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TIME-RESOLVED X-RAY DIFFRACTION STUDIES OF FROG SKELETAL MUSCLE ISOMETRICALLY TWITCHED BY TWO SUCCESSIVE STIMULI USING SYNCHROTRON RADIATION

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In order to clarify the delay between muscular structural changes and mechanical responses, the intensity changes of the equatorial and myosin layer-line reflections were studied by a time-resolved X-ray diffraction technique using synchrotron radiation. The muscle was stimulated at 12–13°C by two successive stimuli at an interval (80–100 ms) during which the second twitch started while tension was still being exerted by the muscle. At the first twitch, the intensity changes of the 1,0 and 1,1 equatorial reflections reached 65 and 200% of the resting values, and further changes to 55 and 220% were seen at the second twitch, respectively. Although the second twitch decreased not only the time to peak tension but also that to the maximum intensity changes of the equatorial reflections (in both cases, about 15 ms), the delay (about 20 ms) between the intensity changes and the development of tension at the first twitch were still observed at the second twitch. On the other hand, the intensities of the 42.9 nm off-meridional and the 21.5 nm meridional myosin reflections decreased at the first twitch to the levels found when a muscle was isometrically tetanized, and no further decrease in their intensities was observed at the second twitch. These results indicate that a certain period of time is necessary for myosin heads to contribute to tension development after their arrival in the vicinity of the thin filaments during contraction.

1. Introduction

Vertebrate skeletal muscle exhibits two prominent equatorial reflections, which arise from the $(10\bar{1}0)$ and $(11\bar{2}0)$ planes of the hexagonal array of thick and thin filaments. They are simply called the 1,0 and 1,1 reflections, respectively. When the muscle contracts, the intensity of the 1,0 reflection, $I(1,0)$ decreases by about 50%, while that of the 1,1 reflection, $I(1,1)$ increases about 2-fold. This phenomenon has been interpreted as being due to mass transfer from the thick to the thin filaments, suggesting that the change in intensities might represent the number of cross-bridges formed between the thick and thin filaments [1–6]. Recent time-resolved X-ray diffraction studies have been shown, however, that the intensity changes occur before tension changes [1,2,7–10]. The results suggest that a certain period of time is needed for the myosin heads to contribute to tension development after their arrival in the vicinity of thin filaments. The time lag may include the time required for changes in conformation and/or orientation of myosin heads in order to generate tension [1,2]. In addition, Yagi et al. [11] found that there was a difference between the time course of the fall of tension and that of the return of the equatorial intensity to the resting level. The slow return of the intensity also suggests that myosin heads in the vicinity of the thin

filaments might not be attached to actin or if they are still attached, might not produce tension. Thus, these results indicate that the intensity change in the equatorial reflections might not directly give the number of myosin heads contributing to tension development.

On the other hand, the myosin off-meridional layer-lines with the basic period of 42.9 nm arise in large part from the helical array of myosin heads around the thick filaments. In particular, the intensity change of the strong 42.9 nm layer-line may offer information about the number of myosin heads out of the helical (resting) positions during contraction [3,8,12].

Experiments were undertaken to clarify the delay between the equatorial X-ray intensity change and tension development. The muscle was stimualted by two successive stimuli so as to make the second twitch start while the muscle was still exerting tension. We expected the effects of the series elastic components and of internal shortening on tension development to be less at the second twitch than at the first [13–15].

2. Materials and methods

2.1. Muscle specimens and the double twitch method

The sartorius muscle of the bullfrog (*Rana catesbeiana*) (4.5–6 cm long and about 1 mm thick) was mounted in a specimen chamber with two Mylar windows to allow X-rays through and stimulated with a multi-electrode assembly. The pelvic end of the muscle was clamped to the chamber while the tibial end was connected to a force transducer (Shinkoh Co., Tokyo, type UT). The sarcomere length of the muscle was adjusted to about 2.3 μm by He-Ne laser light diffraction. The muscle was continuously perfused with Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, pH 7.2, adjusted with $NaHCO_3$) and stimulated by two successive pulses (3 ms duration) to produce isometric contraction.

