Phospholamban-to-SERCA2 ratio controls the force-frequency relationship

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Meyer, Markus, Wolfgang F. Bluhm, Huaping He, Steven R. Post, Frank J. Giordano, Wilbur Y. W. Lew, and Wolfgang H. Dillmann. Phospholamban-to-SERCA2 ratio controls the force-frequency relationship. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H779–H785, 1999.—The force-frequency relationship (FFR) describes the frequency-dependent potentiation of cardiac contractility. The interaction of the sarcoplasmic reticulum Ca\(^{2+}\)-adenosine-triphosphatase (SERCA2) with its inhibitory protein phospholamban (PLB) might be involved in the control of the FFR. The FFR was analyzed in two systems in which the PLB/SERCA2 ratio was modulated. Adult rabbit cardiac myocytes were transduced with adenovirus encoding for SERCA2, PLB, and \(\beta\)-galactosidase (control). After 3 days, the relative PLB/SERCA2 values were significantly different between groups (SERCA2, 0.5; control, 1.0; PLB, 4.5). SERCA2 overexpression shortened relaxation by 23% relative to control, whereas PLB prolonged relaxation by 39% and reduced contractility by 47% (0.1 Hz). When the stimulation frequency was increased to 1.5 Hz, myocyte contractility was increased by 30% in control myocytes. PLB-overexpressing myocytes showed an augmented positive FFR (+78%), whereas SERCA2-transduced myocytes displayed a negative FFR (−15%). A more negative FFR was also found in papillary muscles from SERCA2 transgenic mice. These findings demonstrate that the ratio of phospholamban to SERCA2 is an important component in the control of the FFR.

sarcoplasmic reticulum; staircase; contractility; adenovirus; sarcoplasmic reticulum calcium-adenosinetriphosphatase

MORE THAN A CENTURY AGO it was recognized that cardiac contractility can be enhanced by an increase of the stimulation frequency (4). This behavior was termed the “treppe” (staircase) phenomenon or the force-frequency relationship. Although force is not measured as a primary parameter of contractility in most models (shortening in cardiac myocytes, force in papillary muscles, and pressure in isolated hearts), the terminology is still commonly used (17).

The changes in cardiac mechanics that occur during an increase in frequency can be further divided. All mammals studied show an abbreviation of cardiac contractility when stimulation frequency is increased, within the physiological frequency range (3, 5). This has been confirmed in vivo and in vitro (isolated cardiac myocytes, papillary muscles, and isolated hearts) and is also found in myocardium from diseased human hearts (22). This aspect of the force-frequency relation is determined by time parameters, such as the time from maximum contraction to half-maximal relaxation (RT\(_{50}\)).

When the amplitude of the contraction (expressed as shortening, force, or pressure) is used to describe the force-frequency relation, the results seem to vary substantially. Small rodents like mice display a decrease in contractility if the stimulation frequency is increased (negative force-frequency relationship), whereas an increase in contractility or positive force-frequency relationship has been described in other mammals (3, 5). A more negative force-frequency relation occurs also in the diseased heart in both animals and humans (11, 22). Some investigators prefer the usage of velocity-based parameters such as dP/dt to express the force-frequency relationship. Because these parameters depend on time parameters as well as the amplitude, the force-frequency relation appears more positive.

When methods for assessing intracellular calcium transients became available, it was shown that the frequency-dependent changes in force were paralleled by changes in cytosolic calcium concentrations (14). Several interacting calcium-handling systems have been described that contribute to the calcium homeostasis in cardiac myocytes (1). The voltage-dependent calcium channel, the sodium/calcium exchanger, and the sarcolemmal Ca\(^{2+}\)-ATPase mediate the calcium flux between the extracellular space and the cytoplasm, whereas intracellular calcium uptake and release is predominantly mediated by the sarcoplasmic reticulum. The sarcoplasmic reticulum proteins that are involved in calcium transport are important modulators of cardiac contractility. The activity of the cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2), which transports calcium into the sarcoplasmic reticulum, is a predominant factor mediating sarcoplasmic reticulum effects on contractility. The activity of SERCA2 is regulated by its inhibitory protein phospholamban (28). The inhibition can be relieved by four mechanisms: 1) increase of the calcium concentration, 2) cAMP or 3) calcium/calmodulin-dependent phosphorylation, and 4) reduced expression of phospholamban (19, 27, 28).

