Basis of passive tension and stiffness in isolated rabbit myofibrils

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Bartoo, Marc L., Wolfgang A. Linke, and Gerald H. Pollack. Basis of passive tension and stiffness in isolated rabbit myofibrils. Am. J. Physiol. 273 (Cell Physiol. 42): C266–C276, 1997.—By examining the mechanical properties of isolated skeletal and cardiac myofibrils in calcium-free, ATP-containing solution, we attempted to separate the stiffness contribution of titin filaments from that of weakly bound cross bridges. Efforts to enhance weak cross-bridge binding by lowering ionic strength were met by clear contractile responses. Even at low temperature, myofibrils bathed in low-ionic-strength relaxing solution generated increased force and exhibited sarcomere shortening, apparently caused by active contraction. At normal ionic strength, myofibril stiffness, estimated from the force response to rapid sinusoidal oscillations, increased steadily with sarcomere extension up to a strain limit. No obvious stiffness contribution from weak cross bridges was detectable. Instead, the stiffness response, which was frequency dependent at all sarcomere lengths, was apparently generated by the viscoelastic titin filaments. During imposed stretch-hold ramps, both peak force/stiffness and the amount of subsequent stress relaxation increased with higher stretch rates, larger stretch amplitudes, and longer sarcomere lengths. We conclude that, for a truly relaxed myofibril, both passive force and dynamic stiffness principally reflect the intrinsic viscoelastic properties of the titin filaments.

weak cross-bridge binding; connecting filaments; titin; connectin; stress relaxation; viscoelasticity; ionic strength

RELAXED MUSCLE EXHIBITS a viscoelastic response to sarcomere length (SL) change. During stretch, passive tension increases. After stretch stops, tension decays to a lower value. The majority of this viscoelasticity appears to be caused by titin (also known as connectin, e.g., Refs. 17, 41), the giant filamentous protein of the vertebrate sarcomeric cytoskeleton (Refs. 23, 29, 42; for recent review, see Ref. 24). The $>1$ μm-long titin filaments are functionally stiff in the A band, due to a tight association with thick filament proteins, but they are viscoelastic in the I band (13, 20, 38). Although it is accepted by many that, at relatively low rates of stretch, titin may be the sole source of myofibrillar viscoelasticity, it has been proposed that, during rapid stretch, a significant portion of the viscoelastic behavior observed is due to weak binding interactions of myosin heads with thin filaments (4, 5, 16, 17, 22, 35). The relative viscoelastic tension contribution from titin filaments compared with that from weak cross-bridge binding is undefined. Further uncertainty has recently been added to this issue, for some reports have not confirmed that weak actin-myosin interactions contribute to viscoelasticity during quick stretch (2, 31). In an effort to understand what part of the viscoelastic tension response to stretch is supported by titin filaments and what part is generated by weak cross bridges, we have investigated isolated cardiac and skeletal myofibril preparations under a variety of experimental conditions.

In the isolated myofibril, viscoelastic properties of titin are determined, for example, by examining tension transients in response to slow length perturbations, by performing measurements at SLs beyond thick and thin filament overlap, or, indirectly, by damaging the titin-thick filament supramolecular assembly while leaving thick and thin filaments per se unaffected. Determination of the viscoelastic contribution from weak cross-bridge binding is done by measuring the force response to rapid length perturbations and by varying ionic strength (IS). Those experiments may be performed at full filament overlap, under which condition weak cross-bridge binding should be maximal, and beyond overlap, under which condition cross bridges cannot bind. Results from such experiments are reported in this study. They generally support the conclusion that the viscoelastic tension response of relaxed muscle fibers does not have a significant weak cross-bridge component. Although the possibility remains that a few percent of weak cross bridges are bound to actin under physiological relaxing conditions, our findings point to the role of titin filaments in determining both passive force and dynamic stiffness of relaxed myofibrils.

METHODS

Myofibril preparation. Male New Zealand rabbits (2.25 kg) were killed by cervical dislocation. For preparation of skeletal myofibrils, the musculus psoas was isolated, and small strips (0.5 × 4–5 cm) were tied to sticks and skinned in 1% Triton-rigor buffer for 1 h. After the first skinning, the muscle strips were soaked in glycerol-rigor solution (50:50 vol/vol) for 2 h, followed by 1% Triton-rigor solution for 1 h and glycerol-rigor solution (50:50 vol/vol) overnight, and were then placed in a freezer for at least 10 days at −20°C. Throughout all solution exchanges, the muscle was kept on ice. For isolation of cardiac myofibrils, thin muscle strips from right ventricular wall tissue were dissected for storage in glycerol-rigor solution (50:50 vol/vol) for a minimum of 5 days at −20°C (cf. Ref. 26).

