Biophysical Chemistry, 39 (1991) 287–297

BIOCHE 01549

Time-resolved synchrotron X-ray diffraction studies of a single frog skeletal muscle fiber

Time courses of intensity changes of the equatorial reflections and intracellular Ca^{2+} transients

Masato Konishi [a], Katsuzo Wakabayashi [b], Satoshi Kurihara [a], Hideo Higuchi [a], Noboru Onodera [a], Yoshiki Umazume [a], Hidehiro Tanaka [c,*], Toshiaki Hamanaka [b] and Yoshiyuki Amemiya [d]

[a] *Department of Physiology, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minato-ku, Tokyo 105,* [b] *Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560,* [c] *Department of Physiology, School of Medicine, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173 and* [d] *Photon Factory, National Laboratory for High Energy Physics, 1-1 Oho, Tsukuba 305, Japan*

Received 20 August 1990
Revised manuscript received 27 October 1990
Accepted 30 October 1990

Time-resolved X-ray diffraction; Muscle contraction; Skeletal muscle fiber; Aequorin; Ca^{2+} transient; Synchrotron radiation; (*Rana temporaria*)

Time-resolved X-ray equatorial diffraction studies on a single frog skeletal muscle fiber were performed with a 10 ms time resolution using synchrotron radiation in order to compare the time courses of the molecular changes of contractile proteins and the intracellular Ca^{2+} transient during an isometric twitch contraction at 2.7°C. Measurements of the Ca^{2+} transient using aequorin as an intracellular Ca^{2+} indicator were conducted separately just before and after the X-ray experiments under very similar experimental conditions. The results, which showed a similar time course of tension to that observed in the X-ray experiment, were compared with the aequorin light signal, tension and the intensity changes of the 1,0 and 1,1 equatorial reflections. No appreciable change in both reflection spacings indicated that the effect of internal shortening of the muscle was minimized during contraction. The intensity change of the equatorial reflections generally occurred after the aequorin light signal. In the rising phase, the time course of increase in the 1,1 intensity paralleled that of the rise of the light signal and the intensity peak occurred 20–30 ms after the peak of the light signal. The decrease in the 1,0 intensity showed a time course similar to that of tension and the intensity minimum roughly coincided with the tension peak, coming at 80–90 ms and about 60 ms after the peaks of the light signal and the 1,1 intensity change, respectively. In the relaxation phase, the 1,1 intensity seemed to fall rapidly just before the tension peak and then returned to the original level in parallel with the decay of tension. The 1,0 intensity returned more slowly than the tension relaxation. Thus, the change of the 1,1 intensity was faster than that of the 1,0 intensity in both the rising and relaxation phases. When the measured aequorin light signal was corrected for the kinetic delay of the aequorin reaction with a first-order rate constant of either 50 or 17 s^{-1}, the peak of the corrected light signal preceded that of the measured one by approx. 30 ms. Thus, the peak of the Ca^{2+} transient appeared earlier than the peaks of the 1,1 and 1,0 intensity changes by 50–60 and 110–120 ms, respectively. The time lag between the extent of structural change and the Ca^{2+} transient is discussed in relation to the double-headed attachment of a cross-bridge to actin.

Correspondence address: K. Wakabayashi, Dept. of Biophysical Engineering, Faculty of Engineering Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka. 560, Japan.
* Present address: Department of Physiology, Teikyo Heisei College of Nursing, Ichihara, Chiba 290-01, Japan.

1. Introduction

Contraction and relaxation of skeletal muscle fibers are regulated by a change in the free Ca^{2+}

concentration ($[Ca^{2+}]$) in the myoplasm. Measurement of the intracellular $[Ca^{2+}]$ change in vertebrate skeletal muscle fibers shows a transient change in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) prior to tension development [1].

After this $[Ca^{2+}]_i$ increase, molecular changes occur before tension development, e.g., Ca^{2+} binding to troponin, movement of myosin projections toward the actin filaments, their binding to actin, and structural changes of actomyosin cross-bridges leading to force generation or filament sliding. However, the time courses of the sequential molecular changes are still not fully understood. One powerful technique for exploring time-dependent molecular changes of contractile proteins is time-resolved X-ray diffraction. This method has been applied to skeletal muscle bundles and/or whole muscle to explore the molecular mechanism of muscle contraction. However, muscle bundles contain different types of muscle fibers. The time course of molecular changes in contractile proteins might differ in fast and slow muscle fibers, since the time courses of the Ca^{2+} transient and tension differ in these two types of muscle [2]. Therefore, the time-dependent molecular changes of contractile proteins must be clarified using a single muscle fiber.

