Phospholamban: a major determinant of the cardiac force-frequency relationship

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Bluhm, Wolfgang F., Evangelia G. Kranias, Wolfgang H. Dillmann, and Markus Meyer. Phospholamban: a major determinant of the cardiac force-frequency relationship. Am. J. Physiol. Heart Circ. Physiol. 278: H249–H255, 2000.—The cardiac force-frequency relationship has been known for over a century, yet its mechanisms have eluded thorough understanding. We investigated the hypothesis that phospholamban, a potent regulator of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), determines the cardiac force-frequency relationship. Isolated left ventricular papillary muscles from wild-type (WT) and phospholamban knockout (KO) mice were stimulated at 2 to 6 Hz. The force-frequency relationship was positive in WT but negative in KO muscles, i.e., it was inverted by ablation of phospholamban (P < 0.01, n = 6 mice). From 2 to 6 Hz, relaxation accelerated considerably (by 10 ms) in WT muscles but only minimally (by 2 ms) in KO muscles (WT vs. KO: P < 0.0001, n = 6). To show that the lack of frequency potentiation in KO muscles was not explained by the almost maximal basal contractility, twitch duration was prolonged in six KO muscles with the SERCA inhibitor cyclopiazonic acid to WT values. Relaxation still failed to accelerate with increased frequency. In conclusion, our results clearly identify phospholamban as a major determinant of the cardiac force-frequency relationship.

The myocardial contractile response to changes in frequency is reflected in two separate, but related, observations. First, the magnitude of developed force or pressure may either increase or decrease with frequency, depending most notably on the species (small rodents vs. larger mammals) and origin of cardiac tissue (atrial vs. ventricular) (14) but also on other factors such as the range of frequencies, temperature, calcium concentration, muscle dimensions, etc. In contrast, the time course of contraction and relaxation is unanimously accelerated with increased stimulation frequency across all preparations and species. This frequency-dependent twitch acceleration is therefore a well-preserved and previously unexplained basic phenomenon of cardiac muscle physiology.

Earlier studies on the mechanisms of the force-frequency relationship have identified some contributing factors, such as changes in intracellular sodium and calcium and adrenergic control (7, 8, 14, 17–19, 29). Our own work has focused on the roles of the calcium pump of the sarco(endo)plasmic reticulum (SR Ca\(^{2+}\)-ATPase, SERCA) and its inhibitory protein, phospholamban, together perhaps the most potent regulators of cardiac contractility.

SERCA is clearly recognized as a major determinant of myocardial contractility (21, 26). Because SERCA activity determines the rate of Ca\(^{2+}\) sequestration from the cytoplasm into the SR, it directly affects the speed of myocardial relaxation. SERCA activity may also indirectly influence the speed of contraction and the developed force or pressure by altering SR Ca\(^{2+}\) content.

In cardiac muscle, SERCA activity is potently regulated by the inhibitory protein phospholamban (16, 32). In its unphosphorylated state, phospholamban strongly attenuates SERCA activity. However, this inhibition is relieved through protein kinase A (PKA)- or calcium/calmodulin-dependent protein kinase-mediated phosphorylation of phospholamban or through increases in Ca\(^{2+}\) concentration (12, 31, 32). Several studies with phospholamban knockout mice have firmly established the great importance of phospholamban in regulating basal contractility and in mediating the adrenergic response (13, 20, 22–24, 30, 33). However, a role of phospholamban in the cardiac force-frequency relationship had not been clearly established.

In a recent study we were able to implicate the ratio of phospholamban to SERCA as a contributing factor in determining the frequency response of cardiac muscle (27). In the current study, using papillary muscles from phospholamban knockout mice allowed us to directly determine the role of phospholamban in the cardiac force-frequency relationship. In addition, we examined the mechanisms of postrest potentiation (1–3), i.e., the increased force development following a rest period without stimulation.

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Whereas previous work has clearly established the role of increased calcium loading of the SR in the force-frequency relationship (19), the mechanism was thought to originate from sarcolemmal processes, such as a primary increase in sodium influx due to more frequent stimulation followed by a secondary increase in calcium influx (sodium pump lag hypothesis). Our work now examines and confirms the hypothesis that major control of the force-frequency relationship occurs directly at the level of the SR through phospholamban.