The glycerinated strips were minced, and (in the case of cardiac muscle) the pieces were further skinned in 4°C rigor solution containing 0.5% Triton X-100 for 0.5–1 h. To obtain single myofibrils, we homogenized the tissue pieces in a blender (Sorvall Omni Mixer) at low speed for 3–6 s, after washing them with fresh rigor solution. A droop of this suspension was placed in the specimen chamber (volume, ~300 μl), and myofibrils were allowed to settle. From myofibrils...
brils that adhered lightly to the chamber bottom, one specimen was picked up by two microelectrodes that were tip coated with a silicone adhesive (3145 RTV, Dow Corning). Although we used mainly single myofibrils, we sometimes selected doublets.

**Solutions.** Normal relaxing solution had a composition (in mM) of 3 (sometimes 6) total magnesium, 4 NaATP, 15 ethylene glycol bis(aminooxyethyl) N,N',N'',N''' tetraacetic acid (EGTA; pH 7.1), 100 potassium (methanesulfonate used as major anion), and 3-(N-morpholino)propanesulfonic acid buffer to give total IS of 200 mM. Rigor solution had a composition in mM of 75 KCl, 10 tris(hydroxymethyl)aminomethane-HCl, 2 EGTA, and 2 MgCl₂ (pH 7.0). Relaxing low-IS solution (20 mM) had a composition (in mM) of 1 K₂EGTA, 10 imidazole, 3 MgCl₂, and 1 Na₂ATP. Relaxing mid-IS solution (35 mM) was low-IS solution plus 15 mM KCl. Relaxing high-IS solution (170 mM) was low-IS solution plus 150 mM KCl. All solutions contained at least 20 μg/ml protease inhibitor leupeptin, added just before use. In control experiments (sodium dodeyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)), we had verified that this concentration of leupeptin is sufficient to largely protect the titin from degradation (25).

**Experimental apparatus.** A temperature-controlled apparatus developed to measure SL and tension in isolated myofibrils has been described (3, 26, 28). The myofibril is suspended between two glass needles that are attached to a piezoelectric motor and a force transducer, respectively. The transducer operates on the basis of a stiff, displaceable optical fiber (beam diameter, 70 μm) that emits a cone of white light. When force is exerted on the emitting fiber by an attached myofibril, the beam is deflected according to the amount of force applied (10). Deflection of the beam is monitored by a pair of receiving optical fibers. Differential illumination of the two receiving fibers is used to generate a voltage proportional to deflection of the emitting fiber and hence to force. Voltage signals are digitized and stored at a rate of 12.5 kHz. Two different transducer beams were used in this study. The longer beam had a resonant frequency of 750 Hz (for characteristics, see Ref. 25) and was used for all experiments except those in which a sinusoidal oscillation of 1,300 Hz was applied to the muscle. For high-frequency stiffness measurements, a shorter beam was used that had a resonant frequency of 1,500 Hz.

When the resonant frequency of the force transducer was raised to measure applied oscillations of 1,300 Hz, the transducer’s sensitivity dropped significantly. This drop in sensitivity interfered with our ability to accurately measure stiffness at short SLs, because of the low signal-to-noise ratio. Hence, we chose to measure stiffness mostly at 500 Hz. Although application of length perturbations at frequencies above 1 kHz has been a standard method to detect weak cross-bridge binding in relaxed muscles (e.g., Refs. 4, 5), it has been stated that length perturbation amplitudes above 12 nm/half-sarcomere cause the force response to level off, for sarcomeres will be stretched beyond the reach of weak cross bridges (35).

We measured the force response of relaxed cardiac myofibrils to 500-Hz sinusoidal oscillations of increasing amplitude to investigate whether weak cross bridges may be detectable under nearly physiological conditions (200 mM IS; room temperature). We reasoned that, if a significant population of weak cross bridges were present in cardiac myofibrils, oscillation amplitudes above 10–20 nm/half-sarcomere should result in the postulated “break” in force response because filaments would slide beyond the limits of the weak acto-myosin bond.

Figure 1A shows the typical relative force response of a cardiac myofibril as a function of oscillation amplitude (for raw data, cf. Ref. 25). Sinusoidal oscillations were imposed on cardiac myofibrils (total n = 5) at a series of different SLs. At any given SL, the force response increased nearly proportionately as the amplitude of oscillation increased from 2.5 to 80 nm/half sarcomere. A best fit of the data points at a given SL was selected to force level off, for sarcomeres will be stretched beyond the reach of weak cross bridges (35). We measured the force response of relaxed cardiac myofibrils at 500-Hz sinusoidal oscillations of increasing amplitude to investigate whether weak cross bridges may be detectable under nearly physiological conditions (200 mM IS; room temperature). We reasoned that, if a significant population of weak cross bridges were present in cardiac myofibrils, oscillation amplitudes above 10–20 nm/half-sarcomere should result in the postulated “break” in force response because filaments would slide beyond the limits of the weak acto-myosin bond.

