J. exp. Biol. ***187****, 275–293 (1994)*
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CONTRACTILE PROPERTIES OF A HIGH-FREQUENCY MUSCLE FROM A CRUSTACEAN

II. CONTRACTION KINETICS

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Accepted 22 November 1993

Summary

1. The flagella (small appendages on the maxillipeds) of the crab *Carcinus maenas* beat regularly when active at about 10 Hz (15 ˚C). The beat of a flagellum is due to contraction of a single small muscle, the flagellum abductor (FA). The optimal stimulus frequency for tetanic contraction of the FA was about 200 Hz. When the muscle was stimulated at 10 Hz with paired stimuli per cycle, the interstimulus interval that maximized peak force was 2–4 ms, which corresponded well to the interspike intervals within bursts recorded from motor axons during normal beating.

2. Contraction of the isolated FA showed pronounced neuromuscular facilitation and many stimuli were needed to activate the muscle fully. The dependence on facilitation in isolated muscles appeared to be greater than that *in vivo*. It is suggested that neuromodulators in the blood of the crab enhance neuromuscular transmission and reduce the dependency on facilitation in intact animals.

3. The FA had a narrow length–tension curve. Tetanic tension became vanishingly small at muscle lengths less than about 90 % of the maximum *in vivo* length. The maximum length change of the muscle during *in vivo* contraction was about 5 %.

4. The maximum isometric force of the FA was low (about 6 N cm^{-2}) but its shortening velocity was high. V_m, the maximum shortening velocity determined from isotonic shortening, was 4.0 muscle lengths s^{-1}; V_0, the maximum shortening velocity from slack test measurements, was about 8 lengths s^{-1}.

5. The structure and physiology of the FA are compared with those of locust flight muscle, chosen because it too is a muscle capable of long-duration, high-frequency performance.

Introduction

The maxillipeds of decapod crustaceans have small appendages, the flagella, which often beat rhythmically. The flagella are noteworthy for the high frequency at which they

Key words: *Carcinus maenas*, muscle, flagellum abductor, shortening velocity, contraction kinetics, slack test.

operate, 10–16 Hz in different species (Burrows and Willows, 1969). Each flagellum is powered by a single muscle, the flagellum abductor (FA). The FA is a small, parallel-fibred muscle with simple innervation. The muscle receives two axons, both of which are excitatory and each of which fires up to five times for each stroke of the flagellum (Burrows and Willows, 1969; Charlton, 1971; Josephson and Stokes, 1994*a*).

The FAs are quite specialized muscles with a single mode of operation – high-frequency, twitch-like contraction. Correspondingly, the fibres which make up the muscle are structurally a homogeneous set with obvious specializations for both brief contraction (well-developed sarcoplasmic reticulum) and fatigue resistance (extensive mitochondria and glycogen stores; Stokes and Josephson, 1992). Because of fibre homogeneity, mechanical measurements made from whole muscles should reflect well the contractile physiology of the individual fibres of the muscle. Performance should not be distorted by simultaneous activity in fast and slow fibres or in readily fatigued and fatigue-resistant fibres. The FA, therefore, provides a useful preparation in which to characterize the mechanical performance of a fast crustacean muscle.

The flagellar muscles are functionally similar in several ways to insect flight muscles, the best studied of all invertebrate muscles. Both the FA and insect flight muscles operate at a high and relatively constant frequency. Both are capable of continuous activity for long periods without obvious fatigue. And for both kinds of muscle the load – the resistance of an appendage moving in water for the FA, in air for flight muscles – is constant and predictable. It was thought that a comparison of the contractile properties of the FA and insect flight muscles might give insight into which features of muscle are malleable and tailored through evolution for high-frequency performance and which features are conservative and reflect crustacean or insect origins.

Materials and methods

The experiments were performed at the Marine Biological Laboratory in Woods Hole, MA. Green crabs, *Carcinus maenas* (L.), were collected by the laboratory supply department and were maintained in running sea water at 15–21 ˚C. The animals used were all male and ranged in mass from 25 to 102 g.

The basic preparation consisted of a second maxilliped and its exopodite, attached portions of the thoracic phragma, and a length (0.5–1 cm) of the nerve to the muscles of the maxilliped. The distal flagellum was removed, leaving a small remnant of exoskeleton at the insertion of the FA to serve as an attachment point for a force transducer. The exoskeleton surrounding the FA was removed. The sclerotized basal region where the muscle originates was left intact. The initial dissection to expose the nerve and to isolate it and the maxilliped was carried out in continuously flowing, chilled (10–15 ˚C) sea water. The final dissection to expose the muscle was carried out in crab saline. The distance between sclerites at the base of the muscle and the proximal edge of the fully adducted flagellum was measured with an ocular micrometer before dissecting the muscle. The muscle length when these landmarks were at the same separation as *in vivo* was termed the reference length. The reference length is the longest muscle length reached *in vivo*.

The experimental preparation was placed in a dish of saline and the exoskeleton

housing the origin of the FA was mounted firmly to a block of resin using staples made from insect pins. The distal portion of the muscle was free for attachment to a force transducer. A cooling coil in the experimental chamber maintained the temperature of the saline surrounding the muscle at 15±0.5 °C. A motor-driven paddle in the chamber gently circulated the saline.

Muscle responses were evoked by stimulating the motor nerve with a suction electrode. The stimuli were 0.5 ms voltage pulses at an intensity 2–3 times that required to activate the motor unit with the highest threshold. Trials in all experiments were presented regularly at 2 min intervals. Several pacing trials were always given before the onset of data collection in order to bring the preparation more-or-less into a steady state.

In experiments in which the measured muscle response was isometric tension (investigations of length–tension relationships, effects of stimulus number and frequency), the muscle was mounted horizontally, and the distal end was attached to a stiff insect pin mounted on a force transducer. The force transducer was held in a manipulator. The transducer was constructed from a pair of semiconductor elements (see Fig. 4.4 in Miller, 1979) and it, together with the attached insect pin, had a resonant frequency of about 1.5 kHz. Generally the muscle was positioned so that it was at the reference length, as judged by the distance between cuticular landmarks at the base and distal end of the muscle segment. In measurements involving rapid length change, for example in determinations of force–velocity characteristics, the muscle was attached to a Cambridge model 300H ergometer (Cambridge Technology, Cambridge, MA 02140, USA). In these experiments, for technical reasons, it was most convenient to mount the muscle vertically. In the vertical orientation, the muscle length could not be determined easily with an ocular micrometer, so the length at which the muscle was set during experiments was based on measured performance. The optimum muscle length was defined as that length at which the muscle force was greatest when the muscle was stimulated for 0.4 s at 200 Hz. At the end of experimentation, the muscle was held at the optimum length and fixed with 70 % ethanol before being released from the ergometer. The muscle length after fixation was measured with an ocular micrometer in order to determine the relationship between the optimum length, determined during the experiments with physiological measurements, and the reference length, measured optically before the muscle was dissected.