Further information on the interaction of phospholamban and SERCA2 was gained from genetically modified animal models. In transgenic mice overexpressing phospholamban, cardiac relaxation and contractility are depressed compared with control animals (12). In contradistinction, SERCA2-overexpressing or phospholamban-ablated mice exhibit accelerated relaxation

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and augmented contractility (9, 19). Because altered expression of SERCA2 and phospholamban is shown to have such profound effects on cardiac performance, we hypothesized that the expression levels of these proteins might also be an important component of the force-frequency relationship.

To test this hypothesis, two approaches were pursued. Isolated rabbit cardiac myocytes were transduced with recombinant adenoviral vectors coding for SERCA2 or phospholamban. After the confirmation of transgene expression and confirmation that the phospholamban-to-SERCA ratio could be readily modulated with this approach, infected myocytes were subjected to a contractile study that included an increase in stimulation frequency. In a second approach, the force-frequency relationship was investigated in papillary muscles from transgenic mice overexpressing SERCA2.

Because cAMP is an important modulator of sarcoplasmic reticulum function and adrenergic stimulation is known to have strong effects on the force-frequency relationship (25), we postulated that cAMP might contribute to the frequency-dependent changes of contractility. To investigate this, isolated rabbit cardiac myocytes were subjected to different stimulation frequencies, and the cAMP formation was determined.

**METHODS**

Rabbit cardiac myocytes and adenoviral infection. New Zealand White rabbits (1.8–2.8 kg) were anesthetized with pentobarbital sodium (50 mg/kg iv). The hearts were rapidly excised and mounted on a Langendorff perfusion apparatus. This was followed by perfusion with Tyrode solution containing 0.75 mg/ml collagenase B (lot 14325222, Boehringer Mannheim, Indianapolis, IN) and 0.16 mg/ml protease (lot 84H0613, Sigma Chemical, St. Louis, MO). After enzymatic digestion, the left ventricles were dissected and dispersed. The resulting myocyte suspension was passed through a nylon mesh and rinsed with solutions containing increasing concentrations of calcium (final, 2 mM). Cells were infected with recombinant adenovirus (500 pfu/cell) for 1 h in modified medium 199 and kept for 72 h in an incubator without being attached to the surface of the dish. The transgenes inserted into the virus are as follows: rat SERCA2a (AdvSerca), mouse phospholamban (AdvPlb), and a bacterial β-galactosidase with a nuclear translocation signal (AdnβGal), which was used as a control. The phospholamban and β-galactosidase constructs are driven by a human cytomegalovirus enhancer-promoter construct. The phospholamban cDNA was generated by RT-PCR from mouse cardiac mRNA and confirmed by sequencing (data not shown). The SERCA2 construct is under control of a thymidine kinase promoter with tandem simian virus 40 enhancers (7). This approach was recently shown to be a valuable tool to assess functional changes due to adenovirus-mediated transgene expression in adult cardiac myocytes (21). Three days after adenoviral infection, aliquots of the cells were transferred into the measuring chamber for shortening measurements. The infection efficiency was confirmed in AdnβGal-infected myocytes with a β-galactosidase-driven color reaction. Of all rod-shaped myocytes, 97% were found to express the transgene (Fig. 1). Myocytes from three rabbits were used in this study.

Edge detection. The cardiac myocytes were placed in a 2-ml superfusion chamber (Bioptech, Butler, PA), visualized with an inverted microscope (Nikon, Diaphot, Tokyo, Japan) and continuously superfused with Tyrode solution (32°C, 2.5 mM Ca2+). The stimulation frequency was stepwise increased from 0.1 to 1.5 Hz. The changes in myocyte length were recorded by a solid-state camera (GP-CD60, Panasonic, Secaucus, NJ) and a video edge detection system (Crescent Electronics, Sandy, UT) connected to a 486 computer with a DI-220 Codas data acquisition system and Windaq software (Dataq Instruments, Akron, OH). The RT50 was determined and myocyte shortening was calculated as the percent change in myocyte length from rest to minimal myocyte length. Data from three consecutive beats at each frequency were averaged. A time course of the contractile function in the rabbit cardiac myocytes revealed a 37% reduction in shortening and a 34% increase of RT50 (both P < 0.05) after 72 h of incubation. A comparison of AdnβGal-infected myocytes with uninfected myocytes showed no difference in contractile performance.

