Synchronous Behavior of Spontaneous Oscillations of Sarcomeres in Skeletal Myofibrils under Isotonic Conditions

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ABSTRACT An isotonic control system for studying dynamic properties of single myofibrils was developed to evaluate the change of sarcomere lengths in glycerinated skeletal myofibrils under conditions of spontaneous oscillatory contraction (SPOC) in the presence of inorganic phosphate and a high ADP-to-ATP ratio. Sarcomere length oscillated spontaneously with a peak-to-peak amplitude of about 0.5 μm under isotonic conditions in which the external loads were maintained constant at values between 1.5×10^4 and 3.5×10^4 N/m^2. The shortening and yielding of sarcomeres occurred in concert, in contrast to the previously reported conditions (isometric or auxotonic) under which the myofibrillar tension is allowed to oscillate. This synchronous SPOC appears to be at a higher level of synchrony than in the organized state of SPOC previously observed under auxotonic conditions. The period of sarcomere length oscillation did not largely depend on external load. The active tension under SPOC conditions increased as the sarcomere length increased from 2.1 to 3.2 μm, although it was still smaller than the tension under normal Ca^{2+} contraction (which is on the order of 10^5 N/m^2). The synchronous SPOC implies that there is a mechanism for transmitting information between sarcomeres such that the state of activation of sarcomeres is affected by the state of adjacent sarcomeres. We conclude that the change of myofibrillar tension is not responsible for the SPOC of each sarcomere but that it affects the level of synchrony of sarcomere oscillations.

INTRODUCTION

The study of the molecular mechanism of muscle contraction has been advanced by the advent of a nanoscopic analysis technique measuring the motion of and generation of force by single molecular motors (cf. Kishino and Yanagida, 1988). From a physiological viewpoint, on the other hand, the structural and functional unit is a single myofibril. To elucidate the mechanisms regulating tension development in the higher-order structure of muscle, we have therefore devised a simple method for investigating both the mechanical properties and fine structure of a single myofibril (cf. Anazawa et al., 1992; Yasuda et al., 1995).

Recently, a new type of spontaneous oscillation of tension and sarcomere length in vertebrate skeletal myofibrils was found and was named SPOC (for spontaneous oscillatory contraction; Okamura and Ishiwata, 1988). The SPOC occurs under conditions intermediate between relaxing and contracting conditions induced either by high concentrations of MgADP and inorganic phosphate in the absence of Ca^{2+} (ADP-SPOC; Ishiwata et al., 1991; Shimizu et al., 1992) or by submicromolar concentrations of free Ca^{2+} (Ca-SPOC). Whereas ADP-SPOC is observed irrespective of the muscle type, Ca-SPOC has not been observed in fast-type skeletal muscle but has been observed in slow-type skeletal muscle (Iwazumi and Pollack, 1981; Stephenson and Williams, 1981, 1982) and cardiac muscle (Fabiato and Fabiato, 1978; Sweitzer and Moss, 1990; Linke et al., 1993).

The SPOC phenomenon has been studied under isometric conditions, where the lengths of constituent sarcomeres oscillate while maintaining the total length of myofibrils (muscle fibers) constant, as well as under auxotonic conditions, in which the tension increases as the sarcomere length decreases such that the external load and the length of myofibrils are inversely related (cf. Anazawa et al., 1992). Under isometric conditions, oscillation of one part of the myofibril does not appear to be related to that of other parts. Under auxotonic conditions, on the other hand, the yielding phase of sarcomeres repeatedly propagates from one end of a myofibril to the other (we previously called this the *organized state of SPOC*; see figure 6 of Anazawa et al., 1992; hereafter we call this *metachronal SPOC*, in contrast to synchronous SPOC). These observations imply that the activation state of sarcomeres is affected by the states of adjacent sarcomeres.

In the present study, we used a newly developed microscopy system to investigate the length-tension relation of SPOC in a single skeletal myofibril under isotonic conditions for the purpose of understanding the role of the external load in SPOC.