We used isolated left ventricular papillary muscles from wild-type and phospholamban knockout mice as a preparation ideally suited to studying these mechanisms. The results establish that phospholamban strongly regulates the frequency response of cardiac muscle and almost solely determines the frequency-induced twitch acceleration.

**MATERIALS AND METHODS**

Force measurements. Experiments were performed on 32 mice in accordance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

The methods for measuring force in isolated mouse papillary muscles are similar to those described previously (10). Twelve wild-type mice and twenty phospholamban knockout mice were anesthetized with ketamine (140 mg/kg ip) and xylazine (10 mg/kg ip). Hearts were removed and rinsed in oxygenated dissecting solution. Left ventricular papillary muscles were excised, inserted into $\Omega$-shaped clamps made from strips of platinum foil, and tied with 6-0 braided silk suture. The muscles were transferred to a 0.5-mL muscle chamber, where they were mounted on hooks of platinum wire.

Muscles were perfused with oxygenated Tyrode solution at 37°C and stimulated at frequencies between 2 and 6 Hz through the platinum clamps. Force was measured with an isometric force transducer (model OPT1L, Scientific Instruments, Heidelberg, Germany) and recorded on a chart recorder or a digital data acquisition system. Muscles were stretched over a period of 30–60 min to the length at which active force development was maximal ($L_{max}$). Forces were normalized by muscle cross-sectional area, which was calculated for each muscle as the ratio of muscle volume (determined by weighing) to muscle length at $L_{max}$.

Muscle length was 2.55 ± 0.15 mm for muscles from wild-type mice and 2.17 ± 0.07 mm for muscles from phospholamban knockout mice. Muscle cross-sectional area was 0.43 ± 0.02 mm² for muscles from wild-type mice and 0.39 ± 0.02 mm² for muscles from phospholamban knockout mice. There was no statistically significant difference of cross-sectional areas between groups ($P = 0.21$).

Time to peak tension was determined as the time from 10% of maximum developed tension to the peak of contraction. The relaxation time $RT_{50}$ was determined as the time from the peak of contraction to 50% of tension decline during relaxation.

$df/dt_{max}$ and $df/dt_{min}$ are the maximum rate of force development during contraction and the maximum rate of force decay during relaxation, respectively. Postrest potentiation was studied by stopping stimulation for intervals ranging up to 15 s and then resuming regular stimulation.

In six muscles from phospholamban knockout mice, the regular protocol was followed by incremental additions of cyclopiazonic acid (CPA) to the Tyrode solution until the relaxation time $RT_{50}$ was similar to that of wild-type mice. Before repeating the force-frequency protocol, we verified that the CPA effects were sufficiently equilibrated to guarantee stable twitch parameters during the remaining protocol duration of ~5–10 min.

SR Ca²⁺ content. Assessment of SR Ca²⁺ content with rapid cooling contractures was adapted from a previously described method (4) as follows. Regular stimulation was stopped, and after a rest interval of either 1 or 15 s, the perfusate was switched to a Na⁺- and Ca²⁺-free Tyrode solution at 0°C to minimize Na⁺/Ca²⁺ exchange. This solution was perfused at ~2.5 mL/s (~5 chamber volumes/s) to ensure rapid solution changes and cooling of the muscle. The maximum magnitude of the following contracture was measured and taken as an index of SR Ca²⁺ content. Muscles were rewarmed by perfusion with normal Tyrode solution at 37°C, and normal stimulation was resumed.

**RESULTS**

The primary objective of this study was to investigate the role of phospholamban in the frequency response of cardiac muscle. Muscles from wild-type mice and phospholamban knockout mice were stimulated at frequencies between 2 and 6 Hz. The protocol is illustrated by the original tracings from individual muscles shown in Fig. 1. Several key results, which are representative of the average data from all muscles, are readily apparent.

First, the muscle from the phospholamban knockout mouse displays a higher contractility as evidenced by larger developed forces and shorter twitch duration. However, the developed force clearly declines with increasing stimulation frequency, whereas there is very little change in twitch duration. In comparison, the force-frequency response of the wild-type muscle is only slightly negative, and there is a very noticeable acceleration of the twitch with increased stimulation frequency.

