

ACTIVATION OF MULTIPLY INNERVATED FAST AND SLOW MYOTOMAL MUSCLE FIBRES OF THE TELEOST *MYOXOCEPHALUS SCORPIUS*

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Summary

A nerve–muscle preparation from the sculpin *Myoxocephalus scorpius* was used to study the membrane response of fast and slow muscle fibres to stimulation of the spinal nerves. There was no significant difference between resting potential in fast (-81.9 mV) and slow fibres (-80.8 mV). Fast fibres responded to a supra-threshold stimulus in the spinal nerve with an action potential. Overshoots of up to $+32$ mV were recorded. Both junction potentials and overshooting action potentials were observed in the slow fibres.

The twitch/tetanus characteristics of myotomal muscle were investigated using isolated bundles of 'live' fast and slow fibres. Both fibre types responded to a single stimulus with a mechanical twitch. Fused tetani were obtained at around 50 Hz in fast fibres and 20 Hz in slow fibres. In the slow fibres, tetanic tension increased with frequency up to around 50 Hz. At frequencies giving maximum tetanic tension, the twitch/tetanus ratio was 0.70 for fast fibres and 0.29 for slow ones.

These results are discussed with reference to the polyneuronal/multiterminal innervation pattern of the myotomal muscle in teleost fish and its role in locomotion.

Introduction

With few exceptions [notably in amphibians (Lannergren, 1979) and birds (Ginsborg, 1960)], the twitch muscle fibres of higher vertebrates are focally innervated by one or occasionally two motor axons, and propagate action potentials (APs) (e.g. Lannergren, 1987). Fast muscle fibres in elasmobranchs are focally innervated, and have similar properties to those of frog fast twitch fibres (Stanfield, 1972; Curtin & Woledge, 1987). In teleosts, however, there is evidence for strong selective pressure for a distributed pattern of innervation in fast fibres (Ono, 1983). Fast fibres are focally innervated in primitive teleosts, but multiply innervated in higher groups (Bone, 1964; Bone & Ono, 1982). Probable transitional forms exist, with both focal and polyneuronal patterns of innervation

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(e.g. in the stomiiformes, Bone & Ono, 1982). Hudson (1969) found that fast myotomal fibres in the sculpin (*Myoxocephalus scorpius*) receive up to five axons from each of four spinal nerves. Single stimuli through a spinal nerve could induce action potentials from a single junctional potential (JP), although JPs were seen in the absence of APs. We have recently reported that isolated fast fibres from sculpin myotomal muscle respond to direct stimulation with all-or-none twitches (Altringham & Johnston, 1987, 1988).

The slow muscle fibres of vertebrates can be divided into three basic classes. Class 1 fibres are multiply innervated, and are activated by junction potentials (JPs) only. Fibres of this type are found in all vertebrates (Morgan & Proske, 1984; Johnston, 1985), and have a postural or tonic role in terrestrial animals. They have large irregular myofibrils in cross-section, relatively few mitochondria, a sparse sarcoplasmic reticulum and lack M-lines (Hess, 1970; Smith & Ovalle, 1973). Similar fibres have been described in the myotomes of elasmobranchs and teleosts, where they may have a role in holding a bend in the trunk (Bone *et al.* 1986; Kilarski & Kozłowska, 1983).

Class 2 fibres are also multiply innervated, but possess M-lines, and have intermediate to high mitochondrial densities. They have been found in the locomotory muscles of fish and some reptiles (Bone, 1978; Johnston, 1981; Gleeson & Johnston, 1987). The electrophysiological and contractile properties of these fibres have been little studied. Baretz (1961) provided evidence that slow myotomal muscles in teleosts are activated by JPs, although Stanfield (1972) suggested that similar fibres in elasmobranchs may be capable of generating APs. Recent studies have shown that the respiratory (Granzier *et al.* 1982; Akster *et al.* 1985) and pectoral fin (Johnston, 1987) muscles of teleosts contain slow twitch fibres. Proske & Vaughan (1968) also reported a polyneuronally innervated slow fibre in lizards in which both JPs and APs were recorded.

