Hystereses in the force-length relation and regulation of cross-bridge recruitment in tetanized rat trabeculae

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Levy, Carmit, and Amir Landesberg. Hystereses in the force-length relation and regulation of cross-bridge recruitment in tetanized rat trabeculae. Am J Physiol Heart Circ Physiol 286: H434–H441, 2004.—Various mechanisms have been suggested to explain cardiac force-length Ca\textsuperscript{2+} relations. The existence of a cooperativity mechanism, whereby cross-bridge (XB) recruitment is affected by the number of active XBs, suggests that the force response to length oscillations should lag length oscillations. Consequently, the oscillatory force response should be larger during shortening than during lengthening. To test this prediction, force responses to large-sarcomere length (SL) oscillations (36.7 ± 16.0 nm) at different SLs (n = 6) and frequencies (n = 7) were studied in intact tetanized trabeculae dissected from rat right ventricle (n = 13). Stable tetani were obtained by utilizing 30 μM cyclopiazonic acid in Krebs-Henseleit solution containing 6 mM extracellular Ca\textsuperscript{2+} at 25°C. SL was measured by laser diffraction techniques (Dalsa). Force was measured by silicone strain gauge. Instantaneous dynamic stiffness during large oscillations was measured by superimposing additional fast (50 or 200 Hz) and small-amplitude (2.25 ± 0.25 nm) oscillations. The force responses lagged the SL oscillations at slow frequencies (112 ± 41 ms at 1 Hz), and counterclockwise hystereses were obtained in the force-length plane: the force was higher during shortening than during lengthening. The delay in the force response decreased as the frequency of the SL oscillation was increased. Clockwise hysteresis, where the force preceded the SL, was obtained at frequencies >4 Hz. Similar hysteresis characteristics were obtained in the force-SL and stiffness-SL planes. Maximal lag was observed at the shortest SL, and the delay decreased with sarcomere elongation: 131.1 ± 31.7 ms at 1.78 ± 0.03 μm vs. 14.7 ± 18.5 ms at 1.99 ± 0.015 μm. The results establish the ability of cardiac fiber to adapt XB recruitment to changes in prevailing loading conditions. This study supports the stipulated existence of a cooperativity mechanism that regulates XB recruitment and highlights an additional method to characterize regulation of the force-length relation.

excitation-contraction coupling; Frank-Starling law; regulated actin; troponin

The force-length relation of cardiac muscle at partial activation is steeper than the force-length relation of skeletal muscle at full activation (1). Shortening cardiac sarcomere length (SL) by 10% causes a 50% drop in isometric force (1, 17). Lengthening the cardiac sarcomere shifts the sigmoidal force-Ca\textsuperscript{2+} concentration relation to the left, toward lower free Ca\textsuperscript{2+} concentration in intact and skinned fibers, a phenomenon designated “length-dependent Ca\textsuperscript{2+} sensitivity” (1, 14). The steep force-length relation and the length-dependent Ca\textsuperscript{2+} sensitivity cannot be explained solely by the sliding filament theory (10) and are attributed to one or more length-dependent regulatory mechanisms that affect myofilament activation (1, 19).

Isometric force is proportional to the number of force-generating (“strong”) cross bridges (X Bs). At full activation, the number of strong XBs is determined solely by the single overlap length between actin and myosin filaments (10). At partial activation, XB recruitment is modulated by “regulated actin,” i.e., actin-troponin-tropomyosin complexes (5, 22). Ca\textsuperscript{2+} binding to troponin C (TnC) shifts the regulated actin toward the activated state, i.e., the “on-actin” state (22), and allows strong XB recruitment. The number of on-actins is determined by free Ca\textsuperscript{2+} concentration, the affinity of troponin for Ca\textsuperscript{2+}, and the interactions between the XBs and the regulatory proteins (11, 15, 30).

