Cybernetics).

*Immunocytochemical Staining—* Whole hearts were taken from NTG and TG mice. Tissue-Tek O.C.T compound was added to merge the

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**Fig. 1. Structure of the a-MHC-AIP$_4$-SRL transgene construct and determination of copy number and FLAG expression in TG mice.** A, the transgene consists of cardiac a-MHC promoter, AIP coregulator gene (ds oligos, double-strand oligonucleotides), FLAG epitope, and truncated PLB transmembrane domain (SR localization sequence, SRL). B, Southern blot analyses of tail DNA from transgenic founders. The samples were digested with KpnI and hybridized with the SalI to SalI released fragment as probe. The control bands representing a series of copy numbers (1–20) were calculated according to the formula under “Experimental Procedures.” Only transgenic founders show 5.8- and 1.0-kb bands. C, Western blot analysis of FLAG expression in TG hearts. Tissue homogenate protein derived from whole hearts isolated from NTG and TG mice was immunoblotted with anti-FLAG antibody. DNA construct of AIP$_4$-FLAG-SRL driven by cytomegalovirus promoters was used to transient transfect BHK-21 cells and was served as a positive control. Quantification of the FLAG protein showed 2–3-fold higher level in line 46 than in line 35.
hearts into a chamber, and then the hearts were immediately frozen in liquid N2. Frozen tissue sections (4 μm) were stained as described previously (28). The FLAG expression was detected using mouse anti-FLAG antibody (1:10 dilution) followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Cappel, 1:200 dilution). The same tissue sections were used to detect SERCA2a protein using polyclonal anti-SERCA2a antibody (1:100 dilution) followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Cappel, 1:200 dilution). The tissue sections were observed by a Nikon 135 optiphot fluorescence microscope.

**Immunodetection of Site-specific Phosphorylation of PLB and RyR**—To determine the basal phosphorylation level of PLB and RyR, polyclonal antibodies raised against a PLB peptide phosphorylated at Ser16 (PLB-phosphoserine 16) or at Thr17 (PLB-phosphothreonine 17) (Cyclacel, Dundee, UK) and polyclonal antibody against phosphorylated RyR-2809 antibody (Badrilla, Leeds, UK) were used. The mouse hearts were excised and immediately freeze-clamped using instruments pre-cooled with liquid N2 and homogenized. Twenty μg of homogenate protein was subject to 15% SDS-PAGE followed by the detection of the protein on the membrane. Homogenates were prepared, and the following procedures were performed according to the methods described previously (29).

In vitro phosphorylation was performed using cardiac homogenates from mouse heart, as described previously (30). For endogenous CaMKII phosphorylation, 10 μL (25 μg) of the cardiac homogenates was added to 10 μL of reaction mixture containing 20 mM imidazole (pH 7.0), 10 mM MgCl2, 10 mM NaF, 0.5 mM EGTA, 0.1 mM ATP. Additionally, 0.5 mM CaCl2, 2 μM calmodulin, and 1 μM protein kinase inhibitor peptide 5–24 amide (Sigma) were included. The reaction was carried out at 30 °C. PKA phosphorylation of the cardiac homogenates was carried out in the above-described reaction mixture with 20 units of the PKA catalytic subunit. Reactions were terminated with 4 μL of 6X SDS sample buffer after a 5- (CaMKII) and 2-min (PKA) incubation, which was associated with optimal phosphate incorporation in PLB. Twenty μg of protein was subject to 15% SDS-PAGE followed by the detection of PLB Ser16 and PLB Thr17 using the appropriate phosphorylated PLB antibodies as described above. In vivo phosphorylation was carried out by perfusion with increments from 1 to 32 ng/g/min dobutamine only or followed by 50 mg/ml Ca2+ via left jugular vein. The hearts were rapidly freeze-clamped with liquid N2 and homogenized. Twenty μg of cardiac homogenate protein was used for the detection of PLB Ser16 and PLB Thr17 as described above.

To detect phosphorylated RyR, 20 μg of homogenate protein was subjected to 6% SDS-PAGE followed by probing with anti-phosphorylated RyR-2809 antibody. After stripping the membrane, the same blot was used to probe with anti-RyR antibody to confirm the right location of the protein on the membrane.

