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Parvalbumin correlates with relaxation rate in the swimming muscle of sheepshead and kingfish

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Summary

Parvalbumin is a muscle protein that aids in relaxation from contraction. Parvalbumin binds myoplasmic Ca^{2+} during contractions, reducing calcium concentration and enhancing relaxation. Different isoforms of parvalbumin have varying affinities for calcium, and relaxation rates in skeletal muscle may be affected by variations in the isoforms of parvalbumin expressed. This study examines the effect of expression levels of parvalbumin isoforms on relaxation rate in the sheepshead, *Archosargus probatocephalus* (Pisces, F. Sparidae). We measured relaxation rate of each of the three fiber types, white (fast-twitch), red (slow-twitch) and pink (intermediate), from three longitudinal body positions. Sheepshead show a significant longitudinal shift in relaxation rate in red muscle, with anterior muscle displaying faster rates of relaxation than posterior, but this pattern was not significant in the pink and white muscle. We hypothesized that patterns of parvalbumin expression determine relaxation rate along the length of the fish. The prediction is that total parvalbumin content and the relative expression of parvalbumin isoforms will differ between the anterior and posterior red muscle, but little longitudinal variation will be observed in parvalbumin expression in white and pink muscle. We successfully employed protein electrophoresis (SDS–PAGE) with western blots to identify two parvalbumin isoforms in each muscle fiber type. SDS–PAGE and densitometry were used to determine the relative expression levels of the two parvalbumin isoforms and total parvalbumin expression. Red muscle displays a significant shift, from anterior to posterior, in the relative expression of the two isoforms, both in their relative contribution and in total parvalbumin content, but white and pink muscle did not. The red muscle of southern kingfish, *Menticirrhus americanus* (Pisces, F. Scianidae) showed a pattern similar to the red muscle of sheepshead.

Key words: parvalbumin, sheepshead, *Archosargus probatocephalus,* southern kingfish, *Menticirrhus americanus*, SDS–PAGE, protein analysis.

Introduction

Fish vary in terms of swimming form and can employ a variety of swimming gaits. The gaits are recruited depending on performance level and power needs. Typically, the median and paired fins work together to propel fish at lower speeds whereas the body and caudal fins are used for undulatory swimming at higher speeds (Webb, 1998). Undulatory swimming that employs the body involves sinusoidal waves of body bending that travel longitudinally down the axis of the body. The amplitude of the wave typically increases as it passes to the tail, with thrust generation ultimately based on either drag- or lift-based mechanisms (Biewener, 2003). Swimming mode depends on the physiological properties and recruitment of the various myotomal muscle fiber types. Red, slow-twitch fibers provide the propulsive energy to propel fish at low undulatory swimming speeds, since they have limited oscillatory frequency (Rome et al., 1984). At higher swimming speeds, the pink and/or white muscle fibers are recruited (Coughlin and Rome, 1999). White fibers, which are recruited for faster swimming speeds, have a high power output but fatigue readily in most fish species (Webb, 1994).

Red, pink and white muscles represent fiber types that vary in terms of physiological, morphological and biochemical properties. This variability is responsible for the diversity found in the structure and function of muscles (Berchtold et al., 2000) and contributes to the diversity of fish swimming forms. The variation in the speed of contraction between the fiber types is attributable to a variety of elements in the structure of muscle fibers. The protein myosin heavy chain, part of the myosin hexamer, defines the maximal (as well as optimal) shortening velocity of muscle (Moss, 1995). Muscle activation (rate of increase in force) is dependent on the rates of release of Ca^{2+} from the sarcoplasmic reticulum, Ca^{2+} binding affinity of troponin isoforms and on the myosin-ATPase activity (Rome et al., 1984). Muscle contraction begins when a muscle is stimulated by a motor neuron and the sarcoplasmic reticulum

releases Ca^{2+}. The Ca^{2+} is bound by the troponin complex, leading to conformational changes in the troponin complex and tropomyosin and eventually leading to the exposure of the myosin binding sites on the actin filament and cross-bridge formation. Muscle relaxation depends on the on–off rate of Ca^{2+} from troponin and on the rate that Ca^{2+} is returned to the sarcoplasmic reticulum *via* Ca^{2+}-ATPase pumps.

Physiological properties of muscles such as contraction and relaxation are highly dependent on the types or isoforms of various muscle proteins (Berchtold et al., 2000). Different muscle fiber types display different physiological properties resulting from variations in the isoform(s) of myosin, troponin and other myofibrillar proteins. In fishes, variations can also be seen within a given muscle fiber type along the length of a myotomal swimming musculature (Coughlin, 2002). Subtle variations in the relative contribution of different isoforms of a given muscle protein appear to account to this intra-fiber type variation. For instance, Coughlin et al. (2005) showed that the relative expression of two isoforms of troponin T, a member of the troponin complex, varied along the length of the fish. The study demonstrated that there was a significant shift in the relative expression of two TnT isoforms from the anterior to the posterior in the red muscle of rainbow trout *Oncorhynchus mykiss*. In addition, this variation in TnT expression correlated with muscle contraction properties along the length of the fish: the anterior muscle had faster rates of activation (Coughlin et al., 2005).

Fish also commonly show longitudinal variation in muscle relaxation rate (Coughlin, 2002). This study focuses on the contribution of a myoplasmic protein, parvalbumin, in aiding relaxation. Parvalbumin is a low molecular mass protein (9-11 kDa) that binds free Ca^{2+}, thereby reducing intracellular [Ca^{2+}] in muscle and neurons. It aids relaxation from contraction in muscle and increases the rate of firing of neurons. Each molecule of parvalbumin has two binding sites, and these sites have high affinity for Ca^{2+} and moderate affinity for Mg^{2+}. Parvalbumin binds Ca^{2+} with a higher affinity than troponin C, but less affinity than the sarcoplasmic reticulum Ca^{2+} ATPase pumps (Berchtold et al., 2000). As Ca^{2+} is pumped back into the sarcoplasmic reticulum by Ca^{2+}-ATPase pumps and the myoplasmic [Ca^{2+}] decreases, parvalbumin competes with TnC to bind to the sarcoplasmic Ca^{2+}, accelerating the relaxation of muscle. There is a wide range in total parvalbumin content in fish muscle, from zero to >1.5 mmol l^{-1} (Gillis, 1985). The amount of parvalbumin present in a given muscle will affect relaxation. Greater parvalbumin content is typically associated with fast-twitch muscle of various vertebrates – muscle with relatively high rates of relaxation (Heizmann et al., 1982; Hou et al., 1991; Berchtold et al., 2000).