Replacement of cardiac TnC with skeletal TnC decreased the Ca\textsuperscript{2+} sensitivity of the cardiac muscle, suggesting that cardiac TnC plays a key role in regulation of the length-dependent Ca\textsuperscript{2+} sensitivity (2). This hypothesis was contradicted by expressing the cardiac TnC in skeletal muscle. This substitution study did not alter the skeletal muscle sensitivity (24). Moreover, the cardiac length-dependent Ca\textsuperscript{2+} sensitivity was found to be independent of the isoform of TnC (23): myocytes from transgenic mice, in which the fast skeletal isoform of TnC was expressed in the heart, had length-dependent Ca\textsuperscript{2+} sensitivity of tension identical to that of myocytes with the cardiac isoform of TnC. These studies (23, 24) suggest that additional regulatory proteins are involved in modulation of the cardiac force-length relation. The role of XBs in the regulation of Ca\textsuperscript{2+} affinity in cardiac muscle was established by Hofmann and Fuchs (15), who showed that Ca\textsuperscript{2+} affinity decreased and became length independent in the presence of vanadate, which inhibits XB cycling.

Three main types of cooperativity mechanisms have been suggested to explain the regulation of the force-length-Ca\textsuperscript{2+} relations in striated muscles (11, 30) and to describe the interactions between the nearest regulated actin, XBs, and Ca\textsuperscript{2+}-binding sites: 1) interactions between the nearest neighboring Ca\textsuperscript{2+}-binding sites (Ca-Ca cooperativity), whereby Ca\textsuperscript{2+} affinity is affected by the amount of Ca\textsuperscript{2+} bound to the nearest TnC proteins (11, 30), 2) interactions between the nearest myosin-binding sites along the actin filament (XB-XB cooperativity), whereby the strongly bound XBs cooperatively shift the neighbor regulated actins toward the on-actin state (12, 30), and 3) interactions between strong XBs and their neighbor TnC proteins (XB-Ca cooperativity), whereby strong XBs increase the affinity of TnC for Ca\textsuperscript{2+} (1, 15).

The force-length-Ca\textsuperscript{2+} relations are usually measured at steady isometric conditions or at peak isometric force (1, 17).
However, analysis of the force response to SL oscillation allows more precise characterization of the kinetics involved in regulation of XB recruitment (4). The existence of any cooperativity mechanism suggests that changes in the generated force should lag SL perturbations because of the limited rate kinetics of Ca$^{2+}$ binding to troponin, regulated actin turnover to the activated state, and XB cycling. When the force response lags the SL oscillations at constant Ca$^{2+}$ concentrations, a counterclockwise (CCW) hysteresis is anticipated at the force-length plane. It is also reasonable to expect that if the rate kinetics of Ca$^{2+}$ binding or XB cycling are length dependent, then the delay between the force and the SL oscillations and the consequent shape of the hysteresis in the force-length plane depend on SL and the frequency of oscillation.

This study aims to test these hypotheses and to analyze the force and the stiffness responses to large SL oscillations (36.7 ± 16.0 nm) at different oscillation frequencies and at different SLs in tetanized intact trabecula from rat right ventricle. We know of no data showing the effects of large oscillations at a constant activation level, in intact cardiac trabeculae and on the effect of different SLs on the force response to large SL oscillations.

As shown here, the force lagged the SL oscillations at low oscillation frequencies (≤2 Hz), and CCW hystereses were obtained in the force-length plane. The phase delay between the force and the SL oscillations was found to depend on SL and was larger at shorter SLs. The observed CCW hysteresis in response to large SL oscillations, which depend on SL and frequency, improves our insight into the regulation of XB recruitment and the mechanisms underlying the cardiac force-length relation.

**METHODS**

*Experimental setup.* Thin trabeculae from rat right ventricle (Sprague-Dawley, 250–300 g body wt) were isolated by the method described by Backs and ter Keurs (3). The rats were anesthetized with diethyl ether. The hearts were quickly removed and transferred to a dissection dish. The hearts were perfused through the aorta with a solution to 6 mM. CPA blocks Ca$^{2+}$ uptake by the sarcolemmal reticulum Ca$^{2+}$-ATPase. To prevent premature exchange of the solution, the perfusion was reduced to 0.2 Hz, and tetani were elicited every 10 regular twitches (i.e., every 50 s) by utilizing 8-Hz stimulation (40-ms pulse width). The tetanus duration was 3.5 s. Steady fused tetanic contractions (Fig. 1A) were achieved by raising Ca$^{2+}$ concentration in the solution to 6 mM.

