The Transgenic Expression of Highly Inhibitory Monomeric Forms of Phospholamban in Mouse Heart Impairs Cardiac Contractility*

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Transgenic mice were generated with cardiac-specific overexpression of the monomeric, dominant-acting, superinhibitory L37A and I40A mutant forms of phospholamban (PLN), and their phenotypes were compared with wild-type (wt) mice or 2-fold overexpressors of wt PLN (wtOE). The level of PLN monomer in cardiac microsomes was increased 11-13-fold, and the apparent affinity of the sarco(endo)plasmic reticulum Ca2+-ATPase for Ca2+ was decreased from pCa 6.22 in wt or 6.12 in wtOE to 5.81 in L37A and 5.72 in I40A. Basal physiological parameters, measured in isolated myocytes, indicated a significant reduction in the rates of shortening (+dL/dt) and relengthening (∼dL/dt). Hemodynamic measurements indicated that peak systolic pressure was unaffected but that pressure changes (+dP/dt and −dP/dt) were lowered significantly in both mutant lines, and relaxation time (τ) was also lengthened significantly. Echocardiography for both mutants showed depressed systolic function and an increase in left ventricular mass of over 1.4-fold. Significant decreases in left ventricular shortening fraction and velocity of circumferential shortening and increases in ejection time were corrected by isoproterenol. The use of antibodies specific against Ser16- and Thr17-phosphorylated PLN peptides showed that phosphorylation of both pentameric and monomeric PLN were increased between 1.2- and 2.4-fold in both the L37A and I40A lines but not in the wtOE line. These observations show that overexpression of superinhibitory mutant forms of PLN causes depression of contractile parameters with induction of cardiac hypertrophy, as assessed with echocardiography.

The contraction of cardiac muscle depends, in part, on the release of Ca2+ from the lumen of the sarcoplasmic reticulum; relaxation depends on removal of Ca2+ from the myoplasm by the combined activity of Ca2+ pumps in the sarcoplasmic reticulum (SERCA1 pumps), the plasma membrane pumps, and by Na+/Ca2+ exchange at the plasma membrane (1, 2). The activity of SERCA2a, the cardiac slow-twitch isoform of the Ca2+ pump, is regulated by the integral membrane protein phospholamban (PLN) (3). Dephosphorylation of PLN inhibits the activity of SERCA2a at low Ca2+ concentrations by lowering its apparent affinity for Ca2+. Inhibition is overcome by phosphorylation of PLN by either protein kinase A or Ca2+-calmodulin kinase (3, 4).

That PLN regulates left ventricular basal contractile parameters and their responses to β-agonists has been demonstrated through the analysis of the function of myocytes infused with an antibody against PLN (5), of PLN-null mice (6, 7), and of mice overexpressing wild-type (wt) PLN (8). Hemodynamic tests proved that the ablation of PLN is associated with significant increases in intraventricular systolic pressure, in the rate of change of both positive (+dP/dt) and negative (−dP/dt) hemodynamic pressure, and in τ, a measure of the rate of cardiac muscle relaxation. These elevated parameters could be stimulated only minimally with the β-adrenergic agonist, isoproterenol (9). By contrast, the 2-fold overexpression of wt PLN in transgenic mice resulted in an inhibition of Ca2+ transport by the sarcoplasmic reticulum, decreased Ca2+ kinetics, contractile parameters in transgenic ventricular myocytes, and depressed basal left ventricular systolic function in vivo (8).

Wild-type PLN exists in pentameric and monomeric forms (10), but mutations in one face of the PLN transmembrane helix, including Leu37 to Ala (L37A) and Ile40 to Ala (I40A), lead to monomer formation (11, 12). These mutations also enhance the inhibitory function of PLN, leading to the proposal that monomeric PLN is the inhibitory species (13, 14). This view has been supported by measurement of the monomer concentration in membranes (15). If the monomer is the inhibitory species, the superinhibitory function of monomeric mutant forms of PLN can be explained, at least in part, by mass.

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The abbreviations used are: SERCA2a, sarco(endo)plasmic reticulum Ca2+-ATPase isoform 2a; PLN, phospholamban; L37A, a mutant form of phospholamban in which Leu37 is mutated to Ala; I40A, a mutant form of phospholamban in which Ile40 is mutated to Ala; wt, wild-type; wtOE, a transgenic mouse line in which wild-type phospholamban is overexpressed by 2-fold in the heart; α-MHC, α-myosin heavy chain promoter; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; PAGE, polyacrylamide gel electrophoresis; LV, left ventricular.
action, since the normal PLN pentamer to monomer ratio of about 10:1 is reduced to less than 1:10 (13). PLN mutants N27A and N30A are also superinhibitors, even though they are not monomeric (16). We have proposed that these mutant PLN pentamers dissociate normally and inhibit as monomers, but the monomer has enhanced affinity for SERCA2a (13, 16). Anomalous mutants have also been described. The C41A mutant is about 75% monomeric but does not gain inhibitory function at 25 °C (13). Furthermore, the C41F mutant is largely monomeric at 37 °C (17) but loses partial inhibitory function in vitro (18) and in vivo (19), when compared with controls.

