Correlation of electrophysiological, histochemical, and mechanical properties in fibres of the coxa rotator muscle of the locust, *Locusta migratoria*

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Accepted August 17, 1991

Key words: Skinned muscle fibres – Neuromuscular properties – Histochemistry – Mechanical properties – *Locusta migratoria*

Introduction

In vertebrate muscles, fibres composing a motor unit are assumed to have uniform properties in many respects (Nemeth et al. 1981). In arthropod muscles, by contrast, considerable heterogeneity may exist among the fibres of a motor unit. This has been studied most thoroughly in crustaceans [reviews in Govind and Atwood (1982) and Rathmayer and Maier (1987)]. The recruitment of different muscle fibre populations and the grading of muscular mechanical performance are achieved in vertebrates by the recruitment of motoneurons, varying in number and size (Henneman et al. 1965). The situation is more complex in the sparsely innervated arthropod muscles, where inhibitory control of tonic muscle fibres and fibre heterogeneity within a given motor unit appear to play crucial roles in the control of mechanical performance (Rathmayer and Maier 1987; Wiens 1989).

With regard to insect muscles, most previous studies of muscle heterogeneity concentrated on single aspects such as innervation pattern (Hoyle 1978), ultrastructure (Jahromi and Atwood 1969 a, b; Cochrane et al. 1972; Stokes et al. 1975; Morgan and Stokes 1979; Josephson and Young 1987), electrophysiology (Hoyle 1978) and enzymatic composition (Hart and Fourtner 1979; Stokes et al. 1979; Morgan et al. 1980). A comparison of ultrastructural and histochemical features of cockroach leg muscles has revealed a correlation between assumed contractile type and morphological and metabolic parameters (Stokes 1987). Mechanical properties were correlated with different compositions of contractile proteins in *Drosophila* and *Lethocerus* flight and leg muscles (Peckham et al. 1990). In general, however, previous studies were restricted to measurements on whole-muscle preparations. A detailed analysis on the level of single,

Summary. The fibre composition of the anterior coxa rotator muscle of the locust middle leg (M92) was examined. The muscle is composed of 90–100 fibres. Muscle fibres were characterized with regard to innervation pattern, electrophysiological properties, and morphological parameters. Activity and isoenzyme composition of myofibrillar ATPase, succinic acid dehydrogenase (SDH) activity and glycogen content were examined employing histochemical techniques. Shortening velocity and the dependence of tension on intracellular Ca^{2+} were determined in skinned fibre experiments. A close match was observed between the innervation pattern of the muscle fibres and their histochemical and physiological properties. The combination of all parameters examined allowed an accurate classification of the muscle fibres into three types. Within a given type, broad variability of some properties was observed (SDH activity, Ca^{2+} sensitivity) while others assumed distinct values (innervation pattern, shortening velocity). The comprehensive characterization of muscle fibre properties permits a functional interpretation of fibre heterogeneity with regard to muscle performance. Fibres with the same innervation pattern may be recruited specifically, according to their electric properties and Ca^{2+} sensitivities. The resulting specific recruitment of fibres with different mechanical responses should allow a subtle control of muscular force, with regard to force amplitude, temporal characteristics of contraction, and metabolic cost.

Abbreviations: Cl_1 common inhibitory neurone one; ejp, ijp excitatory, inhibitory junctional potential; EGTA ethylene glycol-bis[β -aminoethyl ether] N,N,N',N'-tetraacetic acid; mATPase myofibrillar adenosinetriphosphatase; MOPSO 3-[N-morpholino]-2-hydroxypropanesulfonic acid; M92 anterior rotator muscle of the coxa; n Hill coefficient; pCa_{50} pCa corresponding to half-maximal tension; P_0 maximal isometric tension; SDH succinic acid dehydrogenase; $V_{\max}$ maximal shortening velocity

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identified fibres is still lacking. Such an analysis should permit a direct correlation of electrophysiological and mechanical properties of individual muscle fibres with their morphological and histochemical characteristics.

In the present investigation, identified fibres of the mesothoracic M92 in the locust (Snodgrass 1929) were examined. We determined their morphological and electrophysiological parameters and subsequently investigated their histochemical properties by staining serial cross sections of the muscle for myofibrillar ATPase (mATPase) activity, for the occurrence of isoforms of this enzyme, and for SDH activity and glycogen content. Finally, we studied mechanical properties of these fibres by measuring shortening velocities under constant load conditions and tension-pCa relationships. This allowed the direct correlation of morphological and histochemical parameters with physiological properties of individual fibres and provided the basis for a detailed analysis of the fibre composition of the muscle. This, in turn, permitted an interpretation of the role the different fibre types play in muscle performance. Some of the results have previously been reported in abstract form (Müller et al. 1990).

Materials and methods

Animals. Adult locusts (*Locusta migratoria*) were obtained from the University of Konstanz breeding stock. Animals of either sex were used 1–3 weeks after their imaginal moult.