For parvalbumin to bind Ca^{2+}, Mg^{2+} must not be bound. The dissociation rate for Mg^{2+} is thought to determine the physiological properties of parvalbumin and determine its contribution to relaxation rate, particularly in sub-maximal tetanic contractions (Hou et al., 1991, 1993). Dissociation rates of Mg^{2+} from parvalbumin might vary between isoforms of parvalbumin. However, this has not been tested. Prolonged muscle stimulation leads to saturation of the available parvalbumin, diminishing its contribution to relaxation (Hou et al., 1991; Raymackers et al., 2000). Dissociation rates for Ca^{2+} determine how quickly parvalbumin is able to recover from saturation (Hou et al., 1991). Although Gillis (1985) suggested that parvalbumin isoforms show little variation in terms of Ca^{2+} and Mg^{2+} binding, recent work has demonstrated variations in Ca^{2+} binding affinity in the form of differences in Ca^{2+} dissociation constants for parvalbumin from different fish species (Erickson et al., 2005). This lends credence to the hypothesis that different forms of parvalbumin might also vary in terms of Mg^{2+} binding affinity and, potentially, Mg^{2+} dissociation rates.

In the muscle of fishes, parvalbumin is reportedly only abundant in fast-twitch muscle (Zawadowska and Supiková, 1992; Berchtold et al., 2000; Chauvigné et al., 2005). However, Hamoir (1978) did suggest that the slow-twitch and cardiac muscle of an Antarctic fish, *Champsocephalus gunnari*, contained parvalbumin. The slow fiber types of this fish were termed ‘yellow’ because of the lack of hemoglobin or myoglobin in the animal, and may represent a special case for the role of parvalbumin in slow-twitch muscle. Other work (Gerday, 1982) suggests that fish red muscle does contain parvalbumin of a fiber-type specific form, although Gillis (1985) indicates that red muscle contains only ‘trace’ amounts of parvalbumin. The differing accounts in the literature as to the role of parvalbumin in slow fish muscle require clarification. Fish muscle, at least white muscle, commonly expresses two to three isoforms of parvalbumin in a given fish (Sanuki et al., 2003). Some teleosts appear to express three to five isoforms of parvalbumin in their white muscle throughout development from larval to adult forms (Gillis, 1985; Chikou et al., 1997; Huriaux et al., 2002; Focant et al., 2003).

In this study, we tested the hypothesis that patterns of parvalbumin expression determine the relaxation rate of the swimming muscle along the length of sheepshead, *Archosargus probatocephalus* (Pisces, F. Sparidae). Our goals were: (1) to characterize the physiological properties of the three myotomal muscle fiber types in sheepshead, red, pink and white, along the length of the fish; (2) to identify parvalbumin isoforms expressed in the myotomal muscle; and (3) to relate protein parvalbumin expression to contractile properties such as relaxation rate. Since it has been reported previously that parvalbumin only exists in fast skeletal muscle fibers and its concentration decreases in larger animals (Berchtold et al., 2000), we added a second fish for the study of red muscle: muscle relaxation and parvalbumin expression was also studied in the red muscle of southern kingfish, *Menticirrhus americanus* (Pisces, F. Scianidae).

Materials and methods

Animals

Sheepshead (*Archosargus probatocephalus* Walbaum 1792) and southern kingfish (*Menticirrhus americanus* Linnaeus 1758) were obtained at Dauphin Island Sea Laboratory,

Dauphin Island, Alabama, USA, during a sabbatical stay by D.J.C. The fish were maintained in marine aquaria at 25°C and fed frozen shrimp. All handling of experimental animals was in accordance with the Widener University Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Physiology experiments

Red, pink and white muscle bundles of sheepshead fish, and red muscle bundles of southern kingfish were used to examine the contractile properties at different positions along the length of the fish. The muscle bundles were extracted from a total of seven sheepshead fish (total length *TL*=26.9±10.1 cm, mass=400.0±319 g) and five southern kingfish (*TL*=22.9±5.2 cm, mass=154.7±96.0 g). The large size range in the sheepshead fish was due to the inclusion of a few large fish (>35 cm *TL*) to permit pink muscle preparations. To perform mechanics experiments, the fish were killed by spinal transection and pithing. The scales were removed and strips of muscles (~1.0 mm wide) were extracted from just above and below the lateral line of the fish. Muscle preparations were taken from three longitudinal body positions: anterior (ANT, 35% *TL*); middle (MID, 55% *TL*); and posterior (POST, 75% *TL*). Subsequent dissection was carried out in physiological saline at 4°C with the use of a stereomicroscope (Coughlin et al., 2005). Live muscle bundles were the length of one myomere (~5–10 mm in sheepshead, 4–5 mm in southern kingfish) with a muscle fiber cross-sectional area of 0.25–1.0 mm^2. Using a muscle mechanics system comprising a servomotor (Cambridge Technology 300S, Cambridge, MA, USA) and a force transducer (Aurora Scientific 404A, Aurora, ON, USA), the muscle bundles were tied into the system and maintained at a temperature of 20°C for all experiments. The physiological saline was aerated gently to supply oxygen and to induce circulation. Experimental control and data collection were carried out using a PC, Keithley-Metrabyte DAS-1601 input/output board (Cleveland, OH, USA) and custom software.

Activation conditions (muscle length, pulse length and amplitude for twitch contractions, stimulus duration and frequency tetanic contractions) for each bundle were optimized to generate the maximal tetanic force. The duration of the stimulus was 200–250 ms for red and pink muscle and 100–125 ms for white muscle and was composed of 2–3 ms pulses at a frequency of 100–200 Hz. The amplitude of each pulse was typically 7–9 V. For tetanic contractions, time of activation (TA) was defined as the time from 10–90% of maximum isometric stress. Time of relaxation (TR) was the time from 90–10% of peak isometric stress. Twitch time (TW 90) was defined as the time from stimulation to 90% recovery (10% of peak isometric stress) in twitch contractions.

Physiological results for red and white muscle are reported only for fish from which a data set could be generated that include ANT, MID and POST bundles from the same fish for red muscle (*N*=5 for each species) and ANT and POST bundles from the same fish for white muscle (*N*=4 for sheepshead). It was difficult to consistently get pink muscle samples from two to three body positions from each fish. Therefore, the pink muscle data presented here represent a collection of measurements with some fish included for more than one body position and others included for only one body position.

At the end of each experiment, the fiber area of the live muscle bundles was estimated based on the width and depth of bundles as measured in the muscle mechanics apparatus. Live fiber area was estimated from bundle cross-section area by multiplying by 0.49 for red and pink muscle and 0.63 for white muscle. These factors are estimates of the live fiber area of a bundle, accounting for ~30% dead fiber area and ~30% connective tissue in red muscle and ~30% dead fiber area and ~10% connective tissue in white muscle. These relatively conservative factors are based on prior experience with histological analysis of muscle (e.g. Coughlin, 2000; Thys et al., 2002). Tension (force per unit area) calculated from measures of force production and the estimated live muscle bundle area ranged from 70–150 kN m^{-2}. No additional analysis of force production between muscle samples was carried out.

