Cardiac-directed parvalbumin transgene expression in mice shows marked heart rate dependence of delayed Ca\(^{2+}\) buffering action


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Day SM, Coutu P, Wang W, Herron T, Turner I, Shillingford M, LaCross NC, Converso KL, Piao L, Li J, Lopatin AN, Metzger JM. Cardiac-directed parvalbumin transgene expression in mice shows marked heart rate dependence of delayed Ca\(^{2+}\) buffering action. *Physiol Genomics* 33: 312–322, 2008. First published March 11, 2008; doi:10.1152/physiolgenomics.00302.2007.—Relaxation abnormalities are prevalent in heart failure and contribute to clinical outcomes. Disruption of Ca\(^{2+}\) homeostasis in heart failure delays relaxation by prolonging the intracellular Ca\(^{2+}\) transient. We sought to speed cardiac relaxation in vivo by cardiac-directed transgene expression of parvalbumin (Parv), a cytosolic Ca\(^{2+}\) buffer normally expressed in fast-twitch skeletal muscle. A key feature of Parv’s function resides in its Ca\(^{2+}/\)Mg\(^{2+}\) binding affinities that account for delayed Ca\(^{2+}\) buffering in response to the intracellular Ca\(^{2+}\) transient. Cardiac Parv expression decreased sarcoplasmic reticulum Ca\(^{2+}\) content without otherwise altering intracellular Ca\(^{2+}\) homeostasis. At high physiological mouse heart rates in vivo, Parv modestly accelerated relaxation without affecting cardiac morphology or systolic function. Ex vivo pacing of the isolated heart revealed a marked heart rate dependence of Parv’s delayed Ca\(^{2+}\) buffering effects on myocardial performance. As the pacing frequency was lowered (7 to 2.5 Hz), the relaxation rates increased in Parv hearts. However, as pacing rates approached the dynamic range in humans, Parv hearts demonstrated decreased contractility, consistent with Parv buffering systolic Ca\(^{2+}\). Mathematical modeling and in vitro studies provide the underlying mechanism responsible for the frequency-dependent fractional Ca\(^{2+}\) buffering action of Parv. Future studies directed toward refining the dose and frequency-response relationships of Parv in the heart or engineering novel Parv-based Ca\(^{2+}\) buffers with modified Mg\(^{2+}\) and Ca\(^{2+}\) affinities to limit systolic Ca\(^{2+}\) buffering may hold promise for the development of new therapies to remediate relaxation abnormalities in heart failure.

physiological feature of Parv rests in its “delayed” Ca\(^{2+}\) buffering function. Parv has a markedly higher affinity for Ca\(^{2+}\) binding (K\(_{Ca}\) 10\(^9\) M\(^{-1}\)) than for Mg\(^{2+}\) binding (K\(_{Mg}\) 10\(^4\) M\(^{-1}\)). But, because free [Mg\(^{2+}\)] is ~10\(^4\) times greater than [Ca\(^{2+}\)] in resting muscle, the majority of the Parv metal binding sites are occupied by Mg\(^{2+}\) at baseline prior to contraction. Upon contraction, the intracellular Ca\(^{2+}\) concentration rises from ∼nM to ∼μM, causing a fraction of the metal binding sites on Parv to switch from the Mg\(^{2+}\) to the Ca\(^{2+}\) bound state. However, the unbinding of Mg\(^{2+}\) is relatively slow (K\(_{OFF,Mg}\) 3.42 s\(^{-1}\) at 20°C), causing the binding of Ca\(^{2+}\) to Parv to be delayed relative to the rise of the Ca\(^{2+}\) transient (23, 24). This delayed Ca\(^{2+}\) buffering by Parv is critical for minimizing buffering of systolic activating Ca\(^{2+}\), allowing it to be available for binding to troponin C in the sarcomere during contraction. Thus, Ca\(^{2+}\) buffering by Parv is maximal in the early part of the Ca\(^{2+}\) transient decay, which allows for faster relaxation. There is a strong correlation between the absolute speed of muscle relaxation and the concentration of Parv in skeletal muscle. An extreme example of this relationship is found in the muscles of the toadfish swim bladder, which contract at ~200 times a second and contains the highest reported [Parv] (1.5 mM) (40).

