Identification of myosin light chains in \textit{Rana pipiens} skeletal muscle and their expression patterns along single fibres

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Accepted 1 October 2001

Summary

Isoforms of myosin heavy chain (MHC) and myosin light chain (MLC) influence contractile kinetics of skeletal muscle. We previously showed that the four major skeletal muscle fibre types in \textit{Rana pipiens} (type 1, type 2, type 3 and tonic; amphibian nomenclature) contain four unique MHC isoforms. In the present study we defined the MLCs expressed in each of these \textit{R. pipiens} fibre types. The MLC composition of single MHC-typed fibres was determined from western blots using a panel of monoclonal MLC antibodies. A total of seven MLCs were identified, including four types of MLC1, two of MLC2 and a single MLC3. Twitch fibre types (types 1, 2 and 3) expressed MLC1\textsubscript{f} and MLC2\textsubscript{f}, while tonic fibres contained a unique set of isoforms, MLC1\textsubscript{Ta}, MLC1\textsubscript{Tb} and MLC2\textsubscript{T}. MLC3 was expressed primarily in type 1, type 1-2 and type 2 fibres. Surprisingly, some frogs displayed a striking pattern of MLC expression where a unique isoform of MLC1 (MLC1\textsubscript{x}) was coexpressed along with the normal MLC1 isoform(s) in all fibre types. MLC1\textsubscript{x} was either expressed in all fibres of a given frog or was completely absent. The intraspecific polymorphism in MLC1 expression is likely to have a genetic basis, but is unlikely to be caused by allelic variation. The ratio of MLC3/MLC1 increased in direct proportion to the percentage of type 1 MHC, but was only weakly correlated. The variability in MLC3/MLC1 within a fibre type was extremely large. Both the MHC isoform and MLC3/MLC1 ratio varied significantly between 1 mm segments along the length of fibres. For all segments combined, MLC3/MLC1 increased with the percentage of type 1 MHC, but the correlation between segments was weaker than between fibres.

Key words: frog, \textit{Rana pipiens}, fibre type, skeletal muscle, electrophoresis, contractile protein, isoform.

Introduction

The motor protein myosin in skeletal muscle is a hexameric polypeptide consisting of two myosin heavy chain (MHC) and four myosin light chain (MLC) subunits. Each MHC is associated with one essential light chain (ELC) and one regulatory light chain (RLC; also called alkali light chain). All vertebrates, including humans, express a family of MHC, ELC and RLC isoforms in their skeletal muscles. In a given species, variable expression of multiple MHC and MLC isoforms results in a diverse population of muscle fibres with a wide range of shortening velocities and power capabilities. Thus, myosin isoforms have a critical impact on functional diversity in muscular systems and influence how muscles are adapted to meet the specific motor requirements of an organism.

The influence of MHC and MLC isoforms on the force–velocity relationship remains controversial and may vary among vertebrates (for reviews, see Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). Mechanical studies on single skinned fibres established that MHC isoforms influence maximal shortening velocity ($V_{\text{max}}$) and maximal power (Sweeney et al., 1988; Larsson and Moss, 1993; Bottinelli et al., 1994a, 1996; Hilber and Galler, 1997). In some studies, $V_{\text{max}}$ was also affected by the ratio of MLC3/MLC1\textsubscript{f} (Moss et al., 1982; Sweeney et al., 1988; Bottinelli et al., 1994a). However, Larsson and Moss found no relationship between MLC3/MLC1\textsubscript{f} and $V_{\text{max}}$ in human fibres (Larsson and Moss, 1993). The influence of MHC and MLC isoforms on maximal isometric tension also remains the subject of considerable debate (Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000).

Frog muscle is unique in that relatively long, single, intact ‘living’ fibres can be isolated that retain excellent mechanical stability and from which sarcomere length transients can readily be measured by laser diffraction. For this reason,
frog muscle offers an exceptional opportunity to study the relationship between myosin isoforms and contractile function in living single cells. However, inadequate definition of both MHC and MLC isoforms in frog muscle limits our ability to characterize this relationship.