The studies of Luo et al. (6) have shown that PLN ablation, which results in chronic relief of the inhibitory properties of PLN, alters cardiac function but does not lead to cardiomyopathy. As a corollary, it was of interest to determine whether chronic high inhibition of SERCA2a by superinhibitory, monomeric forms of PLN might affect cardiac function and possibly lead to cardiomyopathy. Accordingly, we created transgenic mice in which L37A and I40A are overexpressed. Overexpression results in a dramatic shift to lower affinity in the range of 5 to 10 μM (13). Furthermore, we found that the reverse primer (MHC, 5′-CAG CCT CTG CTA CTC CTC TTC CTG CCT-3′) and the forward primer (MHC, 5′-CAT CTT GAG GAC GTT GAG CTA TTA CTC GAG GCC ACC ATG GAG AAA GTT C-3′) used as a forward primer in PCR amplification of the clone, was 5′-CTG TTA CTC GAG GCC ACC ATG GAG AAA GTT C-3′. A HindIII restriction endonuclease site was introduced into the 3′ end of the clone using the reverse PCR primer 5′-CTG TCA AGA AGC TTA GTC AGA GAA GCA TGA TGA CCA TG-3′ by modification of the plasmid to a consensus translation initiation sequence (20) for efficient mRNA translation. The 5′ nucleotide sequence, used as a forward primer in PCR amplification of the clone, was 5′-CTG TTA CTC GAG GCC ACC ATG GAG AAA GTT C-3′. A HindIII restriction endonuclease site was introduced into the 3′ end of the clone using the reverse PCR primer 5′-CTG TCA AGA AGC TTA GTC AGA GAA GCA TGA TGA CCA TG-3′ by modification of the region to a consensus translation initiation sequence (20) for efficient mRNA translation. The 5′ nucleotide sequence, used as a forward primer in PCR amplification of the clone, was 5′-CTG TTA CTC GAG GCC ACC ATG GAG AAA GTT C-3′.

**Experimental Procedures**

**Construction of PLN L37A and I40A Transgenic Mice**—The mutations L37A and I40A were introduced into rabbit PLN cDNA as described previously (13). The construct used previously was modified by the addition of a BamHI restriction enzyme site at the 5′ end of the clone using the BamHI restriction endonuclease digestion, gel-purified, and extracted from the gel using a kit (Qiaquick DNA purification kit, QIagen). DNA samples were then introduced by microinjection into the pronuclei of one-cell-stage C57BL/6 × CBA murine embryos (in the laboratory of Dr. F. Jirik, University of British Columbia) to generate transgenic mice.

Mice carrying the mutated PLN transgene were identified by the presence in DNA extracted from tail tips of a 6.243-kb BamHI restriction fragment that included most of the α-MHC promoter sequence, the 159-bp PLN coding sequence (including the stop codon), and the 3′ end of the human growth hormone cDNA sequence. A 1.108-kb DNA fragment, cut from the middle of the α-MHC promoter sequence between restriction sites SpII and EcoRI, was used as a probe for non-radioactive genomic Southern and slot-blot analysis. The probe was labeled (22) with dioxxygenin-11-DUTP following the recommendations of the manufacturer (Roche Molecular Biochemicals) and hybridized to DNA on positively charged membranes, as described by Hauge and Goodman (23), except that 1% bovine serum albumin was replaced by 1% blocking reagent (Roche Molecular Biochemicals). Alkaline phosphatase activity was detected on membranes with CDP-Star as described by the manufacturer (Roche Molecular Biochemicals). Since the endogenous α-MHC gene yielded a 6035-bp BamHI fragment in which 5439 bp were from the promoter region and thus were identical to the transgene, this fragment hybridized to the same probe in wild-type animals. The copy number of the construct was estimated by comparison of densitometric scans of lanes in Southern blots of DNA samples from transgenic mice with those from wild-type mice in which the copy number of the endogenous α-MHC gene was assumed to be 2.

A 500-bp PCR product that covered the junction between the α-MHC promoter and PLN-encoding sequences was generated using the forward primer (MHC, 5′-CAG CCT CTG CTA CTC CTC TTC CTG CCT-3′) and the reverse primer (PLN, 5′-GAG CTG TTT GTT GAG GTA TTA CTA TGC-3′) and used for the same identification of L37A, I40A, and wtOE transgene-positive animals.

**Control Animals**—Transgenic mice in which wt PLN is overexpressed 2-fold (wtxOE) were used as a control for the overexpression of superinhibitory mutant PLN (8). The PLN L37A and PLN I40A transgenic mice, produced in the laboratory of Dr. F. Jirik, were primarily on an FVB/N genetic background. Southern blot analysis with the C57BL/6, whereas transgenic mice overexpressing the murine wt PLN, produced in the laboratory of Dr. E. Kranias, were on a FVB/N background. Thus only first generation C57BL/6 × FVB/N heterozygotes overexpressing either wild-type or superinhibitory transgenes were studied wherever a common genetic background was required for comparison of the effects of the transgene. In most experiments, first generation C57BL/6 × FVB/N wt animals expressing normal amounts of wt PLN were included as controls.