In contrast to fast-twitch skeletal muscle, Parv is not normally expressed in mammalian cardiac muscle. Sequestration of Ca\(^{2+}\) in the heart during diastole relies on two primary mechanisms: active pumping of Ca\(^{2+}\) back into the SR via SERCA2a and extrusion of Ca\(^{2+}\) via the sarcolemmal sodium-calcium exchanger (NCX) (1). Under normal circumstances, these mechanisms are sufficient to allow the heart to rapidly and fully relax between beats. However, Ca\(^{2+}\) homeostasis is often dramatically altered in the failing heart (49). Impaired Ca\(^{2+}\) reuptake into the SR can arise in part from reduced activity and content of the SERCA2a pumps in energetically compromised failing hearts (17, 29). Several investigators have demonstrated improved contractility and relaxation by modifying or overexpressing SR Ca\(^{2+}\) regulatory proteins (10, 11, 31, 41, 51). However, directly altering SR Ca\(^{2+}\) regulation can also have detrimental effects (2, 20, 26, 43, 45). An alternative approach would be to introduce an exogenous cytosolic Ca\(^{2+}\) buffer, such as Parv, into cardiac myocytes to act as a delayed Ca\(^{2+}\) buffer and accelerate Ca\(^{2+}\) sequestration during relaxation. We and others have previously demonstrated that short-term in vitro and regional in vivo gene transfer of Parv accelerates relaxation in normal and diseased cardiac myocytes (3, 5, 7, 20, 25, 42, 44, 46). However, before contemplating application of such an approach as a genetic therapy to remediate diastolic dysfunction in heart failure, we need a more

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comprehensive understanding of how sustained expression of Parv in the heart affects cardiac structure and function over the long term. Furthermore, a careful examination of how Parv expression affects cardiac performance in the whole heart at varying rates of contraction is essential for translating experimental findings in rodents with high intrinsic hearts rates to larger mammals with lower heart rates.

Accordingly, we tested here for the first time the hypothesis that Parv’s Ca\(^{2+}\) buffering capacity, and thus its positive lusitropic effects, would be inversely proportional to heart rate at the whole heart organ level. The long-term physiological effects of cardiac Parv expression in several lines of transgenic mice in vivo is also examined. It was previously shown that Parv transgene expression accelerated relaxation in isolated myocytes in vitro from these mice (3). Here, we report that expressing high levels of Parv in the heart, in the range typically observed in mammalian fast skeletal muscle, has no untoward effects on heart structure or function and did not suppress cardiac hypertrophic growth in response to aortic banding. Cardiac Parv expression decreased SR Ca\(^{2+}\) buffering capacity, and thus affecting other aspects of normal intracellular Ca\(^{2+}\) homeostasis. At physiological heart rates in the mouse in vivo, Parv modestly accelerated relaxation without affecting contractility. Ex vivo pacing of the heart to frequencies below the normal intrinsic mouse heart rate and within the dynamic range of the human heart demonstrates for the first time that the organ level physiological effects of Parv’s Ca\(^{2+}\) buffering capacity are highly frequency dependent, with greater Ca\(^{2+}\) buffering and relatively faster relaxation times at pacing frequencies. These studies also reveal a limitation of wild-type Parv in buffering systolic Ca\(^{2+}\) and thus decreasing contractility as pacing rates were slowed. To complement our experimental results, we present mathematical models that provide further insight into the concentration and frequency dependence of Parv’s Ca\(^{2+}\) buffering in the heart.

MATERIALS AND METHODS

Ethical approval. All animal care and experimental procedures complied with the Principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

Transgenic animals. The transgenic constructs used the murine Myh6 promoter (a gift from J. Robbins, Cincinnati Children’s Hospital) to direct expression of human α-Parv cDNA to the heart. Constructs were microinjected into the male pronuclei of C57BL/6 × SJL F\(_2\) fertilized eggs and implanted in pseudopregnant females. Transgenic mice genomic DNA were detected by PCR using the promoter and 5′-banding. Cardiac Parv expression decreased SR Ca\(^{2+}\) buffering systolic Ca\(^{2+}\) transient analysis by loading with fura-2 AM (2 \(\mu\)M) as previously described (5, 7) and using an infrared calcium fluorimeter (Perkin Elmer Life Sciences) with filters at 340/380 nm. Digital images were captured using a Zeiss axioskop microscope.

Adult mouse myocyte isolation and analysis. Adult mouse cardiac myocytes were isolated from 3–6 mo old mice as described (3). Between 5 × 10\(^5\) and 1 × 10\(^6\) rod-shaped cells were obtained from a single mouse heart and subjected to sarcromere shortening and Ca\(^{2+}\) transient analysis by loading with fura-2 AM (2 \(\mu\)M) as previously described (5, 7) and using an infrared calcium fluorimeter (Perkin Elmer Life Sciences) with filters at 340/380 nm. Digital images were captured using a Zeiss axioskop microscope.

Echocardiography. We carried out echocardiography on mice anesthetized with 1% inhaled isoflurane in oxygen using a Vivid 7 ultrasound system and an S10-MHz phased-array transducer (GE Healthcare) as previously described (9).