Preliminary experiments were done using the crab saline of Mercier and Wilkens (1984). Later experiments were performed using the crustacean saline of Govind and Lang (1981; pH adjusted to 7.4 with NaOH before use). The Govind/Lang saline contains more calcium (16 *versus* 12 mmol l^{-1}) and less magnesium (7 *versus* 20 mmol l^{-1}) than does the Mercier/Wilkens saline. The responses of the flagellar muscle to stimulation of its nerve were more vigorous, and more like those that we assume to pertain in intact animals based on flagellar movements, in the Govind/Lang saline than in the Mercier/Wilkens saline. The Govind/Lang saline was used in all the experiments to be reported here.

Those muscles used in isometric tension measurements were dissected free and weighed at the end of an experiment. The muscles were then stored for several weeks in 70 % ethanol, after which they were rehydrated overnight in saline and reweighed. This

was done in order to determine the amount of weight loss associated with alcohol fixation. The mass of the rehydrated muscles was 76 % (S.E.=1.3 %, *N*=14) of the original wet mass. This factor was used to estimate the original wet mass of those muscles used with the ergometer, which had been fixed in ethanol while still in the experimental chamber. The muscles that were fixed *in situ* were subsequently stored in ethanol before they were later rehydrated and weighed. Muscle area was estimated from the ratio of muscle mass to muscle length (muscle density assumed to be $1\,g\,cm^{-3}$). The average dimensions of the muscles used in this paper and in the work described in the following paper (Josephson and Stokes, 1994*b*), which sometimes involved the same preparations were (*N*=35): length 9.13 mm (S.D.=0.83 mm); mass 5.07 mg (S.D.=1.45 mg); area $5.34\times10^{-3}\,cm^2$ (S.D.=$1.56\times10^{-3}\,cm^2$).

The amount by which the muscle shortens *in vivo* was determined by excising a maxilliped, removing a window of exoskeleton overlying the distal insertion of the muscle, and measuring the displacement of the insertion when the muscle was stimulated tetanically. At rest, the elasticity of the joint at the base of the flagellum pulls the flagellum into a fully adducted position, with the FA fully extended. During stimulation, and seemingly also during normal beating, the flagellum abducts until it reaches a natural stop and the length of the muscle at full flagellar extension is that of maximal shortening.

Results

Isometric contraction

Stimulus frequency and pattern

The response to a single stimulus ranged from small to undetectable. Multiple stimuli were needed to evoke substantial force. The effects of interstimulus interval (ISI) on isometric tension were investigated using bursts of 50 stimuli at constant ISI. The ISIs tested were 2, 5, 10, 20, 50, 100 and 200 ms. Two series of trials, in mirror order, were obtained with each preparation. In three preparations, the first set of trials was with progressively decreasing ISI, the second set with progressively increasing ISI. In two preparations the orders were reversed: the first series had increasing intervals and the second decreasing. With each preparation, the values for maximum force at a given ISI were averaged for the increasing and the decreasing series.

The evoked tension responses were smoothly fused for ISIs of 10 ms or less. Progressive increase of the ISI above 10 ms resulted in increasingly greater fluctuation of tension on each cycle. At an ISI of 50 ms, the tension fluctuation was about 10 % of the peak force at the end of the stimulus burst (mean 11.3 %, S.D.=4.2 %). The peak tension during the bursts was maximal at an ISI of 5 ms, and tension declined with increase in ISI above 5 ms (Fig. 1). Peak tension also declined with reduction of the ISI from 5 to 2 ms. With the shorter ISIs tested (2, 5, sometimes 10 ms), the muscle tension was generally still increasing at the end of the stimulus burst, whereas with the longer ISIs the maximum tension reached a plateau. The experimental protocol, therefore, somewhat understates the increase in muscle tension with decreasing ISI.

During normal flagellar beating, the two motor units to the FA muscle are activated in

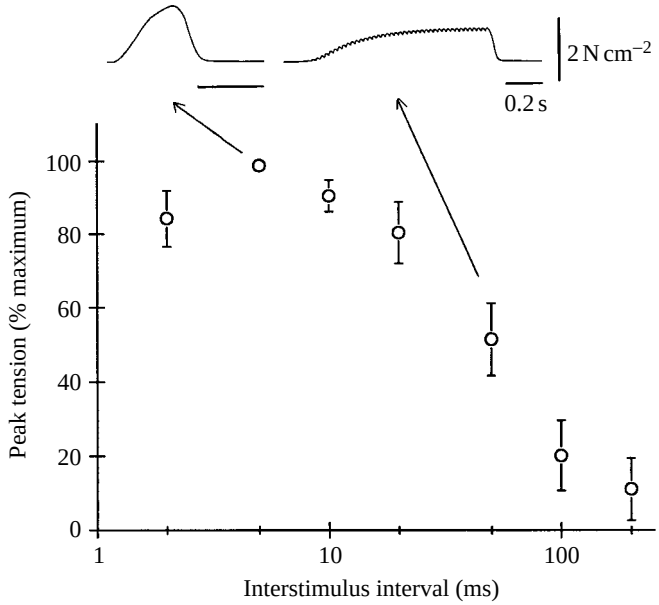

Fig. 1. Peak isometric tension (mean ± S.E., *N*=5) during bursts of 50 stimuli as a function of interstimulus interval (ISI). Values are plotted as a percentage of the tension recorded with the optimum ISI, which was 5 ms for four of the preparations used and 10 ms for the remaining one. The traces above are examples of the mechanical responses at two of the interstimulus intervals examined.

bursts. The frequency of the bursts is about 11 Hz, and each of the two motor units typically fires 1–3 times at intervals of 3–4 ms during a burst (Josephson and Stokes, 1994*a*). The following protocol was used to examine the effects of ISI when the muscle was activated with pairs of stimuli as it often is with pairs of neurone impulses during normal beating. Muscles were stimulated for 50 cycles at a cycle frequency of 10 Hz. In each cycle, the muscle was activated with single shocks or with paired stimuli at ISIs of 2–20 ms. Each series began with a trial of a single stimulus per cycle, continued with trials of paired shocks at 2–20 ms ISIs in increasing order, and then returned in a mirror series through decreasing ISIs to single shocks. The values obtained with a given ISI were averaged for the increasing and the decreasing interval series.

At the 10 Hz cycle frequency, paired stimuli at any ISI between 2 and 20 ms gave appreciably more tension than did single stimuli (Fig. 2). Among the ISIs tested, 2 ms and 4 ms were approximately equal in effectiveness and gave the greatest peak tension per cycle. The ISI giving maximum tension per cycle thus matches well the usual interval between pulse pairs *in vivo*.

