J Mol Cell Cardiol **33**, 1031–1040 (2001)
doi:10.1006/jmcc.2001.1370, available online at http://www.idealibrary.com on IDEAL®

The Enhanced Contractility of the Phospholamban-deficient Mouse Heart Persists with Aging

J. P. Slack[1], I. L. Grupp[1], R. Dash[1], D. Holder[2], A. Schmidt[1], M. J. Gerst[1], T. Tamura[3], C. Tilgmann[1], P. F. James[4], R. Johnson[2], A. M. Gerdes[1] and E. G. Kranias[1]

[1]*Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA,* [2]*Merck Research Laboratories, West Point, PA 19486, USA,* [3]*South Dakota Health Research Foundation, Cardiovascular Research Institute, 1400 West 22nd Street, Sioux Falls, SD 57105, USA,* [4]*Department of Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA*

(Received 10 January 2001, accepted in revised form 22 February 2001)

J. P. Slack, I. L. Grupp, R. Dash, D. Holder, A. Schmidt, M. J. Gerst, T. Tamura, C. Tilgmann, P. F. James, R. Johnson, A. M. Gerdes and E. G. Kranias. The Enhanced Contractility of the Phospholamban-deficient Mouse Heart Persists with Aging. *Journal of Molecular and Cellular Cardiology* (2001) **33**, 1031–1040. Phospholamban ablation in the mouse is associated with significant increases in cardiac contractility. To determine whether this hyperdynamic function persists through the aging process, a longitudinal examination of age-matched phospholamban-deficient and wild-type mice was employed. Kaplan–Meier survival curves indicated no significant differences between phospholamban-deficient and wild-type mice over the first year. Examination of cardiac function revealed significant increases in the rates of contraction ($+dP/dt$) and relaxation ($-dP/dt$) in phospholamban-deficient hearts compared with their wild-type counterparts at 3, 6, 12, 18 and 24 months of age. Quantitative immunoblotting indicated that the expression levels of the sarcoplasmic reticulum Ca^{2+}-ATPase were not altered in wild-type hearts, while they were significantly decreased at 12 months (40%) and 18 months (20%) in phospholamban-deficient hearts. These findings on the persistence of hyperdynamic cardiac function over the long term suggest that phospholamban may constitute an important target for treatment in heart disease.
© 2001 Academic Press

Key Words: Phospholamban; Aging; Sarcoplasmic reticulum; Myocardium; Contractility.

Introduction

Aging is often associated with diminished cardiac performance characterized mainly by a longer relaxation phase, although a prolongation in the time to peak tension has also been reported in some studies.[1–6] The depressed contractile parameters have been postulated to be due to alterations in the expression levels of the key calcium-handling proteins in the heart. Studies in papillary muscles of young and senescent rats indicated a switch in the myosin isoforms from the faster (α-myosin heavy chain) to the slower (β-myosin heavy chain) protein, resulting in slower actomyosin ATPase activity and improved efficiency of muscle mechanics in the senescent myocardium. The transcript and protein levels of the SERCA2a isoform of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase were also shown to decrease, and this was associated with decreases in SR Ca^{2+}-ATPase content and depressed Ca^{2+}-transport rates in the senescent rat heart.[2,7–9] However, other reports indicated no change in

Please address all correspondence to: Evangelia G. Kranias, Ph.D., Department of Pharmacology and Cell Biophysics, 231 Bethesda Ave., Cincinnati, OH 45267-0575, USA. Fax: 513-558-2269; Tel: 513-558-2377; E-mail: Litsa.Kranias@uc.edu

0022–2828/01/051031+10 $35.00/0 © 2001 Academic Press

SERCA2a mRNA and protein levels in the aging rat heart.[10,11] Additional biochemical studies suggested that decreases in SR Ca^{2+} transport were associated with a compensatory increase of the sarcolemmal Ca^{2+}-ATPase,[12] and the Na^{+}/Ca^{2+} exchanger.[13] Although the physiological factors underlying the regulation of gene expression in the aging heart are not known, there is evidence that chronic exercise could reverse the prolongation in contraction and relaxation times, assessed in isometrically contracting rat papillary muscle.[8,14] The improved relaxation was associated with increases in the expression levels of SERCA2a and increased SR Ca^{2+} transport rates, while there were no alterations in the depressed function of the calcium-activated myosin ATPase activity in these hearts.[8,14]

Collectively, these studies indicate that the depressed contractile parameters of the senescent myocardium are mainly due to decreased expression of the SR Ca^{2+}-ATPase. However, there is little information on the role of phospholamban, the regulator of the SR Ca^{2+}-ATPase affinity for Ca^{2+}, in the aging process. Studies on phospholamban mRNA[15] or phosphorylated phospholamban levels[16] did not observe any alterations upon senescence in the rat. In the mouse, a recent study indicated increases in phospholamban protein without alterations in SERCA2a in the aging heart.[17] These findings on decreased SERCA or increased PLB levels suggest elevation of the relative phospholamban/SERCA2 ratio, which is expected to result in inhibition of cardiac contractility,[18] and may contribute to the prolonged relaxation and contraction rates in senescent hearts.

The importance of the phospholamban/SERCA2 ratio in determining the affinity of the SR-Ca^{2+} transport for Ca^{2+} and cardiac contractile parameters in isolated myocytes, perfused hearts or intact mice, was recently elucidated using genetically engineered mouse models with altered expression levels of phospholamban. Specifically, ablation of phospholamban was associated with highly enhanced basal contractility, which could be minimally stimulated by β-agonists.[19,20] However, it is not clear whether this hyperdynamic phenotype may persist over the long-term or whether compensatory changes may develop in the aging phospholamban-deficient myocardium, which may diminish its enhanced function. The question of long-term maintenance of the enhanced cardiac function becomes especially important, since recent studies have demonstrated the beneficial effects of phospholamban deficiency in the impaired cardiac function associated with hypertrophy or failure in the mouse, suggesting that phospholamban may represent a potential therapeutic target in heart disease.[21,22] Thus, a longitudinal study was carried out in the phospholamban-deficient and its isogenic wild-type mouse to determine whether the hyperdynamic contractile parameters of phospholamban-deficient hearts persist through the aging process.