Conductance micromanometry. We obtained in vivo hemodynamic measurements by conductance micromanometry using a 1.4 French Millar pressure-conductance catheter inserted into the left ventricular apex as previously described (30). Mice were anesthetized with 1.5% isoflurane via a tracheotomy.
**Left coronary artery ligation model.** The mice were sedated with intraperitoneal pentobarbital sodium (45 mg/kg), intubated orally, and ventilated via a pressure-controlled ventilator with 1% isoflurane in 100% oxygen at a peak inspiratory pressure of 15 cmH2O and a respiratory rate of 60 breaths/min. With the aid of a dissecting microscope, the heart was exposed via a left thoracotomy, and a 7-0 silk suture was tied around the proximal portion of the left coronary artery (LCA) 1–2 mm from the left atrium. The chest was filled with warm sterile saline to evacuate air, and the incision was closed in layers using 5-0 silk suture (9). Sham-operated mice underwent thoracotomy without LCA ligation.

**Suprarenal abdominal aorta coarctation model.** Animals were anesthetized with 2–3% inhaled isoflurane in 100% oxygen via nose cone. The mice were prepped and placed in left lateral decubitus position, and a 2 cm para-median incision was made on the left flank. Blunt dissection was used to reach the peritoneum. The kidney was bluntly separated from the surrounding connective tissue to view the suprarenal aorta and branching arteries. A 7-0 silk suture was tied around the vessel over a 27-gauge needle. The needle was removed after the suture was secured, effectively reducing the diameter of the vessel lumen to \( \frac{1}{10} \) mm. The retroperitoneal incision was closed in running fashion with 7-0 Prolene suture, and skin was closed with 5-0 silk.

**Langendorff isolated heart model, atrio-ventricular node ablation, and experimental pacing protocol.** We used an isovolumic-contracting mouse heart preparation as previously described (9). Before insertion of the intraventricular balloon pressure catheter, the atrio-ventricular node was ablated by direct application of mechanical force to the interatrial septum using blunt forceps. The ablation procedure was repeated as necessary to achieve complete atrio-ventricular block, confirmed by electrocardiography. The heart was externally paced from the LV apex using a mini-coaxial stimulation electrode (Harvard Apparatus) at varying pacing frequencies from 7 Hz, decreasing in 1 or 0.5 Hz increments down to 2.5 Hz. Pacing <2.5 Hz could not be routinely achieved due to an overriding intrinsic escape rhythm.

**Mathematical model simulations.** A mathematical model describing the predicted relationship between Parv concentration and stimulation frequency was implemented to aid in data interpretation (6). Sodium and potassium intracellular concentrations were clamped at initial values of 12.7 and 140 mmol/l, respectively, to avoid model instability at high pacing frequencies. The model assumed that measurements were taken 200 s after pacing initiation, to allow for establishment of a steady-state relationship.

**Statistical analysis.** All results are expressed as means ± SE. Continuous variables were compared using one-way analysis of variance (ANOVA) with either Tukey post hoc between-group comparisons or Dunnett’s method (when data from multiple Parv transgenic lines were compared with ntg controls). Two-way ANOVA with repeated measures and Bonferroni post hoc between-group comparisons was used to analyze data from the paced heart Langendorff experiments.

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**Fig. 1.** Protein expression and localization of parvalbumin (Parv) in cardiac tissue. **A:** Western blot analysis of homogenized heart samples (5 μg/lane) obtained from mice representing 4 independent Parv transgenic lines and a nontransgenic littermate (ntg) as a negative control. Calsequestrin (CSQ) is shown as loading control. In lowest panel, an increased amount of protein (100 μg/lane) was loaded to show expression in the lowest expressing line 277. Protein purified from rat fast skeletal muscle samples was used as a positive control. SVL, superficial vastus lateralis; EDL, extensor digitorum longus. **B:** Parv concentration was estimated by establishing a standard curve using published concentrations of Parv in SVL and EDL skeletal muscle. Values are expressed as means ± SE. **C:** Immunofluorescent detection of Parv in representative heart sections from 3 independent transgenic lines and a ntg littermate as a control. Inset, far right: from a ntg control with no primary antibody. Scale bar is 50 μm. **D:** Heart-to-body weight ratios (HW/BW, mg/g) for 4 independent Parv transgenic lines compared with ntg hearts. There were no statistical differences between groups (average \( n = 24 \)). **E:** HW/BW ratios (mg/g) for ntg and Parv transgenic mice 4 wk after abdominal aortic banding. *P < 0.001 vs. sham, †P < 0.05 vs. ntg banded; \( n = 8–13 \) per group.
Fig. 2. Shortening, relaxation, and calcium transients in single isolated myocytes. A: representative normalized tracings of sarcomere length (SL) shortening in isolated myocytes from ntg and 2 Parv transgenic lines. B: relaxation kinetics in Parv and ntg myocytes paced at 0.2 Hz, expressed as the time from peak amplitude to 25, 50, and 75% relaxation. Results are displayed in increasing order of Parv protein expression (left to right). C: relationship between relaxation times and Parv protein concentration using an exponential smoothed curve fitting function. $R^2$ values = 0.9239 (25% relaxation), 0.9309 (50% relaxation), and 0.9573 (75% relaxation). D: mean shortening amplitude in ntg and Parv transgenic myocytes. E: representative normalized Ca$^{2+}$ transient tracings in isolated myocytes loaded with a fluorescence indicator (2 μM fura-2 AM) from ntg and 2 Parv transgenic lines. F: decay kinetics of the Ca$^{2+}$ transient in Parv and ntg fura-2-loaded myocytes. G: relationship between Ca$^{2+}$ transient decay kinetics and Parv protein concentration using an exponential smoothed curve fitting function. $R^2$ values = 0.9537 (25% decay), 0.9401 (50% decay), and 0.9218 (75% decay). H: mean Ca$^{2+}$ transient amplitude in ntg and Parv myocytes ($n = 65–70$ myocytes/per group for all panels). *$P < 0.05$ vs. ntg. Values are means ± SE.