Protein analysis

Parvalbumin identification

Prior to analysis of parvalbumin expression, parvalbumin isoforms were identified using SDS–PAGE and western blots. Representative muscle samples were extracted from the myotome of sheepshead (all three fiber types) and southern kingfish (red and white muscle, pink is not evident). The muscle fibers were homogenized using a protocol adapted from that of Lutz et al. (1998) upon the advice of Dr Fred Schachat, Duke University. Muscle samples were weighed, and a homogenization solution [250 mmol l^{-1} sucrose, 100 mmol l^{-1} KCl, 20 mmol l^{-1} Tris-base, 5 mmol l^{-1} EDTA, 1000 μmol l^{-1} phenylmethylsulphonyl fluoride (PMSF), 10 ng μl^{-1} leupeptin, and 10 ng μl^{-1} pepstatin] was added to the sample in a 1:1 v/w ratio. Homogenization was performed using 7.0 ml glass-in-glass grinders. Samples were spun at 11 750 ***g*** for 10 min. The parvalbumin-rich supernatant was removed. The supernatant was partially purified by raising it to 95°C in a water bath for 5 min, after which it was placed on ice and then spun for 10 min at 11 750 ***g***. The resulting supernatant contained parvalbumin and a little other protein (F. Schachat, personal communication). SDS–PAGE samples were prepared using Tricine buffer (BioRad, Hercules, CA, USA).

For western blots, 25 μl of sample were loaded onto a 16.5% Tris-tricine/peptide precast gel (BioRad). The gel was kept at 4°C and was run at 50 V for 30 min and 100–125 V for 3–4 h. Parvalbumin from the SDS–PAGE gel was transferred to the polyvinylidene difluoride (PVDF) membrane using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad) using Towbin buffer. After the application of the parvalbumin, the PVDF membrane was blocked using 3% gelatin in Tris-buffered saline (TBS) and rinsed in Tween 20–Tris-buffered saline

(TTBS). An antibody solution (1:1000 dilution of anti-parvalbumin antibody; Sigma, P3088) in antibody buffer (1% gelatin in TBS) was applied. After 2 h of incubation with gentle agitation, the membrane was washed with TTBS, and the secondary antibody solution was added for 30 min [1:1000 dilution of goat anti-mouse IgG (Sigma A-3688) in antibody buffer]. The membrane was washed in TTBS and TBS, and an alkaline phosphatase color development solution (AP Color Development; BioRad) was used. The membrane was allowed to incubate until bands were fully visible. Membranes were scanned for further analysis using Kodak 1-D gel analysis software. Subsequent to the transfer of protein to the membrane, the gel was stained with either silver stain or SyproRuby stain. This permitted determination of the apparent molecular mass (in daltons) of parvalbumin identified by western blot.

Analysis of parvalbumin expression

Subsequent to any muscle mechanics experiments, muscle samples were extracted for analysis of parvalbumin expression. For sheepshead on which muscle physiology measurements were made, red, pink and white muscle samples were extracted from seven body positions (25, 35, 45, 55, 65, 75, 85% *TL*). For kingfish, samples were obtained of the red muscle from three body positions (35, 55, 75% *TL*). For red and white muscle of sheepshead and red muscle of southern kingfish, subsequent analysis of parvalbumin expression was carried out only for fish from which physiological data are reported. For pink muscle, parvalbumin expression is described for three fish.

The SDS–PAGE gel was washed in SyproRuby buffer for 30 min (10% methanol, 7% acetic acid in dH_2O), and then incubated with gentle agitation in SyproRuby overnight at room temperature. The gel was then de-stained using SyproRuby buffer for a minimum of 30 min. To determine the relative expressions of the parvalbumin isoforms, densitometry analysis was performed on the SDS–PAGE gels using Kodak 1-D gel analysis software. The program permitted estimation of the relative intensity of expression of the two parvalbumin isoforms (Parv1 and Parv2) observed in muscle samples at each body position along the length of the fish. For each sample, the background was subtracted using the Kodak software, and a Gaussian algorithm was used to fit a curve to the absorption pattern of the peaks for the two parvalbumin isoforms. In practice, the two peaks could be readily resolved. The proportion of the larger isoform (Parv1) was quantified as a proportion of total parvalbumin for each muscle sample. The method of parvalbumin isolation did not permit rigorous, calibrated determination of total parvalbumin, but relative amounts of total parvalbumin were determined for each body position of a given muscle fiber type for each fish. To control for variations in loading between samples of a given muscle fiber type from a given fish, actin expression was used to correct total parvalbumin. The sum of intensity of expression of the two parvalbumin isoforms was divided by the intensity of expression of actin. Actin was used under the assumption that total actin expression would not vary with longitudinal position for a given muscle fiber type and has been used previously in studies of parvalbumin expression (e.g. Thys et al., 1998, 2001). Actin was readily identified based on molecular size (~42 kDa). To facilitate comparison between individuals for a given fiber type, corrected total parvalbumin expression for a given fish was normalized relative to the body position of maximum parvalbumin expression for that fish.

Statistical analysis

Contractile properties, total parvalbumin expression and the relative expression of parvalbumin isoforms were examined relative to body length using two-factor ANOVA without replication. Parvalbumin expression data, which were proportions, were log-transformed to permit their analysis with ANOVA. Body position and individual fish were the two factors. For all but a few tests, the individual fish factor had no significant effect ($P>0.05$) on the dependent variable (i.e. parvalbumin expression or relaxation rate). The exceptions are reported in the Results. Pink muscle contraction kinetics were analyzed using a *t*-test because the anterior and posterior data were not from the same fish. The results of ANOVAs and *t*-tests are given as the test statistic with degrees of freedom in parentheses and the *P* value of the test. Simple linear regression and multiple regression were used to relate the parvalbumin expression variables to relaxation time.

Results

Muscle physiology

Sheepshead and southern kingfish red muscle show a longitudinal shift in relaxation time (Fig. 1). For both species, the posterior muscle has a relaxation time of close to twice that of the anterior muscle. However, only in the sheepshead is this relationship significant (two factor ANOVA without replication, $F_{(2,8)}=4.65$, $P=0.046$ for sheepshead; $F_{(2,6)}=3.97$, $P=0.079$ for kingfish). There was a significant difference of relaxation rate in individual kingfish ($F_{(3,6)}=9.15$, $P=0.011$ for kingfish); one fish showed the opposite pattern of faster relaxation in the posterior muscle. Both white and pink muscle of sheepshead show modest trends towards slower relaxation rates in the posterior, but the relationship is not significant in for either muscle fiber type ($F_{(1,3)}=0.815$, $P=0.433$ for white muscle; $t_{(11)}=0.465$, $P=0.651$ for pink muscle).

In terms of time of activation, only kingfish red muscle showed a pattern of longitudinal variation, with the posterior muscle activating significantly slower than the anterior ($F_{(2,6)}=6.105$, $P=0.036$ for kingfish red; $F_{(2,8)}=0.39$, $P=0.684$ for sheepshead red; $F_{(1,3)}=5.16$, $P=0.107$ for sheepshead white; and $t_{(11)}=0.625$, $P=0.544$ for sheepshead pink). Only sheepshead red muscle showed a significant affect of body position on twitch time ($F_{(2,8)}=5.78$, $P=0.023$ for sheepshead red; $F_{(2,6)}=2.36$, $P=0.175$ for kingfish red; $F_{(1,3)}=0.663$, $P=0.475$ for sheepshead white; and $t_{(11)}=0.205$, $P=0.841$ for sheepshead pink).