In the present study, we measured the time-dependent changes of X-ray equatorial reflections and of Ca^{2+} transients in single frog skeletal muscle fibers during an isometric twitch contraction and then compared the time courses of the Ca^{2+} transient, cross-bridge change and tension. In the X-ray experiment, the use of synchrotron radiation made it possible to carry out time-resolved measurement of the 1,0 and 1,1 equatorial reflections (which arise from the hexagonal array of myofilaments) from a single fiber with a time resolution of 10 ms during a twitch contraction. In the Ca^{2+}-transient experiment, aequorin, a Ca^{2+}-sensitive photoprotein which emits light (peak wavelength 465 nm) on binding Ca^{2+} was used as an intracellular Ca^{2+} indicator. Although the response of aequorin is not fast enough to permit the monitoring of the rapid change in $[Ca^{2+}]i$ in frog skeletal muscle fibers [3], the light signal of aequorin by Ca^{2+} binding is known to be relatively unaffected by the movement of muscle contraction, and Ca^{2+} transients and tension can be measured simultaneously at a physiological sarcomere length [1,4]. This is an advantage of the aequorin method compared to those using Ca^{2+}-sensitive dyes, in which the muscle is usually stretched to a considerable sarcomere length to eliminate movement artifacts in Ca^{2+}-related signals (e.g., see ref. 5). In order to estimate the time to the peak of the myoplasmic Ca^{2+} transient, the aequorin light signal was corrected for the kinetic delay of the aequorin reaction (lag-corrected light based on in vitro measurements. The result was compared with X-ray diffraction studies which were carried out in separate experiments.

A preliminary report was presented at the Annual Meeting of the Biophysical Society of Japan [6] and in the Photon Factory Activity Report [7].

2. Materials and methods

2.1. Specimens and stimulation

Single fast twitch muscle fibers were carefully dissected from m. tibialis anterior of cold-adapted frogs (*Rana temporaria*) (kept at 4°C for 1–4 weeks) and used for the studies. The fibers were about 150–200 μm in diameter and 5–7 mm long. In the Ca^{2+}-transient and X-ray diffraction experiments, using chambers of different types, a single fiber was mounted in a narrow channel in a chamber. One end of the fiber was clamped to a hook while the other end was connected to a thin steel rod extending from a force transducer (Akers AE801, Norway). The sarcomere length of the fiber was adjusted to about 2.3 μm by observing the laser light diffraction pattern. The fiber was continuously perfused with Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, pH 7.1, at room temperature). The temperature of the solution inside the chamber was kept at 2–3°C and measured with a thermistor thermometer. The fiber was stimulated with a square pulse of 500 μs duration at 1.5 × threshold through a pair of platinum wire electrodes (diameter, 0.3 mm). The stimulation rate was 0.1 Hz in the aequorin and X-ray experiments. At this stimulation frequency,

peaks of the aequorin light signal and tension declined rapidly for a few minutes after the stimulation started, but did not change markedly after they had reached 'steady state' (see below). All data (X-ray diffraction patterns, aequorin light signal and tension) were collected after the peak tension and the peak light signal had reached steady state. The fiber condition was frequently checked by observing the sustained plateau of tetanic contraction.

2.2. Time-resolved X-ray equatorial diffraction

The X-ray diffraction experiments were performed with the beamline 15A1 on the storage ring at the Photon Factory, Tsukuba, Japan. A focused and monochromatized X-ray beam of wavelength 0.155 nm was produced by a double focusing camera [8,9] from synchrotron radiation of the storage ring operated at an electron energy of 2.5 GeV with the current between 65 and 120 mA. The camera consisted of seven 20-cm long segments of totally reflecting curved mirrors and a bent silicon crystal monochromator cut at 7.8° to the 111 planes. The specimen-to-detector distance was 180 cm, and the beam size at the specimen was collimated to 0.3 mm (vertical) × 2 mm (horizontal) with sacrifice of an appreciable fraction of the total flux to reduce undesired scatter around the beam stop.

A muscle fiber was mounted in the experimental chamber with two windows covered with thin Mylar sheets to pass X-rays through the middle of the fiber. The chamber was set with the fiber axis horizontally in the camera. The fiber position in the X-ray beam was carefully checked by telescope. The equatorial patterns were recorded by a linear position-sensitive detector using a 400 ns internal delay line (Rigaku Denki Co., Tokyo). The spatial resolution of the detector was about 0.33 mm. The detector opened was 85 mm long and the width was reduced by an 8 mm slit placed just before the detector. This slit was enough to accept the full width of the equatorial reflections across the equator. The entire course of twitch tension including the resting phase was divided into 100 frames, each of 10 ms time width. The signals of one-dimensional patterns and tension were simultaneously stored as a function of time in a CAMAC memory (LeCroy, Research Systems, Geneva, Switzerland) linked to a Micro 11/F23 computer (ASR, Tokyo) and the memory was divided into 100 time frames of 256 channels.

To record the X-ray pattern, the twitch contraction was repeated 100 times and usually five cycles of this type were carried out with a rest time of about 15 min between cycles. The data of five cycles were added to obtain reasonable counting statistics. A shutter in front of the specimen interrupted the X-ray beam except for the periods of data collection in order to minimize the radiation damage. Integrated intensities of the 1,0 and 1,1 reflections in the pattern were measured as the area under the peaks above the background. The background curve was determined by a polynominal fit through minimum points on the intensity curve. The reflection intensities were taken as the mean value of the intensities on both sides of the origin of the pattern.

2.3. Aequorin experiment

A glass micropipette was used to inject a small volume of the aequorin solution (2 mg/ml) and the aequorin light was detected with a photomultiplier (9789A, EMI, Ruislip, U.K.). The light signal was recorded as a photomultiplier current. Light and tension signals were stored in an FM tape recorder and the memory of a digital computer through a 12-bit A/D converter (sampling interval 150 μs) for later analysis. Details of the procedure have been described elsewhere [10].