MATERIALS AND METHODS

Solutions

Rigor solution A: 60 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-maleate (pH 6.8), and 1 mM EGTA. Rigor solution B: 0.1 M KCl, 5 mM $MgCl_2$, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0), and 1 mM EGTA. Relaxing solution: 0.12 M KCl, 4 mM $MgCl_2$, 4 mM ATP, 20 mM MOPS (pH 7.0), and 4 mM EGTA. SPOC solution: 0.12 M KCl, 4 mM $MgCl_2$, 0.2 mM ATP, 4 mM ADP, 4 mM K_2HPO_4, 20 mM MOPS (pH 7.0), and 4 mM EGTA. The pH values were adjusted for each preparation of solution. ATP and ADP were purchased from Boehringer Mannheim

Received for publication 8 August 1995 and in final form 4 January 1996.
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0006-3495/96/04/1823/07 $2.00

GmbH (Mannheim, Germany); MOPS and EGTA were from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were also of reagent grade.

Preparation of myofibrils

Single myofibrils and small bundles of myofibrils were prepared by homogenizing rabbit psoas fibers glycerinated in 50% (v/v) glycerol containing 0.5 mM $NaHCO_3$, 5 mM EGTA, and 1 mM leupeptin for more than 3 weeks at −20°C as described previously (Ishiwata and Funatsu, 1985). Glycerol and undissolved large aggregates in the suspension of myofibrils were removed by centrifugation at 3000 × *g* in rigor solution A at 4°C.

Microscopy system

The microscopy system and methods for analysis were basically the same as those reported previously (Anazawa et al., 1992; Yasuda et al., 1995), except for a piezo element that was used for feedback control to establish isotonic conditions. The system (Fig. 1) consisted of the following four parts: 1) A temperature-controlled cell (solution capacity of about 200 μl) in which a myofibril was fixed to a pair of glass microneedles, one of which was flexible (Hooke's constant of 10–40 mN/m) and the other of which was rigid (Hooke's constant of 3 N/m). The displacement of the rigid needle was controlled by the piezoelectric element (PSt 150/50/5, Dr. Lutz Pickelmann, Munich, Germany). 2) An inverted phase-contrast microscope (DIAPHOT-TMD; Plan Apo 60× oil DM objective lens (1.40 NA; Nikon Co., Tokyo, Japan) equipped with a CCD camera (C3077H; Hamamatsu Photonics K. K., Hamamatsu, Japan). 3) A feedback control system for keeping the position of the flexible needle constant (i.e., for maintaining the isotonic condition) by controlling the displacement of the rigid needle (cf. Kamimura and Kamiya, 1989; Yagi et al., 1994). 4) A video-computer image analysis system for recording the positions of the microneedles and for making micrographs and density profiles of the myofibrils. The position of the flexible needle was monitored every millisecond by using two opposing elements of a quadrant-type photodiode (S1557; Hamamatsu Photonics K. K.). The position of the rigid microneedle was feedback controlled so as to keep the differential output voltage of the two elements of the photodiode constant. The video-computer system was used to estimate the average sarcomere length by measuring the separation between the inner edges of the two microneedles (i.e., the length of myofibril) by using a double-channel position detector (width analyzer C3161; Hamamatsu Photonics K. K.). The tension developed (strictly, the external load) under isotonic conditions was estimated with the video-computer system by measuring the deflection of the flexible needle. The time resolution for data acquisition by the video analysis system was 33 ms.

Microscopic analysis of SPOC

A single myofibril or a small bundle of myofibrils was held with a pair of glass microneedles according to the procedure reported previously (Anazawa et al., 1992). The cross-sectional area of the myofibrils was estimated to be from 2×10^{-12} to 7×10^{-12} m^2, and the number of sarcomeres per myofibril was between 20 and 30. After the myofibril or myofibril bundle was set on the microneedles, the rigor solution B in the cell was replaced by the relaxing solution. The central area of the quadrant-type photodiode was set at the position of the flexible microneedle by moving the photodiode manually, and feedback control of the rigid microneedle was started. After an appropriate load was imposed by moving the photodiode manually, the relaxing solution was replaced by the SPOC solution. During feedback control of the rigid microneedle, the position of the flexible microneedle was kept in the central area of the photodiode such that the myofibrils were maintained under isotonic conditions. Thus the level of external load was determined by the position of the photodiode and was changed by moving the photodiode.

