Robust Adenoviral and Adeno-Associated Viral Gene Transfer to the In Vivo Murine Heart
Application to Study of Phospholamban Physiology

Hunter C. Champion, MD, PhD; Dimitrios Georgakopoulos, PhD; Saptarsi Haldar, MD; Lili Wang, PhD; Yibin Wang, PhD; David A. Kass, MD

Background—Viral gene transfer to the whole heart in vivo has been achieved in several mammalian species but remained difficult to accomplish in murine hearts. We postulated that a key impediment derives from the use of proximal aortic occlusion during virus injection, because this eliminates coronary perfusion gradients in mice as aortic root and left ventricle pressures equalize.

Methods and Results—Pressure-volume analysis confirmed these mechanics. In contrast, descending aortic occlusion with whole-body cooling (20°C) preserved transmyocardial perfusion gradients and allowed for sustained (∼10-minute) dwell times in an upper-body perfusion circuit. This approach yielded robust cardiac transfection with adenovirus (AdV) and adeno-associated virus (AAV) injected into the left ventricle cavity or more simply via a central vein. Cardio-specific expression was achieved with a myocyte-specific promotor. Optimal AdV transfection required 9-minute aortic occlusion, versus 5-minute occlusion for AAV. Using this method, we examined the in vivo function of phospholamban (PLB) by stably transfecting PLB-null mice with AAV encoding PLB (AAVPLB). AAVPLB restored PLB protein to near control levels that colocalized with SERCA2A in cardiomyocytes. At baseline, PLB-null hearts exhibited enhanced systolic and diastolic function, but frequency-dependent reserve was blunted versus wild-type controls. These properties, particularly the frequency response, returned toward control 3 months after AAVPLB transfection.

Conclusions—The new simplified approach for murine whole-heart viral transfection should assist molecular physiology studies. (Circulation. 2003;108:2790-2797.)

Key Words: gene therapy ■ viruses ■ aorta ■ cardioplegia ■ contractility

The use of viral-based vectors for myocardial gene transfer is an attractive approach for studying fundamental mechanisms of cardiac function and developing novel disease treatments.1–3 Although cardiomyocytes are good vector targets, efforts have been impeded by low transfection efficiencies and difficulties in culturing and studying mature cells.1–5 In vivo studies have generally used left ventricle (LV) cavity or aortic root virus injection during proximal (ascending) aortic constriction, with successes reported in rat, rabbit, and Syrian hamster.6–11 The addition of total body cooling has facilitated longer cross-clamp times, enhancing gene transfer in smaller animals.11,12

Despite these advances, successful translation of gene transfer methods to the murine heart has remained problematic. One problem may lie with use of proximal aortic occlusion, because this could greatly reduce aortic compliance in the mouse to effectively eliminate a transmyocardial perfusion gradient. Coronary perfusion would depend solely on manual pressures generated during injection or require sustained ventricular asystole that could heighten procedural risks. By contrast, mid-descending aortic occlusion might preserve a perfusion gradient by generating an isolated upper-body circuit and, combined with whole-body cooling, facilitate sustained clamp times to enhance gene transfer. More distal occlusion would also allow intravenous virus injection, simplifying the approach. The present study tested this methodology for both adenovirus (AdV)-mediated and adeno-associated virus (AAV)-mediated gene transfer to the in vivo murine heart. To provide proof of principal for stably altering in vivo cardiac function, we restored murine phospholamban (PLB) to mice lacking the PLB gene and provide novel insights regarding its role to the in vivo modulation of cardiac function by beat frequency.

