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Excitation–contraction coupling in skeletal and caudal heart muscle of the hagfish *Eptatretus burgeri* Girard

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Summary

Hagfishes are regarded as the most primitive living craniates. Excitation–contraction (E–C) coupling mechanisms were studied in skeletal and caudal heart muscle fibres of the hagfish *Eptatretus burgeri*. In white (fast) skeletal muscle fibres from the *musculus tubulatus*, force generation in response to electrical stimulation was maintained in nominally Ca^{2+} free artificial seawater (ASW) (0Ca^{2+}-ASW) containing 10 mmol l^{-1} Co^{2+} (a blocker of Ca^{2+} currents). Similarly, in red (slow) fibres from parietal muscle bathed in 0Ca^{2+}-ASW containing 10 mmol l^{-1} Co^{2+}, force generation occurred in association with K^+ depolarisation when the external K^+ concentration was increased to 100 mmol l^{-1}. Therefore, external Ca^{2+} is not required for muscle contraction. Hence, both white and red fibres possess the function of depolarisation-induced Ca^{2+}-release from intracellular Ca^{2+} stores. This function is the same as in the skeletal muscle of all other vertebrates. In caudal heart muscle fibres, twitches in response to electrical stimuli were maintained in 0Ca^{2+}-ASW containing 30 mmol l^{-1} Co^{2+}. In fibres loaded with fluo-3 bathed in 0Ca^{2+}-ASW containing 30 mmol l^{-1} Co^{2+}, an increase in the intracellular free Ca^{2+} level associated with K^+ depolarisation was observed after the external K^+ concentration was increased to 100 mmol l^{-1}. Thus E–C coupling in the caudal heart muscle is also of the vertebrate skeletal muscle type.

Key words: excitation–contraction coupling, Ca^{2+}, skeletal muscle, caudal muscle, hagfish, *Eptatretus burgeri*.

Introduction

Hagfishes are now considered, on both morphological and molecular grounds, to belong in the Cyclostomata as a sister-group to lampreys, the Cyclostomata itself being the sister-group to the vertebrates. They are less evolved than lampreys and so are the most primitive craniates available for study (Forey and Janvier, 1993; Mallat et al., 2001; Zardova and Meyer, 2001). Consequently, most studies on these animals have addressed phylogenetic aspects (see books edited by Brodal and Fänge, 1963; Jørgensen et al., 1998; Ahlberg, 2001), but they have also attracted a variety of other studies as they are specialized animals with a unique physiology. For example, hagfish blood is iso-osmotic to seawater. Our studies on hagfish were focused on the mechanisms of excitation–contraction coupling (E–C coupling) of skeletal and caudal heart muscles. This topic is of some phylogenetic interest, since the mechanism of E–C coupling is quite different in invertebrates and vertebrates.

In the wide spectrum of invertebrate and protochordate twitch muscle fibres so far examined, influx of extracellular Ca^{2+} is necessary for E–C coupling under physiological conditions (Hagiwara et al., 1971; Inoue et al., 1994, 1996, 1997; Bone et al., 1997, 1999; Tsutsui et al., 2000). However, as the vertebrate group evolved, skeletal muscle gained a new unique function, and E–C coupling in vertebrates is different: external Ca^{2+} is not required (Armstrong et al., 1972). The fibres contract in response to electrical excitation of the muscle membrane using only intracellular Ca^{2+} stored in the sarcoplasmic reticulum (SR). Depolarisation of the transverse tubules directly triggers Ca^{2+} release from the SR, known as depolarisation-induced Ca^{2+} release (DICR) (Ríos and Brum, 1987). Hence this ‘vertebrate type’ of E–C coupling is in striking contrast to the E–C coupling of invertebrate twitch muscles.

An interesting question is where in phylogeny this change in the mechanism of skeletal muscle E–C coupling took place, i.e. when the DICR function was acquired. We have previously shown that in the skeletal muscle of the lamprey *Lampetra planeri*, E–C coupling is of the ‘vertebrate type’, whilst in the acraniate *Branchiostoma lanceolatum*, E–C coupling is of the ‘invertebrate type’, requiring influx of extracellular Ca^{2+} (Inoue et al., 1994). Based on these studies, we proposed that the DICR function was acquired somewhere in phylogeny

between the acraniate and agnathan levels (Inoue et al., 1994). Unfortunately there is no living intermediate between the two levels; however from molecular analyses, both lampreys and hagfish should be regarded as a natural group of Agnatha, forming the sister group to the Gnathostomata. In order to test our hypothesis, we examined whether E–C coupling of the hagfish skeletal muscle is of the 'vertebrate type' (no requirement for Ca^{2+} influx) or the 'invertebrate type' (requiring Ca^{2+} influx), bearing in mind that the body fluid of hagfishes is iso-osmotic to seawater (Alt et al., 1981) and contains a much higher concentration of Ca^{2+} than the body fluid of any other vertebrate. We studied two different types of skeletal muscle fibres, one with white (fast) fibres and the other with red (slow) fibres.

