Modulation of force-frequency relation by phospholamban in genetically engineered mice

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Kadambi, Vivek J., Nancy Ball, Evangelia G. Kranias, Richard A. Walsh, and Brian D. Hoit. Modulation of force-frequency relation by phospholamban in genetically engineered mice. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H2245–H2250, 1999.—Phospholamban levels regulate cardiac sarcoplasmic reticulum Ca2+ pump activity and myocardial contractility. To determine whether and to what extent phospholamban modulates the force-frequency relation and ventricular relaxation in vivo, we studied transgenic mice overexpressing phospholamban (PLBOE), gene-targeted mice without phospholamban (PLBKO), and isogenic wild-type controls. Contractility was assessed by the peak rate of left ventricular (LV) isovolumic contraction (±dP/dtmax), and diastolic function was assessed by both the peak rate (−dP/dtmax) and the time constant (τ) of isovolumic LV relaxation, using a high-fidelity LV catheter. Incremental atrial pacing was used to generate heart rate vs. −dP/dtmax (force-frequency) relations. Biphasic force-frequency relations were produced in all animals, and the critical heart rate (HRcrit) was taken as the heart rate at which dP/dtmax was maximal. The average LV +dP/dtmax increased in both PLBKO and PLBOE compared with their isogenic controls (both P < 0.05). The HRcrit for LV +dP/dtmax was significantly higher in PLBKO (427 ± 20 beats/min) compared with controls (360 ± 18 beats/min), whereas the HRcrit in PLBOE (340 ± 30 beats/min) was significantly lower compared with that in isogenic controls (440 ± 25 beats/min). The intrinsic heart rates were significantly lower, and the HRcrit and the ±dP/dtmax at HRcrit were significantly greater in FVB/N than in SvJ control mice. We conclude that 1) the level of phospholamban is a critical negative determinant of the force-frequency relation and myocardial contractility in vivo, and 2) contractile parameters may differ significantly between strains of normal mice.

treppe; myocardial contractility; hemodynamics

THE ADVANCES OF MOLECULAR biology and the development of gene targeting and transgenic technologies in the mouse have exponentially increased our understanding of cardiovascular physiology and pathophysiology (4, 13, 15, 31). Although a number of sophisticated in vitro and in vivo techniques are currently available to assess cardiac function in genetically engineered murine models, these methods are not without limitations (4, 15). The recent development of miniaturized catheter-based approaches to evaluate left ventricular (LV) function in both the open- and closed-chest mouse allows a more precise evaluation of in vivo cardiac function (12, 23, 28, 30). However, studies from several laboratories have shown that heart rate is an important determinant of cardiac contractility (1, 2, 8, 20, 27, 28, 32), and thus caution must be exercised when evaluating cardiac function in genetically engineered mice with variable heart rates.

The ability to systematically assess the effects of varying heart rates on cardiac contractility has demonstrated that the force-frequency relation is a powerful modulator of myocardial function in intact open- and closed-chest anesthetized mice (12, 28). We and others (12, 20) recently demonstrated that the relationship between paced heart rate and the peak rate of isovolumic contraction (±dP/dtmax) is biphasic, with both ascending and descending limbs, and that the force-frequency relation can be characterized by the heart rate at which dP/dtmax is maximal [the critical heart rate (HRcrit)]. Although altered Ca2+ homeostasis has been implicated in the genesis of the force-frequency relation (8, 12), the underlying molecular mechanisms remain uncertain.

Phospholamban is a 52-amino acid integral sarcoplasmic reticulum (SR) membrane phosphoprotein, which in its dephosphorylated state is an inhibitor of the apparent affinity of the SR Ca2+-ATPase for Ca2+; phosphorylation by protein kinases relieves this inhibition (16). The role of phospholamban in modulating cardiac contractility was recently elucidated by the generation of phospholamban gene-targeted and transgenic mice with cardiac specific overexpression of phospholamban (17, 24). An inverse correlation exists between the levels of phospholamban and cytosolic Ca2+ transients such that ablation of phospholamban results in an increase, whereas twofold cardiac-specific overexpression of phospholamban results in a decrease, in the amplitude of the Ca2+ transient (17, 21, 24).