**Calcium Uptake Assays**—Animals 12–16 weeks old were sacrificed by cervical dislocation. Hearts were excised and frozen within seconds in liquid nitrogen and stored at −70 °C. The frozen hearts were powdered under liquid N2 with a chilled mortar and pestle, and microsomal membranes were prepared as described previously (24), except that the homogenizing buffer contained 10 mM Tris-HCl, pH 7.4, 20 mM CaCl2, 0.3 mM sucrose, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and Complete™ protease mixture. The apparent Ca2⁺ affinity of SERCA2a was determined from measurement of the Ca2⁺ dependence of Ca2⁺ transport, as described previously (18). Protein concentrations were determined by the Bio-Rad method using bovine serum albumin as a standard.

**Western Blot Analysis**—Relative levels of expression of pentameric and monomeric PLN were determined by SDS-PAGE of measured amounts of cardiac microsomes at several dilutions, followed by densitometric analysis of Western blots using monoclonal antibody 1D11 against PLN (a gift from Drs. Robert Johnson and Edward McKenna, Merck). Binding of primary antibody was detected by peroxidase-conjugated secondary antibodies and ECL detection reagents (Amersham Pharmacia Biotech). The composition of Tyrode was as follows: 118 mM NaCl, 5.4 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4, 0.33 mM NaHPO4, 2 mM MgCl2, and 30 mM taurocholate. Hearts were perfused at 2 ml/min at 37 °C in oxygenated Tyrode containing 2 mM CaCl2, 2-fold (wtOE) were used as a control for the overexpression of superinhibitory mutant PLN (8). The PLN L37A and PLN I40A transgenic mice, produced in the laboratory of Dr. F. Jirik, were primarily on an FVB/N genetic background. Southern blot analysis with the C57BL/6, whereas transgenic mice overexpressing the murine wt PLN, produced in the laboratory of Dr. E. Kranias, were on a FVB/N background. Thus only first generation C57BL/6 × FVB/N heterozygotes overexpressing either wild-type or superinhibitory transgenes were studied wherever a common genetic background was required for comparison of the effects of the transgene. In most experiments, first generation C57BL/6 × FVB/N wt animals expressing normal amounts of wt PLN were included as controls.

**Cardiac Myocyte Studies**—Ventricular myocyte isolation procedures were modified from a previously described protocol (25). Briefly, mice were anesthetized with sodium pentobarbital (6.5 mg, intraperitoneal); aortas of excised hearts were cannulated in ice-cold Ca²⁺-free Tyrode buffer, and the heart was attached to a Langendorff perfusion apparatus. The composition of Tyrode was as follows: 118 mM NaCl, 5.4 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4, 0.33 mM NaHPO4, 2 mM MgCl2, and 30 mM taurocholate. Hearts were perfused at 2 ml/min at 37 °C in oxygenated, Ca²⁺-free Tyrode for 3 min, followed by 25 mM CaCl2 ⁄ Tyrode containing a mixture (2:1) of type 2 and type 1 collagenase (Worthington) totaling 0.5 mg/ml and bovine serum albumin (1 mg/ml). Cells were isolated by enzymatic digestion followed by gentle mechanical dissociation and filtered through 250-μm nylon mesh. Cells were washed sequentially in 100 mM CaCl2, 200 mM CaCl2, and 1 mM CaCl2, and resuspended in 1.8 mM CaCl2-Tyrode solution. Typical isolation yielded at least 30% rod-shaped cells in 1 mM CaCl2-Tyrode.

Ventricular myocytes, suspended in 1.8 mM CaCl2-Tyrode, were plated in a Flexiglas chamber on the stage of an inverted epi-fluorescence microscope (Nikon Diaphot 200). Myocytes were stimulated at 0.5 Hz with a Grass S5 stimulator (pulse duration of 4 ms), and a video edge detector (Crestden Electronics) was used to track myocyte contractions. Signals were recorded and analyzed using a Delta Scan-1 dual-beam spectrophotofluorimeter and Felix software (Photon Technologies Inc.). Myocyte length, fractional shortening, shortening rate (dL/dt), and shortening rate (−dL/dt), and relengthening rate (L/dt) were determined for an average of nine myocytes from each heart. A single heart was considered as a single sample. The average age of the mice was 17.6 weeks.