The hagfishes possess a unique circulation system, using a secondary caudal heart. The caudal heart is a bilaterally symmetrical organ below the notochord, consisting of two chambers on each side of a median caudal cartilage. Both sides of the caudal heart muscle contract alternately, bending the caudal cartilage to the contracting side. These rhythmic movements cause the chamber of the other side to expand and flatten, so that blood is returned to the central veins (see reviews by Johansen, 1963; Lomholt and Franko-Dossar, 1998). The caudal heart muscle is extrinsic to each heart chamber. The muscle fibres are long and cross-striated, and innervated from the spinal cord. The hagfish *Myxine glutinosa* the caudal heart muscle was examined by Retzius (1890, 1892), who found that it is derived from parietal muscle and innervated by spinal nerves terminating in similar nerve endings to those of red parietal muscle fibres. The fibres of the caudal heart muscle are smaller in diameter than any in the myotomes. Our study reveals that E–C coupling in the caudal heart muscle as well as in both white and red skeletal muscle fibres is of the 'vertebrate type'.

Materials and methods

Materials

Adult hagfishes *Eptatretus burgeri* Girard (30–50 cm long) were caught with net traps at the bottom of the Wakasa Bay, off the coast of Ine, Kyoto, Japan. Animals were maintained in a laboratory tank filled with filtered seawater at 18°C. Each animal was killed by decapitation, the skin was removed, and muscle tissue used for experiments was dissected out in nominally Ca^{2+}-free artificial seawater ($0Ca^{2+}$-ASW) at 15°C.

White skeletal muscle fibres were dissected from the *musculus tubulatus* that protrudes the dental plate cartilage from the mouth. Approximately ten fibres (approx. 400 μm diameter and 3–5 cm long) were dissected under a binocular microscope. The fibres were separated (except for a portion at both ends) using a pair of fine needles. The tendon at one end of the fibres was pinned down to the Sylgard base of a small chamber. The other end of the fibres was connected to a strain gauge (SensoNor N801, Norway) *via* a small dissecting pin (Seirin Hinaishin No 2, Japan) as described below. The volume of the chamber was 4 cm^3, and the bathing solution continuously flowed at a rate of 10 ml min^{-1}. A sheet of red skeletal muscle fibres (100–150 μm thick, approx. 3 mm wide and 7 mm long) was sliced off from the parietal muscle using a vibrotome (Campden Instruments Vibroslice, UK), and was pinned down to the Sylgard base of the chamber under the binocular microscope.

Caudal heart tissues were dissected using a small pair of scissors. The caudal heart muscle was carefully dissected out from each heart and pinned down to the Sylgard base of the chamber under the binocular microscope. Epithelial tissue and connective tissue were removed. The caudal muscle fibres were 40–50 μm diameter and 5–7 mm long.

Aqueous solutions

Artificial seawater (ASW) consisted of 450 mmol l^{-1} NaCl, 9 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 50 mmol l^{-1} $MgCl_2$ and 15 mmol l^{-1} Hepes-Na buffer, pH 7.8. Nominally Ca^{2+}-free ASW ($0Ca^{2+}$-ASW) was made by replacing $CaCl_2$ with $MgCl_2$. Na^+-free ASW ($0Na^+$-ASW) consisted of 450 mmol l^{-1} choline chloride, 9 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 50 mmol l^{-1} $MgCl_2$ and 15 mmol l^{-1} Tris-HCl buffer, pH 7.8.

Chemicals

Tetrodotoxin (TTX) (Sankyo, Japan), acetylcholine (ACh) (Sigma, USA) and d-tubocurarine (d-TC) (Sigma, USA) were dissolved in ASW to make 1 mmol l^{-1}, 100 mmol l^{-1} and 1 mmol l^{-1} stock solutions, respectively. Each stock solution was kept at 4°C in the dark, and used by adding appropriate amounts to the bathing solutions. $CoCl_2$ was added to bathing solutions to block ionic currents through calcium channels.

Stimulation and recordings of the intracellular potential and muscle twitches

Fibres of either skeletal muscle or caudal heart muscle were stimulated electrically using a pair of electrodes connected to the isolating unit of an electric stimulator (Nihon Kohden SEM3201, Japan). One of the electrodes was a suction electrode made of fine polyethylene tubing (100 μm inner diameter). A microelectrode (10–20 MΩ) filled with a 3 mol l^{-1} KCl solution connected to a head stage of an electrometer (World Precision Instruments Duo773, USA) was inserted in a fibre whose twitches had been observed in response to electrical stimuli. For direct stimulation of a caudal heart muscle fibre, a microelectrode was inserted to the fibre and transmembrane current was applied to the fibre through the microelectrode using the current injection circuit of the electrometer.

Twitches were monitored with a CCD camera and recorded on videotape (Video recorder: Sony FS10, Japan). Records of the twitches were obtained subsequently by placing a CdS photocell on the video monitor screen on the twitching muscles. The photocell signal was recorded through a low-pass filter (<33 Hz). Force generation of skeletal muscle fibres in response to electrical stimulation was measured with a strain gauge. A dissecting pin fixed to the gauge was inserted in the tendon at one end of the fibres.