Class 3 slow fibres are focally innervated and are activated by APs. This fibre type is common in most vertebrates (e.g. Lannergren, 1987), but not in fish, with the possible exception of the intermediate fibres of elasmobranchs identified by Bone & Chubb (1978). However, these fibres are more likely to be analogous to the fast oxidative fibres of other vertebrates.

In the present study we have investigated the electrophysiological and mechanical properties of both fast and slow fibres in the teleost *Myoxocephalus scorpius*.

Materials and methods

Animals and solutions

Specimens of *Myoxocephalus scorpius* L. were caught in the Firth of Forth, and kept in seawater tanks at 5°C for 1–21 days before use. The lengths of the fish ranged from 17 to 23 cm. Fish were killed by a blow to the head followed by decapitation. All experiments were performed in Ringer's solution at 2.5–3.5°C. The composition of the Ringer was (mmol l⁻¹): NaCl, 132.2; sodium pyruvate, 10; KCl, 2.6; MgCl₂, 1; NaHCO₃, 18.5; NaH₂PO₄, 3.2; CaCl₂, 2.7; pH 7.2 at 10°C.

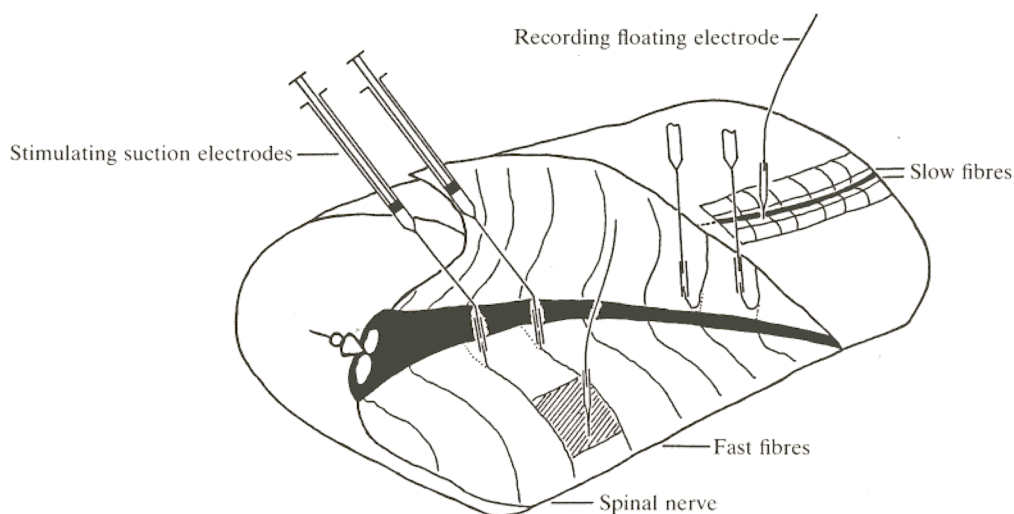


Fig. 1. A schematic diagram of the preparation showing the location of the stimulating and recording electrodes, and of the fast and slow muscle fibres studied.

Electrophysiology

The preparation consisted of the entire trunk of the fish, after removal of the head and tail (Fig. 1). The abdomen was opened and the viscera removed. The preparation was pinned out in a cooled Perspex chamber with a silicone elastomer base (Sylgard 184, Dow Corning), and covered in Ringer's solution, which was changed at regular intervals. The upper (left) abdominal wall was folded back to reveal the abdominal cavity. For the study of fast fibres two or more spinal nerves on the lower (right) wall were cut close to their point of emergence from the spinal column, and dissected away from the underlying tissue. The nerves were then drawn into suction electrodes. The peritoneum and underlying connective tissue were removed from large areas of the abdominal wall innervated by the dissected spinal nerves. To gain access to the slow fibres, spinal nerves of the left wall were drawn into electrodes, and the abdominal wall folded back to give access to the lateral line area. The skin was removed, and the underlying connective tissue layer was cut with fine scissors along the lateral line canal, and gently teased away from the ventral slow muscle fibres. The axons innervating the slow muscle in several species studied lie in the medial ramus of the ventral spinal nerve (Fetcho, 1986). This branches close to the point of exit from the vertebral column, so it was essential to cut the nerve as close to the emergence point as possible. The branch was difficult to locate, and it was therefore not possible to stimulate it alone.

