Effect of Stretch and Release on Equatorial X-Ray Diffraction During a Twitch Contraction of Frog Skeletal Muscle

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ABSTRACT Time-resolved intensity measurements of the x-ray equatorial reflections were made during twitch contractions of frog skeletal muscles, to which stretches or releases were applied at various times. A ramp stretch applied at the onset of a twitch (duration, 15 ms; amplitude, ~3% of muscle length) caused a faster and larger development of contractile force than in an isometric twitch. The stretch accelerated the decrease of the 1,0 reflection intensity ($I_{1,0}$). The magnitude of increase of the 1,1 reflection intensity ($I_{1,1}$) was reduced by the stretch, but its time course was also accelerated. A release applied at the peak of a twitch or later (duration, 5 ms; amplitude, ~1.5%) caused only a partial redevelopment of tension. The release produced clear reciprocal changes of reflections toward their relaxed levels, i.e., the $I_{1,0}$ increased and the $I_{1,1}$ decreased. A release applied earlier than the twitch peak had smaller effects on the reflection intensities. The results suggest that a stretch applied at the onset of a twitch causes a faster radial movement of the myosin heads toward actin, whereas a release applied at or later than the peak of a twitch accelerates their return to the thick filament backbone. The results are discussed in the context of the regulation of the myosin head attachment by calcium.

INTRODUCTION

A living skeletal muscle shows a phasic contraction called a twitch, in response to a single electrical stimulation. At low temperatures (5–10°C), the twitch contraction of a frog muscle reaches a peak at ~150–200 ms after the stimulation and is completed within ~500 ms. It has been suggested, however, that the activation level of the contractile machinery follows a time course different from that of isometric tension.

A. V. Hill (1949) reported that a quick stretch imposed at the onset of a twitch abruptly brings the tension to the level of isometric tetanus. Later experiments by Ritchie (1954) showed that releases applied in the relaxation phase of a twitch result in only a partial redevelopment of tension. These results were interpreted to indicate that the "intensity of active state" reaches a maximum immediately after stimulation and decays faster than the isometric tension. Here the "active state" refers to the tension exerted by the "contractile element" when it is not influenced by the shortening or lengthening of the "series elastic component."

The notion of active state is based on an implicit assumption that a stretch or a release does not affect the time course of the activity of the contractile machinery. It is increasingly clear, however, that length perturbations alter the time course of the activity of the contractile machinery. Haugen and Sten-Knudsen (1987) reported that the abrupt rise in tension caused by a stretch is accompanied by a concomitant increase in fiber stiffness, which is usually taken to reflect the number of attached myosin heads. Briden and Alpert (1972) reported that the amplitude of releases affects the time course of the active state decay. A restretch of a muscle fiber after a release does not restore its isometric tension, suggesting that the number of attached heads has been reduced (Iwamoto et al., 1990a).

These observations raise the following questions. 1) Does a stretch applied at the onset of a twitch accelerate the attachment of the myosin heads to the thin filament, or simply accelerate the transition from low-force to high-force states? 2) Do the myosin heads detached by a release immediately return to the thick filament backbone, or stay in the vicinity of the thin filament for a while? The first approach to these questions would be to monitor the movement of myosin heads between the thick filament backbone and the thin filaments. This can be achieved by a millisecond time-resolved intensity measurement of the two low-angle x-ray equatorial reflections (1,0 and 1,1), which are generally considered to reflect the radial movement of myosin heads within the hexagonal lattice of filaments (Haselgrove and Huxley, 1973; Haselgrove et al., 1976). The recording with a high time resolution is made possible by the use of synchrotron radiation as an intense x-ray source.

The present results suggest that the activities of the myosin heads are more sensitive to length changes during a twitch than during a tetanus. The results provide an insight into the properties of the attached, low-force myosin heads, which abound at the beginning of contraction, as well as those of the force-bearing heads, which remain attached after the intracellular calcium has reduced. Preliminary reports have been published (Iwamoto et al., 1990b; 1993).

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MATERIALS AND METHODS

Preparation

Whole sartorius muscles of a frog, *Rana japonica*, were dissected. The pelvic end of the muscle was fixed to the force transducer, and its tibial end

was tied to the extension of the servo motor with a silk thread. Muscle lengths ranged from 20 to 35 mm. Sarcomere lengths were ~2.2–2.4 μm as determined by He-Ne laser diffraction. Muscles were bathed in Ringer's solution having the following composition in mM: NaCl, 115, KCl, 1.8; $CaCl_2$, 2.5; Tris-maleate, 10 (pH = 7.2). Temperature was controlled to 5–8°C by circulating cooled Ringer's solution.

Experimental setup

The experimental chamber was made of acrylic resin and had windows covered with Mylar film to pass x-ray beams. Muscles were stimulated by passing square electrical pulses of supramaximal intensity (duration up to 3 ms) through an array of platinum wire electrodes placed in the chamber. The force transducer was a pair of semiconductor strain gauges KSPH-2000-E4 (Kyowa Dengyo, Tokyo, Japan) attached to both sides of a phosphor-bronze beam. The servo motor was a mechanical stimulator (Dia-Medical, Tokyo). This was able to complete a 10-mm step length change within 5 ms. Timing of stimulation and length change was controlled by a Macro 11/F34 minicomputer, which was also used for x-ray data collection.