**RESULTS**

Myofibrillar force response to increasing amplitude of sinusoidal oscillation. Measuring the force response of a muscle preparation to imposed rapid, small amplitude sinusoids or step length changes (5–12 nm/half-sarcomere) has been used as a standard method to detect weak cross-bridge binding in relaxed muscle (e.g., Refs. 4, 5). It has been stated that length perturbation amplitudes above 12 nm/half-sarcomere cause the force response to level off, for sarcomeres will be stretched beyond the reach of weak cross bridges (35).

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When the force values of Fig. 1A were converted to stiffness, it was also apparent that with increasing
amplitude of sinusoidal oscillation the myofibrillar stiffness response decreases. This decrease was not linear but relatively steep at small oscillation amplitudes and much shallower at larger amplitudes (Fig. 1B and inset). The manner in which stiffness decreased with increasing oscillation amplitude was similar in all specimens investigated.

Stiffness and force in low-IS solution. To more reliably assess a possible stiffness contribution from weakly binding cross bridges, we measured the change in myofibrillar stiffness when the relaxing solution bathing the specimen was changed from physiological (high) to low IS. Decreased IS has typically been used to elicit a larger stiffness contribution from weakly bound cross bridges (5, 17). We investigated stiffness in high-IS (170 mM), mid-IS (35 mM), and low-IS (20 mM) solutions at both room temperature (20–22°C) and 5°C. Also, experiments were done at full actin-myosin filament overlap and beyond overlap. Rabbit psoas myofibrils were used for these experiments because titin-based stiffness and force are much lower than in cardiac specimens (3, 28).

When the relaxing solution was changed from 170 to 20 mM IS, initially relaxed sarcomeres shortened and often developed large length inhomogeneities. At either room temperature or 5°C, myofibrils developed increased stiffness and force when subjected to 20 mM IS solution. The behavior of myofibrils bathed in 35 mM IS solution was more complex. When rest-length myofibrils (SL, 2.3–2.4 μm) were exposed to 35 mM IS solution, sarcomeres did not measurably shorten or exhibit increased force. However, in myofibrils whose sarcomeres were initially >2.6 μm, immersion in 35 mM IS solution caused sarcomere shortening (Fig. 2A, compare top and bottom traces). Whenever sarcomere...
shortening was observed, we also found a significant increase in stiffness and force. IS dependent changes in passive force, stiffness, and SL were absent when sarcomeres were stretched beyond thick and thin filament overlap.

To accurately measure the presence of any contractile activity in low-IS relaxing solutions, we performed ramp stretch-hold-release experiments on >10 skeletal myofibrils altogether, with similar results. Figure 2 shows the response of the same myofibril to three identical motor ramps (temperature 22°C). The myofibril was first bathed in 170 mM IS solution and next in 35 mM IS solution and was then returned to 170 mM IS solution. Initial SLs are different in the three traces (Fig. 2A) because sarcomeres shortened and generated higher force when exposed to 35 mM IS solution. Baseline force (Fig. 2B) and SL returned to their initial values after the myofibril was returned to physiological IS solution. Sarcomere shortening observed during the ramp-hold plateau, in conjunction with increasing force generation, indicates that in 35 mM IS solution the muscle was actively contracting.

Figure 3 shows the typical response of a myofibril bathed in 35 mM IS solution to ramp stretch at a temperature of 5°C. Low temperature was used in an effort to suppress contractile activity, a standard method (see Discussion for rationale). On sarcomere stretch, force rapidly increased and then fell precipitously just as the stretch stopped (Fig. 3C, inset). During the ramp-hold plateau, SL decreased while force increased, indicating that the myofibril was actively contracting. Further indication of contractile activity can be seen after specimen release to shorter length, when force fell below the initial baseline and then slowly recovered while sarcomeres shortened slightly.

Twelve skeletal myofibrils were tested in low-IS solution (the solutions were made three different times to test for errors in composition), and four specimens were tested at 5°C. Ramp stretch or force measurements were repeated 3–15 times, with excellent reproducibility. All five cardiac myofibrils tested in low-IS solution exhibited spontaneous contractile behavior and developed inhomogeneous SLs. In sum, due to active contraction observed in low-IS relaxing buffer even at low temperature, we were unable to characterize the contribution of weak (non-force-generating) cross bridges to passive stiffness under these conditions.

Sarcomere length and frequency dependence of relaxed stiffness. Stiffness measurements were made on myofibrils in 200 mM IS relaxing solution over a wide range of SLs. We performed the experiments by applying usually 500-Hz or 1,300-Hz sinusoidal oscillations to one end of the specimen and recording the amplitude of force oscillations at the other end (cf. Ref. 25). Sometimes, the active force-suppressing drug, 2,3-butanedione monoxime (30–40 mM), was added to the relaxing solution to test for the possibility of residual force generating cross bridges contributing to myofibril stiffness. No significant effect on stiffness was found, and thus strong actin-myosin interactions did not appear to be a factor under the conditions chosen.

