Spontaneous Oscillatory Contraction without Regulatory Proteins in Actin Filament-Reconstituted Fibers

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ABSTRACT Skinned skeletal and cardiac muscle fibers exhibit spontaneous oscillatory contraction (SPOC) in the presence of MgATP, MgADP, and inorganic phosphate (P_i), but the molecular mechanism underlying this phenomenon is not yet clear. We have investigated the role of regulatory proteins in SPOC using cardiac muscle fibers of which the actin filaments had been reconstituted without tropomyosin and troponin, according to a previously reported method (Fujita et al., 1996. *Biophys. J.* 71:2307–2318). That is, thin filaments in glycerinated cardiac muscle fibers were selectively removed by treatment with gelsolin. Then, by adding exogenous actin to these thin filament-free cardiac muscle fibers under polymerizing conditions, actin filaments were reconstituted. The actin filament-reconstituted cardiac muscle fibers generated active tension in a Ca^{2+}-insensitive manner because of the lack of regulatory proteins. Herein we have developed a new solvent condition under which SPOC occurs, even in actin filament-reconstituted fibers: the coexistence of 2,3-butanedione 2-monoxime (BDM), a reversible inhibitor of actomyosin interactions, with MgATP, MgADP and P_i. The role of BDM in the mechanism of SPOC in the actin filament-reconstituted fibers was analogous to that of the inhibitory function of the tropomyosin-troponin complex (-Ca^{2+}) in the control fibers. The present results suggest that SPOC is a phenomenon that is intrinsic to the actomyosin motor itself.

INTRODUCTION

Muscle is generally in one of two possible states, relaxation or contraction, depending on the Ca^{2+} concentration in the presence of MgATP (Ebashi and Endo, 1968; Weber and Murray, 1973). On the other hand, skinned cardiac muscle fibers are in an oscillatory state at intermediate Ca^{2+} concentrations (Fabiato and Fabiato, 1978; Sweitzer and Moss, 1990; Linke et al., 1993; Fukuda et al., 1996). This is also the case in slow-type skeletal muscle (Iwazumi and Pollack, 1981; Stephenson and Williams, 1981), but not in fast-type skeletal muscle (Ishiwata and Yasuda, 1993).

Nearly 10 years ago, we established a new autooscillation condition, that is, the coexistence of MgATP and its hydrolytic products, MgADP and P_i, in myofibrils (Okamura and Ishiwata, 1988; Ishiwata et al., 1991; Anazawa et al., 1992; Yasuda et al., 1996) and skinned muscle fibers (Shimizu et al., 1992; Ishiwata et al., 1993; Fukuda et al., 1996). We termed this phenomenon spontaneous oscillatory contraction (SPOC). SPOC was classified into ADP-SPOC, which occurs under the coexistence of MgATP, MgADP, and P_i in the absence of Ca^{2+}, and Ca-SPOC, which occurs in the presence of micromolar concentrations of Ca^{2+} under normal activating conditions. Under SPOC conditions, isometric tension oscillates spontaneously. When myofibrils are observed under a phase contrast microscope, repetition of rapid lengthening followed by a slow shortening of sarcomeres occurs reproducibly, resulting in an oscillation of sarcomere length with a sawtooth waveform. SPOC conditions are sandwiched between relaxation and contraction conditions, indicating that SPOC is a third state of muscle, occurring between the two major states (Ishiwata and Yasuda, 1993; Ishiwata et al., 1993; Fukuda et al., 1996).

Although the chemical and mechanical properties of SPOC have been studied for nearly a decade, the molecular mechanism of SPOC is not yet clear. In the present study, to clarify the role of regulatory proteins in SPOC, we investigated tropomyosin-troponin complex-free actin filament-reconstituted cardiac muscle fibers. Thin filaments in skeletal and cardiac muscle fibers can be selectively removed using calf plasma gelsolin, an actin filament-severing protein (Funatsu et al., 1990, 1993; Yasuda et al., 1995). By adding exogenous actin to the thin filament-free cardiac muscle fibers under polymerizing conditions, actin filaments can be fully reconstituted (Fujita et al., 1996). Because of the lack of regulatory proteins, these actin filament-reconstituted fibers generate active tension in a Ca^{2+}-insensitive manner, and tension oscillation was not observed under standard SPOC conditions. Herein we have succeeded in generating spontaneous tension oscillation in actin filament-reconstituted fibers by adding 2,3-butanedione 2-monoxime (BDM), an inhibitor of actomyosin interactions (Li et al., 1985; Horiuti et al., 1988). These results indicate that regulatory proteins are not necessarily required for autooscillation of the contractile system and that the actomyosin motor itself has autooscillatory properties.

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MATERIALS AND METHODS

Muscle fibers and proteins

Bovine cardiac muscle bundles (~5 mm in diameter) were excised from a straight portion of left ventricular papillary muscle while the muscle was

still warm. Both ends of the muscle bundle were tied to a glass rod and incubated overnight in glycerol solution composed of 50% (v/v) glycerol, 0.5 mM $NaHCO_3$, 5 mM EGTA, and 2 mM leupeptin at 0°C. Fibers were then stored in fresh glycerol solution at −20°C. Glycerinated fibers were used between 2 and 8 weeks after storage. Bovine plasma gelsolin was prepared according to the method of Kurokawa et al. (1990). Actin was extracted from acetone powder (Kondo and Ishiwata, 1976) of bovine cardiac muscle according to the method of Spudich and Watt (1971). Purified G-actin was stored at 0°C and used within 2 weeks.