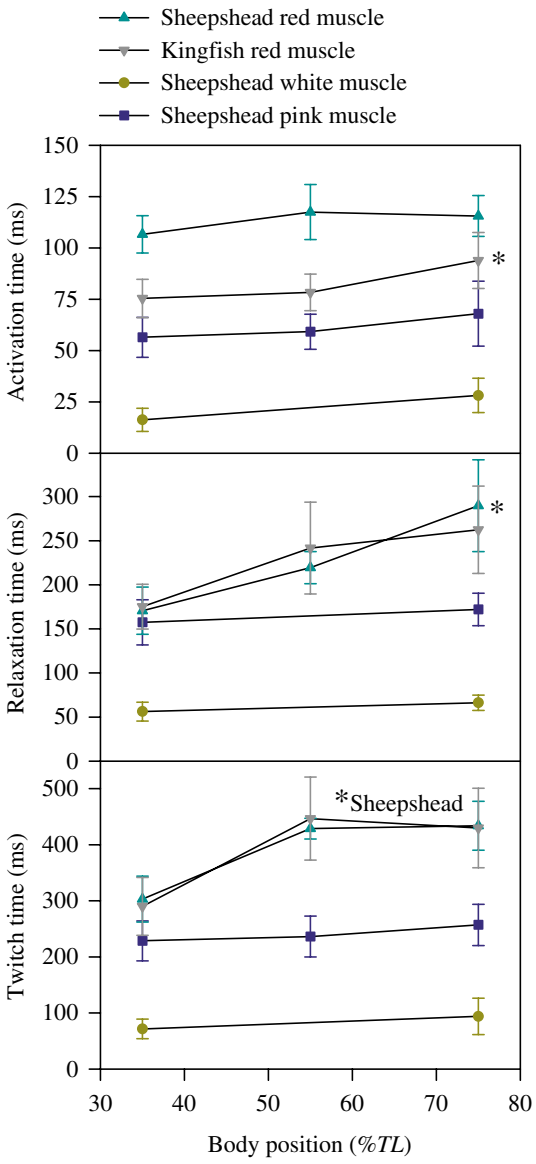

Fig. 1. Tetanic activation and relaxation time and twitch time for isometric contractions in sheepshead and kingfish muscle. Values (means ± s.e.m.) are given at three body positions for red muscle and two body positions for white and pink muscle. In kingfish red muscle there is a significant effect of longitudinal position on activation time (asterisk). Sheepshead red muscle there is a significant effect of longitudinal position on relaxation time and twitch time (asterisk). Definitions of variables and statistical analysis can be found in the text. *N*=5 (sheepshead red muscle), *N*=4 (kingfish red muscle and sheepshead white muscle), *N*=6 (sheepshead pink muscle).

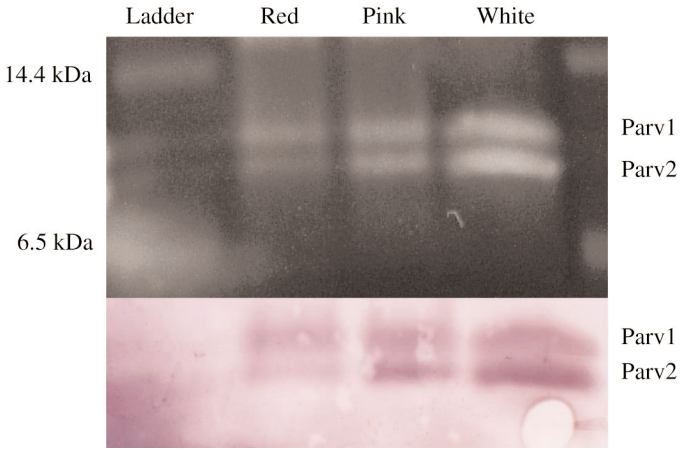

Fig. 2. Identification of parvalbumin in sheepshead muscle. Upper image is a SyproRuby-stained SDS–PAGE gel showing a pair of bands in the 10–11 kDa range in red, pink and white muscle of sheepshead. The lower image is a western blot of the same gel employing an anti-parvalbumin antibody. All three muscle fiber types appear to express the same two parvalbumin isoforms.

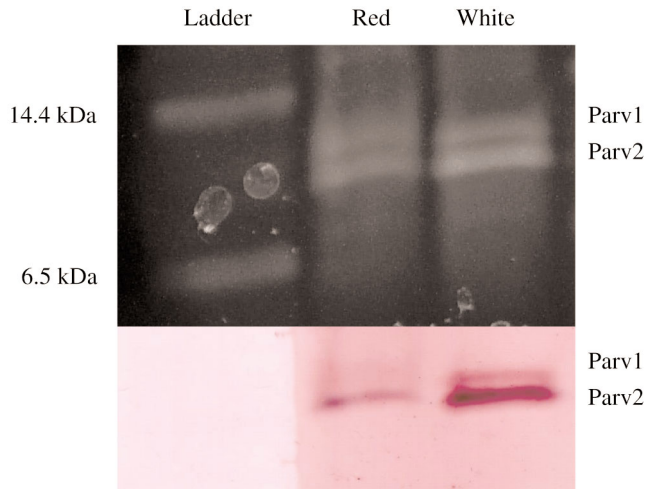

Fig. 3. Identification of parvalbumin in kingfish muscle. Upper image is a SyproRuby-stained SDS–PAGE gel showing a pair of bands in the 10–11 kDa range in red and white muscle of kingfish. The lower image is a western blot of the same gel employing an anti-parvalbumin antibody. Both muscle fiber types appear to express the same two parvalbumin isoforms. The larger (upper) isoform has relatively low affinity for the antibody.

Parvalbumin analysis

Two isoforms of parvalbumin were identified in red, pink, and white muscle of sheepshead fish (Fig. 2) and red and white muscle of southern kingfish (Fig. 3). All samples of red, pink and white muscle from sheepshead appeared to contain the same size parvalbumin isforms. The estimated size of the larger parvalbumin isoform (Parv1) in Sheepshead was 11.6 kDa and the smaller (Parv2) was 10.3 kDa. For southern kingfish, the estimated parvalbumin sizes were 11.4 kDa for Parv1 and 9.5 kDa for Parv2. For both fish species, the greater staining intensity of the white muscle samples relative to red (and pink) are indicative of a higher parvalbumin content in this muscle.

For sheepshead red, pink and white muscle and for kingfish red muscle, the relative contribution of Parv1 and Parv2 to total parvalbumin was determined by examining parvalbumin expression in seven positions along the body length of sheepshead and three positions in kingfish (e.g. Figs 4 and 5).