**Microsurgical Methods and in Vivo Hemodynamic Measurements**—Mice were anesthetized using a mixture of ketamine (50–100 mg/kg) and xylazine (3–6 mg/kg) by intraperitoneal injection. After achieving full anesthesia, mice were placed in a supine position. The right carotid artery was isolated and an incision was made and cauterized with a cautery probe (PE 200) thickness of 150–300 μm and a 20–30-μm wall thickness. The polyethylene catheter was connected to a TXD-310 low compliance pressure transducer (MicroMed, Louisville KY) and amplified by a blood pressure analyzer (BPA model 300, MicroMed, Louisville, KY). The pressure recording system had a 28 ± 2-Hz roll-off frequency measured at −3 db, which might be expected to produce some
distortion of our pressure recordings. After insertion of the catheter into the carotid artery, the catheter was advanced into the aorta and then into the left ventricle to record the aortic and ventricular pressures. The parameters measured and analyzed were heart rate, aortic pressure, left ventricular (LV) systolic pressure, LV diastolic pressure, and the maximum and minimum first derivatives of the LV pressure (\(+\frac{dP}{dt}_{\text{max}}\) and \(-\frac{dP}{dt}_{\text{max}}\), respectively). The \(+\frac{dP}{dt}_{\text{max}}\) and \(-\frac{dP}{dt}_{\text{max}}\) give a measure of the contractility of the heart, whereas \(-\frac{dP}{dt}_{\text{max}}\) is also strongly influenced by the rate of pressure relaxation. To complement the \(-\frac{dP}{dt}_{\text{max}}\) estimates, the time constants for pressure relaxation were also estimated by fitting the relaxation phase of the pressure traces (from 30 to 80% of the peak systolic pressure) to a monoeXponential.

Some pressure recordings \((n = 3 \text{ or } 4 \text{ in each group})\) were also made using a 1.4 French Millar catheter (Millar Inc., Houston) connected to an amplifier (TCP-500, Millar Inc.). The pressure signals were filtered at 300 Hz (3 db), recorded using an A/D data acquisition board (2801A, Data Translation, Marlboro, MA) at a 1-kHz sampling rate and stored in the computer for later analysis. No significant difference in peak pressure was observed between the two recording methods. However, the maximum \(\pm \frac{dP}{dt}\) was about 18% lower for the fluid-filled catheters than the Millar-based measurements. Only the pressure recordings made using the fluid-filled catheters were included in the analyses reported below.

In Vivo Echocardiographic Assessment of Cardiac Function—M-mode and Doppler echocardiography were performed for non-invasive assessment of left ventricular (LV) function and dimensions, using previously described methods (26). Briefly, mice were lightly anesthetized with 2.5% avertin (0.01 ml/g) intraperitoneally and were allowed to breathe spontaneously. The chest was shaved, acoustic coupling gel was applied to the left precordium, and a warming pad was used to maintain normothermia. Mice were imaged in a shallow left lateral decubitus position using an Interspec Apogee X-200 ultrasonograph with a 9-MHz imaging and a 5–7.5-MHz pulsed-wave Doppler transducer. Studies were performed at base line and after the administration of 2.0 \(\mu\)g isoproterenol intraperitoneally. Left ventricular percent fractional shortening, velocity of circumferential shortening corrected for heart rates at matched heart rates, and end diastolic wall thickness/cavity radius ratio were calculated as described previously (8, 26). LV mass was calculated using M-mode LV measurements according to American Society of Echocardiography conventions and the modified American Society of Echocardiography-cube LV mass equation (27), as described previously (8, 26). The feasibility of the use of cardiac ultrasound in the \textit{in vivo} assessment of LV mass in mice has been shown in a necropsy validation study (28).

Phosphorylation of PLN—Hearts were excised and frozen within seconds in liquid nitrogen and stored at \(-70^\circ\) C. The frozen hearts were powdered under liquid N2 with a chilled mortar and pestle, and micro
dilutions were made using the fluid-filled catheters than the Millar-based measurements. Only the pressure recordings made using the fluid-filled catheters were included in the analyses reported below.

Results

Construction and Identification of Transgenic Animals—To create the PLN L37A founder mice, 290 embryos were injected; 142 embryos were implanted, and 10 pups were born. One of two positive pups died early, and the other founded the line used in this study. For PLN I40A mice, 286 embryos were injected, 159 embryos were implanted, and 20 pups were born. Of these, 5 were positive, but 3 did not transmit or showed poor expression levels; 1 line could not be propagated, and the other founded the line used in this study. This resulted in one transgenic line for each of the PLN mutant constructs that could be propagated for further analysis. However, the L37A and I40A lines have similar phenotypes in \textit{vivo} and \textit{in vitro}, so that creation of two comparable lines from two comparable mutants makes insertional mutagenesis and inter-line variability of phenotypes unlikely. The founders of both transgenic lines and their progeny did not appear to differ from their littermates in either behavior or reproductive ability.

In the Southern blots shown in Fig. 1B, the presence of a heavily stained 6243-bp BamHI fragment representing most of the a-MHC promoter, the PLN coding sequence, and part of the human growth hormone sequence in the transgene vector was used to identify transgenic mice. In control lanes, a weakly stained 6035-bp BamHI fragment, which overlaps the mobility of the 6243-bp transgene fragment, represents the endogenous murine a-MHC gene, and the level of staining is equivalent to two copies of the gene. The level of hybridization of the probe to the endogenous a-MHC gene fragment in wt mice compared with the level of hybridization of the probe to the transgenic a-MHC gene fragment revealed that the L37A and I40A transgenics were integrated into the mouse genome at copy numbers of about 20 and 15, respectively.