The effects of stimulus number per burst were evaluated with the following protocol. Muscles were stimulated for 20 cycles with a constant number of stimuli per cycle. The cycle frequency was 10 Hz. The first trial had a single stimulus per cycle. Subsequently, the number of stimuli per cycle was first increased in successive trials to five and then decreased to a single stimulus per cycle. Thus, trials with 1–4 stimuli per cycle were repeated twice, once in the ascending series and again in the descending series. Results

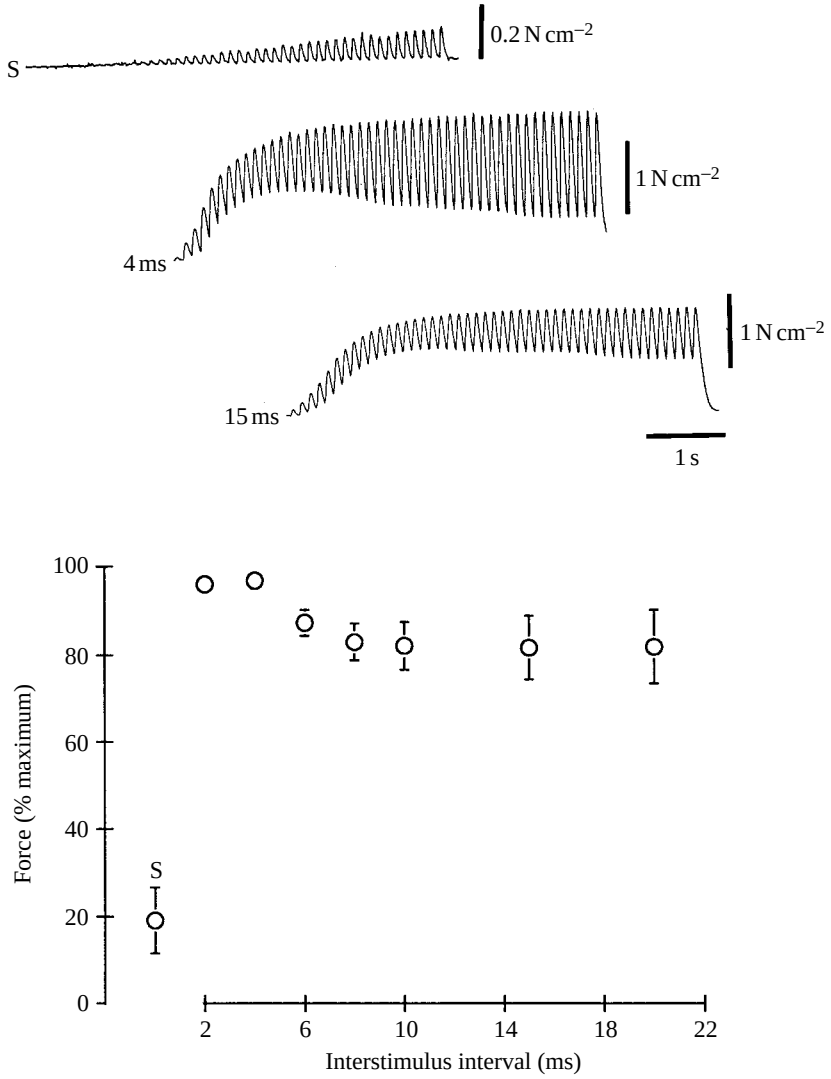

Fig. 2. Isometric contraction in response to stimulation at 10 Hz with a single stimulus (S) and with pairs of stimuli at varied ISI. The plotted values are the peak tension reached during a 5 s burst (mean ± S.E. where this is larger than the symbol, *N*=5). The traces above show responses to single stimuli, paired stimuli at an ISI of 4 ms and paired stimuli at an ISI of 15 ms. Note that the trace with a single stimulus has a different amplitude scale.

from the ascending and the descending series were averaged to obtain a single set of values describing changes in force throughout successive cycles for that number of stimuli per cycle.

The peak force per cycle tended to increase progressively throughout the set of 20 cycles for all numbers of stimuli per cycle. The force at any cycle in the series increased with increasing number of stimuli per cycle (Fig. 3). The rate of force rise in successive cycles was both absolutely and relatively greater the greater the number of stimuli per

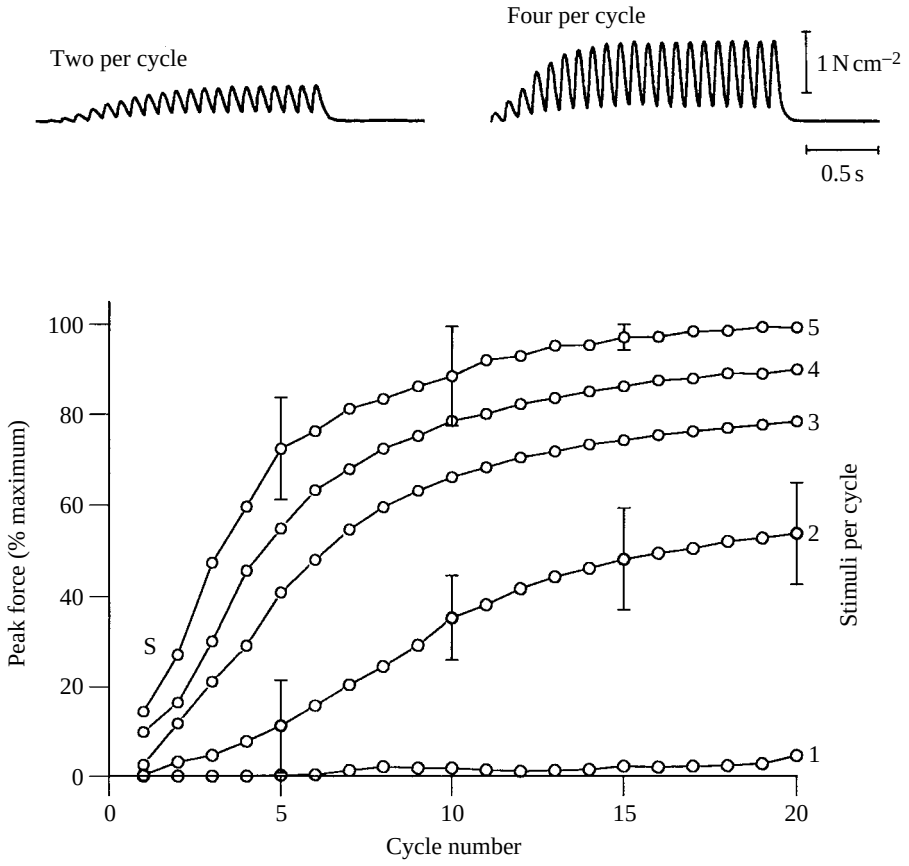

Fig. 3. Peak force reached during successive cycles with differing numbers of stimuli per cycle. Values plotted are means (N=5). Vertical lines show standard deviations for a few representative points. The traces above are representative of those from which measurements were made.

cycle. On average, force reached or exceeded that on the twentieth cycle by the fourth cycle with four or five stimuli per cycle, by the fifth cycle with three stimuli per cycle, and only by the ninth cycle with two stimuli per cycle.

