Abstract: We developed an electromagnetic apparatus to perform a quick change in load in the motility system, using magnetizable beads on which myosin thick filaments from molluscan smooth muscle or green algae, Chara, myosin were attached. The quick change in load to beads (diameter 4.5 μm) was applied in the range of 0–85 pN. The movement of beads was recorded by a video-system and analyzed with special software. When the quick increase in load was applied during the movement of beads under no load, the beads showed a transient movement to the reverse direction before the steady slower movement to the normal direction. When the application of load was stopped, the beads showed a transient fast phase of movement. The change in load-sustaining ability was measured by a double load step. The backward velocity at the second constant test load was smaller when the first preceding step was increased, suggesting that the ability to sustain load was higher with a higher preceding step. These phenomena were observed both in molluscan thick filaments and in Chara myosin, and the time course of the movement of a bead was quite similar to those observed previously in frog single muscle fibers. This suggested that the velocity transients are the intrinsic properties induced by the interaction between actin and myosin, irrespective of the hexagonal lattice structure of filaments, the regular sarcomere structure, and myosin type, namely, that the molecule of myosin itself has the ability to adjust to mechanical circumstances.

Key words: myosin, cross-bridge, actin, muscle, motility assay, velocity transients, force–velocity relation.

Throughout the long history of muscle research, muscle physiologists have attempted to gain information concerning the interactions between actin and myosin responsible for muscle contraction [1–3]. These works have been studied by applying rapid step changes in force or in length to isometrically tetanized fibers and by recording the resultant length or force changes, respectively [4–7]. The former methods provided information on isotonic velocity transients, the latter on isometric force transients.

Civan and Podolsky [5] constructed a mechanical system consisting of a light metal lever and a spring that imposed a constant isotonic load on a tetanized fiber. With this apparatus, they found that a quick decrease in load was followed by an initial oscillatory shortening while the load remained constant, contrary to the concept that shortening velocity is instantaneously determined by the load [2]. The shortening velocity they observed was initially fast, but it then slowed and finally reached a constant velocity. Similar oscillatory velocity transients following step changes in load have been recorded by other investigators, including ourselves [1, 8–14].

On the other hand, in a study of isometric force transients, Huxley and Simmons [4] applied small step length changes to an isometrically tetanized fiber and analyzed the resultant transient force changes. The changes following a step decrease in length of a middle fiber segment consisted of three phases. These phases in the isometric force transient were considered comparable to, and to correspond with, the three phases recorded in the isotonic force step experiments. That is, the two types of experiments clearly displayed two manifestations of the same underlying behavior of muscle fibers, and from the detailed analyses of the force transients, Huxley and Simmons [4] presented their well-known “cross-bridge rotation theory.” This Huxley-Simmons model has been central to muscle physiology, and many attempts have been made to prove that the tilting motion of the myosin head is responsible for producing force and motion in muscle. At the current time, discussions continue about whether the new cross-bridge rotation theory, which assumes rotation of the light-chain domain of myosin, is correct [15].

The above experiments investigating mechanical transients, however, have all been performed on living frog single striated muscle fibers, which are composed of complex structures, e.g., myofibrils, sarcomeres, hexagonal lattice, Z-discs, elastic proteins, and so on. Furthermore, as the concomitant structural changes that occur during contraction, such as “the staggering,” i.e., the irregular change in the shape of Z-discs, take place, the possibility...
that these structural changes may contribute to the mechanical transients in load or in length step cannot be ruled out. Edman and Tsuchiya [11] indicated that the strain of passive elements could be involved in isotonic velocity transients during force enhancement by stretch. The present experiment was performed to determine whether velocity transients could be observed in a simple reconstituted motility system in which the load was applied by an electromagnetic apparatus to magnetizable beads, on which myosin filaments from molluscan smooth muscle or myosin of Chara were attached, moving along actin filaments in algal internodal cells. The development of the electromagnetic apparatus was already reported [16].