Single 0.1 ms square pulses were applied to the cut end of a spinal nerve from a Grass S48 stimulator. Intracellular recordings were made using 20–30 M Ω electrodes filled with 3 mol l⁻¹ potassium acetate, floating on fine chlorided silver wire. The myotomes lying immediately caudal or ventral to the stimulated nerve were studied. Stimulation voltage was increased incrementally until a response

was obtained from a fibre, reduced to just below threshold, and increased in very small steps. Responses were recorded using Simmonds amplifiers connected to a Nicolet 3091 digital oscilloscope, and measurements taken from the screen. Results were then dumped to a printer *via* a BBC microcomputer.

Mechanics

The isometric contractile properties of isolated live fibres were determined as previously described (Altringham & Johnston, 1988). Briefly, small bundles of fibres (1–10 fast, 10–20 slow) were isolated, and foil clips placed around the trimmed myosepta, to allow attachment to the apparatus. The fibres were mounted on a servo system, one end of which was a silicon strain gauge (AME 801, Horten, Norway), in a flow-through chamber. Stimulation was by parallel platinum wire electrodes. Fast fibres were taken from the abdominal myotomes as previously described. Slow fibres were isolated by following the procedure described for the nerve–muscle preparation, and then removing a thin layer of fibres for further dissection. In addition to electrical stimulation, K^+ contractures were studied in both fibre types, by replacing a variable amount of NaCl with KCl in the Ringer's solution.

Results

Electrophysiology

Penetration and recording from fast fibres was trouble-free, with the electrodes remaining in place, and resting membrane potential remaining steady for multiple contractions. Penetrating slow muscle presented problems, since fibres are smaller and the overlying connective tissue is more difficult to remove. Stimulating the spinal nerve activated axons to both fast and slow fibres, but the fast fibre axons were recruited first. Contractions of the fast fibres often dislodged electrodes from the overlying slow fibres. Several approaches were tried in an effort to overcome this. The first two led to some improvement. (1) The spinal nerve was cut at varying points along its length. This reduced fast fibre activity, but not in the immediate vicinity of the slow fibres. (2) Fast fibres were disabled by cutting peripheral branches of the spinal nerves, and directly damaging fast fibres in myotomes adjacent to the one under study. (3) 2 mol l^{-1} formamide, which can block excitation–contraction coupling (Herrera, 1984), lowered the resting membrane potential of the fibres on the surface before blocking contraction of those below, and was therefore ineffective.

Fast fibres

Intracellular recordings were made from 46 fibres from seven animals. Resting membrane potential was $-81.9 \pm 0.7 \text{ mV}$ (mean \pm s.e.). Four fibres did not appear to be innervated by the nerve stimulated, and in three the electrode jumped out on stimulation.

The remaining fibres all responded to spinal nerve stimulation with APs.

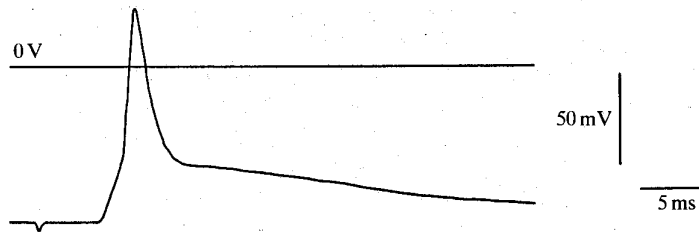


Fig. 2. Action potential from a fast fibre.

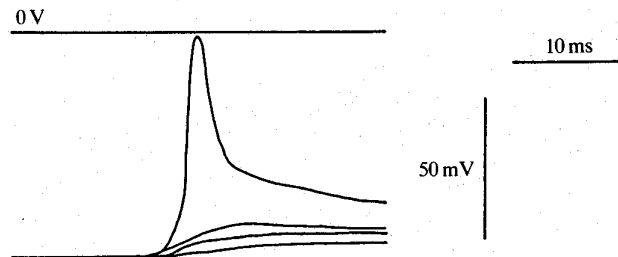


Fig. 3. Action and junction potentials recorded from a fast fibre with low resting potential from a gravid female fish.