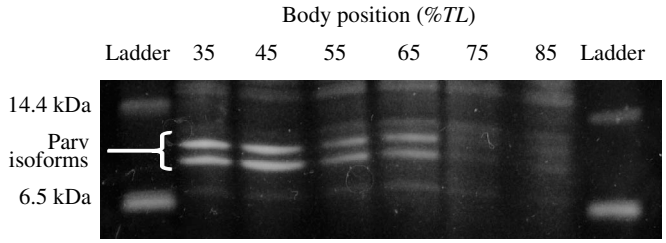

Fig. 4. SDS–PAGE showing parvalbumin expression in sheepshead red muscle from six body positions, ranging from 35–85% of the body length from the snout. The two isoforms occur in all body positions. In this animal, there is a gradual increase in the relative contribution of Parv1, from 50% Parv1 at 35% of body length to 67% Parv1 at 85% of body length. There is also a gradual decrease in total parvalbumin expression from anterior to posterior.

The proportion of Parv1, the larger isoform of parvalbumin, increased from anterior to posterior in all muscle types (Fig. 6), although this effect was only significant in sheepshead and kingfish red muscle ($F_{(4,16)}$=3.56, P=0.029 sheepshead red; $F_{(2,8)}$=7.24, P=0.016 kingfish red; $F_{(5,20)}$=2.39, P=0.074 sheepshead white; and $F_{(4,8)}$=3.46, P=0.063 sheepshead pink). As indicated in the Materials and methods, there were a few cases of individual effects. For instance, there were significant effects of individual fish on relative isoform expression (proportion of Parv1) for sheepshead red, pink and white muscle ($F_{(4,16)}$=7.31, P=0.002 for sheepshead red; $F_{(2,8)}$=2.91, P=0.092 for kingfish red; $F_{(5,20)}$=4.67, P=0.008 for sheepshead white; and $F_{(4,8)}$=15.52, P=0.002 for sheepshead pink). Sheepshead red and white and kingfish red muscle gels were also analyzed for total parvalbumin at each body position, which was expressed as a normalized proportion along the length of each fish for each fiber type (Fig. 7). The process of normalization was described above. For all three muscle types, there was a trend for more parvalbumin expression in the anterior (Fig. 7). However, this was significant only for sheepshead red muscle ($F_{(4,16)}$=3.82, P=0.023 for sheepshead red; $F_{(5,20)}$=1.65, P=0.192 for sheepshead white; and $F_{(2,8)}$=3.011, P=0.106 for kingfish red).

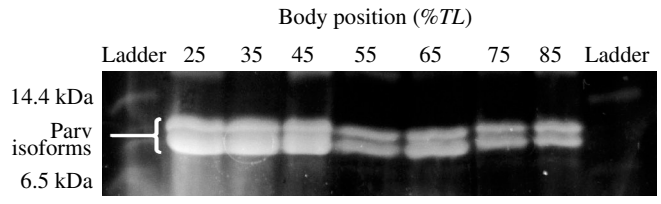

Fig. 5. SDS–PAGE showing parvalbumin expression in sheepshead white muscle from seven body positions ranging from 25–85% of the body length from the snout. The two isoforms are evident in all body positions. In this animal, there is a gradual increase in the relative contribution of Parv1, from 45% Parv1 at 25% of body length to 60% Parv1 at 85% of body length. There is a clear decrease in total parvalbumin expression from anterior to posterior.

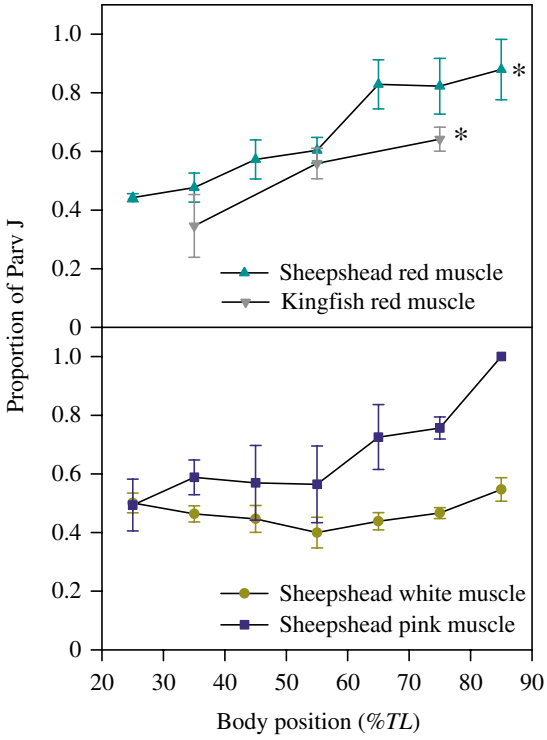

Fig. 6. Expression of the larger parvalbumin isoform, Parv1, relative to total parvalbumin content (expressed as a proportion). Values (mean ± s.e.m.) are given for 7 body positions for all sheepshead muscle types and three body positions for red muscle of kingfish. In sheepshead and kingfish red muscle, there is a significant longitudinal shift in parvalbumin expression, with a higher proportion of Parv1 in the posterior swimming muscle. There is also a similar pattern of Parv1 content in the other sheepshead muscle fiber types but this is not statistically significant. N=6 (sheepshead red muscle), 4 (kingfish red muscle and sheepshead white muscle), 3 (sheepshead pink muscle).

Regression analysis was used to relate parvalbumin expression to relaxation rate of the muscle. Because of the significant individual effects, relaxation rate was plotted as a function of either relative expression of parvalbumin isoform or total parvalbumin for individual fish (Figs 8 and 9). For kingfish red muscle, all four individuals showed the same general pattern of relaxation, time increasing with the relative contribution of the Parv1 (Fig. 8, top). Further, all four fish showed the pattern of decreasing relaxation time with increasing total parvalbumin expression (Fig. 9, top). The same pattern was observed in the red muscle of the five sheepshead (Figs 8 and 9, middle) except for one individual that showed little variation in relaxation time relative to expression of Parv1. Lastly, sheepshead muscle shows little variation in relaxation time relative to either parvalbumin expression variable (Figs 8 and 9, bottom).

