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ROSTRAL–CAUDAL VARIATION IN TROPONIN T AND PARVALBUMIN CORRELATES WITH DIFFERENCES IN RELAXATION RATES OF COD AXIAL MUSCLE

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Summary

Relaxation rate is an important determinant of axial muscle power production during the oscillatory contractions of undulatory locomotion. Recently, significant differences have been reported in the relaxation rates of rostral *versus* caudal white muscle fibers of the Atlantic cod *Gadus morhua* L. The present study investigates the biochemical correlates of this rostral–caudal physiological variation. Using denaturing gel electrophoresis, a series of fresh muscle samples from the dorsal epaxial muscle region was analyzed and several differences were detected. First, a gradual shift occurs in the expression of two troponin T isoforms along the length of the body. Second, rostral muscles were found to contain significantly greater amounts of parvalbumin than caudal muscles. Third, two soluble Ca^{2+}-binding proteins, in addition to parvalbumin, were also detected in the rostral muscle samples yet were absent from the caudal samples. This suite of rostral–caudal variations provides a strong biochemical basis for regional differences in the relaxation rates of cod white muscle.

Key words: axial muscle, relaxation, Atlantic cod, *Gadus morhua*, contractile protein, parvalbumin, troponin T, regional variation.

Introduction

Relaxation rate has been hypothesized to be an important determinant of contractile frequency (Altringham and Johnston, 1990) and power generation in the axial muscles of fish (Moon *et al.* 1991; Rome and Swank, 1992; Rome *et al.* 1993; Johnson *et al.* 1994). After completing the shortening phase of its contraction cycle, a muscle must relax rapidly if it is to be extended with little resistance (Josephson, 1981). Hypothetically, maximum power can be achieved if muscle is 'turned on' instantaneously at the start of shortening and 'turned off' instantaneously just before lengthening (Marsh, 1990). A longer caudal relaxation rate can increase differences in the phase relationship between muscle strain and excitation. Differences in this phase relationship can have profound effects on the mechanical behavior of skeletal muscle (Josephson, 1985; Altringham and Johnston, 1990; Johnson and Johnston, 1991; Altringham *et al.* 1993). The resistance of active caudal muscles to being stretched (negative work) can act to stiffen the tail region, which can increase tail thrust. Thus, the rate at which caudal muscle relaxes can play a vital role, not only in the amount of negative work the muscle performs but also in determining the overall thrust produced by the tail region, which ultimately affects the performance of the whole organism (Swank *et al.* 1997).

Recent work by Davies *et al.* (1995), comparing rostral *versus* caudal mechanical properties of Atlantic cod *Gadus morhua* L. axial muscle fibers, documents a significant rostral–caudal difference in relaxation rates: the time required to relax to half peak isometric tension averaged 54.0±2.9 ms (mean ± S.E.M.) for rostral single fibers and 88.3±8.0 ms for caudal fibers. Similar differences have been reported for the white muscle fibers of pollack *Pollachius virens* (Altringham *et al.* 1993) and the red muscle fibers of scup *Stenotomus chrysops* (Rome *et al.* 1993). The present study explores the biochemical basis of rostral–caudal variation in cod axial muscle relaxation rate.

Relaxation of skeletal muscle after a single twitch or tetanic contraction is initiated by a reduction in myoplasmic Ca^{2+} concentration. Four major mechanisms are implicated in controlling relaxation. The first mechanism involves dissociation of Ca^{2+} from troponin C. Troponin T is believed to affect this rate of dissociation, as has been shown in frogs (Baylor *et al.* 1983; Gillis, 1985) and rabbits (Schachat *et al.* 1987). The second mechanism involves facilitated diffusion of Ca^{2+} from the myofibrils to regions near the sarcoplasmic reticulum. Parvalbumin, a low-molecular-mass Ca^{2+}-binding protein, has been shown to play a key role in this process in both frogs (Hou *et al.* 1991) and mice (Müntener *et al.* 1995). A third mechanism involves uptake of Ca^{2+} into the sarcoplasmic reticulum by the Ca^{2+}-ATPase pump (e.g. frog, Baylor *et al.* 1983; numerous vertebrates, Gillis, 1985; teleosts,

Rome *et al.* 1996; Swank *et al.* 1997). The number and/or efficiency of Ca^{2+}-ATPase pumps determines the rate of Ca^{2+} uptake. A fourth mechanism involves changes in the actomyosin crossbridge detachment kinetics (rabbit, Greaser *et al.* 1988; Sweeney and Stull, 1990). Myosin isoforms can be a strong determinant of detachment kinetics.

The present study implicates the first two of these regulatory mechanisms in controlling rostral–caudal variation in the relaxation rate of cod axial muscle.

Materials and methods

Experimental animals

Three specimens of fresh Atlantic cod *Gadus morhua* L. (cod GM01, 653 mm; cod GM02, 635 mm; cod GM03, 775 mm standard length) were obtained commercially from Maine waters during the months of May and June 1997 and March 1998. A fourth specimen (cod GM04, 470 mm standard length) was captured off the Massachusetts coast, maintained in captivity for several days at Northeastern University's Marine Science Laboratory in Nahant, MA, USA, and killed by pithing prior to axial muscle dissection.