Table 1. *Osmolarity and electrolyte concentrations of the blood plasma*

Sample	Osmolarity (mosmol kg^{-1})	Na^+ (mmol l^{-1})	K^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	Mg^{2+} (mmol l^{-1})
1	996	504	16.5	429	7.4	12.0
2	1017	597	13.2	516	8.2	14.8
3	981	525	11.1	420	5.3	7.2
4	996	453	9.9	360	5.8	7.7
5	1037	483	10.5	390	5.8	8.8
6	1031	629	13.2	268	6.5	10.1
Mean	1009.7	531.8	12.40	397.2	6.50	10.10
S.D.	22.1	68.0	2.43	82.2	1.11	2.88

Measurements of intracellular Ca^{2+} transients

Each muscle bundle was pinned down to a silicone pad in a culture dish. The fibres were loaded with the Ca^{2+} indicator dye fluo-3 by incubating them in each test solution containing 2 μmol l^{-1} fluo-3-AM (Dojin, Japan) for 1.5 h at 14°C. After washing off the fluo-3-AM, the chamber was placed on the inverted microscope fitted with an epi-illumination apparatus, and a laboratory-built fluorescence photometer system (Bone et al., 1997). A small area of the specimen (100×100 $μm^2$) was illuminated by excitation wavelength light (470 nm with half-bandwidth of 20 nm), and the intensity of emitted light (520–600 nm wavelength) was measured with a photomultiplier tube (Hamamatsu Photonics R268, Japan). The detecting area (10×10 $μm^2$) was determined by a slit, and background level measured on the area off the muscle.

Signals from the photocell, the strain gauge, and the photomultiplier were recorded with a VHS video system *via* a laboratory-modified PCM converter (Sony PCM501ES, Japan) at a sampling frequency of 15 kHz. Data were analysed off-line using an IBM/PC compatible computer and pCLAMP software.

Collection of blood plasma

After decapitation of the animals, blood was collected (1–2 ml from each), and centrifuged at 500 revs min^{-1} for 5 min. The supernatant was taken and centrifuged at 3,000 revs min^{-1} for 10 min. 250 μl of the supernatant was used for measurements of the osmolarity with an osmometer (Fiske Mark 3, USA), and the remainder for measurements of the electrolyte concentrations with a biochemical analyser (Fuji Film FDC3500, Japan).

Numerical values are presented as means ± S.D. Experiments were carried out at a room temperature of 21±1°C.

Results

Osmolarity and ionic composition of the blood plasma

Table 1 shows the osmolarity and electrolyte concentrations of the blood plasma of the hagfish *Eptatretus burgeri*. Each value is similar to that of the blood plasma of the Atlantic hagfish *Myxine glutinosa* (Alt et al., 1981), and close to that of seawater in the laboratory tank (993 mosmol kg^{-1}), being mainly composed of electrolytes.

Electro-mechanical properties of skeletal muscle

Membrane potentials were recorded from white muscle fibres of the *musculus tubulatus* using microelectrodes. The resting potentials in ASW recorded from 16 fibres were –55 to –80 mV, mean –67.4±7.0 mV. This is similar to that recorded from the white muscle fibres of the *musculus longitudinalis*

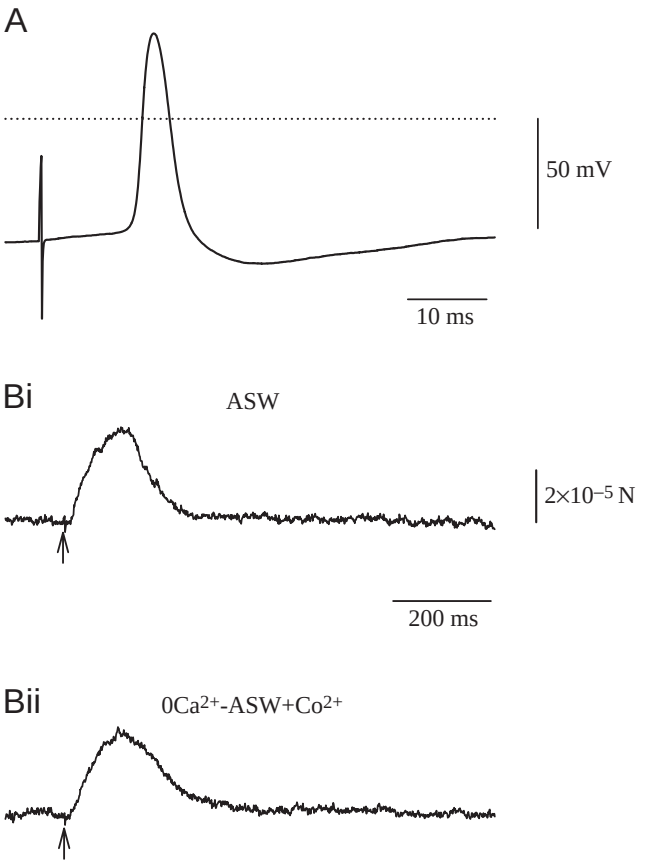

Fig. 1. (A) Propagating action potential recorded from a white muscle fibre of *musculus tubulatus* using a microelectrode. The dotted line indicates 0 mV. External solution, artificial seawater (ASW). (B) Force generated by a bundle of seven white muscle fibres in response to electrical stimulation measured with a strain gauge. Ten traces were averaged. Arrows indicate when stimulation was applied with a suction electrode. (Bi) Recorded in ASW, (Bii) recorded 10 min after the external ASW was switched to $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+}.