Segment length recording

The length of the x-ray-irradiated segment of the sartorius muscle was measured optically. Two black-stained pieces of aluminum foil were attached with cyanoacrylate glue to the part of the muscle just beneath the Mylar window, with a longitudinal separation of ~3 mm. Collimated laser beams emitted from a laser diode (wavelength = 780 nm) were scanned along the length of the muscle at a frequency of ~1.5 kHz by rotating a polygon mirror placed on top of the chamber. The incident angle of the laser beams was made perpendicular to that of the x-ray beams so as to enable x-ray diffraction and segment-length recordings simultaneously. The segment length was determined as the time required for the beams to travel the gap between the markers, and it was measured with a pin photodiode placed beneath the window.

X-ray diffraction studies

All experiments were done at the beamline BL15A of the Photon Factory, National Laboratory for High Energy Physics at Tsukuba, Japan. The positron storage ring was run either at 2.5 GeV with a ring current of 170–360 mA, or at 3.0 GeV with a ring current of 130–180 mA. The radiation emitted from the positron storage ring was monochromatized (wavelength = 1.507 Å) and focused by a camera consisting of an assembly of glass mirrors and a bent germanium crystal (Amemiya et al., 1983; Wakabayashi and Amemiya, 1991). The specimen-to-detector distance was 2300 mm. The diffracted beams were collected by a one-dimensional position-sensitive proportional counter (Rigaku, Tokyo, Japan), which had a spatial resolution of 0.3 mm. The time resolution was 5 ms. The x-ray data were stored in a CAMAC memory system and transferred to the minicomputer.

Experimental protocol and data analysis

A single muscle was stimulated at an interval of 60 s. This frequency did not cause a significant decay of peak isometric tension or a significant deceleration of relaxation. X-ray diffraction data for 10 consecutive contractions were accumulated to create a single data file. Each muscle was made to contract up to 80 (usually 60) times. A series of isometric control and stretch or release runs were alternated for each muscle. The integrated intensities of the 1,0 and 1,1 reflections ($I_{1,0}$ and $I_{1,1}$) were calculated by the method of Podolsky et al. (1976), as follows. The accumulated data were smoothed by calculating the 1,2,1 running average, and the background was subtracted. The signals of overall muscle length, segmental length, and tension from several of the series of contractions were stored in a Nicolet 310 digital oscilloscope (Nicolet Instrument Corp., Madison, WI) and then transferred to a Toshiba T-1000 portable computer. Besides these recordings, the tension signals from all contractions were accumulated in the CAMAC memory system. All analyses were done using a personal computer (model 5530, IBM Japan, Tokyo).

RESULTS

Effect of step length changes in the time course of twitch tension

The timing and amplitudes of the length changes were selected to reproduce the effect of a stretch or a release reported by Hill (1949) and Ritchie (1954). Representative records of tension are shown in Fig. 1, together with the displacement signal from the servomotor and the segment length. A stretch (duration, 15 ms; amplitude, ~3% muscle length) resulted in a concomitant rise in tension to a level much higher than isometric control when it was applied at 30 ms after the stimulus (Fig. 1 *a*). A release (duration, 5 ms; amplitude, ~1.5%) was brought to, or close to, the base line level, and was followed by a partial redevelopment of tension. The extent of tension redevelopment became progressively smaller as the timing of the release was delayed (Fig. 1, *b–d*).

The segment recording showed that the length of the x-ray-irradiated segment reduced by ~1–2% upon activation, whereas in other muscles it stayed almost constant or increased slightly. The segment length reflected the length change imposed on the tibial end of the muscle by the servo motor.

Effect of a stretch applied at the onset of twitch contractions on the time course of intensity changes of the equatorial reflections

Under isometric conditions, the intensities of the two principal equatorial reflections 1,0 and 1,1 ($I_{1,0}$ and $I_{1,1}$) showed approximately reciprocal changes after stimulation as has been reported earlier (Huxley, 1975; Sugi et al., 1978; Matsubara and Yagi, 1978): the $I_{1,1}$ increased and the $I_{1,0}$ decreased, leading to a drop of the $I_{1,0}/I_{1,1}$ ratio from ~1.3 to ~0.5.

The effect of a stretch on the equatorial reflections is readily observed when the time courses of the $I_{1,0}$, $I_{1,1}$ and tension changes are expressed as fractions of their maximal changes observed during isometric contractions. In Fig. 2, such normalized changes during contraction with a 15-ms stretch are compared with those during isometric contraction. The changes of the intensities ahead of tension rise ($I_{1,1}$ being faster than $I_{1,0}$) are consistent with the previous reports (Huxley, 1975; Matsubara and Yagi, 1978; Cecchi et al., 1991). The $I_{1,0}$ decrease was clearly accelerated by the stretch (Fig. 2 *a*), and this is also evident from the visual comparison of the intensity profiles of the frames before the stretch with those in the late phase of the stretch (Fig. 3, *a* and *b*, taken from the frames indicated by arrowheads in Fig. 2 *a*). The difference appeared as clear dips in the difference diagram at the positions of the 1,0 peaks (Fig. 3 *a*). In isometric control, the difference was much smaller for the same period of time (Fig. 3 *b*). The stretch reduced the absolute magnitude of intensity change of the 1,1 reflection after the stretch was completed (Fig. 2 *b*). This direction of the intensity change is consistent with the reported effect of increasing sarcomere length (Elliott et al., 1963) and also the effect of slow stretches applied at the plateau of a tetanus (Amemiya et al., 1988). A tendency of the $I_{1,1}$ to accelerate upon stretch is also recognized when the $I_{1,1}$ is normalized to control (Fig. 2 *c*).