Muscle length strongly affects peak isometric tension and the time course of muscle relaxation (see below). A series of trials like those illustrated in Fig. 3 was carried out at different muscle lengths to determine whether muscle length changed the dependence of the muscle on facilitation. The muscles were stimulated with two or with three stimuli per cycle at muscle lengths ranging from 95 % of reference length to 105 % of reference length. Increasing muscle length increased the peak force per cycle and the relative rate at which force approached a maximum during successive cycles, but there was still a strong dependence on facilitation at all lengths examined, with three or more cycles being required before the peak isometric force reached half that on the twentieth cycle.

A strong dependence of muscle force on facilitation is a striking feature in each of the

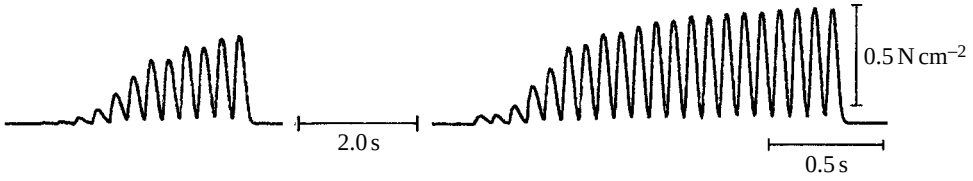

Fig. 4. Initial development of tension during a stimulus train and redevelopment of tension following a break in the train. The muscle was activated at 10 Hz with two stimuli per cycle.

experiments whose results are illustrated in Figs 1–3. In fact, facilitation seems to be much more pronounced in these isolated muscles bathed in a saline solution than it is in muscles of intact animals bathed in crab blood as judged by the force produced by the flagellum of intact crabs during spontaneous beating (see Fig. 2 in Josephson and Stokes, 1994*a*). We will return to the comparison of isolated muscles and those of intact animals in the Discussion.

The decay rate of facilitation has not been examined in detail, but it is clearly quite fast. Interrupting a sequence of stimulation for a few seconds essentially abolishes the enhanced excitability resulting from facilitation, and the recovery of force when the stimulation is restarted has a time course quite similar to that at the onset of contraction (Fig. 4).

Muscle length and tetanic tension

The relationship between muscle length and maximum isometric tension was determined using a procedure similar to that employed earlier with a crab respiratory muscle (Josephson and Stokes, 1987). The muscle was mounted horizontally and its length was monitored with an ocular micrometer. Muscle lengths were expressed as a fraction of the reference length, which is the longest length reached by the muscle *in vivo*. The nerve to the muscle was stimulated with 0.5 s bursts of stimuli. The stimulus frequency within a burst was 200 Hz. Stimulus bursts were regularly paced at 2 min intervals. The passive force of the muscle was measured as the force immediately before stimulation, and the active force was the maximum increase in force above the passive level during stimulation. Length changes were made immediately after each stimulus burst, so the passive force was that after almost 2 min at the new length. The muscle cross-sectional area used in calculating stress (=force per unit area) was the area at the reference length. Thus, the actual stress in a stretched muscle was greater than that plotted in length–tension curves (see Fig. 6) because the cross-sectional area of the stretched muscle was less than that at the reference length.

Passive and active forces were determined at a series of increasing lengths, beginning at 80–90 % of reference length and continuing to about 150 % of reference length. Stretching the muscle to longer lengths resulted in impairment of performance and obvious visual damage. In order to monitor the condition of the muscle and to assay possible damage resulting from stretch, trials at a control length, typically about 105 % of the reference length, were alternated between trials at experimental lengths.

Passive tension, which was very small at muscle lengths less than the reference length,

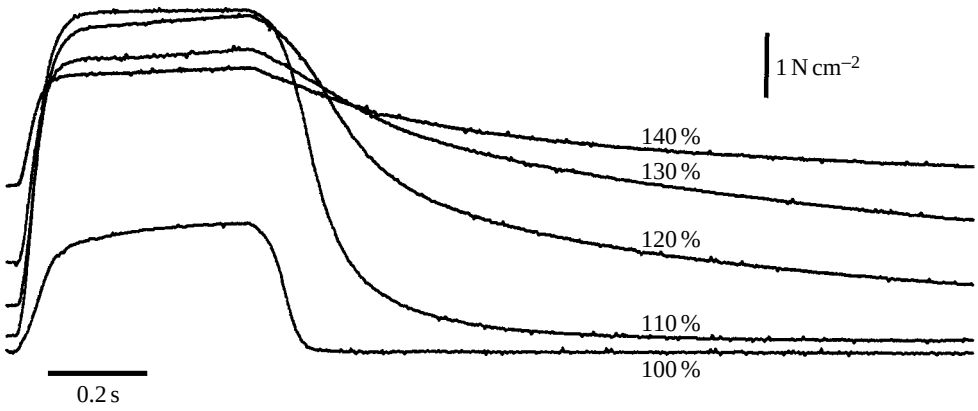

Fig. 5. Isometric tetanic contractions at different muscle lengths. The muscle length at which the response was measured, expressed as a percentage of the reference length (=maximum *in vivo* length), is indicated beside each curve. The force was essentially zero before and after the contraction at 100 % of the reference length, so these portions of that trace provide a force baseline.

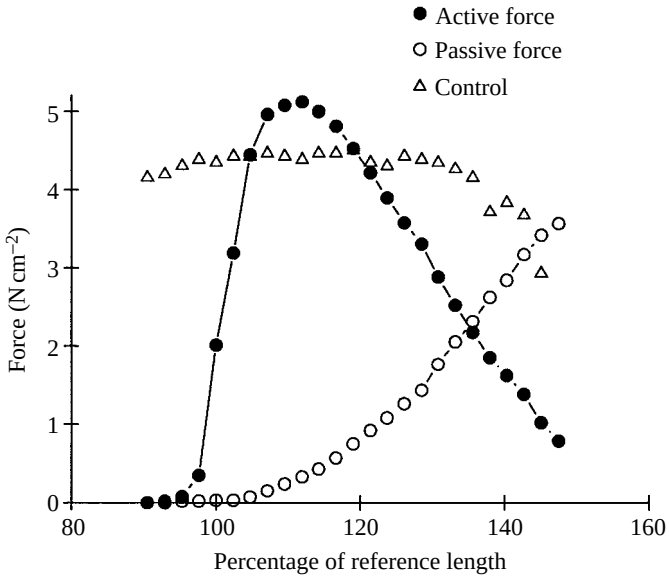

Fig. 6. Active and passive length–tension curves. The passive force was that immediately before stimulation, the active force was the maximum increase from the passive force level during tetanic stimulation. The control force values were the active forces recorded at 105 % of the reference length between trials at different experimental lengths. The control forces are plotted at the muscle length of the preceding trial.