*Oscillations at various frequencies.* Large sinusoidal length oscillations of 35.5 ± 14.8 (SD) nm (n = 7) were imposed at four different frequencies: 1, 2, 4, and 8 Hz (Figs. 2A, 3A, and 4A). The lower frequency of oscillation was limited by the duration of the steady tetanic contraction. Steady force and SL were reached within 1 s (Fig. 1A). To ensure the existence of steady force and SL before the beginning of the length oscillations, the large length perturbations started 1.5 s after the beginning of the tetanic stimulation (Figs. 2–4), leaving only 2 s until the end of the tetanus. To obtain at least two cycles, the slowest possible oscillation frequency was 1 Hz. The maximal frequency of oscillation was limited by the shortening velocity and the effect of the shortening velocity on the generated force. The shortening velocity increases with the increase in the oscillation frequency. The maximal shortening velocity at oscillation amplitude of 40 nm and oscillation frequency of 8 Hz is already 15% of the sarcomere unloading velocity (13.6 μm/s at 25°C) (7).

*Dynamic stiffness.* Variations in the number of strong XBs during the oscillations were evaluated by measuring the muscle dynamic stiffness (8, 16, 29). The dynamic stiffness was measured by superimposing additional high-frequency (50 or 200 Hz) and small-amplitude oscillations (2.25 ± 0.25 nm) on the large oscillation (Figs. 2B, 3A, and 4A). Measurements of force and dynamic stiffness during the large oscillations allowed us to determine whether oscillations in the force response resulted from variations in the number of XBs or from the effect of shortening velocity on the average force per XB (8). The dynamic stiffness depends on the oscillation frequency and on the number of strong XBs (8, 16). At constant oscillation frequency (50 or 200 Hz), changes in the dynamic stiffness are proportional to variations in the number of strong XBs (4, 8). Therefore, only one frequency (50 or 200 Hz) of the small oscillation was utilized during the large-amplitude oscillations in each of the trabeculae to assess the relative instantaneous changes in the dynamic stiffness and in the number of XBs (Fig. 2, B–D).

*Different SLs.* Various SLs were utilized in the second group of trabeculae (n = 6) to determine whether the delay in the force response to SL oscillation is length dependent. The stable SL during the tetanus contraction was maintained by stretching the trabeculae during the first second of the tetanic contraction (Fig. 1B). Stable SL...
was reached ~0.5 s before the large SL oscillations were imposed. The amplitude of the SL oscillations was 37.6 ± 17.0 nm in this group, and the oscillation frequency was 1 Hz. Four to five different SLs were studied in each trabecula (n = 6).

Data analysis. The muscle length, force, and SL were sampled at 5,000 Hz. Each oscillation was repeated 5–10 times, and the results were averaged to reduce the random noise. The force response included oscillations at two frequencies when the instantaneous dy-

Fig. 1. A: stable titanic contractions for 3.5 s were achieved by utilizing 30 μM cyclopiazonic acid and 8-Hz stimulations at 6 mM extracellular Ca2+. B: desired sarcomere length (SL) during tetanus was obtained by stretching the fiber during the initial 0.5 s of tetanic contraction. Stresses were normalized by mean tetanic stress obtained at SL of 2.0 μm. ML, muscle length; Nor Stress, normalized stress.