Solutions

The solutions used were rigor solution (170 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0)); relaxing solution (117 mM KCl, 5 mM $MgCl_2$, 4 mM ATP, 1 mM EGTA, 10 mM MOPS (pH 7.0), 20 mM 2,3-butanedione 2-monoxime (BDM)); contracting solution (117 mM KCl, 4.25 mM $MgCl_2$, 2.2 mM ATP, 2 mM EGTA, 20 mM MOPS (pH 7.0), 1.9 mM $CaCl_2$); and standard SPOC solution (41 mM KCl, 14.2 mM $MgCl_2$, 2.2 mM ATP, 16.4 mM ADP, 2 mM EGTA, 10 mM MOPS (pH 7.0), 10 mM P_i, 0.1 mM P^1,P^5-di(adenosine-5′)pentaphosphate (AP_5A)). ATP (Na-salt), ADP (K-salt), and AP_5A were purchased from Boehringer Mannheim (Mannheim, Germany); EGTA and MOPS were from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of reagent grade. The concentrations of free Mg^{2+}, MgATP, MgADP, P_i, and other chemicals were calculated by computer, using the published values for stability constants (Horiuti, 1986).

Tension measurement

For the experiments, a glycerinated thin bundle (~1 mm in length, ≤60 μm in diameter) was carefully stripped from a glycerinated fiber with a pair of forceps with the aid of a stereomicroscope just before use. To prepare a suitably thin bundle, dissection was carried out in glycerol solution at around −10°C (Fukuda et al., 1996). Both ends of the muscle bundle were fixed to thin tungsten wires with enamel, one of which was attached to a tension transducer (AE-801; SensoNor a.s, Holten, Norway). The muscle was then immersed in rigor solution containing 1% Triton X-100 for 20 min to remove residual portions of the membrane system. After the Triton X-100 was washed out with rigor solution, the muscle was immersed in relaxing solution. Active tension was measured by immersing the muscle bundle in activating solution, and the measurements were recorded with a pen recorder (VP-6533A; National, Tokyo). Tension was first measured in the standard solution and then, after immersion in the relaxing solution, in the assay solution. The tension was then measured again in the standard solution. The relative tension was estimated from the ratio of the tension measured in the assay solution to the average of the standard tension before and after the assay measurement. The measurement chamber used was a silicon-coated aluminum block (10 cm × 10 cm × 1 cm) with several small holes (5 mm in diameter) filled with ~0.4 ml of various solutions, designed for tension measurements (Horiuti, 1986). The muscle was immersed in the piled-up portion of the solution, such that only 1–2 s was required for transfer of the muscle from one solution to another.

Removal and reconstitution of actin filaments

Fig. 1 schematically illustrates the process of removal and reconstitution of thin filaments in a sarcomere. Cardiac muscle fibers were immersed in contracting solution containing 20 mM BDM (to suppress tension development during gelsolin treatment) and 0.3 mg/ml gelsolin at 2°C for 80 min to remove thin filaments. We confirmed that no active tension developed after the gelsolin treatment in contracting solution. Fibers were then immersed in actin-polymerizing solution (80 mM KI, 4 mM $MgCl_2$, 4 mM ATP, 4 mM EGTA, 20 mM BDM, and 20 mM K-phosphate, pH 7.0) containing 1 mg/ml purified G-actin that had been mixed just before use. The polymerizing solutions containing actin were newly prepared and exchanged every 7 min. The actin polymerizing treatment was applied for a total of 28 min (7 min × 4), shorter than the 42 min (7 min × 6) reported in our previous paper (Fujita et al., 1996), so that the average tension developed in the actin filament-reconstituted fibers was nearly equal to that in the control fibers. All procedures were carried out at 2°C. Relaxation of the actin filament-reconstituted fibers was achieved by immersing the fibers in relaxing solution containing 20 mM BDM. We confirmed that Ca^{2+} sensitivity was not observed in actin filament-reconstituted fibers.

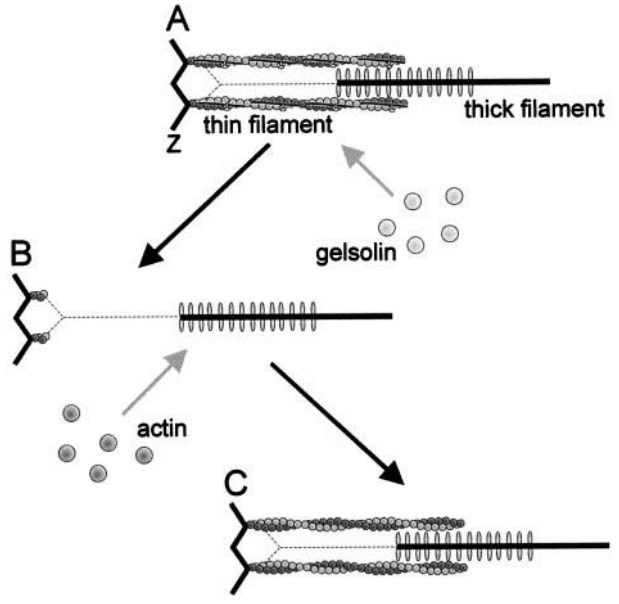

FIGURE 1 Schematic diagram illustrating how to remove and reconstitute actin filaments in a half-sarcomere of muscle fibers. Thin filaments in glycerinated cardiac muscle fibers (*A*) can be removed by gelsolin, yielding thin filament-free muscle fibers (*B*). (*C*) Actin filaments can be reconstituted by adding exogenous G-actin under polymerizing conditions. The actin filament-reconstituted fibers do not have regulatory proteins in reconstituted actin filaments and thus generate active tension in a Ca^{2+}-insensitive manner. Dotted lines schematically show connectin/titin that connects the thick filament and the Z line.