When the interval of the two successive stimuli was less than the tension fusion interval (40 ms under the present conditions), supernormal changes occurred in tension in response to the second stimulus. But equatorial X-ray intensity responses to the second twitch were only very slightly supernormal, making it difficult to determine accurately the time to the maximum intensity change at each twitch. When the stimulus interval was longer than the tension fusion interval, both the tension increment and intensity changes of equatorial reflections in response to the second twitch could be clearly discerned from those in response to the first one (see fig. 2). Since tension development and intensity changes of equatorial reflections also depended upon the temperature, the stimulus interval and temperature were chosen to enable clear observation of both the tension increment and the equatorial intensity changes in response to the second stimulus.

Contractions were repeated 60 times for each muscle at a 10 s interval. After the experiments, the muscles were blotted and weighed to calculate tension per cross-section area as $P_0 l/m$, where P_0, l and m are the maximum tetanic tension, muscle length and blotted weight, respectively. The calculated values ranged from 2.0 to 2.6 kg/cm^2, and the peak tension at the second twitch ranged from 60 to 70% of the tetanic tension.

2.2. X-ray diffraction

A focused and monochromatized X-ray beam of wavelength 0.15 nm was produced by a double-focusing camera [19,20] from synchrotron radiation of a storage ring at the Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan. The storage ring was operated at an energy of 2.5 GeV with the beam current between 70 and 150 mA. This system has also been used in other experiments on muscular contraction [21,22]. The X-ray diffraction pattern was recorded with a 20 cm long linear position-sensitive proportional counter (Rigaku Denki, Tokyo), giving a spatial resolution of about 0.33 mm. The signals of the one-dimensional patterns were stored as a function of time in a CAMAC memory (LeCroy, Research System S.A., Geneva, Switzerland, 3588) linked to a computer (Micro 11/F23, Automation system Research, Japan). The memory was divided into 60 frames of 256 channels. Singals from 60 contractions were accumulated in

the relevant frame to obtain diffraction patterns with reasonable counting statistics. A shutter in front of the specimen interrupted the X-ray beam except during the period of data collection so that the radiation damage could be minimized. In the present experiments, the exposure time for one specimen did not exceed 40 s.

Data analysis was carried out on the computer using an interacting graphic facility [19]. The times to the peak tension and to the maximum X-ray intensity change were determined by calculating a polynomial fit through data points around their peak values using a standard least-square fitting routine.

3. Results

3.1. Time courses of changes in tension and equatorial X-ray intensities

Fig. 1 shows the relationship between the levels of the two peak tensions and the times to peak tensions at each twitch for the various stimulus intervals at 13°C. The level of peak tension is expressed as the ratio of the peak tension (F_2) at the second twitch to that (F_1) at the first one, while Δt denotes $T_1 - T_2$, where T_1 and T_2 are the times to the peak tensions at each twitch measured from each onset of the stimulus as shown in fig. 2. Fig. 1 shows that as the stimulus interval was increased, both Δt and the tension ratio (F_2/F_1) decreased. Thus, the time to the peak tension at the second twitch (T_2) became shorter as the stimulus interval decreased, suggesting that the effects of series elastic compliance and of internal shortening on tension development can be reduced by applying the second twitch at a suitable stimulus interval to muscles that are exerting tension.

Based on the results shown in fig. 1, the stimulus interval (80–100 ms) and temperature (around 13°C) were chosen to enable clear observation of both the tension increment and the equatorial X-ray intensity changes in response to the second stimulus.