Overexpression of Monomeric PLN—The expression of monomeric mutant PLN in cardiac microsomes from transgenic mice was assessed by Western blotting with the PLN-specific monoclonal antibody, 1D11. In previous experiments on the transient expression of L37A and I40A in HEK-293 cells, L37A was 94% monomeric, whereas I40A was 100% monomeric (13). Thus any increase in PLN monomer content in L37A and I40A transgenic mice could be attributed to the expression of these mutant transgenes. Data presented in Fig. 2 and Table I show that content of pentameric PLN was constant in microsomal fractions from wt, L37A, and I40A transgenic lines, whereas the content of pentameric PLN was increased about 2-fold in the wtoE line, as described previously (8). The content of monomeric PLN was also constant for wt and wtoE lines at about 5.5–5.7% of total PLN. In the L37A line, however, the distribution between monomer and pentamer was increased to 40.5% and in I40A to 46.2% of total PLN (Table I). The overall increase in PLN expression for the L37A line was about 1.54-fold, and the increase in monomeric PLN was about 11-fold. The overall increase in PLN expression for the I40A line was
ventricular pressures for the L37A and I40A mice compared with age-matched littermate control mice. Consistent with Ca\(^{2+}\) uptake measurements and cell shortening studies described above, the magnitude of both left ventricular \(+\frac{dP}{dt}\) \(_{\text{max}}\) and \(-\frac{dP}{dt}\) \(_{\text{max}}\) was significantly depressed in L37A and I40A transgenic mice compared with controls. Neither heart rates nor end diastolic pressures were significantly different between the groups, suggesting that the differences in \(-\frac{dP}{dt}\) \(_{\text{max}}\) between the groups were not due to changes in preload or heart rate. These differences in hemodynamic parameters between the groups were not the result of the limited frequency response of our pressure recording system, since signal filtering will tend to reduce (not enhance) the observed differences between L37A/I40A and wt control mice. As expected from the differences in \(-\frac{dP}{dt}\) \(_{\text{max}}\) between the groups, the relaxation times (\(\tau\)) of the pressure traces were significantly prolonged in the I37A and I40A mice compared with either wt or wtOE littermate controls.

As with our studies of single myocytes and our echocardiographic measurements described below, measurements carried out with wtOE mice were indistinguishable from control mice. This was unexpected, since 2-fold overexpression of wild-type PLN was found to be inhibitory when compared with wild-type controls in two earlier studies (8, 19). However, our findings may be a consequence of the genetic background of all of the animals in our study (C57BL/6/FVB/N), which was different from the genetic background in the earlier studies (FVB/N).

Echocardiographic Properties—Echocardiographic studies revealed that, when compared with wt or wtOE mice, L37A and I40A mice displayed significant reductions in left ventricular fractional shortening and velocities of circumferential shortening (Table V). The relative magnitude of these differences in the extent and velocity of shortening between the groups was remarkably similar to the reductions in contractility as measured by \(+\frac{dP}{dt}\) \(_{\text{max}}\) and \(-\frac{dP}{dt}\) \(_{\text{max}}\). Ejection times were also prolonged in the L37A and I40A mice, relative to wt and wtOE mice, but this difference did not reach significance for I37A mice. The reductions in indices of cardiac contractility were accompanied by increases in left ventricular mass to body weight ratios and left ventricular dimensions in both L37A and I40A mice, when compared with wtOE mice. A 1.47-fold increase in LV mass for the L37A mice and a 1.44-fold increase in LV mass for the I40A mice suggest that compensatory hypertrophy occurred in these two lines of mice. Reduced contractility in L37A and I40A mice, compared with wtOE mice, could be completely overcome by treatment with isoproterenol (Table VI).

PLN Phosphorylation in Transgenic Animals—The lack of mortality in most of the L37A and I40A transgenic mice suggests that compensatory mechanism(s) may be activated to counteract the superinhibitory effects of mutant PLN. The most likely compensatory change would result in a sustained increase in PLN phosphorylation, leading to dissociation of PLN from the inhibited SERCA2a complex. To test this hypothesis, the content of phospho-PLN in pooled hearts from three animals was determined by its reaction with anti-Ser(P) and anti-Thr(P) antibodies. Increased phosphorylation was observed in both pentameric and monomeric PLN in all three of the transgenic samples (Fig. 3). Changes could also be deduced from the alterations in mobility that are characteristic of phosphorylation following immunoblotting with antibody 1D11 (Fig. 2).

The key question, however, was whether the increase in phosphorylation was greater than the increase in PLN expression. The most straightforward measurement of the increase in phosphorylation levels was through measurement of the phos-

![Fig. 2. Western blot analysis of PLN expression in L37A (I40A) PLN transgenic and wild-type mice. Microsomal proteins (100 ng) were isolated from pooled hearts of three animals, separated on 12.5% PAGE, transferred onto a nitrocellulose membrane (0.05-\(\mu\)m pore size), and probed with anti-PLN monoclonal antibody, 1D11. Further details are provided in the legend to Table I. Arrows indicate pentameric (\(p\)) and monomeric (\(m\)) PLN forms. Multiple bands in monomeric PLN are likely due to different levels of phosphorylation.](http://www.jbc.org/)