Recently, β-adrenergic-mediated increases in HRcrit were demonstrated in the anesthetized mouse (28); shifts in the force-frequency relation may have been mediated by catecholamine-induced phosphorylation...
(and hence disinhibition) of phospholamban. However, the effects of catecholamines are not specific. Thus the present study was designed to specifically investigate the effect of ablation and overexpression of phospholamban [with well-characterized concomitant alterations in intracellular Ca\(^{2+}\) homeostasis, myocardial contractility, and relaxation (17, 24)] on the force-frequency relation in anesthetized closed-chest mice. Specifically, we tested the hypothesis that the level of phospholamban is an important negative determinant of the force-frequency relation in anesthetized closed-chest mice.

**METHODS**

Generation of genetically engineered mice. The phospholamban knockout mouse was produced with gene-targeting technology as previously described (24). Wild-type mice of identical mixed background were bred simultaneously with the phospholamban knockout animals. Mice with cardiac-specific overexpression of phospholamban were generated as previously described (17).

Inasmuch as the genetic background of the phospholamban knockout differs from that of the phospholamban overexpression mouse, we thought it was important to compare hemodynamic data from each animal model with the appropriate wild-type controls. Thus the phospholamban knockout mice were processed in parallel with age-matched wild-type littermates (129SvJ), whereas the phospholamban overexpression mice were compared with their isogenic wild-type littermates (FVB/N).

Animal preparation. Phospholamban knockout and phospholamban overexpression mice along with their respective isogenic wild-type littermates were instrumented as previously described (12). Briefly, mice were anesthetized with a mixture of ketamine (100 mg/kg), xylazine (5 mg/kg), and morphine (2.5 mg/kg). 0.1 ml/10 g intraperitoneally, intubated with a 24-gauge J eco intravenous catheter, and ventilated at 100 cycles/min. The right jugular vein was isolated, and a 1-Fr bipolar pacing wire (NuMED, Nicholville, NY) was positioned in the right atrium. Flame-stretched Nalgene tubing was advanced into the right jugular vein for intravenous access. The right carotid artery was isolated and cannulated with a 1.4-Fr high-fidelity micromanometer (Millar, Houston, TX), which was then advanced into the left ventricle and secured with 6-0 silk.

Experimental protocol. Studies were performed in 26 animals (23–32 g) of either sex. There were six phospholamban overexpression and eight FVB/N controls, and six phospholamban knockouts and six 129SvJ controls.

After hemodynamic stability was ensured, baseline data were obtained in the intrinsic state and with the atrial pacemaker set at 5 Hz (300 beats/min). The sinus node fuzzy channel current inhibitor DKAH-0269 (0.1 ml iv; Boehringer Ingelheim, Ridgefield, CT) was given if the intrinsic heart rate was >300 beats/min.

Atrial pacing was initiated just above intrinsic heart rate (or 300 beats/min) using a stimulator (model S88, Grass, West Warwick, RI), which was set at 3–4 V, 2 m/s pulse-width duration, and increased by 12 beats/min increments until LV dP/dt \(_{\text{max}}\) was visually decreased or until atrioventricular block unresponsive to 0.04–0.08 ng/g atropine supervised (HR\(_{\text{crit}}\)).

Western blotting. Cardiac homogenates from 129SvJ and FVB/N wild-type mice were processed in parallel for quantitative immunoblotting analysis as previously described (17). Briefly, homogenates were separated on a 13% SDS polyacrylamide gel, electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH), probed with either a SR Ca\(^{2+}\)-ATPase polyclonal antibody or an anti-phospholamban monoclonal antibody (Affinity BioReagents), and visualized using the enhanced chemiluminescence kit (Amersham). The