A nomenclature for amphibian fibre types has been established based on morphological, physiological, immunohistochemical, histochemical and biochemical analysis (Smith and Lannergren, 1968; Smith and Ovalle, 1973; Rowlerson and Spurway, 1988; Lutz and Lieber, 2000). In general, anuran skeletal muscles are composed of twitch fibres (types 1, 2 and 3) and tonic fibres (Rowlerson and Spurway, 1988; Lutz and Lieber, 2000). Also, a fibre type with properties intermediate between twitch and tonic fibres (type 4) has been described in Xenopus laevis (Lannergren, 1979), but not in Rana pipiens (Rowlerson and Spurway, 1988). Detailed mechanical analysis of intact single fibres of X. laevis by Lannergren and colleagues revealed differences in $V_{\text{max}}$ and maximal power in the order: type 1>type 2>type 3>type 4>tonic (Lannergren and Hoh, 1984; Lannergren, 1987). The differences in contractile properties were correlated with MHC isoforms, while the ratio of MLC3/MLC1f did not influence the force–velocity relationship. However, interpretation of these results was complicated by the fact that MHC content in the different fibre types was not measured directly but was estimated from a combination of isomyosin banding patterns after native polyacrylamide gel electrophoresis (PAGE) and MLC content after SDS-PAGE. In addition, the identification of MLC isoforms in the various fibre types was incomplete.

We recently characterized the MHC isoform composition of Rana pipiens fibre types at both the mRNA and protein levels (Lutz et al., 1998a,b, 2000; Lutz and Lieber, 2000). Four major fibre types (type 1, type 2, type 3 and tonic), were differentiated by their reactivity to a panel of monoclonal MHC antibodies. Four novel MHC mRNA transcripts were cloned from whole muscle, and using single-fibre reverse transcription–polymerase chain reaction (RT-PCR), each transcript was found to be expressed predominantly in one of the four fibre types. SDS-PAGE of single immunotyped fibres showed that the MHCs from the three twitch fibre types (type 1, type 2 and type 3) were resolved into three separate isoform bands, while tonic MHC comigrated with type 1. Coexpression of two MHC isoforms in single fibres was often observed at both mRNA and protein levels.

The purpose of this report was to identify the MLC isoforms corresponding to the various fibre types in R. pipiens and to determine the relationship between the expression of MLC and MHC isoforms at the subcellular level. Unequivocal identification of MLC isoforms by SDS-PAGE can be more difficult to achieve than for MHCs because of the presence of numerous other proteins that migrate at similar positions on gels. In this report we identified the MLCs in the full range of R. pipiens fibre types using western blot analysis with a panel of MLC monoclonal antibodies. Western blots of single fibres provided clear delineation of seven unique MLCs; four types of MLC1, two types of MLC2 and one MLC3. The detailed identification of MLC isoforms presented here, along with our previous description of MHCs, enables the precise relationship between MHCs, MLCs and contractile function in intact single muscle cells to be elucidated.

**Materials and methods**

**Animals and muscles**

Adult male frogs, Rana pipiens, were purchased commercially (Charles Sullivan Inc, Nashville, TN, USA) and were kept at room temperature in 901 aquaria containing dry, dark surfaces and recirculating filtered water. Frogs were fed live crickets twice weekly. Frogs were killed by double pithing and various hindlimb muscles were removed. To ensure that examples of all fibre types were obtained, a wide variety of different muscles were harvested. Typically, these were the anterior tibialis, gracilis and tonus region of the cruralis muscle, but in some cases the gluteal, sartorius and semimembranosus were also acquired. Large muscles (gracilis, gluteal and semimembranosus) were cut into strips. All muscles and strips were pinned to parafilm-coated cork and immediately frozen in isopentane cooled in liquid N2 then stored at –80 °C. For isolation of single fibres, frozen muscles were freeze-dried for at least 1 h and brought to room temperature under vacuum. Single fibres were teased out of freeze-dried muscles with fine forceps under a stereomicroscope and were transferred into 200 µl microfuge tubes. SDS-PAGE sample buffer consisting of diethiothreitol (DTT; 100 mmol L$^{-1}$), SDS (2 %), Tris-base (80 mmol L$^{-1}$) pH 6.8, glycerol (10 %) and Bromphenol Blue (0.012 % w/v) was added to the tubes and samples were frozen immediately in liquid N2 and stored at –80 °C. Samples were boiled for 2 min prior to loading onto gels. Because our purpose was to obtain adequate numbers of each fibre type, no attempt was made to standardize the number of muscles obtained from each frog or the number of fibres harvested from each muscle.