Methods

Animals
Control mice (C57Bl6, 2 to 4 months) and littermate SVJ129/CF-1 mice with and without a null mutation for the PLB gene (PLB−/−, 6 to 8 months) were used.
Viral Vectors
Replication-deficient recombinant adenovirus (ST5) encoding nuclear-targeted β-galactosidase (AdV-β-gal) driven by a cytomegalovirus (CMV) promoter13–14 or cardiomyocyte-specific promoter (mouse α-myosin heavy chain [MHC]) was used. Replicant AdV was plaque purified, and titers were determined by plaque assay on HEK293 cells in culture. Viral titers for AdV-β-gal and AdMHC-β-gal were 1.7 × 10^12 and 1.3 × 10^12 particles (pt)/mL, respectively. After purification, virus was suspended in PBS (pH 7.4) with 3% sucrose and kept at −80°C until use. Amplification and purification were done by the University of Iowa Gene Transfer Vector Core. Replication-deficient recombinant adeno-associated virus (ST2) encoding nuclear-targeted β-galactosidase (AAV-β-gal; titer, 2.1 × 10^13 pt/mL) or phospholamban (PLB; titer, 3.7 × 10^13 pt/mL) driven by CMV promoter was also prepared.15–16

In Vivo Myocardial Gene Transfer
Mice were anesthetized with isoflurane induction and endotracheally intubated. Animals were placed supine on a thermoregulated table (37°C) and ventilated with isoflurane (2%) vaporized in 100% O2 using a custom constant-flow ventilator (6.7 mL/1 tidal volume at 120 minutes−1). ECG and core temperature were monitored. The left external jugular vein was cannulated, and modest volume expansion was provided (100 to 150 mL of 12.5% human albumin) at 30 μL/min−1. Topical 2% lidocaine gel was applied to the chest, and the thorax was entered by 1 of 2 techniques. For the first method, virus was injected into the LV chamber. Mice were placed on their right sides with the left forepaw affixed to the surgical table. The point of maximal intensity was visualized (approximately 0.5 to 0.75 cm below the axilla), and an incision was made to the left side of the point of maximal intensity. The thorax was carefully entered, avoiding cardiac injury, and the thoracotomy was widened by blunt dissection between the ribs to a total length of 0.5 to 0.75 cm. This provided direct visualization of the descending aorta immediately after the takeoff of the right subclavian artery and the course of the aorta to the diaphragm. The pericardium was opened, and a 7-0 suture was placed at the apex of the left ventricle. The aorta and pulmonary artery were identified. The anesthetized mouse was then cooled with a water jacket to a core temperature of 18 to 21°C (heart rate −80 to 100 bpm). The aorta was clamped distal to the takeoff of the right subclavian artery using a microserrefine (18055-03, Fine Science Tools). Because the IVC was not easily accessed through the lateral incision, it was not routinely occluded. IVC clamping was used, however, if the right ventricle (RV) became clearly dilated. Modified St Thomas cardioplegia (20 mL, 20°C) was injected into the LV cavity via an apically inserted 27G needle, followed by 20 μL of lipofectamine 2000 (Invitrogen), 1 μg/kg histamine (Sigma), and 20 μL of AdV-β-gal, AAV-β-gal, or AAVr+LR. The second transfection technique introduced virus intravenously. The thorax was accessed by right lateral incision approximately 0.5 cm above the diaphragm. Both descending aorta and inferior vena cava were cross-clamped, and virus was injected into the right internal jugular vein. For both methods, the aortic clamp was maintained for up to 15-minute durations.

After releasing the aortic clamp, isoproterenol (3 to 10 ng/kg per min IV) or transesophageal pacing (NuMed, Hopkinton)17 was used for cardiac support, if needed, and mice were warmed to 37°C over 30 to 40 minutes. The chest was closed with 6-0 prolene, negative thoracic pressure was restored, and the animals were extubated. Subsequent analysis was made 1 to 180 days after transfection. Using this method and a typical aortic occlusion time of 9 minutes, surgical survival was 71%. The most common identifiable causes of perioperative mortality were excessive bleeding, complete heart block, and ventricular arrhythmias. Postoperative mortality was 6%, being principally attributable to infection.

Expression of β-Galactosidase
β-galactosidase expression was assessed by enzyme activity in tissue samples and histochemical staining. Tissues were minced, lysed (75 mL per sample; 0.2% Triton X-100 and 100 nmol potassium phosphate; pH 7.8), and centrifuged (12 000 for 10 minutes), and supernatant was removed. Lysate was assayed for β-galactosidase activity by commercial test (Galacto-Light Plus, Tropix) with light emission measured by luminometer (Luminoscan RS, Labsystems) calibrated to a standard curve based on purified E. coli β-galactosidase. Activity was normalized per milligram of protein.

