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Slow type muscle cells in the earthworm gizzard with a distinct, Ca^{2+}-regulated myosin isoform

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Summary. Histochemical staining of the gizzard from the earthworm, *Lumbricus terrestris*, reveals low ATPase and high succinic dehydrogenase activity for all muscle cells as compared to the main part of the body wall. In accordance with the presence of slow type muscle cells in the gizzard, isolated actomyosin shows an ATPase activity three times lower than the body wall actomyosin.

Gizzard myosin represents an isoform, distinct from those of the body wall muscle, by comparison of the light chain pattern in isoelectric focusing. No difference was observed in the Ca^{2+}-regulatory properties between gizzard and body wall actomyosin. Gizzard actomyosin is dual-regulated, and the myosin contains a regulatory light chain which is reversibly dissociated by EDTA. Isolated gizzard binds two molecules of Ca^{2+} per molecule, in the same range of free Ca^{2+} concentrations over which actomyosin is activated, suggesting that the myosin-linked regulatory system is mediated by direct binding of Ca^{2+}.

The molar ratios of the major contractile proteins of body wall and gizzard actomyosins differ considerably, indicating a structural diversity of fast and slow type muscle cells.

Introduction

The presence of myosin isoforms in vertebrate skeletal and cardiac muscles has been widely investigated, demonstrating that differences in the ATPase activity of a particular muscle fibre type can be roughly correlated with certain myosin isoforms. These isoforms differ in the composition of their light chains as well as in their heavy chains (review: Weeds 1980). In a recent study, at least 15 different myosin light chains were described for adult rabbit skeletal muscle (Pernelle et al. 1986). Single skeletal muscle fibres were found to contain different myosin heavy and light chain isoforms (Billeter et al. 1981). All these reports indicate that the polymorphism of the myosin component reflects the complexity of vertebrate muscle contraction. Several investigations have demonstrated that the diversity of muscle contraction in invertebrates is not only realized by a different organization of the myofilaments in the muscle cells, but also by heterogeneous isoforms of the contractile proteins (Kondo and Morita 1981; Levine et al. 1986; Miller et al. 1986). Little information exists about the heterogeneity of invertebrate muscle proteins compared to vertebrates.

As recently demonstrated (D'Haese and Carlhoff 1987) two distinct regions can be discriminated in the body wall muscle of the earthworm, *Lumbricus terrestris*, by histochemical, fibre typing, methods, indicating the presence of fast and slow type muscle cells. Each region is characterized by its individual homodimeric myosin isoform. So far it has not been possible to separate these regions for a detailed study of their contractile proteins. As fast type muscle cells exist in a 5 to 6-fold excess over the slow type, the properties determined on isolated proteins of the whole body wall muscle account for the main part only.

Fibre typing methods, applied to cross sections of the earthworm at the region of the gizzard, revealed similar staining pattern as found for those regions of the body wall probably containing slowly contracting muscle cells. Furthermore, SDS-PAGE of gizzard muscle showed that the apparent

Abbreviations: *DTNB* 5,5′-dithio-bis-(2-nitrobenzoic acid); *DTT* dithiothreitol; *EDTA* ethylenediaminetetraacetic acid; *EGTA* ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; *HC* myosin heavy chain(s); *HMM* heavy meromyosin (product of limited proteolytic cleavage of myosin); *IEF* isoelectric focusing; *LC* myosin light chain(s); *PAGE* polyacrylamide gel electrophoresis; *PMSF* phenylmethylsulfonyl fluoride; *SDH* succinic dehydrogenase; *SDS* sodium dodecyl sulfate; *Tris* tris(hydroxymethyl)aminomethane

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molecular masses of the gizzard myosin light chains were identical with those of the slow type myosin isoform of the body wall muscle. On the assumption that both muscular tissues are functionally related, the earthworm gizzard muscle was investigated more extensively to obtain further information on the properties of the contractile proteins of 'slow type' muscle cells in the earthworm.

Materials and methods

Preparation of earthworm muscle contractile proteins. Actomyosin and myosin from earthworm body wall, gizzard muscle as well as the light chains of gizzard myosin were prepared according to D'Haese and Carlhoff (1987) with the exception, that the last purification step including DEAE-Sepharose chromatography was omitted for the gizzard myosin light chain preparation. Special care was taken to avoid proteolytic degradation of gizzard contractile proteins by drastically reducing the preparation time and by increasing the concentration of phenylmethylsulfonyl fluoride (PMSF) to 1 mmol l^{-1} in the wash and extraction solution.

Thin filaments were extracted from body wall and gizzard muscle homogenates in 3 mmol l^{-1} Na_2ATP, 4 mmol l^{-1} $MgCl_2$, 30 mmol l^{-1} Tris-maleate buffer, pH 6.3, 1 mmol l^{-1} DTT, 0.5 mmol l^{-1} PMSF. The suspension was centrifuged at 30000 *g* for 60 min. 1 mmol l^{-1} Na_2ATP was added to the resulting supernatant and thin filaments were sedimented by ultracentrifugation at 100000 *g* for 3 h. The pellet was resuspended in a small volume of 30 mmol l^{-1} Tris, 1 mmol l^{-1} DTT, homogenized and slowly titrated to pH 7.0 with 30 mmol l^{-1} maleate, 1 mmol l^{-1} DTT.