A typical length-dependent force response of cardiac myofibrils to 500-Hz, small amplitude sinusoids is shown in Fig. 4A (a total of 12 myofibrils was studied). On SL increase, stiffness rose approximately proportionally to passive force. After sarcomere stretch to beyond 2.9–3.0 μm, a distinct flattening of the stiffness curve was observed in all cardiac myofibrils investigated. There were only slight variations in the SLs at which the stiffness curve became flat (cf. Ref. 25). This SL has been shown to represent a cardiac myofibril’s “yield point,” corresponding to the release of previously stiff A band titin into the I band (25, 39). Psoas myofibrils (n = 4) generated a similar stiffness vs. SL response, although the yield point was shifted to longer lengths (Fig. 4B). Figure 4C shows a comparison of cardiac myofibril stiffness measured at sinusoidal driving frequencies of 500 and 1,300 Hz. Also at the higher frequency, we found a distinct break in the stiffness curve near an SL of 3 μm. Notably, passive stiffness increased with oscillation frequency, even at zero actin-myosin overlap. On average, stiffness at 1,300 Hz was almost twice as high as that at 500 Hz, and the ratio of 1,300-Hz to 500-Hz stiffness magnitude remained similar over the entire SL range investigated; at the two frequencies, the shapes of the curves were essentially the same. Figure 4C, inset, illustrates that a frequency dependence of stiffness became detectable already at sinusoidal driving frequencies of ~50 Hz. Because the
speed-dependent stiffness increase was apparently independent of SL and persisted without thick and thin filament overlap, it was not due to weak cross-bridge binding.

To further assess the separate stiffness contributions from weakly binding cross bridges and titin filaments, we utilized the property of the A band portion of titin to become extensible at extremely long SLs (25, 30, 38, 40). Mobilization of previously stiff titin segments will affect the titin-borne contribution to passive force and stiffness. This contribution can thus be characterized when stiffness is measured on small to extreme myofibril stretch and release. Figure 5 shows a summary of results of 500-Hz stiffness measurements on five cardiac myofibrils during three cycles of stretch and release, with the maximum SL being progressively increased in each cycle. The specimens were stretched in steps from slack length to a series of SLs. After each stretch, the myofibrils were allowed to rest for 3 min to achieve stress relaxation. After several measurements made at sequentially longer SLs, an identical series of measurements was made on releasing the myofibrils to shorter and shorter SLs. By the end of each cycle of sarcomere stretch and release, the specimens had been returned to slack length. SL homogeneity in the myofibrils usually remained excellent throughout all stretch maneuvers, and contrast in the phase image did not

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Fig. 4. Sarcomere length and frequency dependence of myofibril stiffness. A: stiffness and force as a function of SL in relaxed rabbit cardiac myofibrils. Stiffness was measured by dividing amplitude of force response to 500-Hz sinusoidal oscillations by peak-to-peak magnitude of imposed SL perturbation (7 nm/half-sarcomere). B: stiffness response of psoas myofibril (500 Hz). C: comparison of stiffness response to 500-Hz and 1,300-Hz perturbation of isolated cardiac myofibrils (peak-to-peak amplitude, 7-12 nm/half-sarcomere) relative to 500-Hz stiffness at 

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Fig. 5. Stiffness during stretch-release cycles. Relative passive stiffness as a function of SL in relaxed cardiac myofibrils is shown. Stiffness values at a given SL (±0.1 µm) are means ± SD relative to stiffness at strain limit SL of ~3.0 µm (100%); ○, cycle 1 of measurements during stretch and release from 1.9 µm (average slack length) to ~2.6-µm SL; ●, cycle 2 of stretch and release from slack length to ~2.85-µm SL; □, cycle 3 of stretch to ~3.2 µm SL and release. Note that some data points measured at short SLs are not shown, because stiffness was below sensitivity limitations of force transducer (noise; hatched area). Solid lines, stretch; dashed lines, release. Conditions: 500-Hz sinusoidal oscillations, 8–12 nm/half-sarcomere; 200 mM IS; 20–22°C.
BASIS OF PASSIVE TENSION AND STIFFNESS

Fig. 6. Force and SL during stress relaxation. Passive force and SL response of psoas myofibril to imposed stretch and hold at 3 different initial SLs (indicated at right) are shown. A: passive force; relative force response during imposed motor ramp (offset to same baseline). B: change in SL (not to scale, offset to allow comparison with correspondingly numbered force traces). Conditions: 200 mM IS; 20–22°C.