β-galactosidase histochemistry was performed on hearts fixed by 2% paraformaldehyde and 0.2% glutaraldehyde in PBS perfused through the RV for 10 minutes and on isolated myocytes. Tissue was incubated in X-gal stain (in PBS, 20 mM/L K3Fe(CN)6, 3H2O, 20 mM/L K4Fe(CN)6, 2 mM/L MgCl2, and 1 mg/mL X-gal [Promega] in DMSO) for 2 hours at 24°C, rinsed in PBS, and postfixed in 7% buffered formalin for 6 hours.

Isolation of Cardiac Endothelial Cells and Myocytes
To assess cell targeting of viral vectors, murine coronary endothelial cells were isolated using anti-endothlin (CD-106) antibodies (Transduction Laboratories) in a mini-MACS separation unit (Miltenyi Biotec [MB], Bisley, No. 421-01) with confirmation of cell isolation performed by flow cytometry.14 Labeled cells were incubated with MACS magnetic goat anti-rat IgG (H1L) (MB-485-01). MicroBeads and streptavidin (MB-481-01). MicroBeads were then separated using a high-gradient magnetic separation column 1 (MS columns, MB-422-01).18 Myocytes were isolated as described.19

Expression of Phospholamban and Colocalization With SERCA
Western blot analysis, immunoprecipitation of PLB, and colocalization of PLB with SERCA2a were performed as described.20–24 Immunohistochemistry was performed on isolated myocytes fixed in 4% paraformaldehyde and 0.5% Triton X-100. Primary overnight incubation with mouse monoclonal PLB and or SERCA2a antibody was followed by 1-hour secondary incubation with anti-rabbit Alexa 488 and anti-mouse Alexa 568 (Molecular Probes). Imaging was performed on a Nikon Diaphot 300 inverted epifluorescence microscope with a PCM-2000 laser confocal scanning microscope (Nikon).

Hemodynamic Analysis
Age-matched littermates (PLB+/−, PLB+/−, and PLB−/− + AAVr+LR, 3 months after transfection) were anesthetized, intubated and ventilated, and studied by pressure-volume catheterization.17 A 1.8F catheter (SPR-719) was advanced via the LV apex to lie along the longitudinal axis and connected to a stimulation/analysis system to yield pressure-volume loops. To assess heart-rate modulation of cardiac function, a 2F pacing catheter (NuMed) was placed in the esophagus to achieve atrial pacing.17 Spontaneous sinus rate was first slowed using the I1 inhibitor ULFS-49 (Boehringer Ingelheim; 15 to 20 mg/kg−1 IP).17

Statistical Analysis
Data are expressed as mean±SEM and analyzed by ANOVA followed by Tukey post hoc test to determine statistical significance between groups or by Student’s t test.

Results
Influence of Ascending Aortic Versus Descending Aortic Occlusion
Figure 1A displays aortic and LV pressure and their difference (transmyocardial gradient) during descending aortic occlusion (DAO) versus ascending aortic occlusion (AAO). Diastolic myocardial perfusion gradients were maintained by DAO, whereas with AAO, aortic root and LV pressures equilibrated to equalize a perfusion gradient. Figure 1B displays the data as pressure-volume loops. Application of AAO to the cooled heart resulted in near isovolumic contraction.
with high systolic pressures but also marked elevation of diastolic pressure (near 45 mm Hg). In contrast, DAO yielded an ejecting beat with augmented systolic yet normal diastolic pressures. Thus, the hemodynamics of more distal occlusion in the beating heart were far more favorable for sustained occlusion and for generating adequate coronary perfusion to facilitate gene transfer.