The anterior tibialis muscle was used exclusively for experiments investigating MHC and MLC content along the length of single cells. For these experiments care was taken to insure that full-length fibres were dissected from freeze-dried muscle. Fibres were cut into 1 mm segments along their full length and segments were suspended in SDS-PAGE sample buffer.

**SDS-PAGE and western blots**

Single fibre samples were split; 10 % of the sample was used for analysis of MHCs and the remaining 90 % for MLCs. The MHC composition was determined for a large number of fibres to obtain sufficient numbers of each type and then MLC analysis was performed on a subset of these same fibres.

**Myosin heavy chains**

MHC isoforms were separated by SDS-PAGE, based on methods of Talmadge and Roy (1993) as previously described (Lutz et al., 1998a). Total acrylamide concentration was 4 %...
Myosin light chains

Western analysis was used to identify the MLCs present in the various fibre types. Single fibre samples for which MHC composition had been determined were split into two parts and subjected to identical SDS-PAGE. Total acrylamide concentration was 4% and 14% in the stacking and resolving gels, respectively (acrylamide:bis-acrylamide, 38:1). Gels (7.5 cm×10 cm, 0.75 mm thick) were run at a constant current of 20 mA for 2.5 h at 4°C. One gel was silver stained and its counterpart was transferred to nitrocellulose for western analysis. Typically, two thirds of the single fibre sample was run on the silver-stained gel and the remaining one third was used for western blots. Western blots were reacted with three different anti-avian MLC monoclonal antibodies, T14, MF5 and F310. The T14 and F310 antibodies were a generous gift from Dr Frank Stockdale (Stanford University). T14 has been shown to react with MLC1f, MLC2f and MLC3 in avian muscle, while F310 reacts with MLC1f and MLC3 (Crow et al., 1983). The MF5 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) reacts with MLC2f in avian muscle (Shimizu et al., 1985). Primary antibodies were applied overnight at 4°C at dilutions of 1:5, 1:500 and 1:100 for T14, MF5 and F310, respectively. The secondary antibody (diluted 1:5000) was peroxidase-labeled, anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA). Bands were visualized using ECL western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The specific MLC reactivity of the antibodies is shown in Table 1. In most cases, blots were stripped and reprobed sequentially through the series of antibodies. Banding patterns on western blots were compared with silver-stained gels to assess the position of MLC bands relative to other bands. Apparent molecular masses were calculated for all MLCs by comparison with standards of known molecular mass.

Quantitative assessment of the MLC isoform ratios within single fibres was performed on silver-stained gels using densitometry and NIH Image software. Molar ratios were calculated from the band density and molecular mass of each isoform. For this purpose we used molecular mass derived from amino acid sequence analysis, rather than apparent molecular mass calculated from gel mobility, which are prone to significant error. This is especially true of MLC1, which is known to migrate much more slowly on gels, and hence at higher apparent molecular mass, than its actual molecular mass deduced from its amino acid composition (Frank and Weeds, 1974). We used molecular masses of 20.7, 19.0 and 16.5 kDa for MLC1f, MLC2f and MLC3, based on the derived amino acid composition of cloned full-length cDNAs for each of these isoforms (G. J. Lutz and R. L. Lieber, unpublished data).

Troponins

To identify the other myofibrillar proteins present in the various fibre types in the MLC region of the gels, western blots were performed with monoclonal antibodies against troponin I (MAB1691; Chemicon International Inc., Temecula, CA, USA) and troponin C (Novocastra NCL-TROPC; Vector Laboratories, Burlingame CA, USA). Western blots were reacted with anti-TnI (diluted 1:100) and anti-TnC (diluted 1:40) as described for MLCs. MHC isoform content was determined from a sample of each fibre as described above.

