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Electrical and mechanical properties and mode of innervation in scorpionfish sound-producing muscle fibres

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Summary

To obtain information about the neural mechanism underlying sound production in teleost fish, we studied the electrical and mechanical properties and mode of innervation in the swimbladder muscle (SBM) fibres of scorpionfish *Sebastiscus marmoratus*. Action potentials of the SBM fibres in response to direct electrical stimulation neither exhibited overshoot nor propagated along the fibre. Stimulation of the motor nerve, however, uniformly evoked action potentials along the fibre. When neuromuscular transmission was blocked by curare, motor nerve stimulation uniformly evoked endplate potentials along the fibre. These results indicate that action potentials propagate along the nerve branches but not along the SBM fibre membrane. In accordance with the above results, histochemical studies showed that motor nerve branches run along the SBM fibres to form many endplates with cholinesterase activity, indicating multiterminal innervation. The SBM consisted of about 600 fibres, while its motor nerve contained about 100 axons, giving an innervation ratio of about 1:6. Like mammalian fast muscle fibres, the SBM fibres exhibited a low succinic dehydrogenase activity and a high ATPase activity. These results are discussed in connection with the function of the SBM fibres in producing sound.

Key words: sound-producing muscle, teleost fish, action potential conduction, multiterminal innervation, innervation ratio, endplate potential, scorpionfish, *Sebastiscus marmoratus.*

Introduction

Some species of teleost fish are capable of producing long-lasting sound production by vibrating their swimbladder at high frequencies (Fänge, 1966; Blaxter and Tytler, 1978). The swimbladder muscle (SBM) producing sound exhibits rapid twitches, which do not fuse even at stimulus frequencies above 100–200 s^{-1} (at 20–30°C; Skoglund, 1961; Gainer et al., 1965). Recently, Suzuki et al. (2003) showed that the SBM fibres of scorpionfish contain extremely well developed sarcoplasmic reticulum (SR), indicating that the rapid twitch associated with rapid relaxation may be due to a rapid rate of Ca^{2+} uptake by the SR. Using techniques of quick freezing and energy dispersive X-ray microanalysis of cryosections, Suzuki et al. (2004) also studied the mode of intracellular Ca^{2+} translocation in the SBM fibres. Nevertheless, how the mechanical activity of the SBM fibres is controlled by motor nerve impulses during sound production still remains obscure.

The aim of the present work was to investigate the mode of neural control of mechanical activity in the SBM fibres during sound production. It will be shown that, in the SBM fibres, motor nerve branches run in parallel with the muscle fibres to form many cholinergic neuromuscular junctions, so that action potentials propagate rapidly along the nerve branches but not along the fibre membrane. The SBM consists of about 600 muscle fibres, with low succinic dehydrogenase activity and high ATPase activity, and is innervated by a motor nerve containing about 100 nerve fibres. These results are discussed in connection with the mode of neural control of the SBM during sound production.

Materials and methods

Physiological studies

Adult scorpionfish *Sebastiscus marmoratus* Cuvier and Velenciennes (body length, 16–20 cm) were collected at Sagami Bay, Japan. Animals were killed by decapitation, and the swimbladder in the abdomen exposed. A bundle consisting of 20–30 muscle fibres (slack length, ~20 mm; diameter, 90–200 μm) was carefully removed together with the motor nerve, which entered the SBM. A piece of cranium was attached to one fibre end, while a piece of swimbladder tissue was attached to the other end. The nerve-fibre bundle preparation was mounted horizontally by pinning both ends to the bottom of an experimental chamber filled with

experimental solution (fish Ringer), containing (in mmol l^{-1}): NaCl, 167.5; KCl, 4.4; $CaCl_2$, 2.2; $MgCl_2$, 1.3 (pH adjusted to 7.2 with 10 mmol l^{-1} Hepes-Tris buffer). Curare (10^{-6} g ml^{-1}) was added to block neuromuscular transmission.