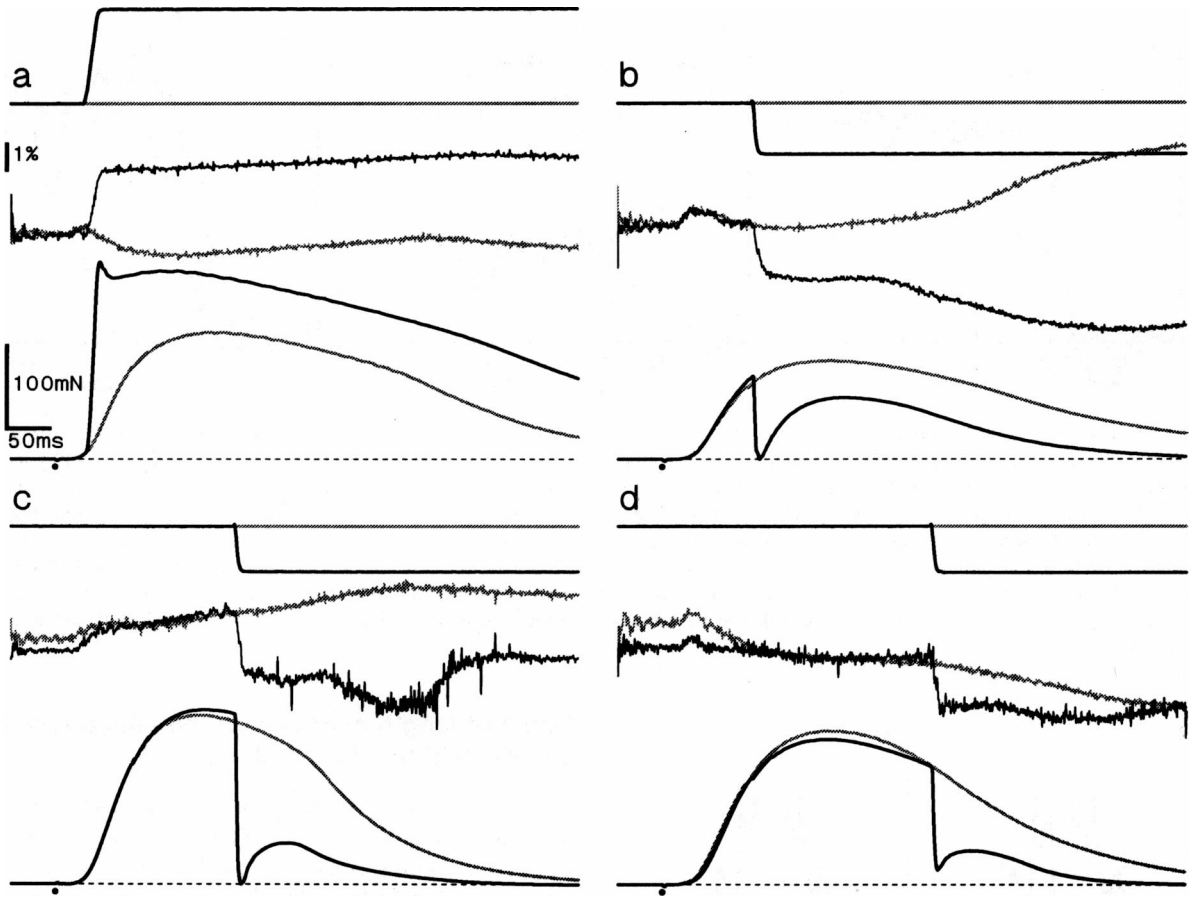

FIGURE 1 Time courses of twitch tension and the accompanying segment length change of a whole frog sartorius muscle, to which a stretch or a release was applied at various times. (*a*) ~3% stretch (duration, 15 ms) was applied at 30 ms after the stimulus (dot beneath the base line). (*b*, *c*, *d*) ~1.5% releases (duration, 5 ms) were applied at 100, 200, and 300 ms after the stimulus, respectively. Top traces, overall muscle length (displacement signal from the servo controller); middle traces, length of the segment at which x-ray was irradiated (distance between two markers attached to the surface of the muscle); bottom traces, tension. The muscle and segmental lengths are expressed as the fraction of the lengths before stimulation. Solid lines, with a stretch or a release; stippled lines, isometric control from the same muscle. All the records are from single contractions. The periodic noise due to the rotation of the polygon mirror was removed from the segmental signal by digital filtering. The apparently large noise at the beginning of the record is an artifact derived from the filtering.

In the difference diagram in Fig. 3 *a*, peaks are observed at the positions of the 1,1 reflections. On close examination, these peaks are slightly shifted toward the high angle side of the 1,1 reflections and are accompanied by smaller negative peaks on the low angle side of the 1,1 reflections. This suggests that the peaks arise because of the combined effects of the increased $I_{1,1}$, a change in the lattice spacing, and an increase in the width of the 1,1 reflections (see Figs. 5 and 6). These peaks are again less obvious in control (Fig. 3 *b*).