Anatomy. Somata of motoneurons were stained by immersing their axons in the peripheral nerves in solutions of CoCl_2 or $\text{Co}(\text{NH}_4)_6\text{Cl}_2$, with concentrations ranging from 2% to 6% (Pitman et al. 1972). After 1–3 days, the mesothoracic ganglion was removed from the animal and cobalt ions were precipitated with ammonium sulphide solution. Ganglia were fixed in buffered formaldehyde solution (4% paraformaldehyde, 10 g CaCl_2 and CaCO_3 powder added to saturation into 1 l solution, pH adjusted to 7.2 with NaOH), dehydrated and cleared in methyl salicylate for microscopic inspection. If necessary, whole mounts were silver intensified (Bacon and Altman 1977) and embedded in Canada balsam.

Electron microscopy. For electron microscopy preparations were dissected, prefixed for 2 h in 3% glutaraldehyde and 3% formaldehyde, postfixed for 1 h with 2% osmium tetroxide and embedded in Spurr's resin. Thin sections (80–90 nm) were contrasted with uranyl acetate and lead citrate and were examined on a Zeiss EM9s electron microscope.

Electrophysiology. For neuromuscular studies, the neck connectives of the locusts were severed. Animals were pinned in a plasticine-coated petri dish, dorsal side up. To gain access to the M92 (Snodgrass 1929) the cuticle of the sternellar lobe was excised and removed, together with part of the meso-metathoracic suture and muscle 93 attaching to it. After the removal of tracheal sacs, a pair of hook electrodes (stainless steel pins, diameter 50 μm) were placed on nerve 3C₁ (Fig. 1A) in bipolar configuration and insulated with a drop of vaseline. With an appropriate setting of the stimulus voltage, this permitted selective stimulation of the fast excitatory axon innervating M92. For selective stimulation of the common inhibitory neurone (CI₁) a second pair of electrodes was attached to nerve 3A₃. Voltage pulses applied to this bipolar electrode activated CI antidromically. Spontaneous and stimulus-evoked junction potentials were recorded intracellularly from the muscle fibres. In most experiments microelectrode recordings were made from two

muscle fibres simultaneously in order to compare differences in junctional responses and to permit their identification. One microelectrode was kept in a superficial fibre, while the other was advanced from dorsal to ventral, recording from all fibres encountered during the passage. Recordings were stored on magnetic tape and displayed on a digital storage oscilloscope for evaluation.

Histochemistry. Coxa rotator muscles were isolated from decapitated animals. The muscle's apodeme at the sternellar suture and its insertion at the coxa were excised and left attached to the muscle. After removal of connective tissue and trachea, these cuticular structures served to mount the muscle between two pairs of forceps and stretch it to resting length. The preparation was superficially dried with cellulose tissue, embedded in Tissue TEK medium (Miles, Elkhart, USA) and shock-frozen in liquid nitrogen. Preparations were mounted with Tissue TEK on the cold chuck of a cryotome (Frigocut 2000, Reichert/Jung) at -30°C . Serial cross sections (21 μm) were made and placed on dry, glycerin-albumin-coated cover slides. They were quickly thawed and air dried on a heating plate at 40°C for about 30 min. After staining (see below), sections were dehydrated, cleared in xylene and mounted in Permount. All assays were run at room temperature.

Staining for mATPase activity was performed according to Padykula and Hermann (1955), with modifications adopted from Maier et al. (1984), except that pH was adjusted to 8.4. Sodium azide (10 mM) was added to the reaction medium in order to inactivate mitochondrial Ca^{2+} -ATPase.

To detect possible isoforms of mATPase on the basis of different pH stability, sections were preincubated for 20 min at different pH values between 4.0 and 5.0 and at pH 10.2, before transfer to the reaction medium at pH 9.4 (Brooke and Kaiser 1970; Guth and Samaha 1970).

Activity of muscular SDH was determined according to the method of Nolte and Pette (1972). Single sections were incubated in the reaction medium for 1–2 min at 35°C . As a control, the procedure was repeated without adding succinate to the medium.

Muscular glycogen content was determined by the periodic-acid-Schiff reaction (PAS) as described by Romeis (1968). Counterstaining with Harris' haematoxylin was not necessary.

Muscle mechanics. The coxa rotator muscle was isolated as described above. It was frozen in liquid nitrogen and stored on silica-gel at -22°C for 6–8 weeks (Stienen et al. 1983). Single fibres were dissected from freeze-dried muscles under the microscope. Samples were taken from various regions and different layers of the muscle, and included fibres with different innervation type and histochemical properties. Dissected fibres were used for experiments immediately or were again frozen on silica gel at -22°C for not more than 2 weeks.

One end of the freeze-dried fibre was glued to a force transducer (SensoNor AE801 Horten, Norway), with nitrocellulose dissolved in acetone, the other to a motor-driven glass peg. Fibres were immersed in a relaxing solution (composition see below) and adjusted to a length corresponding to just slack position. Sarcomere lengths were determined from the diffraction patterns generated by a He-Ne laser beam focused on the fibre [$\lambda = 632.8$ nm, 4 mW, Spectra Physics, Eugene, USA, Zite-Ferenczi and Rüdel (1978)]. Fibre length and fibre diameter were measured under the microscope with an ocular scale.