**Ca2+ Transport Assays**—Oxalate-facilitated Ca2+ uptake into SR vesicles in cardiac homogenates was determined by the Millipore filtration technique following an established method (31). Cardiac homogenates (100 μg/ml) were incubated (37 °C) in uptake medium containing various concentrations of CaCl2 to yield 0.03–3 μM free Ca2+ (containing 1 μCi/μmol 45Ca2+), as determined by the computer program (32). The reaction was initiated by the addition of 5 mM ATP. The rate of Ca2+ uptake was calculated by least squares linear regression analysis of uptake at 30, 60, and 90 s. Data were analyzed by Origin 7.0 (Microcal Software).

**SR Protein Phosphatase Activity Assay**—The SR protein phosphatase activity was determined with the Ser/Thr phosphatase assay kit from Upstate Biotechnology. The reaction was initiated by adding SR (10 μg) or cytosol (5 μg) proteins prepared as described above to microtiter wells with and without the synthetic substrate (200 μM) in a total assay volume of 25 μL for 20 min. The reaction was terminated by the addition of 4X SDS-PAGE sample buffer.
of 100 μl of Malachite Green solution. The absorbance was read at 630 nm to determine the inorganic phosphate released. The phosphatase activity was calculated by subtracting the OD values in the absence of the substrate from those in its presence (24).

**Hemodynamic Measurements**—Hemodynamic function was measured as described previously (33). Briefly, animals were anesthetized with 50 mg/g body weight ketamine and 100 mg/g body weight thiobutabarbital (Inactin, Research Biochemicals International, Natick, MA) and placed on a thermally controlled surgical table. Following tracheostomy, the right femoral artery and vein were cannulated with polyethylene tubing (OD 0.3–0.5 mm). The arterial catheter was connected to a COBE CDXIII fixed dome pressure transducer (Cobe Cardiovascular, Arvada, CO) for measurement of arterial blood pressure, and the venous catheter was connected to a syringe pump for the infusion of experimental drugs. A high fidelity, 1.4 French (0.5 mm) micromanometer (SPR-671, Millar Instruments, Houston, TX) was then inserted into the right carotid artery and advanced across the aortic valve and into the left ventricle. After completion of the surgery animals were allowed to stabilize for 30–45 min. Cardiovascular responses to separate doses of dobutamine, delivered as a constant infusion (0.1 μg/min/g body weight) over a 3-min period, were then determined; animals were allowed to recover to base line for 5–10 min between doses. After completion of the dose-response protocol, 50 mg/ml CaCl2 was infused.
at a rate of 0.2 μl/min/g body weight, and measurements of heart function were repeated after 5 min. To evaluate left ventricular function, the following measurements and calculations were made: the maximal positive first derivation of LV pressure (peak + dP/dt), an indicator of myocardial contractility; the rate of LV pressure increase determined at an LV pressure of 40 mm Hg (dP/dtmax), an indicative of isovolumic contractility (34, 35); the time constant of relaxation (τ), a measure of LV relaxation, calculated from the exponential decay of the LV pressure trace to a zero asymptote LV; end-diastolic pressure (LVEDP), an index of LV pre-load and compliance (36).

Pressure signals were recorded and analyzed using a PowerLab 4/8 data acquisition system (ADInstruments, Colorado Springs, CO). Average values and each variable were determined for 20–30-a periods at the end of each 3-min dose.

Analytical and Statistical Procedures—Data are reported as the means ± S.E. The statistical analysis was performed using unpaired Student’s t test or one factor (within) or mixed, two-factor analysis of variance (for cardiac function data). A p value of <0.05 was considered statistically significant.

RESULTS

Design, Generation, and Identification of CaMKII-AIP4 Transgenic Mice—Autocamtide, a 13-amino acid peptide (KKALRRQETVDAL), is an ideal substrate for CaMKII. Autocamtide inhibitor peptide, AIP, based on the autocamtide sequence (KKALRQEQAVDAL) is a potent and specific competitive inhibitor for CaMKII (25). In this study the cardiac specific α-MHC promoter was used to direct the concatamer AIP4 genes to the heart. In order to target the expression of AIP4 to the longitudinal SR, a truncated PLB transmembrane domain was used as SR localization signal. To prevent the inhibition of PLB transmembrane domain on SERCA activity, mutations were made at Leu(31A) and Asn(34) (N34A) (26).