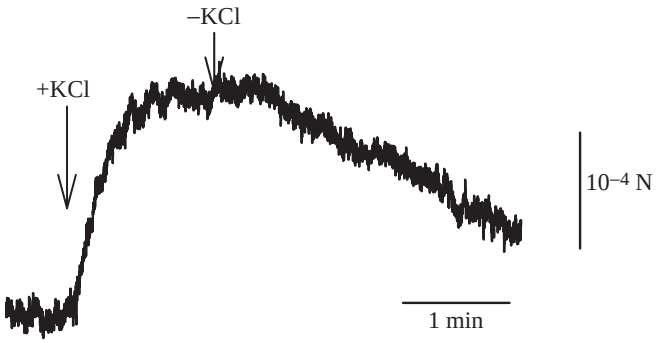

Fig. 2. Force generation measured with a strain gauge in response to depolarisation produced by an elevation of external K^+ concentration to 100 mmol l^{-1} in red skeletal muscle fibres. The red muscle fibre tissue was pre-soaked in $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} for 1.5 h. 0.6 mol l^{-1} KCl solution was added to the bath at the time indicated (+KCl), and the external solution was switched back to the original $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} at time (–KCl).

linguae in *Myxine glutinosa*, which is used for retracting the dental plate into the mouth (Andersen et al., 1963; Nicolaysen, 1966; Flood, 1998).

Electrical stimulation with a suction electrode at the tendon evoked a propagating action potential and a muscle twitch. A propagating action potential recorded from one of those twitching fibres overshot to +40 mV, followed by undershoot beyond resting potential (Fig. 1A). Externally applied TTX (1 μmol l^{-1}) blocked both action potential and twitch (not shown). In contrast, both action potential and twitch were maintained after switching the external ASW to $0Ca^{2+}$-ASW that contained 10 mmol l^{-1} Co^{2+} (N=6). Electrical stimulation in ASW, using a bundle with seven fibres, evoked a transient force reaching 3×10^{-5} N (Fig. 1Bi). The same force was evoked even 10 min after switching the external ASW to $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} (Fig. 1Bii). Hence, external Ca^{2+} was not involved in E–C coupling.

Although we did not measure the membrane potential of the red fibres, we assumed that, like the red fibres of *Myxine glutinosa*, they do not propagate overshooting action potentials (Jansen and Andersen, 1963) and, like them, operate by slow and graded depolarisations. We mimicked this effect with K^+ depolarisation. To test the mechanism of E–C coupling, bundles of red fibres were pretreated with $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} for 1.5 h, then pinned down in the chamber filled with the same solution. Contraction of red fibres was observed under the binocular microscope when the external K^+ concentration was increased to 100 mmol l^{-1}, to be sure that the function of DICR by intracellular Ca^{2+} release (see Fig. 5) is definitively present in the red fibres. The force generated by the K^+ depolarisation was measured with a strain gauge, and an example of the results obtained from six specimens is shown in Fig. 2.

Caudal heart muscle properties

Membrane potentials were recorded from caudal heart muscle fibres with microelectrodes. The resting potential was between –30 and –60 mV, mean –43.6±8.9 mV (N=53).

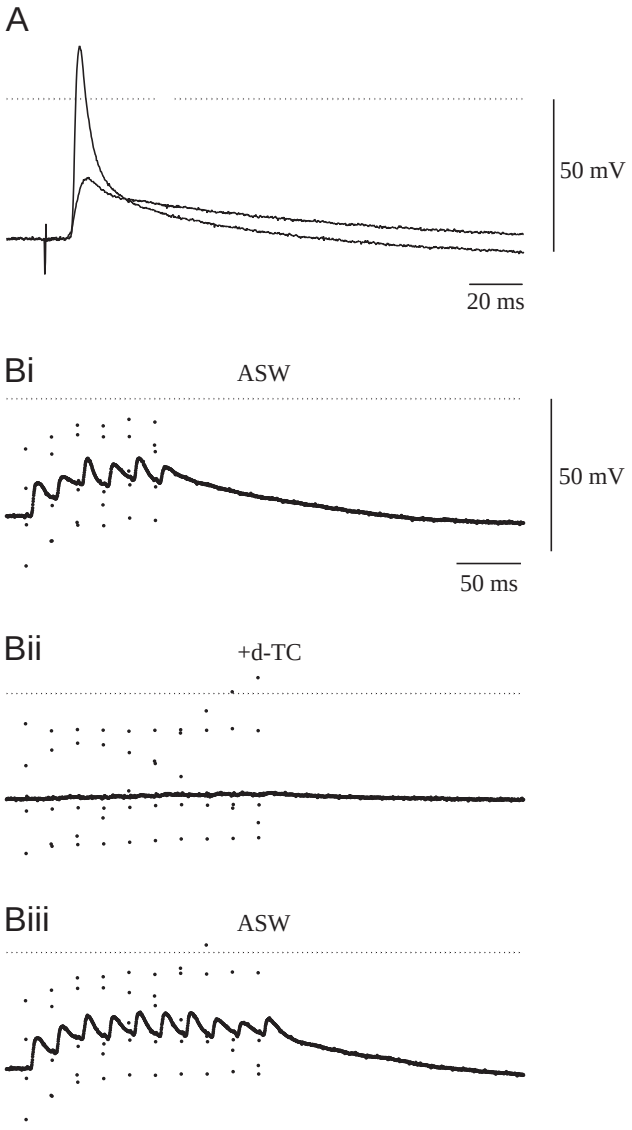

Fig. 3. (A) Subthreshold response and all-or-none spike recorded from a caudal heart muscle fibre in response to electrical stimulation by a suction electrode. Two traces are superimposed. External solution, artificial seawater (ASW). The dotted line indicates 0 mV. (Bi–iii) Reversible suppression by externally applied d-tubocurarine (d-TC; 20 μmol l^{-1}) of subthreshold responses evoked by a train of electrical stimuli by a suction electrode. The dotted lines indicate 0 mV. The recordings in A and in B were obtained from different individual caudal heart muscle fibres.