Myofibril preparation

Fresh white muscle samples (0.5–1.0 g) were dissected from the epaxial arm and cone region of every sixth myomere and from one rostral and one caudal hypaxial cone site from cod GM01 (Fig. 1). Each muscle sample was minced on a chilled glass plate, transferred to an Eppendorf tube and suspended in 1 ml of buffer A, containing 15 mmol l^{-1} Tris, pH 7.6, 100 mmol l^{-1} NaCl, 4 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 7 mmol l^{-1} β-mercaptoethanol, 0.1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF) and 75 ng ml^{-1} each of antipain, leupeptin and pepstatin A. Samples were vortexed and centrifuged at 14 000 revs min^{-1} (12,500 ***g***) for 12 s in an Eppendorf 5415 microcentrifuge, and the resulting pellet was resuspended in 1 ml of buffer B (composition as for buffer A but containing only 0.3 µl ml^{-1} leupeptin, antipain and pepstatin). The samples were vortexed and centrifuged (at 12,500 ***g***), and the resulting pellets were incubated in 1 ml of buffer C (buffer B plus 2 % Triton X-100) for 10 min. After recentrifugation at (12,500 ***g***), the pellets were washed twice more with buffer B and resuspended in 1 vol of buffer B.

To prepare myofibrillar homogenates, fresh superficial white muscle samples were dissected from the epaxial arm region of every sixth myomere from cod GM02 and GM03. All samples were minced as described above and suspended in approximately 5 vols of buffer A supplemented with 0.6 mol l^{-1} NaI. Samples were incubated on ice for 10 min and centrifuged at room temperature (21 °C) for 2 min at 12,500 ***g***, and the supernatants were reserved for further characterization.

SDS–PAGE

Sample preparation

Gel samples were prepared by 1:1 dilutions with 2× Laemmli reducing buffer (2 % SDS, 50 mmol l^{-1} Tris, pH 6.8, 20 % glycerol, 1 % β-mercaptoethanol, Bromophenol Blue). Samples were then heated for 3 min at 100 °C, frozen until solid, reheated for 2 min at 100 °C, and centrifuged. Supernatants were diluted as necessary in Laemmli reducing buffer (Laemmli, 1970).

Preparation of gels

SDS–PAGE was performed according to Laemmli (1970) using 10.5 %, 12.5 % and 15 % polyacrylamide–SDS gels as indicated. Mini-gels were run at room temperature on a BioRad Protean II cell, and full-sized gels were electrophoresed at 8 °C using Hoefer Scientific Instruments SE500 apparatus. Unless otherwise specified, all gels were fixed and stained with Coomassie Brilliant Blue G250 as described by Neuhoff *et al.* (1988).

Gels were scanned into Adobe Photoshop v. 3.05 with an Agfa Arcus II Scanner and quantification was performed with NIH Image v. 1.61. When necessary, intensity plots were fitted

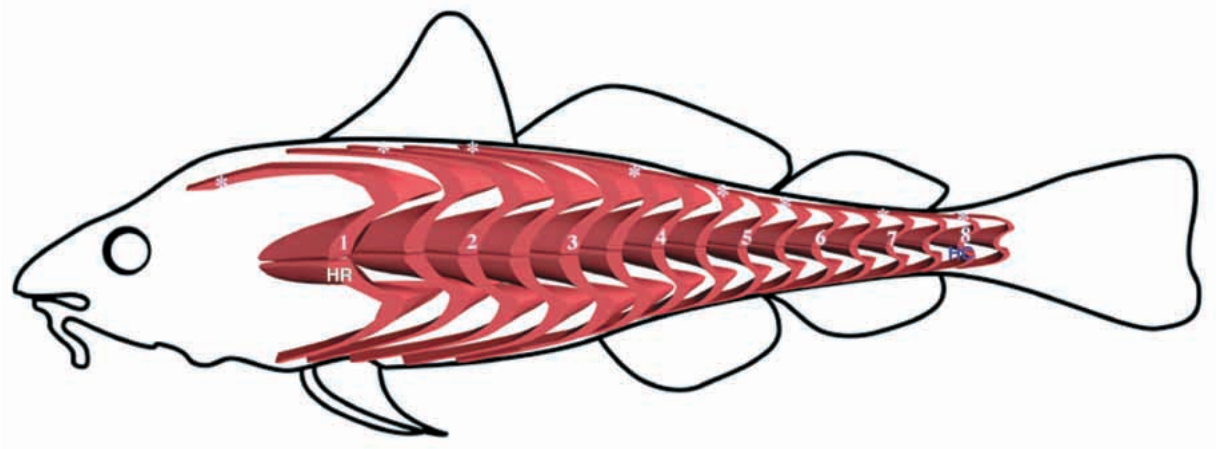

Fig. 1. Schematic drawing of cod axial muscle indicating sampling sites. Isolated white muscle myomeres are depicted in pink. Every sixth myomere is numbered. Arm sample sites are depicted by asterisks, and cone sample sites are depicted by numbers. Hypaxial cone samples are labeled: HR, hypaxial rostral cone site; HC, hypaxial caudal cone site. The drawing was constructed using Strata Studio Pro v. 1.75 with the help of Mark Johnston.

using Jandel PeakFit v. 4. The scanner was calibrated with a Kodak density step tablet (CAT 1523406), and dilutions of myofibril samples were run to ensure linearity of staining.