Stress relaxation at short and long SLs. We also examined the dependence of stress relaxation on the amplitude and rate of imposed ramp lengthening (stretch duration, 0.01–6.2 s) and of SL. We found that SL changes closely followed the imposed ramp stretch, while force increased steadily. Then, with the specimen length held constant after the stretch, force decreased by variable amounts, depending on experimental conditions. Representative results of experiments on skeletal myofibrils (total \( n = 9 \)) are shown in Fig. 6. As initial SL increased, the magnitude of peak force and decay response (stress relaxation) increased, confirming previous findings in cardiac myofibrils (25). Such behavior was found despite the fact that the actual change in SL decreased slightly at longer lengths, due to the force transducer’s compliance and higher levels of passive force. Furthermore, it became clear that, at any SL from full actin-myosin filament overlap to well beyond, increasing the amplitude of sarcomere stretch increased the stress relaxation response (cf. Ref. 28). Figure 7 illustrates that the rate of sarcomere stretch also affected the amplitude of stress relaxation in a predictable manner. For a given stretch amplitude, faster stretches induced larger stress relaxation responses, and this too was found at SLs both with and without filament overlap (data not shown).

The relationship of stiffness to passive force during stress relaxation was examined by superimposing small amplitude (7–12 nm/half-sarcomere), 1,300-Hz sinusoids onto a sarcomere stretch-hold-release ramp. Figure 8 demonstrates a typical experimental protocol with a cardiac myofibril. Altogether, five specimens were examined, with reproducible results. The shape of the imposed motor ramp is shown in Fig. 8A. The amplitude and time course of stress relaxation are shown both with and without the superimposed sinusoidal perturbations in Fig. 8, C and B, respectively. The amount of stress relaxation appeared to be unaffected by the superposition of small amplitude perturbations. Expanded segments of the force trace (Fig. 8C, insets) obtained with superimposed oscillations can be used to assess myofibril stiffness during the ramp stretch protocol. It can be seen that the peak-to-peak amplitude (representing stiffness) was much higher just after sarcomere stretch ended and then decayed away proportionally to the fall in passive force. In this particular experiment (stretch duration, 0.06 s), both passive force and stiffness decreased during the \( \sim 1.5 \) s-long hold period by \(-30\%\) (whereas the absolute amounts of peak force/stiffness and of subsequent stress relaxation were larger at 3.4 \( \mu \text{m} \) than at 2.5- \( \mu \text{m} \) SL). Thus high-frequency stiffness followed the same decaying time course as the passive force during stress relaxation. The manner in which stiffness changed during the ramp stretch-hold protocol was qualitatively similar at both 2.5- \( \mu \text{m} \) and 3.4- \( \mu \text{m} \) SL. These results suggest that changes in dynamic stiffness are closely related to changes in passive force, regardless of the amount of thick and thin filament overlap.

DISCUSSION

Myofibril quality and structure. The single myofibril appears to be a desirable preparation for examining the “passive” mechanical properties of muscle: because it lacks extraneous parallel structures, measured force/stiffness can be unambiguously ascribed to the elements within the sarcomere. With thicker preparations, interpretation is less direct. This holds especially true for isolated cardiac myofibrils, since they do not contain, for example, collagen or microtubules, which contribute to stiffness in larger preparations, particu-
Fig. 8. Proportional response of passive force and stiffness during ramp stretch. Force and stiffness response of cardiac myofibril to ramp stretch and hold are shown. Measurements were made at SLs of 2.5 μm (left) and 3.4 μm (right). A: imposed motor ramp. B: force response of myofibril to imposed motor motion, without added sinusoidal perturbation. C: force response of myofibril to motor ramp with added sinusoidal oscillations (1,300 Hz, 12 nm half-sarcomere). Insets: oscillatory force measured early and late in stress relaxation (expanded scale). Conditions: 200 mM IS; 20–22°C.

larly at the high end of the physiological SL range (15, 28, 37). At the shorter length, passive tension per cross-sectional area is similar in larger preparations and isolated myofibrils from both cardiac and skeletal muscle (3, 28). Furthermore, single myofibrils perform as well as or better than larger specimens during active contraction (3, 28), which leads us to believe that our specimens are “healthy” and contain all pertinent contractile proteins in an ordered state. This is supported by electron micrographs made of fixed, single myofibrils recovered after active tension measurements in our apparatus (28). Such myofibrils show normal filament structure and lack of external sarcomeric elements such as sarcoplasmic reticulum. Moreover, SDS-PAGE of suspensions of myofibrils prepared as described in METHODS reveals the typical bands for contractile and regulatory proteins, titin, and nebulin, in a manner indistinguishable from that of larger, skinned preparations (25). In sum, the single myofibril is a model well suited for mechanical studies at the level of the smallest structural unit of muscle, the sarcomere.

Possible sources of stiffness in relaxed muscle. At least three different elements can potentially be responsible for the force response of a relaxed striated muscle fiber to length perturbations. Primary candidates are the titin (connectin) filaments (29, 42), which run along the half-sarcomere from the Z line to the M line (13). The mechanical properties of titin filaments are not fully understood yet (cf. Ref. 24). Although their I band portion can apparently act as an elastic element (e.g., Refs. 13, 17, 20, 40), there is also evidence that titin exhibits frequency-dependent stiffness (8, 17, 41).