Fig. 2. Effects of 1-Hz length oscillations on stress and dynamic stiffness. A: ML oscillations (1 Hz) were imposed during tetanic contraction and produced oscillations in SL and stress. B: additional high-frequency (50 Hz) and low-amplitude (2.25 ± 0.25 nm) oscillations were superimposed 1 s after onset of contraction to measure instantaneous dynamic stiffness. C: time course of normalized SL (thick solid line), stress (thin solid line), and dynamic stiffness (dashed line) during large 1-Hz oscillations. Stress and stiffness lagged SL oscillations by 101 and 122 ms, respectively. D: counterclockwise hystereses in phase plots of stiffness and stress vs. SL at 1-Hz oscillations. Stresses were normalized by mean steady tetanic stress.
namic stiffness was measured (Figs. 2B and 3A). The high-frequency component was riding over the large and slow oscillation in the force. The amplitude of the high-frequency (50 or 200 Hz) force oscillations, used for quantifying the instantaneous dynamic stiffness, was modulated by the slow oscillations in the number of XBs (Fig. 2, B and C).

The force response to the large oscillations was detected by filtering out the high-frequency oscillations. The high-frequency oscillations were filtered by using a moving average window, where the width of the window was equal to the periodicity of the high-frequency oscillations (20 or 5 ms). Next, the high-frequency component was derived from the difference between the original force response and the filtered force response, which included only the slow oscillation component. The instantaneous dynamic stiffness was calculated from the high-frequency oscillations as the ratio of the amplitude of the force to the amplitude of the SL oscillation. The delays between the force or the stiffness and the SL large oscillations were best presented in phase plots of stiffness and stress vs. SL at 2-Hz oscillations. Stresses were normalized by mean steady tetanic stress.

RESULTS

The force responses to length oscillations at various frequencies were studied in seven trabeculae, and six more trabeculae were used to study the effect of SL on the force responses to length oscillations. The trabeculae were 144.4 ± 58.2 μm wide and 106.2 ± 49 μm thick at slack length. The peak isometric twitch stress was 40.2 ± 20.55 mN/mm² (1.5 mM extracellular Ca²⁺), while the steady stress during tetanus, using 30 μM CPA and 6 mM Ca²⁺, was 40.64 ± 25.12 mN/mm² for the same initial SL of 2 μm.

Oscillations at various frequencies. Figure 2A presents five superimposed responses to 1-Hz length oscillations acquired with one trabecula. The stress responses were reproducible and sensitive to the SL oscillation. The initial SLs were 2.03 ± 0.03 μm, but at steady tetanus the SLs were 1.79 ± 0.07 μm.

Figure 2B shows the effect of superimposing high-frequency (50 Hz) and small-amplitude (2.25 nm) oscillations on the large oscillations for quantification of the instantaneous dynamic stiffness. Ten superimposed recordings are displayed in Fig. 2B. The same stress responses to the large oscillations were observed with and without the high-frequency oscillations (Fig. 2, A and B). Addition of the high-frequency oscillations did not alter the stress response to the large oscillations. Hence, the oscillations in the instantaneous dynamic stiffness (Fig. 2, C and D) represent the variations in the number of strong XBs during the large oscillations.

The phase delays between SL, stress, and stiffness are shown in Fig. 2C after the averaged signals were normalized by their amplitudes. The stress response lagged the SL oscillations by 101 ms. The oscillations in stress and stiffness were closely aligned (Fig. 2C), but stiffness lagged stress by an additional 21.8 ms and slugged SL by 122.8 ms. The stress responses lagged the SL oscillations by 111.9 ± 41 ms [40.3 ± 14.7 (SD) degrees] for the six trabeculae studied at 1-Hz oscillations, and the stiffness lagged the stress by 21.8 ± 3.4 ms (n = 3).

The phase delays between stress or stiffness and SL are better presented in Fig. 2D using phase plots of stiffness and

Fig. 3. Effects of 2-Hz length oscillations during tetanus on stress and dynamic stiffness. A: small (2-nm) 50-Hz length oscillations were superimposed on large (40-nm) 2-Hz oscillations for measurement of instantaneous dynamic stiffness. B: time course of normalized SL (thick solid line), stress (thin solid line), and dynamic stiffness (dashed line) during large length oscillations. Stress and stiffness lagged SL oscillations by 29 and 18 ms, respectively. C: counterclockwise hystereses in phase plots of stiffness and stress vs. SL at 2-Hz oscillations.
stress against SL. Hystereses were obtained between stress or stiffness and SL. At a given SL, the generated stress was larger during sarcomere shortening than during lengthening. These hystereses were in the CCW direction for stiffness and stress versus SL. The hystereses in stiffness and stress are similar in shape and direction. Although only two cycles of 1-Hz oscillation could be performed during the tetanus, no significant difference in stress was observed between the two cycles (Fig. 2D).