RESULTS

Ectopic expression of Parv protein in transgenic mouse hearts does not affect heart morphology or growth. Parv was expressed in transgenic mice using the murine Myh6 promoter to direct expression to adult myocardium. Parv protein expression was detected in hearts of four independent transgenic lines by Western blotting, and Parv concentration was estimated based on known concentrations of Parv in rat fast-twitch skeletal muscle tissue from the SVL and EDL (14). A wide range of estimated concentrations of Parv were observed among the four lines: 0.015 mmol/l (line 277), 0.316 mmol/l (line 268), 0.568 mmol/l (line 253), and 0.617 mmol/l (line 299) (Fig. 1, A and B). The three highest expressing lines achieved concentrations of Parv in the heart comparable to or exceeding those found naturally in rat fast-twitch skeletal muscle. Indirect immunohistochemistry and confocal imaging of myocardial cryosections revealed homogeneous cytoplasmic localization of Parv throughout the heart (Fig. 1C).

All transgenic mice expressing Parv were viable, reproducitively fit, with no overt phenotype and apparent normal life expectancy (>2 yr of age). Heart-to-body weight ratios were not different between Parv mice and their ntg controls, even in mice with estimated [Parv] at or above that of fast twitch skeletal muscle (Fig. 1D). To ascertain whether driving Parv expression to supraphysiological levels would have effects on heart growth or morphology, we interbred mice from lines 253 and 268. These mice had estimated [Parv] of 1.02 mM, essentially additive of the estimated [Parv] in lines 253 and 268 (Fig. 1B), and displayed no overt phenotype with structurally normal hearts and heart-to-body weight ratios (5.0 ± 0.13, $n = 8$) not significantly different from controls. Cardiac Parv expression also did not suppress pathologic heart growth in response to pressure overload, induced by suprarenal coarctation of the abdominal aorta (Fig. 1E). Parv mice from line 268, but not the higher expressing line 299, showed a small but significant increase in heart-to-body weight ratio in response to banding compared with ntg mice. By echocardiography, abdominal aortic coarctation resulted in significant increases in mean left ventricular end-diastolic dimension, end-diastolic volume, and cardiac index as well as a shortening of isovolumic relaxation time (Supplemental Table S1).1 We propose that these are compensatory responses to maintain systolic and diastolic function, as no differences were seen in other measures of contractility or relaxation. Expression of Parv in the heart did not have a significant impact on any structural of functional echocardiographic parameters.

Cardiac Parv expression speeds relaxation and decay of the Ca$^{2+}$ transient in isolated myocytes. Sarcomere length shortening and relengthening were measured at 0.2 Hz pacing in isolated myocytes from all four transgenic mouse lines. Ectopic expression of Parv in transgenic cardiac myocytes accelerated relaxation in a concentration-dependent fashion (Fig. 2, A–C). Similarly, the Ca$^{2+}$ transient decay was faster in fura-2-loaded myocytes expressing Parv and directly proportional to the concentration of Parv (Fig. 2, E–G). At higher concentrations, Parv expression caused a decrease in sarcomere shortening amplitude (Fig. 2D) and a decrease in the peak height of the Ca$^{2+}$ transient (Fig. 2H). These results indicate that in single cardiac myocytes, Parv is an effective diastolic Ca$^{2+}$ buffer that speeds relaxation by accelerating the decline

1 The online version of this article contains supplemental material.
in cytosolic Ca\(^{2+}\) levels in a concentration-dependent manner. However, at higher concentrations, Parv buffers systolic Ca\(^{2+}\) resulting in a decline in the peak Ca\(^{2+}\) transient and a decrease in force generation.