Some contribution to a stretch-induced force response in relaxed fibers could also come from a viscous resistance to filament sliding (2, 8, 31). This force response would be exclusively dependent on stretch rate. The viscous component is much reduced in skinned fibers, compared with intact specimens (2), and thus should also be relatively small in single myofibril preparations.

Another possible source of viscoelastic force in relaxed muscle fibers is weak binding cross bridges, which in vitro are characterized by a rapid equilibrium between attached and detached states (7). They have been proposed to be present in relaxed muscle and detectable mechanically in the force response of a myofiber to imposed rapid length perturbations (4, 5, 34). The concept of weakly bound cross bridges is primarily based on the finding that, in low-IS relaxing solution, muscle fibers have a relatively high stiffness that increases with stretching speed and decreases with the reduction of actin-myosin filament overlap, suggesting the presence of rapidly cycling cross bridges (4, 5, 35). In conflict with this, some recent studies have reported that stiffness in low-IS solution in fact increases with SL (2, 17). Clearly, the question of whether weak cross-bridge binding does or does not contribute to the viscoelastic behavior of relaxed muscle is not settled yet.

Activating effects of low-IS solution. In an attempt to define the contribution of weakly binding cross bridges to dynamic stiffness, we examined single myofibrils in high- and low-IS solutions, whose respective compositions were as in Ref. 5. Additionally, we tested the effects of 35 mM IS. Under most conditions, we found a low IS induced increase in myofibril stiffness (and force), confirming previous findings by others (5, 17). IS changes had no effect on force/stiffness in sarcomeres stretched beyond filament overlap. However, at physiological SLs, the data shown in Figs. 2 and 3 imply that increased myofibril stiffness in low-IS solution was due to active contraction.
Active contraction of fibers (5, 14, 17, 43) and myofibrils (33) bathed in low-IS relaxing buffer at room temperature has been consistently reported. Tension generated under these conditions is a few percent of active tension. The nature or cause of such contractile activity is unclear. To avoid the activation effect, many researchers have lowered specimen temperature to 5°C. In skinned fibers held at 5°C, contractile activity reportedly ceased (5, 17). By contrast, when isolated myofibrils from either cardiac or psoas muscle were bathed in low-IS solution at 5°C, contractile activity persisted (Fig. 3). Active contraction could not be suppressed even when, in some preliminary experiments, the free magnesium ion concentration was raised to >5 mM (for rationale, cf. Ref. 18).

Was the behavior of myofibrils observed at low-IS and 5°C indeed an active contraction? Our apparatus allows careful measurement of force and length changes at the level of the individual sarcomere, and low levels of contractile activity can be unequivocally detected. We found the clearest evidence of contractile activity when specimens underwent a ramp stretch protocol in 35 mM IS relaxing solution, as demonstrated in Fig. 3. Figure 3, inset, shows an expanded scale of the force peak measured as sarcomeres were stretched. Interestingly, this two-phase peak is strikingly similar to that reported by Edman et al. (Ref. 9; see also Ref. 36), who studied the response of activated fibers to sarcomere stretch. The early, steep slope of this peak has been attributed to increased force development by negatively strained cross bridges, whereas the flatter, second slope in this peak has been attributed to the maximum force a cross bridge could bear, before being ripped free of the thin filament (9). When these similarities between stretched, active fibers and stretched myofibrils in low-IS buffer are considered, it appears that the myofibrils bathed in 35 mM IS relaxing solution were indeed actively contracting.

Possibly the low IS contractile behavior of myofibrils at low temperature is due to some problem with the preparation. However, previous work suggests that myofibrils behave similarly to larger specimens both under relaxing and activating conditions (3, 28). It is also unlikely that some component of the regulatory system became lost during specimen isolation, because SDS gels suspensions of myofibrils show no abnormalities in the band pattern and band intensity expected for the regulatory proteins (25). Furthermore, single myofibrils exhibit a calcium sensitivity comparable to that measured in larger skinned muscle preparations (3, 28). Therefore, we do not think there was a problem with the preparation. Rather, we argue that in low-IS relaxing buffer, lowering temperature to 5°C will suppress the active force observed at room temperature to some degree, but there is no verifiable justification as to why the preparation should change from its activated state to a relaxed state. Even minute amounts of contractile activity could account for the increased stiffness in low-IS solution. A necessary experiment then will be to carefully test whether low levels of activation remain at 5°C in whole fibers also. For further study of the validity of the weak cross bridge hypothesis, it will be interesting to measure in both myofibrils and whole fibers the ratio between force and stiffness, to test whether this ratio is different in activating solution and in low-IS relaxing buffer.