Effect of a release applied at various times of twitch contractions on the time course of intensity changes of the equatorial reflections

Fig. 4 shows the effect of a release on the equatorial intensities. The effect was most clearly observed when it was applied at 200 ms after the stimulus (close to the peak of the twitch tension). The release caused an increase in the $I_{1,0}$ toward the resting level, and the effect persisted for the rest of the period (Fig. 4 *b*). The $I_{1,1}$ also decreased toward the resting level, although the effect was less prominent (Fig. 4 *e*).

Fig. 3, *c* and *d* compare selected frames of the intensity profiles taken before and after the application of a release at 200 ms (taken from the frames indicated by arrowheads in Fig. 4 *b*. The $I_{1,0}$ increased substantially after the release (Fig. 3 *c*), and this is also clear from the large peaks in the difference diagram at the positions of the 1,0 reflections. In isometric control, the change of $I_{1,0}$ that occurred in the same period of time was relatively small (Fig. 3 *d*).

A release applied while the tension was already falling (at 300 ms after the stimulus) increased the $I_{1,0}$ and decreased the $I_{1,1}$ in a manner similar to the case of the release at 200 ms (Fig. 4, *c* and *f*). When a release was applied while the tension was still rising (at 100 ms after the stimulus), a similar effect was observed on the $I_{1,0}$, but the effect was transient and the time course of the $I_{1,0}$ in the rest of the period did not differ appreciably from isometric control (Fig. 4 *a*). The $I_{1,1}$

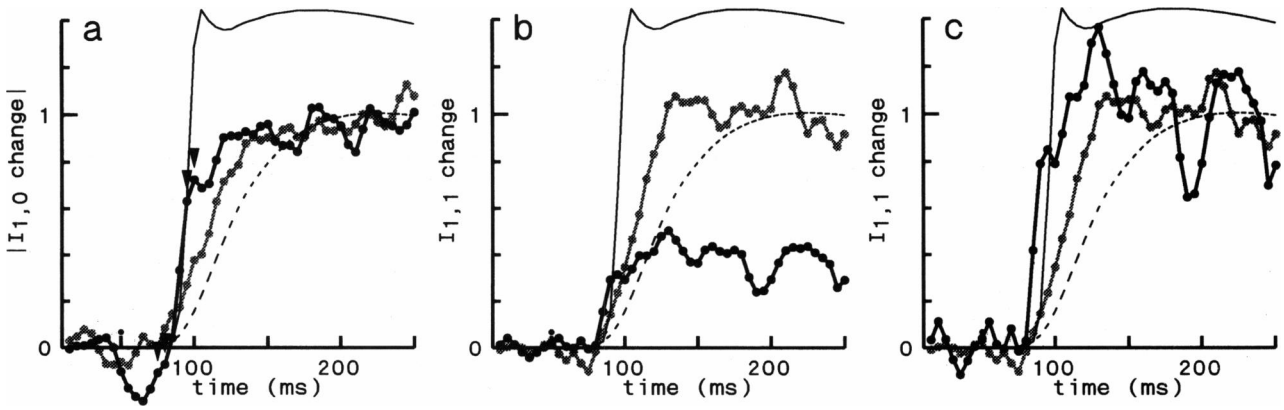

FIGURE 2 Time courses of the intensity changes of the 1,0 and 1,1 equatorial reflections during the rising phase of a twitch contraction of frog sartorius muscle, with or without a stretch at the beginning of contraction. The time resolution is 5 ms. The stretch (duration, 15 ms; amplitude, ~3%) was applied at 30 ms after the stimulation. Sum from 70 contractions of two muscles. (*a*) $I_{1,0}$. (*b*) $I_{1,1}$. In (*a*) and (*b*), the intensity changes are plotted so that they are 0 before stimulation and 1 at the peak of isometric twitch tension. The actual change of the $I_{1,0}$ is a decrease. (*c*) Same data as in (*b*), but the intensity change for stretch is also normalized to its maximal value to allow direct comparison of the time courses. The solid lines are with stretch, the stippled line without stretch (isometric control). The thin lines represent tension (solid line, with stretch; broken line, without stretch) accumulated in the CAMAC system from all contractions. These records also have 5 ms time resolution. The abscissa represents the time elapsed from the start of the x-ray recording. The stimulus was given at 50 ms from the start (dot above the abscissa). The arrowheads in the top diagram of (*a*) indicate the frames from which the intensity profiles in Fig. 3, *a* and *b* are taken.