Methods

Animals

The generation of the phospholamban-deficient mouse was previously described.[20] Animals of either genotype or sex were sacrificed at specified time intervals. Mice used for biochemical and physiological characterization were of the 129SvJ/CF-1 genetic background and housed at the University of Cincinnati in an IACUC approved murine pathogen free barrier facility, monitored by sentinel testing. Animals used for the survival curve studies were of the 129SvJ background and were housed in a murine pathogen free isolated barrier unit system at Taconic Labs, Germantown, NY, USA. Ablation of phospholamban was associated with similar enhancement of cardiac contractility in both strains (data not shown).

Survival curves

Survival curves for each sex and genotype were estimated separately using the Kaplan–Meier procedure. The studies included populations of 276 wild-type and 106 phospholamban-deficient mice. The 50% mortality time is estimated as the first time when the estimated survival curve falls below 0.50. The 95% confidence interval for the 50% mortality time was calculated by finding the times where the upper and lower 95% confidence interval for the proportion alive first intersects the horizontal line at 0.50. Differences in the distribution of survival times for the wild-type and the phospholamban-deficient groups were tested using the log rank test.

Western blots

The relative tissue levels of phospholamban, SR Ca^{2+}-ATPase, and calsequestrin were assessed by quantitative immunoblotting.[23] Cardiac homogenates from pools of four to five hearts for each

age group were assayed in triplicate using 10 μg of protein (within the linear range of detection for all of the proteins measured), which were separated on 13% SDS polyacrylamide gels and transferred to nitrocellulose membranes. A sample of 3-month-old hearts was processed on the same gels as the aging groups, and the values observed in 3-month-old hearts were taken as 100%. The membranes were incubated with either a phospholamban monoclonal antibody (1:5000 dilution, Affinity Bioreagents), a SR Ca^{2+}-ATPase polyclonal antibody (1:500 dilution), or a calsequestrin antibody (1:2000 dilution), kindly provided by Dr Larry Jones, Krannert Institute of Cardiology. Antibody binding was visualized with a horseradish peroxidase-labeled secondary antibody (Amersham, Inc.) and the degree of labeling was determined with ImageQuant (Molecular Dynamics) software.

Working heart preparations

The experimental conditions for the work-performing mouse heart preparations were described previously.[20,24] After Langendorff mode retrograde perfusion, anterograde work-performing perfusion was initiated at a workload of 250 mmHg/ml/min, which was achieved with a venous return of 5 ml/min and an aortic pressure of 50 mmHg. Coronary and aortic flows were separately measured. Pressure and volume loadings were carried out in all work-performing heart preparations. First, afterload (aortic resistance) was kept constant at 50 mmHg, and venous return was increased until the contractility ($+dP/dt$) was no longer elevated. Then the venous return was kept constant at 5 ml/min, and the aortic pressure (afterload) was increased to the point where the $+dP/dt$ was not further elevated. The cardiac work at different venous return or afterload conditions was calculated and expressed as mmHg $\times$ ml/min.

In vivo echocardiograph assessments

M-mode and Doppler studies were performed to assess non-invasively left-ventricular (LV) function and dimensions, using an Interspec Apogee X-200 ultrasonograph with a 9 MHz imaging and 5–7.5 MHz pulse-waved Doppler transducer, as previously described.[25] Mice were anesthetized with 2.5% Avertin (0.01 ml/g) i.p., and were allowed to breathe spontaneously. The chest was shaved, acoustic coupling gel was applied to the left hemithorax and a warming pad was used to maintain normothermia. Mice were imaged in a shallow left lateral supine position. Left-ventricular percent fractional shortening (LVFS) and the velocity of circumferential shortening corrected for heart rate differences (Vcf_c) were calculated as previously described.[18,25]

Statistical analysis

Values represent the mean $\pm$ S.E.M. Statistical significance was assessed using the one-way repeated measure of variance (ANOVA) and the Student–Neuman–Keuls test. $P<0.05$ was considered statistically significant.

Results

Figure 1 shows the Kaplan–Meier survival curves for wild-type and phospholamban-deficient mice. We detected no overall significant differences for the survival times of phospholamban-deficient mice as compared to their wild-type cohorts. The estimated 50% and 75% mortality times for the wild-type mice were 108 (101, ∞) and 121 (121, ∞) weeks *v* 95 (84, 105) and 126 (105, ∞) weeks for the phospholamban-deficient mice, suggesting no significant differences ($P=0.15$) between the two groups (Fig. 1). However, in the course of this study, there were five clusters (each with $\geq$ three mice) of deaths in the wild-type group, involving mice that were likely housed together in the same cages and died in the same week. If the estimate for the wild-types is adjusted such that the mice, which died in clusters are treated as censored, the estimated 50% mortality time was 113 (108, ∞) weeks for this group, which is significantly ($P<0.05$) longer than that of their phospholamban-deficient counterparts for the entire 2 year period. However, even after this adjustment, there is no evidence of increased survival of wild types *v* phospholamban-deficient mice for the first year. Furthermore, the estimated 75% mortality time for the adjusted wild-type data, was 121 (121, ∞) weeks, which is not significantly different from that of the phospholamban-deficient animals.