Relative molecular mass (M_r) was determined by comparing the relative mobility of the cod myofibrillar proteins with that of proteins from rabbit psoas myofibrils, whose molecular masses are known from direct sequencing or cDNA analysis.

Immunoblotting (western blotting)

Gel samples were prepared from homogenates, myofibrils or supernatants as indicated. Based on the method of Davies *et al.* (1995), four sample sites were selected; one epaxial rostral site, one epaxial caudal site, one hypaxial rostral site and one hypaxial caudal site from the right side of two individuals (cod GM02 and GM03). To normalize to levels of actin, two lanes were run for each sample: one for Coomassie staining, and the second for immunoblotting.

Parvalbumin was identified following transfer to nitrocellulose using monoclonal anti-parvalbumin mouse ascites fluid PA-235 (Sigma 3171) and the protocol outlined in the Vectastain ABC kit. The blot intensities were quantified densitometrically and normalized to actin using NIH Image.

Ca^{2+}-binding proteins

Two-dimensional electrophoresis was performed using standard 12.5 % SDS–PAGE with all solutions supplemented with 1 mmol l^{-1} EDTA in the first dimension and with 12.5 % SDS–PAGE with all solutions supplemented with 1 mmol l^{-1} $CaCl_2$ in the second dimension. Lanes from the first dimension were excised and mounted horizontally on the stacker for the second dimension using an agarose solution containing 0.125 mol l^{-1} Tris, pH 6.8, 1.25 % low-melting-point agarose (FMC Corp.), 0.1 % SDS, 1:4000 β-mercaptoethanol and 1 mmol l^{-1} $CaCl_2$.

The gel was stained with Coomassie Brilliant Blue as described above. A right shift of the proteins away from the diagonal is an indicator of Ca^{2+} binding. Ca^{2+} binding to proteins displaces SDS, reducing the charge and mobility of Ca^{2+}-binding proteins in the second dimension and resulting in an upward shift in position in the second dimension (Burgess *et al.* 1980).

Protein purifications: myosin and troponin

Owing to the inherent thermal instability of gadoid myosins (Connell, 1960; Castell *et al.* 1973; Laird and Mackie, 1981), special precautions were taken to ensure successful purification of the myosin and troponin myofibrillar subfractions. Axial muscle tissue samples were dissected from cod GM04, immediately frozen in liquid nitrogen and stored at −80 °C for 2 days.

Approximately 2 g of muscle from the arm region of site 3 (see Fig. 1) were pulverized in liquid nitrogen. The resulting powder was washed in 10 vols of chilled solution 1 [50 mmol l^{-1} Tris/HCl, 30 mmol l^{-1} Tris base, 50 mmol l^{-1} LiCl, 15 % glycerol (BRL Molecular Biology Grade), 7 mmol l^{-1} β-mercaptoethanol, 0.1 mmol l^{-1} PMSF, 1/10000 (25 mg ml^{-1}) leupeptin] and centrifuged for 5 min at 3000 ***g*** in a Jouan CR412 refrigerated centrifuge. LiCl was incorporated into solution 1 since it has been shown to be more effective at extracting protein from fish muscle than either sodium chloride or potassium chloride under most conditions (Kelleher and Hultin, 1991). Glycerol was added to further stabilize against denaturation (Gekko and Timasheff, 1981). The supernatant from the first centrifugation was saved for the analysis of soluble proteins, and the pellet was washed a second time in 10 vols of solution 1. Myosin, troponin and tropomyosin were prepared by incubating the pellet in 3 vols of chilled extraction buffer (1 mol l^{-1} LiCl, 25 mmol l^{-1} Tris/HCl, 7.5 mmol l^{-1} Tris base, 15 % glycerol, 7 mmol l^{-1} β-mercaptoethanol, 0.1 mmol l^{-1} PMSF) for 10 min at 0 °C. Following incubation, the sample was centrifuged for 75 min at 250,000 ***g*** at 2 °C in a 100Ti rotor in a Beckman tabletop ultracentrifuge. The supernatant, containing solubilized myofibrillar proteins, was dialyzed overnight at 4 °C against a low-salt solution (15 % glycerol, 25 mmol l^{-1} LiCl, 30 mmol l^{-1} Tris/HCl, 10 mmol l^{-1} Tris base, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EGTA, 14 mmol l^{-1} β-mercaptoethanol, 0.1 mmol l^{-1} PMSF) to precipitate the myosin. The dialysis solution was changed after the first 4 h and replaced with a fresh solution devoid of EGTA. Following dialysis, the sample was centrifuged at 4 °C, and the pelleted myosin and the supernatant (containing mainly actin, tropomyosin and troponin) were separated. The supernatant was supplemented with 5 mmol l^{-1} phosphate buffer and loaded onto a 5 ml hydroxyapatite column. The column was washed with 2 vols of 5 mmol l^{-1} phosphate buffer (5 mmol l^{-1} phosphate, 0.6 mol l^{-1} LiCl, 1 mmol l^{-1} $MgCl_2$, 14 mmol l^{-1} β-mercaptoethanol, 0.1 mmol l^{-1} PMSF, pH 7) and eluted with a linear 5 mmol l^{-1} to 200 mmol l^{-1} phosphate gradient in the same salt at 4 °C. Fractions (1 ml) were collected and analyzed using SDS–PAGE.