The muscles were stimulated by two successive stimuli at an interval of 80 ms at 13°C. The time

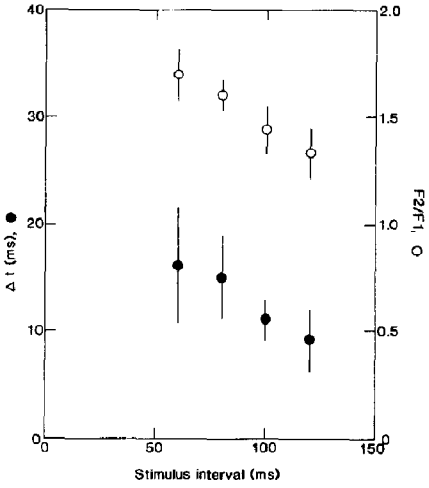

Fig. 1. Changes in level of peak tension and time to peak tension with various stimulus intervals. The muscles were stimulated by two successive pulses at various intervals at 13°C. Tension is expressed as the ratio of peak tension at the second twitch (F_2) to that at the first twitch (F_1). Δt ($= T_1 - T_2$) denotes the time difference between two peak tensions, where T_1 and T_2 are the times to peak tensions at each twitch measured from each onset of stimulus (see fig. 2). Each data point represents a mean value (±S.D.) from nine muscles ($N = 9$). T_1 was about 70 ms (see table 1).

courses of the intensity changes of the 1,0 and 1,1 equatorial reflections measured with a 4 ms time resolution are shown in fig. 2, together with the isometric tension record. Each data point was an average of the data on seven different muscles. Each muscle was contracted 60 times, and the peak tension at the 60th twitch was about 80% of that of the first. Tension started to increase at approx. 10 ms after the onset of the first stimulus ($t = 0$ in fig. 2B) and reached its first peak at 70.8 ± 2.0 ms (mean ± S.D.) (T_1). $I(1,0)$ and $I(1,1)$ also started to change approximately at the same time as the onset of tension development. $I(1,0)$ and $I(1,1)$ reached their first maximum levels at 51.6 ± 5.3 and 51.6 ± 3.2 ms, respectively. Thus, at the first twitch, the intensity changes reached the maximum levels about 20 ms earlier than the peak of the first twitch tension. The difference was statistically significant ($P < 0.1$, $N = 7$). At the second twitch, the tension peaked at 56.5 ± 4.3 ms (T_2) after the onset of the second stimulus. The

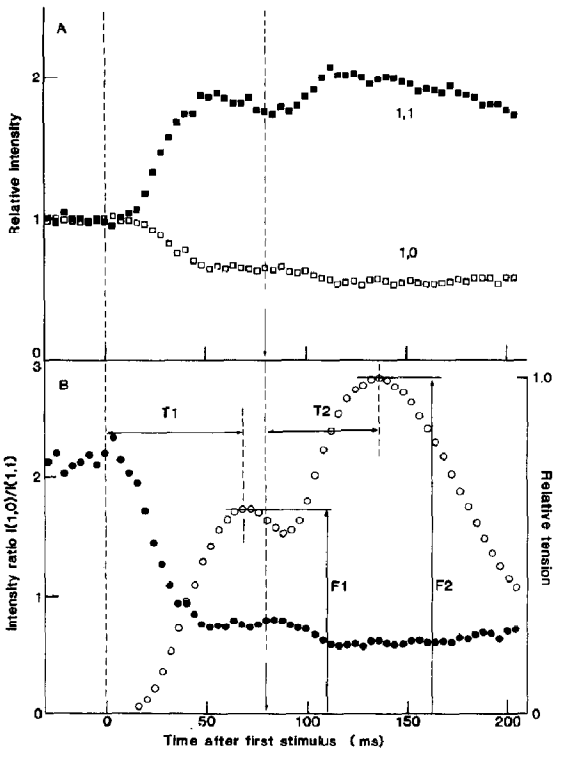

Fig. 2. Time courses of changes in equatorial reflections and tension when the muscles were stimulated by two successive pulses at an interval of 80 ms at 13°C. The first stimulus was given at $t = 0$ and the second at the time shown by the vertical arrow with a dashed line. The entire period of data collection (240 ms) was divided into 60 frames of 4 ms duration. The counter with an entrance slit of 8 mm width was placed on the equator, and measurement was made in the range of the reciprocal radial coordinate of $R \lesssim 0.011$ nm^{-1}. Each data point represents a mean value from experiments on seven muscles. In A, $I(1,0)$ (□) and $I(1,1)$ (■) denote the intensities of the 1,0 and 1,1 reflections, respectively. They are expressed relative to the averaged resting values obtained in each experiment. In B, $I(1,0)/I(1,1)$ (●) denotes the intensity ratio of these reflections, and the isometric tension record (○) is expressed relative to the peak tension of the second twitch of each muscle. Each point is an average from seven muscles. Notations of T_1, T_2, F_1 and F_2 defined in the legend to fig. 1 are illustrated.