Furthermore, the FLAG epitope was included to identify the targeted expression of AIP4 in cardiac SR. The design for the transgene is shown in Fig. 1A. TG mice expressing FLAG-tagged CaMKII-AIP4 were generated as described under “Experimental Procedures.” Six transgenic founders were identified by Southern blotting and PCR analysis (Fig. 1B). Out of six transgenic founders, three lines (lines 35, 36, and 46) transmitted the transgene to offspring; lines 32 and 45 did not transmit the transgene to offspring, and line 42 failed to generate litters. Line 46 carried an estimated two copies of transgene, and line 35 carried one copy (Fig. 1B). To compare the AIP4 protein expression, cardiac homogenates were subjected to competitive immunoblotting by using anti-FLAG antibody. As shown in Fig. 1C, the FLAG protein is only expressed in TG hearts. Quantitation of the FLAG protein levels revealed that the expression level in line 46 is 2–3-fold higher than in line 35. Line 46 was chosen for the following functional experiments.

To confirm the localization of AIP4 expression on cardiac SR, immunofluorescent staining was performed. The tissue sections from TG hearts using both anti-FLAG and anti-SERCA2a antibody showed the similar striated pattern of staining (Fig. 2A). This result indicated that the AIP4 protein was, as predicted based on its SR localization signal, present and highly concentrated in the SR. Furthermore, by using isolated SR and cytosolic fractions, Western blot analysis showed the FLAG epitope was exclusively expressed in cardiac SR in transgenic mice (Fig. 2B). To determine whether AIP4 expression affects CaMKII expression in cardiac SR, isolated SR proteins were electrophoresed, blotted, and probed with anti-CaMKII antibody. There is no significant difference in CaMKII protein level between NTG and TG mice (arbitrary units: 0.983 ± 0.002 in NTG versus 1.017 ± 0.015 in TG, n = 5, p = 0.287) (Fig. 3, A and B).

The 12–16 week-old TG mice were capable of producing healthy litters that exhibited no overt manifestations of a diseased phenotype. There was no evidence of cardiac hypertrophy in the TG mice. The heart weight/body weight ratio (mg/g) was 5.35 ± 0.03 for NTG and 5.33 ± 0.11 for TG in line 46 (n = 10, p > 0.05).

Cardiac Expression of AIP4 Results in Decreased Phosphorylation of Phospholamban—PLB is regulated by phosphorylation of Thr17 by CaMKII and of Ser16 by PKA (7–9). To determine the effect of SR-targeted expression of AIP4 on PLB phosphorylation, the PLB phosphorylation levels under either basal or stimulated conditions were examined. As shown in Fig. 4A, the basal levels of both high and low molecular forms of Thr17-phosphorylated PLB were significantly decreased by 59.7% in extracts prepared from 14- to 16-week-old TG mouse hearts (arbitrary units: 1.426 ± 0.075 in NTG versus 0.574 ± 0.085 in TG).

**TABLE I**

| Protein phosphatase protein levels and activity in TG and NTG mice |
|---------------------------------------------------------------|
| Parameter                     | NTG (n = 3) | TG (n = 4)  |
| PP1a protein level, arbitrary unit | 1.035 ± 0.03 | 0.964 ± 0.04 |
| PP2A protein level, arbitrary unit | 0.992 ± 0.075 | 1.008 ± 0.057 |
| SR phosphatase activity, pmol/μg/min | 3.855 ± 0.215 | 3.985 ± 0.085 |
| Cytosolic phosphatase activity, pmol/μg/min | 34.31 ± 0.78 | 35.09 ± 0.40 |