Figure 1. A, Simultaneous aortic and LV pressure tracings at baseline (top left) and with ascending aortic occlusion (top right) and their difference (transmural pressure gradient, bottom) during DAO versus AAO. With AAO, aortic and ventricular pressures virtually equalize, eliminating a coronary perfusion gradient, whereas this is not the case for DAO. B, LV pressure-volume loops in the mouse before (control) and after cooling (cool) and with subsequent occlusion of the DAO or AAO. Unlike DAO, AAO results in a near-isovolumic contraction with very high end-diastolic pressures. This can additionally contribute to reduced myocardial flow and thus efficacy of viral transfection.

Figure 2. A, Influence of aortic occlusion time on of expression of β-gal activity in whole-heart homogenate after transfection of adult mouse hearts with viral vehicle (control), AdVCMV β-gal (3 days after transfection), or AAVCMV β-gal (7 days after transfection). *P<0.05 vs control. **P<0.05 vs AdVCMV β-gal. B, Influence of aortic occlusion time on of expression of β-gal activity in coronary vascular endothelium after transfection of adult mouse hearts with viral vehicle (control), AdCMV β-gal, or AAVCMV β-gal. *P<0.05 vs control. **P<0.05 vs AdVCMV β-gal. C, Influence of aortic occlusion time on of expression of β-gal activity in isolated myocytes after transfection of adult mouse hearts with AdV β-gal (3 days after transfection) or AAV β-gal (7 days after transfection). *P<0.05 vs baseline value for β-gal expression after 0 minutes of occlusion time.
Influence of Aortic Occlusion Time on Transfection Efficacy

We next determined the impact of aortic occlusion time (AOT) on gene transfer to determine the optimal duration or if this time varied between AdV and AAV. Animals were cooled similarly, and AOT varied between 1 and 12 minutes (Figure 2). Whole heart, isolated myocytes, and isolated coronary endothelial cells were examined for β-galactosidase activity 3 to 4 or 7 to 10 days after transfection with AdV and AAV, respectively (time points of near-maximal expression of reporter gene determined in pilot studies). There was little to no β-gal activity in AdV-transfected hearts until AOT was ≥9 minutes, at which point a sudden marked rise in activity was observed. For AAV, AOT could be shortened, although optimal activity still required 9 minutes of occlusion. Figure 2B shows results of a similar analysis performed on isolated coronary endothelial cells. For AdV, there was virtually no β-gal activity detected until 15 minutes of aortic clamping. In contrast, AAV resulted in endothelial expression at much earlier time points, with values approximately 60% of those in myocytes at the longest cross-clamp durations. Figure 2C shows results for isolated myocytes in which significant transfection was achieved after 5 minutes of aortic occlusion time with AAV, but 9 minutes of occlusion time was required for AdV. Thus, for a 9- to 10-minute clamp time, transfection with AdV vectors was principally myocyte-targeted, whereas AAV transfected both cell types.

Distribution and Chronicity of β-Galactosidase Expression

Figure 3 shows whole-heart and isolated myocyte histochemistry for β-gal expression. For AdV transfection (using either LV or intravenous injection routes), hearts were stained 3 days after transfection, whereas those transfected with AAV were stained at 7 days. Staining was diffuse but patchy in localized regions and likely reflects some overstaining (ie, leakage of β-gal/X-gal product into the cytosol), because it was difficult to fully rinse X-gal from the intact heart to stop the reaction in a whole-heart preparation. Figure 3B shows myocytes isolated from transfected hearts, revealing nuclear staining in approximately 50% to 65% of cells transfected with AdV, with similar amounts with AAV. Mouse myocytes frequently display polyploidy, so cells rather than nuclei were counted for this analysis. Both healthy and no longer viable (ie, rounded) cells were counted, because the latter were presumably alive when transfected in vivo.

AdV β-gal transfection yielded significant marker gene activity within 24 hours, peaking at 3 days and declining by 2 weeks (Figure 4A). This time course was independent of the route of viral introduction (LV cavity or intravenous, data not
AAV coupled to the CMV promoter, activity was greatest and similar in heart and lung but also observed at lower levels in liver and other organs. AdV transfection with the α-MHC promoter selectively targeted the heart and yet produced similar enzyme activity to that with the CMV promoter. Thus, although use of a descending aortic clamp increased the tissue distribution of transfection, this could be obviated by use of a tissue-specific promoter.