Conventional glass capillary microelectrodes, filled with 3 mol l^{-1} KCl and connected to a high impedance amplifier, were used either to record membrane potentials or to pass rectangular current pulses across the fibre membrane. The motor nerve innervating the SBM was stimulated with single or repetitive 1 ms current pulses given through a pair of platinum wire electrodes. Tension was recorded using a tension transducer (Akers 801, resonance frequency, 5 kHz; Holten, Norway). Current, membrane potential and tension were recorded using an oscilloscope (Tektronix 5113, Beavertown, USA). In each type of experiment, 8–10 different preparations were used with similar results. All experiments were performed at 18–22°C.

Anatomical and histochemical studies

Golgi silver impregnation of nerve branches

The whole SBM and the whole motor nerve entering it were incubated for 24 h in a mixture of 3% $K_2Cr_2O_7$, 20% OsO_4 at a ratio of 3:1 (v/v), and then in 0.75% silver nitrate for 24 h (Kobayashi et al., 1989). After the above procedure, the tissues were dehydrated with ethanol, embedded in celloidin, and longitudinal sections (thickness, 90–110 μm) were cut for microscopic observation.

Cholinesterase activity at the neuromuscular junction

The SBM fibers were fixed in 10% formalin, incubated in a reaction solution (Karnovsky and Roots, 1964) at 37°C for 60 min, and observed microscopically.

Succinic dehydrogenase activity

The SBM fiber bundle was quickly frozen at –78°C, and the cryosections (thickness, ~10 μm) were incubated in a reaction solution at 37°C for 60 min (Barka and Anderson, 1963). Then the cryosections were fixed in 10% formalin, and thin sections were cut for microscopic observation.

ATPase activity

The SBM fiber bundle was fixed in 10% formalin, and then quickly frozen at –78°C. Cryosections (thickness, ~10 μm) were preincubated in a reaction solution (pH 10.4 or 4.6) at 4°C for 20 min (Kahn et al., 1974). Thin transverse sections were observed microscopically.

Results

Membrane electrical properties

The resting membrane potential of the SBM fibres was 75±0.5 mV (mean ± S.D., N=20). Based on the linear cable theory (Hodgkin and Rushton, 1946), membrane constants were determined using a pair of microelectrodes inserted into the fibre at varying distances between them; one passed rectangular current pulses across the membrane, while the other recorded the resulting changes in membrane potential (Fig. 1). Fibre circumference and cross-sectional area were roughly calculated by measuring fibre diameter under the microscope, assuming a circular cross-section. Since the organization of the transverse tubules and the SR in the SBM fibres is different in the middle and the end regions (Suzuki et al., 2003), the electrical constants were determined in both regions.