An electrical stimulus supplied by a suction electrode evoked a twitch of several fibres simultaneously. Subthreshold stimuli evoked graded depolarising responses that lasted >50 ms. However, the subthreshold stimuli did not evoke muscle twitches. When stimulation exceeded the threshold, a fibre generated an action potential in an all-or-none manner (Fig. 3A), and the fibre twitched.

When a train of subthreshold stimuli at the same intensity

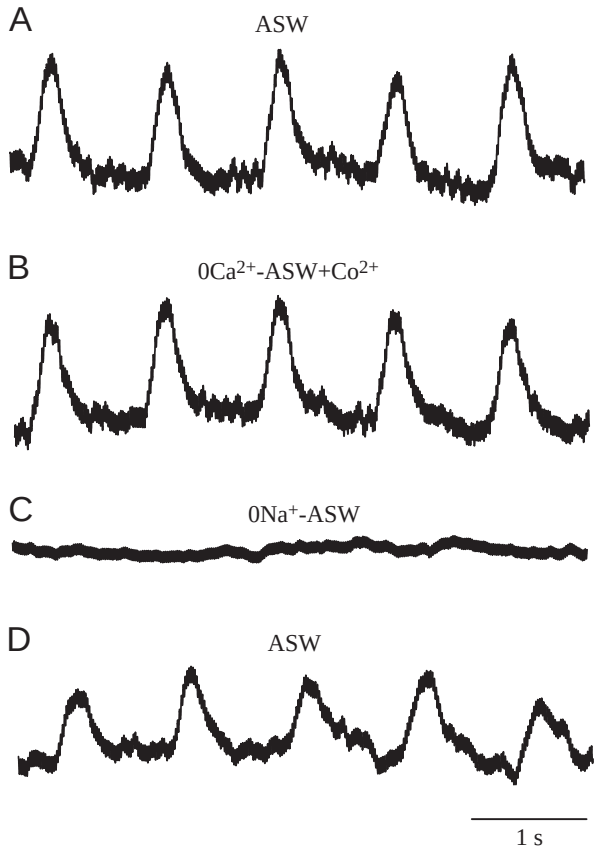

Fig. 4. Effects of $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} and of $0Na^{+}$-ASW on twitches of a single caudal heart muscle fibre electrically stimulated every 2 s with a microelectrode inserted into the fibre. Twitches were detected with a CdS photocell from a video monitor screen. ASW, artificial seawater.

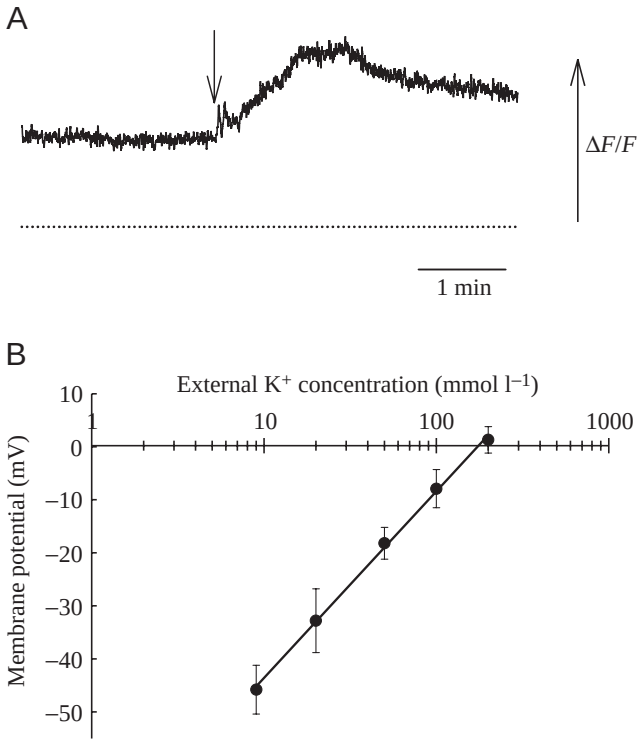

Fig. 5. (A) An increase in the fluorescence intensity ($\Delta F/F$) indicating an increase in the intracellular Ca^{2+} concentration of a single caudal heart muscle fibre associated with depolarisation produced by an increase in the external K^{+} concentration to 100 mmol l^{-1} at the time marked by arrow. The fibres had been loaded with fluo-3 by immersing in $0Ca^{2+}$-ASW containing 30 mmol l^{-1} Co^{2+} and 2 μmol l^{-1} fluo-3-AM for 1.5 h. The bath solution was $0Ca^{2+}$-ASW containing 30 mmol l^{-1} Co^{2+}. The dotted line indicates the background fluorescence measured off the cell. (B) Effect of changing the external K^{+} concentration on the membrane potential of caudal heart muscle fibres. Values are means ± S.D. obtained from 10 fibres. The slope was obtained by linear regression to the mean values, and is 35.2 mV per tenfold change in the K^{+} concentration.