Laser scanning confocal microscopy

Both ends of glycerinated fibers (~2 mm in length, ≤60 μm in diameter) were attached, via double-sided adhesive tape, to a glass slide so that fibers were suspended in solution without touching the glass surface. Fibers were stained with 8 μM rhodamine-phalloidin (RhPh) (Molecular Probes, Eugene, OR) in relaxing solution for 2 h at 0°C, and then free RhPh was washed out with relaxing solution. For microscopic observation, 20 mM dithiothreitol, 0.22 mg/ml glucose oxidase, 0.036 mg/ml catalase, and 4.5 mg/ml glucose were added to the solution. The preparation was observed with an inverted microscope (TMD-300; Nikon, Tokyo) equipped with a real-time confocal scanning unit (CSU10; Yokogawa Electric Co., Tokyo), and fluorescence images were recorded on a videotape recorder.

RESULTS

Effects of MgADP on isometric tension development

We first examined the effects of MgADP on the tension developed by actin filament-reconstituted cardiac muscle fibers, because SPOC observed in the absence of Ca^{2+} is a phenomenon that occurs under activation by MgADP. As

previously reported (Fukuda et al., 1996), skinned cardiac muscle fibers (control) generated isometric tension with the addition of MgADP under relaxing conditions (Fig. 2, *open circles*), suggesting that MgADP functions as an activator, probably because the AMADP (A, actin; M, myosin) complex acts as a desuppressor of the inhibitory state of thin filaments (Shimizu et al., 1992). In Ca^{2+}-activated fibers, tension augmentation up to ~20% occurred with the addition of low concentrations of MgADP (0.5–3 mM), reaching a maximum at ~1 mM (Fig. 2, *filled circles*). Active tension was decreased by further addition of MgADP up to 10 mM, and was below the original level. These results are consistent with those previously reported (Cooke and Pate, 1985; Kawai, 1986). In the actin filament-reconstituted fibers, active tension developed irrespective of the presence or absence of Ca^{2+} when MgATP was present, because of the lack of regulatory proteins. Tension augmentation was not observed in the actin filament-reconstituted fibers at low concentrations of MgADP; rather, only monotonous inhibition was observed regardless of the Ca^{2+} concentration. Active tension decreased by ~10% with the addition of 10 mM MgADP in both the presence and absence of Ca^{2+} (Fig. 2, *triangles*).

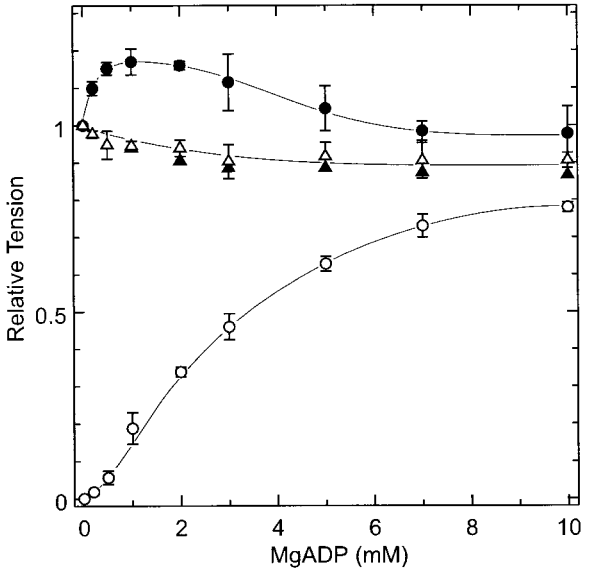

FIGURE 2 Effects of MgADP on active tension in control (○, ●) and actin filament-reconstituted (△, ▲) fibers. ●, ▲, With (pCa < 5) Ca^{2+}; ○, △, without (pCa > 8) Ca^{2+}. Active tension was measured at 20°C. Relaxation of actin filament-reconstituted fibers was obtained by the addition of 20 mM BDM. Tension was normalized to that of Ca^{2+}-activated fibers without MgADP. The ratio of the normalized tension level between control and actin filament-reconstituted fibers was nearly 1. Vertical bars show SD calculated from three to five data points. Solvent conditions: 4.25–15.6 mM $MgCl_2$ (2 mM free Mg^{2+}), 2.2 mM ATP (2 mM MgATP), 2 mM EGTA, 10 mM MOPS (pH 7.0), 0.1 mM AP_5A, ±2 mM $CaCl_2$ (+Ca^{2+}: pCa < 5; -Ca^{2+}: pCa > 8), and various MgADP concentrations (0–15.6 mM ADP). Ionic strength (I.S.) was maintained at 150 mM by the addition of KCl (61–120 mM KCl).