In aequorin experiments, tension and light signals of 200 twitch contractions were averaged to improve the signal-to-noise ratio (S/N). Peak amplitudes of tension and light did not significantly change during these 200 contractions. Limited space for injection and signal detection of aequorin in the X-ray experimental hutch has obliged us to perform the aequorin experiments separately from the X-ray work. Just before and/or after the X-ray experiments, they were conducted under very similar experimental conditions by using frogs from the same batch. We chose the fiber which showed a similar time course of tension to that in the X-ray experiment in order to compare the time

courses of the aequorin light signal and changes in the X-ray diffraction pattern (see fig. 5).

In vitro study on the kinetics of aequorin has revealed that the kinetic limitations of the aequorin reaction are those of a first-order filter; the aequorin light signal tracks the change of free $[Ca^{2+}]$ with a first-order rate constant [3]. Therefore, the measured aequorin light signal was corrected for the kinetic delay of aequorin reaction with the following equation:

$$L_c = 1/K(dL/dt) + L$$

where L and L_c denote the measured and lag-corrected light signals of aequorin, respectively, and K is a first-order rate constant. The rate constant measured in vitro is 100–116 s^{-1} at 20 °C (ref. 11, Neering, personal communication) and about 50 s^{-1} at 2–3 °C (Neering, personal communication), if aequorin is not equilibrated with Mg^{2+} before the change of $[Ca^{2+}]$. However, if aequorin is pre-equilibrated with 3 mM Mg^{2+} (this corresponds to the resting myoplasmic $[Mg^{2+}]$ reported by Hess et al. [12]), the rate constant is 37 s^{-1} at 20 °C, which is one-third of the 110 s^{-1} obtained without pre-equilibration with Mg^{2+} [11]. If it is assumed that the rate constant at 2–3 °C is also reduced to one-third by pre-equilibration with 3 mM Mg^{2+}, the rate constant is expected to be about 17 s^{-1} in 3 mM Mg^{2+} at 2–3 °C. Since there is uncertainty about the resting $[Mg^{2+}]_i$ in the frog skeletal muscle fibers, two different rate constants of aequorin light emission were used to correct the measured light signals for the delay of the aequorin reaction; 50 s^{-1} for $[Mg^{2+}]_i = 0$ mM and 17 s^{-1} for $[Mg^{2+}]_i = 3$ mM. If the resting $[Mg^{2+}]_i$ is in the range of 0–3 mM, the aequorin light signals corrected with those two rate constants would be the extreme cases of the light signal without the kinetic delay.

3. Results

3.1. Time-resolved X-ray equatorial diffraction from a single fiber during twitches

Changes in the intensities of the 1,0 and 1,1 equatorial reflections were studied with a time resolution of 10 ms over the entire course of a twitch at 2.7 °C. In these experiments, 500 twitch contractions of a single muscle fiber were required to obtain X-ray diffraction patterns suitable for analysis. Fig. 1 shows a typical example of the equatorial pattern averaged over 500 contractions, in which three intensity curves are depicted. The records corresponded to a resting frame, a contraction frame around the peak tension and the last frame of relaxation. Diffraction data were obtained with reasonable counting statistics and the S/N ratio. The intensity curve of the last frame of relaxation almost coincided with that of the resting phase. Fig. 2A shows the tension records of the first and last twitches in each 100 contractions of five cycles during an X-ray experiment. The peak value of tension at the beginning of each 100 contractions was almost the same. Although the peak tension gradually decreased in each 100 contractions, the peak value of the last twitch response was approx. 80–90% of that of the first. The average peak tension of 100 twitches was almost the same in five cycles, being about 70% of the maximum tetanic tension of the fiber. Fig. 2B shows the normalized average tensions of five cycles; the times of onset of contraction, peak tension and duration of contraction did not change significantly during each cycle of the experiment. Therefore, the intensity and tension data of 500 contractions were summed and averaged for comparison purposes.

Fig. 3 shows the changes of integrated intensities of the 1,0 and 1,1 reflections (denoted by $I_{1,0}$ and $I_{1,1}$) and the tension change as a function of time. The tension started to rise at approx. 10 ms after the onset of stimulation, reaching a peak at 140–150 ms and returning to the resting level at 850–900 ms. $I_{1,0}$ and $I_{1,1}$ started to change rapidly approximately at the same time as the onset of tension development. $I_{1,0}$ decreased to about 50% and $I_{1,1}$ increased up to about 220% of the resting values. None of the intensities at maximal level were significantly different from the values obtained for the whole muscle during the isometric twitch [13,14] and tetanus [15,16]. They returned rather slowly to their resting levels with tension relaxation. We examined the spacing of these two reflections as a function of time (fig. 4). No appre-

ciable change was observed in both reflection spacings, indicating that the effect of internal shortening of the muscle was very small during contraction. Separate experiments on three other single fibers showed behavior similar to that in fig. 3.