Results are mean ± S.E. of 3–4 experiments for each group. Each experiment for the measurement of phosphatase activity was from 10 to 15 pooled mouse hearts. p > 0.05 as compared with NTG controls.
FIG. 7. Dobutamine dose-response relationships for heart rate, LV systolic pressure, maximum dP/dt, dP/dt<sub>40</sub>, and relaxation time constant. Heart rate (A), LV systolic pressure (B), maximum dP/dt (dP/dt<sub>max</sub>) (C), isovolumic contractility dP/dt<sub>40</sub> (rate of contraction at intraventricular pressure of 40 mm Hg) (D), and isovolumic relaxation (relaxation time constant, τ) (E) of 3–4-month-old NTG and TG female mice were examined under control conditions and in response to increasing levels of β-adrenergic and 50 mg/ml CaCl<sub>2</sub> stimulation. Values were measured during the final 30 s of each 3-min period in which increasing concentrations of the β-adrenergic agonist, dobutamine, were infused. Data are derived from 8 NTG and 6 TG mice. *, p < 0.05, significantly different from NTG at corresponding dose levels; #, significantly different from NTG at all dose levels (p < 0.05), as determined by analysis of variance. Cont, control; Ca<sup>2+</sup>, perfuse with 50 mg/ml CaCl<sub>2</sub>.
0.094 in TG, n = 6, p < 0.01). However, Ser16-phosphorylated PLB in TG mice was not significantly altered (1.041 ± 0.024 in NTG versus 0.959 ± 0.052 in TG, n = 4, p = 0.104). Our results indicate that functional expression of AIPα is evidenced by exclusively blocking the CaMKII pathway. Furthermore, we examined the PLB phosphorylation under in vitro stimulated conditions. Cardiac homogenates from NTG and TG hearts were incubated with calmodulin (CaM) in the presence of Ca2+ under optimal conditions and subjected to SDS-PAGE followed by Western blot using PT-17 antibody. As shown in Fig. 4B, in the absence and presence of calmodulin and Ca2+, Thr17-phosphorylated PLB, were decreased by 63.9 (1.469 ± 0.060 in NTG versus 0.530 ± 0.017 in TG, n = 3, p < 0.01) and 47.8% (1.315 ± 0.039 in NTG versus 0.686 ± 0.096 in TG, n = 3, p < 0.01), respectively, in TG hearts compared with NTG controls, indicating the endogenous CaMKII activity in TG hearts is dramatically decreased. On the other hand, PKA-dependent phosphorylation of PLB was not significantly altered as represented by the similar pattern of Ser16-phosphorylated PLB in the presence of PKA catalytic subunit between NTG (increased by 48.4%, n = 4) and TG (increased by 50.4%, n = 4) hearts (Fig. 4B). This result suggested that the PKA pathway is not affected by AIP expression on SR.

It has been reported that β-adrenergic stimulation was associated with significant PLB phosphorylation at both Ser16 and Thr17 sites. To observe the effect of β-adrenergic stimulation on phosphorylation of PLB, the NTG and TG mouse hearts were perfused with increments from 1 to 32 ng/g/min dobutamine. Rapidly frozen hearts were homogenized to detect the PLB phosphorylation with site-specific antibodies to phosphorylated Ser16 and Thr17 PLB. The maximal phosphorylation level of the PKA site in response to β-adrenergic stimulation as represented by Ser16-phosphorylated PLB remained unaltered in TG mice (Fig. 4C). However, the level of Thr17-phosphorylated PLB was decreased by 73.6% in TG (0.418 ± 0.096 in NTG, n = 3, p < 0.01) following β-adrenergic stimulation (Fig. 4C). Thus, maximal PLB phosphorylation of the CaMKII site could not be achieved in the TG animals. These results further demonstrate that due to expression of AIPα, the SR CaMKII pathway is significantly blocked, thus resulting in a significant decrease in PLB phosphorylation at Thr17.

It has been reported that Ca2+ entry through L-type Ca2+ channels triggers the activation of CaMKII pathway in cardiomyocytes (37). To determine the stimulation of CaMKII pathway by Ca2+, 50 mg/ml Ca2+ was perfused in vivo after the cardiac function recovered from the response to β-adrenergic stimulation. The maximal level of Thr17-phosphorylated PLB was decreased by 74.1% (1.589 ± 0.053 in NTG versus 0.411 ± 0.030 in TG, n = 3, p < 0.01) in TG mice (Fig. 4C). Quantitation of the signals using anti-PLB antibody did not show the alteration of the total PLB protein in TG mice (data not shown).