Examination of the heart and body weights in 3, 6, 12, 18 and 24-month-old wild-type and phospholamban-deficient mice revealed no alterations in the heart/body weight ratios in either genotype throughout the aging process (Table 1). There were no significant alterations in left ventricular cell length or sarcomere length[26] observed either (data not shown).

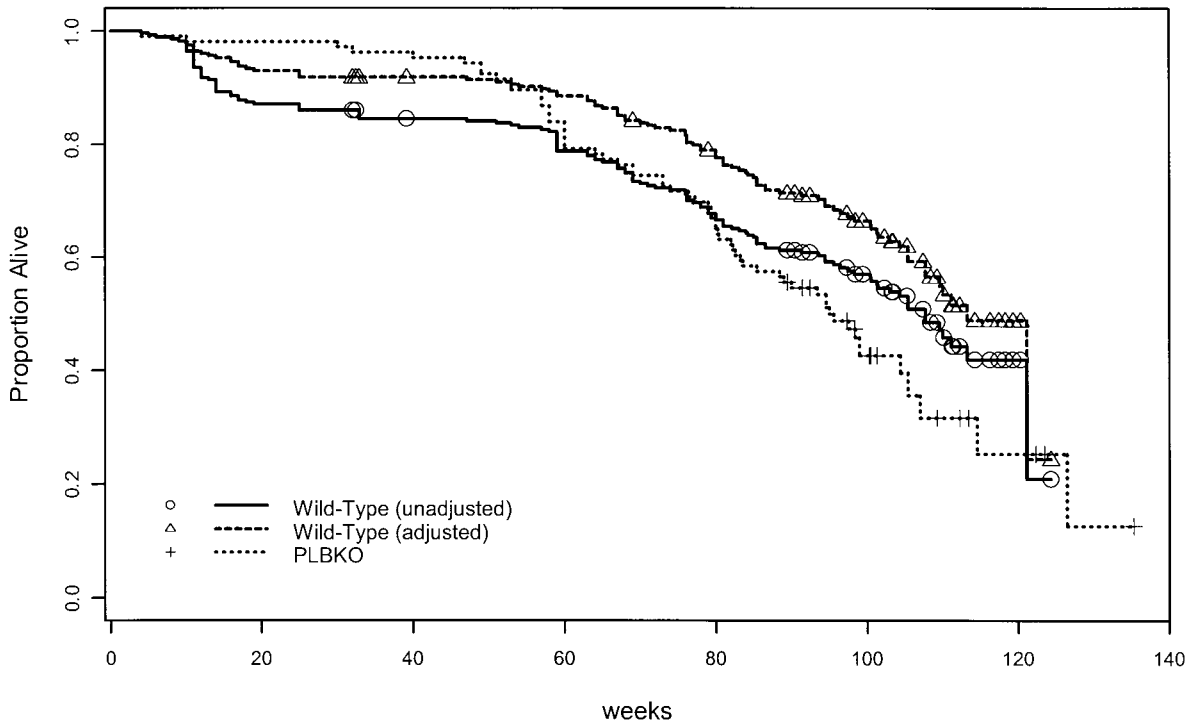

Figure 1 Kaplan–Meier survival curves are plotted for 276 wild-type and 106 phospholamban-deficient mice. The plotting symbols mark censoring times (animal withdrawn from the study prior to death) for wild-type, unadjusted (○), and phospholamban-deficient (+) mice. For the "adjusted estimate" (△), wild-type mice that died as part of a cluster (three or more in the same week and same cage) were censored.

Table 1 Relationship of heart weight to body weight in wild-type and phospholamban-deficient mouse hearts at different ages

Age/genotype	*n*	HW (mg)	BW (g)	HW/BW Ratio
3 mo WT	5	223±25	29.89±3	7.5±0.7
6 mo WT	4	275±17	36.2±3.9	7.7±0.4
12 mo WT	7	326±14	40.6±3	7.6±0.3
18 mo WT	6	271±16	34.5±1.4	7.5±0.2
24 mo WT	6	197±13	26.9±0.7	7.3±0.5
3 mo KO	4	225±12	29.5±1	7.6±0.2
6 mo KO	5	240±12	32.2±1.3	7.5±0.4
12 mo KO	6	289±11	40.8±1.1	7.1±0.4
18 mo KO	6	276±17	37.9±2.3	7.2±0.4
24 mo KO	6	220±33	26.6±1.4	8.0±0.9

WT denotes wild-type mice; KO, phospholamban-deficient mice. Values represent the mean±S.E.M. for *n* different hearts. ANOVA analysis of HW/BW ratios did not reveal any statistically significant differences in any of the age groups, when compared to the 3-month controls.

To assess the effects of age on cardiac performance in wild-type and phospholamban-deficient mice, the isolated work-performing heart preparation was used. Cardiac contractility at identical loading conditions was examined in mice of both genotypes at 3, 6, 12, 18 and 24 months of age. In this preparation, the isolated mouse heart generates intraventricular pressures greater than 100 mmHg, assuring adequate coronary filling during diastole, which allows these hearts to function outside the body for 4 h without decline in cardiac function. We observed no age-dependent changes in the rates of contraction or relaxation in the hearts of wild-type mice at an afterload (mean aortic pressure, MAP) of 50 mmHg and at a venous return of 5 ml/min (Fig. 2). Furthermore, the times to peak pressure (TPP) and half relaxation ($RT_{1/2}$) were also unchanged with age (data not shown). While it was of interest to examine the effects of age on heart function in the wild-type mouse, a central question to this study was whether the hyperdynamic cardiac function, observed previously in 3-month-old phospholamban-deficient mice,[20] would persist with age. As illustrated in Figure 2, the contractile parameters in phospholamban-deficient mice appeared to progressively become less hyperdynamic between 3 months and 12 months of age, and subsequently increased between 12 and 24 months of age. At 6 months of age, the contractile parameters in phospholamban-deficient hearts were not significantly different than those of their