Skinned fibres

To determine which bands on silver-stained gels were cytoplasmic (soluble) proteins and thus not incorporated into myofibrils (myofibrillar proteins), freeze-dried fibres were cut into two segments. One segment was skinned to remove soluble proteins and the other was placed directly in SDS-PAGE sample buffer. For skinnning, fibre segments were pinned in Sylgard dishes, skinned for 30 min (1% Triton X-100 in standard relaxing solution), washed twice with relaxing solution, transferred to dry tubes and immersed in SDS-PAGE sample buffer. Skinned and non-skinned segments of individual fibres were subjected together to SDS-PAGE.

All values are reported as mean ± s.e.m. unless otherwise indicated. Comparison of means was performed by Student’s t-test.

Table 1. MLC isoform reactivity to monoclonal antibodies

<table>
<thead>
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<th>Antibody</th>
<th>MLC1s</th>
<th>MLC1Ta</th>
<th>MLC1f</th>
<th>MLC1Tb</th>
<th>MLC2f</th>
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Results
Identification of myosin light chains

Western analysis was used to identify the MLCs in the various fibre types of *R. pipiens* skeletal muscle. Single fibres were isolated from freeze-dried muscles and fibre types were determined by SDS-PAGE analysis of MHC isoform content. Fibres representing each of the major and intermediate fibre types (types 1, 1-2, 2, 2-3, 3 and tonic), were then analyzed on western blots for their reactivity to three monoclonal MLC antibodies. A representative example of the silver-stained banding pattern and corresponding western blots is shown for a complete series of fibres obtained from one frog (Fig. 1). The specific reactivity of the antibodies is provided in Table 1. As indicated by the summary representation in Fig. 1, in this frog, a total of six different MLC bands were identified in the various fibre types by the three antibodies combined: three distinct MLC1 bands, two MLC2 bands and a single MLC3 band. All twitch fibre types (types 1, 1-2, 2, 2-3 and 3) contained only one common MLC1 and an MLC2 band, designated as MLC1 and MLC2 (where f denotes fast). A single MLC3 band was observed in all twitch fibre types except type 3, and was not present in tonic fibres.

Tonic fibres expressed two unique MLC1 bands and one unique MLC2 band not found in twitch fibres (Fig. 1). The MLCs expressed exclusively in tonic fibres are designated with the subscript T as MLC1T, MLC1Tb and MLC2T. The MLC1Tb and MLC2T bands in tonic fibres are clearly different from the bands designated as MLC1 or MLC2 in twitch fibres, based both on their differential gel mobility and their differential reactivity to MLC antibodies. The MLC1Tb band could not be differentiated from MLC1 by antibody reactivity, and appeared to comigrate with MLC1 on most gels (Figs 1, 2); however, on several gels the MLC1Tb band clearly migrated more slowly than MLC1, strongly suggesting they are unique MLC1 isoforms.

A strikingly different, and very unusual pattern of MLC expression was observed across the full range of fibre types in some frogs (Fig. 2). As indicated by the schematic summary of the western blots in Fig. 2, in certain frogs a unique MLC1 band, designated as MLC1x, was expressed in all fibre types. In each of the fibre types, the MLC1x band was coexpressed

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**Fig. 1.** Identification of MLCs in single fibres representing the full range of fibre types in *R. pipiens*. (A) MHC isoform composition of single fibres was determined by SDS-PAGE. Shown are MHC bands from single fibres representing each of the major and intermediate fibre types (types 1, 1-2, 2, 2-3, 3 and tonic T). All fibres were obtained from one frog. Fibres were typed by comparison with the known positions of MHC isoforms from a standard whole muscle homogenate (anterior tibialis muscle). Fibre types, based on MHC content, are indicated at the bottom of each gel lane. (B) Analysis of MLCs in each of the MHC-typed fibres shown in A (fibre types labeled at bottom). (Left) SDS-PAGE (silver stained) of each fibre. Gel markers in leftmost lane have molecular masses (from top to bottom) of 45, 31, 21.5 and 14.5 kDa. (Middle) Western blots of the gel on left using monoclonal MLC antibodies T14, MF5 and F310. (Right) Schematic representation based on western blots of the MLC composition of the fibre types from this frog and 23 other frogs with a similar MLC composition.
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along with the usual MLC1 band (the MLC1 bands in Fig. 1). Thus, in these frogs, all twitch fibre types contained both MLC1f and MLC1x, while tonic fibres contained a triplet of MLC1 bands (MLC1Ta, MLC1Tb and MLC1x). In qualitative terms, the only difference between the MLC expression in the frogs of Figs 1 and 2 was the presence or absence of the MLC1x band. In all other respects, the MLC expression patterns were qualitatively the same. There was no indication that the band designated as MLC1x was actually a different isoform in twitch and tonic fibres as the antibody reactivity of this band and its migration rate appeared to be identical in all fibre types (Fig. 2, Table 1).