The spatial resolution for monitoring the position of the microneedle was 51 nm, and when a flexible needle with a Hooke's constant of 2.37×10^{-2} N/m was used, the resolution with which the developed tension was measured was 1.2×10^{-9} N ($(51 \times 10^{-9}$ m) × $(2.37 \times 10^{-2}$ N/m)).

RESULTS

Performance of the feedback system

Figs. 2 and 3 show how accurately isotonic tension was maintained (see *left arrow* in Fig. 2 and *left arrowheads* in Fig. 3) during the shortening and yielding of sarcomeres in SPOC. The feedback control of the rigid needle was rapid enough to follow the change in tension. In Fig. 4 *b*, for example, we can see that the external load imposed on the myofibril was kept almost constant (i.e., within 1.4% of the average tension: 2.15×10^4 N/m^2). The deviation could be

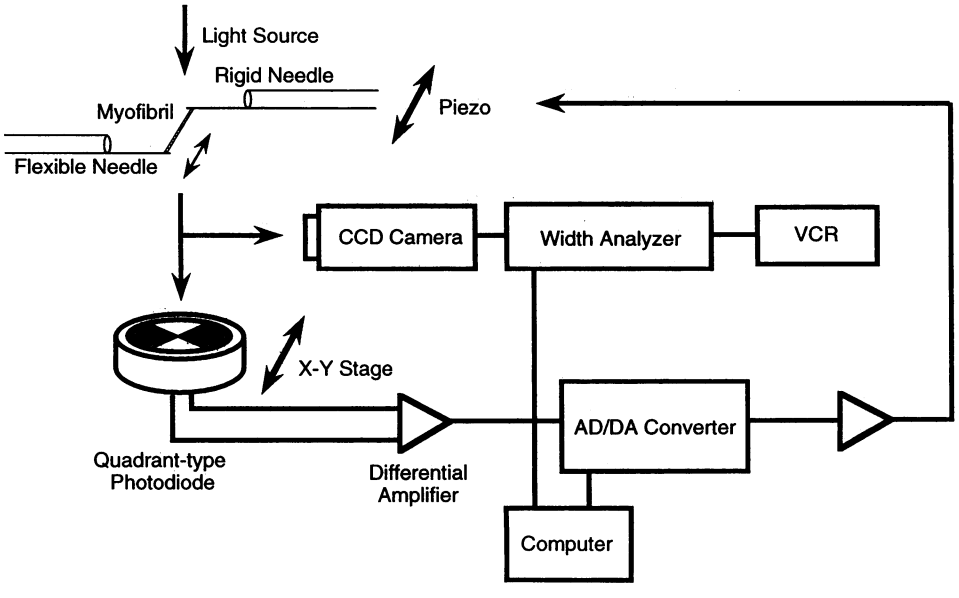

FIGURE 1 The microscopic analysis system. (For details, see Materials and Methods.)