Parv decreases SR Ca\(^{2+}\) load without altering expression of Ca\(^{2+}\) handling proteins or Na\(^{+}\)-Ca\(^{2+}\) exchange current. This set of experiments was designed to determine if Parv expression in the heart affects normal Ca\(^{2+}\) homeostasis. Caffeine-induced SR Ca\(^{2+}\) release was measured in acutely isolated mouse myocytes from ntg and Parv (line 268) transgenic hearts. There was a significant reduction in SR Ca\(^{2+}\) load in Parv myocytes compared with ntg myocytes (Fig. 3, A–C). However, there were no differences in NCX current density (Fig. 3D) or in protein expression of several key Ca\(^{2+}\) handling proteins (Fig. 3, E and F). These data indicate that Parv acts as an intracellular Ca\(^{2+}\) sink that effectively competes with the SR to sequester Ca\(^{2+}\) during diastole without otherwise altering normal Ca\(^{2+}\) homeostasis.

Parv has modest effects on cardiac function at physiologic mouse heart rates in vivo. We focused experiments on intermediate Parv-expressing lines 268 and 253 for in vivo heart function studies. Echocardiographic analysis of Parv mice from line 268 revealed no differences in left ventricular chamber sizes or systolic function compared with ntg mice (Fig. 4, A and B). Deceleration times of the mitral valve inflow, the interval from the peak velocity of early filling velocity (E) to the time it reaches baseline, were shorter in Parv mice compared with ntg controls (Fig. 4, C). Interestingly, mean heart rates and the rate of pressure development (+dp/dt) were also higher in Parv transgenic mice (Fig. 4, F and G). Other parameters, including the LV peak pressure (Fig. 4H), rate of pressure decline, cardiac output, and stroke work, were not different between groups (data not shown). Overall, these data indicate a modest effect of Parv to speed relaxation of the mouse heart without compromising systolic function at physiological heart rates in vivo.

To investigate the effects of cardiac Parv expression in vivo in response to pathologic stress, mice underwent ligation of the LCA. Four weeks after myocardial infarction, significant left ventricular dilation and systolic and diastolic dysfunction were evident in both ntg and Parv (line 268) transgenic mice (Supplemental Table S2). However, Parv had little effect on postinfarction cardiac remodeling or hemodynamics, with an increase in mean left ventricular diastolic volume only compared with ntg mice. Importantly, Parv did not further depress systolic function at physiological heart rates in vivo in this pathologically relevant model.

![Fig. 3. Analysis of Ca\(^{2+}\) homeostasis. Ca\(^{2+}\) transients in isolated ntg and Parv (line 268) mouse myocytes at baseline with 1 Hz pacing (A) and after application of 20 mM caffeine to release sarcoplasmic reticulum (SR) Ca\(^{2+}\) (B). C: summary of Ca\(^{2+}\) transients and caffeine-induced SR Ca\(^{2+}\) load data. *P < 0.001 vs. ntg (n = 22–25 myocytes per group). D: current-voltage relationships for Ni\(^{2+}\)-sensitive currents (I_{NCX}) in ntg and Parv 268 myocytes measured at the end of voltage steps. There is no significant difference in currents between the 2 groups. E and F: immunoblot analysis of Ca\(^{2+}\) handling proteins. No differences in expression were observed between ntg and Parv (line 268) for any of the proteins. PLN, phospholamban; p-PLN, phosphorylated phospholamban; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; NCX, sodium-calcium exchanger.](http://physiolgenomics.physiology.org/)

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Parv speeds relaxation and decreases contractility in the isolated perfused whole heart in a frequency-dependent manner. Previous myocyte gene transfer studies and mathematical modeling suggest that Parv’s dose-response relationship is highly dependent on contraction frequency (3, 7). Parv’s intrinsic on/off binding rates for Ca\(^{2+}\)/H\(^{+}\) and Mg\(^{2+}\)/H\(^{+}\) sets limits on the buffering capacity of Parv as contractile frequency is increased (4). Therefore, the rapid physiological heart rate in the mouse in vivo (∼600 beats/min, ∼10 Hz) is mathematically modeled to limit Parv from exerting its full Ca\(^{2+}\) buffering capacity for a given level of expression (7). This is distinct from results in paced single myocyte studies where diastole is typically extended to nonphysiological time frames of 1–5 s. To address the relationship between pacing frequency and contractile function in the whole heart in the presence of Parv, we used an ex vivo isolated heart preparation with atrioventricular node ablation and extrinsic cardiac pacing over a range of frequencies. We tested the hypothesis that Parv’s