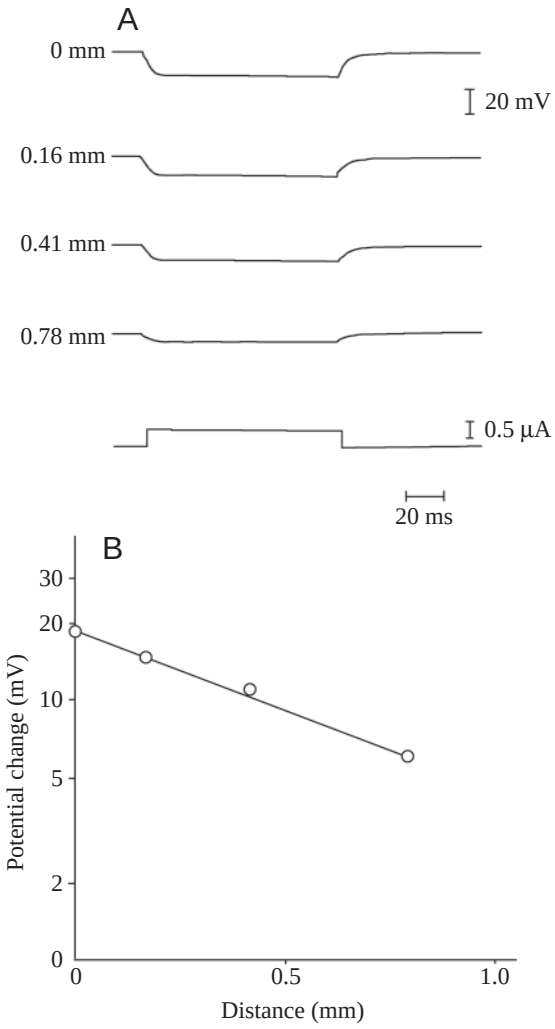

Fig. 1. Measurement of membrane electrical constants. (A) Membrane potential changes recorded at the indicated distances from the point of application of inward current pulses (bottom trace). (B) Typical relationship between the membrane potential change (logarithmic scale) and the distance from the current electrode.

The results are summarized in Table 1. In both the middle and end regions of the SBM fibres, the length constant (λ), time constant (τ) and specific membrane resistance (R_m) were much smaller, and specific membrane capacitance (C_m) was much larger, than the corresponding values for frog skeletal muscle fibres (λ=2.4 mm, C_m=8 μF cm^{-2}, τ=34.5 ms, R_m=4100 Ωcm; Fatt and Katz, 1951). The large C_m values in the SBM fibres reflect the extremely well developed SR (Suzuki et al., 2003).

Table 1. *Membrane electrical constants of the swimbladder muscle fibres*

Fibre region	λ (mm)	τ (ms)	R_m (Ωcm)	C_m (μF cm^{-2})
Middle	0.53±0.09	5.37±1.3	188±37	32±10
End	0.82±0.08	5.80±0.7	212±43	16±4

λ, length constant; τ, time constant; R_m, specific membrane resistance; C_m, specific membrane capacitance.

Values are mean ± S.D. (N=15–20).

C_m is about twofold larger in the middle region than in the end region of the fibre, which may be explained by the different triadic junction distribution in each sarcomere of the SBM fibres; each sarcomere contains two triadic junctions in the middle region, and one triadic junction in the end region (Suzuki et al., 2003).

Action potentials in response to direct and indirect stimulation

Depolarization of the SBM fibre membrane by 40–50 mV, by passing outward current pulses, elicited an action potential. The action potential did not show overshoot, as has been the case in other fish skeletal muscle fibres (Takahashi, 1959; Barets, 1961; Hagiwara and Takahashi, 1967; Hidaka and Toida, 1969), and its amplitude increased with increasing current intensity (Fig. 2), indicating that the action potential in the SBM fibre is not of an all-or-none type but graded, depending on the stimulus intensity. The action potential elicited by the intracellularly applied current pulse was localized around the current electrode and did not propagate along the fibre.

If, on the other hand, the motor nerve innervating the SBM was maximally stimulated, the action potential was uniformly recorded along the fiber, and the SBM showed a distinct twitch tension development (Fig. 3). The interval between the time of motor nerve stimulation and the time of onset of action potential increased with increasing distance between the point of stimulation and the point at which the recording electrode was inserted into the fiber. The apparent conduction velocity of the action potential along the SBM was roughly estimated by comparing the time of onset of action potential recorded at different distances from the point at which motor nerve just entered into the SBM, and was found to be 7.0±1.2 m s^{-1} (N=8). The action potential in response to both direct and indirect stimulation disappeared when the external Na^+ was

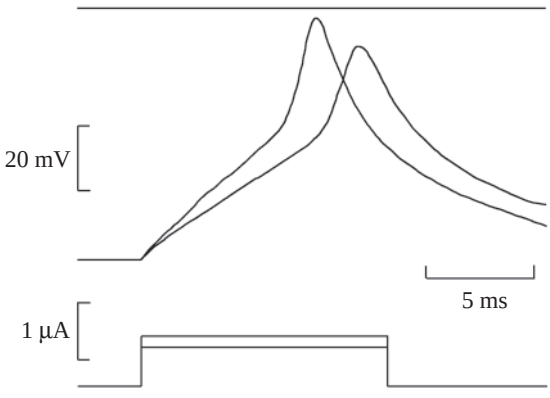

Fig. 2. Superimposed records of action potentials (upper traces) and intracellulary applied outward current pulses (lower traces). Action potentials were recorded close to the insertion point of the current electrode. The horizontal line indicates zero potential level in this and Figs 3 and 5.