3.2. Comparison of the time courses of changes in $I_{1,0}$, $I_{1,1}$ and the measured aequorin light signal

In aequorin experiments, a single fiber which showed a similar time course of tension to that observed in the X-ray experiment was chosen to compare the light signal, tension and the intensity changes of reflections. The light signal (L) was normalized by L_{max}, the maximal light signal of injected aequorin, which was estimated by lysing the cell with Triton X-100 (1%) in a solution with saturating [Ca^{2+}] (1.8 mM) at the end of each experiment (see ref. 17). The peak value of L/L_{max} during the isometric contraction was about 0.0003. The time courses of tension in aequorin and X-ray experiments were not significantly different (see fig. 5).

In fig. 5, the results from aequorin and X-ray experiments were superimposed to compare the time courses of changes in the measured aequorin light signal and $I_{1,0}$ and $I_{1,1}$ together with tension. In each case, the changes are expressed as a percentage of the average maximum value. The aequorin light signal started to rise with a short lag and peaked at 60 ms after stimulation. It then immediately decreased and disappeared after 450–500 ms. This decay was due mainly to the decrease in $[Ca^{2+}]_i$ by binding of Ca^{2+} to troponin and pumping of Ca^{2+} back to the sarcoplasmic reticulum (SR). Tension developed after the light signal and the peak was about 150 ms after stimulation. The tension delay was 80–90 ms at the peak after the measured aequorin light signal. The intensity change of the equatorial reflections generally occurred after the aequorin light signal. In

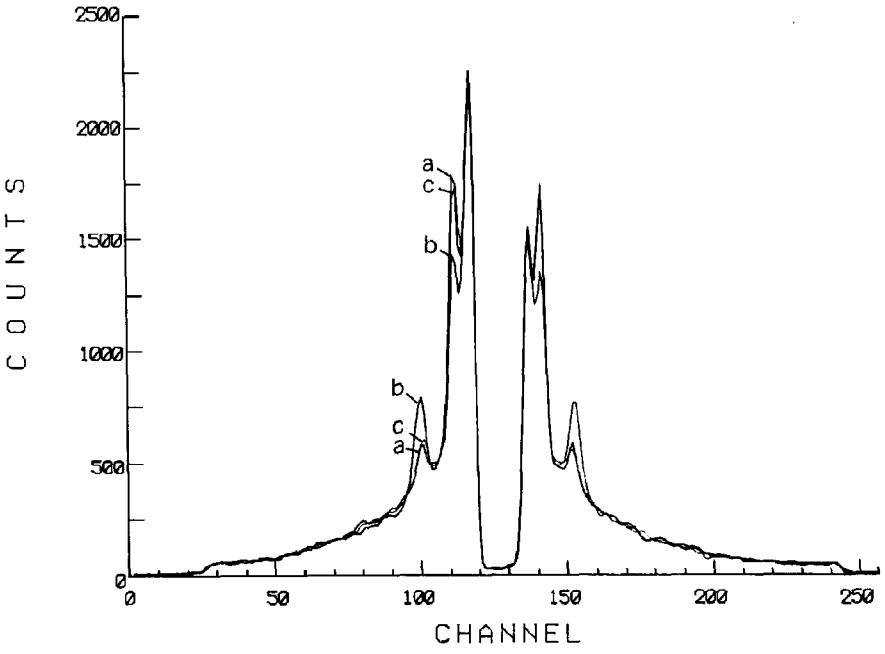

Fig. 1. Equatorial X-ray intensity distributions recorded from a single frog skeletal muscle fiber at a sarcomere length of 2.3 μm at 2.7 °C. The pattern is averaged over 500 twitch contraction cycles. Effective exposure time, 5 s. (a) Resting frame, (b) contracting frame around the peak tension, (c) the last relaxation frame. In each pattern, two peaks are seen on each side of the origin at the 127th channel. 1,0 and 1,1 denote, respectively, the 1,0 and 1,1 reflections from the hexagonal lattice of the myofilaments.

the rising phase, the time course of increase in $I_{1,1}$ paralleled that of the rise of the light signal and the intensity peak came at 20–30 ms after the peak of the light signal. On the other hand, the decrease in $I_{1,0}$ showed a time course similar to that of tension and the intensity peak roughly coincided with the tension peak, thus coming at 80–90 ms and about 60 ms after the peaks of the light signal and $I_{1,1}$, respectively. Although there was a considerable scatter of data points in the relaxation phase, $I_{1,1}$ seemed to fall rapidly just before the tension peak, and at about 100 ms after the tension peak, it slowly returned to the original level, roughly in parallel with the decay of tension. The return of $I_{1,0}$ behaved similarly to that of $I_{1,1}$ but seemed to be slower than the tension relaxation. By the time the tension had fallen to zero, $I_{1,1}$ had recovered almost to the resting level, but $I_{1,0}$ was slightly higher than the resting level. Thus, the 1,1 intensity changed faster than the 1,0 intensity both in the rising and relaxation phases.