In conclusion, the presence of contractile activity in myofibrils at low IS and low temperature did not allow us to subtract the stiffness in high-IS buffer from that in low-IS solution to obtain the stiffness contribution of weakly bound cross bridges. For myofibrillar specimens, assessment of any stiffness contribution from weakly binding cross bridges apparently must come from other experimental approaches.

Weak cross-bridge detection by increasing length perturbation amplitude. Small amplitude, rapid length perturbations have been used to study weak cross-bridge binding in relaxed muscle, in order to strain bound cross bridges but not pull them free from thin filaments. It has been postulated (35) that larger amplitude perturbations lead to a break in the force response curve, presumably caused by stretching sarcomeres beyond the reach of a normal cross-bridge stroke. We wished to capitalize on this property to examine the force response supported by weak cross bridges in relaxed cardiac myofibrils.

We found no clear break or change in force response for peak-to-peak oscillations from 2.5 to 80 nm/half-sarcomere (Fig. 1A). As the sinusoidal amplitude was increased, the amplitude of force oscillations increased proportionally, both at short SLs and with negligible thick and thin filament overlap. This result can be compared with a number of literature reports about perturbation-dependent force measurements in relaxed muscle (1, 2, 11, 19). Large amplitude perturbations (to >40 nm, as in Fig. 17 of Ref. 11) generated a linear increase in force, just as found by us. Another study demonstrated that any discontinuity in force response occurs within the first 1–2 nm/half-sarcomere of stretch and not for larger perturbations (2). Our data indirectly suggest the same result. Examination of the curves in Fig. 1A reveals that they all cross the force axis well above zero when extrapolated back toward zero amplitude oscillations. Because the force response to zero amplitude oscillation must start at the origin, the slope of the curve from the origin to the region we were able to measure must be significantly steeper: the response to stretch is highest for very small perturbations. However, measurements on sarcomeres with essentially no thick and thin filament overlap exhibit a nonzero intercept as well (Fig. 1A, top curve). Therefore, instead of being due to weak cross-bridge binding, it seems more plausible that the initial steepness is caused by viscoelastic behavior presumably originating from the titin filaments. In this context, it will be of interest to study why the stiffness shown in Fig. 1B (which is apparently titin based) is largest for small sinusoidal oscillation amplitudes. More insights into the molecular mechanism of titin viscoelasticity are needed to understand this phenomenon.

To explain our and others' apparent inability to measure a break in the force response curve with
increasing length perturbation amplitude $>2.5$ nm/half-sarcomere, we consider the following possibilities: 1) Weak actin myosin binding is a stochastic process, and to completely abolish possible weak cross-bridge action based merely on geometric considerations might not be feasible. 2) The attachment/detachment rate constants of weak cross bridges in cardiac muscle at room temperature could be so high that these bridges cannot be detected with $500$-Hz sinusoidal oscillations. However, because in preliminary experiments with $1,300$-Hz sinusoids we were also not able to measure a break in the force response curve (data not shown), we consider it unlikely that detection of such a break was hampered by speed limitations. 3) As suggested for skeletal muscle at physiological IS and room temperature (22), $<10\%$ of all cross bridges might be weakly attached to actin in cardiac myofibrils and may therefore not be detectable with our mechanical method. 4) Binding of weak cross bridges is not detectable mechanically because these bridges are absent in relaxed muscle (2, 31, 32). Altogether, we conclude that, in relaxed myofibrils under physiological conditions, weak cross bridges may either occur in insignificant numbers only or be completely absent.

**Weak cross-bridge vs. titin filament stiffness.** To further assess the relative stiffness contributions from weak cross-bridge binding and from titin filaments at the shorter SLs, we applied an experimental protocol to damage the titin-thick filament supramolecular assembly while retaining thick and thin filament overlap (cf. Refs. 25, 41). The specimens were repeatedly stretched to beyond normal physiological SLs and then returned to a shorter length (Fig. 5). We used mostly cardiac myofibrils for these experiments, to explore the possibility of weak cross-bridge binding in this muscle type, in which titin-based stiffness is relatively high. Different stiffness values can be found at a given SL, depending on which stretch or release cycle we choose. After stretch to beyond the "strain limit" ($2.9-3.0$ $\mu$m; Refs. 25, 39), stiffness does not increase much further; clearly, some element contributing to passive stiffness changes. Evidence has accumulated over the past years that this element is the titin filaments; mobilization of previously stiff A band titin segments by "sarcomere overstretch" decreases stiffness (17, 25, 39–41). On the other hand, because sarcomeres were never pulled beyond filament overlap, it is unlikely that the permanent changes in stiffness evident in Fig. 5 can be attributed to disarray of thick and thin filaments. At a given SL, both before and after specimen stretch, the same number of cross bridges should be able to interact with the thin filament. After titin-borne stiffness is reduced greatly, the stiffness remaining at SLs with full thick and thin filament overlap should principally indicate that of weak cross bridges (Fig. 5, end of "release" curve of cycle 3). Because stiffness at these SLs was so low that it fell below our resolution limit, we reasoned that weak cross-bridge binding contributed at best very little to relaxed cardiac muscle stiffness. Most or all of the stiffness seen before overstretch of the myofibrils appeared to be that of titin filaments.