was applied through a suction electrode, subthreshold responses were intensified until they reached a constant height (Fig. 3Bi). The amplitude of the subthreshold responses was different between fibres. These subthreshold responses were reversibly suppressed by addition of a number of agents, such as TTX (1 μmol l^{-1}, *N*=6), d-TC (20 μmol l^{-1}, *N*=8), or Co^{2+} (5 mmol l^{-1}, *N*=7), and by a substitution of Na^{+} with choline^{+} (*N*=1), or Ca^{2+} with Ba^{2+} (*N*=4). Fig. 3Bii,iii shows the reversible suppression of subthreshold depolarisations by d-TC. Depolarisation was also induced by extracellular application of ACh (*N*=3, not shown). The subthreshold depolarisations represent junction potentials responding to neuromuscular transmitters released from nerve endings by electrical stimulation of the nerves innervating the muscle fibre. Hence, action potential and associated twitch were evoked when the junction potential exceeded the threshold for the Na^{+} spike.

In order to stimulate a muscle fibre directly, electric current was applied to a fibre through a microelectrode inserted to the fibre. Application of a 0.2 ms outward-directed current pulse evoked twitches in an all-or-none manner when the pulse intensity exceeded a threshold level (*N*=14). No other fibres twitched, indicating that there is no electrical coupling between fibres. The force generated by one fibre in the bundle of fibres was insufficient to be detected by the strain gauge. We detected muscle twitches from a video monitor screen using a CdS photocell. Fig. 4 shows the effects of $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} and of $0Na^{+}$-ASW on twitches in response to electrical stimulation applied every 2 s. Twitches were maintained in $0Ca^{2+}$-ASW containing 10 mmol l^{-1} Co^{2+} (Fig. 4B). In contrast, twitches declined gradually in the $0Na^{+}$-ASW, and eventually disappeared at 15 min after the Na^{+} substitution (Fig. 4C). The twitches recovered to near the original level within a short time (5 min) after replacing the external $0Na^{+}$-ASW with ASW (Fig. 4D). These results indicate that the twitch does not require influx of extracellular Ca^{2+}, but does require Na^{+} for action potential generation.

To examine the presence of DICR further, changes in the intracellular Ca^{2+} level in response to depolarisation by

increased external K^+ concentration were measured in fibres loaded with fluo-3 (N=4). The fibres were bathed in $0Ca^{2+}$-ASW containing 30 mmol l^{-1} Co^{2+} for 30 min before fluorescence measurements were done. On increasing the external K^+ concentration to 100 mmol l^{-1}, the fluorescence signal increased (Fig. 5A), indicating that the K^+ depolarisation had induced intracellular Ca^{2+} release without the influx of extracellular Ca^{2+}.

Fig. 5B shows the effect of external K^+ concentration on the membrane potential in caudal heart muscle fibres. Each point indicates mean ± S.D. obtained from 10 fibres. Between 9 and 200 mmol l^{-1} K^+, the membrane potential changed linearly against log[K^+]. The slope was approximately 35 mV per tenfold change, indicating that the membrane does not behave as a pure K^+ electrode, and permits permeation of other ions. Therefore, the intracellular K^+ concentration could not be estimated in this way.

Discussion

Phylogeny of skeletal muscle E–C coupling

The present experiments revealed that in the hagfish *Eptatretus burgeri*, E–C coupling with depolarisation-induced Ca^{2+} release (DICR) is present in white and red skeletal muscle, and in the caudal heart muscle. This supports our hypothesis that evolution to acquire the function of DICR for muscle E–C coupling occurred in phylogeny between the acraniate and agnathan levels (Inoue et al., 1994).

Skeletal muscles in vertebrates are large and multinucleated. The SR is coupled in a regular way to the transverse (T)-tubular membrane. The couplings comprise end-feet consisting of dihydropyridine (DHP) receptors in the T-tubular membrane and ryanodine receptors in the SR membrane (Inui et al., 1987; Block et al., 1988; Bers and Stiffel, 1993). In the E–C coupling of skeletal muscle, depolarisation of the T-tubular membrane is sensed by a voltage sensor in the DHP receptor, and directly transferred to the ryanodine receptor (the intracellular Ca^{2+} release channel) *via* the end-feet to trigger Ca^{2+} release (Schneider and Chandler, 1973; Huang 1989; Ríos and Pizarro, 1991). Hence E–C coupling does not require influx of external Ca^{2+} (Armstrong et al., 1972). The voltage sensor is thought to be a part of the α1 subunit of the DHP receptor, a superfamily of L-type calcium channels (Tanabe et al., 1987; Brum et al., 1988). Thus the DHP receptor of skeletal muscle possesses a dual function: as a voltage sensor for E–C coupling and as an L-type calcium channel.