3.3. *Lag-corrected light signal of aequorin*

As mentioned previously, the measured aequorin light signal was corrected for the kinetic delay of the aequorin reaction with the first-order rate constant of either 50 or 17 s^{-1} (see section 2), and the time courses of the corrected light signal together with that of the measured light signal are shown in fig. 6 where each trace was normalized to its own peak value for a temporal comparison. Interestingly, the peak of the light signal corrected with the rate constant of either 50 or 17 s^{-1} preceded that of the measured light signal by approx. 30 ms (25 ms with 50 s^{-1} and 32 ms with 17 s^{-1}). Note that the lag between the peaks of the corrected and measured light signals did not

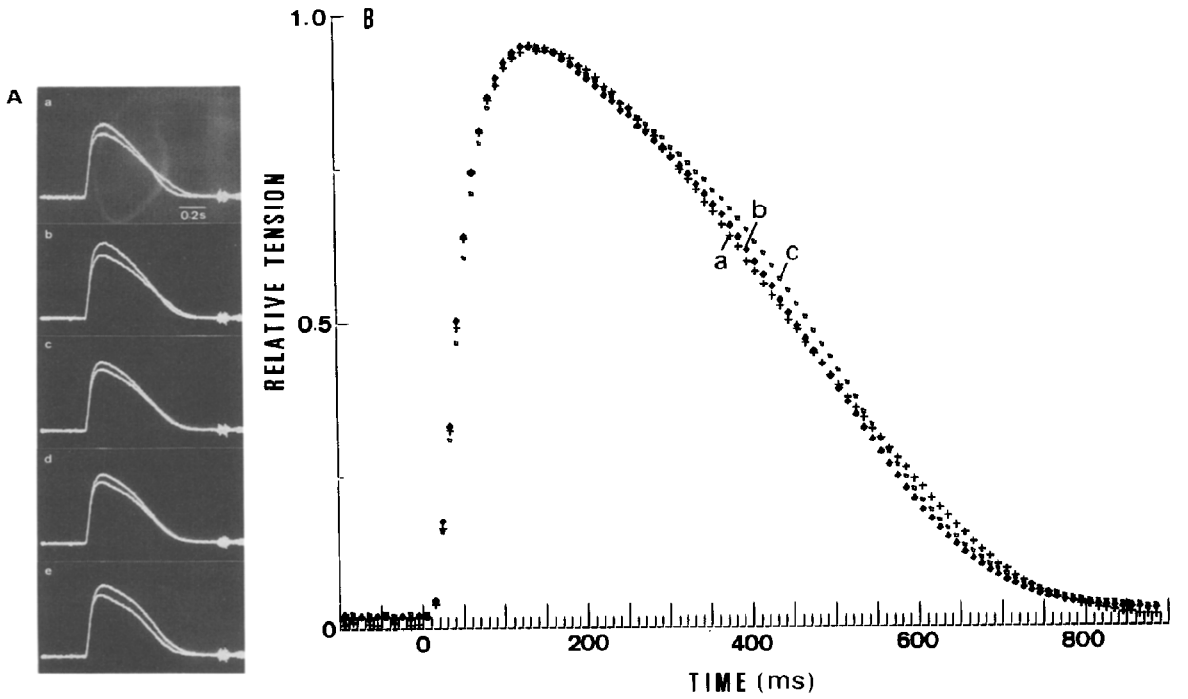

Fig. 2. (A) Typical records of the first and last tensions for each 100 twitch contractions of five cycles (a–e) from a single fiber during an X-ray experiment at 2.7 °C. (B) Normalized average twitch tensions of A. (a) The first cycle; (b) the third cycle; (c) the fifth cycle. The maximum value for each cycle is normalized as 1.0.

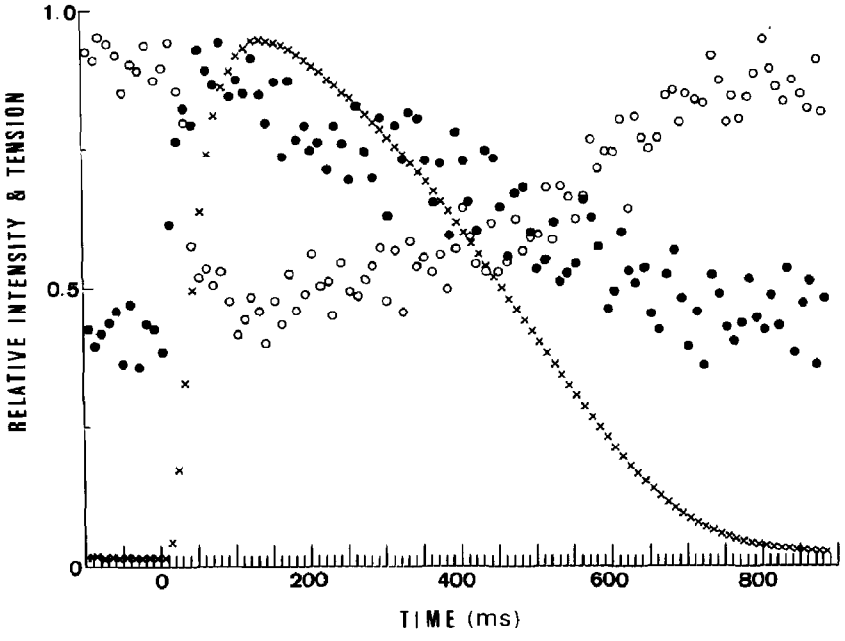

Fig. 3. Changes of integrated intensities of the 1,0 (○) and 1,1 (●) equatorial reflections and twitch tension (×) as a function of time at 2.7 °C. Time 0, the onset of stimulation. Time resolution, 10 ms. Each point represents a mean value of 500 contractions. The maximum values of data in each cycle are normalized as 1.0.