The relaxing solution, modified after Moiescu and Thieleczek (1979), contained (mM) $\text{Na}_2\text{H}_2\text{ATP}$ 8.0, creatine-Na-phosphate 10.0, caffeine 15.0, MOPSO buffer 60.0 (pH 6.2–7.6), K_4 -EGTA 25.0, Mg-propionate 7.3. Prior to measuring Ca^{2+} dependency of force development, 1% Triton X-100 was added to the relaxing solution. Skinned muscle fibres were incubated for 15 min in this solution in order to destroy internal Ca^{2+} stores. The activating solution contained (mM): $\text{Na}_2\text{H}_2\text{ATP}$ 8.0, creatine-Na-phosphate 10.0, caffeine 15.0, MOPSO buffer 60.0, Ca-EGTA- H_2 25.0, Mg-propionate 6.74, and K-propionate 23.82. pH was adjusted to 6.7 with KOH. In both solutions, total ionic strength was 0.16 M. Free Mg^{2+} concentration was 1 mM calculated on the basis of equi-

Fig. 1A–C. Morphology and innervation of the coxa rotator muscle of *Locusta migratoria*. (A) Innervation of the anterior coxa rotator muscle (M92) by nerve 3C₁ of the mesothoracic ganglion and its attachment sites in the mesothoracic segment of the locust. (B) Camera lucida drawing of the mesothoracic ganglion showing the neurones stained by a cobalt backfill from N3C₁. Up to five smaller

neurones listed by Martell and Smith (1977). The pCa (negative log of free [Ca²⁺] concentration) of the relaxing solution was 7.06, and that of the activating solution 4.25 as determined by Ca²⁺-sensitive electrodes based on the ionophor ETH 1001 [exact procedure described in Galler et al. (1990)]. Fifty units creatine kinase per ml were added to the solutions immediately before use.

Shortening velocity. At the beginning of each experiment, maximal isometric tension (P_0) was achieved by exposure to activating solution. Using a feedback control system (Scientific Instruments, Gth, Heidelberg, FRG) the load on the fibre was decreased instantaneously from P_0 to lower, constant and pre-adjustable values so that the fibre would shorten isotonically. The load could be clamped at any fraction of P_0 . The clamp was maintained for 500 ms. Contraction speed was determined between 100 and 200 ms after the beginning of each clamp, when the fibre exhibited almost linear isotonic shortening (inset, Fig. 5). Experimental data were displayed on a digital oscilloscope (Nicolet 4094) and stored on magnetic disks. Shortening velocities (% fibre length $\cdot$ s⁻¹) were plotted versus relative tension (% P_0). Maximum shortening velocities (V_{max}) were extrapolated from these force-velocity curves by a nonlinear least-squares fit of the Hill equation (Woledge et al. 1985).

Tension–pCa relationship. To measure tension–pCa relationships, single freeze-dried fibres were immersed successively in solutions containing different pCa (obtained by mixing different proportions of relaxing and activating solutions). Calculated pCa values of these mixtures were verified by Ca²⁺-sensitive electrodes (see above). At the beginning and end of each experiment, fibres were fully activated in order to correct for possible decreases in maximal tension during the experimental period. Two different protocols were used for the actual determination of tension–pCa relationships. In the first, fibres were initially immersed in relaxing solution and then exposed to activating solutions of increasing pCa. In the second, fibres were fully relaxed before each transfer to higher pCa. Both protocols yielded similar results. The tension produced at different Ca²⁺ levels (P) was compared with maximal tension (P_0). Relative tension (% P_0) was then plotted versus pCa and sigmoid curves were fitted to the data points. pCa₅₀ values (pCa at half-maximal activation) were read from the curves obtained from the data set of each fibre. Hill coefficients (n , characterizing the steepness of the tension-versus-pCa graph) were calculated by linear regression from Hill plots.

Results

Morphology

The M92 arises from the sternellar suture (Snodgrass 1929), its tendon inserts at the anterior dorsal rim of the coxa [Fig. 1A; see also Albrechts (1953)]. The muscle consists of 90–100 fibres, is approximately club-shaped and has a pennate structure. In accordance with this shape, the muscle fibres are arranged in a fan-shaped fashion along the tendon. The central fibres, termed type I, insert at the most proximal segment of the tendon. They are the shortest fibres in the muscle, with lengths between 1300 and 1700 μ m. The outer layers contain fibres ranging from 1500 to 2400 μ m. In general, the short

somata that were stained only occasionally are not drawn (details see text). Numbers indicate peripheral nerves (Campbell 1961). (C) Electron micrograph of a cross section through nerve 3C₁, close to M92. The three large axon profiles belong to the motoneurones innervating M92 (“fast” F, “slow” S, common inhibitor 1 CI₁, in the order of their diameters)

fibres are also of smaller diameter, while the long fibres at the dorsal surface of the muscle have the largest cross-section areas in the muscle profiles (Fig. 4). Sarcomere lengths are equal in all fibre types ($5.5 \pm 1.1 \mu\text{m}$, $n = 121$, $P > 0.1$).

M92 is innervated by nerve $3C_1$ (Campbell 1961) which contains three larger axon profiles (diameters 16.5, 6.0, and $3.8 \mu\text{m}$) and five smaller axons ca. $0.7 \mu\text{m}$ in diameter (Fig. 1C). The larger axons belong to the two excitatory neurones (one "fast" and one "slow") and the common inhibitory neurone (CI_1). Origin and function of the five small axons are unknown.