increased monotonically with stretch beyond the reference length (Figs 5, 6). The active tension, the increase in tension that resulted from stimulation, rose rapidly with increasing length to a peak at about 110 % of the reference length (mean=109.4 %, S.E.=1.3 %) and fell thereafter. The control tension, the tension measured in a trial at the

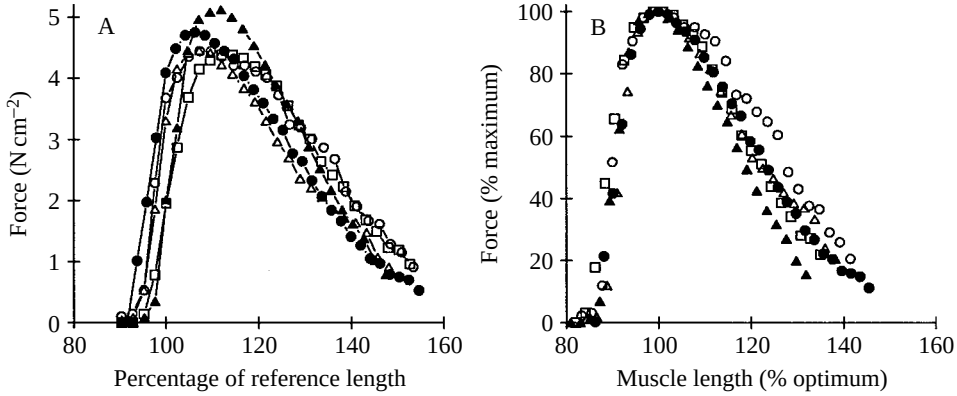

Fig. 7. (A) Muscle length and active force in five preparations. (B) The data in A, normalized to peak force and to optimum length for each preparation.

control length following the trial at an experimental length, remained reasonably constant up to stretches of 130–140 % and then declined rather quickly with further stretch. Judging by the control contractions, we conclude that some part of the fall in active tension at long muscle lengths was a consequence of muscle damage, but the early decline in force with increasing length, up to at least 130 % of the reference length, was not attributable to damage.

Length–tension curves were obtained from five preparations. The shapes of these curves were quite similar from preparation to preparation (Fig. 7A), especially when allowance was made for differences between preparations in the maximum stress and in the actual value of the optimum length for force production (Fig. 7B). The peak stress (F_0) in these preparations averaged 4.6 N cm^{-2} (S.D.=0.3 N cm^{-2}), and the average length at which stress was maximal was 109 % of the reference length (S.D.=3 %). The width of the length–tension curves, measured at 50 % F_0, averaged 35.7 % of the reference length (S.D.=3.7 %; average boundaries of the 50 % width were from 98.6 % of the reference length at the low end to 134.4 % at the upper end).

There were changes in the shape as well as in the amplitude of the tension response with increasing muscle length. At short lengths, up to about 100 % of the reference length, the rise time became shorter with increasing length, presumably because less time was required to take up existing slack. Increasing the length above the reference length led to increasingly slower relaxation (Fig. 5).

In vivo *strain*

The muscle extension associated with the transition from full adduction to full abduction was measured for nine muscles from six animals (animal mass range 25–44 g). The maximum muscle strain averaged 5.16 % (S.E.=0.19 %) of the reference length.

Shortening velocity

The force–velocity characteristics of the FA muscle were determined from the rate of isotonic shortening following release from isometric contraction. The muscle was

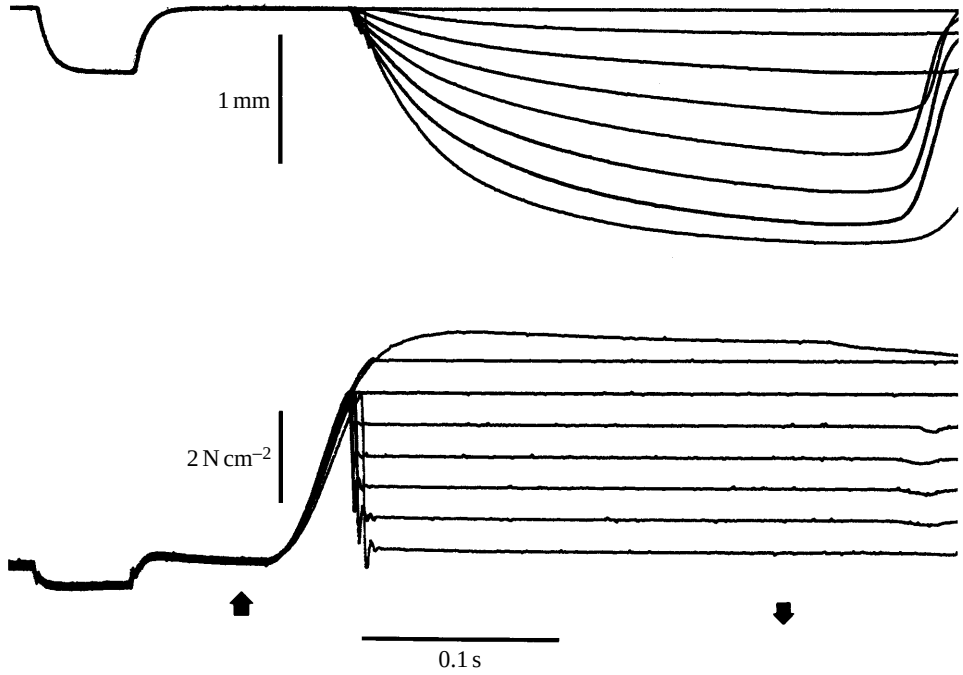

Fig. 8. Muscle shortening (upper set of traces) following release from isometric to isotonic contraction. The lower set of traces shows the corresponding muscle force. The arrows below the lower traces mark the beginning and the end of stimulation (0.5 ms shocks at 5 ms ISI). Each tetanic contraction was preceded by a short release in order to determine better the zero force level.

stimulated tetanically at 200 Hz. Velocity was measured by linear regression of the values of muscle length against time. The interval analyzed was that from 5 to 20 ms immediately after the cessation of the force and length transients associated with the transition from isometric to isotonic contraction. Transducer drift created some difficulties for accurately measuring the small muscle forces needed to characterize the low-force, high-velocity portion of a force–velocity curve. To increase measurement accuracy, each tetanic contraction was preceded by a short (50 ms), small (about 5 % of reference length) release (Fig. 8). The force recorded during the release was taken as the zero force baseline for measurement of the force during shortening. A Hill curve was fitted to the data points using the procedure of B. Wohlfart and K. A. P. Edman (unpublished results). The force–velocity curves obtained in this series of experiments were of quite typical shape (Fig. 9). The estimated maximum shortening velocity (V_m, velocity intercept at zero force) was 4.0 muscle lengths s^{-1} (S.E.=0.2 muscle lengths s^{-1}, N=5). The maximum tetanic tension in this series averaged 5.6 N cm^{-2} (S.E.=0.9 N cm^{-2}).