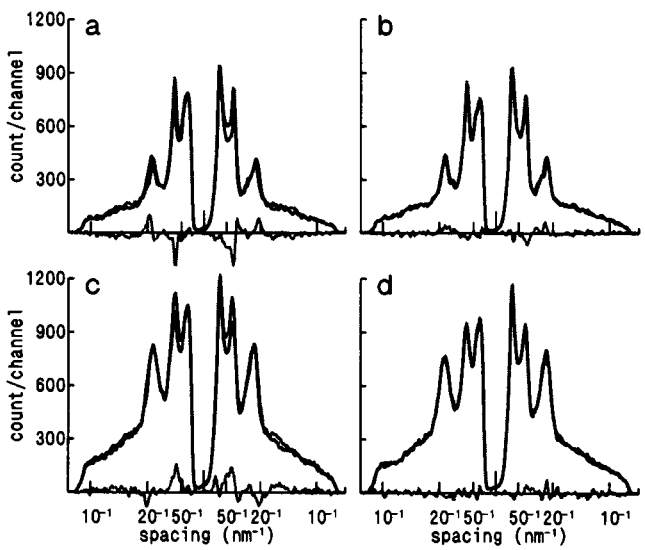

FIGURE 3 Selected frames of equatorial diffraction intensity profile. (*a*) Profiles before (stippled line) and after (solid line) the major intensity changes accompanying a ~3% stretch (duration, 15 ms) applied at 30 ms after the stimulus (Fig. 2 *a*). The frames are chosen so that the effect of stretch is most prominent. Each line represents the sum of the two frames indicated by arrowheads in Fig. 2 *a*, top. The stippled line along the abscissa is the difference in the intensities (*after* minus *before* the major intensity change). (*b*) Frames taken at the same timing as in (*a*) but from isometric control. (*c*) Profiles before (stippled line) and after (solid line) the application of a ~1.5% release (duration, 5 ms) at 200 ms after the stimulus (Fig. 4 *b*). Each line represents the sum of the two frames indicated by arrowheads in Fig. 4 *b*, top. The stippled line along the abscissa is the difference in the intensities (*after* minus *before* the release). (*d*) Frames taken at the same timing as in (*c*) but from isometric control.

increased slightly upon release (Fig. 4 *d*), in accord with the effect of a slow release applied during a tetanic plateau (Amemiya et al., 1988).

Effect of length changes on the lattice spacing and the widths of reflections

Upon length changes, the spacing of the reflections changed in a manner expected from the tendency of muscle fibers to keep their volume constant (Fig. 5). The full width at half maximum (FWHM) of the reflections tended to increase slightly upon stretch, indicating that the stretch induced an increase in lattice disorder (Fig. 6 *a*). When a release was applied at 200 or 300 ms, a small decrease of the FWHM was observed, indicating that the release slightly alleviated the lattice disorder (arrows in Fig. 6, *c* and *d*).

DISCUSSION

Interpretation of x-ray diffraction data

The present study showed that the time course of the intensity changes of the equatorial reflections are affected by a stretch or a release applied during the course of a twitch contraction. A stretch applied shortly after the stimulus had two effects: accelerated changes of the $I_{1,0}$ and $I_{1,1}$, and the reduced magnitude of the $I_{1,1}$ change at the peak of tension. A release applied at various times during a twitch generally brought the intensities (especially the $I_{1,0}$) toward the resting values, and the most prominent effect was observed when the muscle was released near the peak of the twitch.

The interpretation of equatorial diffraction data by analyzing the two innermost reflections alone (1,0 and 1,1) is limited because they are influenced by many factors. Although these reflections are most sensitive to the mass transfer between the thick and thin filaments (Elliott et al., 1963), they are also affected by the configurational changes of the myosin heads while attached to actin. The interpretation is further complicated by the changes in the spacing or the order of lattice that accompany the length change. We will discuss