| Table 1. Hemodynamic parameters in PLBKO and 129SvJ mice at baseline and atrially paced at 300 beats/min |
|---|---|---|---|---|---|
| **HR, beats/min** | **LVSP, mmHg** | **+dP/dt\(_{\text{max}}\), mmHg/s** | **−dP/dt\(_{\text{max}}\), mmHg/s** | **τ, ms** |
| **Baseline** | | | | |
| 129SvJ | 330 ± 29 | 85 ± 8 | 5,837 ± 767 | 6,111 ± 699 | 7.8 ± 0.6 |
| PLBKO | 447 ± 36* | 117 ± 11* | 14,546 ± 1,150* | 12,817 ± 1,800* | 3.6 ± 0.7* |
| **Atrially paced** | | | | |
| 129SvJ | 300 ± 0 | 80 ± 12 | 5,085 ± 560 | 4,280 ± 425 | 8.2 ± 0.8 |
| PLBKO | 300 ± 0 | 122 ± 18* | 14,570 ± 950* | 11,100 ± 620* | 3.9 ± 0.6* |

Values are means ± SE; n = 6 mice/group. PLBKO, phospholamban knockout mice; 129SvJ, isogenic wild-type mice; HR, heart rate; LVSP, left ventricular systolic pressure; +dP/dt\(_{\text{max}}\), peak rate of isovolumic contraction; −dP/dt\(_{\text{max}}\), peak rate of isovolumic relaxation; τ, time constant of isovolumic relaxation. *P < 0.05 vs. 129SvJ.

| Table 2. Basal hemodynamic parameters in PLBOE and FVB/N mice at baseline and atrially paced at 300 beats/min |
|---|---|---|---|---|
| **HR, beats/min** | **LVSP, mmHg** | **+dP/dt\(_{\text{max}}\), mmHg/s** | **−dP/dt\(_{\text{max}}\), mmHg/s** | **τ, ms** |
| **Baseline** | | | | |
| FVB/N | 218 ± 24 | 80 ± 4.5 | 6,979 ± 522 | 6,180 ± 551 | 8.2 ± 0.6 |
| PLBOE | 223 ± 22 | 72 ± 2 | 5,726 ± 244 | 5,289 ± 194 | 9.8 ± 0.6 |
| **Atrially paced** | | | | |
| FVB/N | 300 ± 0 | 92 ± 8 | 9,180 ± 330 | 7,920 ± 250 | 7.9 ± 0.6 |
| PLBOE | 300 ± 0 | 86 ± 7 | 7,210 ± 836* | 7,310 ± 300 | 10.2 ± 0.5* |

Values are means ± SE; n = 8 FVB/N and 6 PLBOE mice/group. PLBOE, phospholamban overexpression mice. FVB/N, isogenic wild-type mouse. *P < 0.05 vs. FVB/N.
levels of Ca\textsuperscript{2+}-ATPase and phospholamban were quantified using the ImageQuant analysis software.

Methods of analysis. Analog signals of LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) and peak rates of isovolumic contraction (+dP/dt\text{max}) and relaxation (−dP/dt\text{max}) obtained by electronic differentiation of the LV pressure signal were recorded online using a Gould Window-Graf four-channel recorder (Gould, Cleveland, OH) and digitized via an analog-to-digital board at 1,000 Hz.

The time constant of LV isovolumic relaxation (\(\tau\)) was derived from high-fidelity LV tracing, assuming a monoexponential decay of LV pressure to a zero asymptote (37).

Statistical analysis. Data are expressed as means ± SE. Hemodynamic data were compared between FVB/N and phospholamban overexpression mice and phospholamban knockout and 129SvJ mice using unpaired t-tests, respectively. ANOVA was used to compare the LV +dP/dt\text{max} at initial-, critical-, and final-paced heart rates. Phospholamban and Ca\textsuperscript{2+}-ATPase were expressed as a ratio (to account for variability in gel loading) and compared with unpaired t-tests. P < 0.05 was considered statistically significant.