Results

Identification of the myosin, troponin and tropomyosin subunits

The abundance of myofibrillar actin and myosin heavy chain makes them readily identifiable on denaturing polyacrylamide gels (Fig. 2). Unambiguous identification of the remaining myofibrillar proteins requires purified or enriched preparations. Myosin, troponin and tropomyosin were purified from the arm region of site 3 (Fig. 1), a site where all the major muscle proteins and their isoforms are expressed. Initial attempts at protein purification using standard techniques, however, resulted in aggregated and denatured proteins. Both formylation and thermal denaturation have been implicated in inducing aggregation of gadoid muscle proteins (Connell, 1960; Tokunaga, 1964; Castell *et al.* 1973; Laird and Mackie, 1981). To combat potential formylation, muscle samples were dissected immediately after the fish had been killed, flash-frozen in liquid nitrogen and stored at −80 °C. The samples were then pulverized in liquid nitrogen and placed in a homogenization buffer supplemented with Tris, a primary

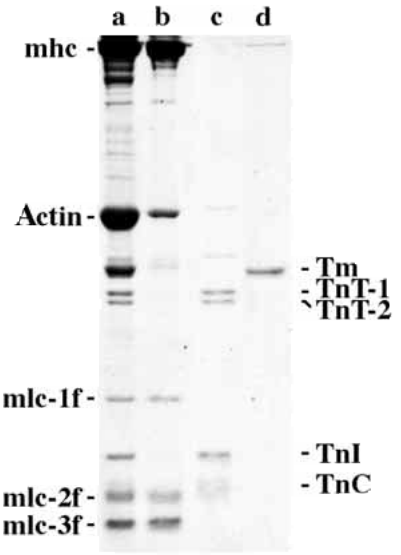

Fig. 2. Electrophoresis of purified cod contractile proteins. 12.5 % SDS–PAGE was used to identify the subunits of cod axial muscle proteins. Myofibrils (lane a) were run for comparison with an enriched myosin preparation (lane b) and purified fractions of troponin (lane c) and tropomyosin (lane d). The three fast myosin light chains in the purified myosin sample are labeled mlc-1f, mlc-2f and mlc-3f, and the two fast troponin T species are labeled TnT-1 and TnT-2, where the numbers reflect the relative mobility of the proteins in ascending order. mhc, myosin heavy chain; TnI, troponin I; TnC, troponin C; Tm, tropomyosin.

amine that inhibits protein formylation by mass action. Further protein denaturation was prevented by (1) supplementing solutions with glycerol, (2) using the leiotrophic salt LiCl in place of NaCl or KCl, and (3) performing all procedures at or below 0 °C, except for the hydroxyapatite chromatography which was performed at 4 °C.

From the hydroxyapatite column chromatography fractions, the major myofibrillar protein subunits were readily identified using SDS–PAGE. Comparison of the homogenate with the myosin-enriched fraction (Fig. 2, lanes a and b, respectively) revealed the presence of three myosin light chain species designated mlc-1f, mlc-2f and mlc-3f. The relative mobility of these light chains (compared with the mobility of rabbit fast myosin light chains) indicates that their relative molecular masses (M_r) are 21 100, 18 670 and 18 240, respectively.

The purified troponin fraction (Fig. 2, lane c) revealed the presence of two TnT species (TnT-1 and TnT-2) with M_r values of 32 110 and 30 900, respectively, a troponin I (M_r 19 040) and a troponin C (M_r 16 940), which migrates just above mlc-2f and is often difficult to resolve. Tropomyosin (M_r 34 580) is shown in Fig. 2, lane d.

Arm versus *cone*

SDS–PAGE of myofibrils from the arm and cone regions of the same myomere did not demonstrate significant differences in myofibrillar protein expression (Fig. 3), although a rostral–caudal transition in TnT isoforms was apparent. Because of the overall similarity between the arm and cone regions, subsequent investigations focused on rostral–caudal differences in the arm region alone. The less complicated morphology of the arm region also simplified the dissection of muscle samples from individual myomeres.

Rostral versus *caudal*

Investigation of both myofibrils (Fig. 3) and homogenates (Fig. 4) along the rostral–caudal axis by SDS–PAGE revealed a marked difference in the ratio of the TnT isoforms. The faster-migrating rostral TnT isoform, TnT-2, is gradually replaced by the slower-migrating caudal isoform, TnT-1. Fig. 5 quantifies the shift in TnT isoforms as a function of myomere position in the three cod sampled. Each animal exhibits a rostral to caudal shift from TnT-2 to TnT-1 expression; at the level of the third dorsal fin, site A6 (Fig. 1) (approximately two-thirds of the way down the body), all the TnT in both the arm and cone regions is composed of the slower-migrating TnT-1 isoform.