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**Observed in** V of I40A and L37A myocytes (Table III). Fractional shortening of I40A and L37A myocytes was decreased (\(p < 0.05\)) by 46 and 33%, whereas rates of contraction (+\(\frac{dL}{dt}\)) of I40A and L37A myocytes were depressed by 53 (\(p < 0.05\)) and 40% (\(p < 0.05\)), respectively. Finally, myocyte relengthening rates (−\(\frac{dL}{dt}\)) were decreased by 63 (\(p < 0.05\)) and 47% (\(p < 0.05\)) in I40A and L37A myocytes, respectively. These results support in vitro data and suggest that I40A mutant PLN is a more potent inhibitor of SERCA2a than L37A. By contrast, the mechanical function of myocytes from wtOE mice expressing 2-fold elevated levels of pentameric wt PLN showed no functional depression compared with wt cells. Since one of our previously published studies (8) showed significant decreases in mechanical parameters of myocytes overexpressing wt PLN in the FVB/N genetic background (at 0.25 Hz), we reassessed the functional parameters of myocytes from this transgenic model. We observed significant decreases in fractional shortening (18%), +\(\frac{dL}{dt}\) (31%), and −\(\frac{dL}{dt}\) (36%) in myocytes (at 0.5 Hz) overexpressing wt PLN in the FVB/N genetic background, consistent with our previous report (9). However, the background of the mice used in the current study reflects an F1 cross between FVB/N and C57BL/6. This suggests that the lack of functional depression in wtOE myocytes may be a function of the altered genetic background.

**Hemodynamic Properties—**Hemodynamic characteristics of L37A and I40A mice were examined at 8–12 weeks of age. The results are summarized in Table IV. There were no significant differences in mean aortic pressures and peak systolic left
**DISCUSSION**

Inhibitory interactions between PLN and SERCA2a establish the range of myoplasmic Ca\(^{2+}\) concentrations over which elevated Ca\(^{2+}\) can shift SERCA2a Ca\(^{2+}\) transport from base line to full activity. When PLN is phosphorylated, these inhibitory interactions are abolished, and the Ca\(^{2+}\) activation range is shifted, temporarily, toward higher apparent Ca\(^{2+}\) affinity. Following the ablation of PLN, the Ca\(^{2+}\) activation range is shifted chronically toward higher apparent Ca\(^{2+}\) affinity, and apparent Ca\(^{2+}\) affinity is unaffected by \(\beta\)-adrenergic stimulation. Under these conditions, an enhanced rate and extent of Ca\(^{2+}\) uptake into the sarcomplasmic reticulum can be correlated with enhanced cardiac contractility and with a decrease in relaxation time. These sustained changes in the performance of the heart do not induce cardiomyopathy, and the PLN-null animals enjoy a normal life span (29).

In earlier studies (13, 16), we showed that several dominant-acting mutant forms of PLN, including L37A, I40A, and N27A, gain inhibitory function by inducing dramatic decreases in the apparent affinity of SERCA2a for Ca\(^{2+}\). We predicted that this shift in Ca\(^{2+}\) affinity might compromise cardiac function, leading to cardiomyopathy (13). In order to test this hypothesis, we have created animal models in which the dominant, highly inhibitory L37A and I40A PLN mutant proteins are overexpressed. We obtained one transgenic line for each PLN mutant, but since the L37A and I40A mutants have similar phenotypes in vitro and in vivo, creation of two comparable lines from two comparable mutations would rule out insertional mutagenesis and inter-line variability of phenotypes and, in this way, would be comparable to creation of more than one line from a single mutant. The L37A mice showed a 1.54-fold increase in overall PLN expression and an 11-fold increase in monomeric PLN expression; the I40A mice showed a 1.71-fold increase in overall PLN expression, and a 13-fold overexpression of monomeric PLN in cardiac microsomes. Measurement of the Ca\(^{2+}\) dependence of Ca\(^{2+}\) uptake in these microsomes recreated with high fidelity the observations made earlier in HEK-293 cells co-expressing SERCA2a and PLN (13). Thus we were successful in transferring the experimental system from heterologous cell culture to intact animals where physiological correlations could be measured.

Chu et al. (19) investigated the effect of transgenic overexpression of the C41F mutant of PLN. This mutant is anomalous in that it was largely monomeric in SDS gels (17) but did not gain inhibitory function in in vitro tests (18). In line with the in vitro observations, the transgenic overexpression of the C41F mutant in mice did not lead to superinhibition of Ca\(^{2+}\)-ATPase function in isolated cardiac microsomes (19). The level of inhibition was slightly greater than that observed in wild-type mice but less than that observed in mice expressing a 2-fold excess of wild-type PLN. Analysis of cardiomyocyte mechanics and Ca\(^{2+}\) kinetics indicated that the inhibitory effects of C41F mutant PLN overexpression were also less pronounced than those of 2-fold overexpression of wild-type PLN. Thus, the C41F mutant mouse has not proven to be a suitable model for the investigation of the effects of overexpression of highly inhibitory monomeric forms of PLN such as the mutants L37A and I40A.