Developed forces at 2 and 6 Hz were determined in 12 wild-type mice and 20 phospholamban knockout mice (Fig. 2). Force declined from 4.24 ± 0.96 to 2.96 ± 0.76 mN/mm² in knockout muscles and from 3.29 ± 0.55 to...
2.62 ± 0.35 mN/mm² in wild-type muscles. The larger force development in the knockout muscles was therefore more apparent at the lower frequency than at the higher frequency. The ratio of forces between 2 and 6 Hz was significantly higher in phospholamban knockout muscles than in wild-type muscles (P < 0.05), i.e., the force-frequency relationship was significantly more negative in muscles from phospholamban knockout mice.

In addition to changes in developed force, the frequency response of cardiac muscle is further characterized by changes in the twitch duration. We measured the time to peak tension and relaxation time (RT₅₀) as a function of stimulation frequency (note: error bars for KO mice are too small to show). Right: percent acceleration of time to peak tension and RT₅₀ from 2 to 6 Hz. Twitch acceleration is greatly diminished by ablation of phospholamban.

This difference was equally striking for RT₅₀ with an acceleration (from 2 to 6 Hz) of 9.7 ± 0.7 ms or 22.1 ± 1.9% in wild-type muscles and merely 1.8 ± 0.3 ms or 6.8 ± 1.0%, in knockout muscles (P < 0.0001). In other words, the effects of phospholamban ablation on the time course of contraction and relaxation were greatest at lower frequencies and gradually diminished at higher frequencies. The small remaining acceleration of time to peak tension and RT₅₀ in the phospholamban knockout mice was still significant (P < 0.01, knockout muscles 2 Hz vs. 6 Hz).

The frequency response of cardiac contractility is often assessed by dF/dₜ_max and dF/dₜ_min. These parameters are of a composite nature, reflecting both the absolute magnitude as well as the time course of force development. In six wild-type and six phospholamban knockout muscles, we determined dF/dₜ_max and dF/dₜ_min for stimulus frequencies between 2 and 6 Hz (Fig. 4). In muscles from wild-type mice, both dF/dₜ_max and dF/dₜ_min increased with greater stimulus frequencies (positive force-frequency relationship), except for a small decrease at the highest frequencies. In contrast, in muscles from phospholamban knockout mice, both dF/dₜ_max and dF/dₜ_min decreased with each successive increase in stimulus frequency. Wild-type muscles and phospholamban knockout muscles showed a significantly different frequency response of both dF/dₜ_max (P < 0.001) and dF/dₜ_min (P < 0.01). Therefore, ablation of phospholamban inverted the force-frequency relationship when...
dF/dt_max and dF/dt_min were used as indexes of contractile function.

The lack of twitch acceleration and the inverted force-frequency relationship demonstrate that muscles lacking phospholamban are also lacking the normal frequency potentiation observed in wild-type mice. A related phenomenon of cardiac muscle physiology is observed in postrest potentiation (Fig. 5). After a rest period of 1–15 s in wild-type muscles, the first postrest contraction was larger than the previous steady-state contraction. The magnitude of this potentiation slowly and progressively increased with the duration of the rest period. As shown previously (5), the time course of postrest potentiation in wild-type muscles was greatly accelerated when it followed regular stimulation at 6 Hz (results not shown here). After a 15-s rest, the relative potentiation of wild-type muscles was 3.35 ± 0.31 at 2 Hz and 4.21 ± 0.44 at 6 Hz.

In stark contrast, this three- to fourfold postrest potentiation was nearly abolished in muscles lacking phospholamban. After a 15-s rest period, the relative potentiation of knockout muscles was only 1.19 ± 0.03 at 2 Hz (P < 0.0001 compared with WT) and 1.50 ± 0.11 at 6 Hz (P = 0.0001). Whereas absolute forces at steady state tended to be larger in knockout muscles when compared with wild-type muscles, muscles from both groups appeared to reach similar forces after the longest rest periods.

To further investigate the mechanism of postrest potentiation, we used rapid cooling contractures as an established procedure (2) to assess the SR Ca\textsuperscript{2+} content following 1- and 15-s rest periods in four wild-type and four phospholamban knockout muscles. Almost immediately after the onset of cold perfusion, a large contracture developed with a magnitude that exceeded the steady-state stimulated contractions. In wild-type muscles, the cooling contracture after a 15-s rest increased to 121 ± 4% of that after a 1-s rest. In contrast, no increase was observed in phospholamban knockout mice (99 ± 2%). The cooling contractures therefore indicated an increase in SR calcium load during rest in wild-type muscles but none in phospholamban knockout muscles.