DTNB-extractable light chain of gizzard myosin was isolated at low ionic strength according to Kendrick-Jones et al. (1976) and concentrated by precipitation at 80% ammonium sulfate saturation. Excess DTNB was subsequently removed by dialysis against 20 mmol l^{-1} KCl, 5 mmol l^{-1} 2-mercaptoethanol, 30 mmol l^{-1} Tris-maleate buffer, pH 7.0 with several changes of the dialysis solution.

Actomyosin desensitization and resensitization. Earthworm gizzard and body wall muscle actomyosin were treated for desensitization and resensitization as described (D'Haese 1980a) with the exception that the second desensitization step was performed at 30 °C (Chantler and Szent-Györgyi 1980).

Preparation of rabbit skeletal muscle proteins. Rabbit skeletal muscle myosin isolated by the method of Margossian and Lowey (1982) was proteolytically cleaved according to Weeds and Taylor (1975) to obtain heavy meromyosin (HMM). Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (1971).

Ca^{2+} binding. Ca^{2+}-binding studies were performed by equilibrium dialysis in a Dianorm equilibrium dialysis apparatus (Bachofer, Reutlingen, FRG) equipped with 20 microdialysis cells (2 × 0.2 ml). One side of the dialysis cells was filled with 150 µl of myosin (3–5 mg ml^{-1}) in equilibrium dialysis buffer (50 mmol l^{-1} KCl, 50 mmol l^{-1} imidazole-HCl, pH 7.0, 4 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} $Na_4P_2O_4$). The other side contained 150 µl equilibrium dialysis buffer supplemented with 0.2 mmol l^{-1} $^{40}CaCl_2/^{45}CaCl_2$ and EGTA to adjust the free Ca^{2+} concentration. Different free Ca^{2+} concentrations were obtained by the addition of appropriate amounts of EGTA to the constant amount of $CaCl_2$ (30 nmol, 100000 cpm), adopting a dissociation constant for Ca-EGTA of 2×10^{-7} mol l^{-1} at 25 °C (Weber 1969). Dialysis was performed at 4 °C overnight and for a further 2 h at 25 °C. The dialysate was removed from each side of the cell and retained solution was washed out with 2 × 250 µl of 0.5 mol l^{-1} KCl. Radioactivity was counted in 15 ml aliquots of Quickszint 212 (Zinsser Analytic, Frankfurt, FRG) using a Beckman LS 8000 liquid scintillation counter.

ATPase activity determination. ATPase activity measurements were usually performed in a medium containing 20 mmol l^{-1} KCl, 30 mmol l^{-1} Tris-maleate, pH 7.0, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} MgATP, in the presence of 0.1 mmol l^{-1} $CaCl_2$ or 2 mmol l^{-1} EGTA. The reaction was initiated by the addition of ATP and stopped after 1 to 3 min with 50% trichloroacetic acid (5% final concentration). Liberated phosphate was determined by the method of Fiske and SubbaRow (1925). Ca^{2+} sensitivity is expressed as 1-(ATPase activity at 2 mmol l^{-1} EGTA/ATPase activity at 0.1 mmol l^{-1} Ca^{2+}) × 100 (%). The thin filament fractions were immediately used, without dialysis, to determine the Ca^{2+}-sensitive activation of the HMM ATPase (see Fig. 4). Therefore the ionic conditions were carefully adjusted to standard ATPase assay conditions (see above) and the values obtained were corrected to account for possible myosin impurities and phosphate contaminations from ATP hydrolysis during the preparation of the thin filaments.

For direct comparison with the Ca^{2+}-binding properties the Ca^{2+} dependency of the ATPase activity was measured at the same free Ca^{2+} concentrations using the equilibrium dialysis buffer (see above), omitting $Na_4P_2O_4$.

Histochemical staining. Histochemical staining for Ca^{2+}-activated myosin ATPase, Mg^{2+}-activated actomyosin ATPase, and succinic dehydrogenase (SDH) was carried out essentially as previously described (D'Haese and Carlhoff 1987).

Gel electrophoresis. Electrophoresis on polyacrylamide slab gels in the presence of sodium dodecyl sulfate (SDS-PAGE) and isoelectric focusing (IEF) in the presence of urea was carried out as described (D'Haese and Carlhoff 1987). In some cases focused proteins were identified in the second dimension by SDS-PAGE. For re-electrophoresis, protein bands were visualized by precipitation with 10% trichloracetic acid, after omitting detergent in IEF gels and sample buffer (Hirabayashi 1981).