Figure 3A presents stress and SL responses to 2-Hz oscillations. A smaller time delay was observed at 2-Hz than at 1-Hz oscillations. The normalized stress and the stiffness lagged the SL oscillations (Fig. 3B) by 29.4 and 18.2 ms, respectively. The hystereses were in the CCW direction for stiffness and stress (Fig. 3C). For all trabeculae at 2-Hz oscillations, the stress responses lagged the SL oscillation by 34.6 ± 28.1 ms (n = 5).

The opposite phenomenon was observed at 4- and 8-Hz oscillations. Stable large oscillations in stress were observed with only a brief transient response of less than one cycle. The stress responses preceded the SL oscillations (Fig. 4B), and the hystereses between stress or stiffness and SL were in the clockwise (CW) direction (Fig. 4C). At a given SL, stress was larger during sarcomere lengthening and smaller during sarcomere shortening. For all trabeculae, stress preceded SL by 47.2 ± 19.5 ms at 4-Hz oscillations (n = 7) and by 27.85 ± 6.58 ms (n = 4) at 8-Hz oscillations.

**Oscillations at different SLs.** SL before the length oscillations were imposed had a significant effect on the phase of force response. Figure 5 presents four different SLs on which 1-Hz length oscillations of similar amplitude were imposed. CCW hystereses were obtained at SL < 1.95 μm. The phase delay decreased as SL was increased. For SL oscillations of ~1.95 μm, the delay disappeared, and at SL of 2.02 μm the...
force preceded the SL oscillations, and the opposite CW hysteresis was observed.

A similar effect of SL on the force response was observed in all trabeculae (n = 6; Fig. 6). The amplitude of the SL oscillations was 37.6 ± 17.0 nm. The force responses to 1-Hz oscillations lagged the length oscillations, and CCW hystereses were observed at short SLs. The largest delay in the force response was observed at the shortest SL (131.16 ± 31.75 ms at SL = 1.78 ± 0.03 μm). There was a monotonic decrease in the delay with an increase in SL. At SL of 1.99 ± 0.015 μm the delay in the force response to 1-Hz length oscillation was reversed, and the force preceded the SL oscillation (14.73 ± 18.5 ms), leading to the opposite CW hysteresis.

**DISCUSSION**

This study identified hysteresis in the force response to large SL oscillations at constant Ca²⁺ concentration and shows the dependence of the shape and direction of the hysteresis on the frequency of oscillation and the SL. At slow oscillation frequencies, i.e., <4 Hz (6 mM Ca²⁺ and 30 μM CPA at 25°C), the stress response lagged SL, and the hystereses between stress and SL were in the CCW direction. Thus two stress levels were obtained at the same SL: the higher stress was observed during SL shortening, and the lower stress was reached during SL lengthening. At oscillation frequency >4 Hz, the opposite phenomenon was observed: the stress responses preceded the SL changes, leading to CW hysteresis in the stress-SL plane, where the stresses were larger during lengthening than during shortening.

The hystereses were in the CCW direction at SL <1.99 ± 0.015 μm and in the CW direction at longer SL for the same oscillation frequency of 1 Hz (Fig. 6). The largest phase delay was observed at the shortest SL. The delay decreased as SL was increased (Fig. 6).

The various hystereses represent the effect of SL and the frequency of oscillation on regulation of XB recruitment. The area inside the phase plot of stress against SL is equal to the external work done. Hysteresis in the CW direction indicates that external work is done on the fiber. A CW hysteresis is observed with viscoelastic elements, where more force is required to stretch the material than the force gained during the recoil. Similarly, the observed CW hysteresis at high frequencies (>4 Hz) may relate to the viscoelastic properties of the XBs (8, 20) and to the effect of the shortening velocity on the XB duty cycle (20). The CCW direction, observed at low frequencies (<4 Hz), infers that external work is done by the fiber on the system and that more stress is generated during shortening than is needed for stretching the fiber. These CCW hystereses (Figs. 2D and 3C) are opposite in direction from the hysteresis expected on the basis of the simple force-length and force-velocity relations, where the force is expected to be smaller during shortening than during lengthening at any given SL.