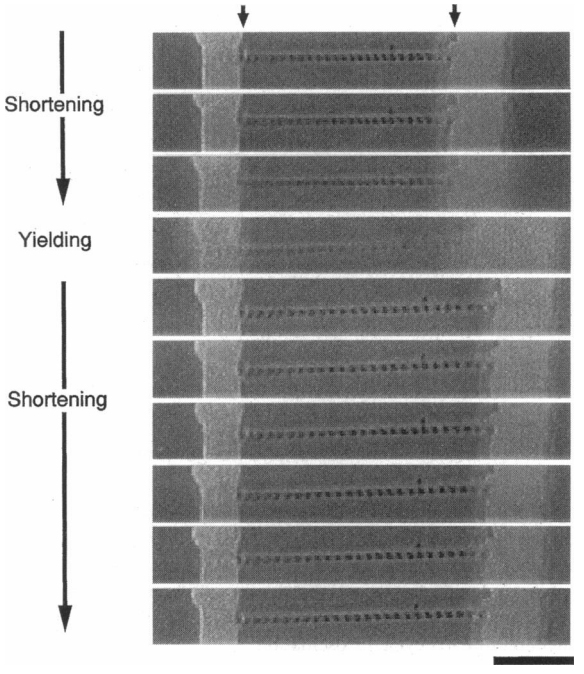

FIGURE 2 Phase-contrast micrographs taken every 33 ms and showing the time course of the isotonic SPOC of a single myofibril. The displacement of the flexible needle (*left arrow*) indicates the level of the external load imposed on the myofibril. At the fourth micrograph from the top, the synchronous yielding of sarcomeres occurred within one video frame (33 ms); note that the flexible microneedle was kept at almost the same position even during the yielding phase. The average external load was 2.17×10^4 N/m^2 (see Fig. 4 *b*). Scale bar, 20 μm.

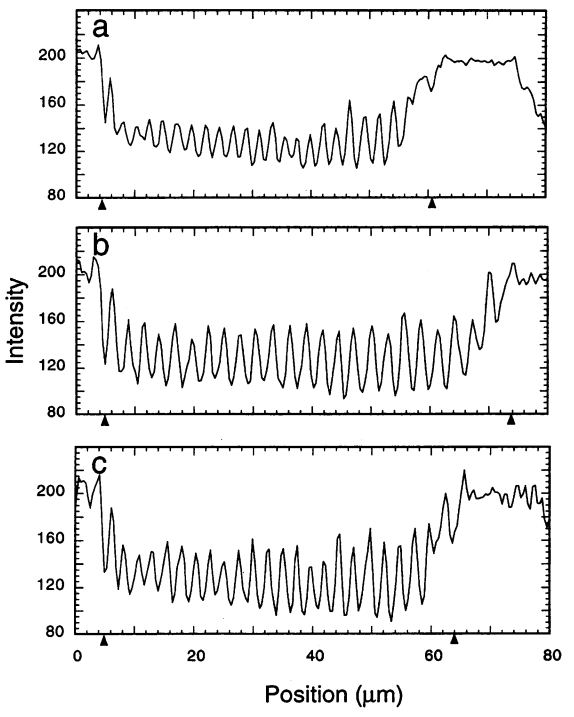

FIGURE 3 Image profiles of myofibrils showing isotonic SPOC. Horizontal and vertical axes represent the position along a myofibril and the brightness of the phase-contrast image. The arrowheads on each side correspond to the inner edges of microneedles (see *arrows* in Fig. 2): (*a*) just before the yielding (the third micrograph from the top in Fig. 2), (*b*) just after the yielding (the fifth micrograph from the top in Fig. 2), and (*c*) during the shortening (micrograph not shown). The flexible needle corresponding to the arrow on the left-hand side was kept at the same position by manipulating the rigid needle with feedback control. Peaks and valleys of the image profile respectively correspond to the I-bands and the A-bands of the sarcomeres.

reduced by increasing the gain of the feedback amplifier but was limited by the 1-ms time resolution of feedback control. Thus in this system we accepted a fluctuation of about 1% of the external load.

The transient response to a sudden change of load was estimated to take place within 10 ms because the image profile of the flexible needle, which is averaged over 33 ms, was broadened only slightly (see the fourth micrograph from the top in Fig. 2).

Microscopic analysis of SPOC

As shown in Figs. 2 and 3, the shortening and yielding of sarcomeres tended to be synchronized along the length of the myofibril (synchronous SPOC). Every sarcomere yielded within 33 ms, and all of them yielded at almost the same time (Fig. 2). This means that the yielding velocity of sarcomeres was greater than 12 μm/s. Such a coordination of yielding was never observed when sarcomeres under auxotonic conditions were investigated (Anazawa et al., 1992). The image profiles of the sarcomeres (Fig. 3) show the uniformity of sarcomere lengths. The average sarcomere length was estimated by dividing the distance between the inner edges of two microneedles by the number of sarcomeres. Because the shortening and yielding of sarcomeres were in concert, this could be regarded as an estimate of the length of each sarcomere. The metachronal SPOC occurred reproducibly without changing the oscillation period when the condition was changed from isotonic to auxotonic (data not shown).