Cobalt backfills from nerve $3C_1$ stain three large somata (diameters 35–75 μm) in the mesothoracic ganglion (Fig. 1B) and occasionally 3–5 smaller cell bodies in the same cluster as two of the larger ones. These two belong to the excitors of M92 and are located ipsilaterally, at the ventral edge of the ganglion close to the root of nerve 1. The third soma lies ventrally and posteriorly, just contralateral to the midline. It has been identified morphologically and physiologically as CI_1 (Hale and Burrows 1985; Watson et al. 1985). The general appearance of the two excitatory motoneurones is similar to those of other leg motoneurones whose axons leave via nerve 3 (Wilson 1979). Main branches of the two neurones run parallel, sometimes twisting around one another. Two prominent neurites branch off in the lateral region of the neuropilar segment, extending posteriorly. A third neurite, characteristic of the M92 motoneurones, extends medially from the initial neuropilar segment. Dendrites of higher order arborize profusely in the dorsal neuropil of the ganglion.

Neuromuscular properties

The fibres of M92 were classified into three distinct groups according to their innervation patterns (Fig. 4). The first fibre population, type I, is situated centrally in the muscle. These fibres are innervated by the "slow" axon and by CI_1 . Another group, the type IIa fibres, forms a single continuous layer of about 30 fibres surrounding the type I fibres. They receive triple innervation by the "slow", "fast" and CI_1 axons. The third group, termed type IIb fibres, receives innervation through the "fast" axon only. They are located in the dorsal half and on the lateral surfaces of the muscle.

Electric stimulation of nerve $3C_1$ always activated the "fast" axon first, because it has the largest diameter and, consequently, the lowest threshold. Single stimuli applied to the nerve produced large postsynaptic responses of about 40.0 mV amplitude (Fig. 2). These potentials consisted of a synaptic component (ejp) and an electrically elicited response. The fibres supplied by the "fast" axon cover about 75% of the muscle's cross-sectional area.

Activity of the "slow" axon produced only small junctional responses. They were analyzed from spontaneous discharges of this motoneurone. Amplitudes of the "slow" ejps usually ranged from 7.5 to 11.0 mV (Fig. 2). The fibres innervated by the "slow" motoneurone accounted for about 50% of the muscle's cross section area.

Fig. 2. Intracellular recordings from muscle fibres belonging to the three different types (I, IIa, IIb). Junctional potentials were recorded upon spontaneous motoneurone discharges. Small ejps (elicited by "slow" motoneurone activity) and ijps are visible in type I and IIa fibres. Large potentials (elicited by "fast" motoneurone discharges) are present in IIa and IIb fibres

Inhibitory innervation of muscle fibres was established by antidromic stimulation of CI_1 via nerve $3A_3$ (see Materials and methods). The ijp amplitudes were dependent on membrane resting potential. In fibres which had more negative resting potentials, ijps were observed only after application of high-potassium saline (20–30 mM K^+). CI_1 innervation was found to strictly parallel the innervation pattern of the "slow" motoneurone (Fig. 4).

Junctional responses recorded from the three fibre types during spontaneous discharges of all three motoneurones are presented in Fig. 2. In the intracellular recording shown for fibre type I, facilitation of "slow" ejps and summation of ijps, due to long membrane time constants of this fibre type, are discernible.

Membrane potentials differed between the three fibre populations. The average resting potentials were $-38.0 \pm 8.8 \text{ mV}$ ($n = 75$) for type I, $-47.5 \pm 8.9 \text{ mV}$ ($n = 49$) for type IIa, and $-45.3 \pm 8.4 \text{ mV}$ ($n = 114$) for type IIb fibres. Differences between type I and type II fibre populations were statistically significant ($P < 0.001$), while resting potentials of type IIa and type IIb fibres could not be distinguished by statistical analysis ($P > 0.05$).

Histochemistry

Serial cross sections were obtained from an area of the muscle proximal to the tendon where all fibres were encountered. Histochemical determination of mATPase activity produced a distinct staining pattern (Fig. 3A) closely matched with the classification derived from the innervation pattern (Fig. 4). Type IIa and IIb fibres stained dark, indicative of high mATPase activity. While type IIb fibres revealed homogenous, strong reactions, type IIa fibres showed more variability. The centrally

Fig. 3A–D. Histochemistry of the coxa rotator muscle of *Locusta migratoria*. **A** Overall mATPase activity. **B** pH-stability of mATPase; mATPase was tested after alkaline preincubation at pH 10.2. **C** Succinic acid dehydrogenase (SDH) activity. **D** Glycogen content. Dark stains indicate high enzymatic activities/glycogen content. Dorsal is to the *top*

located type I fibres stained only lightly due to low mATPase activity.