The expression of MLC1x was clearly ‘all or none’ as it was either expressed in all fibres of a given frog or it was completely absent. A total of 7 of 31 frogs studied contained the MLC1x band. From these 7 frogs, 52 fibres were analyzed, and each contained the MLC1x band. The 52 fibres included all fibre types as follows: type 1 (8), type 1-2 (9), type 2 (7), type 2-3 (8), type 3 (5) and tonic (15). Conversely, MLC1x was absent from each of 206 fibres obtained from the remaining 24 frogs. The 206 fibres were distributed among all fibre types as follows: type 1 (39), type 1-2 (63), type 2 (34), type 2-3 (16), type 3 (11) and tonic (43).

Overall, bands reacting positively to MLC antibodies could clearly be categorized as MLC1, MLC2 or MLC3 based on a comparison of their apparent molecular mass with those of other species. The apparent molecular mass of each of the identified MLC bands was as follows (in kDa): MLC1x (26.8), MLC1Ta (26.2), MLC1f (26.1), MLC1Tb (25.5), MLC2f (22.8), MLC2Tb (21.7), and MLC3 (15.0). The apparent molecular masses for both MLC2 isoforms were slightly larger than reported in previous studies of frog myosin (18–20 kDa) (Giambalvo and Dreizen, 1978). These relatively small differences (5–10 %) could easily be due to species differences, as previous studies did not include R. pipiens. The differences could also result from subtle differences in the SDS–PAGE system. Also, most previous studies of frog muscle have reported molecular masses of MLCs purified from whole muscle where proteolytic degradation could have been a complicating factor.

Identification of other myofibrillar proteins

To avoid potential ambiguities in qualitative and quantitative analysis of the MLCs, it was important to identify...

Fig. 2. In some frogs, a novel MLC, MLC1x, is expressed in all fibre types. (Left) SDS-PAGE (silver stained) of single fibres representing most of the different fibre types. All fibres (fibre types labeled at bottom of gel lanes) were obtained from two frogs and were typed by their MHC banding pattern by SDS-PAGE, as in Fig. 1 (not shown). (Middle) Western blots of the gel on left using monoclonal MLC antibodies T14, MF5 and F310. (Right) Schematic representation based on western analysis of the MLC composition of the fibre types from this frog and six other frogs with similar MLC composition. A unique type of MLC1, designated as MLC1x, was expressed in all fibre types, producing a strikingly different expression pattern from those seen in fibres examined in Fig. 1.

Fig. 3. Identification of tropomyosin I (TnI) and troponin C (TnC) isoforms in the various R. pipiens fibre types. (Left) SDS-PAGE (silver stained) banding pattern of single skinned fibres representing most of the different fibre types. All fibres (fibre types labeled at bottom of gel lanes) were obtained from one frog and were typed by MHC banding pattern after SDS-PAGE as in Fig. 1 (not shown). (Right) Western blots of the gel on left using anti-TnI and anti-TnC antibodies. Anti-TnI labeled one isoform in fast twitch fibres (TnIf) and a unique isoform in tonic fibres (TnIT). Anti-TnC labeled an isoform in tonic fibres (TnCT) that was not expressed in any other fibre type, but did not react with the TnC isoform present in fast twitch fibres (TnCF). The position of TnCT was deduced from indirect evidence (see Results for details). The lightly stained band below MLC2T in twitch fibres is an unidentified contaminant that is not typically present (see Figs 1, 2 and 4). Note, this frog did not contain the MLC1x isoform in any of its fibres.
membrane permeabilization (skinning), the only remaining
much lighter per unit mass compared to other proteins). Second,
migrated just ahead of MLC2 T (Fig. 3). Anti-TnC did not react
(designated TnC T ; apparent molecular mass 20.6 kDa) that
influence the conclusions in this study.