Determination of protein ratio. For protein ratio determination SDS-PAGE slab gels were used containing a 9 to 17% and 13 to 17% linear polyacrylamide gradient, respectively. Various amounts (50 to 150 µg) of high ionic strength extracts of earthworm gizzard and body wall muscle were applied to the gels. These extracts were used, rather than homogenized muscle pieces, as total muscle revealed an increased number of bands and background staining but almost the same relations between the major contractile proteins. The relative protein content of the different bands was determined according to Fenner et al. (1975). The molar ratios were calculated adopting the following molecular masses in kDa: single myosin heavy chain, 220; single paramyosin chain, 100; actin, 42; LC28, 28; LC18, 18. The LC18 of both extracts appeared as a single, well separated band in the SDS-gels and was chosen as the reference (see Table 1), assuming that the myosin molecule is composed of subunits (HC and the two different types of LC) in a ratio of 2:2:2. The calculated ratios of HC to the accompanying LC fractions were about equimolar for both extracts demonstrating the reliability of the method used.

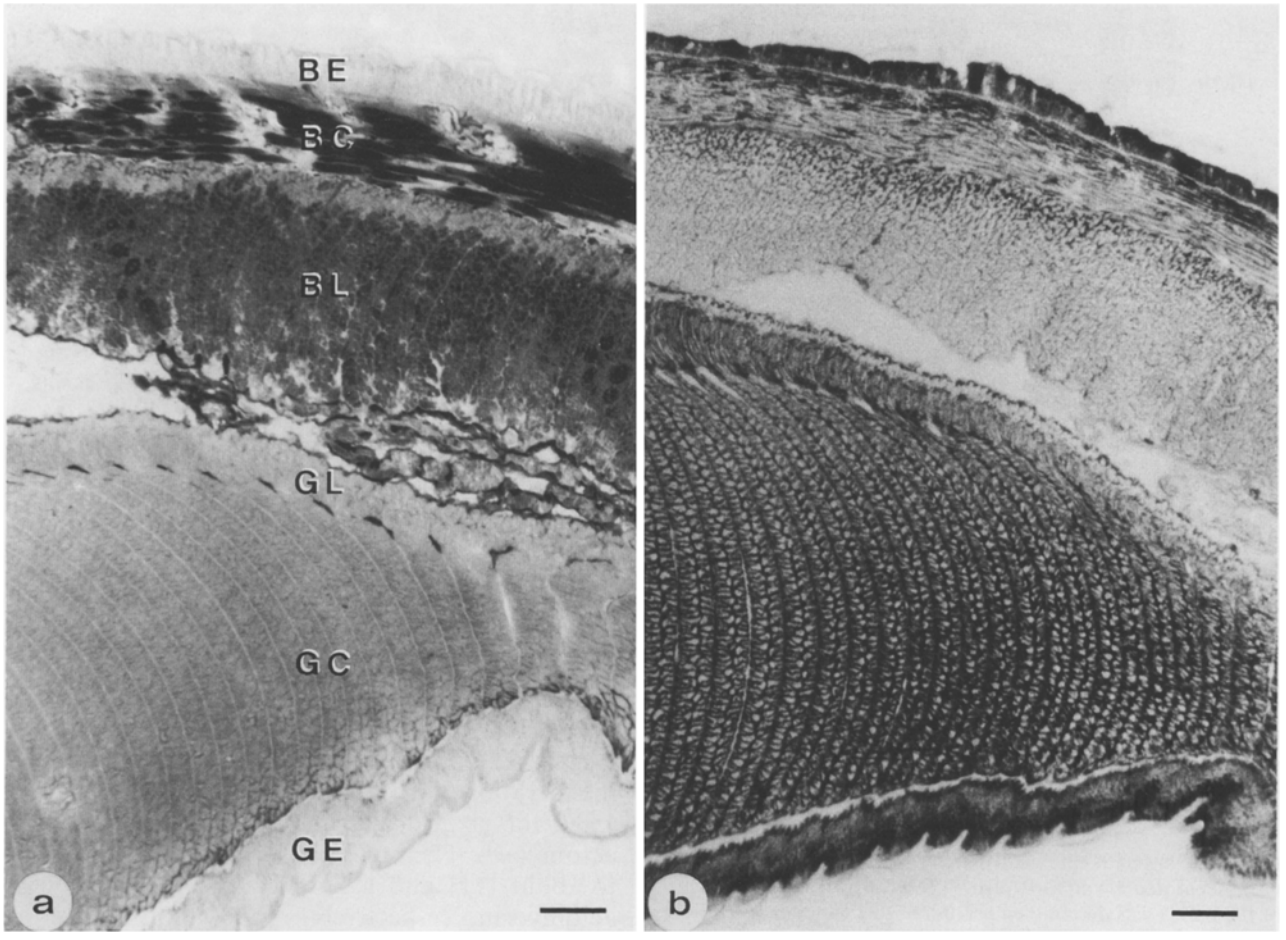

Fig. 1a, b. Histochemical staining of transverse sections from the earthworm at the region of the gizzard showing the main components of body wall and gizzard muscle: *BE* body wall epithelium; *BC* circular muscle layer of the body wall; *BL* longitudinal muscle layer of the body wall; *GL* longitudinal muscle layer of the gizzard; *GC* circular muscle layer of the gizzard; *GE* gizzard epithelium. Cryostat sections were treated histochemically to demonstrate the activities of Ca^{2+}-activated myosin ATPase (**a**) and succinic dehydrogenase (SDH) (**b**). Gizzard muscle revealed low ATPase and high SDH activity in comparison with the main part of the body wall muscle. Magnification × 85. Bars 0.1 mm

Protein concentration. Protein concentration was determined by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard.