second stimulus caused significant decrease in the time ($\Delta t \sim 15$ ms) to the peak tension ($P < 0.1$, $N = 7$). On the other hand, $I(1,0)$ reached a maximum level at 37.2 ± 3.9 ms, the value for $I(1,1)$ being 36.4 ± 2.5 ms after the onset of the second stimulus. Therefore, the second twitch decreased not only the time to peak tension but also that to the maximum intensity change of the equatorial reflections by about 15 ms. These results are summarized in table 1. It should be noted here that nearly the same delay between the structural change and the mechanical response was still observed at the second twitch.

3.2. Relationship between equatorial X-ray intensity changes and tension levels

As shown by fig. 2A, $I(1,0)$ decreased to 0.651 $\pm$ 0.06 of the resting value at the first twitch and even further to 0.543 ± 0.04 in response to the summated tension response. On the other hand, $I(1,1)$ increased to 2.06 ± 0.22 of the resting value at the first twitch and further to 2.24 ± 0.20 at the second twitch. Although the value of F_2/F_1 was in the range 1.5–1.7 (see fig. 1B), the second stimulus did not cause graded changes in the equatorial intensities, suggesting that the intensities are not a linear function of the isometric tension (see ref. 23 and table 2). The change in intensity ratio, $I(1,0)/I(1,1)$ is also given in fig. 2B and table 2 to aid comparison of the present results with those of other reports. Before stimulation, the intensity ratio was 2.14 ± 0.22. The minimum of the intensity ratio attained at the first twitch was 0.675 ±

Table 1

Time constants of equatorial X-ray intensity changes and tension during two successive twitches

The values listed are the times (mean ± S.D.) needed to reach maximum changes after the the onset of each stimulus. $I(1,0)$ and $I(1,1)$ represent the intensities of the 1,0 and 1,1 equatorial reflections, respectively. The delay, ΔD, denotes the time difference between peak tension and maximum intensity changes. Each value is the mean of seven values.

	First twitch (ms) (1)	Second twitch (ms) (2)	Difference (ms) (1)–(2)
$I(1,0)$	51.6 ± 5.3	37.2 ± 3.9	14.4
$I(1,1)$	51.6 ± 3.2	36.4 ± 2.5	15.2
$I(1,0)/I(1,1)$	52.4 ± 2.5	35.6 ± 3.2	16.8
Tension	70.8 ± 2.0	56.5 ± 4.3	14.3
ΔD	19.2	19.7	

Table 2

Equatorial X-ray intensity changes and tension levels at two successive twitches

Intensities (mean ± S.D.) are expressed relative to the resting values. $I(1,0)/I(1,1)$ denotes the intensity ratio of the 1,0 and 1,1 reflections. Tension is expressed relative to the peak value of the first twitch. The asterisk corresponds to F_2/F_1 (see fig. 2B). Each value is the mean of seven values.

	First twitch	Second twitch
$I(1,0)$	0.651 ± 0.06	0.543 ± 0.04
$I(1,1)$	2.059 ± 0.22	2.237 ± 0.20
$I(1,0)/I(1,1)$	0.675 ± 0.06	0.521 ± 0.07
Peak tension	1.0	1.59 ± 0.14 *

0.06 and that at the second twitch was 0.521 ± 0.07. The intensity ratio of the minimum level at the second twitch was not significantly different from the values obtained obtained for the muscles during the maximum isometric tetanus [11,23]. $I(1,0)$ and $I(1,1)$ showed a small recovery between the two twitches, resulting in a small increase in $I(1,0)/I(1,1)$.