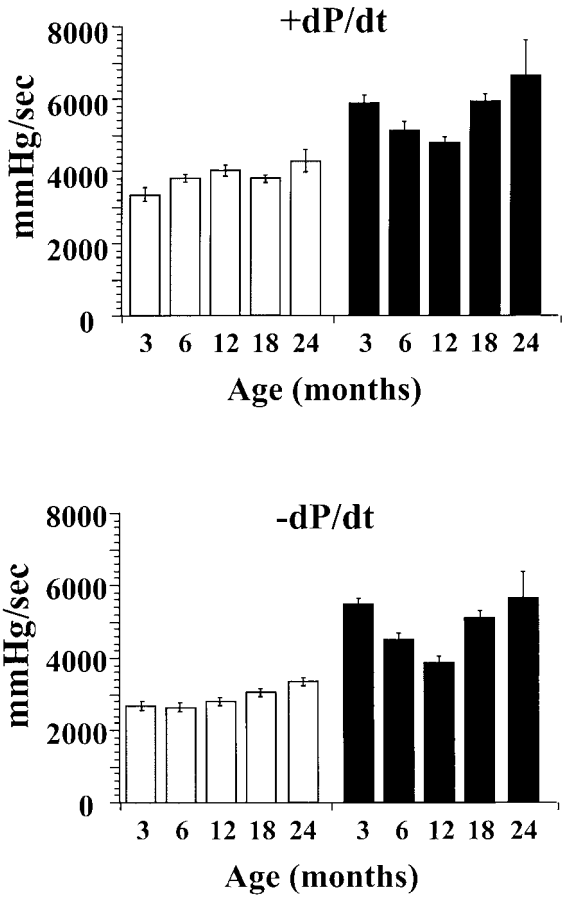

Figure 2 Basal cardiac function in isolated hearts from wild-type (white bars) and phospholamban-deficient (black bars) mice. Hearts were taken from mice at 3, 6, 12, 18 and 24 months of age and studied under identical, basal venous return, afterload, and similar heart rates. The rates of contraction and relaxation are significantly increased in phospholamban-deficient hearts relative to those observed in their age-matched wild-type cohorts. The values for maximal rates of pressure development (+dP/dt) and relaxation (−dP/dt) are shown in the panels as mean ± S.E.M. The number of mice used (n) for each group was: 3 months ($n=5$), 6 months ($n=4$), 12 months ($n=7$), 18 months ($n=6$) and 24 months ($n=3$) wild-type; and 3 months ($n=4$), 6 months ($n=5$), 12 months ($n=5$), 18 months ($n=6$) and 24 months ($n=3$) phospholamban-deficient.

3-month-old counterparts. However, at 12 months of age, the rates of contraction and relaxation were significantly lower than those in the 3-month-old phospholamban-deficient hearts, yet still hyperdynamic when compared with age-matched wild-type mice. This was observed in three independent groups of 3-month-old and 12-month-old animals studied in parallel, and did not appear to correlate with any subcellular increases in the expression levels of a fetal gene program. Examination of the atrial natriuretic factor and β-myosin heavy chain transcript levels in 3- and 12-month-old hearts from wild-type and phospholamban-deficient mice indicated that there were no detectable increases in the transcript levels of either gene (data not shown). It is interesting to note that by 18 months of age, all indices of cardiac function (+dP/dt, −dP/dt, TPP, and $RT_{1/2}$) in phospholamban-deficient hearts were similar to those observed at 3 months of age.

Table 2 Echocardiographic measurements in aged wild-type and phospholamban-deficient mice

	WT ($n=3$)	KO ($n=3$)
Heart rate (beats/min)	412.6 ± 25.9	390 ± 18.9
LV end-diastolic dimension (mm)	3.79 ± 0.16	3.78 ± 0.08
LV end-systolic dimension (mm)	2.48 ± 0.12	2.23 ± 0.11
LV posterior wall thickness (mm)	0.71 ± 0.02	0.70 ± 0.01
LV shortening fraction (%)	34.56 ± 0.79	40.93 ± 1.73*
Vcf_c (circ/s)	5.98 ± 0.56	8.55 ± 0.54*

The wild-type (WT) and phospholamban-deficient (KO) mice used for these studies were 24 months old. Values represent the mean ± S.E.M., * indicates $P<0.05$ v wild-type mice. LV: left ventricular; Vcf_c: velocity of circumferential fiber shortening corrected for heart rate differences.

To quantify myocardial function in the live animal within the context of an intact circulatory system, we also examined cardiac contractility in 24-month-old wild-type and phospholamban-deficient mice using M-mode echocardiography. The aged phospholamban-deficient mice exhibited greater fractional shortening and increases in the left ventricular velocity of circumferential fiber shortening, Vcf_c, compared to their wild-type counterparts (Table 2). These alterations are indicative of increased rates of myocardial contractility, similar to previous observations in 3-month-old mice.[25] There were no differences observed in heart rate or left ventricular measurements of end systolic and end diastolic dimensions. Furthermore, the left ventricular posterior wall thickness was similar between the two groups, in agreement with the lack of changes in heart/body ratios (Table 1).