Results

Slow type muscle cells in earthworm gizzard

For comparative histochemical staining of gizzard and body wall muscle cryostat cross sections at the region of the gizzard were incubated to demonstrate Ca^{2+}-activated myosin ATPase (Fig. 1a), Mg^{2+}-activated actomyosin ATPase (result not shown), and SDH activity (Fig. 1b). Both ATPase reactions resulted in very similar staining intensities. The body wall is seen in the upper parts of both micrographs with its characteristic staining pattern (D'Haese and Carlhoff 1987). The gizzard is mainly composed of an outer layer of longitudinal muscle (GL), a thick, circular muscle layer (GC), and a 'cuticle' covered epithelium (GE) adjacent to the gut cavity. Histochemical staining revealed homogeneous enzyme activity in all gizzard muscle cells demonstrating low Ca^{2+}-activated myosin and Mg^{2+}-activated actomyosin ATPase activity but high SDH activity. Thus the gizzard muscle showed staining intensities, equal to those of the small distal region of the longitudinal and circular muscle layer of the body wall, which are assumed to contract slowly (D'Haese and Carlhoff 1987).

Composition of the contractile proteins in gizzard muscle

Isolated gizzard muscle protein fractions and the corresponding fractions of the earthworm body

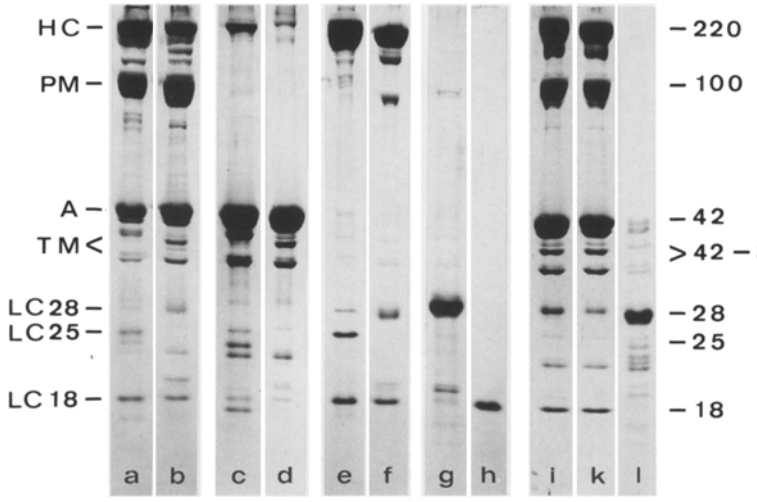

Fig. 2a–l. SDS-PAGE of earthworm body wall and gizzard muscle contractile proteins to illustrate the preparation of actomyosin (**a** and **b**), thin filaments (**c** and **d**), and myosin (**e** and **f**). Gizzard myosin (**f**) contains a 28 kDa DTNB-extractable light chain (LC28) (**g**) and an 18 kDa light chain (LC18) (**h**). Treatment of gizzard actomyosin (**i**) with EDTA led to the extraction of mainly LC28 (**k** and **l**). The bands of myosin heavy chains (*HC*), paramyosin (*PM*), actin (*A*), tropomyosin (*TM*), and myosin light chains (*LC*) are marked. Apparent molecular masses are given in kDa

Table 1. Molar ratios of the contractile proteins extracted from earthworm gizzard and body wall muscle

	Gizzard	Body wall
LC18	2	2
LC28 (LC25 + LC28)	2.02 ± 0.07	1.89 ± 0.27
Actin	11.20 ± 0.60	5.13 ± 0.78
Paramyosin	3.01 ± 0.33	1.75 ± 0.22
Myosin (HC dimer)	0.90 ± 0.04	0.94 ± 0.09

High ionic strength extracts from gizzard and body wall muscle were separated by SDS-PAGE. The relative protein content of the bands was determined according to Fenner et al. (1975). The values obtained for gizzard as well as for body wall myosin LC18 were chosen as the reference assuming a HC to LC molar ratio of 2:4 for the myosin molecule. Values represent the mean ± SD from four independent preparations

wall muscle separated by SDS-PAGE are shown in Fig. 2a–f. Gizzard thin filaments (Fig. 2d) revealed only little contamination with myosin and, besides the predominant bands of actin (A) and tropomyosin (TM), one band with higher mobility and an apparent molecular mass of 23 kDa. This band may correspond to a troponin I-like protein, as a doublet of bands at about 23 and 24 kDa (Fig. 2c) has been characterized as such in the thin filament fraction of the body wall (Ditgens 1983). Gizzard myosin (Fig. 2f) showed two light chain bands of about equal staining intensity with an apparent molecular mass of 28 (LC28) and 18 kDa (LC18). Body wall muscle myosin (Fig. 2e) revealed three different light chains with apparent molecular mass of 28 (LC28), 25 (LC25), and 18 kDa (LC18) according to the presence of two myosin isoforms which differ in their regulatory light chain (LC25 and LC28) (D'Haese and Carlhoff 1987). Thus one of these isoforms – the slow type LC28 myosin – shows an identical band pattern in SDS-PAGE as the gizzard myosin.