Effects of P_i on isometric tension in the presence of MgADP

Next we examined the effects of P_i on the isometric tension developed in the presence of 10 mM MgADP. In the control fibers, the tension was decreased by the addition of P_i and reached a plateau at ~50% of the initial tension (Fig. 3, *open circles*). SPOC was observed when more than 3 mM P_i was added, as previously reported (Fukuda et al., 1996). On the other hand, in the actin filament-reconstituted fibers, no tension decrease was observed in the 1–10 mM range of P_i (Fig. 3, *triangles*). This result was indistinguishable from that of the control fibers in the presence of Ca^{2+} (pCa < 5.0) (Fig. 3, *filled circles*). SPOC was not observed in either the actin filament-reconstituted fibers or the control fibers with Ca^{2+}.

Effects of BDM on isometric tension and SPOC

In the actin filament-reconstituted fibers, not only SPOC but also tension suppression did not occur with the addition of P_i in the presence of MgADP and MgATP. We therefore examined the inhibitory effects of BDM in combination with or instead of P_i. Fig. 4 *A* shows the effects of BDM on isometric tension in the presence of 10 mM MgADP and 10 mM P_i. In control fibers (Fig. 4 *A*, *open circles*), when BDM was added under SPOC conditions, active tension decreased in a dose-dependent manner and became undetectable in the

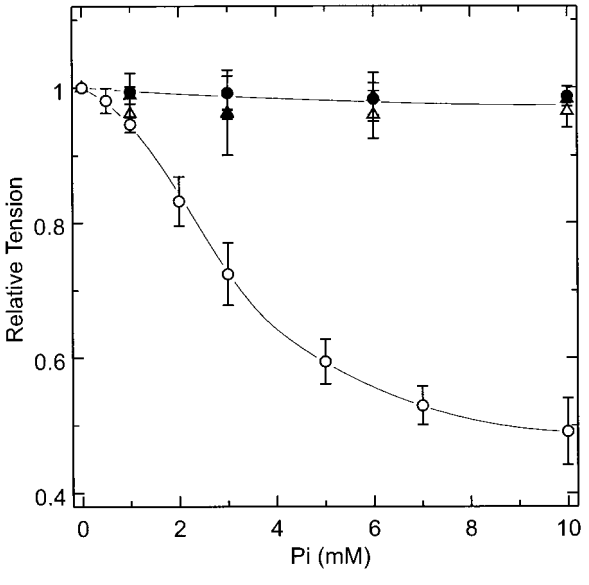

FIGURE 3 Effects of P_i on MgADP-induced tension in control (○, ●) and actin filament-reconstituted (△, ▲) fibers. ●, ▲, With (pCa < 5) Ca^{2+}; ○, △, without (pCa > 8) Ca^{2+}. Active tension was measured at 20°C. Tension was normalized to that obtained at 0 mM P_i. Vertical bars show SD calculated from three to five data points. Solvent conditions: 15.6 mM $MgCl_2$ (2 mM free Mg^{2+}), 2.2 mM ATP (2 mM MgATP), 15.6 mM ADP (10 mM MgADP), 2 mM EGTA, 10 mM MOPS (pH 7.0), 0.1 mM AP_5A, ±2 mM $CaCl_2$ (+Ca^{2+}: pCa < 5; -Ca^{2+}: pCa > 8), and various P_i concentrations. I.S. was maintained at 150 mM by the addition of KCl (38–58 mM KCl).