3.3. Change in reflection widths during two successive twitches

Fig. 3 shows that the changes in integral widths of $I(1,0)$ and $I(1,1)$ during two successive twitches. A very small decrease in integral widths was seen at the beginning of contraction by the first stimulus in both reflections. However, the widths remained approximately constant until the beginning of relaxation of the second twitch and then returned to their resting values. This finding indicates that changes in $I(1,0)$ and $I(1,1)$ caused by the second twitch were not brought about by a change in the filament order.

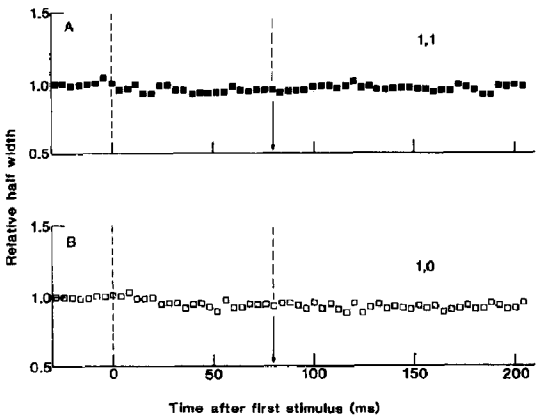

Fig. 3. Changes in integral widths of the equatorial reflections in fig 2A during two successive twitches. The integral width was calculated by dividing the peak area above the background by its peak height. (A) The 1,0 reflection, (B) the 1,1 reflection.

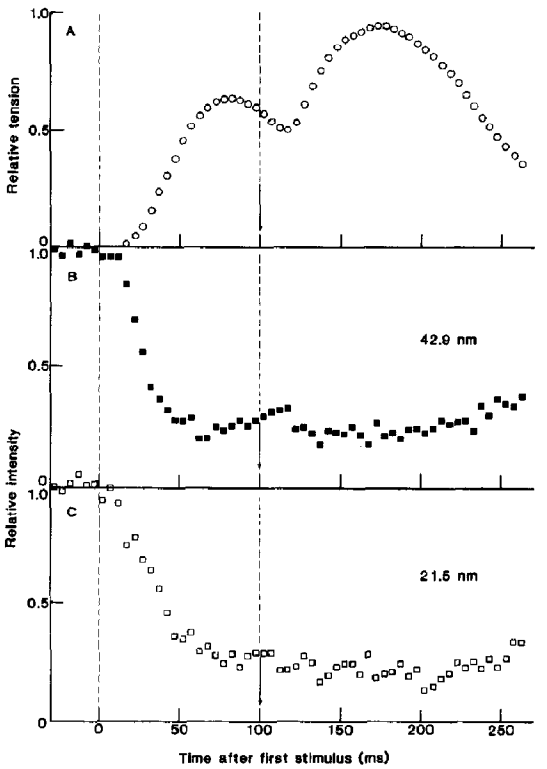

Fig. 4. Time courses of the changes in tension and intensities of the 42.9 nm off-meridional and the 21.5 nm meridional myosin reflections when the muscles were stimulated by two successive pulses at an interval of 100 ms at 12°C. The first stimulus was given at $t = 0$ and the second at the time shown by the vertical arrow. The entire period of data collection (300 ms) was divided into 60 frames of 5 ms duration. For the intensity measurement of the 42.9 nm reflection, the counter with an entrance slit of 8 mm width was placed apart from the meridian with its long axis parallel to the meridian. Measurement was done in the range of radial coordinates of 0.036 $nm^{-1} \lesssim R \lesssim$ 0.058 nm^{-1}. To measure the 21.5 nm reflection, the counter with the entrance slit of the same width was set on the meridian. Each data point represents a mean value from five experiments on four muscles. (A) Tension, (B) intensity of the 42.9 nm reflection, (C) intensity of the 21.5 nm reflection.