Isolated body wall and gizzard actomyosin separated by SDS-PAGE (Fig. 2a and b) showed differences in the relative amounts of the myosin heavy chains, paramyosin, and actin. These differences are summarized in Table 1. The calculated molar ratios of myosin to paramyosin were about 1:3.0 for gizzard and about 1:1.8 for body wall actomyosin. The myosin to actin ratios amounted to about 1:11 and 1:5 for gizzard and body wall actomyosin, respectively. Thus about twice as much paramyosin as well as actin was found in gizzard than in the body wall muscle in relation to myosin.

A distinct myosin isoform in gizzard muscle

Gizzard myosin, which could not be differentiated from the slow type myosin isoform of the body wall muscle in SDS-PAGE, revealed remarkable differences to both body wall myosin isoforms when analysed by IEF (Fig. 3a–c). The differentially charged forms of the light chains, indicated in Fig. 3, were identified by re-electrophoresis of the protein bands on SDS-gels. Focusing of the isolated light chain fractions (Fig. 3d and e) also verified the identification of the predominant bands of gizzard myosin (Fig. 3c). LC28 of the myosin fraction appeared as two bands, a strongly stained more alkaline and a weakly stained more acid one. The purified light chain fraction revealed only the predominant band (Fig. 3d). LC18 of both the purified light chain fraction (Fig. 3e) and the myosin were also focused as two bands with about the same difference in staining intensity like the two bands of the LC28. Interestingly, both mi-

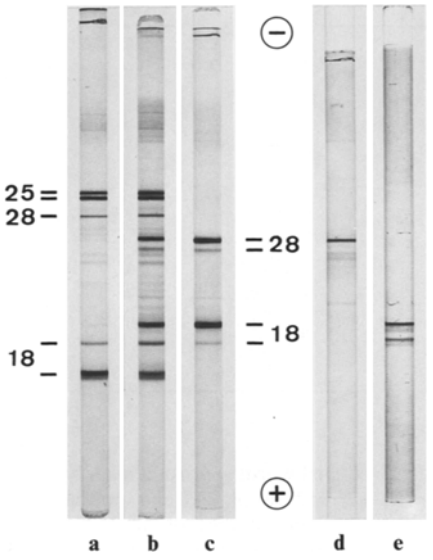

Fig. 3a–e. Isoelectric focusing in the presence of urea of earthworm body wall myosin (**a**), gizzard myosin (**c**), and gizzard myosin light chain fractions (**d** and **e**). The light chain pattern of body wall and gizzard myosin differ considerably, as can be confirmed by a mixture of both myosin fractions run in the same gel tube (**b**). The main bands of isolated gizzard LC28 (**d**) and LC18 (**e**) correspond with those of total gizzard myosin (**c**). The light chains marked on the left for the body wall myosin and in the middle for the gizzard myosin are indicated with their apparent molecular mass in kDa from SDS-PAGE (Fig. 2)

nor components of the gizzard as well as of the body wall LC18 were focused at the same position in the gel (Fig. 3b). According to these results two species of myosin might exist in the earthworm gizzard muscle. Both are obviously different from the body wall muscle myosin isoforms.

ATPase activity and Ca^{2+} regulation of gizzard actomyosin

Isolated gizzard actomyosin revealed a three times lower ATPase activity in comparison with that of isolated body wall actomyosin (cf. Table 2). The activation of the ATPase activity of isolated gizzard actomyosin was Ca^{2+}-dependent with an 80–90% inhibition in the absence of Ca^{2+}. A competitive myosin- or actin-binding assay according to Lehman and Szent-Györgyi (1975) using HMM and purified actin from rabbit skeletal muscle, respectively, has already indicated the presence of an actin-linked as well as a myosin-linked regulatory system (data not shown). To verify dual regulation isolated thin filaments and myosin fractions of gizzard and body wall were tested in parallel. Maximum Ca^{2+} sensitivity of about 60% for the gizzard actin-linked and about 80% for the gizzard myosin-linked regulatory system was determined. These values are lower than those of the body wall