Overshooting APs were produced by all but eight fibres, with a maximum overshoot of +32 mV. The remaining eight gave undershooting APs, but had unstable resting potentials and broad and variable APs. Mean spike height was 94.1 ± 1.8 mV excluding the eight undershooting fibres, and 88.2 ± 2.7 mV with the data from these included (mean \pm s.e.). There was no correlation between resting potential and spike height. Fig. 2 shows an overshooting AP from a typical fibre. Despite small incremental increases in stimulation voltage just below threshold, JPs were not seen in the absence of APs.

Experiments were performed on tissue from three heavily gravid females in early 1988. In all three cases, mean resting potentials were significantly depressed (-75 ± 1.4 mV, $N = 6$, -74.9 ± 2 mV, $N = 12$ and -73.4 ± 3 mV, $N = 7$, \pm s.e.) relative to those reported above, obtained from well-fed specimens in the previous summer and autumn. Stable resting potentials as low as -65 mV were recorded in some fibres. In these three preparations, APs were seen in only 50% of fibres, only one fibre gave an overshoot, and few of the remainder reached zero potential. In most cases, AP duration was greatly prolonged. In these preparations, JPs of 5–15 mV were recorded. With a single stimulus of increasing voltage, 0–4 summing JPs were typically seen before an AP, indicating a minimum of 1–5 axons innervating a fibre from one spinal nerve. Summing JPs leading to an undershooting AP are shown in Fig. 3. Mechanical preparations from the same fish were unstable.

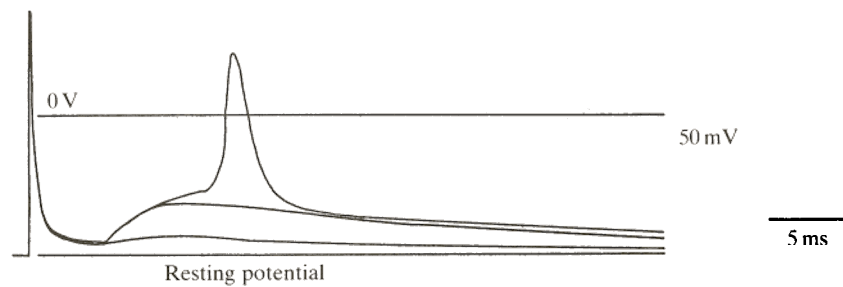


Fig. 4. Action and junction potentials from a slow fibre. A large stimulus artefact was seen, due only in part to the larger voltages needed to recruit the small-diameter axons innervating the slow fibres.

Slow fibres

Resting membrane potentials were recorded in 50 fibres (six animals) and the mean value, -80.8 ± 0.5 mV (s.e.), was not significantly different from that of fast fibres. Contraction of the underlying fast fibres ejected the floating electrode from 28 of these fibres before a response to stimulation was measured. A further nine did not respond to stimulation at high voltage despite stable resting potentials. These were presumably not innervated by the spinal nerves in the suction electrodes, or the nerve had been cut distal to the branch innervating these fibres.

Of the remaining 13 fibres, nine responded to stimulation with APs (seven overshooting). In most cases the movement of underlying fast fibres led to transient changes in resting potential, sufficient to hide any small JPs evoked at lower voltages. In two more stable fibres JPs and APs were recorded (e.g. Fig. 4), and two fibres responded with JPs only. All had stable resting potentials.

Mechanics

Isometric twitch, and tetanus contractions were studied (Fig. 5). Results from fast fibres have already been reported in detail (Altringham & Johnston, 1987, 1988). Briefly, fibres responded to a single suprathreshold stimulus with an all-or-none twitch. Fused tetani were obtained at 40–60 Hz, at which frequency tension was maximal. The twitch/tetanus ratio was 0.70 ± 0.02 (mean \pm s.e.). The slow fibres also responded to single stimuli with twitches. No single-fibre preparations were studied, so we cannot be sure that twitches were all-or-none events. Fused tetani were obtained at a frequency of about 20 Hz, but tension increased by around 20% to a maximum at 50–70 Hz. The twitch/maximum tetanus ratio was 0.29 ± 0.03 (mean \pm s.e., $N = 8$).