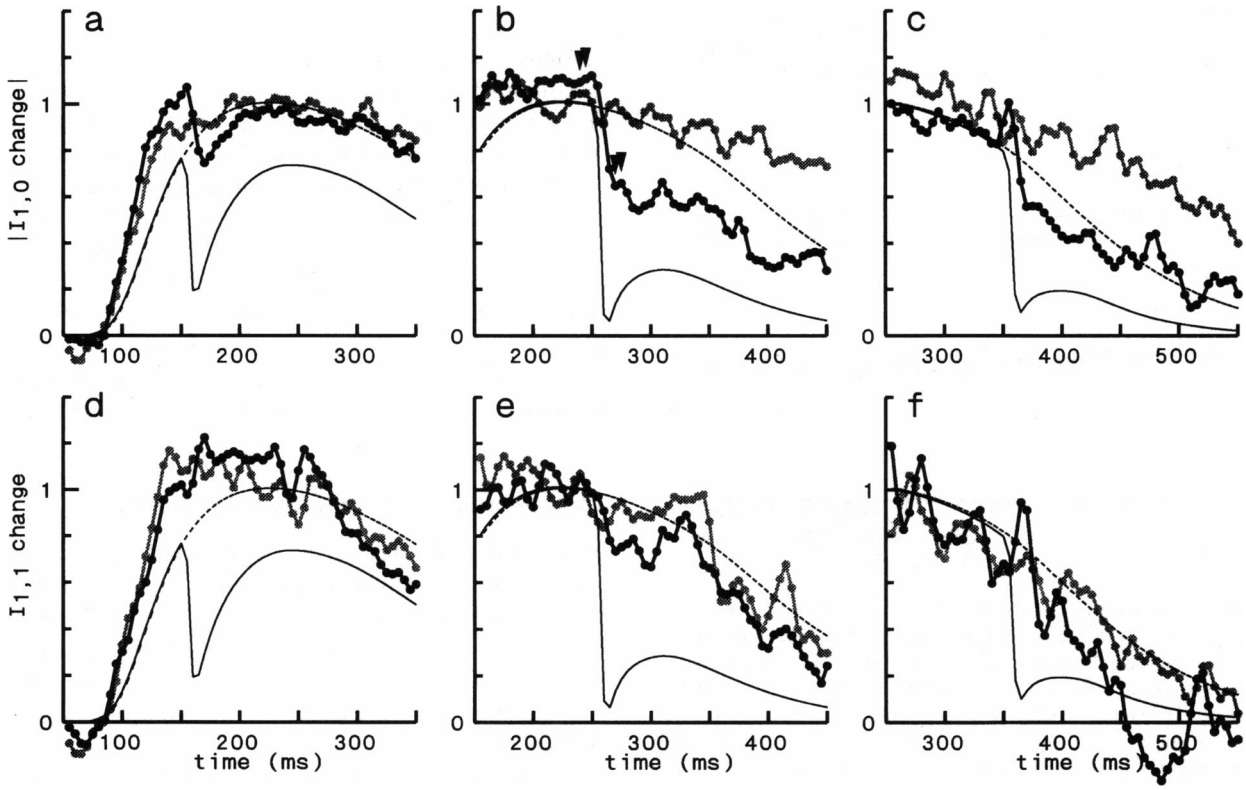

FIGURE 4 Time courses of the intensity changes of the 1,0 and 1,1 equatorial reflections during a twitch contraction of frog sartorius muscle, with or without a release applied at various times. The data are expressed in the same manner as in Fig. 2, *a* and *b*. A release (duration, 5 ms; amplitude, ~1.5%) was applied at 100 (*a*, *d*), 200 (*b*, *e*) and 300 ms (*c*, *f*). (*a*), (*b*), and (*c*) represent the $I_{1,0}$, and (*d*), (*e*) and (*f*) the $I_{1,1}$. (*a*, *d*) Total 120 contractions from four muscles. (*b*, *e*) Total 100 contractions from four muscles. (*c*, *f*) Total 90 contractions from three muscles. The solid lines are with release, the stippled lines without release (isometric control). The thin lines represent tension (solid line, with release; broken line, without release) accumulated in the CAMAC system from all contractions. The abscissa represents the time elapsed from the start of the x-ray recording. The stimulus was given at 50 ms from the start. The arrowheads in the top diagram of (*b*) indicate the frames from which the intensity profiles in Fig. 3, *c* and *d* are taken.

below the influence of each factor on the observed changes of the reflection intensities.

Lattice spacing change

A tendency of a living muscle fiber to keep its volume constant causes a change in the lattice spacing when the fiber length is altered. This affects the intensities of the equatorial reflections due to sampling at a slightly different position of the transform (the structure factor) of the unit cell. Quantitative measurements of this effect on the reflection intensities during isometric contraction were reported by Podolsky et al. (1976) and Sugi et al. (1978). Their data show that, when the sarcomere length was altered from 2.0 to 2.4 μm (20%), the $I_{1,0}/I_{1,1}$ intensity ratio increased only modestly (~10% in the case of Sugi et al., 1978). Although the sarcomere length dependence of intensities is relatively large in resting muscle, it is less marked in a contracting muscle. It is possible that the structure factor of the unit cell in contracting muscle also changes with sarcomere length, resulting in compensation for the intensity changes due to the alteration of a sampling point. Therefore, the observed large changes of the equatorial reflection intensities upon length changes of no more than 3% seem to be too large to be accounted for by altered sampling.

Lattice disorder

When a muscle is activated, the widths of the equatorial reflections increase. The widths are further affected by a stretch or release applied to a contracting muscle (Fig. 6). The increase is larger for the 1,1 reflection than for the 1,0 (data for the 1,0 not shown). Two types of disorder would be involved: disorder due to small dispersity of the sarcomere lengths in different myofibrils, and the second kind or liquid-like disorder of lattice. Both types of disorder cause a widening of reflections with increasing order of reflection. Dispersity of the sarcomere length causes a distribution in lattice spacing, making the width of reflections increase linearly with the order of reflection (see Yu et al., 1985). According to Yu et al. (1985), the standard deviation of the resulting width distribution is a few percent within a contracting muscle fiber. Thus, disorder of this extent, even if it is increased by length changes applied to a contracting muscle, is not expected to affect the integrated intensities significantly. Another type of disorder, i.e., the second kind or liquid-like disorder, widens the reflection width and decreases the peak height proportionately to the square of the order of reflection. However, the integrated intensities of each innermost reflection should remain unchanged provided the background intensity is estimated correctly (for detailed

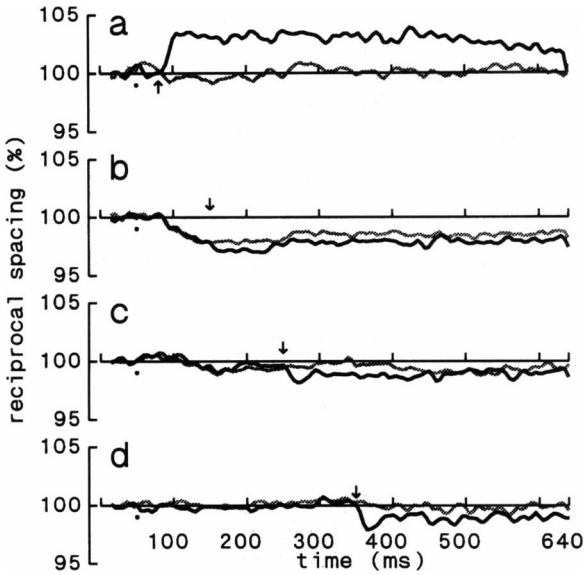

FIGURE 5 Time courses of the myofilament lattice spacing changes in frog sartorius muscle during a twitch, measured as the separation of the two centroids of the 1,1 reflections in the reciprocal space. Expressed as a percentage of the value before the stimulus (dot). Solid line, with a stretch or a release; stippled line, isometric control. (*a*) Stretch (duration, 15 ms; amplitude, ~3%) applied at 30 ms after the stimulus. (*b, c, d*) Release (duration, 5 ms; amplitude, ~1.5%) applied at 100, 200, and 300 ms after the stimulus, respectively. The timing of the length change is indicated by an arrow.