Decreased Vmax of Ca2+ Uptake but No Shift of Affinity for Ca2+ in TG Mice—It is well known that SR Ca2+ uptake is regulated by PLB; PLB phosphorylation disrupts the inhibitory association of PLB with SERCA, producing a shift in SERCA affinity for Ca2+. Furthermore, several studies have shown that SERCA can be phosphorylated by CaMKII resulting in an increase in the Vmax of Ca2+ uptake (12–14). To study whether the decreased phosphorylation of PLB Thr17, due to selective inhibition of SR CaMKII pathway, would influence SR Ca2+ uptake, SR Ca2+ uptake was measured using cardiac homogenates. As shown in Fig. 5, A and B, the Vmax of Ca2+ uptake in TG was decreased by ~30% (nmol Ca2+/mg/min: 120.76 ± 7.38 in NTG, n = 4, versus 84.69 ± 6.39 for TG, n = 5, p < 0.05). However, the apparent affinity for Ca2+, represented as K0.5, was unaltered (µM: 0.192 ± 0.014 in NTG versus 0.198 ± 0.011 in TG, p > 0.05). Our results indicate that decreased PLB phosphorylation at Thr17 did not affect SERCA affinity for Ca2+, suggesting that the intact PKA pathway is sufficient for the PLB regulation of SERCA under the experiment conditions.

### TABLE II

| Parameter                  | NTG (n = 4) | TG (n = 6) |
|----------------------------|-------------|------------|
| Heart/body weight ratio (mg/g) | 5.10 ± 0.22 | 14.46 ± 1.00a |
| Lung/body weight ratio (mg/g)  | 6.39 ± 0.57 | 18.35 ± 2.29a |

*a p < 0.01 as compared with NTG controls.

**Fig. 8.** Morphological and histological analyses of NTG and TG mouse hearts. A, representative example of female mouse hearts at 7 months of age after delivering at least three litters. The heart chamber and atrium from an NTG and a TG mouse hearts are shown. B, paraffin-embedded thin sections of TG and age-matched NTG controls were stained with hematoxylin and eosin. C, enlargement of the left heart chambers is evident, especially left atrium. Atrial thrombi are frequently observed. D, high magnification (×40 microscope objective) of the septum. Increased myocyte hypertrophy and disarray and intracellular spacing are found in the TG ventricles.
However, an ~30% decrease in the $V_{\text{max}}$ of SR $Ca^{2+}$ uptake function was observed in TG mice. It is possible that the decreased $V_{\text{max}}$ of SR $Ca^{2+}$ uptake may be due to the changes of SERCA pump level. To exclude this possibility, Western blot analysis was carried out to measure the SERCA2a protein level using both cardiac tissue homogenates and isolated SR. As shown in Fig. 5C, quantitative immunoblots showed that SERCA2a protein was not significantly altered in TG mice in comparison with NTG mice (arbitrary units: 1.007 ± 0.027 in NTG versus 0.993 ± 0.028 in TG, $n = 8$, $p = 0.365$ for homogenates, and 1.062 ± 0.046 in NTG versus 0.938 ± 0.039 in TG, $n = 5$, $p = 0.042$ for isolated SR). These results suggest a direct inhibition of the $V_{\text{max}}$ of SR $Ca^{2+}$ uptake by AIP4 expression in cardiac SR.

**Effect of Cardiac Expression of AIP4 on Phosphorylation of the Ryanodine-sensitive $Ca^{2+}$ Release Channel—**RyR can be phosphorylated at serine 2809 by either PKA or CaMKII in cardiac SR (17, 18). To observe whether AIP4 expression affects the phosphorylation level of RyR in junctional SR, anti-phosphorylated RyR-2809 antibody was used to detect the phosphorylated RyR under basal conditions as described above. To confirm the identity of CaMKII-phosphorylated high molecular weight protein as RyR, the same blot was stripped and re-probed with anti-RyR antibody. As shown in Fig. 6, the basal phosphorylation level of RyR was slightly decreased by 11% (1.052 ± 0.029 for NTG and 0.930 ± 0.032 for TG, $n = 4$, $p = 0.019$) in TG mice when normalized by RyR protein levels. Our data suggest that RyR phosphorylation was not significantly affected by AIP4 expression in SR, indicating the disruption of SR CaMKII pathway restricted to longitudinal SR.