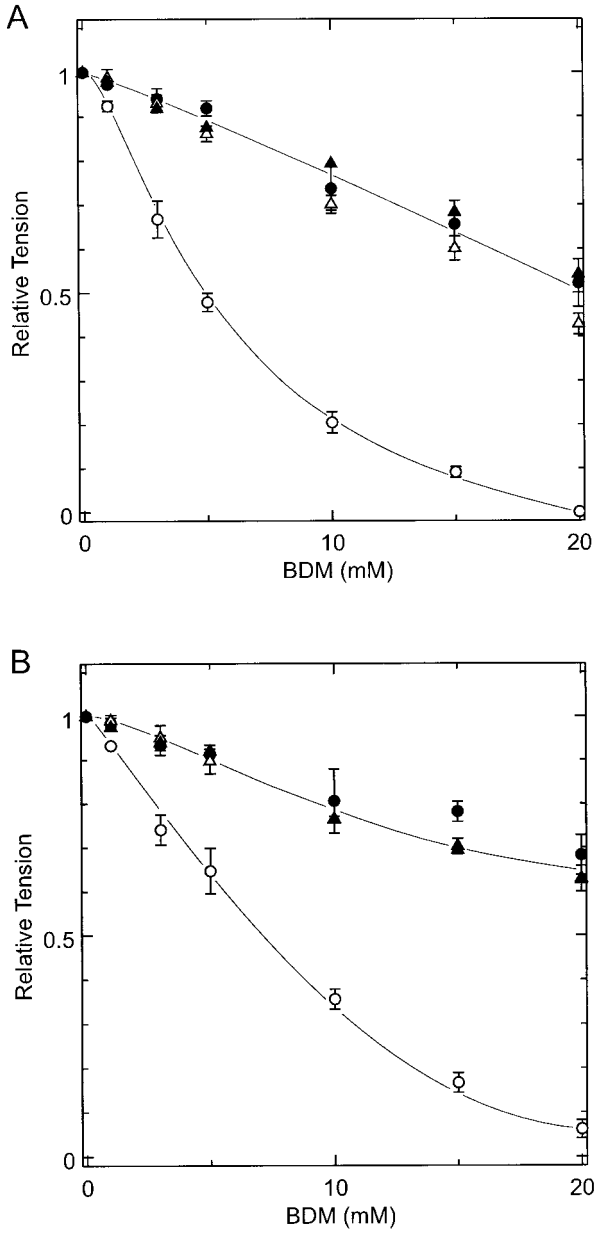

FIGURE 4 Effects of BDM on MgADP-induced tension in control (○, ●) and actin filament-reconstituted (△, ▲) fibers in the presence (*A*) and absence (*B*) of P_i. ●, ▲, With (pCa < 5) Ca^{2+}: ○, △, without (pCa > 8) Ca^{2+}. Active tension was measured at 20°C. Tension was normalized to that obtained in the absence of BDM. Vertical bars are SD calculated from three to five data points. Solvent conditions: 15.6 mM $MgCl_2$ (2 mM free Mg^{2+}), 2.2 mM ATP (2 mM MgATP), 15.6 mM ADP (10 mM MgADP), 2 mM EGTA, 10 mM MOPS (pH 7.0), 0.1 mM AP_5A, ±10 mM P_i, ±2 mM $CaCl_2$ (+Ca^{2+}: pCa < 5; -Ca^{2+}: pCa > 8), and various BDM concentrations. I.S. was maintained at 150 mM by the addition of KCl (39–61 mM KCl).

presence of 20 mM BDM; tension oscillation was observed between 0 and 10 mM BDM but, with further addition of BDM, became undetectable because of the minimal tension. In both control fibers with Ca^{2+} and actin filament-reconstituted fibers, the tension decreased with the addition of BDM, but the degree of the decrease was much smaller, only 50–60%, even at 20 mM BDM.

Surprisingly, in both the control fibers with Ca^{2+} and the actin filament-reconstituted fibers, tension oscillation occurred when 10–20 mM BDM was added. An example of recordings of this tension oscillation is shown in Fig. 5. The period of tension oscillation was ~20 s. The amplitude and frequency of oscillation were almost the same as those of SPOC observed in the control fibers without BDM, i.e., under standard SPOC conditions. These results show that SPOC occurs even without regulatory proteins when the chemical conditions are met.

Next we examined the effects of BDM on isometric tension in the presence of MgADP without P_i (Fig. 4 *B*). With the addition of BDM, MgADP-activated tension decreased monotonously, falling below 10% at 20 mM BDM in the control fibers without Ca^{2+}. In the control fibers with Ca^{2+} and the actin filament-reconstituted fibers, the tension decrease was smaller than that in the presence of P_i (Fig. 4 *A*). The degree of the tension decrease was 30–40% at 20 mM BDM. The smaller tension suppression, as compared to that in the presence of P_i, is attributable to a small population of non-force-generating complexes, such as $AMADPP_i$, stabilized by BDM (Higuchi and Takemori, 1989; Mckillop et al., 1994). When P_i was absent, tension oscillation was not observed, even when active tension was partially suppressed by BDM, indicating that the presence of P_i is essential for SPOC.

Sarcomere length oscillation inside actin filament-reconstituted fibers

To determine the sarcomere length oscillation within fibers, which is one of the characteristics of SPOC, actin filaments were stained with fluorescent rhodamine-phalloidin and observed under a laser-scanning confocal fluorescence microscope.

Fig. 6 *A* shows the internal structure of actin filament-reconstituted fibers under SPOC conditions produced by 10 mM BDM. As shown in a series of micrographs in Fig. 6 *B*, the Z lines moved back and forth, a phenomenon confirmed to last for 5–10 min. The period of this translational movement of sarcomeres was ~20 s, which is consistent with the

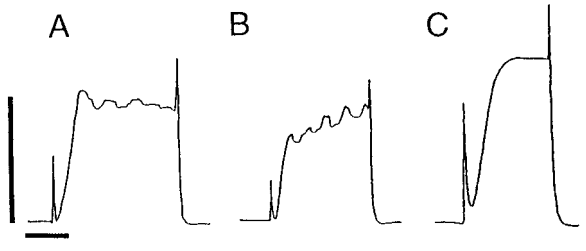

FIGURE 5 Recordings of SPOC in control (*A* and *C*) and actin filament-reconstituted (*B*) fibers. Solvent conditions: (*A*) standard SPOC solution; (*B*) standard SPOC solution with 10 mM BDM; (*C*) standard SPOC solution without 10 mM P_i and with 20 mM BDM. Vertical and horizontal bars indicate 5×10^{-5} N and 30 s, respectively.