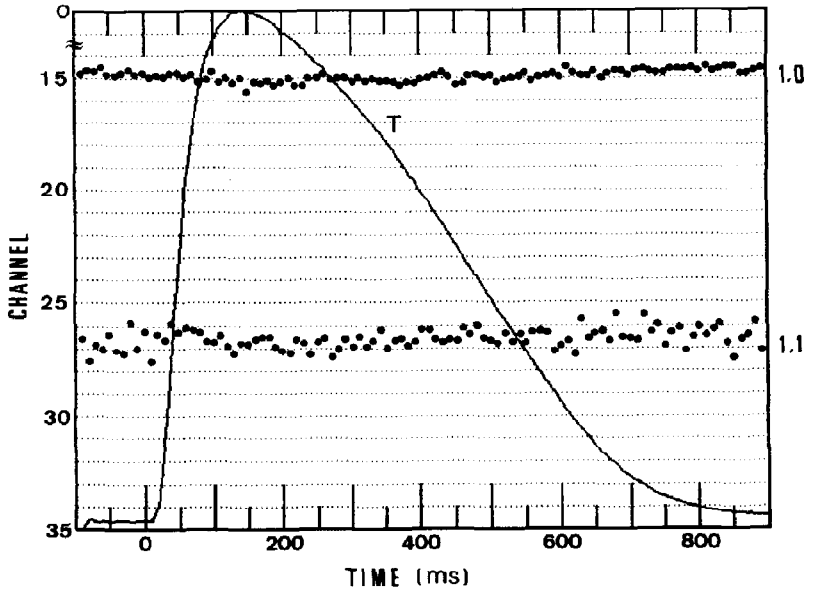

Fig. 4. Spacings of the 1,0 and 1,1 reflections in fig. 3 as a function of time. T, twitch tension. Time 0, the onset of stimulation.

strongly depend on the exact value of the rate constant used for the correction. Thus, the peak of the lag-corrected light signal (the peak of Ca^{2+} transient) clearly appeared earlier than the peaks of the $I_{1,1}$ and $I_{1,0}$ changes by 50–60 and 110–120 ms, respectively. It should be noted, however, that the time course of the lag-corrected light signal is not the same as that of the Ca^{2+} transient because of the nonlinear relationship between aequorin light intensity and $[Ca^{2+}]_i$ [18]. Since our main concern in this study was only the time to the peak of the Ca^{2+} transient rather than its exact time course, we did not convert the aequorin light signal to $[Ca^{2+}]_i$.

4. Discussion

4.1. X-ray equatorial diffraction from a single fiber

Dynamic X-ray diffraction studies for muscle contraction so far have been carried out mainly with whole muscles. The whole muscle is more advantageous than a single fiber with respect to X-ray scattering power, but presents several problems: (1) different types of muscle fibers show different contractile responses to stimulation; (2) it is not certain that all muscle fibers develop uniform tension on stimulation; (3) the sarcomere length among fibers is not uniform, and (4) the

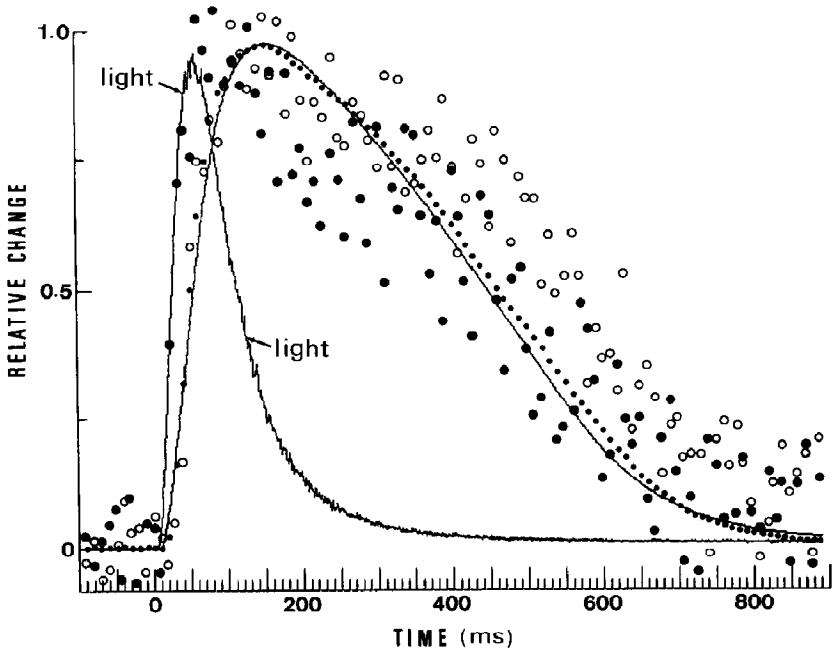

Fig. 5. Comparison of time courses of the intensity changes of the 1,0 (○) and 1,1 (●) reflections in fig. 3 and the measured aequorin light signal (the continuous trace labelled 'light') during the twitches. Tensions: the continuous trace, taken simultaneously with the aequorin light signal and the dotted trace, taken with X-ray data (see fig. 3). Time 0, the onset of stimulation. In aequorin experiments, tension and light signal data were averaged over 200 twitches. The aequorin light signal is expressed in L/L_{max} units (see text). Each time course is normalized to a scale from 0 to 1, where the zero is defined as the intensity at rest and 1 the average maximum value around the peak.