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Fig. 4. Baseline heart structure/function echocardiographic and conductance micromanometry measurements in ntg and 268 Parv mice. Echocardiography: A: left ventricular dimensions in diastole (LVD\(_d\)) and systole (LVD\(_s\)); B: fractional shortening (FS, %). No statistical differences were observed between groups for these parameters (n = 10–15 mice/group). C: pulsed-wave Doppler-derived deceleration time (Dec T) of the early transmitral inflow. Parv transgenic mice had significantly shorter Dec Ts compared with ntg mice, P < 0.01. D: mitral valve annular velocities measured using Doppler tissue imaging. There were no statistical differences between groups in either the early (E\(_a\)) or late (A\(_a\)) annular velocities nor their ratios (n = 10–15 mice/group). Conductance micromanometry: E: tau, the time constant of relaxation. Parv transgenic (tg) mice from 2 lines (268 and 253) were pooled for the analysis, as there were no significant differences in any parameters between these groups. F: heart rates. G: maximum and minimum first derivatives of pressure development. H: maximum systolic pressures (P\(_{max}\)). *P < 0.05. Values are means ± SE and n = 9 (ntg) and 20 (Parv) for all measurements.
effective Ca\(^{2+}\) buffering capacity, and consequently its physiological effects, would be inversely proportional to pacing frequency. Compared with ntg control hearts, transgenic hearts expressing an intermediate level of Parv (line 268) demonstrated accelerated early relaxation (time from peak to 20% relaxation) at all pacing frequencies, ranging from 7 to 2.5 Hz (Fig. 5, A and B). Midrelaxation (time from peak to 50% relaxation) was accelerated in Parv hearts only at slower pacing frequencies. Parv expression did not affect contractility when hearts were paced at 7 Hz but caused a pronounced decline in left ventricular pressure as pacing frequency was lowered from 6 Hz down to 2.5 Hz (Fig. 5, A and C). Transgenic hearts expressing a very low level of Parv (line 277) were no different from ntg control hearts in either contractility or relaxation parameters (Fig. 5, B and C).

Collectively, these results show sustained systolic pressure generation and a statistically significant acceleration in relaxation rates at high heart rates (7–10 Hz). When pacing rates were lowered to nonphysiological values for the mouse heart (<7 Hz), the early phase of relaxation was further accelerated, but systolic pressures progressively declined relative to ntg hearts.

**Mathematical modeling of Parv’s dose and frequency response in the mouse heart.** We utilized a three-compartment cardiac myocyte mathematic model that accurately simulates the adult rodent ventricular cardiac myocyte Ca\(^{2+}\) transient, shortening, and force generation data collected at physiological temperatures (5). We applied this integrated model to address the effects of myocyte pacing on intracellular [Ca\(^{2+}\)] and its relationship to Parv cation buffering action. Simulations reproduced the frequency and Parv concentration-dependent effects on myocyte relaxation performance (Fig. 6A). The model predicts that as pacing frequency is reduced from 10 Hz (physiological for mouse heart) the amount of Ca\(^{2+}\) buffered by Parv during each beat is increased (Fig. 6, B–E). This is explained by the interrelationship between divalent cation binding/unbinding and the time interval between each contractile cycle. With increased time between beats, the fractional Ca\(^{2+}\) buffering capacity of Parv increases (Fig. 6, C and D). Also evident from these simulations is the increased difference in Ca\(^{2+}\) transient amplitude between high-expressing Parv and control conditions when pacing frequency is lowered (Fig. 6E).

The discrepancy in absolute [Ca\(^{2+}\)] between the experimental measurement (Fig. 3, A–C) and that predicted by the mathematical model (Fig. 6E) could be due to a more substantial Ca\(^{2+}\) buffering by fura-2 AM than was accounted for in the model but could also be related to differences between rat and mouse myocardium. Overall, the mathematical simulations qualitatively predict our main experimental findings of both faster relaxation performance and diminished systolic pressure upon lowering the pacing frequency in the isolated heart (Fig. 5).

**DISCUSSION**

There has been considerable interest in developing genetic strategies to manipulate calcium homeostasis in the heart. The appeal of such therapies is based on the hypothesis that abnormal cardiac SR function and altered intracellular Ca\(^{2+}\) cycling contribute to the pathophysiological phenotype of heart failure: decreased contractile and relaxation performance and maladaptive ventricular remodeling (21, 49). Evidence to support this hypothesis stems from a number of animal and human studies showing consistent changes in the expression and/or function of SR and sarcolemmal Ca\(^{2+}\) handling proteins and diminished amplitude and prolonged decay of the intracellular Ca\(^{2+}\) transients in heart failure (17, 29, 33, 36). In the present study, we describe a unique approach to modify Ca\(^{2+}\) cycling behavior in the heart through long-term ectopic expression of Parv, a cytosolic Ca\(^{2+}\) binding protein naturally found in fast-twitch skeletal muscle. Previous reports from our laboratory and others have demonstrated the feasibility of in vitro
gene transfer of Parv into cardiac myocytes (3, 5, 7, 20, 25, 46) and short-term in vivo cardiac gene transfer (42, 44). The present study provides the first detailed analysis of the effects of sustained Parv expression in vivo over the lifespan of an animal. Introduction of Parv into the mouse heart was well tolerated, with no untoward effects on heart growth or morphology, even under pathological conditions of pressure or volume overload, induced by aortic coarctation and myocardial infarction, respectively. These are important observations, because several signaling pathways involved in cardiac growth are regulated by intracellular Ca\(^{2+}\) ions (32, 48).