As shown in Fig. 4, synchronous SPOC occurred under isotonic conditions irrespective of the external load, and the time course of the oscillation of sarcomere length displayed a sawtooth waveform similar to that directly observed by phase-contrast microscopy under isometric (cf. Ishiwata et al., 1991) and auxotonic (cf. Anazawa et al., 1992) conditions. Because of the synchronous oscillation, the waveform of the oscillation of sarcomere lengths could be measured more accurately here than in previous studies. The duration of the yielding of sarcomeres was less than 33 ms, such that the yielding phase represented about 1/60 of the total period of oscillation.

Effect of the external load on the synchronous SPOC

Fig. 5 shows the relation between sarcomere length and average shortening velocity under three different external loads, and the track in this relation was triangular and anticlockwise, irrespective of the external load, and the

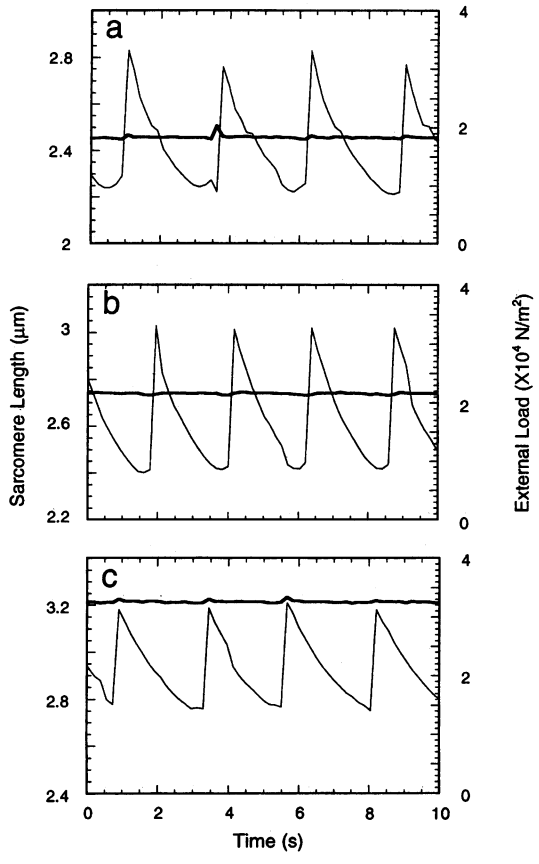

FIGURE 4 Oscillation of average sarcomere length (*thin lines*) during isotonic SPOC under different external loads (*thick lines*). The average external loads were (*a*) 1.83 × 10^4 N/m^2, (*b*) 2.17 × 10^4 N/m^2 (the same as in Figs. 2 and 3), and (*c*) 3.27 × 10^4 N/m^2. The data in these examples were recorded every 150 ms.

same track was followed over many cycles of SPOC (we observed nearly 30 cycles). Just after the yielding of sarcomeres ceased, the shortening of sarcomeres started with its maximum velocity; this initial shortening velocity appears to increase when the external load is reduced. After the start of shortening, the shortening velocity decreased at a nearly constant rate until the shortening stopped. The rate of decrease in shortening velocity was independent of the external load and was about 0.4 μm/s^2.

As summarized in Fig. 6, the peak-to-peak amplitude of the sarcomere length oscillation—that is, the difference between sarcomere length before (*open squares*; shortest length) and after (*open triangles*; longest length) the yielding of sarcomeres—was about 0.50 μm (minimum, 0.40 μm; maximum, 0.58 μm). Developed tension, which should be balanced with the external load at the shortest and the longest sarcomere lengths, increased as sarcomere length increased but was less than tension developed under normal Ca^{2+} contraction ((2–3) × 10^5 N/m^2 at full overlap between thick and thin filaments), even at the greatest sarcomere length. When the external load was less than 1.5 × 10^4 N/m^2 (that is, when the average sarcomere length was less