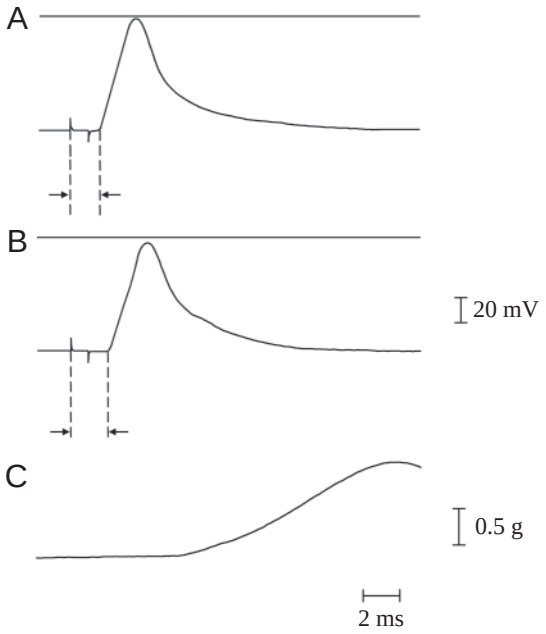

Fig. 3. Action potentials (A,B) and twitch tension (C) in response to a single motor nerve stimulation. The distance between the recording electrode and the point at which motor nerve entered into the SBM was 0 mm in A and 1.4 mm in B. In this and Fig. 4, broken vertical lines and arrows in A and B indicate the interval between the onset of stimulating current pulse and the onset of action potential.

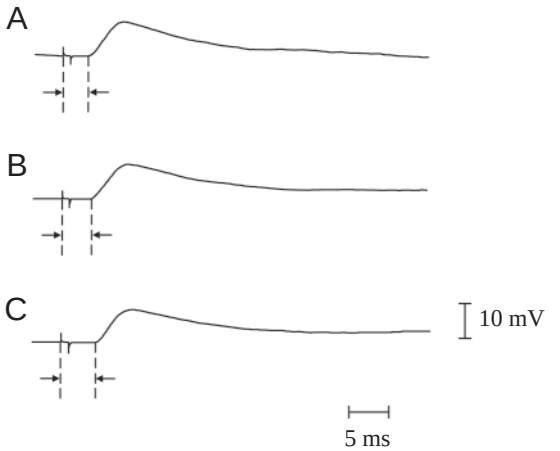

Fig. 4. Endplate potentials in response to a single motor nerve stimulation. The distance between the recording electrode and the point at which motor nerve entered into the SBM was 0 mm in A, 5 mm in B, and 10 mm in C.