### RESULTS

Baseline hemodynamics. Hemodynamic parameters in the phospholamban knockout, phospholamban overexpression, and their isogenic wild-type mice were examined under both basal conditions and at an atrially paced rate of 300 beats/min (Tables 1 and 2). The heart rate under basal conditions, LVSP, and +dP/dt\text{max} were significantly greater, and the time constant (\(\tau\)) was significantly less in the phospholamban knockout mice compared with isogenic controls. In contrast, the heart rate, LVSP, and +dP/dt\text{max} were not significantly different in the phospholamban overexpression mice compared with isogenic controls. However, at matched paced heart rates of 300 beats/min, LVSP and ±dP/dt\text{max} were significantly greater and \(\tau\) was significantly less in the phospholamban knockout mice, and ±dP/dt\text{max} was significantly lower and \(\tau\) significantly greater in the phospholamban overexpression mice compared with their respective isogenic controls.

Force-frequency relation. The effects of incremental atrial pacing on LV +dP/dt\text{max} in representative phospholamban knockout and phospholamban overexpression mice along with their respective wild-type littermates are shown in Fig. 1. Incremental pacing resulted in significant increases in +dP/dt\text{max} in both groups of mice. The average LV +dP/dt\text{max} increased from 9,800 ± 875 to 15,849 ± 1,100 mmHg/s (P < 0.05) in the phospholamban knockout mice compared with 3,300 ± 800 to 5,500 ± 400 mmHg/s in their wild-type SvJ controls (P < 0.05). Similarly, the average +dP/dt\text{max} increased from 5,726 ± 244 to 7,500 ± 400 mmHg/s (P < 0.05) in the phospholamban overexpression mice compared with 6,596 ± 580 to 9,700 ± 600 mmHg/s in their FVB/N wild-types controls (P < 0.05).

The force-frequency relation was shifted upward and to the right in the phospholamban knockout mice, whereas this relation was shifted downward and to the left in the phospholamban overexpression mice compared with their respective controls. Thus the HR\text{crit} for LV +dP/dt\text{max} was significantly higher in the phospholamban knockout mice (427 ± 20 beats/min) compared with controls (360 ± 18 beats/min), whereas the HR\text{crit} in the phospholamban overexpression mice (340 ± 30 beats/min) was significantly lower compared with isogenic controls (440 ± 25 beats/min). In both groups of mice, incremental pacing produced significant increases in LVSP and decreases in LVEDP (data not shown).

### Table 3. Hemodynamic parameters in 2 strains of mice

<table>
<thead>
<tr>
<th></th>
<th>Intrinsic HR, beats/min</th>
<th>HR\text{crit}, beats/min</th>
<th>Intrinsic LVSP, mmHg/s</th>
<th>Intrinsic +dP/dt\text{max}, mmHg/s</th>
<th>+dP/dt\text{max} at HR\text{crit}, mmHg/s</th>
<th>+dP/dt\text{max} at 300 beats/min, mmHg/s</th>
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<tbody>
<tr>
<td>129SvJ</td>
<td>340 ± 20</td>
<td>360 ± 18</td>
<td>80 ± 12</td>
<td>5,200 ± 300</td>
<td>5,500 ± 400</td>
<td>4,800 ± 200</td>
</tr>
<tr>
<td>FVB/N</td>
<td>220 ± 25*</td>
<td>440 ± 25*</td>
<td>88 ± 14</td>
<td>6,800 ± 330</td>
<td>9,700 ± 600*</td>
<td>9,000 ± 445*</td>
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</tbody>
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Values are means ± SE; n = 6 129SvJ and 8 FVB/N mice. HR\text{crit}, critical heart rate. * P < 0.05 vs. 129SvJ.
that the levels of phospholamban/SR Ca\textsuperscript{2+} total cardiac homogenates (Fig. 2). Our results indicate mice, we performed quantitative immunoblotting of dynamic findings in the 129SvJ and FVB/N strains of phospholamban could account for the variable hemodynamic similar in these two strains of mice (1.12 vs. 1.04).