While the difference in TnT expression was the only

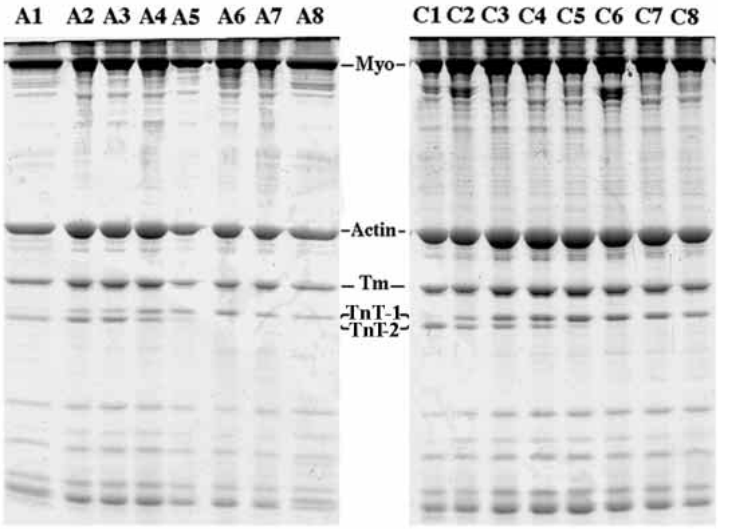

Fig. 3. Comparison of arm and cone myofibrillar proteins in cod myomeres. A 10.5 % SDS gel stained with Coomassie Brilliant Blue G250, comparing samples from arm (A1–A8) and corresponding cone (C1–C8) myofibrils from eight sequential myomeres of cod GM01 (standard length 653 mm). Note that the arm and cone samples show similar muscle protein expression. Both regions show a rostral–caudal shift in two troponin T isoforms. A, arm; C, cone; Myo, myosin heavy chain; Tm, tropomyosin; TnT, troponin T. Note that the faster-migrating rostral troponin T, TnT-2, is gradually replaced by the slower-migrating caudal isoform TnT-1. At the level of the third dorsal fin (site A6, approximately two-thirds of the way down the body), all myofibrillar troponin T in both the arm and cone regions is of the slower-migrating TnT-1 isoform.

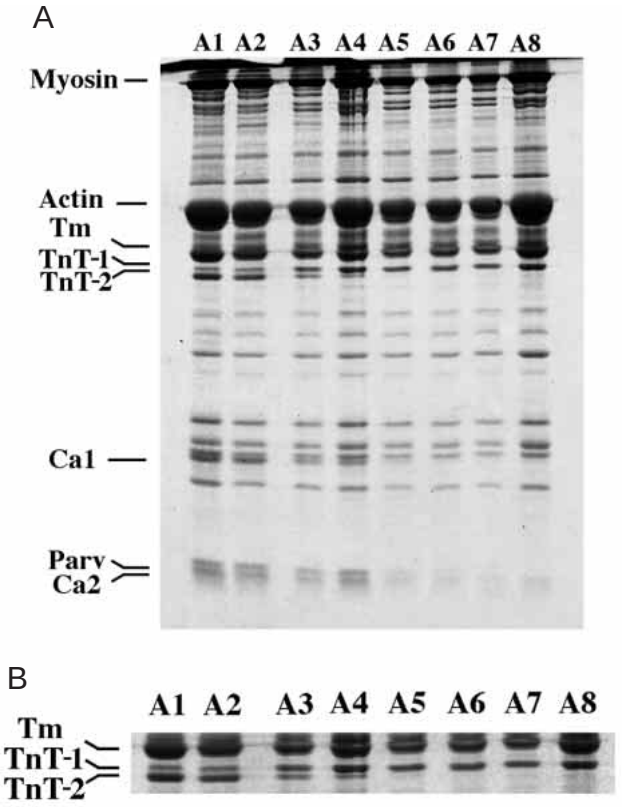

Fig. 4. Rostral–caudal variation in cod axial muscle homogenates. (A) A 12.5 % SDS gel stained with Coomassie Brilliant Blue G250, comparing arm (A1–A8) myofibrillar homogenates from eight sequential myomeres of cod GM02 (standard length 635 mm). (B) Magnification of the portion of the gel containing the troponin T isoforms. Four differences are detectable along the rostral–caudal axis: (1) a shift in the troponin isoforms from TnT-2 rostrally to TnT-1 caudally, (2/3) the presence of two Ca^{2+}-binding proteins (Ca1 and Ca2) in the rostral arm sites (A1–A4) and their absence from the caudal arm sites (A5–A8), and (4) larger amounts of parvalbumin (Parv) in the rostral arm sites (A1–A4) than in the caudal arm sites (A5–A8). Tm, tropomyosin.

myofibrillar variation, three additional rostral–caudal differences were characterized in the muscle homogenates (Fig. 4A). These differences appear to involve differential expression of low-molecular-mass cytosolic Ca^{2+}-binding proteins in the rostral axial white muscle.