The postulation that the hystereses result from regulation of XB recruitment is supported by the measured dynamic stiffness data. Small oscillations (2.25 ± 0.25 nm) at constant frequency (50 or 200 Hz) were used to quantify the variation in the number of strong XBs. At constant frequency of small SL oscillations, variations in dynamic stiffness are proportional only to changes in the number of strong XBs (4, 30). Shapes and directions of hysteresis in stiffness vs. SL were similar to the hystereses in stress vs. SL (Figs. 2D, 3C, and 4C), suggesting that the stress-SL hysteresis results from hysteresis in the number of XBs.

The term hysteresis requires some clarification. Historically, the word hysteresis is of Greek origin meaning etymologically: coming behind or lagging. A physiology textbook defines hysteresis (28) as “a system which fails to follow identical paths upon application and withdrawal of a forcing agent.” The textbook of control system analysis (6) defines hysteresis as a system with multivalued relations, so that the output value depends on the history of the input as well as on its present value. Our observations comply with these classical definitions. If a given input (SL or free Ca²⁺) yields two outputs (force) and the output is a function of the path, then we have a hysteresis. As in the various physical systems, the term hysteresis has mechanical and energetic significance. Here, at low frequencies the force lags the SL changes and the muscle generates external energy (derived from ATP hydrolysis), whereas at high frequency the force precedes SL and the muscle absorbs energy.

Small sinusoidal oscillations of <1% of the muscle length were previously used (4, 16, 27, 29, 33) to measure dynamic stiffness and to characterize the XB cycle. The present study employed larger amplitudes (4% of the SL) to characterize regulation of XB recruitment. Although the phase of the dynamic stiffness was suggested elsewhere (27, 29, 33) to be SL independent, the force-length hysteresis in the present study and the earlier force-Ca²⁺ hysteresis (13) were length dependent because of changes in the number of XBs during the hystereses.

Wannenburg et al. (33) imposed small (2.5 nm per 0.5 sarcomere) length perturbations and derived the stiffness transfer function over a range of Ca²⁺ concentrations at constant SL and at SL of 2.0–2.2 μm. They found that SL had no effect on the phase of the dynamic stiffness. The present study (Figs. 5 and 6) shows significant effects of SL on the phase of the force response. The difference between these two studies may be explained by the different range of SLs and the different amplitude of SL oscillations used in these studies. Wannenburg et al. used SL at the upper range of the force-length relation, from SL of 2.0 to 2.2 μm; in the present study the SL range was 1.72–2.02 μm at the center of the ascending limb of the force-length relation. The amplitudes of oscillations used in the present study were 15-fold larger (5 nm compared with 75.2

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Fig. 6. Delay in force response to length oscillations depend on SL (at 1-Hz oscillations): largest delays were observed at shortest SLs in all trabeculae (n = 6). Delays decreased with increase in SL.

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The phase of the hysteresis depends on SL and decreases with the increase in SL. However, this does not imply that CCW hysteresis is to be attributed to the existence of the double overlap. With short SLs the direction of the hysteresis was a function of the oscillation frequency. For the same SL and the same double overlap, CCW hysteresis was observed at low frequencies (Figs. 2 and 3) and CW hysteresis was obtained at the higher frequencies. Moreover, the passive restoring, repulsive, or compressive forces due to the double overlap generate forces in the direction opposite from the XB s. These forces are expected to resist sarcomere shortening, because the shortening increases the double-overlap length. Therefore, the double overlap is expected to decrease the net force generated during shortening and increase the force during lengthening. This is contrary to the observed CCW hysteresis. Furthermore, CCW hysteresis implies that active external work is generated by the muscle. Passive forces due to the double overlap resist sarcomere shortening and cause energy dissipation as heat. Thus passive forces are expected to produce CW hysteresis. These passive forces impose internal load that was also found to be relatively small compared with the muscle active force (8). The implication is that the effects of the passive forces due to the existence of the double overlap are relatively insignificant.