Measurements of myocyte function provide functional correlates of changes in Ca\(^{2+}\) handling by L37A and I40A overexpression. As expected from the Ca\(^{2+}\) uptake studies, the extent of shortening and the rates of shortening and relengthening for wt or wtOE myocytes, which were not different from each

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**Table I**

| Relative pentamer content | Relative monomer content | Relative phosphorylation of pentameric Ser\(^{16}\) | Relative phosphorylation of pentameric Thr\(^{17}\) |
|---------------------------|-------------------------|-----------------------------------------------|-----------------------------------------------|
| wt                        | 1.00                    | 5.7                                           | 1.00                                          |
| L37A                     | 0.97                    | 40.5                                          | 1.86                                          |
| I40A                     | 0.92                    | 46.2                                          | 2.39                                          |
| wtOE                     | 1.90                    | 5.5                                           | 0.74                                          |

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**Table II**

The effect of I40A and L37A PLN expression on the Ca\(^{2+}\) affinity of SERCA2a

Initial rates of Ca\(^{2+}\) uptake by cardiac sarcoplasmic reticulum from wt and I40A, L37A, and wtOE transgenic animals were assayed over Ca\(^{2+}\) concentrations from 0.15 to 5.5 Ca units for SERCA2a. Data are mean ± S.E.

| K\(_{Ca}\) (in Ca units) for SERCA2a |
|------------------------------------|
| Cardiac microsomes                 |
| wt                                 | 6.22 ± 0.15 (n = 5)            |
| L37A                               | 5.81 ± 0.17 (n = 5)            |
| I40A                               | 5.72 ± 0.06 (n = 5)            |
| wtOE                               | 6.12 ± 0.10 (n = 4)            |

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a K\(_{Ca}\) values reported for wt, I37A, and I40A mutant PLN co-expressed with SERCA2a in HEK-293 cells (13) are included for comparison.

\(p > 0.05\) versus wt control mice.
other, were far greater (11%) than either L37A (7.7%) or I40A (6.2%) myocytes. Consistent with the myocyte studies, both +\(dP/dt_{\text{max}}\) and −\(dP/dt_{\text{max}}\) were found to be depressed in hemodynamic measurements carried out with the L37A and I40A mice, establishing that contractility is impaired in these mice. Associated with +\(dP/dt_{\text{max}}\) changes, there were also reductions in the fractional ventricular shortening, ejection time, and velocity of circumferential shortening. Modulation of these ventricular parameters are all expected to result from reduced [Ca\(^{2+}\)] transient amplitude as a result of reduced sarcoplasmic reticulum Ca\(^{2+}\) uptake and release following impairment of SERCA2a function.

Diminished contractility coincided with a nearly 50% increase in LV heart weight to body weight ratio, a significant increase in LV mass, and an increased chamber size in both L37A and I40A mice (Table IV). These morphological changes are anticipated since cardiac hypertrophy and remodeling is a common compensatory response of the myocardium to alterations in cardiac function or increased hemodynamic loads (30–32). In our hemodynamic recordings, significant slowing of the rate of pressure relaxation was also observed, as reflected in the time constants for pressure relaxation (\(\tau\)) and −\(dP/dt_{\text{max}}\). These changes corresponded with differences in myocyte

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**TABLE III**

Basal physiological parameters of isolated ventricular myocytes

|         | wt, \(n = 10\) | L37A, \(n = 3\) | I40A, \(n = 4\) | wtOE, \(n = 5\) |
|---------|----------------|----------------|----------------|----------------|
| Shortening, % | 11.4 ± 1.09 | 7.65 ± 0.02 | 6.22 ± 0.56 | 10.97 ± 1.38 |
| +\(dL/dt\) (μm/s) | 131.26 ± 11.87 | 79.10 ± 13.30* | 62.22 ± 5.02* | 148.12 ± 27.56 |
| −\(dL/dt\) (μm/s) | 115.45 ± 11.34 | 61.38 ± 15.80* | 42.87 ± 5.06* | 115.09 ± 22.34 |

*\(p > 0.05\) versus wt control mice.

**TABLE IV**

Hemodynamic properties of L37A, I40A, and wtOE transgenic and control mice

|         | wt, \(n = 20\) | I40A, \(n = 8\) | L37A, \(n = 6\) | wtOE, \(n = 5\) |
|---------|----------------|----------------|----------------|----------------|
| Peak systolic pressure (mmHg) | 108 ± 14 | 94 ± 13 | 103 ± 19 | 103 ± 7 |
| +\(dP/dt\) (mmHg/s) | 4247 ± 440 | 3423 ± 408* | 3260 ± 540* | 4503 ± 174 |
| −\(dP/dt\) (mmHg/s) | 4136 ± 415 | 3364 ± 390* | 3171 ± 573* | 4445 ± 156 |
| \(\tau\) (relaxation) (ms) | 15.2 ± 2.2 | 18.7 ± 3.0* | 18.2 ± 1.7* | 16.5 ± 1.7* |

*\(p > 0.05\) versus wt control mice.