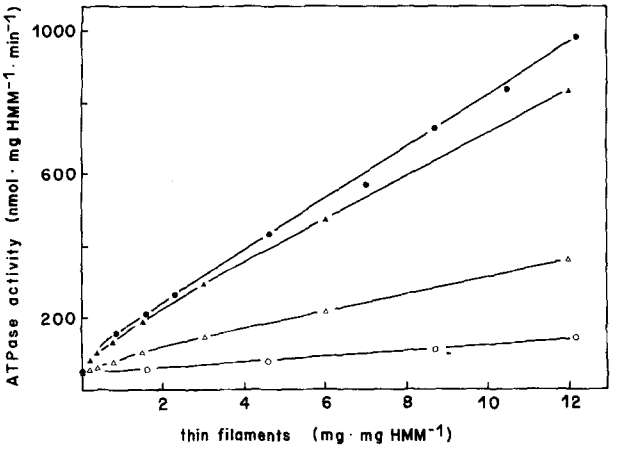

Fig. 4. Ca^{2+}-dependent activation of rabbit skeletal muscle HMM (0.3 mg ml^{-1}) by gizzard thin filaments (▲, △) and body wall thin filaments (●, ○). Closed and open symbols correspond to the activities in the presence (0.1 mmol l^{-1} $CaCl_2$) and absence (2 mmol l^{-1} EGTA) of Ca^{2+}. Gizzard thin filaments form a hybrid, Ca^{2+}-sensitive system together with rabbit skeletal muscle HMM and activate the HMM ATPase in the presence of Ca^{2+} to nearly the same extent as found for the body wall thin filaments

fractions, probably due to a somewhat higher degree of proteolytic degradation of gizzard regulatory proteins. Isolated gizzard thin filaments together with rabbit skeletal muscle HMM formed a hybrid, Ca^{2+}-sensitive system and activated the ATPase of HMM in the presence of Ca^{2+} to nearly the same extent as found for body wall thin filaments. The activation curves (Fig. 4) are nearly linear over a wide range of thin filament amounts. In contrast to the hyperbolic relationship, which is obtained using increasing amounts of purified rabbit skeletal muscle actin for the activation of myosin (Fig. 5), such unusual dependence was reproducibly found as a characteristic property of the earthworm thin filament fractions from the body wall and gizzard muscle. Gizzard myosin was activated by purified rabbit skeletal muscle actin, also in a Ca^{2+}-dependent manner, but revealed a remarkably lower ATPase activity (3 to 6-fold) than body wall myosin. The difference in the ATPase activity also seen in histochemical stainings of earthworm muscle cells obviously depends on the presence of a certain myosin isoform.

Myosin-linked Ca^{2+} regulation

Treatment of gizzard myosin with 10 mmol l^{-1} DTNB led to the extraction of a nearly pure fraction of LC28 (Fig. 2g). LC18 which was not extracted by DTNB was removed from the myosin heavy chain by guanidine-HCl extraction (Fig. 2h). When gizzard actomyosin was treated

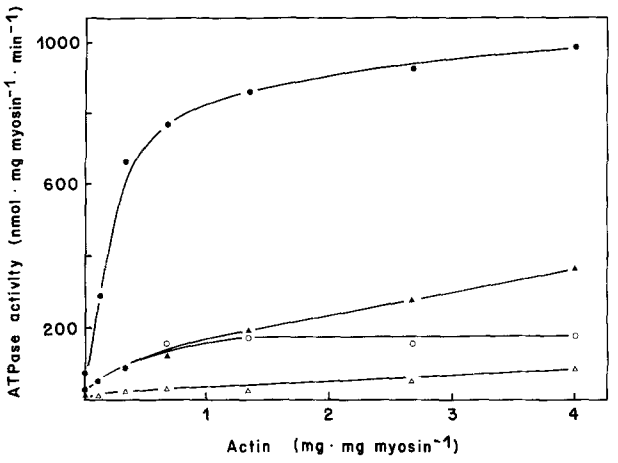

Fig. 5. Ca^{2+}-dependent activation of gizzard myosin (0.45 mg ml^{-1}) (▲, △) and total body wall myosin (0.15 mg ml^{-1}) (●, ○) by purified rabbit skeletal muscle actin. Closed and open symbols correspond to the activation in the presence (0.1 mmol l^{-1} $CaCl_2$) and absence (2 mmol l^{-1} EGTA) of Ca^{2+}, where the activation is depressed by 80 to 90% for both myosins. The ATPase activity of total body wall myosin is about 3 to 6 times higher than that of gizzard myosin