When the data from all fish for a given muscle type were combined, there was a significant linear regression of relaxation rate as a function of the relative amount of Parv1 in sheepshead and kingfish red muscle ($F_{(1,13)}$=5.11, P=0.042

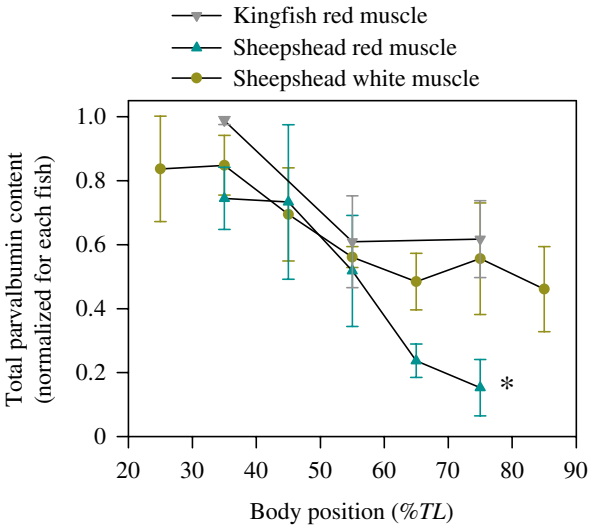

Fig. 7. Total parvalbumin in sheepshead red and white and kingfish red muscle. Values (mean ± s.e.m.) of normalized total parvalbumin are given for each muscle fiber types from several body positions. The means reflect the relative expression for a given muscle fiber type, but comparisons of the values between muscle fiber types are not valid. All fiber types show a longitudinal pattern of higher parvalbumin content in the anterior myotome, but the effect is only statistically significant for the sheepshead red muscle. N=5 (sheepshead red muscle), 4 (sheepshead white and kingfish red muscle).

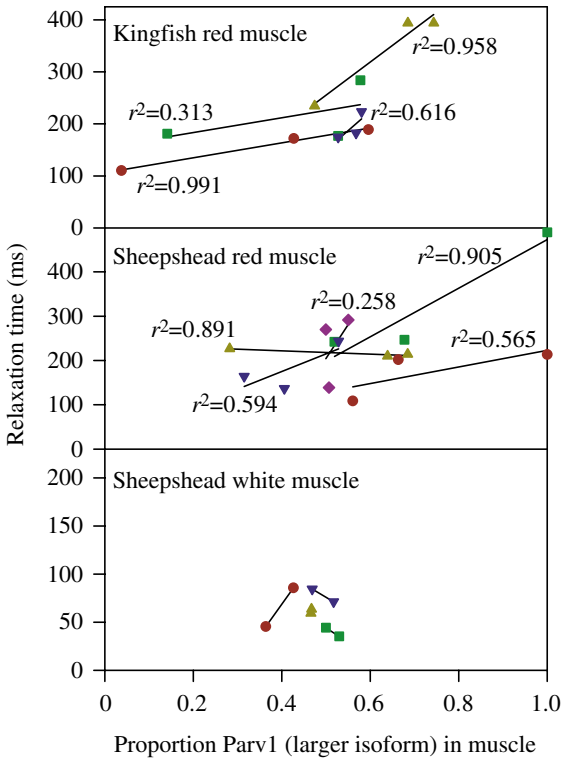

Fig. 8. Relationship of relaxation time to parvalbumin expression in sheepshead and kingfish muscle. For each fish (different colored symbols), the relationship of relaxation time to the relative expression of the Parv1 isoform is shown. r^2 values are given for lines for which there is a substantial variation in the independent variable (and at least three points). All four kingfish show the same pattern of increasing relaxation time with increasing relative contribution of Parv1. Four of the five sheepshead show the same pattern in the red muscle but no clear trend is evident in the sheepshead white muscle data.

for sheepshead; $F_{(1,10)}$=9.44, P=0.012 for kingfish). This relationship was not significant for sheepshead white muscle ($F_{(1,8)}$=0.056, P=0.820). For total parvalbumin, sheepshead red again showed a significant linear regression of relaxation rate as a function of normalized total parvalbumin ($F_{(1,10)}$=7.07, P=0.020), while kingfish red and sheepshead white did not ($F_{(1,10)}$=0.432, P=0.526 for kingfish red; and $F_{(1,8)}$=0.049, P=0.829 for sheepshead white). A multiple linear regression of relaxation rate as a function of relative amount of Parv1 and normalized total parvalbumin was significant for kingfish and sheepshead red muscle ($F_{(2,9)}$=4.255, P=0.050 for kingfish red; $F_{(2,12)}$=4.685, P=0.031 for sheepshead red) but not for sheepshead white muscle ($F_{(2,6)}$=0.429, P=0.670). For both of the red muscle samples, a trend of faster relaxation (shorter relaxation times) is evidently the product of greater parvalbumin content and relatively higher contribution of Parv2 in the muscle (i.e. lower Parv1 contribution, Figs 10 and 11).

Discussion

Relaxation rate and parvalbumin expression in sheepshead and kingfish muscle

This study offers two insights into the role of parvalbumin in modulating contractile properties: (1) ubiquitous expression of parvalbumin in fish skeletal muscle (both slow and fast) and (2) a significant contribution of parvalbumin to the modulation of longitudinal variations in contractile properties. Parvalbumin was found to be expressed in red, pink and white muscle of sheepshead and red and white muscle of southern kingfish (Figs 3 and 4). Several other species examined in our laboratory, such as brook trout *Salvelinus fontinalis*, rainbow trout *Oncorhyncus mykiss*, spadefish *Chaetodipterus faber* and pinfish *Lagodon rhomboids* (D.J.C., unpublished data), also express parvalbumin in both their red and white muscle. To date, no parvalbumin has been detected in the ventricular muscle of any species we have examined (e.g. brook trout). For red and pink muscle, this is the first study to clearly demonstrate parvalbumin expression in slow twitch muscle in fishes.

In sheepshead red muscle, there is a correlation between a significant shift in relaxation rate along the length of the fish and a significant shift in parvalbumin expression (Figs 1, 6 and 7). Southern kingfish red muscle shows similar patterns, but the shift in relaxation rate was not statistically significant. In red muscle of both species, there is a significant relationship between parvalbumin expression and relaxation rate for muscle samples where both contractile properties and parvalbumin content could be assessed (Figs 8 and 9). Multiple regression links total parvalbumin content and the relative expression of the two parvalbumin isoforms to the relaxation rate of the

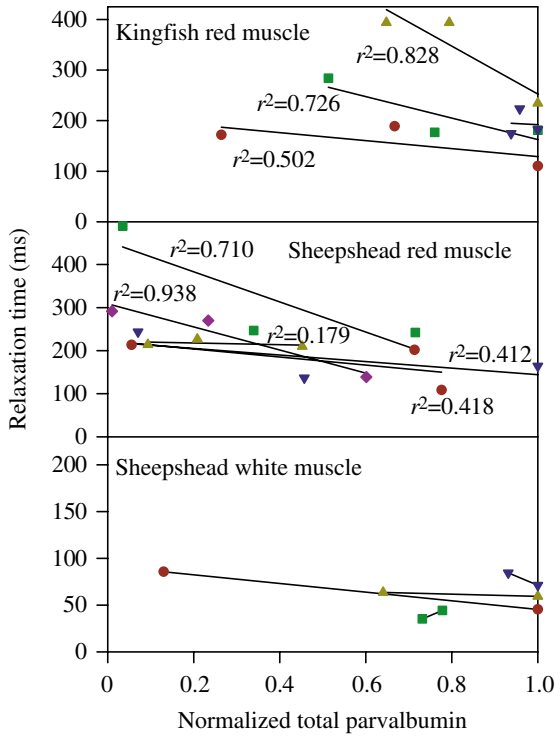

Fig. 9. Relationship of relaxation time to normalized total parvalbumin expression in sheepshead muscle. For each fish (different colored symbols), the relationship of relaxation time to total parvalbumin expression is shown. r^2 values are given for lines for which there is a substantial variation in the independent variable (and at least three points). In the red muscle, all four kingfish and all five sheepshead show the same pattern of decreasing relaxation time with increasing total parvalbumin content. In the white muscle, there is little variation in relaxation time.