**TABLE V**

Base-line echocardiographic measurements of I40A, L37A, wtOE transgenic, and control mice

|         | wt, \(n = 9\) | I40A, \(n = 9\) | L37A, \(n = 8\) | wtOE, \(n = 3\) |
|---------|----------------|----------------|----------------|----------------|
| Heart rate (beats/min) | 398 ± 28.6 | 385 ± 16.2 | 333 ± 27.4 | 400 ± 30.0 |
| LV end diastolic dimension (mm) | 3.62 ± 0.07 | 3.66 ± 0.06 | 3.95 ± 0.06* | 3.98 ± 0.05* |
| LV end systolic dimension (mm) | 2.23 ± 0.07 | 2.27 ± 0.05 | 2.85 ± 0.06* | 2.83 ± 0.05* |
| LV posterior wall thickness (mm) | 0.58 ± 0.03 | 0.56 ± 0.04 | 0.63 ± 0.02 | 0.57 ± 0.02 |
| Calculated LV mass (mg) | 46.1 ± 3.28 | 43.48 ± 2.99 | 55.81 ± 2.41* | 58.78 ± 0.98 (\(p = 0.051\)) |
| LV mass/body mass (mg/g) | 1.83 ± 0.11 | 1.64 ± 0.06 | 2.37 ± 0.13* | 2.41 ± 0.20* |
| h/r | 0.29 ± 0.01 | 0.27 ± 0.02 | 0.26 ± 0.01 | 0.28 ± 0.01 |
| LV shortening fraction (%) | 38.56 ± 1.33 | 37.85 ± 1.89 | 27.98 ± 0.58* | 29.03 ± 1.99* |
| Ejection time (ms) | 76.00 ± 3.43 | 72.22 ± 2.70 | 90.63 ± 6.52* | 77.67 ± 2.40 |
| Vcf\(_{\text{c}}\) (circ/s) | 6.31 ± 0.37 | 6.11 ± 0.36 | 4.33 ± 0.12* | 4.59 ± 0.30* |

*\(p > 0.05\) versus wtOE control mice.

**TABLE VI**

Isoproterenol response of I40A, L37A, wtOE transgenic, and control mice (echocardiographic measurements)

|         | wt, \(n = 9\) | wtOE, \(n = 3\) | I40A, \(n = 4\) | L37A, \(n = 3\) |
|---------|----------------|----------------|----------------|----------------|
| Heart rate (beats/min) | 531 ± 29.1 | 520 ± 39.7 | 455 ± 17.29 | 614 ± 29.9 |
| LV shortening fraction (%) | 61.09 ± 2.35 | 60.21 ± 4.99 | 60.71 ± 3.96 | 68.36 ± 1.66 |
| Ejection time (ms) | 62.50 ± 5.83 | 60.00 ± 4.51 | 62.55 ± 4.42 | 56.00 ± 2.31 |
| Vcf\(_{\text{c}}\) (circ/s) | 10.20 ± 0.80 | 10.82 ± 0.55 | 11.43 ± 1.28 | 12.10 ± 0.08 |

*\(p > 0.05\) versus wtOE control mice.
Reduced contractility in L37A and I40A mice, compared with wtOE mice, was completely overcome by treatment with isoproterenol (Table V). These results indicate that phosphorylation of PLN was not maximal in these animals and that considerable potential for β-adrenergic stimulation remained in the L37A and I40A mice. We used antibodies specific against Ser16- and Thr17-PLN peptides to show that phosphorylation of both pentameric and monomeric PLN was increased in both the L37A and I40A lines but was decreased in the wtOE line (Fig. 3 and Table I). The increases in Thr17 phosphorylation in L37A and I40A might be attributed to activation of Ca2+ calmodulin kinase, but the increases in Ser16 phosphorylation for L37A and I40A would be due to protein kinase A, implying adrenergic activation. These findings suggest that the inhibitory effects of overexpression of L37A and I40A on Ca2+ cycling might be reduced in the transgenic mice by compensatory phosphorylation of PLN. Further studies of compensatory mechanisms will be useful in assessing cross-talk between Ca2+ handling proteins of the sarcoplasmic reticulum and other regulatory proteins in maintaining contractile parameters under basal and isoproterenol-stimulated conditions in the hearts of L37A and I40A mice.

An unexpected finding was that wtOE transgenic animals did not differ in any functional properties from wt animals when the genetic background was an F1 cross between FVB/N and C57BL/6. In earlier studies in which comparisons were made using inbred animals with an FVB/N background, the 2-fold overexpression of wt PLN was found to be inhibitory (8, 19). This suggests that the lack of functional depression in wtOE myocytes may be a function of the altered genetic background; the effect of recessive traits is maximized in inbred lines and minimized in hybrid animals.

In summary, we have found that overexpression of two monomeric, superinhibitory forms of PLN results in significant depression of contractility in mouse hearts. Our findings are particularly relevant, since they are the corollary of the recent findings demonstrating that ablation of PLN can rescue the cardiomyopathic phenotype caused by LIM protein disruption (33). Both of these studies highlight the critical role of SERCA2a function in normal and myopathic hearts.

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The Transgenic Expression of Highly Inhibitory Monomeric Forms of Phospholamban in Mouse Heart Impairs Cardiac Contractility

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