3.4. Intensity changes in the myosin layer-line reflections

Fig. 4 shows the time courses of the intensity changes in the 42.9 nm off-medidional myosin layer-line reflection (B) and the 21.5 nm meridional reflection (C), together with the tension record (A). They were measured with a 5 ms time resolution. The muscles were stimulated by two successive pulses at an interval of 100 ms at 12°C. The intensity of the 42.9 nm reflection started to decrease at the first twitch and reached an intensity level of 0.26 ± 0.03 (mean $\pm$ S.D.) of the resting value. The intensity showed a small recovery between the two twitches, but the minimum level reached at the second twitch was nearly equal to that at the first twitch (0.23 ± 0.04). The difference between the two minima was statistically insignificant ($P \approx 10$, $N = 5$). Thus, in spite of a definite difference in peak tensions between the first and second twitches, the second stimulus did not cause further decrease in the intensity of the 42.9 nm reflection. The intensity of the 21.5 nm meridional reflection also decreased at the beginning of contraction to less than 20% of the resting value, where it remained during the first and second twitches, before recovering when the contraction was over.

Fig. 5 shows the intensity changes of subsidiary peaks on several myosin layer-lines, measured by setting the counter parallel to the meridian at the off-meridional position. Subsidiary peaks also started to decrease on the first stimulus and they began returning to their resting levels with the onset of relaxation of the second twitch.

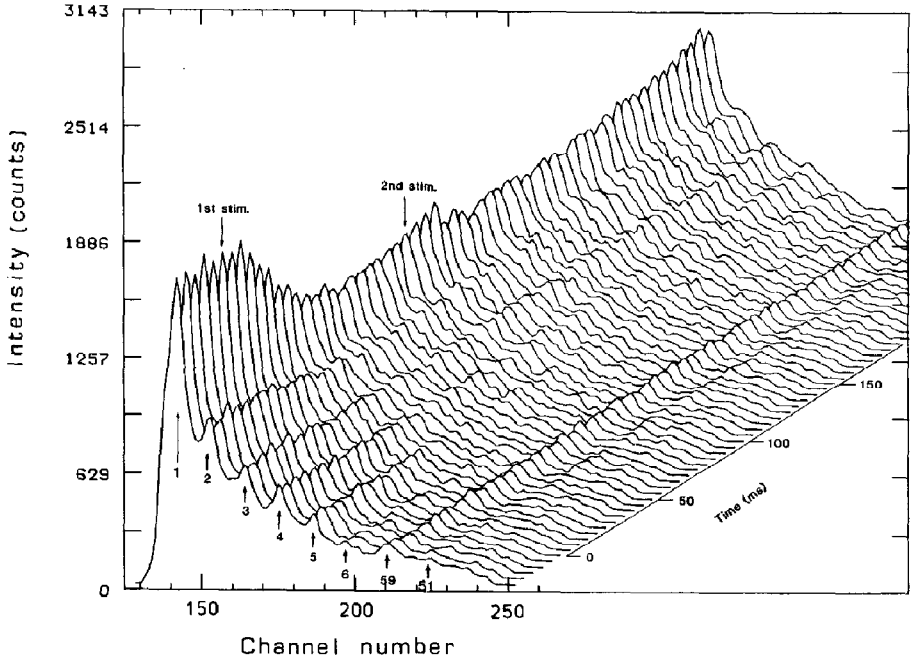

Fig. 5. Time-resolved X-ray diffraction diagram of the off-meridional myosin reflections during two successive twitches. The muscles were stimulated by two pulses at an interval of 100 ms at 12°C. The diffraction patterns were measured by setting the counter with its long axis parallel to the meridian at the off-meridional position around $R \sim 0.05$ nm^{-1}. The conditions for data collection were the same as in fig. 4. The peaks indicated in the first frame by 1–6 are reflections on the myosin layer-lines corresponding to the 1st–6th orders of the 42.9 nm periodicity. The values 5.9 and 5.1 denote the 5.9 nm and 5.1 nm actin layer-line reflections (see ref. 21).