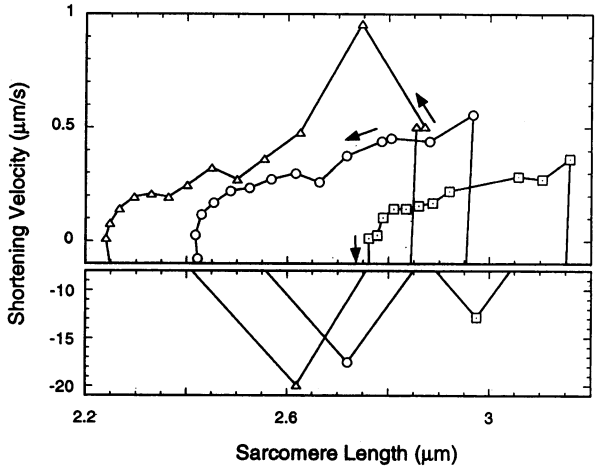

FIGURE 5 Relation between sarcomere length and average shortening velocity of sarcomeres under various external loads. Open triangles correspond to Fig. 4 *a*; open circles, to Fig. 4 *b*; and open squares, to Fig. 4 *c*. Shortening velocities were estimated every 150 ms (actual shortening velocities should be larger than these values; cf. Results). Arrows indicate the direction of each cycle of oscillation.

than 2 μm), synchronous SPOC was not observed and each sarcomere oscillated independently of the others.

We also examined the effects of the transient change of the external load (less than 5% of total load) on SPOC by rapidly (within 10 ms) moving the rigid needle under auxotonic conditions. When we did that, we found that the SPOC waveform was simply shifted to the larger level of tension without a change in the phase or the period of oscillation (data not shown).

Period of isotonic SPOC under various external loads

The period of the SPOC was about 2.3 s (Fig. 7, *open triangles*: minimum period, 1.5 s; maximum period, 2.6 s).

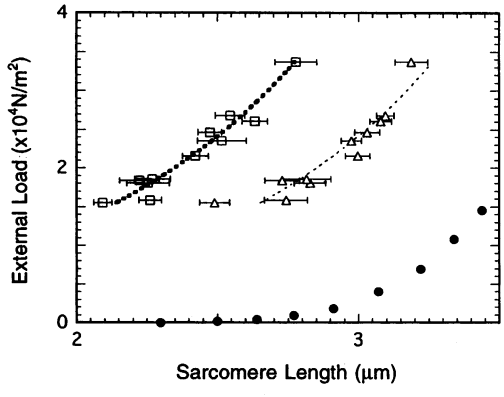

FIGURE 6 Relation between sarcomere length and external load during isotonic SPOC. Open squares and triangles respectively indicate the shortest and the longest sarcomere lengths under various external loads. Error bars indicate the standard deviation of about 15 cycles of oscillation. Closed circles show the resting tension at various sarcomere lengths (taken from figure 7 of Yasuda et al., 1995).

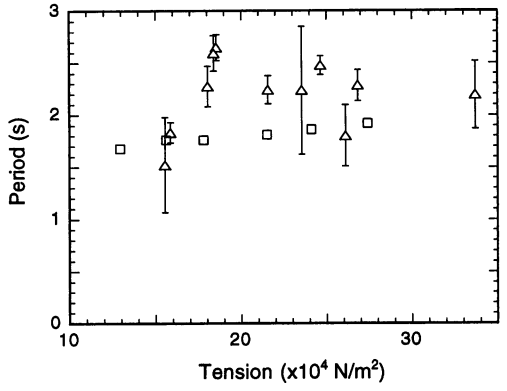

FIGURE 7 Relation between external load and the period of isotonic SPOC. Open triangles indicate average period of sarcomere length oscillation. Error bars indicate the standard deviation of about 15 cycles of oscillation. Open squares indicate average period of auxotonic SPOC (taken from figure 9 of Anazawa et al., 1992).

As shown in Fig. 7, we could not find a clear tension dependence of the period, even when the external load increased by a factor of 2. The period of oscillation for the isotonic SPOC was indistinguishable from that of the auxotonic SPOC (Fig. 7, *open squares*; see figure 9 of Anazawa et al., 1992).