**In Vivo Gene Transfer of PLB to the Myocardium of the PLB<sup>−/−</sup> Mouse**

To test for chronic functionality of gene transfer, we examined expression, localization, and functional influence of AAV<sub>PLB</sub> transfection into myocardium of PLB<sup>−/−</sup> mice. Analysis was performed 3 months after transfection and contrasted to age-matched wild-type (WT) and PLB<sup>+/+</sup> littermate controls. PLB<sup>−/−</sup> hearts had no PLB expression, whereas PLB<sup>+/−</sup> hearts displayed PLB protein levels similar to those of WT controls (Figure 5A). Immunoprecipitation of PLB and subsequent probing for SERCA2a by Western blot revealed normal colocalization of both proteins in WT and PLB<sup>+/−</sup> + AAV<sub>PLB</sub> mice (Figure 5B). PLB<sup>+/−</sup> mice had no detectable expression of PLB by immunolocalization, whereas hearts transfected with AAV<sub>PLB</sub> showed immunostaining for PLB that colocalized to SERCA2a (Figure 5C).

Baseline systolic function (as indexed by dP/dt<sub>max</sub> and preload-normalized maximal LV power) was higher in PLB<sup>−/−</sup> hearts, and systolic kinetics indexed by the time to end systole were faster. The peak rate of pressure decline (dP/dt<sub>min</sub>) was greater, although relaxation time constant was not significantly different (Table). Transfection of AAV<sub>PLB</sub> diminished systolic function modestly, with borderline significant change in dP/dt<sub>max</sub> but significant prolongation of systole.

More striking effects of AAV<sub>PLB</sub> were observed on the frequency dependence of both systolic and diastolic function. Figure 6A shows pooled regression plots for representative

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### Table: Baseline Hemodynamic Parameters in WT, PLB<sup>−/−</sup>, and PLB<sup>+/−</sup> Transfected With PLB Using an Adenoviral Vector (PLB<sup>+/−</sup> + AAV<sub>PLB</sub>)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT, n=5</th>
<th>PLB&lt;sup&gt;−/−&lt;/sup&gt;, n=4</th>
<th>PLB&lt;sup&gt;+/−&lt;/sup&gt; + AAV&lt;sub&gt;PLB&lt;/sub&gt;, n=4</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, min&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>623±11</td>
<td>671±18&lt;sup&gt;*&lt;/sup&gt;</td>
<td>679±20</td>
<td>0.07</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>15.6±1.3</td>
<td>19.5±2.2</td>
<td>15.9±2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>EF, %</td>
<td>52±4</td>
<td>49±6</td>
<td>55±5</td>
<td>0.8</td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;max&lt;/sub&gt;, mm Hg/s</td>
<td>12538±338</td>
<td>21053±1052&lt;sup&gt;†&lt;/sup&gt;</td>
<td>17744±2031†</td>
<td>0.0018</td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;min&lt;/sub&gt;, mm Hg/s</td>
<td>-11954±600</td>
<td>-18080±1263&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-17650±1206&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>Tes, ms</td>
<td>41.4±0.64</td>
<td>35.5±0.2&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>39.6±0.9</td>
<td>0.0002</td>
</tr>
<tr>
<td>PFR&lt;sub&gt;EDV&lt;/sub&gt;, ms</td>
<td>30.2±3.0</td>
<td>28.7±4.3</td>
<td>35.4±1.7</td>
<td>0.4</td>
</tr>
<tr>
<td>PWR&lt;sub&gt;max&lt;/sub&gt;/EDV</td>
<td>33.1±3.7</td>
<td>61.8±6.7&lt;sup&gt;+&lt;/sup&gt;</td>
<td>49.98±4.8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.008</td>
</tr>
<tr>
<td>EDV, mm Hg/s</td>
<td>4.3±0.2</td>
<td>3.5±0.7</td>
<td>3.6±0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Tau, ms</td>
<td></td>
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</tbody>
</table>

HR indicates heart rate; CO, cardiac output; EF, ejection fraction; Tes, time to end systole (QRS to time of maximal LV elastance); PFR<sub>EDV</sub>, peak filling rate normalized to end-diastolic volume; PWR<sub>max</sub>/EDV, maximal LV power normalized to end-diastolic volume; and Tau, time constant of relaxation (logistic fit).