To examine the ability of the aged wild-type and phospholamban-deficient hearts to tolerate increased work, left ventricular Frank–Starling function curves were obtained in 3-, 6-, 12-, 18- and 24-

month-old hearts of either genotype. The hearts were subjected to increased volume (cardiac output, CO) load and/or increased afterload (MAP), which resulted in variable cardiac minute work (MAP × CO, expressed as mmHg × ml/min). Representative plots for cardiac minute work *v* the rates of pressure development (+dP/dt and −dP/dt in mmHg/s) obtained in single hearts from wild-type and phospholamban-deficient mice at 6, 12, 18 and 24 months of age are shown in Figure 3. To assess the effects of increased loading conditions on cardiac function, contractile parameters were examined at an increased workload of 400 (mmHg × ml/min) cardiac work, which was achieved by exposing the hearts to increased venous return or afterload. The effects of this increased workload on cardiac contractility are summarized in Table 3. Similar load-dependent increases in contractile function were observed in wild-type ($125 \pm 11.9\%$ of values obtained at 250 mmHg × ml/min for 24 months of age) and phospholamban-deficient mice ($121 \pm 9.9\%$ of values obtained at 250 mmHg × ml/min for 24 months of age) at all ages studied, suggesting that neither age nor the absence of phospholamban hinders the ability of these hearts to respond to higher workloads. However at all ages studied, the phospholamban-deficient hearts displayed much higher absolute values for +dP/dt and −dP/dt than those obtained in wild-type hearts of the same age, indicating that the phospholamban-deficient myocardium performs at a higher intrinsic contractile state, even in the aged animal.

To determine whether the aging process was associated with any alterations in the levels of the key SR calcium-handling proteins, and whether these alterations were similar between wild-type and hyperdynamic phospholamban-deficient hearts, cardiac homogenates from the two groups of mice at different ages were subjected to quantitative immunoblot analysis, in parallel. The protein levels of the SR Ca^{2+}-ATPase and calsequestrin in both groups, as well as phospholamban in wild-types, were examined at 3, 6, 12, 18, and 24 months of age. As shown in Table 4, protein levels for phospholamban (wild-type only) and calsequestrin remained relatively constant with age in both the wild-type and the phospholamban-deficient mice. The SR Ca^{2+}-ATPase levels were unchanged at 3, 6, 12, 18 and 24 months in wild-types. However, phospholamban-deficient hearts exhibited a 45% decrease at 12 months and a 20% decrease at 18 months in SR Ca^{2+}-ATPase levels, compared with 3-month-old hearts (Table 4). At 24 months, the levels of the SR Ca^{2+}-ATPase were comparable to those observed at 3 months of age (Table 4).

Discussion

This study presents evidence that the hyperdynamic cardiac function of phospholamban-deficient animals persists over the long term. Numerous studies up to now have utilized gene-targeting or transgenic technologies to study the function of specific cardiac genes in the whole animal context. However, there is little information on the effects of aging in these models and specifically the persistence of the observed phenotype in senescent mice. Therefore, the aim of this study was to carry out a longitudinal examination of cardiac function in the phospholamban-deficient mouse model and directly compare it to its wild-type cohort. A central question of this study was whether the hyperdynamic cardiac contractility associated with phospholamban-deficiency would persist with age and, if so, whether it would become detrimental over time. Recently, a transgenic mouse model that exhibits cardiac-specific overexpression of G_{Sz}, a key component of the β-adrenergic receptor signaling pathway, was reported to develop signs of cardiomyopathy at 15 months of age.[27] Since phospholamban is also part of the β-adrenergic receptor cascade, we actually speculated that long-term maintenance of the hyperdynamic contractile state may potentially drive the heart into a state of hypertrophy, possibly leading to heart failure. However, our data indicate that the hyperdynamic contractility, associated with phospholamban ablation, persists over the long term. Furthermore, aging up to 24 months was not associated with any increases in heart/body weight ratios or alterations in myocyte morphometry in either wild-type or phospholamban-deficient mice, similar to previous observations in senescent Sprague–Dawley[28] and Wistar[29] rats. Aging did not alter the cardiac contractile parameters in wild-type mice either, in contrast to other reports indicating a decrease in diastolic function in 30–34-month senescent mice.[17,30] This apparent discrepancy between our findings and previous observations[17,30] may be due to differences in the methodology employed to measure cardiac function, the age of mice (24 months *v* 30–34 months), and the strains of mice used (129SvJ/CF-1 or 129SvJ *v* B6D2F1 or B6C3F1).

While the phospholamban-deficient mouse can live as long as the wild-type mouse, it was of special interest to determine whether the increases in myocyte calcium cycling properties may influence the long-term expression profile of the SR Ca^{2+}-ATPase. Indeed, the levels of the SR Ca^{2+}-transport enzyme were significantly decreased (44.8% decrease, $P<0.0001$) at 12 months, followed by an up-