Western blot analysis. The cells were harvested in solution containing 1% Triton X-100, 150 mM NaCl, and 50 mM Tris-Cl, pH 8.0. Samples were chilled on ice and centrifuged at 12,000 g. The supernatant was recovered, and the protein concentration was determined with a Bradford reagent (BioRad, Richmond, CA). Equal amounts of proteins were incubated in an SDS-containing loading dye for 15 min at 37°C and subsequently resolved on a 4–20% Tris-glycine polyacrylamide gel (Novex, San Diego, CA). After the gel was electrotransferred to a nitrocellulose membrane, the blot was cut at a 70,000-Da marker lane. The top part of the blot was exposed to a polyonal antibody directed against SERCA2 (8), whereas the bottom part of the blot was exposed to monoclonal α-actin antibody and monoclonal phospholamban antibody (Affinity Bioreagents, Golden, CO). This was followed by incubation with horseradish peroxidase-labeled secondary antibodies (Amersham, Arlington Heights, IL). A chemiluminescence reaction was initiated (ECL, Amersham) and then exposed to X-ray film. To quantify protein, the films were scanned with a HP Scanjet et (600 DPI) and analyzed with the NIH Image 1.62 software (National Institutes of Health, Bethesda, MD). Only the high-molecular-weight form of phospholamban was quantified. The linearity of the signal was confirmed by loading different amounts of the samples on each gel. Each sample (n = 3) was determined in duplicate. Incubation of the
myocytes had no significant influence on protein levels of phospholamban, SERCA2a, and actin.

Isolated papillary muscle experiments. Left ventricular papillary muscles were isolated from the hearts of 14 wild-type mice and 14 SERCA2 transgenic mice as described previously (9). Muscles were tied into Ω-shaped clamps made from strips of platinum foil and mounted on hooks of platinum wire in a 0.5-ml muscle chamber. Muscles were superfused with 2.5 mM Ca\(^{2+}\) Tyrode solution at 37°C and stimulated through the platinum clamps. Muscles were stretched over a period of 30–60 min to the length at which active force development was maximal. Forces (in mN) were normalized by the muscle cross-sectional areas. Force-frequency behavior was studied by increasing stimulation frequency from 2 to 6 Hz in 1-Hz increments. The RT\(_{50}\) value was determined as the time from the peak of contraction to 50% of tension during relaxation.

Measurement of cAMP levels in rabbit myocytes. Aliquots of adult rabbit cardiac myocytes (5 × 10^6) in Tyrode solution (2.5 mM Ca\(^{2+}\)) were placed into four stimulation chambers at 37°C. The first group of myocytes was field stimulated at 0.1 Hz for 3 min. The second group was stimulated at increasing frequencies (0.2 Hz for 20 s, 0.5 Hz for 20 s, 1.0 Hz for 20 s, and 1.5 Hz for 30 s). The third chamber remained unstimulated (negative control), and 10 µM forskolin (Sigma Chemical) was added as a positive control to the fourth chamber for 3 min. Incubations were terminated by the addition of 10 vol% TCA. The samples were frozen at −20°C. The amount of cAMP in TCA lysates was quantified by a radioimmunoassay (Calbiochem, La Jolla, CA) as recently described (24). The cAMP was normalized to the unstimulated cardiac myocytes. Myocytes from 10 rabbits were used for this study.

Statistical analysis. Data are expressed as means ± SE. Statistical comparisons were made by repeated-measure ANOVA (force-frequency behavior in isolated cells and muscle strips) and ANOVA followed by a Student-Newman-Keuls post hoc analysis (protein expression in myocytes, frequency ratios in isolated cardiac myocytes, cAMP measurements). Twitch parameters and ratios in isolated papillary muscles were analyzed by a Student's t-test. For the statistical analysis, the StatView software was used.

RESULTS

Characteristics of adenovirus-infected rabbit cardiac myocytes. Immunoblot analysis revealed that adenoviral infection of the cardiac myocytes resulted in a significant overexpression of SERCA2 and phospholamban (Fig. 2). AdvPlb infection increased phospholamban protein expression by 3.3-fold compared with Adn\(\beta\)Gal control myocytes (P < 0.01). In myocytes infected with AdvSerca, SERCA2 protein was increased by 2.4-fold (P < 0.01). AdvSerca overexpression resulted in a 17% reduction in phospholamban protein levels (P < 0.05), whereas phospholamban overexpression had no effect on SERCA2 protein levels. The phospholamban-to-SERCA2 ratio, normalized to 1 in the control, was increased by phospholamban overexpression to 4.5 ± 1.3 (P < 0.01) and decreased by SERCA2 overexpression to 0.5 ± 0.1 (P < 0.01).