Of these three proteins, the protein with an intermediate M_r was identified as parvalbumin by western blotting. Greater amounts of parvalbumin (M_r 10 320) are present rostrally than caudally, as demonstrated by western blotting (Fig. 6) and gels stained with Coomassie Brilliant Blue G250 (Fig. 4; Table 1). The relative abundance of parvalbumin was assessed by determining its concentration relative to that of actin. In cod GM03, the epaxial rostral site, A1, contains 8.3 times more parvalbumin than the caudal site A8. In cod GM02, a similar bias in rostral parvalbumin expression was observed. In the hypaxial muscle samples (HR and HC), both cod GM02 and GM03 show a similar elevation of rostral parvalbumin

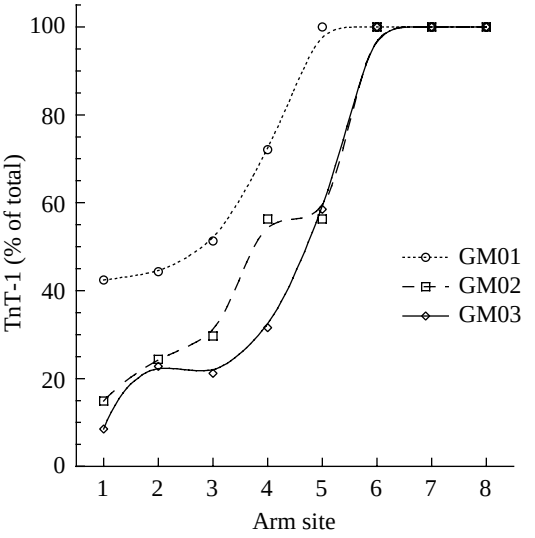

Fig. 5. Rostral–caudal variation in troponin T isoforms. The percentage of TnT-1 to total TnT (TnT1 plus TnT2) is plotted *versus* the axial sampling site (see Fig. 1) for the three cod in this study.

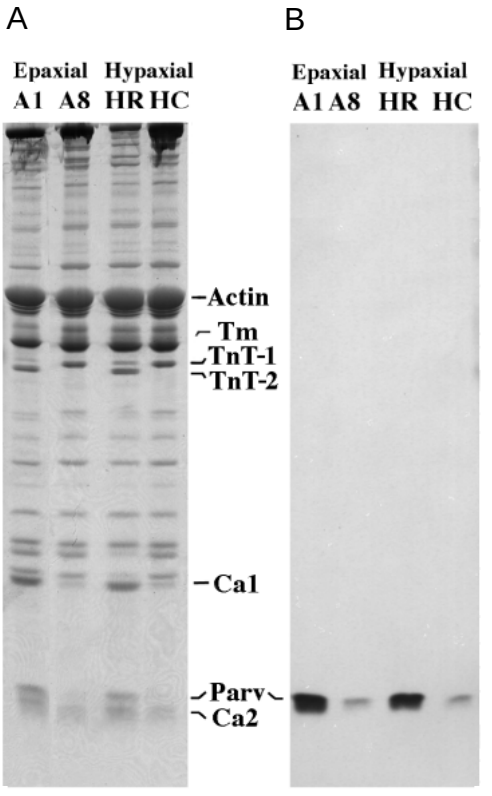

Fig. 6. Identification of parvalbumin by western blotting. (A) A 12.5 % SDS–polyacrylamide gel of rostral arm (A1), rostral hypaxial (HR), caudal arm (A8) and caudal hypaxial (HC) myofibrillar homogenates stained with Coomassie Brilliant Blue G250. (B) Western blot of the same sample. Parvalbumin (Parv) is identified by reaction with a monoclonal antibody. Note that the rostral muscle sites A1 and HR contain significantly greater amounts of parvalbumin than do the caudal sites A8 and HC. Loads were balanced by actin content. Tm, tropomyosin; TnT-1, TnT-2, isoforms of troponin; Ca1, Ca2, Ca^{2+}-binding proteins.

Table 1. *Parvalbumin content of rostral and caudal sites of cod axial muscle*

Sample site	Fish GM02 parvalbumin content	Fish GM03 parvalbumin content
A1	0.143±0.001	0.215±0.026
A8	ND	0.026±0.004
HR	0.169±0.0003	0.176±0.003
HC	ND	0.003±0.001

For details, see Fig. 1.

Values are means ± S.D. (N=3); ND, not detectable.

Levels of parvalbumin are normalized to levels of actin as determined densitometrically using NIH Image from gels stained with Coomassie Brilliant Blue G250.

Note that the rostral sites (A1, HR) have significantly greater amounts of parvalbumin than do caudal sites (A8, HC) (t-test; P<0.05).

expression with exceedingly low caudal parvalbumin levels (Table 1).

In addition to parvalbumin, two cytoplasmic proteins, designated Ca1 and Ca2 (M_r 14 800 and 9 900, respectively), are present in rostral samples (A1–A4) but are absent from caudal samples (A5–A8). Their identification as Ca^{2+}-binding proteins is based on a mobility-shift assay. As shown in the two-dimensional gel in Fig. 7, proteins Ca1 and Ca2 shift to the right of the diagonal when electrophoresed in the presence of 1 mmol l^{-1} Ca^{2+}. Such Ca^{2+}-dependent mobility shifts are indicative of Ca^{2+} binding (Burgess *et al.* 1980). Like parvalbumin, both proteins are expressed at high levels in rostral sites and are undetectable in the most caudal sites (Fig. 4A).