The maximum shortening velocity of the FA muscle was also determined using the slack test method (Edman, 1979). In the slack test, an isometrically contracting muscle is suddenly released to a new length. The release distance is great enough to reduce muscle force to zero, and is varied from trial to trial. The shortening velocity under zero load, often designated V_0, is obtained as the slope of the line relating the distance of release and

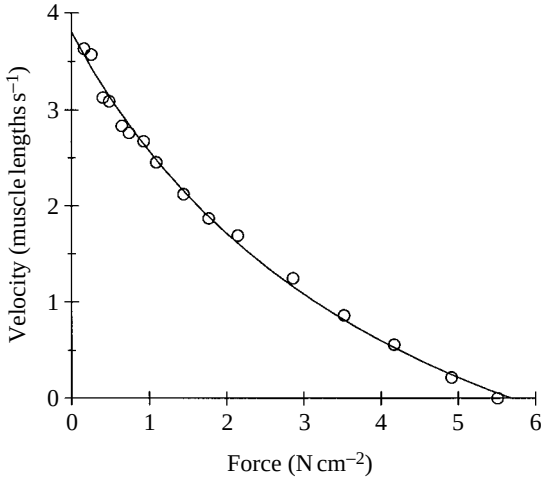

Fig. 9. The initial shortening velocity as a function of muscle tension for the preparation of Fig. 8.

the delay to the onset of tension redevelopment after the release (the reciprocal of the slope of the line in Fig. 11). Slack tests are usually done by releasing the muscle by varied distances from a common starting length. In the FA muscle, the length–tension curve is very narrow (Fig. 6). Releasing the FA muscle from a common starting point by distances which vary by only a few per cent puts the muscle at lengths at which the isometric force is quite different, as is the initial rate of tension redevelopment following the slack period. We were concerned that the different slopes for tension redevelopment might introduce systematic errors in measuring the time at which force onset begins. Therefore, we adopted the procedure suggested by Julian *et al.* (1986), in which the muscle is released from different starting lengths to a common final length at which it redevelops force (Fig. 10). Having force redevelopment occur at a common final length reduced differences in the initial slope of tension redevelopment, which should have made the estimates of slack time more consistent, but it introduced a potential difficulty due to different muscle force and different stretch of series elastic elements at the different initial lengths of the muscle. The consequences of varying stretch of series elastic elements are considered in the Discussion.

In single crayfish muscle fibres, and in skinned muscle fibres from a crab, plots of release distance against slack time obtained from slack test measurements were found to have two segments, an early portion with a high unloaded shortening velocity and a later segment with a distinctly lower shortening velocity (Tameyasu, 1992; Galler and Rathmayer, 1992). Similar plots obtained from the FA muscle of *C. maenas*, in contrast, had a single slope, and therefore a single value for shortening velocity, over the range of release distances tested (Fig. 11). Maximum shortening velocities of the FA measured with the slack test (V_0) averaged 7.8 muscle lengths s^{-1} (S.E.=0.5 muscle lengths s^{-1}, N=8). In five preparations in which both V_0 and V_m were determined, the average ratio of V_0 to V_m was 2.0 (S.E.=0.1).

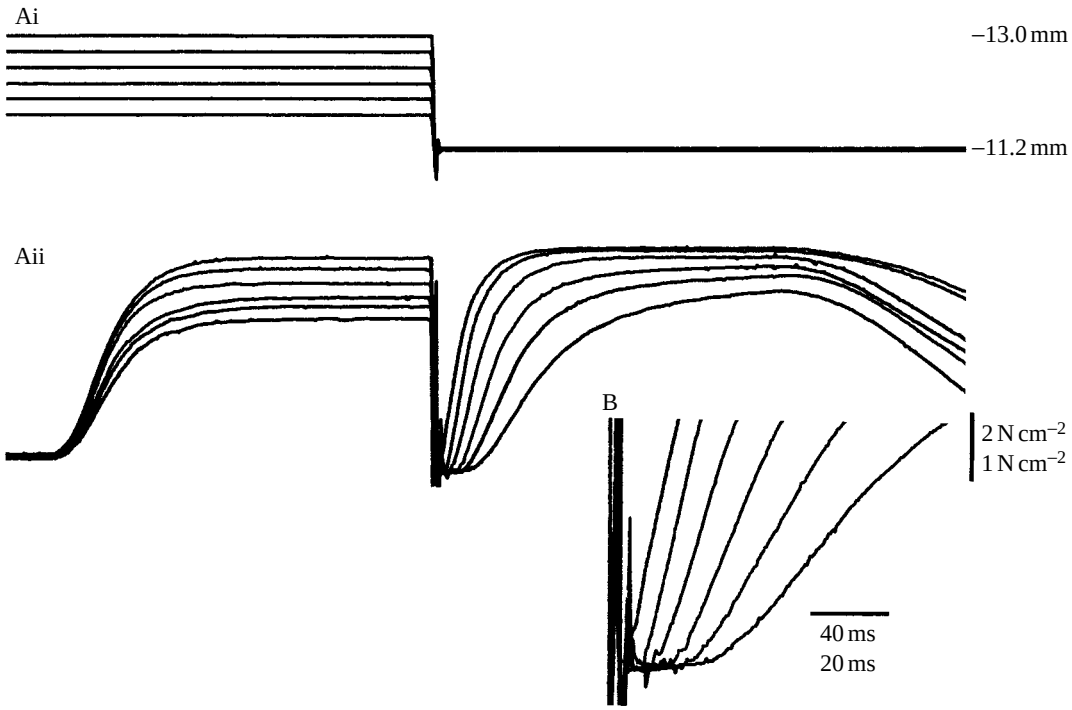

Fig. 10. Slack test measurement. A tetanically stimulated muscle contracted isometrically at a long length and then was released and allowed to redevelop tension at a shorter length. (Ai) Muscle length; (Aii) force. (B) An expanded depiction of the onset of force redevelopment.