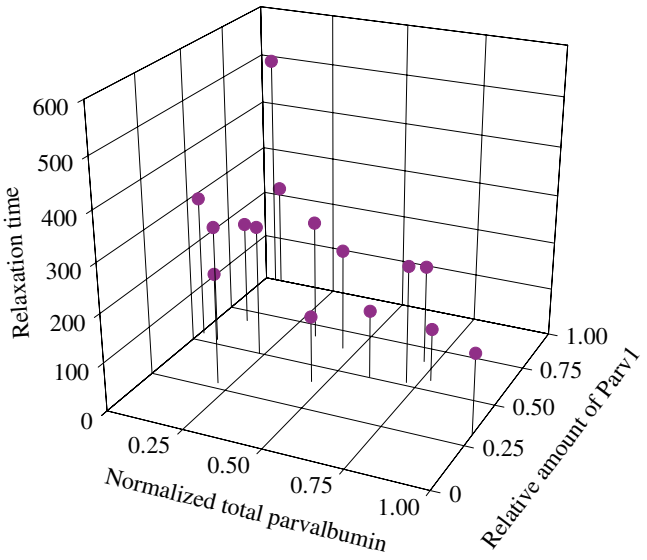

Fig. 10. Relaxation rate as a function of both normalized total parvalbumin and relative Parv1 expression in sheepshead red muscle. The multiple regression had a P value of 0.031. Red muscle equation: relaxation rate=190.3+142.5×relative Parv1 expression–124.0× normalized total parvalbumin.

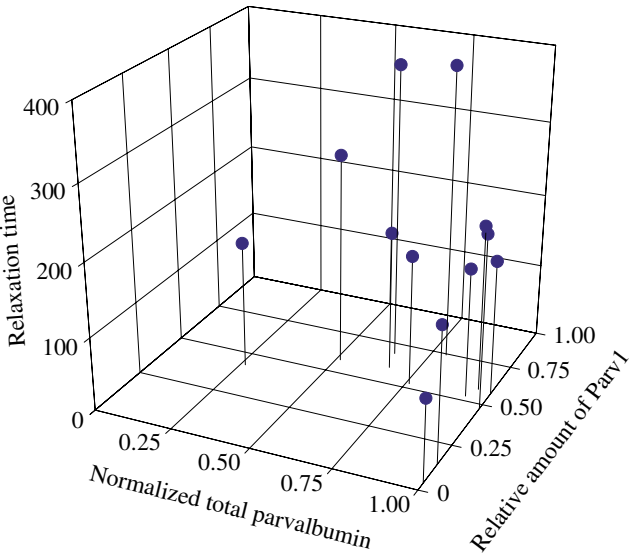

Fig. 11. Relaxation rate as a function of both normalized total parvalbumin and relative Parv1 expression in kingfish red muscle. The multiple regression had a P value of 0.050. Red muscle equation: relaxation rate=86.7+296.5×relative Parv1 expression–7.3× normalized total parvalbumin.

muscle (Figs 10 and 11). In the red muscle fiber types, the faster anterior red muscle expresses a greater level of the smaller isoform of parvalbumin (Parv2). The significant regression of relaxation rate on relative expression of the two parvalbumin isoforms (Fig. 8) suggests that the smaller isoform, Parv2, is kinetically faster.

In pink and white muscle, there were no longitudinal shifts in relaxation time and no statistically significant shifts in parvalbumin expression. This supports our hypothesis, but it was an unexpected result that so little longitudinal variation in relaxation time would be observed in the fast muscle fiber types. Recent work in our lab on brook trout, a fish with a significant longitudinal variation in relaxation rate in the white muscle, suggests a role of parvalbumin in modulating relaxation (D.J.C., unpublished data). As with the red muscle of sheepshead and kingfish, the results on white muscle of brook trout indicate that the smaller isoform of parvalbumin is kinetically faster.

Parvalbumin kinetics in mammalian muscle

A series of elegant experiments with rats and mice have demonstrated the role of parvalbumin in regulating muscle relaxation rate. Schwaller et al. (1999) used parvalbumin knockout mice to demonstrate a connection between relaxation rate and the relative amount of parvalbumin expressed in a fast-twitch muscle in mice, the tibialis anterior. The homozygous knockout mice had prolonged relaxation rate relative to the normal fish. The heterozygous knockout mice had an intermediate level of parvalbumin expression and an intermediate relaxation rate (Schwaller et al., 1999). Similarly, Raymackers et al. (2000) used gene inactivation to show that

rates of relaxation in the extensor digitorum longus (EDL, a fast-twitch muscle) in mice are linked to parvalbumin expression. The parvalbumin-deficient muscle relaxed more slowly.

Using a different approach, Müntener et al. (1995) induced parvalbumin expression in regenerating soleus muscle in cats. The soleus is a slow-twitch muscle that does not normally express parvalbumin. Relaxation rate increased with increasing levels of parvalbumin expression (Müntener et al., 1995). Coutu and Metzger (2002) were able to use gene transfer to induce the expression of parvalbumin in rat cardiac muscle, another muscle that normally does not express parvalbumin. Again, muscle expressing high amounts of parvalbumin relaxed more quickly than normal muscle. In addition, high parvalbumin concentration led to reduced mechanical output, presumably as a result of an attenuated Ca^{2+} transient (Coutu and Metzger, 2002). They were able to demonstrate an 'optimal' parvalbumin content to enhance relaxation but have minimal impact on force production. Chin et al. (2003) reported on a similar experiment involving transgenic mice that expressed parvalbumin in the soleus muscle, a slow muscle that normally contains very little parvalbumin. The expression of parvalbumin did lead to slower contractile properties, and it altered force production in sub-tetanic contractions (Chin et al., 2003). Interestingly, they also reported that transgenic mice also expressed higher levels of fast, type IIa MHC mRNA in their slow muscle than normal mice, but this did not translate into differences in protein composition. Additional evidence for the contribution of the Ca^{2+} binding ability of parvalbumin to relaxation comes from studies employing EDTA (a chelator of divalent cations) as an 'artificial' parvalbumin. In the slow-twitch rat soleus muscle, intracellular EDTA speeds relaxation (Johnson et al., 1999).