So far as is known, influx of external Ca^{2+} is indispensable for physiological E–C coupling in invertebrates and protochordates including the acraniate *Branchiostoma lanceolatum* (Hagiwara et al., 1971; Inoue et al., 1994, 1996, 1997; Bone et al., 1997, 1999; Tsutsui et al., 2000). Ca^{2+} enters the fibres through voltage gated L-type calcium channels (Melzer, 1982; Inoue et al., 1994, 1996, 1997; Bone et al., 1997, 1999; Tsutsui et al., 2000). The activation of those L-type calcium channels in protochordates and invertebrates is fast (<1 ms), whereas that of vertebrate skeletal muscle is much slower (>50 ms) (e.g. Inoue et al., 1994). This suggests that the evolution of E–C coupling may be accompanied by a molecular evolution of the DHP receptor to enhance its function as a voltage sensor. However, at present little is known about the molecular mechanism of the phylogeny of E–C coupling. Future studies from the point of molecular phylogeny may reveal the steps involved in the striking change in the mechanism of skeletal muscle E–C coupling.

E–C coupling of caudal heart muscle

Our experiments revealed that the caudal heart muscle fibres possess the DICR function. Therefore, the caudal heart is a 'vertebrate type' skeletal muscle, perhaps unsurprisingly since it is derived from parietal muscle and similarly innervated.

Na^+ spikes were evoked in an all-or-none manner when the stimulation intensity exceeded a threshold level (Fig. 3). Twitches also occurred in an all-or-none manner when the stimulating current intensity delivered by a microelectrode inserted into the fibre exceeded a threshold level (Fig. 4). These suggest that the sodium spike propagates along the fibre, although there is no direct experimental evidence. Although the resting potential of the caudal heart muscle fibres (–43.6±8.9 mV) is similar to that of red muscle fibres of *Myxine glutinosa* (Andersen et al., 1963; Flood, 1998), the electrical properties of the caudal heart are different from those of the slow fibres. In red fibres of *Myxine glutinosa*, membrane depolarisation is produced by neuromuscular junction potentials, never overshoots, and does not propagate along the fibres (Andersen et al., 1963; Nicolaysen, 1966; Flood, 1998). The electrical responses of the caudal heart muscle fibres rather resemble intermediate fibres of parietal muscle (Flood, 1998). The resting potential of the intermediate fibres is approximately –60 mV, and action potentials overshoot and propagate (Andersen et al., 1963; Flood, 1998). In the dogfish, multiply-innervated red fibres propagate action potentials, though these do not overshoot (Bone et al., 1994).

In living animals, at rest, the rate of contraction of the caudal heart is slow (0.1–0.5 Hz), and fatigue-resistant. However, rather strong and fast contractions appear within a few minutes after swimming. Contractions of the caudal heart stop when they are swimming, as has been reported by Greene (1900). Surprisingly these caudal heart activities are maintained even 24 h after decapitation. The swimming movements of the trunk muscle are also maintained after decapitation; they begin responding to touch stimulation to the skin and continue for 10–30 s. These observations suggest that the caudal heart activities are linked with the swimming movements of the trunk muscle and are principally regulated by cycles of spinal chord reflexes.

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References

Ahlberg, P. E. (ed.) (2001). Major events in early vertebrate evolution. palaentology, phylogeny, genetics and development. *Systematics Association*, special volume series, **61**. London: Taylor and Francis.

Alt, J. M., Stolte, H., Eisenbach, G. M. and Walvig, F. (1981). Renal electrolyte and fluid excretion in the Atlantic hagfish *Myxine glutinosa*. *J. Exp. Biol.* **91**, 323-330.

Andersen, P., Jansen, J. K. S. and Løyning, Y. (1963). Slow and fast muscle fibres in the Atlantic hagfish (*Myxine glutinosa*). *Acta Physiol. Scand.* **57**, 167-179.

Armstrong, C. M., Bezanilla, F. and Horowicz, P. (1972). Twitches in the presence of ethylene glycol-bis(b-aminoethyl ether)-*N,N´*-tetraacetic acid. *Biochim. Biophys. Acta* **267**, 605-608.

Bers, D. M. and Stiffel, V. M. (1993). Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E–C coupling. *Am. J. Physiol.* **264**, C1587-1593.

Block, B. A., Imagawa, T, Campbell, K. P. and Franziani-Armstrong, C. (1988). Structural evidence for the direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.* **107**, 2587-2600.

Bone, Q., Curtin, N. A. and Woledge, R. C. (1994). Action potentials in red muscle fibres from dogfish. *J. Physiol.* **479**, P4.

Bone, Q., Inoue, I. and Tsutsui, I. (1997). Contraction in the absence of a sarcoplasmic reticulum: muscle fibres in the small pelagic tunicate *Doliolum*. *J. Mus. Res. Cell Motil.* **18**, 375-380.

Bone, Q., Carré, C., Tsutsui, I. and Inoue, I. (1999). Calycophoran siphonophore muscle fibres without any sarcoplasmic reticulum but with tubular invaginations morphologically analogous to a T-system. *J. Mar. Biol. Assn. UK* **79**, 1111-1116.

Brodal, F. and Fänge, R. (ed.) (1963). *The Biology of Myxine.* Oslo: Universitetsforlaget.

Brum, G., Fitts, R., Pizarro, G. and Ríos, E. (1988). Voltage sensors of the frog skeletal muscle membrane require calcium to function in excitation-contraction coupling. *J. Gen. Physiol.* **398**, 475-505.