Contribution of connectin (titin) for SPOC

Connectin, an elastic protein of myofibrils, has been shown to have large viscoelastic influence on the mechanical properties of muscle fibers (cf. Magid and Law, 1985). To examine the possible contribution of connectin to SPOC, it was partially digested by adding 0.25 μg/ml trypsin to the SPOC solution under the auxotonic condition. The result (not shown) was that the average tension at an average sarcomere length greater than 2.7 μm gradually decreased with the progress of digestion, but the amplitude of tension oscillation was hardly affected until the level of tension fell by about half.

DISCUSSION

Synchronization of sarcomere oscillations under isotonic conditions

We first observed isometric SPOC in experiments in which both ends of myofibrils were attached to a glass surface (Okamura and Ishiwata, 1988). In such isometric SPOCs, a sawtooth waveform was observed in the oscillation of the length of each sarcomere, but the oscillation was asynchronous or locally metachronal; that is, the yielding phase of sarcomere propagated only locally, extending at most over only several sarcomeres. Thus, in isometric SPOC the oscillation of one part of the myofibril does not appear to be related to that of other parts. Because in those experiments we could neither measure the developed tension nor control the external load, we could not evaluate possible relationships between changes in tension and the oscillation in sarcomere length.

We therefore developed a microscopic analysis system to measure tension and sarcomere length during SPOC under auxotonic conditions (Anazawa et al., 1992). Using that system, we found the organized state of SPOC in which the sarcomere oscillation was not synchronous but in which each sarcomere's oscillation was metachronously related to that of the others. Under auxotonic conditions the yielding of sarcomeres was always accompanied by a decrease in tension and the shortening of sarcomeres was always accompanied by an increase in tension. There was therefore a possibility that the metachronal SPOC was induced by the changes in tension without the boundary condition of isometric SPOC that the total length of myofibril is maintained constant, that is, shortening of one sarcomere should be accompanied by yielding of some other sarcomeres. Thus, clarification of the correlation between the change in tension and SPOC would give us information about the mechanism of synchrony of sarcomere oscillations.

To make the above point clear, we examined the isotonic conditions where the constant external load is always imposed on all sarcomeres in the myofibrils (cf. Schoenberg et al., 1974). As a result, it has been demonstrated that the tendency for the oscillation of adjacent sarcomeres to be synchronized was more pronounced and the synchronous SPOC occurred (Figs. 2 and 3). Thus the metachronal SPOC observed under auxotonic conditions might be regarded as an intermediate state between the asynchronous SPOC under isometric conditions and the synchronous SPOC under isotonic conditions. And the change of the external load during SPOC may cause the decline of synchrony of oscillation from synchronous to metachronal and to asynchronous SPOC.

Under isotonic conditions the period of length oscillation for each sarcomere did not depend on the external load (Fig. 7), and the waveform of each sarcomere in SPOC under isotonic conditions was similar to that under auxotonic and isometric conditions. These results indicate that the change of the myofibrillar tension is not responsible for the SPOC of each sarcomere.

Although the change of the external load may cause the decline of the synchrony of sarcomere oscillations, the transient change of load (mechanical impulse) caused by the delay (1 ms) of our isotonic feedback system might have triggered the synchronous SPOC. We therefore examined the effects of the external impulse by rapidly moving the rigid needle during SPOC; the results show that the synchrony of SPOC is not caused by the impulse.

A possible cause for the synchronization of sarcomeres is the conformational change of the protein components present between half-sarcomeres, such as the Z line and the M line. Linke and his colleagues obtained a dose-dependent decrease of oscillation amplitude by stiffening the Z line structure of cardiac myofibrils through labeling with anti-α-actinin, although the period was not affected (Linke et al., 1993). The structural change of the Z line lattice from