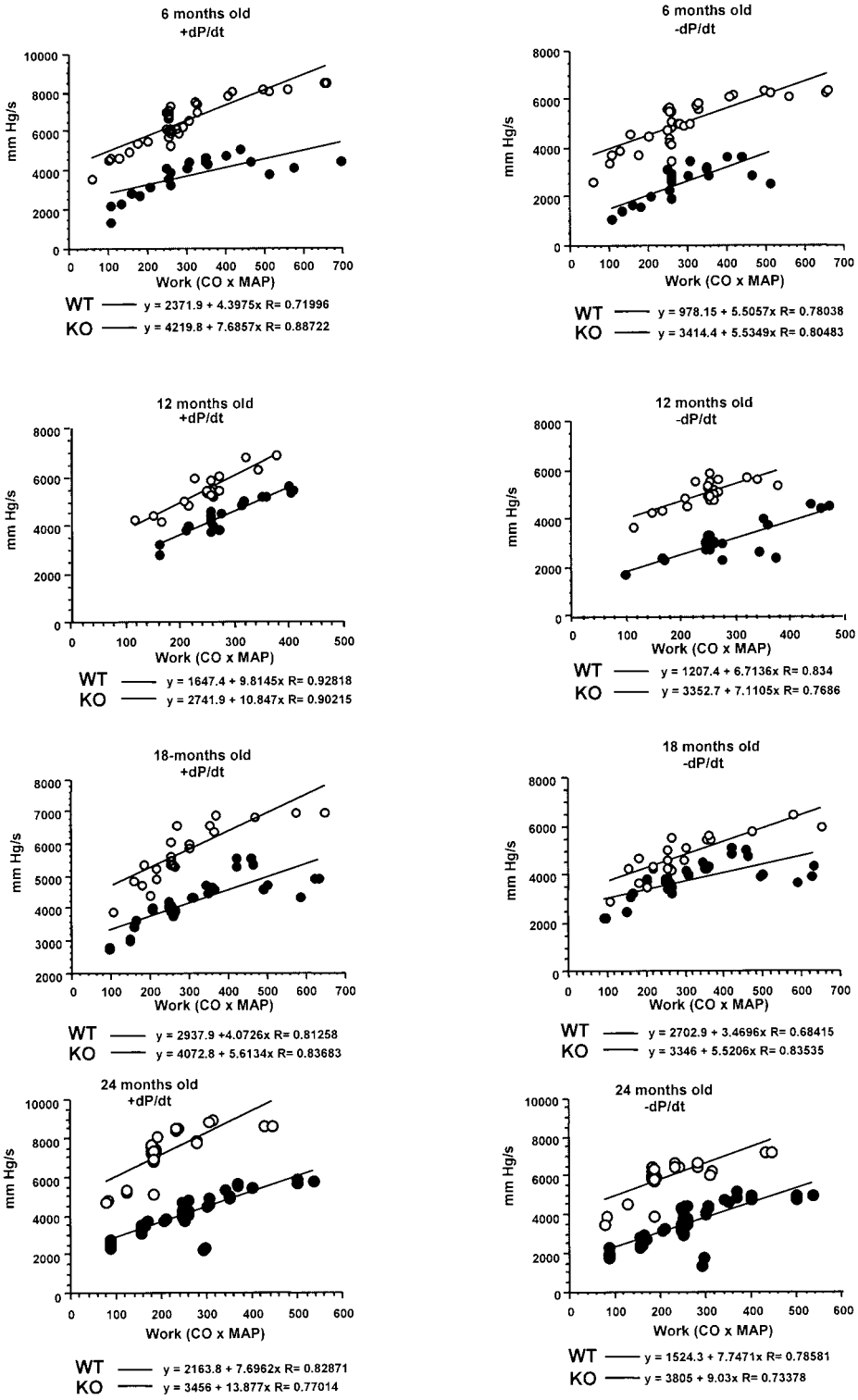

Figure 3 Frank–Starling left ventricular function curves (+dP/dt or −dP/dt *v* cardiac work) in wild-type and phospholamban-deficient mouse hearts at different ages. Each graph represents a single heart from age-matched wild-type and phospholamban-deficient mice, which were examined under varying venous return at constant (50 mmHg) aortic pressure or at various afterloads at a constant (5 ml/min) venous return. The relations between increases in dP/dt and cardiac work are given by the regression lines below each plot. (●) and (○) represent the values obtained in wild-type and phospholamban-deficient mouse hearts, respectively.

Table 3 Cardiac contractile parameters under conditions of increased workload *ex vivo*

Age/genotype	*n*	+dP/dt (mmHg/s)	TPP (ms/mmHg s^{-1})	−dP/dt (mmHg/s)	$RT_{1/2}$ (ms/mmHg s^{-1})
3 mo WT	5	4683±434	0.36±0.03	3837±482	0.42±0.04
6 mo WT	4	4957±624	0.32±0.06	3613±564	0.38±0.07
12 mo WT	7	5005±258	0.35±0.01	3698±128	0.38±0.01
18 mo WT	6	4533±142	0.36±0.01	3746±240	0.42±0.02
24 mo WT	3	4321±327	0.38±0.03	3464±104	0.46±0.06
3 mo KO	4	7147±64†	0.24±0.01†	6690±241†	0.23±0.01†
6 mo KO	5	6618±511	0.26±0.02	5453±197*†	0.26±0.02
12 mo KO	5	5894±479	0.29±0.02†	5051±448*†	0.27±0.02†
18 mo KO	6	6437±516†	0.26±0.01†	5450±429*†	0.27±0.01*†
24 mo KO	3	6680±945†	0.26±0.04†	5704±680†	0.27±0.04†

WT denotes wild-type hearts; KO, phospholamban-deficient hearts. Values were determined at a cardiac minute workload of 400 mmHg × ml/min and represent the mean ± S.E.M. for *n* different hearts, perfused in work-performing mode. Significant differences ($P<0.05$), as assessed by ANOVA, are indicated by * for comparisons *v* 3-month control groups for each genotype or by † for comparisons *v* age-matched wild-type mice.