RT\(_{50}\) was analyzed to determine the effects of SERCA2 and phospholamban overexpression on the relaxation (Fig. 3). At baseline conditions (0.1 Hz), SERCA2 overexpression resulted in a significant abbreviation of RT\(_{50}\) when compared with control myocytes (1.33 ± 0.09 vs. 1.09 ± 0.04, P < 0.05). This indicated that phospholamban overexpression increases the frequency-dependent abbreviation of relaxation.

The effects of transgene expression on shortening as an indicator of the amplitude of the contraction (contrac
tility) showed similar results (Fig. 3). At baseline conditions, phospholamban overexpression resulted in a pronounced decrease of myocyte shortening compared with control myocytes (2.8 ± 0.4 vs. 5.2 ± 0.5%, P < 0.01), whereas the increase in shortening in SERCA2-transduced cardiac myocytes did not reach statistical significance. The ratio of shortening at 1.5 to 0.1 Hz, which indicates the frequency-dependent change in contractility, showed a frequency-dependent potentiation of the contractility in control myocytes (1.30 ± 0.07). In phospholamban-overexpressing myocytes, the positive force-frequency relationship was significantly
more pronounced (1.78 ± 0.15, P < 0.01). In contrast, SERCA2 expression resulted in a frequency-dependent decrease in shortening (0.85 ± 0.05, P < 0.05).

Therefore, the frequency-dependent potentiation of cardiac contraction and relaxation was further enhanced by phospholamban overexpression and diminished by SERCA2 overexpression. However, the increase in frequency-dependent potentiation of RT50 and shortening due to phospholamban overexpression occurred at a lower level of contractility.

Characteristics of papillary muscles from mice overexpressing SERCA2. Immunoblots from cardiac homogenates from transgenic SERCA mice revealed a significant overexpression of cardiac SERCA2. The ratio of phospholamban to SERCA2 was significantly decreased compared with transgene negative control animals (0.83 ± 0.10 vs. 1.00 ± 0.09, P < 0.05).

Isolated papillary muscles from both groups were stimulated at increasing frequencies under isometric conditions (Fig. 4). Similar to the study in isolated cardiac myocytes, the RT50 was used as an index of relaxation. At the baseline frequency (2 Hz), SERCA2-expressing mice displayed a significantly shorter RT50 when compared with control mice (35 ± 1 vs. 40 ± 1 ms, P < 0.05). The increase of the stimulation frequency resulted in a marked abbreviation of RT50 in both groups. If the ratio of RT50 at the highest frequency (6 Hz) to that at the basal frequency (2 Hz) was calculated, it was significantly lower in papillary muscles from SERCA2 transgenic mice in control mice (0.60 ± 0.03 vs. 0.71 ± 0.04, P < 0.05). Therefore, SERCA2 overexpression resulted in a more negative force-frequency relationship with a diminished frequency-dependent abbreviation of relaxation.

cAMP levels in resting and stimulated rabbit cardiac myocytes. Pharmacological interventions that lead to increased cAMP levels have been shown to increase the frequency-dependent acceleration of relaxation in vivo. This was confirmed in force-frequency studies in vitro. We used isolated rabbit cardiac myocytes to investigate the influence of stimulation frequency on cAMP formation. Compared with unstimulated cardiac myocytes (negative control), stimulation at 0.1 Hz as well as 1.5 Hz led to a significant increase in cAMP formation (28 ± 10 and 35 ± 8%, P < 0.05). However, no significant difference between the two frequencies was detected (Fig. 5). Unstimulated forskolin-activated myocytes increased their cAMP levels 4.6-fold (positive control, P < 0.001).

DISCUSSION

Delineation of the underlying mechanisms that lead to the frequency-dependent potentiation of cardiac contractility has been a focus in cardiac research since Bowditch first described this phenomenon in 1871 (4). After the central role of calcium in mediating cardiac contractility was recognized, evidence suggested that the force-frequency relation might be controlled by the
subcellular systems that regulate intracellular calcium levels.