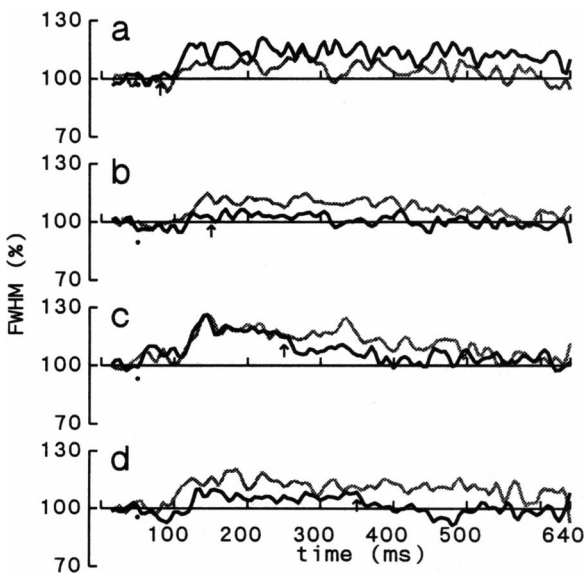

FIGURE 6 Time courses of the changes of the FWHM of the 1,1 reflection in frog sartorius muscle during a twitch. The timing of the stimulus is indicated by a dot. Stippled lines represent isometric control. (*a*) Stretch (duration, 15 ms; amplitude, ~3%) applied at 30 ms after the stimulus. (*b, c, d*) Release (duration, 5 ms; amplitude, ~1.5%) applied at 100, 200, and 300 ms after the stimulus, respectively. The timing of the length change is indicated by an arrow.

explanation, see Vainshtein, 1966). To summarize, the observed intensity changes seem to be too large to be ascribed to increased lattice disorder.

Configurational changes of myosin heads

Various types of configurational changes are conceivable. Theoretically, a certain type of configurational change can result in reciprocal changes of the $I_{1,0}$ and $I_{1,1}$ (e.g., Lymn, 1978), but such configurational change has not been proven in real muscles. Non-reciprocal changes of the $I_{1,1}$ and $I_{1,0}$ may occur as a result of structural changes. During the rising phase of the contraction of fish muscle, the decrease of the $I_{1,0}$ shows a substantial lag behind the $I_{1,1}$ (Harford and Squire, 1992). This lag was ascribed to a type of configurational change of the myosin heads. However, involvement of such a configurational change is unlikely in frog muscle, because the lag of the $I_{1,0}$ behind the $I_{1,1}$ is much smaller.

Mass transfer between thick and thin filaments

This refers to the movement of myosin heads leaving the thick filament backbone and moving toward actin upon activation and vice versa upon relaxation. Nearly reciprocal changes of the equatorial reflections will ensue as has been formulated by Elliott et al. (1963), and the intensity changes induced by a stretch applied at the beginning of a twitch were in fact reciprocal.

These considerations leave an accelerated mass transfer as a most likely explanation for the observed effect of stretch. However, this accelerated movement of the myosin heads may not be the direct effect of the stretch. It would be natural to consider that the primary effect of the stretch is on attached, low-force heads rather than on detached ones. If the stretch converts the low-force myosin heads to high-force ones, the equilibrium between the detached and attached low-force heads will shift toward the latter.

The reduced magnitude of the $I_{1,1}$ change may be due to the effect of reduced filament overlap (Elliott et al., 1963) and the direct effect of a slow stretch (Amemiya et al., 1988). The latter tends to decrease the $I_{1,1}$ more than to increase the $I_{1,0}$. As the stiffness of muscle fibers increases upon stretch (Haugen and Sten-Knudsen, 1987), it is unlikely that the reduced magnitude of the $I_{1,1}$ is caused by a decreased number of attached myosin heads. Possibly some kind of configurational change could be involved. A similar configurational change could also be involved in the skinned muscle fibers treated with a zero-length crosslinker, given that the $I_{1,1}$ decreases preferentially on application of a quick stretch (Iwamoto and Podolsky, 1993).

A release applied at the peak of the twitch or later had clear effects on the equatorial intensities. This is in marked contrast to the ineffectiveness of a quick release applied at the plateau of a tetanic contraction (Huxley et al., 1981; 1983; Irving et al., 1992), and the effect of a slow release applied at the plateau of a tetanic contraction (Amemiya et al., 1988). In the latter case, the release increases the $I_{1,1}$ without affecting the $I_{1,0}$. In the later phase of relaxation, it is known that sarcomere length inhomogeneity develops as detected by laser beam diffraction (Edman and Flitney, 1982). The clearest response of the equatorial reflections was obtained when

the release was applied at the peak of the twitch, i.e., before such sarcomere length inhomogeneity could have developed.

The release applied at the peak of a twitch increased the $I_{1,0}$ prominently and decreased the $I_{1,1}$ to a smaller extent. If the effect of a slow release to increase the $I_{1,1}$ (Amemiya et al., 1988) also operates, it would cancel the reduction of the $I_{1,1}$ toward the resting level. It is likely, therefore, that the intensity changes induced by the release are essentially reciprocal and reflect the detachment of the myosin heads from the thin filament. Still, the effect of the release on the reflection intensities was smaller than that on tension. This suggests that the detached myosin heads stay in the vicinity of the thin filament before they finally return to the thick filament backbone.