Table 4 Relative levels of Ca^{2+}-handling proteins in wild-type and phospholamban-deficient mouse hearts

Age/ genotype	*n*	PLB	SERCA	CSQ
3 mo WT	6	100±10.2	100±7.3	100±8.1
6 mo WT	4	89.3±9.1	98.9±5.2	103.9±3.7
12 mo WT	4	82.3±11.7	96.9±9.7	93.2±7.1
18 mo WT	4	104±13.4	105.4±5.8	91.6±7.6
24 mo WT	5	94.5±9.6	116.5±7.7	94.8±3.5
3 mo KO	6	N/A	100±6.0	100±5.2
6 mo KO	4	N/A	96.8±4.2	115±12
12 mo KO	4	N/A	57.5±3.2*	125±18.5
18 mo KO	4	N/A	80.3±4.1*	109±4.3
24 mo KO	4	N/A	107±3.8	127±12.8

WT indicates wild-type; KO, phospholamban-deficient; PLB, phospholamban; SERCA, SR Ca^{2+}-ATPase; CSQ, calsequestrin; and N/A, not applicable. Values represent the mean ± S.E.M. from three determinations. 3-month S.E.M.s represent the variability of individual determinations relative to the 3-month mean value. The values in the aged samples were expressed as a percentage of the corresponding 3-month values. Significant differences (* $P<0.05$) *v* 3-month control groups for each genotype as assessed by ANOVA.

regulation at 18 months, although the 18-month SERCA levels were still lower than those in 3-month phospholamban-deficient hearts (19.7% decrease, $P<0.05$). Interestingly, by 24 months of age, the levels of SR Ca^{2+}-ATPase were similar to those observed in the 3-month-old group. The mechanisms responsible for altered SR Ca^{2+}-ATPase expression are currently unclear, but may represent a transient response to accommodate the increased diastolic SR calcium content.[20,24] The SR Ca^{2+}-ATPase protein levels were not altered in the aging wild-type mouse hearts in contrast to its rodent counterpart, the rat, which exhibited significant decreases in the expression of this key SR Ca^{2+}-transport protein upon aging.[7–9,14,29] Examination of the calsequestrin protein levels showed no alterations upon aging or between phospholamban-deficient and wild-type mice. These observations are similar to those previously reported for calsequestrin transcript levels in the aging rat hearts.[31]

Aging, importantly, did not diminish the "hyperdynamic" rates of contraction or relaxation in the phospholamban-deficient mouse heart. The mild, yet significantly, lower levels of hyperdynamic contractility observed in phospholamban-deficient hearts at 12 months could be attributed to the depression in SR Ca^{2+}-ATPase levels observed at that age. The subsequent upregulation of SR Ca^{2+}-ATPase levels by 18 months appeared to restore contractile performance to that of 3-month phospholamban-deficient hearts, which persisted in the 24-month age group. However, it is noteworthy that contractility in the 18-month group was similar to that of 3-month group despite a 20% decrease in SR Ca^{2+}-ATPase levels. It is possible that additional compensatory mechanisms, such as alterations in other calcium cycling proteins, may contribute to the restored function in the phospholamban-deficient hearts, at this age. Interestingly, the contractile reserve of these hearts, assessed by their Frank–Starling responses to increased workloads, did not diminish with age. In addition, assessment of cardiac function *in vivo*, using M-mode echocardiography, also indicated that the aged phospholamban-deficient heart functions at a hyperdynamic state, even in the context of an intact circulatory system. These results are most intriguing and provide direct evidence that long-term ablation of phospholamban function is

not detrimental to cardiac performance. Furthermore, it was recently shown that PLB-ablation could rescue the dilated cardiomyopathy phenotype exhibited by the muscle-specific LIM protein knockout mouse and the improved cardiac function persisted with time.[21]

In summary, our findings indicate that the hyperdynamic cardiac function associated with phospholamban ablation persists through aging in the mouse. Thus, it is interesting to speculate that phospholamban may represent an ideal target for pharmacological maintenance of cardiac function in both young and old patients with myocardial dysfunction.

Acknowledgements

We wish to thank Dr Larry Jones for providing us with the calsequestrin polyclonal antibody. This work was supported by National Institutes of Health Grants HL-26057 (E. G. Kranias), HL-52318 (E. G. Kranias), HL07382-17 (J. P. Slack), HL-30696 (A. M. Gerdes) and 1P40RR12358 (E. G. Kranias).