The sarcoplasmic reticulum was recognized as a potent modulator of cardiac contractility, and the interaction of the SERCA2 and its inhibitory protein phospholamban was shown to be an important determinant of sarcoplasmic reticulum function.

Adrenergic substances stimulate cAMP formation and render the force-frequency relationship more positive (25). This suggests that the force-frequency behavior might also be affected by this signal transduction pathway that would subsequently lead to a phosphorylation of phospholamban. We therefore investigated the influence of stimulation frequency on cAMP formation in isolated cardiac myocytes. The data demonstrate that stimulation (independent of the frequency) led to a small increase in cAMP formation. Compared with the dramatic stimulation of cAMP levels after forskolin stimulation, this effect appears to be of minor importance. Similar to earlier studies that investigated the influence of stimulation frequency on cAMP formation (6), we were not able to detect frequency-dependent differences. This finding is in accordance with a recent study that used phosphoserine-16 and phosphothreonine-17 specific phospholamban antibodies to examine phosphorylation of these residues in rat myocytes that were stimulated at two frequencies (10). No frequency-dependent change in phospholamban phosphorylation occurred. This contradicted earlier findings that suggested calcium/calmodulin-mediated threonine-17 phosphorylation might control the activity-dependent acceleration of relaxation (2). Moreover, a recent study provides evidence that phosphorylation of threonine-17 in vivo might occur only with phosphoserine-16 as a prerequisite (18).

Increases in calcium concentrations have been shown to relieve the inhibitory effect of phospholamban on SERCA2 in vitro, and increases of diastolic calcium concentrations due to increased frequencies have been confirmed in different experimental settings (10, 15, 16). Calcium-dependent deinhibition of SERCA2, which in vitro is as potent as the cAMP-dependent phosphorylation (23), cannot be directly assessed in functional cells. Changes in the relative phospholamban-to-SERCA2 ratio can provide some indirect evidence...
about this mechanism in vivo. An increase of the phospholamban-to-SERCA2 ratio was shown to decrease the calcium affinity of the SERCA2. This leads to a decreased SERCA2 activity, a prolonged relaxation time, and a reduced contractility. A decreased phospholamban-to-SERCA2 ratio due to increased levels of SERCA2 or decreased amounts of phospholamban has been shown to increase the calcium transport rates. These approaches differ in that a reduced phospholamban expression changes the affinity of the SERCA2 for calcium, whereas excess expression of SERCA2 increases the maximal pump rate. The importance of the ratio of phospholamban to SERCA2 on the control of myocardial contractility has been repeatedly demonstrated in mouse models with variable expression levels of phospholamban (13, 20).

To test the hypothesis that the phospholamban-to-SERCA2 ratio might also affect the force-frequency relationship, isolated rabbit cardiac myocytes were infected with recombinant adenoviruses encoding for SERCA2 and phospholamban. This approach increased SERCA2 and phospholamban expression levels well above the transgene levels reached in transgenic animals and enabled us to change the relative phospholamban-to-SERCA2 ratio considerably. The contractile study of the myocytes revealed that phospholamban overexpression prolonged relaxation and reduced baseline contractility. An increase in stimulation frequency that led to a small increase in contractility in control myocytes resulted in a marked increase in contractility in phospholamban-overexpressing cardiac myocytes. This accompanied a parallel decrease in the relaxation times in both groups. Although myocytes overexpressing SERCA2 also displayed a frequency-dependent abbreviation of relaxation, contractility decreased and thus inverted the force-frequency relation from being positive in the control myocytes to negative in SERCA2-overexpressing cells. However, contractility levels were higher compared with phospholamban-overexpressing myocytes. The effect of the phospholamban-to-SERCA2 ratio on the force-frequency relation could be confirmed in papillary muscles from transgenic SERCA2-overexpressing mice. Compared with control mice, SERCA2-overexpressing mice displayed a more negative force-frequency relationship.

In summary, the data show that increased phospholamban-to-SERCA2 ratios lead to a more positive force-frequency relationship, independent of the primary course of the force-frequency relationship (negative in the mouse and positive in rabbit). However, the improvement of the force-frequency relationship by increasing the phospholamban-to-SERCA2 ratio is accompanied by a reduced contractility, which is particularly apparent at low frequencies.

Although our findings are not a comprehensive explanation of all the components of the force-frequency behavior, they demonstrate the importance of phospholamban and SERCA2 in the control of this relationship.

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