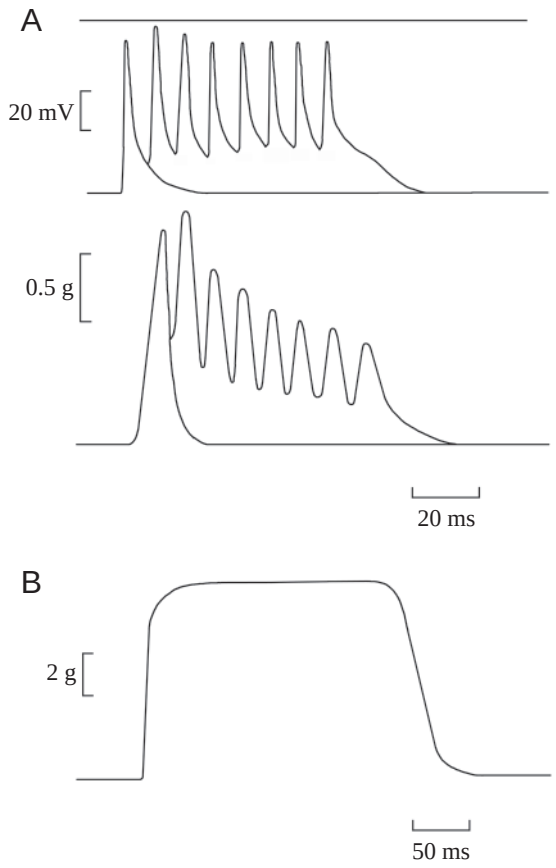

Fig. 5. Electrical and mechanical responses of the SBM. (A) Superimposed recordings of a single isometric twitch in response to a single motor nerve stimulation and a series of twitches in response to repetitive motor nerve stimulation at 100 Hz. The upper and lower traces show action potentials and twitches, respectively. (B) Steady isometric tension in response to transverse a.c. field stimulation (100 Hz).

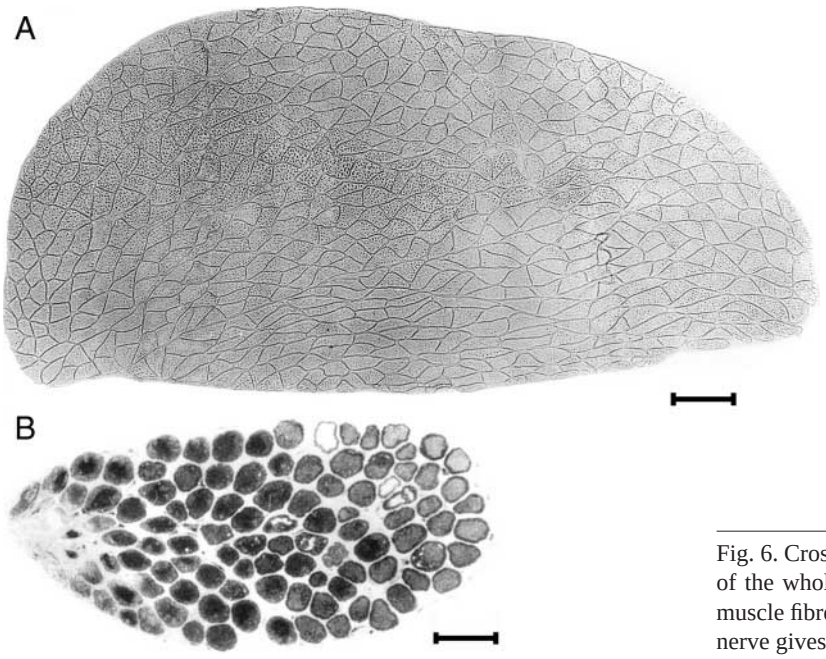

Fig. 6. Cross-section of the whole swimbladder muscle (SBM; A) and of the whole motor nerve innervating the SBM (B). The number of muscle fibres in the SBM relative to the number of axons in the motor nerve gives the innervation ratio of ~6. Bars, 500 μm (A); 50 μm (B).

replaced by choline or in the presence of tetrodotoxin (10^{-7} g ml^{-1}), indicating that the action potential is associated with inward movement of Na^+.

Endplate potentials in response to indirect stimulation

In the presence of curare (10^{-6} g ml^{-1}), which blocks neuromuscular transmission in vertebrate skeletal muscle (Fatt and Katz, 1951), motor nerve stimulation produced endplate potentials uniformly along the fibre (Fig. 4). As with action potentials in response to motor nerve stimulation, the interval between the time of stimulation and the time of onset of endplate potential increased with increasing distance between the point of stimulation and the point of recording. The apparent propagation velocity of endplate potential along the fibre was the same as that of the action potential.