<sup>*</sup>P<0.05 vs WT.

<sup>†</sup>P<0.01 vs PLB<sup>+/−</sup>.
functional parameters at different steady-state heart rates. There was significant enhancement of systolic function and accelerated relaxation with higher heart rate in WT, but this was not significant in PLB⁻/⁻ hearts, with the relations in these hearts being generally flat. Transfection with AAVₚₜ in the null-mutant heart restored the frequency-following response (FFR). Summary data for the slopes of various heart function–heart rate relations are provided in Figure 6B. Pooled regression analysis was performed using dummy variables for each heart to derive the average slope of each relation. Both WT and PLB⁻/⁻ transfected with AAVₚₜ displayed significant relationships, whereas there was little to no heart rate dependence in the PLB⁻/⁻ hearts.

Discussion

Methodologic Issues

We developed a novel method for in vivo gene transfer to the mouse using animal cooling, mid-distal aortic occlusion during virus injection, and substantially longer clamp times than have been generally used in larger rodents. Ascending aortic occlusion, a historically more widely used approach, yielded near isovolumic contraction with elevated systolic pressures but also equalized aortic and LV pressures and markedly raised LV diastolic pressures, compromising myocardial perfusion. Although this approach has been successful in larger rodents, these animals have nearly an order of magnitude (or greater) larger ascending root that may provide adequate compliance to maintain diastolic perfusion. The mouse aortic root is very small, and with AAO, only a trivial compartment remains. Indeed, our initial efforts using an AAO clamp approach consistently yielded low levels of transfection that favored the RV, and occlusion times were much shorter or risked cardiac demise. In contrast, the more distal clamp preserved a coronary perfusion gradient, maintained LV systolic and diastolic pressures, and permitted long clamp times needed for adequate viral-tissue interaction. Importantly, it allowed virus to be introduced intravenously, avoiding the need for proximal aorta and pulmonary artery dissection and LV manipulation.

Several recent studies have combined AAO with marked animal cooling (as first used in neonatal mice) to transfect hamsters with AdV and AAV. These investigators used methods similar to Hajjar et al but prolonged aortic occlusion by cooling to achieve impressive results. Vascular dwell time is a key factor for efficient transfection, and in these hamster studies, 4 to 6 minutes of cross-clamp was adequate at 25 to 26 °C cooling. However, in mice, this duration seems too short with AdV but may be adequate for AAV. The hamster is approximately 5 to 6 times the size of a mouse and probably more similar to guinea pig or rat in this regard.

A potential limitation of distal over proximal aortic occlusion is the enlarged perfused territory encompassing the upper body. However, cardiac specificity could be achieved by use of a myocyte-specific promoter. It is intriguing that marker gene activity was similar whether provided by CMV or α-MHC promoters despite greater potency of the former, but this may relate to relative high transfection levels. One potential limitation may rest in differences in mouse strains. The present study showed successful transfection in 2 broadly
used strains, C57BL/6 and 129SvJ, but this still may not directly translate to other strains that may display different transfection efficiencies.

We did not perform systematic analysis of transfection efficacy as a function of body temperature, although Donahue et al.26 have shown that cooling inhibits AdV transfection. Somewhat higher temperatures may even enhance gene transfer while still permitting prolonged aortic clamp times, but this remains to be tested. It also remains unclear whether myocyte infection occurs during cooling in the present or prior studies11,25 or after normal body temperatures are restored. The colder temperature may assist endothelial permeability to help translocate virus into the interstitium, but then the particles get trapped during rewarming as they become more infectious.