In contrast to the systematic rostral–caudal variation in TnT and Ca^{2+}-binding proteins, SDS–PAGE revealed no rostral–caudal differences in the expression of tropomyosin or alkali myosin light chains (Fig. 3A). The ratio of myosin light chains is virtually invariant rostrocaudally. Low-percentage polyacrylamide gel electrophoresis also failed to detect differences in the rostrocaudal distribution of the myosin heavy chain (data not presented). Given the observations of Davies *et al.* (1995), who report rostral–caudal differences in maximal contraction velocity, it is likely that higher-resolution techniques (e.g. cDNA sequencing) will reveal rostral–caudal variation in myosin heavy chain expression.

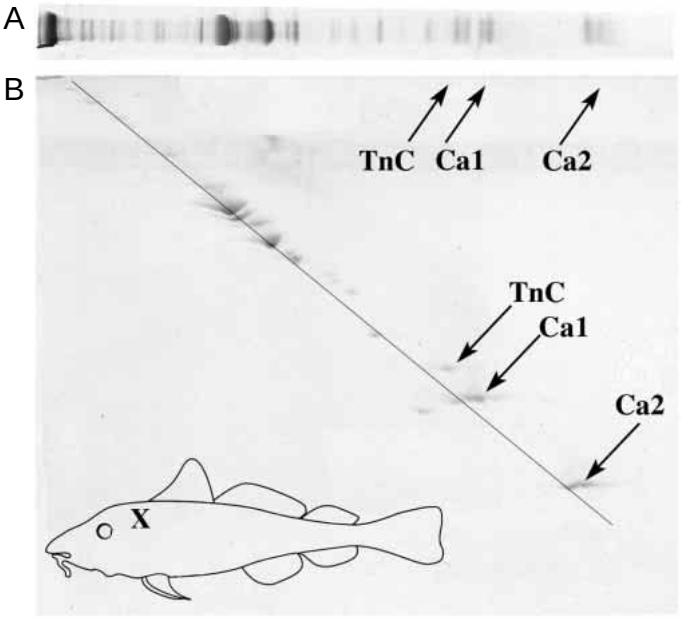

Fig. 7. Identification of two Ca^{2+}-binding proteins by a mobility-shift assay. Two-dimensional gels, each containing 12.5% SDS polyacrylamide, electrophoresed in the presence of 1 mmol l^{-1} EDTA in the first dimension and 1 mmol l^{-1} $CaCl_2$ in the second dimension, were run to identify putative Ca^{2+}-binding proteins. A replicate of the first-dimension gel is shown in A, while B shows the electrophoretogram after the second dimension had been run. The sample was taken from the arm region indicated (X) in the diagram of the fish in B. The diagonal line represents the expected mobility boundary for proteins that are unaffected by Ca^{2+}. Three major proteins whose mobility is shifted to the right are indicated by arrows. They correspond to troponin C (TnC), Ca1 and Ca2. Ca1 and Ca2 are cytosolic proteins that are preferentially expressed in rostral myomeres (see Fig. 4). The reduced mobility in the presence of Ca^{2+} (and the increase in apparent molecular mass) is typical of Ca^{2+}-binding proteins (Burgess *et al.* 1980).

Discussion

The results of this study implicate several cod axial muscle proteins in the regulation of relaxation rates along the length of the body. Changes in the proportions of these proteins are likely to alter the dynamics of Ca^{2+} dissociation from the thin filament and affect the rate at which Ca^{2+} is removed from the myoplasm. The differences detected include (1) a rostral–caudal shift in isoform expression of troponin T, a subunit of the thin filament Ca^{2+} regulatory complex, (2) a significant decrease in the amount of the Ca^{2+}-binding protein parvalbumin in caudal axial muscle sites and (3) the presence of two soluble Ca^{2+}-binding proteins in rostral muscle. Together, these factors provide compelling molecular correlates to measured differences in relaxation rates along the length of cod white axial muscle.

TnT isoforms

Although the properties of the two identified TnT isoforms have not been measured, differences in troponin T have been found to correlate with differences in Ca^{2+}-sensitivity of muscle activation in numerous vertebrates, including rabbits (Schachat *et al.* 1987; Greaser *et al.* 1988; Nassar *et al.* 1991), chickens (Reiser *et al.* 1992), rats (Akella *et al.* 1995) and humans (Mesnard *et al.* 1995). Studies in which mammalian myofibrils were reconstituted with specific TnT isoforms (e.g. bovids, Tobacman and Lee, 1987; human, Wu *et al.* 1995) provide experimental support for a role for TnT in determining the Ca^{2+} concentration needed for half-maximal activation of

the thin filament. Different combinations of TnT isoforms in rabbit axial and limb muscle have also been found to influence thin filament cooperativity, i.e. the ease with which the thin filament switches between the active and inactive states in response to the binding and release of Ca^{2+} (Schachat *et al.* 1987).

Several researchers have attempted to make simple correlations between the sensitivity and cooperativity of Ca^{2+} activation and the length and charge of the variable N-terminal region that defines TnT isoforms (e.g. rabbit skeletal muscle, Greaser *et al.* 1988; dragonfly flight muscle, Fitzhugh and Marden, 1997). Others have found that length and charge are not the critical variables influencing muscle fiber physiology (Schachat *et al.* 1987; Briggs *et al.* 1987). Using rabbit skeletal muscle, they find that what distinguishes fibers with the lowest cooperativity is not a difference in molecular mass or in the charge structure of the muscle fiber TnT isoforms, but rather the regulated expression of TnTs that include a specific amino acid sequence encoded by mammalian exon 4. Similar specificity has been reported for chicken skeletal muscle (Reiser *et al.* 1992; Schachat *et al.* 1995) and would probably hold true for fish skeletal muscle as well.