Mechanical response to direct and indirect stimulation

Repetitive motor nerve stimulation produced a series of brief twitches, which did not fuse even at a stimulus of 100 Hz or more. The twitch tension declined rapidly with time during repetitive stimulation (Fig. 5A), while the action potential amplitude did not change markedly. We roughly estimated the maximum tension per unit cross-sectional area of the SBM (T) by measuring the whole fibre cross-sectional area (S) as:

$$T = W / L , \quad (1)$$

where W and L are the wet mass and length of the SBM, respectively. The value of T was 0.40±0.15 kg cm^{-2} (mean ± S.D., N=25), a value ~5 times smaller than the corresponding value of frog skeletal muscle. If, however, the SBM was subjected to a transverse a.c. field of sufficient intensity (100 Hz, 3.5 V cm^{-1}), using a pair of platinum plates placed at both sides of the SBM, it was possible to produce a steady isometric force (Fig. 5B) amounting to 1.5±0.6 kg cm^{-2} (N=25), a value not much different from the corresponding value in frog skeletal muscle.

Anatomical and histochemical features

The motor nerve innervating the SBM contained about 100 axons, while the SBM consisted of about 600 thick muscle fibers (diameter, 100–200 μm) (Fig. 6A,B), giving a low innervation ratio of 1:6. Golgi silver impregnation of motor nerve showed that nerve branches run along the fibres to form endplates (neuromuscular junctions) at many points (Fig. 7A). The endplates exhibited distinct cholinesterase activity, indicating cholinergic neuromuscular transmission in the SBM (Fig. 7B). The high density of endplates is

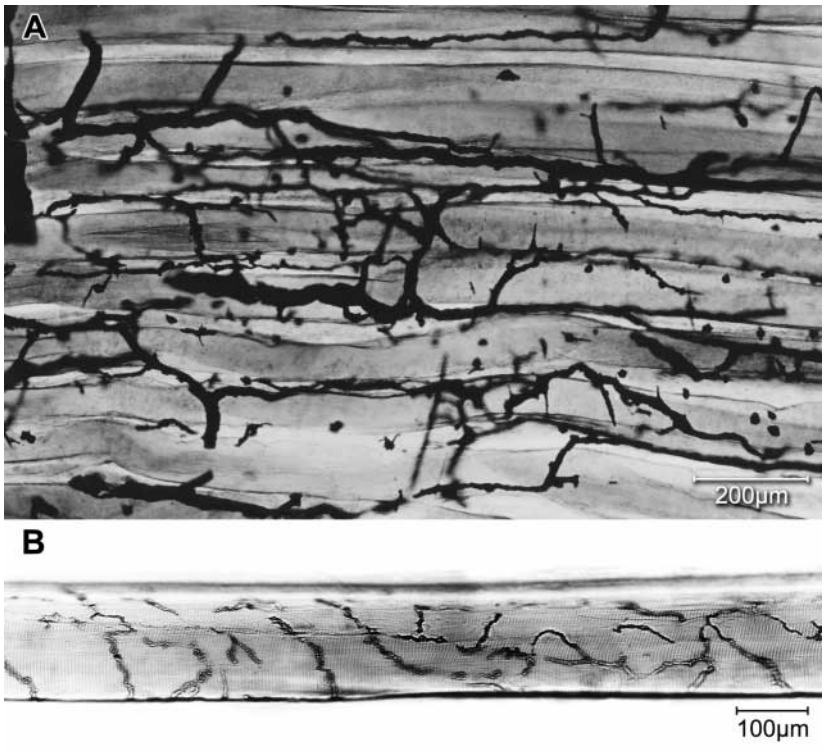

Fig. 7. Motor nerve branches in the swimbladder muscle (SBM). Note nerve branches running along the SBM fibres. (A) Golgi silver impregnation of nerve branches running along the SBM fibres. (B) Dense distribution of endplates with cholinesterase activity in the SBM fibre. Bars, 200 μm (A); 100 μm (B).

entirely consistent with the present result that both action and endplate potentials are uniformly recorded along the fibre length in response to motor nerve stimulation

Succinic dehydrogenase activity in the SBM fibres (Fig. 8A) was much lower than that of superficial muscle fibres in the animal body (Fig. 8B). On the other hand, the ATPase activity of the SBM fibres was high at pH 10.4 (Fig. 9A) and low at pH 4.6 (Fig. 9B).