The fact that the effect of a release applied at 200 or 300 ms after the stimulus persisted for the rest of the period of contraction (Fig. 4, *b* and *c*) suggests that relatively few of the detached heads can rebind to actin. A release applied during the rising phase of a twitch (100 ms after the stimulus) was followed by a substantial redevelopment of tension. At the same time, the effect of the release was transient and smaller in amplitude than that of releases applied later. In this case, the detached heads are probably allowed to rebind to actin. Amemiya et al. (1980) also reported the ineffectiveness of a quick release applied during the rising phase of a twitch.

Regulation of myosin head attachment during a twitch contraction

The difference between the time course of isometric twitch tension and that of the "active state" was a basic question raised by A. V. Hill (1949). His argument was based on the presence of a relatively large series elastic component (SEC) and the hyperbolic force-velocity relation (Hill, 1938), which was assumed to be established in any instant. It has been shown, however, that the amount of the SEC is much smaller than he thought originally (0.4%, Ford et al., 1977, versus 10%, Hill, 1949), and that a steady force-velocity relation is not immediately established during the rising phase of tension (Iwamoto et al., 1990c). Rather, it should be considered that the activity of the contractile machinery is sensitive to externally imposed length changes, and its sensitivity depends on the concentration of intracellular free calcium ($[Ca]_i$).

Contraction of an intact muscle is initiated by the release of calcium from the sarcoplasmic reticulum. Subsequent binding of calcium to troponin C (TnC) turns on the thin filaments (see Ebashi and Endo, 1968). The $[Ca]_i$ reaches a peak much earlier than the peak of tension, and it has already fallen to a low level by the time when the tension reaches a peak (Blinks et al., 1978). After the stimulus, a series of x-ray reflections change their intensities in the following order: second actin layer line, 5.9-nm actin layer line, 42.9-nm myosin layer line, and equatorial reflections (Huxley, 1975; Matsubara and Yagi, 1978; Wakabayashi et al., 1985, 1991; Kress et al., 1986; Cecchi et al., 1991). It is likely that the increase in the 5.9-nm layer line intensity is due to the accretion of the myosin head to the thin filament (Wakabayashi et al., 1991). The rise of stiffness measured with ultrasonic waves rises 40–50 ms ahead of tension rise (Hatta et al., 1988), and it could be as fast as the 5.9-nm reflection. Stiffness measured with conventional methods is 3–4 ms behind the equatorial reflections, but is still ahead of tension rise (e.g., Cecchi et al., 1982; Ford et al., 1986). Taking these observations together, it is inferred that, after the thin filament is turned on, the myosin heads attach to it in a low-force, low-stiffness form. The observed acceleration of the reciprocal changes of the $I_{1,0}$ and $I_{1,1}$ upon stretch is explained if the stretch accelerates the conversion of the attached myosin heads from the low-force, low-stiffness form to a high-force, high-stiffness form, because this conversion is expected to shift the equilibrium between the detached and attached, low-force populations toward the latter. This mechanism would explain the concomitant increase of stiffness upon stretch (Haugen and Sten-Knudsen, 1987), whereas the calcium transient is not affected (Haugen, 1991).

The high tension with a reduced level of $[Ca]_i$ at the peak of a twitch (Blinks et al., 1978) may involve the cooperative binding of myosin heads to actin (Bremel and Weber, 1972; Hill et al., 1980). This mechanism is expected to keep the thin filament "on" even after calcium binding to TnC has reduced to subsaturating levels, causing a substantial delay in the tension peak (and therefore the changes of the equatorial reflections) behind the peak of the calcium transient. A release would then reduce the number of the attached myosin heads, turning off the thin filament more quickly and leading to an accelerated return of the equatorial reflections toward their resting levels. This view is also supported by the observation that the presence of caffeine, which promotes the release of calcium from the sarcoplasmic reticulum, reduces the depressant effect of a release (Edman, 1980).

An interesting development in the recent research on calcium regulation is that the control is bidirectional: evidence is accumulating that the myosin head attachment to actin also controls the calcium binding to TnC. A release of aequorin-injected barnacle muscle fibers during the relaxation phase causes an emission of extra light, suggesting that the affinity of the "activating site" for calcium is reduced (Ridgway and Gordon, 1984; Gordon and Ridgway, 1987). Introduction of fluorescently-labeled TnC to skinned rabbit fibers suggests that the presence of rigor or active myosin heads alters the structure of TnC so as to increase its affinity for calcium (Gordon et al., 1988). It is possible that this mechanism also operates in frog muscle and contributed to the present results.

In summary, the regulation of muscle contraction seems to occur in two steps. The first step is the turning on of the thin filaments by calcium, which allows the myosin heads to move to the vicinity of thin filaments, leading to accumulation of attached low-force heads. The second step is the conversion of the myosin heads from low-force to high-force forms. It is likely that this conversion is accelerated by a stretch, leading to an abrupt rise of tension as observed by A. V. Hill (1949). In the later phase of contraction, a release of a muscle is likely to accelerate the relaxation through the loss of the cooperative binding of the myosin heads and a subsequent reduction of the TnC affinity for calcium.

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