To investigate to what extent the lack of frequency potentiation in phospholamban knockout muscles was related to the near-maximal basal contractility, we incrementally added the SERCA inhibitor CPA in six knockout muscles until twitch duration was prolonged to typical wild-type values (Fig. 6A). The final concentration of CPA was between 3 and 5 µmol/l. Under these conditions of reduced contractility, time to peak tension accelerated by 5.5 ± 1.4 ms (from 2 to 6 Hz), which was significantly less than the acceleration in wild-type muscles (11.5 ± 1.0 ms, P < 0.01). After the addition of CPA to the phospholamban knockout muscles, RT\textsubscript{50} accelerated only by a mere 2.5 ± 0.7 ms, compared with 9.7 ± 0.7 ms in wild-type muscles (P < 0.0001). Addition of CPA greatly reduced steady-state force development (Fig. 6B), but a rest period of 1–15 s was now followed by a strongly potentiated beat. In other words, reducing the basal contractility with CPA restored the relative postrest potentiation in phospholamban knockout mice.
To examine the effects of adrenergic stimulation on our above findings, we challenged four wild-type muscles and four knockout muscles with 10 µM forskolin. This large dose of forskolin accelerated time to peak tension (by 13.6 ± 1.3%), RT₅₀ (by 28.0 ± 2.1%), and developed force (by 155 ± 32%, data at 2 Hz). In comparison, the forskolin effects on all three parameters (time to peak tension, RT₅₀, and force) were smaller in phospholamban knockout mice than those in wild-type mice (P < 0.05 each).

Finally, we compared the frequency-induced twitch acceleration in these muscles before and after the application of forskolin (Fig. 7). Note that the control data (before the addition of forskolin) are very similar to the data shown in Fig. 3 from another set of muscles. In wild-type muscles, forskolin reduced, but did not abolish, the twitch acceleration associated with an increase in stimulus frequency from 2 to 6 Hz. These data suggest the hypothesis that the frequency-induced twitch acceleration is dependent on phospholamban but may be mediated through mechanisms other than PKA-induced phosphorylation.

**DISCUSSION**

Enhanced myocardial contractility at higher rates of contraction, commonly termed the “force-frequency relationship,” has been known for over a century (6), and its physiological importance, including relevance to exercise in conscious subjects and to human heart failure, is now undisputed (9, 28, 29). Nonetheless, the precise mechanisms of this relationship have so far not been fully understood, although some contributing factors have been identified, such as changes in intracellular sodium and calcium and adrenergic control (7, 8, 14, 17–19, 29). Force or pressure may either increase or decrease with higher stimulation frequency, depending on the preparation, species, etc., and the existence of both a negative and a positive inotropic component has been suggested (14). On the other hand, the time course of contraction and relaxation is universally accelerated at higher frequencies in all preparations. This frequency-induced twitch acceleration constitutes therefore a fundamental, and previously unexplained, phenomenon of cardiac muscle physiology.

Our data show twitch acceleration with increasing frequencies to be nearly abolished by ablation of phospholamban. Loss of phospholamban therefore resulted in a loss of twitch acceleration as one component of the frequency potentiation of cardiac contractility. Phospholamban ablation further led to a greater decrease of force from 2 to 6 Hz. In muscles from wild-type mice, the smaller decline in force and the significant twitch acceleration led to a positive force-frequency relationship when contractile function was measured by dF/dt max and dF/dt min. In contrast, in muscles lacking phospholamban, the greater decline in force and the lack of twitch acceleration led to a negative force-frequency relationship. These data therefore demonstrate the powerful role of phospholamban in regulating the frequency response of cardiac muscle.