Discussion

Multiterminal innervation of the SBM fibres

The present experiments provide information about the mechanism of neural control of the SBM. The SBM fiber action potential is graded (Fig. 2) and does not propagate along the SBM membrane; the measured membrane constants (especially the small λ and large C_m; Fig. 1, Table 1) were unfavourable for propagation. The uniform occurrence of action potentials and endplate potentials along the length of the SBM fibres (Figs 3, 4) are completely in accordance with the anatomical and histochemical observations that motor nerve branches run along the fibre to form many endplates containing cholinesterase activity (Fig. 7A,B). It is therefore safe to conclude that, in the SBM fibres, motor nerve impulses propagate along the nerve branches to produce endplate potentials at many points, which in turn set up action potentials around each endplate region to cause contraction. The apparent propagation velocity of action potential (~7 m s^{-1}) reflects the propagation velocity of motor nerve impulses along the nerve branches, which is much faster than that of action potentials along the muscle fibre membrane (1–3 m s^{-1} in frog skeletal muscle fibres; Hiramoto, 1951). The multiterminal innervation therefore provides the means by which motor nerve impulses rapidly stimulate the whole SBM fibres into activity.

Mechanical and metabolic properties of the SBM fibres

In fish, slow swimming movement of the animal body is produced by the mechanical activity of superficial muscle fibres, whereas quick swimming movement is associated with that of inner muscle fibres (Bone, 1966; Rayner and Keenan, 1967; Hudson, 1973). In mammalian skeletal muscle fibres, succinic dehydrogenase activity is high in tonic (red) muscle fibres and low in phasic (white) muscle fibres (Hoyle, 1983; Beckett and Bourne, 1973). The low succinic dehydrogenase activity of the SBM fibres, compared to that of the superficial muscle fibres in the animal's body (Fig. 8A,B), is therefore taken to indicate

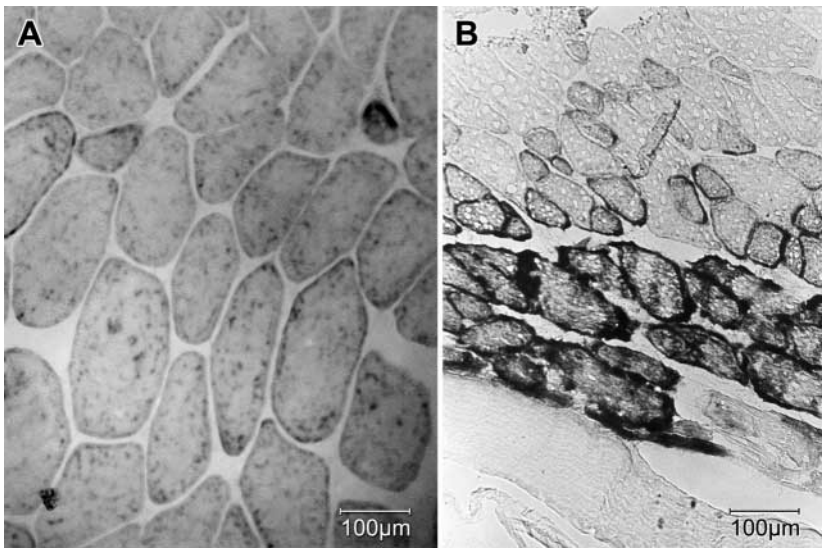

Fig. 8. Cross-sections showing succinic dehydrogenase activity in the swimbladder muscle (SBM) fibres (A) and in the superficial muscle fibres in the animal body (B). Note the weak succinic dehydrogenase activity in the SBM fibres compared to that in the superficial muscle fibres. Bars, 100 μm.