MATERIALS AND METHODS

Electromagnetic apparatus and its operation. Figure 1 shows the electromagnetic apparatus and the analyzing process. The detail was described previously [16]; therefore it is explained only briefly. The electromagnetic apparatus consisted of a magnetic field generator, an electric controller, and an electric power supply (Kikusui, Yokohama; 6-120A). The direct current from the power supply was conical with a diameter of 3.5 mm at the top. A water-circulation tube was wound together with the coil for the electric current to prevent an increase in temperature of the magnet. A current, continuous for 15 s, increased the temperature 1.2°C at the top of the magnet, and a temperature sensor (Omron Tokyo; E5CS) was installed in the magnet so that an increase in temperature would stop the power supply. There was an air gap of 5–20 mm between the top of the magnet and the internodal cell preparation; therefore the influence of increase in temperature was negligible. The magnitude of magnetic field was measured by a gauss meter (Toyo Technica, Tokyo; type 4048), and the maximum magnitude at the top of the magnet was 8,000 Oe. A schematic drawing of the magnet and beads with a myosin filament from the anterior byssal retractor muscle (ABRM) of a mussel Mytilus edulis or with green algae Chara myosin on actin cables is shown in Fig. 1. The chamber in which the preparation was bathed could be moved by a micromanipulator so that the center of the magnet and the internodal cell preparation could be arranged to be in line during the experiment. The distance between the preparation and the top of the magnet could be as short as 2 mm, but measurements were usually performed at a distance of 5–20 mm at various strengths of electric current. The force exerted on the bead was calibrated by measuring the velocity of a bead in silicone oil of known viscosities under a given magnetic field, and it was calculated by Stoke’s law \( F = 6\pi r\eta v \), where \( F \) is force, \( \pi \) is constant, \( \eta \) is viscosity, \( r \) is the radius of a bead, and \( v \) is the velocity of a bead. The response time of the magnet when the electric current started to flow was measured by a force transducer with a very rapid response.

Fig. 1. A schematic drawing of the experimental setup. The electromagnetic apparatus to perform a quick change in load was used in the motility system, consisting of the magnetizable beads with myosin thick filaments from molluscan smooth muscle or with Chara myosin and of actin cables in the Chara internodal cells. A quick change in load was applied to the beads (diameter, 4.5 μm). Circulating water cooled the magnet’s coil, resulting in a negligible increase in temperature on the microscope stage. The movement of the beads was recorded by a video system and analyzed by computer. CI: electric current input; CO: electric current output; M: magnet; MC: magnetic coil; OC: optical condenser; O: objective lens; WI: water inlet; WO: water outlet.
Preparation of the thick filament-attached beads. Native thick filaments were isolated from the anterior byssal retractor muscle (ABRM) of a mussel *Mytilus edulis* by the method of Ishii *et al.* [17]. ABRM fibers were homogenized in the presence of 10^-6 M 5-HT (5-hydroxytryptamine) to keep the fibers relaxed. The isolated thick filaments (length, 10–20 μm) were suspended in a low ionic strength buffer containing 1 mM MgCl₂, 0.2 mM EGTA, 0.5 mM dithiothreitol (DTT), and 2 mM PIPES-KOH (pH 7.0) at a protein concentration of ~1 mg/ml. Thick filament suspensions were used within 12 h. Polystyrene beads (diameter, 4.5 μm or 2.8 μm) (Dynal, Oslo; Dynabeads) were incubated overnight in a 0.4 N KOH solution containing poly-L-lysine (Sigma; 5 mg/ml) at 0°C, and centrifugation (5,000 × g) and resuspension in 1 mM PIPES-KOH solution (pH 7.0) were repeated several times to give a final bead concentration of 3 × 10⁵/ml. The thick filament solution was centrifuged at 5,000 × g for 30 min to precipitate the filaments, which were then resuspended in 1 mM PIPES-KOH at a protein concentration of 1 μg/ml and mixed with the above bead suspension at various volume ratios (from 1:5 to 1:20) to be incubated on ice for 3 h. The thick filament–bead mixture was centrifuged at 5,000 × g for 5 min to obtain beads with one or a few thick filaments attached when observed under a light microscope with dark-field illumination.