Discussion

The contractile performance of the FA muscle from *C. maenas* shares several features with that of a respiratory muscle from this species, scaphognathite levator L2B, which was characterized earlier (Josephson and Stokes, 1987). Both FA and L2B show strong facilitation and give vanishingly small twitches in response to single stimuli. In both muscles, increasing muscle length slows relaxation. In both muscles, the length–tension curves are narrow. The active force becomes negligible at muscle lengths below about 90 % of the reference length and the width of the length–tension curve at 50 % of maximum force is about 35 % of the reference length. For comparison, the equivalent width for length–tension curves from frog sartorius muscle fibres is about 60 % of optimum length (Gordon *et al.* 1966; see Fig. 12 in Josephson and Stokes, 1987).

There are some substantial differences between the FA and the scaphognathite levator. The operating frequency of the FA is more than twice that of L2B. The maximum isometric tension of the FA is about half that of muscle L2B (5.6 N cm^{-2} *versus* 12 N cm^{-2}) and the maximum shortening velocity of the FA, measured either from force–velocity curves or with the slack test, is about twice that of muscle L2B. In fact, the shortening velocity of the FA (V_m = 4.0 lengths s^{-1}, V_0 = 7.8 lengths s^{-1}) is surprisingly high. It is much higher than that of fast fibres from walking legs of the crab

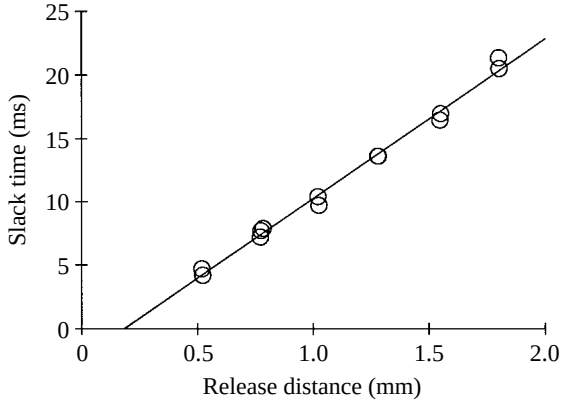

Fig. 11. A plot of slack time as a function of release distance for the preparation of Fig. 10. The slope of the line corresponds to a strain rate of 7.1 lengths s^{-1}.

Eriphia spinifrons (V_m = 0.7 lengths s^{-1} at 20 °C, Galler and Rathmayer, 1992) and somewhat higher than the shortening velocities of the frog sartorius muscle (V_m = 3.6 lengths s^{-1} at 15 °C, Rome, 1983) and the rat extensor digitorum longus (V_m = 3.6 lengths s^{-1} at 15 °C, Ranatunga, 1984), both of which are generally considered to be fast vertebrate muscles.

The performance of the FA is, in general, in good accord with what is known about the ultrastructure of the muscle (Stokes and Josephson, 1992) and the *in vivo* activation pattern of the muscle (Josephson and Stokes, 1994*a*). The sarcomeres of the FA are quite short (about 2 μm), so there are many sarcomeres in series in a given length of muscle, which favours a high shortening velocity (see Josephson, 1975). The I bands of the resting muscle are narrow, which should lead to a steep decline in maximum isometric force with muscle shortening (Gordon *et al.* 1966) and contribute to a narrow length–tension curve. The narrow length–tension curve is also consistent with the small strain experienced by the muscle (about 5 %) during normal contraction. A large part of the muscle cross-sectional area, over 60 %, is non-contractile material, which must be in part responsible for the low isometric stress produced. Finally, the optimal interstimulus interval for force production when using paired shocks at 10 Hz is 2–4 ms, which matches well the usual intervals between the action potentials of a burst (3–4 ms) during normal *in vivo* activation of the muscle.

Facilitation in vivo *and* in vitro

Cycle-to-cycle facilitation of contractile strength early in a bout of activity appears to be much more pronounced in isolated FA muscles than in the muscles of intact animals. Muscle facilitation cannot be easily evaluated from the movement or the mechanical force produced by a beating flagellum in an intact animal. The force produced in a beat cycle can vary because of variability in the number of times each of the two motor units to the muscle fires in a cycle, as well as because of facilitation. In intact animals, flagellar beating may begin at full strength from the onset of a burst, or there may be an initial

cycle-to-cycle increase in force, presumably due at least in part to facilitation (see Fig. 2 in Josephson and Stokes, 1994*a*). In most bursts from intact animals that we have examined, apparently full-strength beating was achieved in 3–5 cycles. This contrasts to the muscle response *in vitro*, where it takes 5–20 or more cycles, depending on the number of stimuli per cycle, for responses to reach a plateau (Fig. 3).

The mechanical responses that we recorded from isolated FA muscles became more vigorous and less dependent on facilitation when we changed from the bathing saline used in preliminary experiments to a saline that contained a higher calcium concentration and a lower magnesium concentration. It is possible that the responses of the FA could be made even less dependent on facilitation, and more like those we assume to be characteristic of muscles *in vivo*, if the calcium concentration in the bathing solution were to be further increased or the magnesium concentration reduced. But increasing calcium concentration or reducing magnesium concentration in the bathing saline cannot be justified from what is known about the concentrations of these ions in normal crab blood. Prosser (1973, Table 2.4) lists values for the ionic composition of haemolymph from 16 crustacean species. In these, the average calcium and magnesium concentrations were $14.2\,\mathrm{mmol\,l^{-1}}$ and $23.1\,\mathrm{mmol\,l^{-1}}$ respectively. Shaw (1955) and Riegel and Lockwood (1961) report calcium concentrations for the blood of *C. maenas* of 17.5 and $14.3\,\mathrm{mmol\,l^{-1}}$, and magnesium concentrations of 47.2 and $21.2\,\mathrm{mmol\,l^{-1}}$, respectively. Thus, in the saline used with the FA muscle, the calcium concentration ($16\,\mathrm{mmol\,l^{-1}}$) is about that expected for crab blood and the magnesium concentration ($7\,\mathrm{mmol\,l^{-1}}$) is already substantially lower than that of crab blood. We suggest that there may be neuromodulators in normal crab blood which enhance neuromuscular transmission and reduce the dependence on facilitation, and it is the absence of these neuromodulators in the saline used that resulted in the obvious facilitation seen in the muscles *in vitro*. Examples of enhancement of neuromuscular transmission and of muscular contraction by neuromodulators in crustaceans are given by Kravitz (1988), Mercier *et al.* (1990) and Pasztor and Golas (1993).