References

1. Capasso JM, Malhotra A, Remily RM, Scheuer J, Sonnenblick EH. Effects of age on mechanical and electrical performance of rat myocardium. *Am J Physiol* 1983; **245**: H72–H81.
2. Froehlich JP, Lakatta EG, Beard E, Spurgeon HA, Weisfeldt ML, Gerstenblith G. Studies of sarcoplasmic reticulum function and contraction duration in young adult and aged rat myocardium. *J Mol Cell Cardiol* 1978; **10**: 427–438.
3. Li Y, Lincoln T, Mendelowitz D, Grossman W, Wei JY. Age-related differences in effect of exercise training on cardiac muscle function in rats. *Am J Physiol* 1986; **251**: H12–H18.
4. Spurgeon HA, Steinbach MF, Lakatta EG. Chronic exercise prevents characteristic age-related changes in rat cardiac contraction. *Am J Physiol* 1983; **244**: H513–H518.
5. Wei JY, Spurgeon HA, Lakatta EG. Excitation-contraction in rat myocardium: alterations with adult aging. *Am J Physiol* 1984; **246**: H784–H791.
6. Lakatta EG, Yin FC. Myocardial aging: functional alterations and related cellular mechanisms. *Am J Physiol* 1982; **242**: H927–H941.
7. Taffet GE, Tate CA. Ca^{2+}-ATPase content is lower in cardiac sarcoplasmic reticulum isolated from old rats. *Am J Physiol* 1993; **264**: H1609–H1614.
8. Tate CA, Helgason T, Hyek MF, McBride RP, Chen M, Richardson MA, Taffet GE. SERCA2a and mitochondrial cytochrome oxidase expression are increased in hearts of exercise-trained old rats. *Am J Physiol* 1996; **271**: H68–H72.
9. Maciel LM, Polikar R, Rohrer D, Popovich BK, Dillmann WH. Age-induced decreases in the messenger RNA coding for the sarcoplasmic reticulum Ca^{2+}-ATPase of the rat heart. *Circ Res* 1990; **67**: 230–234.
10. Buttrick P, Malhotra A, Factor S, Greenen D, Leinwand L, Scheuer J. Effect of aging and hypertension on myosin biochemistry and gene expression in the rat heart. *Circ Res* 1991; **68**: 645–652.
11. Jiang MT, Moffat MP, Narayanan N. Age-related alterations in the phosphorylation of sarcoplasmic reticulum and myofibrillar proteins and diminished contractile response to isoproterenol in intact rat ventricle. *Circ Res* 1993; **72**: 102–111.
12. Narayanan N. Comparison of ATP-dependent calcium transport and calcium-activated ATPase activities of cardiac sarcoplasmic reticulum and sarcolemma from rats of various ages. *Mech Ageing Dev* 1987; **38**: 127–143.
13. Assayag P, Charlemagne D, Marty I, de Leiris J, Lompre AM, Boucher F, Valere PE, Lortet S, Swynghedauw B, Besse S. Effects of sustained low-flow ischemia on myocardial function and calcium-regulating proteins in adult and senescent rat hearts. *Cardiovasc Res* 1998; **38**: 169–180.
14. Tate CA, Taffet GE, Hudson EK, Blaylock SL, McBride RP, Michael LH. Enhanced calcium uptake of cardiac sarcoplasmic reticulum in exercise-trained old rats. *Am J Physiol* 1990; **258**: H431–H435.
15. Cain BS, Meldrum DR, Joo KS, Wang JF, Meng X, Cleveland JC Jr, Banerjee A, Harken AH. Human SERCA2a levels correlate inversely with age in senescent human myocardium. *J Am Coll Cardiol* 1998; **32**: 458–467.
16. Xu A, Narayanan N. Effects of aging on sarcoplasmic reticulum Ca^{2+}-cycling proteins and their phosphorylation in rat myocardium. *Am J Physiol* 1998; **275**: H2087–H2094.
17. Lim CC, Liao R, Varma N, Apstein CS. Impaired lusitropy-frequency in the aging mouse: role of Ca^{2+}-handling proteins and effects of isoproterenol. *Am J Physiol* 1999; **277**: H2083–H2090.
18. Kadambi VJ, Ponniah S, Harrer JM, Hoit BD, Dorn GW 2nd, Walsh RA, Kranias EG. Cardiac-specific overexpression of phospholamban alters calcium kinetics and resultant cardiomyocyte mechanics in transgenic mice. *J Clin Invest* 1996; **97**: 533–539.
19. Koss KL, Grupp IL, Kranias EG. The relative phospholamban and SERCA2 ratio: a critical determinant of myocardial contractility. *Basic Res Cardiol* 1997; **92**: 17–24.
20. Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of β-agonist stimulation. *Circ Res* 1994; **75**: 401–409.
21. Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, Gu Y, Martone ME, Wang Y, Ross J Jr, Kranias EG, Giles WR, Chien KR. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell* 1999; **99**: 313–322.
22. Sato Y, Ferguson DG, Sako H, Dorn GW 2nd, Kadambi VJ, Yatani A, Hoit BD, Walsh RA, Kranias EG. Cardiac-specific overexpression of mouse cardiac

calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *J Biol Chem* 1998; **273**: 28470–28477.
23. Harrer JM, Kiss E, Kranias EG. Application of the immunoblot technique for quantitation of protein levels in cardiac homogenates. *Biotechniques* 1995; **18**: 995–998.
24. Luo W, Wolska BM, Grupp IL, Harrer JM, Haghighi K, Ferguson DG, Slack JP, Grupp G, Doetschman T, Solaro RJ, Kranias EG. Phospholamban gene dosage effects in the mammalian heart. *Circ Res* 1996; **78**: 839–847.
25. Hoit BD, Khoury SF, Kranias EG, Ball N, Walsh RA. In vivo echocardiographic detection of enhanced left ventricular function in gene-targeted mice with phospholamban deficiency. *Circ Res* 1995; **77**: 632–637.
26. Gerdes AM, Onodera T, Tamura T, Said S, Bohlmeyer TJ, Abraham WT, Bristow MR. New method to evaluate myocyte remodeling from formalin-fixed biopsy and autopsy material. *J Card Fail* 1998; **4**: 343–348.
27. Iwase M, Uechi M, Vatner DE, Asai K, Shannon RP, Kudej RK, Wagner TE, Wight DC, Patrick TA, Ishikawa Y, Homcy CJ, Vatner SF. Cardiomyopathy induced by cardiac Gsα overexpression. *Am J Physiol* 1997; **272**: H585–H589.
28. Bai SL, Campbell SE, Moore JA, Morales MC, Gerdes AM. Influence of age, growth, and sex on cardiac myocyte size and number in rats. *Anat Rec* 1990; **226**: 207–212.
29. Besse S, Assayag P, Delcayre C, Carre F, Cheav SL, Lecarpentier Y, Swynghedauw B. Normal and hypertrophied senescent rat heart: mechanical and molecular characteristics. *Am J Physiol* 1993; **265**: H183–H190.
30. Taffet GE, Hartley CJ, Wen X, Pham T, Michael LH, Entman ML. Noninvasive indexes of cardiac systolic and diastolic function in hyperthyroid and senescent mouse. *Am J Physiol* 1996; **270**: H2204–H2209.
31. Lompre AM, Lambert F, Lakatta EG, Schwartz K. Expression of sarcoplasmic reticulum Ca^{2+}-ATPase and calsequestrin genes in rat heart during ontogenic development and aging. *Circ Res* 1991; **69**: 1380–1388.