Previously, we have reported the effects of Parv expression in isolated cardiac myocytes to speed relaxation and, when coexpressed with a hypertrophic cardiomyopathy-causing mutation in α-tropomyosin, to correct cellular relaxation abnormalities (3, 5, 7). These studies, along with mathematical modeling of cardiac Parv expression (6, 7), provide evidence for a critical range of Parv (~0.01–0.1 mM) to accelerate relaxation without attenuating contractile amplitude. However, it is difficult to predict what this concentration range would be in vivo over a wide range of intrinsic heart rates of different species. Therefore, the present study was critical to define both the concentration and frequency dependence of Parv expression in the whole intact heart. We show that organ level physiological effects of Parv’s Ca\(^{2+}\) buffering capacity are fundamentally dependent upon heart rate. At high physiological heart rates in mice (~10 Hz) in vivo, Parv modestly accelerated relaxation, without compromising systolic function. In fact, Parv expression was associated with an increase in the rate of pressure development in vivo, analogous to previously reported results after myocardial gene delivery of Parv into the rat heart (44). The mechanistic basis of this finding is unknown but presumably relates to optimization of SR/Ca\(^{2+}\) handling function by Parv. However, in our ex vivo preparation, gradually decreasing the pacing frequency resulted in a marked acceleration in early relaxation in Parv transgenic hearts relative to ntg control hearts but also caused a significant decrement in contractile function. Thus we were able to uniquely define the physiological effects of Parv’s effective Ca\(^{2+}\) buffering capacity in the intact heart over a wide range of pacing frequencies. Our mathematical modeling studies support our experimental findings, indicating that the lower effective buffering capacity of Parv at high frequencies can be explained by a reduction in the cyclical variation of the fraction of Parv sites occupied by Ca\(^{2+}\) (ΔPvca) in dynamic steady state. As the interbeat duration shortens at high pacing frequencies, the time available for binding and unbinding of Ca\(^{2+}\) and Mg\(^{2+}\) is reduced, thereby limiting Parv’s ability to buffer Ca\(^{2+}\) in a cyclical fashion at fast heart rates.

It is interesting to compare Parv’s Ca\(^{2+}\) buffering characteristics in the intact heart to those in fast-twitch muscle. There is a positive correlation between Parv concentration and the speed of skeletal muscle contraction across the animal kingdom (38). The toadfish (Opsanus tau) swim bladder used for sound production is at the high end of the spectrum and provides an excellent, well-studied model to use for comparison. The swim bladder skeletal muscle is the fastest known vertebrate muscle (>200 Hz at 25°C) and contains the highest concentration of Parv (1.5 mM) ever measured (40). Considering that Parv’s Ca\(^{2+}\) buffering capacity is reduced as fre-
quency of contraction increases, it seems counterintuitive that Parv could accelerate relaxation in muscles contracting so rapidly. However, although in vivo the toadfish call continues for many hours, the duration of the call is ~400 ms, interrupted by 5–15 s intercall intervals. Detailed studies have revealed that Parv binds Ca\(^{2+}\) during the call and then slowly releases it during the intercall interval, allowing for resquestration into the SR by SERCA (40). Thus Parv Ca\(^{2+}\) buffering is essential for enabling the swim bladder muscle to power extremely fast Ca\(^{2+}\) transients. But there is a trade-off for speed. To achieve rapid relaxation, the actin-myosin cross-bridge detachment rate must exceed the cross-bridge attachment rate, thus limiting the number of cross bridges that are attached during contraction. Therefore, the swim bladder muscle is only able to generate ~1/10 the force of locomotory muscle (40). How do these important observations on the kinetics of Parv in the swim bladder inform our experiments in expressing Parv in the rapidly beating mouse heart? First, because the heart must undergo continuous contractile cycles to sustain life, there is minimal opportunity for “catch-up time” for calcium to unbind from Parv. Therefore, Parv’s Ca\(^{2+}\) buffering capacity would saturate at high sustained pacing frequencies in heart muscle, affording only a minimal contribution to Ca\(^{2+}\) sequestration during diastole (Fig. 6). We have found experimentally that the buffering capacity of cardiac-directed Parv expression increases as the pacing frequency is lowered to rates close to those of the dynamic range of the human heart (1–3 Hz). Under these conditions, we observe a similar trade-off for speed of relaxation to that described for the toadfish swim bladder muscle. That is, absolute force decreases as buffering of systolic Ca\(^{2+}\) by Parv increases, reducing the number of force generating cross-bridge attachments during contraction. This represents a limitation of wild-type α-Parv since, unlike the swim bladder, the heart must be able to generate a vigorous force to eject blood and relax quickly enough to be ready for the next contraction.