The results of varying external $[K^+]$ on contracture tension are shown in Fig. 6. Representative contractures from both fibre types are shown in Fig. 7. Maximum tension was obtained at around $80 \text{ mmol l}^{-1} K^+$ in both fibre types, but the slow fibres were more sensitive to lower concentrations. Fast fibres gave only transient contractures, with tension falling rapidly after the maximum was reached. Slow

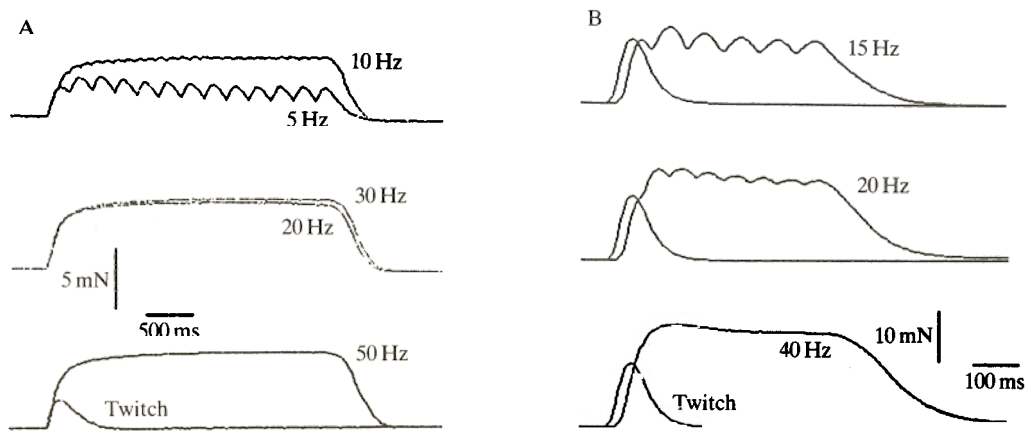


Fig. 5. Twitch and tetanic contractions from slow (A) and fast (B) muscle fibres. Stimulation frequency is given beside each record. Note the difference in time scales.

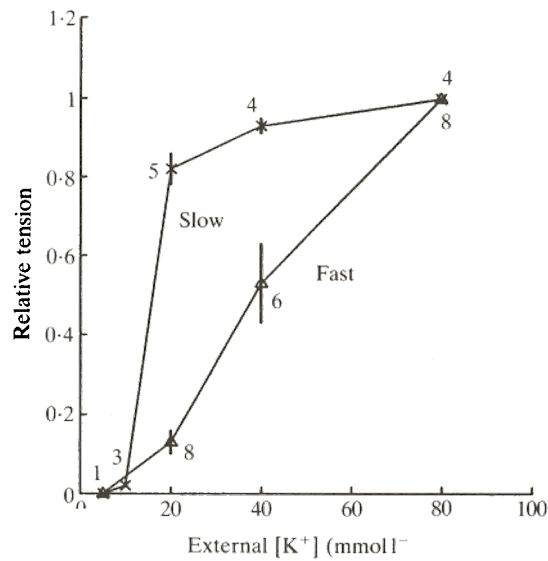


Fig. 6. The dependence of tension on external $[K^+]$, for slow and fast fibres. The numbers beside each point refer to the number of observations. Six fast and four slow preparations were used. Bars show \pm s.e.

fibres could maintain tensions $>80\%$ maximum for many minutes. Maximum tensions in both cases were comparable to maximum tetanic tensions.