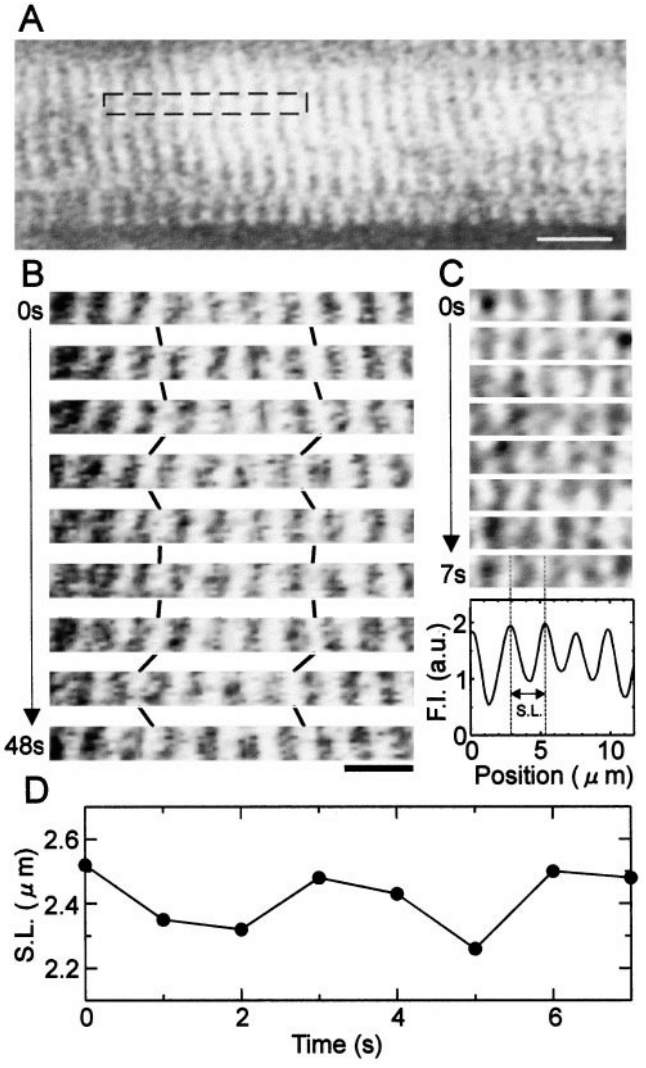

FIGURE 6 Confocal fluorescence image of actin filament-reconstituted fibers stained with rhodamine-phalloidin under SPOC conditions. (*A*) Micrograph of the internal structure of actin filament-reconstituted fibers in standard SPOC solution with 10 mM BDM. The width and thickness of the fibers were ~40 μm and 30 μm, respectively. White scale bar, 10 μm. (*B*) Series of micrographs taken every 6 s of the sarcomeres located in the box surrounded by dashed lines in *A*. The centers of the I-Z-I brushes corresponding to the Z lines are connected by thin lines, allowing observation of the translational movement of sarcomeres. Black scale bar, 5 μm. (*C*) Series of micrographs taken every second, arbitrarily chosen within the box of *A*. *Bottom*: Fluorescence intensity (F.I.) profile of sarcomeres. (*D*) Time course of length change of one sarcomere; the sarcomere length (S.L.) was measured from the separation between two adjacent peaks of the F.I. profile shown in *C*.

period of tension oscillation in fibers. On the other hand, each sarcomere length oscillated in shorter periods (~3 s), as shown in Fig. 6, *C* and *D*, consistent with the period of sarcomere length oscillation observed in myofibrils under standard SPOC conditions. We confirmed that neither periodic translational movement of sarcomeres nor sarcomere length oscillation was observed under either relaxing or contracting conditions (data not shown).

DISCUSSION

Effects of MgADP and P_i

In control fibers, tension augmentation of up to ~20% occurred with the addition of MgADP (Fig. 2). This tension augmentation can be explained as follows. In the control fibers, Ca^{2+} binding is not adequate to fully desuppress the inhibitory function of the regulatory system; desuppression due to strong binding of the AMADP complex is also required to attain full activation of the thin filaments. The decrease in active tension with further addition of MgADP is attributable to competitive binding of MgADP to the MgATP binding site (Shimizu et al., 1992; Seow and Ford, 1997). The above interpretation is supported by the present observation that only a monotonous decrease, without augmentation of tension by MgADP, was observed in the actin filament-reconstituted fibers (Fig. 2); actin filaments without regulatory proteins are considered to be in a fully activated state under the conditions we examined. This is consistent with the previous result that the active tension of the actin filament-reconstituted fibers decreased to some extent (~20%) after the incorporation of regulatory proteins (Figure 8 *a* in Fujita et al., 1996, the vertical scale in which should read "5×10^{-5} N"). These results are also consistent with the binding of myosin subfragment 1 (S1) with the reconstituted F-actin-tropomyosin-troponin complex (Green and Eisenberg, 1980) or thin filaments in myofibrils (Swartz et al., 1996), in the absence of ATP, being cooperative even in the presence of Ca^{2+}. That is, the apparent binding constant increases sigmoidally with the addition of S1, which indicates that the thin filaments are not in a fully activated state, even in the presence of Ca^{2+}.

The tension decrease in response to P_i (Fig. 3), observed in MgADP-activated control fibers ($-Ca^{2+}$), can be interpreted based on the kinetic scheme of actomyosin ATPase. In the MgADP-activated fibers ($-Ca^{2+}$), thin filaments are considered to be activated by the AMADP (strong-binding) complex formed by the association of MgADP with the AM complex. When P_i is added, the population of $AMADPP_i$ complexes increases, whereas that of AMADP complexes decreases, resulting in the deactivation of thin filaments. In the presence of both MgADP and Ca^{2+} in the actin filament-reconstituted fibers, P_i did not act as a deactivator, probably because the AMADP complex described above is not the main factor in activation.