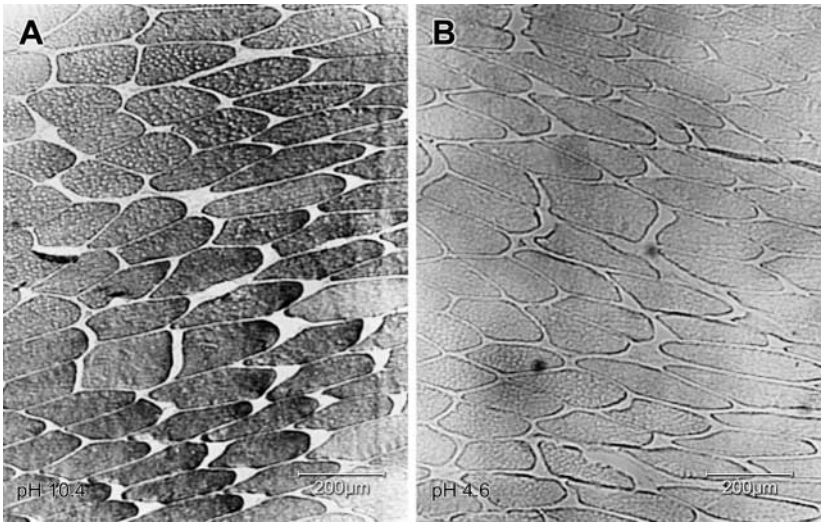

Fig. 9. Cross-sections showing ATPase activity in the swimbladder muscle (SBM) fibres at pH 10.4 (A) and at pH 4.6 (B). Note that the ATPase activity is high at pH 10.4 and low at pH 4.6. Bars, 200 μm.

that the SBM fibres have mechanical and metabolic characteristics analogous to those in mammalian phasic muscle fibres.

Meanwhile, the ATPase activity of mammalian phasic muscle fibres is known to be stable in high pH, and is inhibited at low pH (Kahn et al., 1974). The ATPase activity of the SBM fibres is high at high pH and low at low pH (Fig. 9A,B), which seems to indicate that the properties of the SBM fibres resemble those of mammalian phasic muscle fibres.

Contraction-relaxation cycle in the SBM fibres

The contraction–relaxation cycle in muscle is regulated by the release of Ca^{2+} from, and its uptake by, the SR (Ebashi and Endo, 1968). In fish sound-producing muscles, the maximum frequency of sound produced is determined by the maximum frequency of twitch fusion, which is primarily dependent on the rate of relaxation of twitch tension, which is dependent on the rate of Ca^{2+} uptake by the SR (Skoglund, 1959). In accordance with this view, the fractional SR volume in the sound-producing muscle fibres, including the SBM fibres, is much larger than that in skeletal muscle fibres (Peachey and Porter, 1959; Fawcett and Revel, 1961; Revel, 1962; Franzini-Armstrong, 1972; Appelt et al., 1991; Suzuki et al., 2003).

Twitches produced by repetitive motor nerve stimulation of the SBM tend to decrease rapidly with time (Fig. 5A), as has also been reported by Hidaka and Toida (1969). This may result, at least in part, from the myoplasmic Ca^{2+} concentration gradually decreasing during repetitive motor nerve stimulation, due to a large rate of Ca^{2+} reuptake by the SR. Considering the small innervation ratio of the SBM (~1:6; Fig. 6A,B), the SBM fibres are likely to receive not only multiterminal but also polyneuronal innervation; i.e. there would be ‘functional’ motor units within individual SBM fibres. The alternate activation of these ‘functional’ subcellular motor units by different motor axons would be capable of producing long-lasting sound production, despite the tendency of twitch tension to decrease with time.

The maximum twitch tension per unit cross-sectional area in the SBM fibres was much smaller than the corresponding value in frog skeletal muscle fibres, while the maximum steady tension of the SBM fibres in response to transverse a.c. stimulation was comparable to the latter (Fig. 5B). This may indicate that only a small fraction of cross-bridges within the SBM fibres can be activated to interact with the thin filament during brief twitches evoked by motor nerve impulses, while a much larger fraction of cross-bridges can be effectively activated by transverse a.c. stimulation. This view is supported by the report of Rome and Klimov (2000), who measured rates of ATP utilization by the SR and cross-bridges in toadfish sound-producing muscle fibres.

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