Preincubation of muscle sections in alkaline conditions (pH 10.2) and subsequent staining for mATPase revealed a different, almost reverse, staining pattern (Fig. 3B). Type I fibres now stained dark, their mATPase resisting alkaline treatment. The surrounding layer of

type IIa fibres showed varying degrees of staining intensity ranging from an unimpaired reaction, like that of the type I fibres, through partial degradation, to a complete loss of mATPase activity. Type IIb fibres always remained unstained, due to the inactivation of their mATPase at pH 10.2. Acid preincubation at pH levels between 5.0 and 4.0 resulted in an inactivation of mATP-

Fig. 4. Matching of electrophysiological and histochemical properties. *Left* Schematic diagram of M92, illustrating muscle morphology, innervation pattern, and alkaline stability of mATPase. *Right* Corresponding intracellular recordings from the three fibres types I, IIa, IIb. Characteristic synaptic responses evoked by activity of “fast”, “slow”, and CI axons is shown. Dorsal is to the *top*

Fig. 5. Velocity-tension graphs from constant load experiments with single, identified muscle fibres. Type I (*squares*), type IIa (*circles*), and type IIb (*triangles*). Shortening velocity ($\% \text{ of fibre length} \cdot \text{s}^{-1}$) is plotted versus relative tension (tangential least-square fitting procedure). *Inset* Original recordings of fibre length and tension during three experiments with a type IIb fibre. Force was clamped to 30% (a), 10% (b), and 2% (c) of maximal tension, respectively, for 500 ms, and the fibre was then stretched to resting length again while tension produced an overshoot. Shortening velocity was measured 100–200 ms after the beginning of the force clamp during the period of nearly linear isotonic shortening

ase around pH 4.6, regardless of fibre type (not shown). By comparison, mATPase of crab muscle fibres corresponding to type I tolerates acid preincubation even at pH 4.5 (Maier et al. 1984).

Histochemical determination of SDH activity produced a staining pattern similar to that derived from mATPase activity (Fig. 3C). All gradations between light and intense staining were observed. In general, fibres with a high level of mATPase also stained heavily for SDH, and vice versa.

Examination of the glycogen content of fibres did not reveal any differential staining (Fig. 3D). All fibres responded poorly to the PAS reagent, which is indicative of low glycogen content.

Mechanical properties

Contractile responses were determined in identified skinned muscle fibres. Fibres were activated by exposure to solutions with different Ca^{2+} concentration (pCa, see Materials and methods). Maximum force production was similar in all fibre types, with a value of $4.8 \pm 2.4 \text{ N} \cdot \text{cm}^{-2}$ ($n = 64$, $P > 0.1$). This similarity was consistent with the uniformity in sarcomere lengths reported for the different fibre types (see above).

Maximum shortening velocities were extrapolated from velocity-tension relationships measured under constant load conditions. The inset in Fig. 5 gives three original recording traces of fibre length, with the load being clamped for a period of 500 ms to 30% (a), 10% (b), and 2% (c) of maximal tension, respectively. Velocity–

Fig. 6. Tension–pCa relationship. Steady-state isometric tension (*ordinate*) was measured in identified muscle fibres during exposure to different pCa (*abscissa*). In the tension–pCa plots, averages of all data points ($\% P_0$; n ; pCa_{50}) from individual fibres were used. The summary curves of the types fitted to the equation $P/P_0 = 1/[1 + 10^{n(\text{pCa} - \text{pCa}_{50})}]$. The three fibre types (type I, *squares*; type IIa, *circles*; type IIb, *triangles*) showed different sensitivity to Ca^{2+} and different cooperativity in their contractile response. Mean standard deviations of data points in the direction of the *ordinate* are 0.12 (type I), 0.12 (type IIa) and 0.13 (type IIb)

tension graphs derived from these experiments are presented for the three fibre types. Type I fibres showed slow shortening velocities with an extrapolated V_{max} of $66 \pm 24.4\%$ ($n = 22$) fibre length $\cdot \text{s}^{-1}$, whereas type IIa and IIb fibres were found to be fast-contracting, with extrapolated V_{max} of $106 \pm 40.6\%$ ($n = 23$) and $92 \pm 32.5\%$ ($n = 21$) fibre length $\cdot \text{s}^{-1}$, respectively. Values obtained for type IIa and IIb fibres were not significantly different ($P > 0.05$). However, both fast-contracting fibre types were clearly delimited from the slow-contracting type I fibres ($P < 0.01$ and $P < 0.05$, respectively). These differences between type I and type II fibres held true regardless of whether shortening velocities were related to fibre length or half-sarcomere length (data not shown).

To determine Ca^{2+} sensitivity of tension development, muscle fibres were exposed to solutions with different pCa and their contractile responses were measured under isometric conditions. The graphs presented in Fig. 6 were calculated from the data obtained from 20 type I, 15 type IIa, and 29 type IIb fibres. Tension–pCa relationships were always sigmoidal. Values for pCa_{50} (pCa corresponding to half-maximal tension) were read from these curves. Slow contracting type I fibres started to develop tension and reached maximum activation at lower Ca^{2+} concentrations ($\text{pCa}_{50} = 5.78 \pm 0.18$) than fast-contracting type II fibres. Within the type II population, IIa fibres ($\text{pCa}_{50} = 5.64 \pm 0.19$) exhibited higher Ca^{2+} sensitivities than type IIb fibres ($\text{pCa}_{50} = 5.57 \pm 0.17$). Values for type I fibres were significantly different from those for type IIb fibres ($P < 0.001$) but neither fibre type could be distinguished from type IIa fibres by statistical analysis ($P > 0.05$). The individual