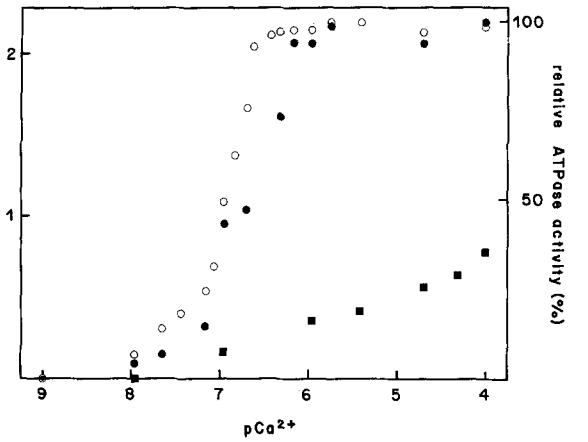

Fig. 6. Ca^{2+} dependence of earthworm gizzard myosin (0.45 mg ml^{-1}) hybridized with purified rabbit skeletal muscle actin (0.6 mg ml^{-1}) (○). Ca^{2+}-binding studies in the presence of 4 mmol l^{-1} Mg^{2+} of earthworm gizzard myosin (●) and rabbit skeletal muscle myosin (■) by equilibrium dialysis with protein concentrations of 3 to 5 mg ml^{-1}. To compare Ca^{2+} binding and ATPase activity, maximum Ca^{2+} binding of 2.2 was equalized with maximum ATPase activity, normalized to 100%. Measured values of 211 and 34 nmol P_i mg myosin^{-1} min^{-1} correspond with 100% and 0% relative ATPase activity, respectively. In contrast to rabbit skeletal muscle myosin two molecules of Ca^{2+} are bound per molecule of earthworm gizzard muscle myosin in the same narrow range of free Ca^{2+} concentrations over which the actin-activated myosin ATPase activity accelerates

Table 2. Recombination of desensitized actomyosin from earthworm gizzard and body wall muscle with gizzard myosin LC28

		ATPase activity (nmol P_i mg^{-1} min^{-1})		Ca^{2+} sensitivity (%)
		$CaCl_2$	EGTA	
Gizzard actomyosin				
Untreated	(0.5 mg)	188	33	82
Densensitized	(0.5 mg)	222	183	18
+LC28	(25 μg)[a]	173	52	70
Body wall actomyosin				
Untreated	(0.5 mg)	525	50	90
Densensitized	(0.5 mg)	552	506	8
+LC28	(25 μg)[a]	570	136	76

Actomyosin was desensitized by EDTA treatment as described in the text. The LC28 fraction used for recombination in this test was obtained by EDTA extraction of gizzard actomyosin (compare with Fig. 2i, k, and l). Assay conditions are as described in 'Materials and methods'

[a] To exclude a restoring effect on the actin-linked regulatory system an excess amount (0.5 mg) of purified actin from the rabbit skeletal muscle was added

with 10 mmol l^{-1} EDTA at 30 °C the majority of the LC28 was released (Fig. 2i, k, and l) and the Ca^{2+} sensitivity was markedly reduced from over 80% to 18% (Table 2). The actomyosin ATPase activity was not reduced. Myosin-linked Ca^{2+} regulation was almost completely restored, when desensitized gizzard as well as body wall actomyosin were recombined with gizzard EDTA-extractable LC28 in the presence of Mg^{2+}. No difference in resensitization was observed using the light chain fraction obtained by DTNB or EDTA treatment of gizzard myosin. The direct influence of this light chain on the Ca^{2+} sensitivity indicates that the LC28 represents the regulatory light chain in gizzard myosin.

To analyse the mode of myosin-linked regulation we measured the amount of Ca^{2+} bound to gizzard myosin at various free Ca^{2+} concentrations by equilibrium dialysis. High affinity, specific binding of Ca^{2+} to myosin occurred in the same narrow range of free Ca^{2+} concentrations, where the actin-activated myosin ATPase activity was accelerated (Fig. 6). About two molecules of Ca^{2+} were bound per molecule of gizzard myosin at a free Ca^{2+} concentration of 1×10^{-6} mol l^{-1}, with a calculated apparent binding constant of approximately 5×10^6 mol^{-1} l. For comparison, Ca^{2+} binding of rabbit skeletal muscle myosin was also determined. At free Ca^{2+} concentrations around 1×10^{-6} mol l^{-1} no significant Ca^{2+} binding was observed and only about 0.8 molecule of Ca^{2+} was bound per molecule of myosin at a free Ca^{2+} concentration of 1×10^{-4} mol l^{-1}. The clear difference obtained for the Ca^{2+} binding between earthworm

gizzard and rabbit skeletal muscle myosin was taken as a strong indication that regulatory properties of gizzard myosin are mediated by direct binding of Ca^{2+}.

Discussion

In this study the gizzard muscle of the earthworm, *Lumbricus terrestris*, was investigated by histochemical fibre typing techniques and biochemical analyses of isolated actomyosin fractions. The results obtained were compared with those of the body wall muscle in order to define the different types of muscle cells with respect to their protein composition and properties of their contractile system.

The molar ratios of the main contractile proteins – myosin, paramyosin, and actin – differed between gizzard and total body wall. The myosin to paramyosin as well as the myosin to actin ratios calculated for the gizzard actomyosin were found to be about half of those for the body wall proteins (Table 1). The relative content of myosin (heavy chains plus light chains), paramyosin, and actin, expressed as percentage of the sum of their total quantity, was 33%, 38%, and 29% for the gizzard and 48%, 32%, and 20% for the body wall, respectively. Thus less myosin but more paramyosin and clearly more actin were extracted from the earthworm gizzard than from the body wall muscle. This may indicate structural differences in the composition and organisation of their myofilaments.