4. Discussion

4.1. Time to peak tension at two twitches

As mentioned in section 1, the internal shortening and the series elastic compliance would slow the development of tension under the externally isometric conditions used in the present experiment. Even if the series compliance other than that within the sarcomeres were eliminated, the remainder, residing in the contractile elements, may still be substantial. However, as suggested by Hill [13,14], the effect of series compliance on tension development can be largely reduced by applying a suitable stretch to a muscle before or during the early stages of contraction. This saves the contractile components from wasting time in stretching the series compliance to the peak tension which the muscle can exert. Therefore, a second twitch can be expected to reduce the time to the peak tension (T_2). In fact, as shown in fig. 1, T_2 is a function of the stimulus interval between two twitches. In the present experiments, T_2 ($\sim$ 55 ms) was about 15 ms shorter than T_1 ($\sim$ 70 ms). The results in fig. 2 and table 1 suggest that the effects of series elastic compliance and of internal shortening on tension development decreased upon application of the second twitch. However, the delay between the time courses of changes in the equatorial intensities and tension was still substantial. At the second twitch, the structural change occurred about 20 ms before the tension response, and the magnitude of the delay was nearly equal to that observed at the first twitch.

4.2. Cross-bridge behavior during two successive twitches

As seen in fig. 4B, the intensity of the 42.9 nm off-meridional myosin reflection decreased to 0.257 ± 0.03 of the resting value at the first twitch, and no further decrease in the intensity (0.228 ± 0.04) was observed at the second twitch ($P \approx 10$, $N = 5$). Since the intensity decreases to a similar level when a muscle is isometrically tetanized [8,12], the intensity probably reaches the minimum level at the first twitch. This suggests that the intensity of the 42.9 nm reflection would be sensitive to the tension change even at lower levels (e.g., less than $0.5P_0$, where P_0 is the tension level attained at the isometric tetanus). Therefore, it is unlikely that most of the myosin heads return to the resting positions and then move again towards the vicinity of the thin filaments during two successive twitches. Although a slight recovery in intensity of the 42.9 nm reflection was seen between two twitches, most of the myosin heads probably remain in the vicinity of the thin filaments until the end of the second twitch. This agrees with the observations that no appreciable intensity recovery of both the 21.5 nm meridional and subsidiary myosin reflections was found until the end of the second twitch (see figs. 3C and 5). Yagi et al. [11] also found that the intensity recovery of the equatorial reflections to the resting level was slower than the tension decay.

4.3. Time delay between structural and tension changes

As mentioned above, if we assume that most of the myosin heads are in the vicinity of the thin filaments during two successive twitches, the fact that the same delay also occurred at the second twitch indicates that a large part of the time lag (about 20 ms, see table 1) is needed for the change in conformation and/or orientation of myosin heads before tension development after their arrival in the vicinity of the thin filaments, as was suggested by Huxley et al. [8].

As shown in fig. 4B, about 20–25% of the original resting intensity remained on the 42.9 nm reflection during contraction (i.e., about 45–50% of the resting amplitude). Concerning this, two extreme possibilities were suggested [8]. One is that about 50% of the total myosin heads still occupy positions in the resting muscle. The other possibility is that most of the myosin heads are in the vicinity of the thin filaments and a portion of the myosin projections (e.g., the S_2 part) conform approximately to the regular helical symmetry to give rise to the residual intensity. In any case, the present results indicate that nearly equal amounts of myosin heads are affected by the two twitches in spite of a definite difference in peak tension between the first and the second twitches. There-

fore, at the first twitch, some of the myosin heads which moved towards the vicinity of the thin filaments probably contributed to tension development, and at the second twitch, the number of myosin heads contributing to tension generation increased, producing a tension increment. Myosin heads have recently been suggested to be attached to actin in a weekly bound state, subsequently undergoing transition to a strongly bound state to generate tension [24]. This case would suggest that the time lag is needed to change the conformation of myosin heads in the transition from the weakly bound to the strongly bound state.

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