Table 1. Properties of muscle fibres composing M92

Fibre type	I	IIa	IIb
Neuromuscular properties			
Innervation	Slow; CI	Fast; slow; CI	Fast
Membrane potential (mV)	-38.0 (± 8.8)	-47.5 (± 8.9)	-45.3 (± 8.4)
Histochemistry			
mATPase	Low	Medium to high	High
mATPase at pH 10.2	Stable	Labile to stable	Labile
SDH activity	Low	Medium to high	High
Glycogen content	Low	Low	Low
Mechanical properties			
pCa ₅₀	5.78 (± 0.18)	5.64 (± 0.19)	5.57 (± 0.17)
Hill coefficient (n)	2.1 (± 0.5)	2.5 (± 0.6)	3.0 (± 1.2)
Shortening velocity (% fibre length · s ⁻¹)	66 (± 24.4)	106 (± 40.6)	92 (± 32.5)

pCa₅₀ values revealed considerable variability, which is indicated by the high standard deviations given above. In fact, the fibres composing M92 exhibited a continuous spectrum of Ca²⁺ sensitivities. Within this spectrum, only the extremes, represented by the type I and type IIb fibres, were clearly delimited.

In contrast to the situation in vertebrate muscles (Moss et al. 1985), the tension-pCa values of locust muscles could be fitted to a single straight line by linear regressions in Hill plots with high correlation coefficients ($r^2 = 0.96 \pm 0.05$, $n = 6$). Hill coefficients (n) calculated from the Hill plots characterize the slope of the tension-pCa relationship and are a measure of cooperativity in the process of force generation. Low cooperativity is indicative of graded contractions in response to changes of intracellular Ca²⁺ level, whereas high cooperativity designates twitch-like responses.

Type I fibres had the lowest Hill coefficients with $n = 2.1 \pm 0.5$. Type IIa fibres showed intermediate properties with $n = 2.5 \pm 0.6$, and type IIb fibres possessed the highest Hill coefficients with $n = 3.0 \pm 1.2$. Like the values obtained for Ca²⁺ sensitivity, Hill coefficients determined in M92 fibres exhibited considerable variability, producing a continuous distribution extending from 1.5 to 4.5. Again, type I fibres and type IIb fibres represented the extremes and statistic analysis revealed significant differences only between these two ($P < 0.05$). Type IIa fibres occupied intermediate values and were not delimited from the two adjacent fibre types ($P > 0.05$ in both cases). Therefore, within the coxa rotator muscle there is a smooth transition from fibres with slow, graded responses (type I) to fibres with a more twitch-like behaviour (type IIb).

Classification of fibre types

In the present investigation, results of morphological, electrophysiological, histochemical, and mechanical studies have been combined for the characterization of the locust anterior coxa rotator muscle. The 90–100 fi-

bres composing this muscle were classified into three distinct types (values see Table 1).

Type I fibres occupy the central portion of the muscle and attach to the most proximal part of the tendon (Fig. 4). They are thus the shortest fibres in the muscle and also the thinnest. They are innervated by the "slow" axon and the common inhibitor and are characterized by relatively positive membrane potentials (Fig. 2). They show low mATPase activity which is resistant to alkaline preincubation, low SDH activity, and low glycogen content (Fig. 3). They are slow contracting (Fig. 5) and have the lowest thresholds for Ca²⁺ activation (Fig. 6). Slow-contracting type I fibres are clearly distinguished from the fast-contracting type II fibres. Type II fibres are subdivided into type IIa and IIb, primarily according to their different innervation patterns.

Type IIa fibres ensheath the type I fibres as a single layer. In many respects they show properties which range in between those of type I and IIb fibres (Fig. 4). This refers to fibre length and diameter, histochemical parameters (Fig. 3 and 4), and Ca²⁺ sensitivity (Fig. 6). However, type IIa fibres have the most negative resting membrane potentials and show the fastest contractile responses (Fig. 5). They are triply innervated by the two excitatory axons and CI₁ (Figs. 2, 4).

Type IIb fibres form the dorsal portion of the muscle. On average, they span the longest distance and have the largest diameters (Fig. 4). Innervated only by the "fast" axon (Fig. 2), they have relatively negative membrane resting potentials. Histochemically, they show high mATPase activity (the enzyme being unstable under alkaline conditions), high SDH activity, and low glycogen content (Fig. 3). The fibres are fast contracting (Fig. 5) and have high thresholds for Ca²⁺ activation (Fig. 6).

Discussion

Morphology. In previous studies concerned with fibre morphology and ultrastructure of arthropod muscle it was reported that muscle fibre length correlates positive-

ly, while sarcomere length correlates negatively, with contraction speed (Jahromi and Atwood 1969b; Atwood 1973; Josephson 1975). In crustaceans, fast-contracting fibres were reported to possess large, slow-contracting fibres small diameters (Jahromi and Atwood 1969b). The present study shows that these correlations also apply to the fibres of locust M92. However, distinct differences in shortening velocity (normalized for fibre length) were found despite identical sarcomere lengths. This raises doubts that this ultrastructural feature is appropriate for a functional classification of locust muscle fibres.