Preparation of *Chara* myosin-attached beads. *Chara* myosin was isolated from the cytoplasm of the internodal cells of *Chara corallina* by the method of Chaen *et al.* [18]. Before experiments, the internodal cells (diameter, approximately 1 mm; length, 10–15 cm) were isolated and stored in artificial pond water containing (mM) 0.1 KCl, 0.1 NaCl, and 0.1 CaCl₂ (pH 5.6). The cell was cut open at both ends, and the cell sap was replaced with EGTA-medium containing (mM) 4 EGTA, 6 MgCl₂, 340 sorbitol, and 60 PIPES-KOH (pH 7.0) by perfusion of the cell interior with three times, and the ATP-medium (EGTA-medium containing 2 mM ATP) was then perfused into the cell. After that, the cells (10 or more) were squeezed by fingers, and the squeezed cytoplasm was mixed with 1 ml ATP-medium. After centrifugation at 3,000 × g for 1 min, the supernatant was used for the preparation of myosin-attached beads. The same procedures as for thick filament–attached beads were used to attach *Chara* myosin.

Motility assay system. *Chara corallina* was cultured by the method of Shimmen and Yano [19]. A single isolated internodal cell segment was cut open at both ends. For the observation of the movement of thick filament–attached beads, the cell was perfused first with a washing solution containing (mM) 190 sorbitol, 5 MgCl₂, 5 ATP, 2 EGTA, 0.5 DTT, and 30 PIPES-KOH (pH 7.0), then with an activating solution prepared by adding 2.1 mM CaCl₂ to fully activate the ATPase activity of the thick filaments. The thick filament-attached beads were suspended in the activating solution and introduced into the internodal cell at the late stage of perfusion. The internodal cell segment (length, ~2 cm) containing the thick filament-attached beads was then ligated at both ends and put into the observation bath filled with artificial pond water in such a way that its chloroplast rows, along which actin cables extended straight, were parallel to the direction of magnetic force. For the observation of *Chara* myosin-attached beads, the cells were perfused first with EGTA-medium several times, then with ATP-medium that contained no Ca²⁺ because it inhibits the movement of *Chara* myosin-attached beads.

Experimental procedures and data analysis. The unidirectional movement of the beads along the actin cables was observed with an inverted microscope (Olympus, Tokyo; IX70) under unloaded conditions. Attention was focused only on the beads that moved smoothly over many seconds. The movement of the beads was recorded at 30 frames/s by a video camera (Sony, Tokyo; CCD-IRIS) and a video recorder (Sony, Tokyo; videoHi8) and analyzed by special software (Swallow series, Digimo). The software can pursue the position of a bead automatically on a video recorder and can display instantaneously the trajectory of a bead movement. A load from 0 to 85 pN was applied to a bead in the opposite (positive load) or in the same direction (negative load) as that of a bead movement. In the curve fitting of the force-velocity relationship (Fig. 7), the regression curve was drawn by the least mean square method. All experiments were performed at room temperature (20–25°C).

RESULTS

Movement of beads under no load

The movements of ABRM thick filament–attached or *Chara* myosin–attached beads have been previously observed to move along actin cables in one particular direction determined by the polarity of the cables [17, 18]. Before the experiments of loaded movements, we carried out several control experiments of unloaded movements. To determine the effects of viscosity on the movement, two beads of different sizes (diameters, 2.8 and 4.5 μm) were introduced into the same internodal cell preparation, and the velocities were compared under no load (Fig. 2). The velocities of the beads of two different sizes were almost the same in the thick filaments and in *Chara* myosin, and there were no significant statistical differences between
them. In subsequent experiments, beads of 4.5 μm in diameter were used in all experiments because it was possible to apply a greater electromagnet load on the larger beads, and the experiments were easier to observe. The mean unloaded velocities of the thick filament–attached and of the Chara myosin–attached beads of 4.5 μm were 0.93 ± 0.23 μm/s (n = 18) and 23.2 ± 6.57 μm/s (n = 10), respectively.

**Velocity transients appeared during both on and off of a load**

Bead movements in response to the rapid application of a load in the direction opposite the initial bead movement (positive load) were observed. The photographs of the typical bead movement are shown in Fig. 3. When a load was suddenly applied to the beads that slid smoothly under no load, they quickly responded and changed immediately the direction of movement to the opposite. When the load was removed, they again moved forward. These movements were observed in ABRM thick filament–attached and Chara myosin–attached beads.