labeled a unique isoform in tonic fibres (designated TnI T ) at a
isoforms.

by the T14 antibody, which strongly labeled all other MLC1
likely to be an MLC isoform, however, as it was not labeled
was not removed from type 2-3 or type 3 fibres (Fig. 3).
and type 2 fibres, indicating that it was a soluble protein, it
was not removed from type 2-3 or type 3 fibres (Fig. 3). Because
this band did not react with any of the MLC or
troponin antibodies we cannot conclusively identify it. It is not
likely to be an MLC isoform, however, as it was not labeled
by the T14 antibody, which strongly labeled all other MLC1
isoforms.

A summary of the MLC and troponin isoforms expressed in
twitch and tonic fibres is shown in Fig. 4. To allow for a direct
comparison, skinned and non-skinned segments of the same
fibres were run in adjacent lanes of the gel. This gel clearly
illustrates the bands that were removed by skinning and the
major differences in myofibrillar protein expression patterns
between twitch and tonic fibres.

Quantification of MLCs in single fibres
In some recent studies, the molar ratio of MLC3/MLC1 f
significantly influenced the mechanical function of single
skeletal muscle fibres (see Discussion). We therefore measured
the MLC3/MLC1 ratio in R. pipiens fibres to determine
whether any significant variability existed in this parameter
among and between the various fibre types. Having established
the identity of the various MLCs and their positions on SDS-
PAGE gels, we quantified the relative levels of MLCs in
individual fibres by densitometry of silver-stained gels. This
quantification was restricted to type 1, type 1-2 and type 2
fibres. All molecular masses used in calculating molar ratios
were based on derived amino acid composition, not gel
mobilities (see Materials and methods). MLC1 content was
taken as the sum of MLC1 f and MLC1 x , and the same
molecular mass was used for both isoforms. The ratio of
MLC3/MLC1 was related to MHC isoform content in single
freeze-dried type 1, type 1-2 and type 2 fibres (N=100)
obtained from various muscles (Fig. 5A, Table 2). Among
pure type 1 fibres (i.e. fibres that expressed only type 1 MHC),
MLC3/MLC1 was extremely variable, ranging between 0.20
and 2.9 (mean=1.20±0.13; N=35). The MLC3/MLC1 ratio was
significantly lower (P<0.0001) in pure type 2 fibres

the other bands that migrated in the MLC region of the gels.
The only myofibrillar proteins known to migrate in the MLC
region under these conditions are troponin I (TnI) and troponin
C (TnC). We performed western blots on the various fibre
types with monoclonal antibodies against TnI and TnC
(Fig. 3). Anti-TnI and anti-TnC western blots were performed
on skinned fibres (soluble proteins removed; Fig. 3) and non-
skinned fibres (not shown). Anti-TnI labeled a single band
(designated TnI f ) in all fast twitch fibres (types 1, 1-2, 2, 2-3
and 3), at a position between MLC1 f and MLC2 f . Anti-TnI also
labeled a unique isoform in tonic fibres (designated TnI T ) at a
position slightly above TnI f . The TnI f band comigrated with
the MLC1 T a isoform band. The apparent molecular masses of
TnI f and TnI T (24.7 and 26.2 kDa, respectively) are in
reasonable agreement with published values for skeletal TnI.
Because we did not perform any quantitative analysis of MLCs
in tonic fibres, comigration of MLC1 T a and TnI T did not
influence the conclusions in this study.
The anti-TnC antibody labeled a single band in tonic fibres
(designated TnC f ; apparent molecular mass 20.6 kDa) that
migrated just ahead of MLC2 f on the gels in
Figs 1–3, based on two observations. First, on all gels of this
type the MLC2 f band appeared diffuse, and on some gels it
appeared as two bands (a tight doublet), composed of a darker
upper and lighter lower portion. The apparent molecular mass
of this band (22.8 kDa) and the fact that it was lightly stained
with TnC in fast twitch fibres. Thus, we could not identify a
putative fast TnC (TnC f ) with western blots. However, it
was not removed from type 2-3 or type 3 fibres (Fig. 3).
Because this band did not react with any of the MLC or
troponin antibodies we cannot conclusively identify it. It is not
likely to be an MLC isoform, however, as it was not labeled
by the T14 antibody, which strongly labeled all other MLC1
isoforms.