One potential strategy to circumvent the compromised systolic function observed with wild-type α-Parv expression in the heart would involve genetically engineering Parv to alter Mg\(^{2+}\) and Ca\(^{2+}\) affinities, analogous to previous manipulations of troponin C, another E-F hand family protein (28). Based on our mathematical simulations from prior reports (6, 7), increasing the affinity of Mg\(^{2+}\) for Parv would slow the Mg\(^{2+}\) off rate from Parv and further delay Ca\(^{2+}\) binding toward early diastole. This approach would theoretically diminish systolic Parv Ca\(^{2+}\) buffering, thus limiting Parv’s effects on systolic function. Mathematical models further predict that increased Mg\(^{2+}\) affinity would broaden the optimal Parv concentration range, a property that would render Parv more suitable for therapeutic use in vivo.

How does Parv compare to other potential therapeutic strategies to manipulate Ca\(^{2+}\) regulation in the heart? Several studies have altered the activity of the SERCA2a pump, either by overexpressing SERCA itself, or by altering its regulation by its endogenous inhibitor phospholamban (PLN). Overexpression of SERCA or ablation of PLN uniformly increases SR Ca\(^{2+}\) uptake, speeds relaxation, and shortens the Ca\(^{2+}\) transient (10, 16, 18), but effects on ventricular function, arrhythmias, and mortality are controversial (2, 12, 13, 21, 22, 26, 31, 43). Moreover, ablation of PLN in a mouse model resulted in increased ischemic energy demand and markedly decreased systolic performance in response to ischemia and reperfusion (8). The notable identification of a human phospholamban null mutation in two families with inherited cardiomyopathies (15) further underscores the need to refine therapeutic strategies of PLN inhibition for heart failure. Parv differs from strategies that alter SERCA or PLN expression or activity in several ways. First, the innate response to β-adrenergic stimulation can be impaired by SERCA overexpression or PLN ablation because of the uncoupling of the normal SERCA/PLN regulation; expression of Parv does not appear to affect this response (5, 20). Second, ATP utilization would be expected to be higher when SERCA ATPase activity is increased (6). In contrast, Ca\(^{2+}\) buffering by Parv is ATP-independent, and mathematical models suggest that Parv may reduce ATP utilization during the Ca\(^{2+}\) transient and redistribute it later in the cardiac cycle at a lower rate. One disadvantage of Parv is that at higher concentrations, it impairs contractility, while increasing expression of SERCA or decreasing expression of PLN enhances systolic function (10, 11, 31). Therefore, limiting the effects of Parv on systolic function will be critical to adapting Parv as a genetic approach to treat heart failure.

It is also important to recognize that the myofilaments play an important role in determining cardiac relaxation performance. There is evidence to suggest a rate-limiting function for the myofilaments in governing relaxation rates in the normal myocardium (27). Likewise, detailed mathematical modeling indicate that relaxation is primarily determined by tight coupling of the Ca\(^{2+}\) transient and the unbinding of Ca\(^{2+}\) from troponin C (34). More recent work shows a significant contribution of troponin I phosphorylation to PKA-dependent cardiac myocyte relaxation (50). Therefore, while the data presented here indicate that introduction of a Ca\(^{2+}\) buffer can accelerate relaxation, complementary effects attributable to the myofilaments are also likely important in determining overall relaxation performance in the normal and diseased heart.

Relaxation abnormalities are pervasive in most cardiomyopathies and heart failure (39). In some cases diastolic and systolic dysfunction coexist, while others are characterized primarily by diastolic dysfunction and preserved or enhanced systolic function (52). Thus it is unlikely that a single molecular therapy for treating impaired relaxation will be universally effective for all cardiomyopathic disorders. Parv may ultimately be most suitably targeted toward conditions associated with diastolic dysfunction and normal or supranormal systolic function. Future studies directed toward refining the dose and frequency-response relationship of Parv in the heart and engineering novel Parv-based Ca\(^{2+}\) buffers to limit systolic buffering of Ca\(^{2+}\) will provide further insight into Parv’s buffering capabilities in the heart and will hopefully generate new Parv-based therapeutic tools for remedying relaxation abnormalities in heart failure.

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