In Fig. 4, the different magnitudes of loads for 10 s were applied three times in ABRM thick filament and for 5 s in Chara myosin, with periods of no load for 5–10 s interposed between the loaded periods. During the positive load, the beads gradually decreased velocity and stopped, then again moved forward. The steady velocity attained during the load was slower than the unloaded velocity. When the load was removed, the beads moved rapidly forward for a short period, and their velocity then returned to the normal unloaded one. These two kinds of velocity transients observed during both conditions, on and off of the load, could be repeatedly reproduced on the same bead in ABRM thick filament (Fig. 4A) and in Chara myosin (Fig. 4B).

**Change in load-sustaining ability**

It was examined how the ability of the beads to sustain
Velocity 1 s after the application of the test load. The backward velocity was applied before the constant test load step in ABRM thick filament (Fig. 5A) and in Chara (Fig. 5B). The ability of sustaining load was judged by the backward velocity 1 s after the application of the test load. The backward velocity lessened at the constant test loads when each preceding load level was increased; namely, the distance of the bead movement during the test load was shorter, suggesting that the ability to sustain load was higher with a higher preceding load step. These phenomena were observed both in ABRM thick filament and in Chara myosin. The similar time courses of movement were observed in intact single muscle fibers [8, 13].

Transient $V_{\text{max}}$

It was found that the velocity of beads under no load could be increased immediately after the end of the application of load (Fig. 6). This phenomenon was observed both in ABRM thick filament (Fig. 6, A and B) and in Chara myosin (Fig. 6, C and D). Unloaded velocities before and after the load ($V_{\text{max}}$) were nearly constant, but the velocity immediately after the end of load (transient $V_{\text{max}}$) was clearly faster than $V_{\text{max}}$. The transient $V_{\text{max}}$ soon returned to $V_{\text{max}}$, and it was dependent on the magnitude of a preceding load just before it appeared (Fig. 6, B and D). The transient $V_{\text{max}}$ observed here is very similar in time course to those observed in the single skeletal muscle fibers [1, 5, 8, 10, 11].
One of the basic characteristics of contracting muscle is that it shortens with a constant velocity during a constant load, thus giving rise to a force–velocity relation. In the present experiment, we examined the force–velocity relation of ABRM thick filament and in Chara myosin in order to compare it with that in single muscle fiber. Figure 7 shows steady-state force–velocity relations of the bead movement obtained by applying various constant positive and negative loads. The force is expressed by the value relative to $P_0$, that is, the load at which beads stop moving. Figure 7, A and B, shows the force–velocity curves in ABRM thick filament and in Chara myosin, respectively. The force–velocity relation obtained was very similar to the one obtained in single muscle fibers of frog [2, 13].

**DISCUSSION**

The use of micro-beads in the analysis of the elemental process of actin-myosin interaction is now very widespread [20–24]. In many cases, beads have been used to support an actin filament, and the molecular interactive force or the length perturbation between actin and myosin has been measured with optical tweezers. In these analytical experiments that use one molecule of myosin, however, the short nanometer range of analysis makes it impossible to directly evaluate the previous physiological experiments that have contributed to the proposition of the basic ideas of cross-bridge movements [2, 4, 5, 7, 9, 14, 22]. To address this problem, Ishii et al. [17] performed experiments in the motility system by using a centrifuge microscope. Their results confirmed the force–velocity relation obtained in muscle fibers [2, 9]. The first
experiment, using magnets to control beads attached to myosin filaments, was performed some years ago [25], but because a permanent magnet was used, the transient behavior of the beads was not recorded.

In the experiment of in vitro motility assay using myosin filaments or myosin molecules, their directions in a system have been discussed. It is known that one myosin filament has opposite polarities at either side, and myosin molecules attached to a bead have random directions. It is probable that only the myosin molecules that fit with actin polarity can interact in a filament or in randomly oriented molecules. The best way is to use myosin and actin of a controlled direction [23]. In the present experiment, however, we used the motility system we used in the past as the first step of this series of experiments, because the method of applying load in the present experiment is quite new, and it is safer for the interpretation of results. In the next step, the use of skeletal and cardiac myosin is planned.