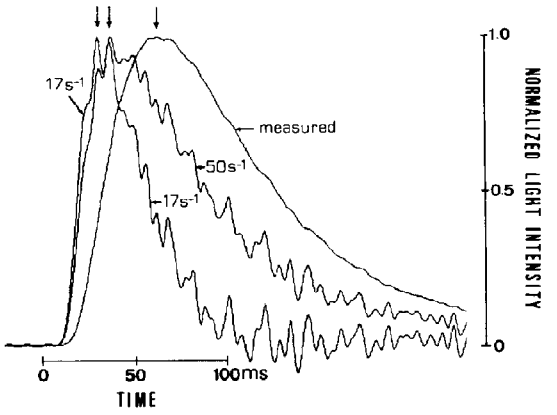

Fig. 6. Correction for the kinetic delay of the aequorin light signal. The aequorin light signal was measured after a single stimulation pulse at time 0 (trace labelled 'measured', the same trace as shown in fig. 5). The 'measured' trace was corrected for the exponential delay with the first-order rate constant of 50 s^{-1} (trace labelled '50 s^{-1}') or 17 s^{-1} (trace labelled '17 s^{-1}'). The corrected traces were digitally smoothed by using 15 points (7 points on both sides), although they are still noisier than the 'measured' trace. The noise is due to differentiation with small rate constants (see the aequorin section in text). The traces are normalized to the same peak height (1 on the ordinate). The peak values in L/L_{max} units (see text) are 0.000831, 0.000411 and 0.000320 for '17 s^{-1}', '50 s^{-1}' and 'measured' traces, respectively. Vertical arrows (from left to right) indicate the time of peaks of '17 s^{-1}', '50 s^{-1}' and 'measured' traces, respectively.

effect of internal shortening is large. Thus, it is not easy to compare directly X-ray diffraction data from a whole muscle with those from physiological experiments in which a single fiber is usually used.

The present results show that X-ray diffraction could be performed with an intact single frog skeletal muscle fiber with a time resolution of 10 ms by using intense synchrotron X-rays. Several hundred contractions were required to improve counting statistics and the S/N ratio. Many such repetitive contractions were possible with a single fiber without appreciable alteration of responses. The result of fig. 4 suggests that the effect of the internal shortening of the fiber could be minimized during the onset of contraction, although any nonuniformity of sarcomere lengths along the fiber might not be avoidable. For technical reasons as mentioned previously, simultaneous measurement of X-ray diffraction and Ca^{2+} transient was not feasible in the present experiment. However, in the fast twitch muscle fiber, we could compare the time course of intensity changes of the equatorial reflections during the isometric twitch contraction with Ca^{2+} transients which were measured in a separate experiment.

In the isometric twitch contraction of a single fiber, we found the following evidence. The intensity change of the equatorial reflections occurred after the aequorin light signal. The changes of 1,0 and 1,1 reflections seemed to be different; the change of $I_{1,1}$ slightly preceded those of $I_{1,0}$ and tension in the rising phase, and in the relaxation phase, the return of $I_{1,1}$ almost paralleled the tension, but $I_{1,0}$ returned more slowly than $I_{1,1}$. When compared with the results of the whole muscle, the time lag between $I_{1,0}$ and $I_{1,1}$ and also that between the intensities and tension seemed to be much smaller in single fibers. This seemed to be due to the reduction of the combined effect of (1) to (4) raised in a whole muscle.

4.2. Delay between peaks of lag-corrected light signal and $I_{1,1}$ change

Since the equatorial intensity change, particularly the change in $I_{1,1}$, can be taken as an indication of the behavior of myosin projections around the thin filaments, initiated by Ca^{2+} binding to troponin, the 50–60 ms delay between peaks of the lag-corrected aequorin light signal and the $I_{1,1}$ change could be mainly attributed to molecular changes in the myofilaments (see below). The molecular process after binding of Ca^{2+} to troponin involves the positional shift of tropomyosin within the thin filament, the movement of myosin projections toward the vicinity of the thin filaments, and some subsequent molecular steps after their arrival.