DISCUSSION

The relation between force and the frequency of stimulation is an important determinant of myocardial contractility; the positive inotropic effect of increasing beat frequency has been demonstrated in a variety of preparations including papillary muscles, ventricular muscle strips, whole hearts, and the intact animal (6, 26, 34). The present study indicates that the level of phospholamban [and therefore the magnitude of SR Ca\textsuperscript{2+} loading (17, 24)] is an important negative determinant of the force-frequency relation and myocardial contractility in the intact closed-chest anesthetized mouse. Another important finding is that frequently measured myocardial contractile parameters are significantly different among various strains of normal mice, emphasizing the importance of using strain-matched littermates for comparative purposes.

Our recent studies have demonstrated that ascending and descending limbs of the force-frequency relation exist in both anesthetized and conscious sedated intact animals and that HR\textsubscript{crit} changes directly with the inotropic state of the heart (12, 20). However, the effects of \(\beta\)-adrenergic receptor stimulation have been controversial (6, 33); discrepant data may be due to species, methodological, and analytic differences. Increased stimulation frequency increases Ca\textsuperscript{2+} entry into the cell per unit time and reduces the time available for SR Ca\textsuperscript{2+} accumulation, ultimately exhausting the capacity of the SR (9). Thus a decreased ability of the SR Ca\textsuperscript{2+}-ATPase to resequester Ca\textsuperscript{2+} is likely to play a critical role in the development of the descending limb of the biphasic force-frequency relation. Our results support this hypothesis insofar as the onset of the descending limb (HR\textsubscript{crit}) and the magnitude of LV dP/dt\textsubscript{max} are inversely related to the relative levels of phospholamban/SR Ca\textsuperscript{2+}-ATPase. Thus with phospholamban ablation there is an increase and with twofold overexpression of phospholamban there is a decrease in both the heart rate and LV +dP/dt\textsubscript{max} at HR\textsubscript{crit}. These data are consistent with those obtained from thin muscle strips in human heart failure (9) and in vivo primates with hyperthyroidism (20); however, these studies relate changes in force-frequency behavior to transriptional and translational changes in Ca\textsuperscript{2+} cycling proteins in settings where additional combinatorial changes occur. The novel transgenic approach and the protocol we employed are free of these confounding influences (and others, such as age, species, stimulation range) and demonstrate unambiguously the effect of phospholamban levels on the force-frequency relation.

The ascending limb of the force-frequency relation has been attributed to the increased amount of systolic Ca\textsuperscript{2+} available to the contractile proteins, the net effect of which is to increase release of SR Ca\textsuperscript{2+} from the ryanodine receptor via a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release; increased Ca\textsuperscript{2+} resesquestration back into the SR results in increased SR Ca\textsuperscript{2+} release during subsequent contractions. In contrast, the potential mechanisms underlying the descending limb of the force-frequency relation are poorly understood. One possible explanation is that the decreased duration of the action potential, which occurs with increased stimulation frequency, may decrease the amplitude of the Ca\textsuperscript{2+} transient and hence cardiac contractility. However, in isolated rat myocytes, changes in the action potential duration have very little effect on the Ca\textsuperscript{2+} transient and contractility (14). Another possible contributor to
the descending limb is the effect of reduced preload (a result of decreased LV filling time) on LV \( +\frac{dP}{dt} \) (22). Although our study does not address this possibility directly, the finding that \( \frac{dP}{dt} \) increased on the ascending limb despite an HR-induced fall in LVEDP and, more importantly, the highly variable heart rates at which the HR\(_{\text{crit}}\) occurred in mice with ablation and overexpression of phospholamban argues against a major contribution of the preload. This is consistent with studies in the isolated ejection dog heart (3) and intact animal (18, 29) that showed a minimal effect of loading conditions on force-interval behavior. Another possible mechanism relates to the timing and effectiveness of atrial contraction (28). Thus it is likely that these mechanisms (i.e., preload, atrioventricular synchrony) act in concert with a frequency-dependent impairment of Ca\(^{2+}\) cycling to produce the descending limb of the force-frequency relation.