Discussion

Fast muscle fibres

Previous physiological studies of polyneuronal innervation in fish have been

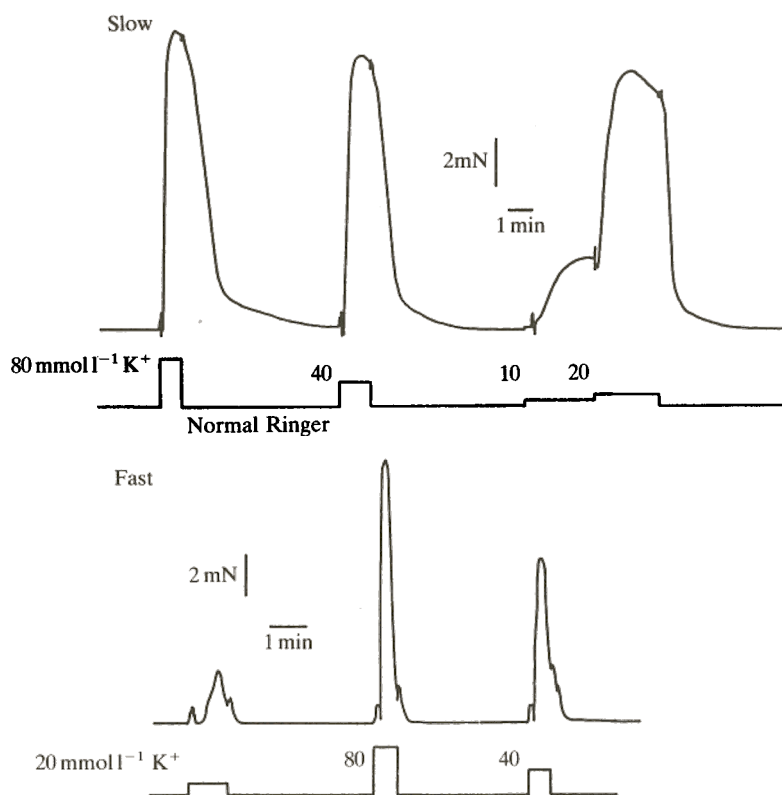


Fig. 7. K^+ contractures from slow and fast fibre bundles. The lines underneath each trace indicate transfer between normal Ringer and high- K^+ solutions (all concentrations given in $mmol\ l^{-1}$).

largely confined to fin and opercular muscles, with the exception of the work done by Barets (1961), Hudson (1969) and Westerfield *et al.* (1986). Early work has been reviewed by Bone (1978), with the tentative conclusion that polyneuronally innervated fast fibres may be capable of responding to stimulation with JPs/local contractions at slow swimming speeds, and APs/twitches at higher speeds. Our experiments suggest that the fast fibres are unlikely to be activated by JPs under normal circumstances; all fibres produced only APs when stimulated through the nerve. Since each spinal nerve contributes only 2–5 axons to a muscle fibre (Hudson, 1969, and the present study), each with a different threshold voltage for recruitment, it is likely that in many cases the APs were elicited by the firing of a single axon. Westerfield *et al.* (1986) recorded only APs in zebrafish fast fibres in response to spinal nerve stimulation in normal Ringer. The fast fibres are innervated by only 2–3 axons in this species, compared with around 20 in sculpin, and may function rather differently. However, Hudson (1969) states that in 'fresh preparations' from sculpin an AP was triggered by a single JP, and suggests that 'spike potentials are normally elicited by a single JP'.

Several papers have been published on the properties of fast fibres from the pectoral muscles of a range of teleost species. Resting membrane potentials are generally similar to those reported for myotomal muscle in the present study and by Westerfield *et al.* (1986) (-80 to -85 mV). APs rarely had overshoots, and have been variously reported as rare (Yamamoto, 1972) or of regular occurrence (Hagiwara & Takahashi, 1967; Hidaka & Toida, 1969). Takeuchi (1959) reported a mean resting potential of only -66 mV and recorded both JPs and APs. A recent paper by Gilly & Aladjem (1987) reports that neither fast nor slow dorsal fin fibres of the Pacific sand dab produce APs, but this is at variance with all other published data known to the authors.

In the experiments on gravid females, membrane potentials were low, APs were slow and undershooting, JPs were small (5–15 mV), and the recruitment of several axons was required to trigger an AP. In some cases APs were not seen. The physiological condition of the fish clearly has some influence on the results. It is well known that fast muscle protein content decreases with both starvation and spawning, and that the insoluble protein fraction of fast muscle increases markedly in preparation for spawning (see Love, 1980, p. 370). Similarities exist between the results from spawning fish and the previous studies discussed above, and may have some bearing on the variability of response in the literature. Furthermore, Hudson (1969) observed that by selecting an appropriate Ringer's solution overshooting APs were recorded in fibres which otherwise gave undershooting potentials. We suspect that in stable preparations, in a physiological Ringer, the normal mode of activation of sculpin fast myotomal fibres is by a propagated, overshooting AP, triggered by a depolarization of around 20 mV by a single JP. The experiments on gravid females confirm Hudson's (1969) finding that each fibre is innervated by 2–5 axons from a single spinal nerve.