To illustrate this regulatory function of phospholamban, we developed a simple conceptual model (Fig. 8). The model depicts the SR, the SERCA pump, and phospholamban as a “brake.” SERCA pumps calcium into the SR, but it is inhibited by phospholamban, i.e., the pump action is slowed down by the brake. Now we speculate that an increase in frequency relieves the inhibition of SERCA by phospholamban, which leads to the enhanced contractile function observed with frequency potentiation. At the low frequency, an increased
amount of phospholamban inhibits SERCA more, because the brake is now bigger. However, when this larger inhibition is relieved, a larger frequency effect is obtained, since taking off a bigger brake has a bigger effect. In other words, more phospholamban leads to more frequency potentiation. This enhanced frequency potentiation was observed following phospholamban overexpression in isolated cardiac myocytes (27). In contrast, less phospholamban inhibits SERCA less at low frequencies, because the brake is smaller. However, less phospholamban also leads to a smaller frequency effect, because taking off a smaller brake has a smaller effect. Less phospholamban leads to diminished frequency potentiation as shown in this study.

Whereas our hypothesis would readily explain the universally observed twitch acceleration, it might also help to explain the species-dependent positive or negative force-frequency relationships. An increase in SERCA activity (through decreased inhibition by phospholamban) directly leads to faster calcium removal from the cytoplasm and hence to faster relaxation. However, the extent to which increased calcium uptake into the SR affects subsequent force development depends on the additional storage capacity of the SR. In rabbits, e.g., the SR appears to be "leaky" as evidenced by postrest decay, and accelerated calcium uptake might help to overcome this leak and hence lead to a positive force-frequency relationship. On the other hand, the postrest potentiation in the rat and mouse indicates more efficient calcium retention by the SR. Therefore, it might be more difficult to further increase SR calcium load and to overcome another, apparently negative, component of the force-frequency relationship. Finally, our hypothesis is consistent with the generally more negative force-frequency relationships of atrial preparations compared with ventricular preparations, since atrial tissue has much lower phospholamban-to-SERCA ratios of mRNA and protein (15, 25).

The exact molecular mechanisms of this phospholamban-mediated frequency potentiation are currently unknown. Our data from forskolin-challenged papillary muscles suggest that it may be mediated through mechanisms other than PKA-induced phosphorylation, because such a high dose of forskolin (which resulted in a 2.5-fold increase in force) would be expected to saturate cAMP-mediated mechanisms. This is consistent with the interpretation of a recent study (20), the reported lack of frequency-dependent change in phospholamban phosphorylation (11), and with our own observation that cAMP levels did not increase with stimulation frequency in rabbit ventricular myocytes (27).

In the mouse, as well as in the rat (3), an unstimulated rest period is followed by a larger, potentiated contraction when regular stimulation is resumed (postrest potentiation). The time course of postrest potentiation is modestly accelerated in mice overexpressing SERCA (10) and is greatly accelerated when it follows regular stimulation at higher frequency (5). We therefore explored the hypothesis that postrest potentiation might also be regulated by phospholamban. Indeed, ablation of phospholamban greatly diminished the three- to fourfold potentiation of wild-type muscles. Using rapid cooling contractures to assess SR Ca\(^{2+}\) load during rest in wild-type muscles that was completely abolished in phospholamban knockout muscles.

Clearly, the lack of frequency potentiation in the phospholamban knockout mice is not solely explained by the greatly increased basal contractility (i.e., the lack of "contractile reserve") as evidenced by the data obtained in the presence of CPA. Even after the addition of CPA to phospholamban knockout mice, both time to peak tension and the relaxation time RT\(_{50}\) accelerated significantly less than that of the wild-type control mice. Furthermore, the suggestion that ablation of phospholamban might merely overwhelm or mask other mechanisms responsible for the frequency response could not explain our earlier observation of increased frequency potentiation following overexpression of phospholamban in cardiac myocytes (27).

Therefore, this study demonstrates that the physiological role of phospholamban extends far beyond depressing basal contractility and mediating the contractile effects of \(\beta\)-adrenergic stimulation and clearly identifies phospholamban as a major component of the cardiac force-frequency response. However, our data also indicate that it is not the only component, because there was a small remaining twitch acceleration even in the phospholamban knockout mice (both before and after CPA). At this time, we cannot identify the remaining components, although possible candidates might include the Na\(^+\)/Ca\(^{2+}\) exchanger, as well as frequency-dependent changes in SR calcium release via the ryanodine channel or via calcium cycling within the SR between the uptake and release compartments.
REFERENCES