Flood, P. R. (1998). The skeletal muscle types of *Myxine glutinosa*. In *The Biology of Hagfishes* (ed. J. M. Jørgensen, J. P. Lomholt, R. E. Weber and H. Malte), pp.173-202. London: Chapman and Hall.

Forey, P. and Janvier, P. (1993). Agnathans and the origin of jawed vertebrates. *Nature* **361**, 129-134.

Greene, C. W. (1900). Contributions to the physiology of the California hagfish, *Polistotrema stouti*. I. The anatomy and physiology of caudal heart. *Am. J. Physiol*. **3**, 366-382.

Hagiwara, S., Henkart, M. P. and Kidokoro, Y. (1971). Excitation-contraction coupling in amphioxus muscle cells. *J. Physiol. (Lond.)* **219**, 233-251.

Huang, C. L.-H. (1989). Intramembrane charge movement in skeletal muscle. *Physiol. Rev*. **68**, 1197-1247.

Inoue, I., Tsutsui, I., Bone, Q. and Brown, E. R. (1994). Evolution of skeletal muscle excitation-contraction coupling and the appearance of dihydropyridine-sensitive intramembrane charge movement. *Proc. Roy. Soc. Lond. B* **255**, 181-187.

Inoue, I., Tsutsui, I., Brown, E. R. and Bone, Q. (1996). A phylogenetic approach to understanding the molecular mechanism of excitation-contraction coupling in striated muscle. In *Basic Neuroscience in Invertebrates* (ed. H. Koike, M. Kidokoro, K. Takahashi and T. Kanaseki), pp. 45-57. Japan Scientific Societies Press, Tokyo.

Inoue, I., Tsutsui, I., Brown, E. R. and Bone, Q. (1997). Evolution of skeletal muscle excitation-contraction coupling. In *Toward Molecular Biophysics of Ion Channels*, Progress in Cell Research **6** (ed. M. Sokabe, A. Auberbach and F. Sigworth), pp. 111-124. Amsterdam: Elsevier Scientific Press.

Inui, M., Sails, A. and Fleischer, S. (1987). Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J. Biol. Chem.* **262**, 1740-1747.

Jansen, J. K. and Andersen, P. (1963). Anatomy and physiology of the skeletal muscles. In *The Biology of Myxine* (ed. F. Brodal and R. Fänge), pp. 161-194. Oslo: Universitetsforlaget.

Johansen, K. (1963). Cardiovascular system of *Myxine glutinosa* L. In *The Biology of Myxine* (ed. F. Brodal and R. Fänge), pp. 289-316. Oslo: Universitetsforlaget.

Jørgensen, J. M., Lomholt, J. P., Weber, R. E. and Malte, H. (ed.) (1998). *The Biology of Hagfishes*. Chapman and Hall, London.

Lomholt, J. P. and Franko-Dossar, F. (1998). The sinus system of hagfishes-lymphatic or secondary circulatory system? In *The Biology of Hagfishes* (ed. J. M. Jørgensen, J. P. Lomholt, R. E. Weber and H. Malte), pp. 259-272. Chapman and Hall, London.

Mallatt, J., Sullivan, J. and Winchell, C. J. (2001). The relationships of lampreys to hagfishes: a spectral analysis of ribosomal DNA sequences. In *Major Events in Early Vertebrate Evolution. Palaeontology, Phylogeny, Genetics and Development*. Systematic Association special volume series, **61** (ed. P. E. Ahlberg), pp. 106-118. London: Taylor and Francis.

Melzer, W. (1982). Twitch activation in Ca^{2+}-free solutions in the myotomes of the lancelet (*Branchiostoma lanceolatum*). *Eur. J. Cell Physiol.* **28**, 219-225.

Nicolaysen, K. (1966). On the functional properties of the fast and slow cranial muscles of the Atlantic hagfish. *Acta Physiol. Scand.* **68 Suppl. 277**, 142(Abstract).

Retzius, G. (1890). Ein s.g. Caudalherz bei *Myxine glutiosa*. *Biologische Untersuchungenm NF* **1**, 94-96.

Retzius, G. (1892). Zur Kentnisse der motorischen Nervendingungen. *Biologische Untersuchungenm NF* **3**, 41-52.

Ríos, E. and Brum, G. (1987). Involvement of dihydropyridine receptors in excitation-contraction coupling. *Nature* **325**, 717-720.

Ríos, E. and Pizarro, G. (1991). Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiol. Rev.* **71**, 849-908.

Schneider, M. F. and Chandler, W. K. (1973). Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature* **242**, 244-246.

Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* **328**, 313-318.

Tsutsui, I., Inoue, I., Carré, C. and Bone, Q. (2000). Activation of locomotor and grasping spine muscle fibres in chaetognaths; a curious paradox. *J. Muscle Res. Cell Motil.* **21**, 91-97.

Zardova, R. and Meyer, A. (2001). Vertebrate phylogeny: limits of inference of mitochondrial genome and nuclear rDNA sequence data due to an adverse phylogenetic signal/noise ratio. In *Major Events in Early Vertebrate Evolution. Palaeontology, Phylogeny, Genetics and Development*. Systematic Association special volume series, **61** (ed. P. E. Ahlberg), pp. 135-155. London: Taylor and Francis.