In the present experiment, an electromagnet was proved to be very useful in the analysis of mechanical transients, as with muscle fibers. Sugi and Tsuchiya [8, 13] studied isotonic velocity transients induced by quick decreases or increases in load in tetanized frog single skeletal muscle fibers. They showed that when quick increases in load were applied during isotonic shortening under a moderate load, the fibers showed an initial transient lengthening before starting to shorten against the new load, suggesting that there is a decrease in the ability of the fibers to sustain load after a period of isotonic shortening, but it recovers with time. A similar result using a servo apparatus was briefly reported by Armstrong et al. [1]. Our present data show that the transient backward movement of the bead attached to thick filament or Chara myosin occurred before forward movement when an increase in load was applied during the movement under no load (Figs. 3 and 4), suggesting that the ability to sustain load changes with time after an application of load. An analogous phenomenon was observed in muscle fibers.

In muscle fibers, the ability to sustain load was proved to change not only with time, but also with the preceding load condition. Sugi and Tsuchiya [13] performed the experiment of double-load steps, in which they showed that the lengthening velocity depended on the preceding load condition. Sugi and Tsuchiya [13] performed the experiment of double-load steps, in which they showed that the lengthening velocity depended on the preceding load condition. In the present experiment, we showed that the backward velocity during a constant test step was slower if the preceding conditioning load was higher (Fig. 5), suggesting that the ability to sustain load changes with the mechanical condition, as in muscle fibers.

Velocity transients following a decrease in load have been demonstrated by several authors in living single muscle fibers [5, 8, 10, 11]. In the present experiment, the velocity of beads showed also the fast phase immediately after the positive load (Fig. 4), and this increase in $V_{\text{max}}$ (transient $V_{\text{max}}$) was proved to change with the preceding load amplitude (Fig. 6).

The similarities mentioned above between the movement of a bead in the present experiment and the previous results in muscle fibers are surprising when the large differences in the two experimental systems are taken into consideration. In living single muscle fiber, complex structures exist, e.g., the lattice composed of numerous actin and myosin filaments, the sarcomeres divided by Z-discs, and the other proteins known to take part in the contractile process directly or indirectly, e.g., connectin (titin), M-protein, $\alpha$-actinin, and others. The other difference between them is the type of myosin, i.e., myosin from smooth muscle and Chara myosin in the present experiment and skeletal myosin in muscle fiber. This fact means that these phenomena can be produced purely by the actin–myosin interaction and that the phenomena appear irrespective of the rate of attachment and detachment between actin and myosin.

These observations can be explained in several ways. Podolsky and Nolan [26] and Sugi and Tsuchiya [8] constructed models by modifying the parameters used in a Huxley model [3]. They demonstrated that the appropriate distributions of “$f$” and “$g$,” the attachment and detachment rate constants, respectively, could explain the velocity transient. The results shown in Figs. 4 and 5 are possible to be explained by means of the Sugi and Tsuchiya model [8].

Huxley and Simmons [4, 7] demonstrated that the fast velocity transient following isotonic release could be explained by their “cross-bridge rotation” hypothesis. Piazzesi et al. [27] also provided evidence in favor of the conformational change of cross-bridge. The fast velocity transients as shown in Fig. 6 may be explained by their models, though their experiments were made on skeletal muscles. The following other ideas also can account for these fast velocity transients. Edman and Curtin [10] studied in detail the synchronous oscillation of length and stiffness during loaded shortening and put forward the hypothesis that the rapid length step at the outset of the load clamp leads to synchronous activity of the myosin cross-bridges along the length of the fiber. Pollack [28] measured a sliding step size between actin and myosin filaments in myofibril and suggested that velocity transients might be simulated by their elemental step-size hypothesi.

In any of these hypotheses, the main part of the velocity transient has been supposed to be induced purely by the interaction between actin and myosin, though the possibility remains that an intracellular elastic element is involved in the phenomena in intact single fibers [11]. Our present data directly proved that the velocity transients in muscle observed so far are brought about by the intrinsic properties of the interaction between actin and myosin, irrespective of myosin type, hexagonal lattice structure of fila-
ments, regular sarcomere structures, and the other intracellular proteins. More experimental works are needed to relate the phenomena observed in single muscle fibers and those observed in artificial motility systems [20, 21, 23, 24]. However, the use of an electromagnet seems to be a useful method to bridge the gap between the above two experimental systems, not only in skeletal muscle, but also in smooth muscle [29, 30].

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