This conclusion obtained from cardiac myofibrils is in agreement with that of a recent study on relaxed skeletal muscle fibers, which has assumed that only a relatively small number ($5-10\%$) of cross bridges may be attached to actin under physiological conditions (22). In this and an earlier, related study (6), depression of passive stiffness by caldesmon (an inhibitor of weak cross-bridge binding in vitro; cf. Ref. 7) was taken as an indication of the presence of weak cross bridges in relaxed muscle. However, whereas these reports have suggested that some fraction of relaxed fiber stiffness may arise from weak cross-bridge binding, it was shown recently that none of the force components during rapid stretch of relaxed skeletal muscle fibers has the properties expected from predictions of weak cross-bridge kinetics (1, 2, 31, 32). Thus, although in both cardiac and skeletal muscle weak cross bridges could determine passive stiffness under physiological conditions to a small degree, it is also possible that such cross-bridge contribution is absent.

In another study relevant to this subject, a large decrease in passive stiffness of skeletal muscle fibers was observed on selective thin filament removal by gelsolin, while passive tension was increased (17). The increase in passive tension was explained by the authors to perhaps result from a closer association between titin and nebulin after actin extraction, which might stiffen the elastic titin. Decreased passive stiffness was attributed to loss of myosin weak binding with the (removed) thin filament. Although these data could be supportive of the existence of weakly binding cross bridges, there is also evidence for interaction of thin filaments with titin (12, 21). Interestingly, this interaction appears to be stronger at low IS than at high IS (21). Loss of actin-titin interactions in relaxed cardiac myofibrils apparently results in decreased (low-frequency) stiffness, which was confirmed to be unrelated to weak cross-bridge binding (27). Thus stiffness changes caused by thin filament removal may not necessarily be evidence for the general presence of weak actin-myosin interactions in relaxed muscle. Clearly, further studies are needed to investigate these issues in more detail and reconcile the differences.

**Stiffness and stress relaxation at different SLs and frequencies.** As reported by others (8, 32), we observed a frequency-dependent stiffness response in relaxed myofibrils (Fig. 4C). Potentially, the presence of weak cross-bridge binding equilibria could imply that the force response to applied SL perturbation is frequency dependent (4, 17, 34). However, not only at full but also at zero filament overlap, stiffness at $500$ Hz was approximately one-half that measured at $1,300$ Hz. Because at zero filament overlap cross bridges cannot bind to thin filaments, other structures must be responsible for the observed stiffness differences: presumably the titin filaments. Titin apparently also contributes to the frequency dependence of stiffness at the shorter SLs, since at $2.5-\mu$m SL we found an increase in stiffness already at frequencies as low as $50$ Hz (Fig. 4C, inset). Because weak cross bridges, due to their high attachment/detachment rate constants (4, 16, 17,
34), should not be detectable at such low frequencies, only the viscoelastic force response from passive elements such as titin (and perhaps a small viscous contribution from filament sliding) should be seen.

Finally, the viscoelastic behavior of titin filaments was also demonstrated by imposing relatively slow ramp stretches onto myofibrils and recording the stress relaxation behavior during the subsequent hold period (Figs. 6 and 7). The observed force decay at SLs both with and without filament overlap implied that titin exhibits a strong elastic and viscous (in series) response to stretch. In a novel experimental protocol, we showed that the time course of stress relaxation is approximately the same for both passive force and high-frequency stiffness, at 2.5 μm and 3.4-μm SLs, respectively (Fig. 8). This again indicated that a common structure is responsible for a relaxed myofibril’s static force and dynamic stiffness. Evidence from this and a related study (25) suggests that the responsible elements are likely to be the titin filaments.

In conclusion, our measurements of stiffness and stress relaxation in relaxed cardiac and skeletal myofibrils have implicated the titin filament as the dominant viscoelastic element within the sarcomere. Stiffness contributions from weak cross-bridge binding were elusive, although very small contributions remain a possibility. A major basis of the weak cross-bridge binding hypothesis, increased stiffness in low-IS relaxation solution, was found to be an inappropriate test in myofibrils, since it caused a weak contractile response even at low temperature. We found that titin filaments apparently contribute to the frequency-dependent stiffness of relaxed muscle at all SLs. In fact, the stiffness of titin filaments alone appeared to be indistinguishable from that widely attributed to weakly binding cross bridges. Thus the concept of measuring weak cross-bridge binding in relaxed muscle by mechanical techniques is not straightforward and should be critically reassessed.

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