To establish a direct molecular explanation for how TnT isoform variation affects the Ca^{2+} responsiveness and relaxation rates of cod axial muscle, sequence data coupled with substitution of specific TnT isoforms into myofibrils from which pCa/tension curves can be generated, is necessary.

Parvalbumin

In the case of parvalbumin, direct correlations between parvalbumin levels and rates of muscle relaxation have been modeled and measured experimentally (Kretsinger and Nockholds, 1973; Müntener *et al.* 1995). Parvalbumin has been shown to affect relaxation rates in a dose-dependent manner in both frogs (Hou *et al.* 1991) and mammals (Müntener *et al.* 1995), and it appears to affect regional relaxation rates in cod axial muscle in a similar manner. The greater the amount of parvalbumin, the faster the muscle relaxes.

Regional expression of parvalbumin, the major component of the soluble fraction of fast teleost muscle (Hamoir, 1974; Zawadowska and Supikova, 1992; Perry, 1996), has been studied by other researchers (Huriaux *et al.* 1996, 1997). In the adult barbel *Barbus barbus*, total parvalbumin concentration decreases caudally, as in cod. Huriaux *et al.* (1992) propose that temporal and spatial variations in total parvalbumin concentration and differential expression of parvalbumin isoforms in barbel reflect the functional requirements of the fish axial musculature according to fish size and myomeric location. To date, however, the present investigation is the first to correlate regional changes in physiology with regional differences in parvalbumin concentration.

We also performed a preliminary investigation of axial muscle proteins from a short-horned sculpin *Myoxocephalus scorpius* (standard length 312 mm). Unlike the cod, the relaxation rate of short-horned sculpin axial muscle remains constant along the body (Johnston *et al.* 1993). Consistent with the effects of parvalbumin in mammals, no significant differences were detected in the parvalbumin content of rostral *versus* caudal white muscle samples in short-horned sculpin axial muscle.

Other Ca^{2+}-binding proteins

The final difference detected between rostral and caudal muscle fibers is the rostral presence of two Ca^{2+}-binding soluble proteins: Ca1 and Ca2 (Figs 4, 6). If and how these proteins affect muscle performance is unknown. Given their relative molecular mass, a possible candidate for the higher-molecular-mass protein, Ca1, is calmodulin, a member of the troponin C family, with four Ca^{2+}-binding sites. Calmodulin plays a pivotal role in many cellular processes controlled by Ca^{2+} (Cheung, 1980); however, its concentration in mammalian skeletal muscle is typically so low ($5\,\mu mol\,l^{-1}$) that it cannot usually alter the cytosolic [Ca^{2+}] by the mere binding of Ca^{2+} (Gillis, 1985).

On the basis of its low relative molecular mass, a possible candidate for the Ca2 protein is another isoform of parvalbumin. While Ca2 does not react with the parvalbumin antibody, it may be parvalbumin-like and have similar effects in relaxation. Multiple parvalbumin isoforms have been reported in numerous species of fish (Gerday, 1982).

To determine the specific identity of both Ca1 and Ca2, micro-sequence data establishing amino acid sequence homologies with other known Ca^{2+}-binding proteins would be informative.

Other mechanisms controlling relaxation

Two other mechanisms may play a part in governing rostral–caudal differences in the relaxation rate of white axial muscle in fishes. These include (1) a difference in the concentration or activity of sarcoplasmic reticulum Ca^{2+}-ATPase (as shown to be the case in the red axial muscle of scup *Stenotomus chrysops*; Swank *et al.* 1997) and/or (2) differences in myosin (as reviewed in Gillis, 1985). Whereas neither the myosin nor the volume and surface densities of T-tubules and sarcoplasmic reticulum (Davies *et al.* 1995) appear to vary significantly from rostral to caudal in cod white axial muscle, it is possible that the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase may differ rostrocaudally. Experiments similar to those conducted on scup red muscle by Swank *et al.* (1997) could address this possibility.

Compared with the red axial muscle system of the scup, however, the white muscle system of the cod appears to regulate regional differences in relaxation rate through distinctly different mechanisms. Interestingly, while the red and white muscle systems of the scup and cod, respectively, may utilize different molecular mechanisms, the end result is similar. Both systems appear to increase the rate at which Ca^{2+} is removed from the myoplasm of rostral muscles. The scup appears to increase the efficiency of the sarcoplasmic reticulum Ca^{2+} pump in its rostral red muscle (Swank *et al.* 1997), while in rostral cod white muscle, increased expression of parvalbumin and differential expression of other

myofibrillar and myoplasmic proteins appear to facilitate the rate of Ca^{2+} transport from the thin filament to the sarcoplasmic reticulum.

In conclusion, few studies provide direct links between molecular differences in Ca^{2+} regulatory proteins and their physiological function in whole muscles and whole-organism performance. This study describes a suite of rostral–caudal biochemical variations that provide compelling molecular correlates to the significantly slower relaxation rates of cod caudal axial white muscle fibers compared with rostral white muscle fibers.

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