Isolated fast muscle fibres responded to suprathreshold stimulation with an all-or-none twitch (Altringham & Johnston, 1987, 1988). The fast fibres therefore appear to be very similar to focally innervated vertebrate muscle fibres. This conclusion is supported by mechanical studies on opercular muscles (Granzier *et al.* 1983; Akster *et al.* 1985).

Slow muscle fibres

Early studies on slow fibres from pectoral muscles usually observed only JPs (Takeuchi, 1959; Yamamoto, 1972), although, Hidaka & Toida (1969) observed APs in the presence of 'excess' calcium (no figure given). Recent experiments (Granzier *et al.* 1983; Akster *et al.* 1985) and the present study have shown that the multiply innervated slow fibres of both opercular and myotomal muscles are capable of twitch responses. In addition, we have shown that myotomal slow fibres produce JPs and overshooting APs, and would thus fall into class 2 as described in the Introduction. Failure to observe APs in some earlier studies may be related to inadequacies in the Ringer composition: Hudson (1967) has shown that resting potential and action potential height are very sensitive to Mg^{2+} and Ca^{2+}

concentration. A better understanding of slow fibre function requires more information on the degree of polyneuronal and/or multiterminal innervation.

Fish slow fibres are comparable to the intermediate fibre type of *Xenopus*, described by Lannergren (1979), in that they have properties common to both twitch fibres (twitches and tetani) and tonic fibres (maintained K^+ contractures). Hidaka & Toida (1969) and Yamamoto (1972) also report sustained K^+ contractures in pectoral slow fibres from fish.

The function of polyneuronal innervation

The focal pattern of innervation is associated with a sharp transition between sustained and burst swimming speeds (Bone, 1966). For example, Pacific herring are able to swim at 3–4 body lengths s^{-1} almost indefinitely, but fatigue within 2 min at >4.5 body lengths s^{-1} , corresponding to the recruitment of the focally innervated fast fibres (Bone *et al.* 1978). In contrast, many teleosts recruit (polyneuronal innervation) fast muscles at sustainable swimming speeds involving either steady (Johnston *et al.* 1977; Bone *et al.* 1978) or flick–glide (Hudson, 1973) swimming behaviour. Capillary and mitochondrial volume densities are several-fold higher in fast muscles with polyneuronal innervation relative to those with the focal pattern (Johnston & Moon, 1981; Johnston *et al.* 1983).

In the zebrafish each myotomal fibre is innervated by only one primary motoneurone, and these do not cross myosepta into adjacent myotomes (Westerfield *et al.* 1986). Most fibres are innervated by 1–4 secondary motoneurons, but only 8% are innervated by secondaries from adjacent segments. The axons branch extensively, forming endplates along the full length of the fibres. This multi-terminal innervation is largely absent in the sculpin, in which most of the endplates arise from separate axons (Hudson, 1969). Patterns of polyneuronal innervation in teleosts are clearly very varied, and future work will no doubt reveal other forms intermediate to those described.

Our results suggest that polyneuronal innervation confers no unique properties on the fast fibres themselves, and its function therefore remains unknown. In a study of synchronous insect flight muscle, Josephson (1985) found that multiple stimuli per oscillatory work cycle increased power output above that generated from single twitch contractions. This suggested to us a possible function for polyneuronal innervation. In the sculpin each muscle fibre receives axons from four spinal nerves, and the myotomal fields innervated by these nerves overlap (Hudson, 1969). Thus, sequential activation of the spinal nerves will lead to multiple stimulation of the muscle fibres. This arrangement may enable the number of stimuli to be varied, in a way determined by the neuromuscular circuitry and the activity patterns of the central nervous system, and thus optimize power output over a range of swimming speeds. The mechanical properties of polyneuronal innervation may therefore be modulated, but by a mechanism entirely different from that proposed by Hudson (1969), who envisaged a simple gradation of contractile force through summing JPs.

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