The period of 50–60 ms should be considered as the upper limit for the molecular changes mentioned above, because some additional steps in the Ca^{2+} transient seem to be also involved in this delay. The first is the diffusion of Ca^{2+} within a sarcomere. If Ca^{2+} is released only from the terminal cisternae of the sarcoplasmic reticulum

and diffuses toward the M-line, a significant Ca^{2+} gradient should be formed within the sarcomere early during the twitch response; the diffusional delay should exist between $[Ca^{2+}]$ near the releasing sites and spatially averaged $[Ca^{2+}]$ around the thin filaments [19]. Since aequorin light emission is proportional to $[Ca^{2+}]^{2.5}$ in the micromolar $[Ca^{2+}]$ range, the light signal from the region with the highest $[Ca^{2+}]$ tends to dominate the total light signal [19]. Thus, the aequorin light signal may mainly reflect $[Ca^{2+}]$ near Ca^{2+}-releasing sites (i.e., terminal cisternae), which reaches a peak earlier than the signal from spatially averaged $[Ca^{2+}]$ around the thin filaments. Cannel and Allen [19] suggested that the peak of the lag-corrected aequorin light signal occurs only slightly earlier (1–2 ms) than that of the spatially averaged myoplasmic $[Ca^{2+}]$ at 20° C (see fig. 8 in ref. 19), but no such estimate was possible at 3° C, the temperature used in this study. Because the diffusion of Ca^{2+} in the myoplasm largely involves binding to myoplasmic Ca^{2+} binding sites such as troponin, parvalbumin, and the sarcoplasmic reticulum Ca-pump [20], the temperature dependence of such a Ca^{2+}-binding process must be clarified in order to estimate the speed of Ca^{2+} diffusion in the myoplasm at 3° C.

Another factor in the delay arises from the kinetic delay of the Ca^{2+}-troponin binding reaction. Based upon the rate constant of the Ca^{2+}-troponin C 'regulatory sites' reaction determined biochemically, several mathematical models [19,21–24] have suggested that the time lag between peaks of (spatially averaged) free Ca^{2+} transient and Ca^{2+}-troponin complex formation is not longer than several milliseconds at 16–25° C. However, the lag due to the kinetics of Ca^{2+}-troponin binding should be somewhat greater at 3° C, if the affinity of troponin C for Ca^{2+} is higher (due to a lower dissociation rate constant, if the association rate constant is assumed to be diffusion-limited) at low temperature as suggested from the p*Ca*-tension relation in skinned amphibian skeletal muscle fibers [25,26]. Godt and Lindley [25] suggested that the dissociation constant of troponin for Ca^{2+} is smaller by a factor of 0.65 at 16° C when compared with that at 25° C. If their Q_{10} ($\sim$ 1.6) is assumed, the affinity of troponin C for Ca^{2+} would be 2.2-times higher at 3° C than at 20° C.

Thus, the delay between the extent of structural change (the change in $I_{1,1}$) and aequorin light signal suggests that a certain interval is needed for the attachment of myosin heads to actin and their conformational change after they move into the vicinity of the thin filaments (see below).

4.3. Delay between changes in $I_{1,1}$ and $I_{1,0}$

When the muscle had become fully active, myosin heads became attached to actin but there was a marked time lag (about 60 ms) between the maximal changes in $I_{1,1}$ and $I_{1,0}$. A similar time lag has been already observed for whole muscle [16]. Although this time lag cannot be clearly explained ($I_{1,0}$ and $I_{1,1}$ are affected by various factors including filament and/or lattice disorder (e.g. see ref 27 and 28), this finding in the single fiber suggests that such a time lag is due to a genuine molecular process. A possible explanation may be related to the double headedness of the myosin cross-bridge. A substantial proportion of cross-bridges are likely to attach first one of their heads to actin with the other remaining unattached (still not immobilized in the 1,1 plane), and the attached heads then facilitate the attachment of their mates to remain close together. The time required for the double-headed attachment of cross-bridges (the attachment configurations of cross-bridges) could be the reason for the time lag between the peaks of the light signal and $I_{1,1}$ change as well as the delayed change in $I_{1,0}$ behind the $I_{1,1}$.

As mentioned previously, the change in $I_{1,0}$ closely followed the initial development of tension. Recently, Chaen et al [29] suggested that the binding of the two heads of a cross-bridge to actin is needed for the development of a substantial fraction of tension. Anderson and Shoenberg [30] suggested from the kinetics of the force decay in the muscle fiber that both heads of a cross-bridge need to bind to actin before the cross-bridges can relax tension. These imply that the progression of the two-headed attachment determines the time course of tension development. Thus, the $I_{1,0}$

change in the rising phase might be correlated closely with the time course of this process.

After the two-headed attachment has been completed, each cross-bridge starts to dissociate [30]. The slightly rapid return of $I_{1,0}$ and $I_{1,1}$ at the early phase of relaxation might be due to the fast detachment of a certain proportion of the cross-bridges [15] and/or to the detachment of one head of the cross-bridge. Most of the detached heads would still remain in the vicinity of the thin filaments, causing the faster change in $I_{1,1}$. The subsequent detachment of myosin heads leads to tension relaxation, accompanied by the $I_{1,1}$ return occurring roughly in parallel with the tension decay. The finding that the $I_{1,0}$ return was slightly slower than the tension decay in the single fiber may be related to what was found with the whole muscle [15]. However, the slow return of $I_{1,0}$ may be due to some longitudinal disordering of thick filaments as well as specific motion of two-headed myosin projections when they move back to their original positions, as shown by the unusual behavior of the intensity relaxation of the myosin meridional reflection [31]. The 1,0 intensity is more sensitive to such disorder of the myosin filaments than the 1,1 intensity. In this sense, the decay in $I_{1,1}$ might be correlated closely with the relaxation of tension.

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