Our notion that the hysteresis in the force-length plane at constant activation results from regulation of XB recruitment is supported by the reported hysteresis in the force-Ca$^{2+}$ relation at constant SL (13, 26). Ridgway et al. (26) were the first to observe hysteresis in the force-Ca$^{2+}$ relation in intact and skinned cardiac fibers. In skinned fibers, they studied the force response to a stepwise increase, followed by a stepwise decrease in Ca$^{2+}$ concentration. They found that at a steady Ca$^{2+}$ concentration the muscle generated more force when the fibers had previously experienced a higher force level. The muscle became more sensitive to Ca$^{2+}$, and the force-length relation shifted to the left by 0.13 pCa as the Ca$^{2+}$ concentration was decreased (26). They concluded that the hysteresis in the force-Ca$^{2+}$ relation at constant muscle length was caused by the dependence of Ca$^{2+}$ affinity on the number of strong XBs. They strengthened this hypothesis by showing, as shown by others (1, 31), that fast length perturbations produced changes in force that were associated with changes in the free Ca$^{2+}$ transient; quick release was associated with an increase in the free Ca$^{2+}$ transient (1, 26, 31), whereas quick stretch was associated with a decrease in the Ca$^{2+}$ transient (31).

Harrison et al. (13) studied the effect of SL on hysteresis in the force-Ca$^{2+}$ relation in skinned rat cardiac fiber. Consistent with our study of hysteresis in the force-length relation at various initial SLs, they found that the width of the hysteresis in the force-Ca$^{2+}$ plane depended on SL. The hysteresis was maximal at short SL and virtually disappeared at SL $\geq$2.3 μm. These results resemble our present findings (Fig. 6). The CCW hysteresis in the force-SL plane also had the maximal phase delay (i.e., width) at the shortest SL, and the phase delay decreased with the increase in SL (Fig. 6).

The CCW direction of hystereses in our force-SL relations at low frequencies and the force-Ca$^{2+}$ hysteresis (13, 26) at quasi-steady-state conditions, as well as the similar dependence on SL (13) of both of these hystereses in the force-length (Fig. 6) and force-Ca$^{2+}$ planes (13), suggest that an identical mechanism underlies these phenomena. The hystereses in the force-SL relations and the dependence on SL can be explained by a direct length-dependent Ca$^{2+}$ sensitivity (1, 9) or an XB-Ca (15, 19) or an XB-XB (11, 12) cooperativity. The hysteresis in the force-Ca$^{2+}$ relation (13, 26) can be explained by Ca-Ca, XB-XB, and XB-Ca cooperativity mechanisms. Hence, hysteresis in the force-Ca$^{2+}$ and force-SL relations does not necessarily indicate the actual underlying mechanisms. Ridgway et al. (26) suggested that the increase in Ca$^{2+}$ sensitivity after the muscle had experienced higher force implies that Ca$^{2+}$ affinity depends on the number of force-generating XBs. This XB-Ca cooperativity was also suggested from the direct measurement of the amount of bound Ca$^{2+}$ in the skinned cardiac muscle (15).

Harrison et al. (13) reported that the addition of high-molecular-weight dextran increased Ca$^{2+}$ sensitivity and decreased the width of the hysteresis in the force-Ca$^{2+}$ relation. This effect is similar to that observed by increasing SL. They suggested that Ca$^{2+}$ sensitivity is mediated by interfilament spacing and not by the number of XBs (length-dependent Ca$^{2+}$ sensitivity). Fuchs and Wang (9) also showed that increasing osmolarity using 5% dextran produced changes in Ca$^{2+}$ sensitivity comparable to those from SL elongation and suggested that Ca$^{2+}$ affinity is mediated by interfilament spacing, rather than by SL, per se. However, the lattice spacing was not measured in these studies (9, 13). Simultaneous measurements of interfilament spacing by X-ray diffraction and fiber width by video microscopy, under various degrees of osmotic compression and at various SLs, revealed (18) that osmotic compression using dextran had a larger effect on the lattice spacing than did sarcomere lengthening. Moderate osmotic compression using 1% dextran, at SL of 2.02 μm, reduced lattice spacing to match uncompressed SL at 2.19 μm without altering Ca$^{2+}$ sensitivity, while sarcomere lengthening from 2.02 to 2.19 μm significantly increased Ca$^{2+}$ sensitivity (18).