How might an elevated dependence on facilitation affect the sorts of physiological responses reported in this paper and alter the relationship between *in vitro* and *in vivo* performance? We think it likely that the properties of fully activated muscle – the force–velocity and the length–tension characteristics of tetanically stimulated preparations – should be relatively unaffected by requirements for facilitation. Similarly, plateau values for force reached after many cycles of cyclic activation may not vary much with the dependency on facilitation. But certainly contraction time course and amplitude in the early cycles, before a steady state is reached, will vary substantially with the extent to which the muscle is dependent on facilitation. Contractile responses to twitch stimulation and short bursts of stimuli in the FA and in other crustacean muscles should be interpreted with some caution and allowance made for the possible influence of neuromodulators in correlating *in vitro* and *in vivo* responses.

Comparison of the FA and locust flight muscle

It was pointed out in the Introduction that the FA is functionally similar in several ways to insect flight muscles. To what extent is this functional similarity reflected in structural

Table 1. *Comparison of the flagellum abductor and a locust flight muscle (the tergocoxal muscle)*

	Flagellum abductor	Tergocoxal muscle
Maximum stress (F_0)		
(N cm^{-2} muscle)	5.6[1]	29.5[2]
(N cm^{-2} fibril)	14.9[3]	62.5[4]
Width (percentage of reference length) of isometric stress *vs* muscle length curve at 50 % F_0	35	50[5]
Strain rate (s^{-1})		
V_m		
15 °C	4.0	
30 °C	11.3[6]	5.8[2]
V_0		
15 °C	7.8	
30 °C	22.1[6]	8.4[2]
Morphometry[7]		
Percentage of muscle fibre as		
Myofibrils	44	55
Mitochondria	18	26
% (SR + T-tubules)/% fibril	0.11	0.33

[1]Average of F_0 values from length–tension and force–velocity series.

[2]From Malamud *et al.* (1988).

[3]Calculated from volume fraction of muscle fibres as fibrils (44.3 %) and volume fraction of muscle as fibres (85.2 %), Stokes and Josephson (1992).

[4]Calculated from volume fraction of fibres as fibrils (55.4 %, Mizisin and Ready, 1986) and an assumed value of 85 %, as for the crab muscle, for the volume fraction of muscle as muscle fibres.

[5]Malamud (1989).

[6]Calculated from value at 15 °C and an assumed Q_{10} of 2.

[7]Flagellum abductor data from Stokes and Josephson (1992); locust data from Mizisin and Ready (1986).

SR, sarcoplasmic reticulum; V_m, maximum shortening velocity; V_0, shortening velocity under zero load.

and physiological similarity? A comparison of the FA and locust flight muscle (Table 1) allows no simple conclusion.

The shortening velocity of the FA is higher and the maximum isometric stress much lower than for the locust flight muscle. The maximum isometric stress of the FA is low even when allowance is made for the small fractional volume of myofibrils in the muscle. Why this should be so is not known.

The insect and the crab muscles do share several structural attributes. In both, myofibrils make up only about half the volume of a muscle fibre and a major part of the remaining volume is taken up by mitochondria. The large mitochondrial volume presumably contributes to the fatigue-resistance of both muscle types. The insect flight muscle has more sarcoplasmic reticulum and transverse tubules relative to the fibril

volume than does the crab muscle, suggesting that the rates of muscle fibre activation and inactivation should be faster in the insect muscle.

Slack test measurements and series elastic shortening

The maximum shortening velocity of a muscle or muscle fibre measured with the slack test (V_0) is generally found to be somewhat greater than that (V_m) determined from the zero-force intercept of a force–velocity curve (e.g. Claflin and Faulkner, 1989; Josephson and Edman, 1988; for a counter example, see Julian *et al*. 1986). The difference between V_0 and V_m determinations from the FA, however, was unusually large, which raises concern as to whether there might be systematic errors in the measurements which have led to overestimation of V_0 or underestimation of V_m.

One possible source of systematic error in our slack test measurements arises from the use of releases which begin at different muscle lengths. Part of the change in muscle length during the rapid release of a slack test is due to shortening of previously loaded and stretched series elastic elements; the remainder is the slack which is subsequently taken up by muscle shortening during the slack time. In most slack test measurements, the release is from a common initial length to a varied final length. This approach has the advantage that the force in the muscle just preceding the release, and therefore the stretch of elastic elements, is similar from trial to trial. In our measurements, the muscle was released from a varied initial length to a common final length. This approach has the advantage that force redevelopment in the set of trials occurs at the same length, which is particularly important for a muscle such as the FA, which has a narrow length–tension curve. But the approach has the disadvantage that the force at release, and therefore the stretch of series elastic elements, is not constant. Varying stretch of series elastic elements can give rise to systematic errors in determinations of maximum shortening velocity.

The slack distance (X_{slack}) produced in a muscle or in a muscle fibre by a quick release is the difference between the total release distance (X_{rel}) and the distance of shortening during the release by the previously stretched series elastic elements (X_{sec}):

$$X_{slack} = X_{rel} - X_{sec}\,.$$

The slack time (ST) is given by:

$$ST = X_{slack}/V_0 = (X_{rel} - X_{sec})/V_0\,,$$

where V_0 is the true shortening velocity of the muscle under zero load. The difference in slack time, ΔST, associated with two release distances separated by ΔX_{rel} and the resulting difference in series elastic shortening ΔX_{sec} is:

$$\Delta ST = (\Delta X_{rel} - \Delta X_{sec})/V_0\,.$$

Letting $\Delta X_{rel}/\Delta ST = V_0'$, and solving for V_0' gives:

$$V_0' = V_0 + (\Delta X_{sec}/\Delta ST)\,.$$

V_0' is the slope of the line formed by plotting X_{rel} against ST, and it is the usual measure of V_0. If the releases in a slack test begin at a common force level, with common stretch of series elastic elements, X_{sec} is the same for all releases, $\Delta X_{sec}=0$ and $V_0'=V_0$. However, if

releases start from different force levels, as is generally the case if the release starts from different muscle lengths, ΔX_{sec} is not zero and the measured value of V_0', based on release distance and slack time, is different from V_0, the true maximum shortening velocity. If the force at the longer distance of the interval separated by ΔX_{rel} were greater than that at the shorter distance, the stretch of series elastic components would be greater at the longer length and ΔX_{sec} would have a positive value. In this case, V_0' overestimates V_0. Conversely, if the releases were from different points on the descending length–tension curve, such that the muscle force at longer distances is less than that at shorter distances, ΔX_{sec} is negative and V_0' underestimates V_0. In the slack test measurements with the FA, the releases were generally from points on the descending limb of the length–tension curve, with declining force at longer lengths. Therefore, there is likely to be a systematic error in the slack test measurements because of differing series elastic stretch, but the error is such as to underestimate V_0 rather than to overestimate it. Because of this systematic error, the actual difference between V_m and the true value of V_0 is even larger than that measured. Why there is such a large difference between values of V_0 and V_m remains unknown.

This study was supported by NSF grants DCB-8811347 and DCB-9104170. We thank D. Syme for helpful comments on the manuscript.

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