Several compensatory biochemical changes in these genetically altered mice potentially influence the force-frequency relation. In the phospholamban knockout mouse, there is an \(-25\%\) reduction in the ryanodine receptor and faster type Ca\(^{2+}\) L-channel inactivation (5, 25); however, both of these would be expected to attenuate, not enhance, the ascending limb of the force-frequency relation. In the phospholamban overexpression mouse, there is a 3.7-fold increase in the Na\(^+\)/Ca\(^{2+}\) exchanger (36); an increase in activity of this exchanger might contribute to the leftward shift of the force-frequency relation in these mice.

Although strain-related differences are not unusual, the marked differences in hemodynamics and force-interval behavior between the SvJ and FVB/N wild-type mice were unexpected. Accordingly, we postulated that the strain differences resulted from variations in the stoichiometric relationship between phospholamban and the SR Ca\(^{2+}\)-ATPase. However, at the protein level, the ratios were similar in the two strains. Thus phospholamban/SR Ca\(^{2+}\)-ATPase stoichiometry cannot be the only determinant of the force-frequency relation because the profound strain differences in the HR\(_{\text{crit}}\) and LV and \( \frac{dP}{dt} \) existed with the same phospholamban/SR Ca\(^{2+}\)-ATPase ratio.

The findings of the present study should be interpreted in the context of several potential limitations. First, studies were performed in anesthetized animals; other data suggest that the augmentation of the contractile state due to increased stimulation rate is greater in the anesthetized than conscious state (11). Although the physiological relevance of the force-frequency relation in conscious animals is established, the magnitude of that importance remains controversial (7, 11, 19, 26); nevertheless, the study of frequency-dependent effects in well-characterized models provides insight into the molecular mechanisms underlying these processes (8, 10, 35). Anesthesia also depresses both the intrinsic and critical heart rate; however, anesthesia is unlikely to account for the differences in the force-frequency relations among transgenic mice overexpressing phospholamban, gene-target mice without phospholamban, and their isogenic controls. Second, the contractile parameter we employed (\( +\frac{dP}{dt_{\text{max}}} \)) is dependent on the LV end-diastolic volume, which varies with the stimulation frequency. However, this effect did not appear to differ between strains and is unlikely to have significantly influenced our findings. In addition, although relatively load-independent indexes based on time-varying elastance have demonstrated the positive inotropic influence of heart rate (22), these measurements are currently impractical to perform in mice. Moreover, the analytic method employed may influence the result, depending on whether the functional index is velocity (e.g., \( \frac{dP}{dt_{\text{max}}} \)) or force (e.g., end-systolic elastance) based (22). Third, because we did not record a simultaneous aortic pressure, it is possible that \( \frac{dP}{dt_{\text{max}}} \) occurred after aortic valve opening. However, in a random sample (n = 9) at HR\(_{\text{crit}}\) the LV pressure at the time of \( \frac{dP}{dt_{\text{max}}} \) did not change (SD 10.4 mmHg) and was at least \( 25 \pm 2.4 \text{ mmHg} \) less than the peak LV pressure.

Despite these limitations, we conclude that, in anesthetized closed-chest mice, HR\(_{\text{crit}}\) is inversely related to the relative phospholamban-to-SR Ca\(^{2+}\)-ATPase ratio, with the HR\(_{\text{crit}}\) being the highest for the phospholamban knockout mice (due to disinhibition of the SR Ca\(^{2+}\) pumps) and the lowest in phospholamban overexpressing mice (due to twofold overexpression of phospholamban and the resultant inhibition of unregulated SR Ca\(^{2+}\) pumps). Thus these data indicate that the level of phospholamban is a critical determinant of the force-frequency relation. However, mechanical factors, such as asynchronous atrial contraction, may contribute the appearance of the force-frequency relation. Finally, strain differences suggest that additional, unknown determinants, modulate the force-frequency relation and urge caution when comparing mice that are not isogenic.

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REFERENCES


H2250 FORCE-FREQUENCY RELATIONS IN TRANSGENIC MICE


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