Effects of BDM

Although the inhibitory effects of P_i and BDM on tension development are apparently similar and additive, significant differences exist between the effects of these two substances: 1) P_i is essential for SPOC, whereas the role of BDM in SPOC can be played by the tropomyosin-troponin complex ($-Ca^{2+}$). 2) The inhibitory effects of BDM (Fig. 4 *B*) on tension development with MgADP of both control fibers and actin filament-reconstituted fibers are larger than

those of P_i (Fig. 3). Furthermore, under normal activating conditions without MgADP, BDM can completely relax the fibers, whereas P_i cannot. These differences between the actions of P_i and BDM in the SPOC mechanism and tension development can be explained as follows. If the $AMADPP_i$ complex is composed of at least two isoforms as previously reported (Dantzig et al., 1992), i.e., non-force-generating ($AMADPP_i(I)$) and force-generating ($AMADPP_i(II)$), P_i must attach to the second isoform, whereas BDM may stabilize the first so as to relax the muscle. Thus the minimum requirements for SPOC, which we previously proposed (Ishiwata et al., 1993; Ishiwata and Yasuda, 1993), that a weak binding (non-force-generating) actomyosin complex (e.g., $AMADPP_i(I)$) and a strong binding (force-generating) actomyosin complex (e.g., AMADP and $AMADPP_i(II)$) coexist over a certain threshold proportion, may be met by the addition of BDM. This explanation is consistent with the idea that the relaxing mechanism of BDM is similar to that of the tropomyosin-troponin complex ($-Ca^{2+}$). The possibility remains that the binding sites of P_i essential for SPOC are not only on myosin catalytic sites, but also on actin nucleotide binding sites. This awaits investigation.

Period of tension and sarcomere length oscillation in SPOC

The period of tension oscillation of actin filament-reconstituted fibers under SPOC conditions with BDM, 10–20 s (Fig. 5 *B*), was consistent with that of control fibers under standard SPOC conditions (~10 s for skeletal muscle: see Shimizu et al., 1992; 10–20 s for cardiac muscle: see Fukuda et al., 1996). On the other hand, the average period of sarcomere length oscillation observed under a confocal fluorescence microscope was much shorter than that of tension oscillation, i.e., 3–4 s, as observed in Fig. 6, *C* and *D*. Such a short period of sarcomere length oscillation is consistent with the period of oscillations of sarcomere length and tension observed in single (or a thin bundle of) myofibrils under a microscope, which is 1–7 s (for skeletal muscle, see Okamura and Ishiwata, 1988; Yasuda et al., 1996; for cardiac muscle, see Fabiato and Fabiato, 1978; Linke et al., 1993).

As for the apparent discrepancy between the long period of tension oscillation in muscle fibers and the short period of sarcomere length oscillation, we suggested in the previous paper (Fukuda et al., 1996) that the long period of tension oscillation may be the effect of interference of a number of oscillations with different periods of sarcomere length oscillation. In fact, a domain consisting of hundreds of sarcomeres showed a translational movement with a period of ~20 s (see the movement of connected thin lines in Fig. 6 *B*). Such a long period of movement of large domains in the fibers coincides with a long period of tension oscillation of fibers. On the other hand, the waveform of sarcomere length oscillation (Fig. 6 *D*) was not so regular as observed in myofibrils, in which the distinct sawtooth waveform of each sarcomere length oscillation was usually observed. We confirmed that the above properties were also the case for control cardiac fibers under standard SPOC conditions (data not shown). Thus the SPOC observed in the actin filament-reconstituted fibers in the presence of BDM was indistinguishable from that observed in the control fibers under standard SPOC conditions.

CONCLUSIONS

We have shown that SPOC occurs even in fibers without regulatory proteins, i.e., tropomyosin-troponin complexes, when the chemical requirements are met, indicating that SPOC is a phenomenon that is inherent to the actomyosin motor itself. In this respect, Jülicher and Prost (1997) showed theoretically that spontaneous oscillation, the waveform of which is similar to that of SPOC, can occur in a system of collective molecular motors elastically coupled to each other, in which regulatory mechanisms are not taken into account. The present results and these theoretical considerations strongly suggest that molecular motor itself (or its assembly) possesses autooscillatory properties, such that SPOC may occur in an in vitro motility assay system consisting of molecular motors and pure actin filaments when an external load is imposed under appropriate conditions.

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REFERENCES

Anazawa, T., K. Yasuda, and S. Ishiwata. 1992. Spontaneous oscillation of tension and sarcomere length in skeletal myofibrils. Microscopic measurement and analysis. *Biophys. J.* 61:1099–1108.

Cooke, R., and E. Pate. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys. J.* 48:789–798.

Dantzig, J. A., Y. E. Goldman, N. C. Millar, J. Lacktis, and E. Homsher. 1992. Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibres. *J. Physiol. (Lond.).* 451:247–278.

Ebashi, S., and M. Endo. 1968. Calcium ions and muscle contraction. *Prog. Biophys. Mol. Biol.* 18:123–183.

Fabiato, A., and F. Fabiato. 1978. Myofilament-generated tension oscillation during partial calcium activation and activation dependence of the sarcomere length-tension relation of skeletal cardiac cells. *J. Gen. Physiol.* 72:667–699.

Fujita, H., K. Yasuda, S. Niitsu, T. Funatsu, and S. Ishiwata. 1996. Structural and functional reconstitution of thin filaments in the contractile apparatus of cardiac muscle. *Biophys. J.* 71:2307–2318.

Fukuda, N., H. Fujita, T. Fujita, and S. Ishiwata. 1996. Spontaneous tension oscillation in skinned bovine cardiac muscle. *Pflügers Arch.* 433:1–8.

Funatsu, T., H. Higuchi, and S. Ishiwata. 1990. Elastic filaments in skeletal muscle revealed by selective removal of thin filaments with plasma gelsolin. *J. Cell Biol.* 110:53–62.