The suggested direct effect of length (or interfilament spacing) (2, 13, 31) on Ca$^{2+}$ affinity cannot explain the existence of force-Ca$^{2+}$ hysteresis at constant SL (13, 26). Moreover, measurements of SL during the force-Ca$^{2+}$ hysteresis (13) revealed that SL was actually shorter (2.04 μm) during the downward stepwise decrease in Ca$^{2+}$ concentration, when Ca$^{2+}$ sensitivity was greater, and longer (2.11 μm) during the stepwise increase in Ca$^{2+}$ concentration, when Ca$^{2+}$ sensitivity was smaller. The force-Ca$^{2+}$ relation was shifted to the left, i.e., to higher sensitivity (13), at the shorter SL, in contrast to the suggested length-dependent Ca$^{2+}$ sensitivity. This observation, however, can be explained by the increase in the number of XBs (despite SL shortening) through XB-Ca cooperativity: Ca$^{2+}$ affinity is higher when the number of strong XBs is larger, although SL is shorter.

XB-XB and XB-Ca cooperativity mechanisms (11, 30) can yield a CCW hysteresis in the force-SL plane and in the force-Ca$^{2+}$ plane. However, only XB-Ca cooperativity can explain the length-dependent Ca$^{2+}$ sensitivity phenomenon, whereby the sigmoidal force-Ca$^{2+}$ relation is shifted to the left at longer SL (1, 14) and “extra” Ca$^{2+}$ release is observed at quick length changes (1, 31). Sarcomere lengthening increases the number of strong XBs and augments Ca$^{2+}$ affinity through...
the XB-Ca cooperativity mechanism. The increase in the amount of bound Ca\(^{2+}\) further increases XB recruitment and the generated force. Shortening reduces the number of strong XBs and decreases Ca\(^{2+}\) affinity through the same XB-Ca cooperativity (1, 26, 32), causing extra Ca\(^{2+}\) transient. Consequently, the XB-Ca cooperativity mechanism is the single cooperativity mechanism that provides the regulation that explains the spectrum of physiological findings in the force-length-Ca\(^{2+}\) relations.

**Summary:** This study describes hysteresis in the stress and the stiffness responses to large sinusoidal SL oscillations in intact tetanized cardiac trabeculae and relates it to regulation of XB recruitment. The shape and direction of the hysteresis depend on the frequency of oscillation and on SL.

CCW hysteresis in the force-length plane is of immense importance, because it infers that work is done by the muscle during the cyclic length changes, while the activation is constant (tetanic contractions). This phenomenon validates the existence of a feedback mechanism, whereby the loading conditions affect XB recruitment (21, 25). The changeover frequency from CCW to CW hysteresis described here defines the maximal rate at which muscle can adapt to changes in the prevailing loading conditions by increasing XB recruitment. The CCW hystereses in the force-Ca\(^{2+}\) (13, 26) and force-length planes, as well as the length-dependent Ca\(^{2+}\) sensitivity, can conceptually be attributed to various mechanisms. However, only the XB-Ca cooperativity, whereby the number of force-generating XBs feed back to the affinity of troponin for Ca\(^{2+}\), complies with all the reported data.

The experimental technique developed here can be utilized for a more precise analysis of the mechanisms underlying the regulation of XB recruitment. Further studies and analysis of the hystereses obtained at the same SL but at various Ca\(^{2+}\) concentrations or at the same stress but at different Ca\(^{2+}\) concentrations and SLs should be pursued to reveal more precisely the role of SL and stress in regulation of XB recruitment.

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**DISCLOSURES**

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