expanded form to contracted form has also been reported to accompany changes in the states of sarcomeres (Goldstein et al., 1988). It has been reported that actin filaments rotate during the sliding movement that occurs when they interact with myosin (Nishizaka et al., 1993). Those observations suggest the following possible mechanism for the transmission of information between adjacent sarcomeres: the generation of active force twists the thin filaments, changing the structure of the Z line lattice (cf. Jarosch, 1987) and thus changing the degree of twist of the thin filaments at the adjacent half-sarcomere. If the twist of thin filaments, for example, accelerates the release of the cross-bridges, it may change the degree of the inhibition of the regulatory proteins. A similar process might occur through the M line. Such a transmission of structural information through the Z line and the M line might be the cause of the metachronal SPOC and the synchronous SPOC. The loss of synchrony during the transition from the synchronous SPOC to the metachronal SPOC may be attributable to the decrease in the external load accompanying the yielding of sarcomeres, which in turn may delay the transmission of structural information to the neighboring sarcomeres. In this respect, we should mention that the transmission of structural information disappeared when the external load was less than a certain threshold value. This is consistent with the idea that the certain degree of strain (such as twist) in constituent proteins is required for the transmission of information that must occur in the organization of SPOC.

The possibility that SPOC is caused by damaged sarcomeres

It has been pointed out that if the tension generation is imbalanced because of damaged sarcomeres, this imbalance might cause a tug-of-war between sarcomeres and, then, SPOC (Iwazumi and Pollack, 1981). The tug-of-war mechanism, however, is excluded by the occurrence of synchronous SPOC because all sarcomeres would not be able to oscillate in concert if tension generation were imbalanced because of the presence of damaged sarcomeres.

Elasticity of connectin (titin) and the mechanism of SPOC

It was previously reported that connectin (titin) is not essential to the SPOC occurring under isometric conditions, because microscopic observation indicated that partial trypsinization of connectin in skeletal myofibril did not affect the oscillation of sarcomere length (Shimizu et al., 1992).

As shown in Fig. 6, the resting tension produced by the elongation of connectin is too small to contribute to the isotonic tension, even under the SPOC condition. This is especially true at sarcomere lengths less than 2.5 μm. There remains a possibility, however, that the large viscoelasticity of connectin may contribute to the tension at the quick yielding phase and thus might determine the greatest sarcomere length during oscillation (cf. Fig. 6, *open triangles*). This possibility was examined by partially digesting connectin, and the result indicates that although the passive tension of connectin may contribute to the level of tension, it is not essential to the oscillation mechanism itself.

The time course of the sarcomere length oscillation during isotonic SPOC was simulated by Smith and Stephenson, who used a model (see figure 11(a) of Smith and Stephenson, 1994) in which the resting tension of connectin was taken into account. The model could reproduce the oscillation of sarcomeres, but the waveform was not of a sawtooth type, and the shortening velocity increased as the shortening of myofibrils proceeded. This change in velocity is opposite that evident in the experimental results shown in Fig. 4.

Finally, we would like to stress that under isotonic conditions the active tension increased as the average sarcomere length increased (Fig. 6). Such a length-tension relation was also observed in the isometric tension analysis under conditions with submicromolar Ca^{2+} concentrations (Endo, 1973; Fabiato and Fabiato, 1978; Iwazumi and Pollack, 1981; Stephenson and Wendt, 1984). Under isotonic conditions, the active tension after yielding (*thin dashed line*) was the same as that before yielding (*thick dashed line*), even though the maximum number of cross-bridges should be smaller. It should be noted that the level of tension under SPOC conditions is much smaller than that in normal Ca^{2+} activation, so that there is room available for a number of force-generating cross-bridges. The number of force-generating cross-bridges may be self-controlled during sarcomere oscillations (cf. Ishiwata and Yasuda, 1993). To confirm this inference, the stiffness of myofibrils should be measured during isotonic SPOC.

We thank Dr. Toshiki Yagi and Dr. Shinji Kamimura of the University of Tokyo for valuable advice, Mr. Tohru Takakura of Waseda University for technical assistance, and Dr. Shin-ichiro Umemura and Dr. Shojiro Asai of Hitachi for their continuous encouragement.

This work was supported in part by Grants-in-Aid for Scientific Research (04402053 to SI) and for Scientific Research on Priority Areas (06213233 to SI) from the Ministry of Education, Sport, Science and Culture of Japan.

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