**SR Protein Phosphatase Activity Is Not Altered in AIP4 Transgenic Mice—**There are dynamic balances between protein kinases and protein phosphatases (PP) in cellular regulation. Therefore, the decrease in SR CaMKII activity resulting from AIP4 expression in TG mice might affect the SR protein phosphatase activity. PP1 and PP2A protein level as well as total protein phosphatase activity were measured in isolated SR membranes from NTG and TG mouse hearts. As shown in Table I, PP1 and PP2A protein levels were not significantly changed in TG mice nor did the protein phosphatase activity.

**Hemodynamic Function in AIP4 TG Mice Expressing CaMKII Inhibitory Peptide—**Under both basal and stimulated conditions, heart rate was significantly lower in AIP4-TG mice compared with NTG ($p < 0.02$), and the maximal heart response to $\beta$-adrenergic stimulation was greater in the NTG animals ($p < 0.03$, Fig. 7A). Mean arterial pressure tended to be increased in the TG mice compared with NTG mice, but the difference was not significant ($p = 0.1$, data not shown). However, as shown in Fig. 7B, LV systolic pressure was consistently elevated in the TG animals, compared with NTG mice ($p < 0.05$), and in response to dobutamine, LV systolic pressure increased in the TG animals but not in NTG mice ($p > 0.05$). Although LV $dP/dt_{\text{max}}$ was not significantly different between the two groups of animals, as shown in Fig. 7C, the $dP/dt_{\text{max}}$, an index of contractility that attempts to correct for the observed differences in afterload, reveals a clear decrement in LV contractile function in the TG mice. As shown in Fig. 7D, $dP/dt_{\text{a0}}$ was reduced by ~18% throughout the dobutamine dose-response range ($p < 0.02$), and the magnitude of the inotropic response to $\beta$-stimulation was blunted in the TG mice ($p < 0.01$). Cardiac relaxation, as indicated by the time constant of pressure decline during diastole (tau, Fig. 7E), was significantly slower by 26.1% in TG animals at base line and over the entire range of $\beta$-adrenergic stimulation ($p < 0.002$). Finally, LVEDP was significantly higher in the TG compared with NTG mice under base-line conditions (3.5 ± 1.8 versus 7.9 ± 2.6, $p < 0.05$), but there was no difference during $\beta$-adrenergic stimulation (data not shown).

Intravenous infusion of $Ca^{2+}$ to elevate serum $Ca^{2+}$ concentration, which is expected to stimulate CaMKII activity independent of adrenergic receptor activation, showed a qualitatively similar pattern of results, as illustrated in Fig. 7.

**Targeted Expression of AIP4 in Cardiac SR Changes the Response to Stress—**Although the 3–4-month-old mice did not show phenotype of cardiac hypertrophy, 7-month-old female TG mice that had produced at least three litters developed severe cardiac hypertrophy and dilated heart failure. As shown in Table II, the heart/body and the lung/body weight ratios in TG mice were dramatically increased by 2.82- and 2.87-fold, respectively, in comparison with NTG mice. In addition, the 12–14-month-old male TG mice developed a similar pattern of heart failure. Morphological studies showed a dramatic enlargement of the left atrium as well as left ventricle. Histological analysis showed that there were multiple large thrombi in the lumen of the left atrium with recanalization and hypertrophy of the myocytes. These samples displayed severe fibrosis with myocyte hypertrophy, especially severe in the left ventricle (Fig. 8).