**PLB Transfection: Functional Studies**

An important component of whole-heart transfection is the capacity to alter net chamber performance. We chose to replace PLB in the PLB<sup>−/−</sup> model because protein expression and localization could only result from transfected gene. Furthermore, although many studies have examined the role of PLB to cardiac function, the present analysis is the first to test the impact of PLB restoration in an intact nonfailing adult PLB-null mouse, providing more direct analysis of its in vivo function. PLB protein levels were similar to WT and colocalized with SERCA2a (shown by immunoprecipitation and confocal microscopy). Our analysis of basal function supports prior data showing enhanced systolic performance in the PLB<sup>−/−</sup> mouse, and this was modestly blunted by restoration of PLB.2,20,22 One difference was the lack of enhanced relaxation in PLB<sup>−/−</sup> hearts that has been reported.29 This may be attributable to the much slower heart rates (~300 minutes<sup>−1</sup>) and reduced contractility present in the earlier study that could favor PLB-dependent relaxation disparities.

More striking hemodynamic results were related to the influence of PLB restoration on the frequency dependence of systolic and diastolic function. This is thought to be principally attributable to enhanced calcium entry and uptake by the sarcoplasmic reticulum, although the exact role of PLB to the process has been somewhat controversial. In some studies,20,28 absence of PLB shifted the FFR from positive to negative, whereas other investigations found the opposite.29 Others found frequency-dependent acceleration of relaxation was preserved in PLB<sup>−/−</sup> but depended on calcium/calmodulin-dependent protein kinase II.30 Some of the discrepancy may relate to the preparation conditions and thus level of PLB phosphorylation, because negative influences of PLB have been found in preparations where optimal systolic function is achieved at subphysiological heart rates, with a negative FFR at more physiological rates.

Our in vivo preparation yielded more physiological basal function with typically positive FFRs that did not decline up to 900 minutes<sup>−1</sup> in normal mice.17 In this setting, lack of PLB elevated dP/dt<sub>max</sub> at lower rates but displayed little rate-dependent rise.17 Relaxation and effects on early peak diastolic filling were similarly largely rate independent in PLB<sup>−/−</sup> animals. The observed restoration of rate responsiveness in both sets of parameters several months after AAV<sub>PLB</sub> transfection argues in favor of in vivo FFR modulation by PLB. The exact mechanism remains to be clarified but may be elucidated by transfection with PLB mutants harboring altered phosphorylation sites. Lastly, it is possible that some other compensatory mechanisms may have played a role, although the duration of expression was only 3 months.

**Figure 6.** A, Force-frequency response for the maximal rate of pressure rise (dP/dt<sub>max</sub>) and isovolumic relaxation time constant (τ) in WT; PLB<sup>−/−</sup>, and PLB<sup>−/−</sup> mice transfected with AAV<sub>PLB</sub>. B, Change in systolic (ΔdP/dt<sub>max</sub>), maximal LV power normalized to preload, ΔPWR<sub>max</sub>/EDV and diastolic function (Δτ and Δpeak filling rate normalized to preload and FFR<sub>EDV</sub> for a given change in heart rate (Δbts/min) were derived from group data, as displayed in A by multiple regression analysis. For each parameter, significant HR dependence was observed in WT animals but not in PLB<sup>−/−</sup> and AAV<sub>PLB</sub> transfection-restored rate responsiveness. *P<0.01; †P<0.001 (P values are for significance of HR dependence in linear regression model). P values shown represent overall ANOVA for comparison of WT, PLB<sup>−/−</sup>, and PLB<sup>−/−</sup> + AAV<sub>PLB</sub>.
Conclusion
The present method for cardiac gene transfer should help the exploration of molecular signaling and development of therapeutic strategies using murine models. Importantly, the systematic identification of key elements for successful transfection, ie, perfusion/clamp time, temperature, and provision of adequate arterial compliance and transmyocardial perfusion gradients, may be relevant to other mammalian species and prove helpful for developing additional approaches for whole-heart gene transfer.

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