The length of each fibre type appears to be adapted to its physiological performance. For example, the fast-contracting type IIb fibres shorten by twice as much as the slow-contracting type I fibres within a given time period. They are specialized for the production of rapid movements during locomotion, whereas short type I fibres may be employed for the maintenance of postural tension.

Neuromuscular properties. The locust M92 was chosen as the object of this investigation because it shows the sparse innervation and muscle fibre heterogeneity typical of arthropod leg muscles. A strictly parallel innervation of muscle fibres by "slow" and the common inhibitory motoneurone has been observed, as previously reported [locust extensor tibiae (Burns and Usherwood 1979); crustacean walking leg muscles (Wiens et al. 1988)]. The fibres' membrane potentials were within the range recorded in other locust muscles by Hoyle (1966) and Usherwood and Grundfest (1965). These and other authors (Werman and Grundfest 1961) also reported that, as in M92, ejps elicited by "fast" axons often initiate graded active responses and are larger than those observed in response to "slow" axon discharges.

Histochemistry. Histochemical properties of the M92 fibres differ from those previously reported for other insect, crustacean or vertebrate leg muscles (Brooke and Kaiser 1970; Stokes et al. 1979; Rathmayer and Maier 1987). For example, the fast-contracting type IIb fibres of the locust coxa rotator are characterized by high mATPase activity, high SDH activity, and low glycogen content. According to the classification scheme established by Peter et al. (1972), these fibres would have to be termed "fast oxidative" (FO). In insects, fast-contracting fibres with high oxidative energy metabolism (β -oxidation) have as yet been described only for flight muscles (Jutsum and Goldsworthy 1976) and not for leg muscles. The fast-contracting Ila fibres show high mATPase, moderate SDH activity and negligible glycogen content, also indicative of a predominantly oxidative metabolism. This suggests that an oxidative energy metabolism is generally of high significance in locust muscles. The high oxidative capacity results from the ample oxygen supply to insect muscle via the tracheal system and, for the locust in particular, it may reflect an adaptation to long-distance flight in terms of weight reduction. The slow-contracting type I fibres exhibited low values in all histochemical parameters examined. This metabolic fibre type is the only one that has previously been described

for insect leg muscles [i.e. coxal muscles of the cockroach, termed type III by Stokes et al. (1979)]. However, myosin isoenzyme composition, as judged from mATPase stability to alkaline preincubation, appears to be different in the respective fibre types of locust and cockroach.

Fibre type I of the locust M92 contains an alkaline-stable myofibrillar ATPase, whereas the isoenzyme of the type IIb fibres is sensitive to alkaline treatment. Type Ila fibres stained variably after alkaline preincubation. The latter observation may be interpreted in two ways: Either (1) the staining pattern of the type Ila fibres is produced by a large number of different myosin isoenzymes with corresponding differences in alkaline resistance, which are distributed throughout the type Ila fibres, or (2) the staining pattern is a result of the coexistence of two isoforms of the myosin heavy chain, one characteristic of the slow-contracting type I, the other of the fast-contracting type IIb fibres, with different fractions of the two being present in individual type Ila fibres. The second interpretation is far more likely to be correct in view of corresponding results obtained from vertebrate muscles. Termin et al. (1989) described the occurrence of three different heavy-chain isoforms in chronically-stimulated rat anterior tibialis muscle. In combination with different light-chain isoforms, these heavy-chain isoforms assemble more than 54 different myosin isoenzymes that may coexist within a single fibre type of the rabbit anterior tibialis (Staron and Pette 1988).

Myosin isoenzyme composition of the three muscle fibre types in M92 of the locust, as judged from alkaline and acid preincubation, is not comparable to that of known vertebrate (Brooke and Kaiser 1970), crustacean (Maier et al. 1984), or insect muscles (Stokes et al. 1979). For instance, the mATPase of the slow-contracting fibres is alkaline resistant and that of the fast-contracting fibres is susceptible to alkaline treatment in the locust, while the reverse is true for cockroach leg muscles. Contrary to the situation in vertebrates, crustaceans and cockroach, none of the locust muscle fibre types possesses an mATPase that is resistant to acid preincubation at pH 5.0–4.0. This underlines the pronounced heterogeneity of myosin isoenzymes in different muscles and species, although muscular proteins are generally considered to be conservative (Harrington and Rodgers 1984).

Muscle mechanics. Mechanical properties of skinned muscle fibres of identified types have not yet been determined in insects. In particular, contraction speed is usually inferred from mATPase activity and not measured directly. According to Barany (1967), high mATPase activity is correlated with rapid muscle fibre contraction. This correlation has been confirmed by our direct measurements of contractile responses in identified fibres of type I and type II fibre populations in a locust muscle.