**DISCUSSION**

The functional relevance of subcellular localization of CaMKII in cardiomyocytes is not clear. In this study, we utilized the $\alpha$-MHC promoter and SR localization signal to target the expression of a potent CaMKII inhibitory peptide, AIP4, into cardiac longitudinal SR. This approach provides rational to study the function of subcellular CaMKII in the hearts. Chemical inhibitors of CaMKII, KN-62 and KN-93, globally disrupt cellular function by inhibiting CaMKII in all regions of the cell. Gene knockout techniques are used to knockout specifically a selective CaMKII isoform, but the consequences affect the entire cells. The advantage of utilizing inhibitory peptides to define the role of CaMKII is that specific cell expression can be controlled by the appropriate gene promoter, and intracellular localization can be directed through the use of targeting sequences. This approach was previously applied to calmodulin inhibitory peptide genes in TG lungs in our laboratory (38). In the present report we observed a targeted expression of AIP into cardiac SR. Furthermore, we assessed a 59.7% decrease in basal phosphorylation of PLB threonine 17 in longitudinal SR, but a mild (11%) decrease in the phosphorylation of RyR serine 2809 in junctional SR, indicating that the expressed AIP is mainly functional at longitudinal SR.

The 3- and 4-month-old TG hearts displayed a moderate decrease in both heart rate and the maximal rate of contraction under both basal and stimulated conditions (see Fig. 7, A and D). Furthermore, there was a significant increase in the relaxation time constant ($\tau$) and an elevation in basal LVEDP, indicating a deficit in myocardial diastolic function. Despite these differences, blood pressure tended to be elevated in the TG animals. These results indicate the contraction and relaxation function of TG hearts was moderately impaired due to targeted inhibition of CaMKII in longitudinal SR. On the other hand, the response to $\beta$-adrenergic stimulation was maintained in TG mice, suggesting that PKA-mediated PLB Ser16 phosphorylation plays an important role in the regulation of heart function under $\beta$-adrenergic stimulated conditions in intact hearts. Considering the dramatic decrease at PLB Thr17 phosphorylation, our results support the concept that the effect of PLB Thr16 phosphorylation is not dominant in the regulation of cardiac function under physiological conditions (7, 39). A recent report by Chu et al. (39), using PLB T17A mutant mice under PLB knockout background which totally aminate the PLB phosphorylation site by CaMKII, demonstrated that PLB phosphoryl-
The important role of SR CaMKII pathway in mediating the protein level in TG mice. Additionally, the unchanged apparent peptide in cardiac SR, because there is no decrease in SERCA activity by targeted expression of CaMKII inhibitory molecular mechanism. The AIP4 transgenic mice will be the unique opportunity to study the role of SR CaMKII in the hypertrophy, ventricular dilation, and decreased contractile failure (40, 41). In this study, in addition to the significant decrease at PLB Thr17 phosphorylation, we did observed an ~30% decrease in the $V_{\text{max}}$ of SR Ca$^{2+}$ uptake without changing the apparent affinity for Ca$^{2+}$ in TG mice. The decreased $V_{\text{max}}$ of SR Ca$^{2+}$ uptake might be due to the direct inhibition of SERCA activity by targeted expression of CaMKII inhibitory peptide in cardiac SR, because there is no decrease in SERCA protein level in TG mice. Additionally, the unchanged apparent affinity for Ca$^{2+}$ suggests that the decreased SR Ca$^{2+}$ uptake may not be due to the expression of the mutant PLB transmembrane domain (SR localization signal) which has been shown to lose its inhibitory effect on SERCA activity by double mutation (26). Recent physiological studies also provide supportive data of direct regulation of SERCA activity by CaMKII mutation (26). Recent physiological studies also provide supportive data of direct regulation of SERCA activity by CaMKII.

In summary, the functional relevance of SR CaMKII in the regulation of SR Ca$^{2+}$ handling under physiological and pathological conditions requires further investigation to define the molecular mechanism. The AIP4 transgenic mice will be the first mouse model in which SR CaMKII activity is specifically inhibited as a result of targeted expression of CaMKII inhibitory peptide in the longitudinal SR. This model provides a unique opportunity to study the role of SR CaMKII in the regulation of excitation-contraction coupling and in the development of heart failure.

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Note Added in Proof—In transgenic mice overexpressing cytoplasmic CaMKII c8, increased phosphorylation of inositol phosphates, ryanodine receptor and phospholamban Thr17, leads to altered myocyte calcium handling (45) and the development of heart failure (46). Our data further support that CaMKII activity must be precisely regulated in order to maintain normal cardiac function.

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