The relation between the structure and the protein composition of adductor muscle myofilaments from seven different molluscan species was investigated in detail by Margulis et al. (1979). It was shown that concurrent with an increase of the relative myosin content the number of thick filaments increases. At the same time a decrease of the relative paramyosin content and a concomitant reduction of the thick filament diameter were observed. An inverse relationship of the relative myosin and paramyosin content was also found, when striated and smooth adductor muscle of the same molluscan species, *Chlamys nipponensis akazara*, were compared (Nishita et al. 1979). Similar findings were made in the translucent and opaque portion of the scallop smooth adductor muscle (Rüegg 1961; Kondo and Morita 1981). A clearly lower myosin but higher paramyosin content was determined for the opaque portion. Furthermore, it was shown that ATPase activity of actomyosin (Rüegg 1961) and of myosin in the presence of rabbit skeletal muscle actin (Kondo and Morita 1981) was about twice as high with protein fractions isolated from the translucent portion than with those from the opaque portion of smooth muscle. The latter is known to be involved in catch contraction. As the ATPase activity and the myosin light chain composition changes gradually between both portions (Morita and Kondo 1982), it was assumed that scallop smooth muscle contains three myosin isoforms. These isoforms, each with a different combination of the two kinds of regulatory light chains, are present in different proportions in both parts of this muscle.

In addition, the ultrastructural organization of the myofilaments and the speed of contraction were correlated by Lanzavecchia et al. (1977) among different obliquely striated muscle fibres in some leeches. It was assumed that the smaller the diameter of the myosin filaments and the higher the ratio of myosin to actin, the faster the fibres contract.

Preliminary evaluations of electron micrographs from earthworm gizzard and body wall muscle cells also indicated that thick filaments with a larger diameter, and a pronounced higher quantity of thin filaments are present in slow type muscle cells of the gizzard and the body wall (unpublished data).

Histochemical staining (Fig. 1a and b) clearly showed that the gizzard contains a different type of muscle cell than the main part of the body wall muscle. The difference in staining intensity of the histochemical ATPase activity is probably not due to the different myosin content in both muscle tissues, since we could demonstrate that these different properties are correlated with biochemical characteristics of the myosin isoforms. A three to six times lower ATPase activity was determined for isolated gizzard actomyosin and myosin fractions in comparison with those of the body wall muscle. Bárány (1967) has shown for vertebrate skeletal muscle that the speed of contraction is correlated with the ATPase activity of myosin extracted from the individual muscles. We therefore conclude that the earthworm gizzard contains only slowly contracting muscle cells in contrast to the body wall muscle which consists mainly of rapidly contracting cells.

Like the body wall actomyosin the gizzard actomyosin was found to be dual-regulated with slightly lower Ca^{2+} sensitivity for the actin- and myosin-linked regulatory system (Figs. 4 and 5). The Ca^{2+} sensitivity of the myosin component was found to be dependent on the presence of a regulatory light chain which could be reversibly removed by EDTA treatment (Table 2), as in the earthworm body wall muscle (D'Haese 1980a) and in some

molluscan muscles (Szent-Györgyi et al. 1973; Kendrick-Jones et al. 1976; Nishita et al. 1979; Konno et al. 1979). The regulatory properties of earthworm gizzard myosin are obviously mediated by high affinity, direct binding of Ca^{2+}. Two molecules of Ca^{2+} are bound specifically per molecule of myosin in the same range of free Ca^{2+} concentrations where actomyosin is activated (Fig. 6). In contrast, parallel experiments with skeletal muscle myosin revealed only low affinity, unspecific binding of Ca^{2+}. Similar differences in the Ca^{2+}-binding properties were obtained by a comparison of myosin from various molluscan adductor muscles with that from rabbit skeletal muscle (Kendrick-Jones et al. 1970; Hirata et al. 1980). For the nematode, *Ascaris suum*, phosphorylation of a particular myosin light chain was reported by Donahue et al. (1985). They assumed that the mechanism of regulation in *Ascaris* muscle contraction is similar to that established for vertebrate skeletal muscle. Experiments using the light chain kinase of chicken gizzard muscle did not show any phosphorylation of earthworm myosin (D'Haese 1980b).

Together with the previously presented investigations our results demonstrate the existence of at least three homodimeric myosin isoforms in earthworm muscle. They also demonstrate that these isoforms are mutually exclusively distributed. Two of them have been found in strictly separated regions of both the circular and the longitudinal muscle layer of the body wall and the third one is located solely in the gizzard muscle. The agreement of histochemical staining and ATPase activity measurements indicate that the body wall muscle contains a fast as well as a slow type myosin isoform and that gizzard myosin belongs to the latter type. Furthermore it strongly suggests a correlation of particular myosin isoforms to slowly and rapidly contracting muscle cells. Despite these differences of myosin isoforms a myosin-linked regulation, mediated by direct binding of Ca^{2+}, can be assumed for both cell types of earthworm muscles.

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