Funatsu, T., E. Kono, H. Higuchi, S. Kimura, S. Ishiwata, T. Yoshioka, K. Maruyama, and S. Tsukita. 1993. Elastic filaments in situ in cardiac

muscle: deep-etch replica analysis in combination with selective removal of actin and myosin filaments. *J. Cell Biol.* 120:711–724.

Greene, L. E., and E. Eisenberg. 1980. Cooperative binding of myosin subfragment-1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. USA.* 77:2616–2620.

Higuchi, H., and S. Takemori. 1989. Butanedione monoxime suppresses contraction and ATPase activity of rabbit skeletal muscle. *J. Biochem.* 105:638–643.

Horiuti, K. 1986. Some properties of the contractile system and sarcoplasmic reticulum of skinned slow fibers from *Xenopus* muscle. *J. Physiol. (Lond.).* 373:1–23.

Horiuti, K., H. Higuchi, Y. Umazume, M. Konishi, O. Okazaki, and S. Kurihara. 1988. Mechanism of action of 2,3-butanedione 2-monoxime on contraction of frog skeletal muscle. *J. Muscle Res. Cell Motil.* 9:156–164.

Ishiwata, S., and K. Yasuda. 1993. Mechano-chemical coupling in spontaneous oscillatory contraction of muscle. *Phase Transitions.* 45: 105–136.

Ishiwata, S., N. Okamura, H. Shimizu, T. Anazawa, and K. Yasuda. 1991. Spontaneous oscillatory contraction (SPOC) of sarcomeres in skeletal muscle. *Adv. Biophys.* 27:227–235.

Ishiwata, S., T. Anazawa, T. Fujita, T. Fukuda, H. Shimizu, and K. Yasuda. 1993. Spontaneous tension oscillation (SPOC) of muscle fibers and myofibrils. Minimum requirements for SPOC. *In* Mechanism of Myofilament Sliding in Muscle Contraction. H. Sugi and G. Pollack, editors. Plenum, London. 545–556.

Iwazumi, T., and G. H. Pollack. 1981. The effect of sarcomere nonuniformity on the sarcomere length-tension relationship of skinned fibers. *J. Cell. Physiol.* 106:321–337.

Jülicher, F., and J. Prost. 1997. Spontaneous oscillations of collective molecular motors. *Phys. Rev. Lett.* 78:4510–4513.

Kawai, M. 1986. The role of orthophosphate in cross-bridge kinetics in chemically skinned rabbit psoas fibres as detected with sinusoidal and step length alterations. *J. Muscle Res. Cell Motil.* 7:421–434.

Kondo, H., and S. Ishiwata. 1976. Uni-directional growth of F-actin. *J. Biochem.* 79:159–171.

Kurokawa, H., W. Fujii, K. Ohmi, T. Sakurai, and Y. Nonomura. 1990. Simple and rapid purification of brevin. *Biochem. Biophys. Res. Commun.* 168:451–457.

Linke, W. A., M. L. Bartoo, and G. H. Pollack. 1993. Spontaneous sarcomeric oscillations at intermediate activation levels in skinned isolated cardiac myofibrils. *Circ. Res.* 73:724–734.

Li, T., N. Sperelakis, R. E. Teneick, and J. Solaro. 1985. Effects of diacetyl monoxime on cardiac excitation-contraction coupling. *J. Pharmacol. Exp. Ther.* 232:688–695.

Mckillop, D. F. A., N. S. Fortune, K. W. Ranatunga, and M. A. Geeves. 1994. The influence of 2,3-butanedione 2-monoxime (BDM) on the interaction between actin and myosin in solution and in skinned muscle fibers. *J. Muscle Res. Cell Motil.* 15:309–318.

Okamura, N., and S. Ishiwata. 1988. Spontaneous oscillatory contraction of sarcomeres in skeletal myofibrils. *J. Muscle Res. Cell Motil.* 9:111–119.

Seow, C. Y., and L. E. Ford. 1997. Exchange of ATP for ADP on high-force cross-bridges of skinned rabbit muscle fibers. *Biophys. J.* 72:2719–2735.

Shimizu, H., T. Fujita, and S. Ishiwata. 1992. Regulation of tension development by MgADP and P_i without Ca^{2+}. Role in spontaneous tension oscillation of skeletal muscle. *Biophys. J.* 61:1087–1098.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Stephenson, D. G., and D. A. Williams. 1981. Calcium-activated force response in fast- and slow-twitch skinned muscle fibres of the rat at different temperatures. *J. Physiol. (Lond.).* 317:281–317.

Swartz, D. R., R. L. Moss, and M. L. Greaser. 1996. Calcium alone does not fully activate the thin filament for S1 binding to rigor myofibrils. *Biophys. J.* 71:1891–1904.

Sweitzer, N. K., and R. L. Moss. 1990. The effect of altered temperature on Ca^{2+}-sensitive force in permeabilized myocardium and skeletal muscle. *J. Gen. Physiol.* 96:1221–1245.

Weber, A., and J. M. Murray. 1973. Molecular control mechanisms in muscle contraction. *Physiol. Rev.* 53:612–673.

Yasuda, K., T. Anazawa, and S. Ishiwata. 1995. Microscopic analysis of the elastic properties of nebulin in skeletal myofibrils. *Biophys. J.* 68:598–608.

Yasuda, K., Y. Shindo, and S. Ishiwata. 1996. Synchronous behavior of spontaneous oscillations of sarcomeres in skeletal myofibrils under isotonic conditions. *Biophys. J.* 70:1823–1829.