Several research groups are examining the use of parvalbumin as a therapeutic agent in aging or diseased muscle. Gene transfer has been used to increase the speed of contraction of cardiac muscle in mice, rats and dogs (e.g. Coutu et al., 2004; Hirsch et al., 2004; Michele et al., 2004; Schmidt et al., 2005). Coutu et al. (2004) showed that in rodents with a hypertrophic cardiomyopathy linked to mutation in tropomyosin, relaxation rate of myocytes can be improved by inducing the expression of parvalbumin. Similarly, Huq et al. (2004) and Schmidt et al. (2005) used gene transfer to induce parvalbumin expression in rat models of aging. Overexpression (or *de novo* expression) of parvalbumin reduces the level of diastolic dysfunction, a trait of aging hearts (Huq et al., 2004). The aged rats that expressed parvalbumin in their cardiac muscle as a result of transgenesis had lower diastolic blood pressure and improved performance, particularly at higher beat frequencies as compared to control aged rats (Schmidt et al., 2005). Although Coutu et al. (2004) expressed concern that working on isolated myocytes might not reflect the true physiological response of muscle *in vivo*, Michele et al. (2004) and Schmidt et al. (2005) showed that parvalbumin expression does have a significant impact on cardiac function in the intact heart. Hirsch et al. (2004) also showed that transgenic expression of parvalbumin enhances relaxation rate in larger mammals (dogs) with diastolic dysfunction. The benefit of transgenic parvalbumin on the activity of myocyctes was similar to that resulting from overexpression of SR Ca^{2+}-ATPase pumps, and the benefit of parvalbumin expression was maintained under physiological stress (B-adrenergic stimulation) while the benefit of expression of SR Ca^{2+}-ATPase was lost (Hirsch et al., 2004). This provided support for the potential role of parvalbumin expression as a therapeutic agent in humans.

Parvalbumin and relaxation in fish muscle

The role of parvalbumin in relaxation of fish muscle has not been studied directly before. Previous attempts to determine the molecular mechanism(s) of variations in relaxation rate were not successful. For instance, Swank et al. (1997) examined the longitudinal variation in relaxation rate in scup *Stenotomus chrysops*, a member of the same family of fishes (Sparidae) as sheepshead. They suggest that relaxation rate does not vary as a function of the number sarcoplasmic Ca^{2+}-ATPase pumps or the myosin heavy chain isoforms expressed at different body positions. They were unable to determine a mechanism of longitudinal variation in relaxation rate in scup. Although they did suggest that parvalbumin might be important, their preliminary results did not suggest that parvalbumin was present in scup red muscle. The present study suggests that parvalbumin is found in red muscle in a variety of teleosts, including several members of the family Sparidae.

Does parvalbumin play a role in modulating relaxation rate in fishes? The present study is the first to suggest that longitudinal variations in relaxation rate in red muscle (a slow fiber type) are explained by variations in parvalbumin content. Other studies have linked parvalbumin in fishes to adaptations for high relaxation rates, particularly in fast muscle fiber types. Parmentier et al. (2003) reported that pearl fish *Carapus acus* have a variety of adaptations in their sonic muscle to permit high frequency oscillations, including a unique isoform of parvalbumin. The functional explanation for their suggestion is that the sonic muscle isoform of parvalbumin would have a faster Mg^{2+} dissociation rate – the same explanation suggested here for the possible differing kinetic properties of the two isoforms of parvalbumin found the swimming musculature of fishes (see below). Hamoir et al. (1980) reported that the oyster toadfish *Opsanus tau* has high parvalbumin concentrations in its sonic muscle, a trait that Feher et al. (1998) suggest facilitates the high frequency oscillations necessary for sound production. Indeed, the latter report indicates that toad fish sonic muscle does not display specific adaptations to increase Ca^{2+} activity, as indicated above for myotomal muscle of scup, leaving parvalbumin expression as the main mechanism of faster relaxation. Thys et al. (1998, 2001) showed correlations between longitudinal patterns of parvalbumin isoform expression and muscle contractile properties of myotomal white muscle in cod and bass. Both species show greater parvalbumin content in the anterior white muscle, which corresponds to faster muscle contractile properties.

We suggest that variation in relaxation rate is *in part* modulated by parvalbumin expression in sheepshead and other fishes. This modulation appears to be a function of both (1) total parvalbumin content and (2) the relative contribution of two different isoforms of parvalbumin, with one isoform being kinetically faster than the other. Evidence from research on mammalian systems and on white muscle in fishes supports the conclusion that the relative total amount of parvalbumin affects relaxation rate (e.g. Schwaller et al., 1999; Thys et al., 2001). To test the suggestion that the relative contribution of the two isoforms of parvalbumin affects relaxation rate, the kinetics of Ca^{2+} binding and Mg^{2+} dissociation of the two parvalbumin isoforms need to be determined. We predict that the two isoforms of parvalbumin would have differing Mg^{2+} dissociation rates, with the smaller isoform (Parv2) having faster kinetics. This prediction would correspond to the observation that muscle containing a greater proportion of Parv2 relative to the larger isoform (Parv1) has a faster rate of relaxation (Fig. 8). Erickson et al. (2005) recently reported on variations in Ca^{2+} dissociation constants (K_D) of parvalbumin from several fish species – two species of Antarctic and two species of temperate fishes. The Ca^{2+} K_D values of all species were similar at their natural environmental temperatures and quite different when tested at the same temperature for all species. This suggests that there are parvalbumin adaptations to maintain function at low temperature in the Antarctic fishes. Importantly, Erickson et al. (2005) showed that fish parvalbumins do vary in terms of binding affinity. It remains to be seen if there are clear differences in Mg^{2+} dissociation constants and, more importantly, Mg^{2+} dissociation rates between fish species or between isoforms of parvalbumin within a given fish.

The role of parvalbumin in fish skeletal muscle opens many opportunities for study. Why might parvalbumin be employed in fish as a means to modulate relaxation rate? Parvalbumin may be a cost-efficient means to alter rates of muscle relaxation without altering the myofibrillar proteins or a host of other muscle contractile properties. The expression of more or less parvalbumin would presumably require a modest level of cellular control. Why does the relaxation rate alter along the length of fish? Work loop experiments on fish suggest that faster rates of relaxation are important to the production of power by anterior red muscle during swimming at maximal sustainable speeds (Rome et al., 1993; Coughlin, 2000). The selective increase in relaxation rate in the anterior permits that muscle to overcome, in part, disadvantageous activation conditions, such as long stimulation time and low levels of oscillatory length change. Why some fish show longitudinal variation in their white muscle relaxation rate and parvalbumin expression (e.g. bass, cod and brook trout) and others do not (e.g. sheepshead) is not clear. However, longitudinal patterns of white muscle function during swimming have not been studied for very many fish species. Further, these species display different swimming forms (i.e. levels of body curvature during swimming). The role of parvalbumin in white muscle contractile properties and *in vivo* power production merits further study.

Lastly, fish muscle may provide a useful animal model for the examination of the impact of parvalbumin on muscle function. The wide variation in parvalbumin content (e.g. anterior *vs* posterior muscle in Fig. 4) and shifts in relative expression of the two isoforms provide opportunities for transgenic manipulation of parvalbumin expression and subsequent examination of contractile properties in swimming musculature.

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