Maximum tension development observed in the locust coxa rotator muscle ($4.78 \text{ N} \cdot \text{cm}^{-2}$) is lower than that reported for intact crustacean muscle fibres [$60 \text{ N} \cdot \text{cm}^{-2}$; Griffiths et al. (1990)]. However, this value was obtained from the specialized holding muscle in the giant barnacle. Ca^{2+} sensitivities of the three fibre types in the locust are in the range of those of other insects

(Stephenson and Williams 1980; Peckham and White 1989; Peckham et al. 1990) but less than in vertebrate muscle fibres (Stephenson and Williams 1982). Shortening velocities of single fibres of the locust leg muscle investigated are twice as high as in crab leg muscle fibres of comparable physiological type [$V_{\max}$ of the slowest type I fibres = 27.8%, and that of the fastest contracting type IV fibres = $74.4\% \text{ fibre length} \cdot \text{s}^{-1}$; Galler and Rathmayer (1991)]. Shortening velocities measured in the locust muscle were smaller than in vertebrate twitch muscle fibres [$V_{\max}$ of slow twitch fibres = 76%, $V_{\max}$ of fast twitch fibres is up to $299\% \text{ fibre length} \cdot \text{s}^{-1}$; Reiser et al. (1985)]. It must be kept in mind, however, that pH, temperature, and ionic strength play important roles in the performance of skinned muscle fibres (Galler and Rathmayer 1991), and these parameters must be taken into account when comparing values obtained for different muscle fibre types studied under different experimental conditions.

A clear correlation was observed between mechanical properties and histochemical features. For instance, mATPase stability to alkaline preincubation and speed of contraction are closely correlated (Figs. 3B, 5). This is explained by the fact that shortening velocity is primarily dependent on the type of myosin heavy chain constituting the myosin head in the respective fibre (Reiser et al. 1985). However, other components regulating mATPase activity, such as myosin light chains (Pette and Schnez 1977), may also influence mechanical properties. There is also a correlation between oxidative capacity of fibres and their contraction speed. Oxidative metabolism is generally higher in fast-contracting than in slow-contracting fibres, ensuring appropriate energy supply to the respective fibre types (Fig. 3C).

Fibre typing. The combination of mechanical measurements with electrophysiological, histochemical, and morphological studies allowed a comprehensive characterization of the muscle fibre types composing M92. From the data presented above, it is evident that some of the parameters used for classification occupy discrete values while others exhibit mutually overlapping or even continuous distributions. Muscle fibres can be classified unequivocally according to their innervation pattern or by myosin isoenzyme content. On the other hand, histochemical staining of mATPase and SDH activity, as well as Ca^{2+} sensitivity and shortening velocity, yielded variable results even in fibres with identical innervation patterns. Because of this pronounced diversity of certain fibre properties, only combinations of several parameters can lead to a meaningful classification of functionally different muscle fibre types.

It has to be kept in mind, however, that each muscle fibre may be regarded as an individual with distinct physiological properties. This is suggested by the fact that even within a distinct population of muscle fibres, some properties show continuous distributions. This aspect is of importance for a functional interpretation of fibre types and fibre diversity, in particular with regard to fibre recruitment.

Fibre recruitment. Type I fibres are activated by the "slow" motoneurone which elicits ejps of small amplitudes. However, they also possess the most positive membrane resting potentials of all fibres and long membrane time constants. Thus, they should reach excitation-contraction threshold at modest depolarization levels. Due to the long decay-time constants of the ejps, summation may occur (Fig. 2) and contribute significantly to activation of these fibres. As indicated by the pCa_{50} values, type I fibres also possess the lowest threshold for Ca^{2+} activation ($\text{pCa}_{50} = 5.78$). In addition, they show little cooperativity ($n = 2.1$). This demonstrates that they are specialized for "slow" contractions in response to "slow" motoneurone activity.

Triply innervated type IIa fibres possess a dual mode of activation. They require higher frequencies of "slow" motoneurone impulses for activation than do the type I fibres. They have more negative membrane resting potentials and, since "slow" ejps are of similar size in both fibre types, IIa fibres will be recruited by higher discharge frequencies of the "slow" motoneurone. In addition, they can be activated by the "fast" motoneurone. The electrically excited, active responses elicited by this neurone always reach excitation-contraction threshold. The different status of the type IIa fibres is also reflected by a more pronounced cooperativity ($n = 2.5$) and lower pCa_{50} values, resulting in twitch-like contractions when compared to the "slow"-contracting type I fibres.

Type IIb fibres are innervated only by the "fast" motoneurone. An ejp elicited by the "fast" motoneurone will usually lead to an electrically excited active response at the membrane and to a twitch contraction of the fibre. Corresponding to their "fast" contractions, they exhibit high cooperativity ($n = 3$) and low Ca^{2+} sensitivity ($\text{pCa}_{50} = 5.57$).

In summary, fibre diversity subserves the versatile mechanical performance in a sparsely innervated insect muscle. For instance, with increasing spike frequency of the "slow" axon, type I fibres will be activated first, followed by a stepwise recruitment of different type IIa fibres. Activity of the common inhibitor will further modulate muscle fibre recruitment in these two groups by restricting the activation of "slow" innervated fibres (Rathmayer 1990). Discharges of the "fast" motoneurone will result in twitch-like muscle responses caused by type IIa and IIb fibres. The mechanical properties of the muscle are thus adapted to the various behavioral requirements, such as maintenance of posture and rapid locomotion.

Acknowledgements. This study was supported by the Deutsche Forschungsgemeinschaft, SFB 156, and by a scholarship from the Boehringer Ingelheim Fonds to A. Müller. We thank C. Dittrich for excellent technical assistance, C. Giele for drawing the figures, and M.A. Cahill for correcting the English text.

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