A summary of the MLC and troponin isoforms expressed in
twitch and tonic fibres is shown in Fig. 4. To allow for a direct
comparison, skinned and non-skinned segments of the same

Fig. 4. Summary of the expression pattern of MLC and troponin
isoforms in fast twitch and tonic fibres. This figure combines the
information from Figs 1 and 3 to indicate the positions of each of the
identified myofibrillar proteins (except MLC1 x ) on a silver-stained
gel after SDS-PAGE. Skinned (S) and non-skinned (NS) segments of
the same fibres ( fibre type indicated below each lane) are shown in
adjacent lanes for visualization of the bands containing soluble
proteins (i.e. the bands present in non-skinned segments but absent
from skinned segments). For clarity, a type 1 fibre only was used to
represent the fast twitch fibres, although significant differences in the
quantitative and qualitative expression of MLC and troponin
isoforms did exist among the twitch fibres. Note that skinning
removed a prominent soluble protein band just above the MLC1
region in both fibre types. Skinning also removed a prominent
soluble protein band between MLC1 f and TnC f , as well as a band
below TnI T in tonic fibres. Note that this frog did not contain the
MLC1 x isoform in any of its fibre types.

Quantification of MLCs in single fibres
In some recent studies, the molar ratio of MLC3/MLC1 f
significantly influenced the mechanical function of single
skeletal muscle fibres (see Discussion). We therefore measured
the MLC3/MLC1 ratio in R. pipiens fibres to determine
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taken as the sum of MLC1 f and MLC1 x , and the same
molecular mass was used for both isoforms. The ratio of
MLC3/MLC1 was related to MHC isoform content in single
freeze-dried type 1, type 1-2 and type 2 fibres (N=100)
obtained from various muscles (Fig. 5A, Table 2). Among
pure type 1 fibres (i.e. fibres that expressed only type 1 MHC),
MLC3/MLC1 was extremely variable, ranging between 0.20
and 2.9 (mean=1.20±0.13; N=35). The MLC3/MLC1 ratio was
significantly lower (P<0.0001) in pure type 2 fibres
and the percentage of type 1 MHC was similar in fibres that weak (Table 2). Of type 1 MHC and the correlation was significant but very weakly positively correlated with the percentage of type 1 fibre types were harvested), MLC3/MLC1 ratio increased only slightly with the percentage of type 1 MHC. Overall, the highest levels of MLC3/MLC1 were found in fibres obtained from the gracilis muscle. When fibres from all muscles except the gracilis were considered as a group, the MLC3/MLC1 ratio was markedly the MLC3/MLC1 ratio. Fibres with MLC1x were distributed across the full range of type 1, type 1-2 and type 2 fibres. On average, MLC1x was expressed in nearly equal molar amounts to MLC1f in type 1, type 1-2 and type 2 fibres as a group (MLC1x/MLC1f=1.10±0.07), but the range of values was substantial (0.64–1.71; Fig. 6). MLC1x/MLC1f was slightly higher in type 2 (1.30±0.30) than in type 1 fibres (0.98±0.06; P<0.05). Regression analysis of type 1, type 1-2 and type 2 fibres showed that MLC1x/MLC1f was inversely related to the percentage of type 1 MHC (P<0.05; Fig. 6).

To estimate the extent to which variability in the MLC3/MLC1 ratio between fibres was due to inherent inaccuracy in the quantification procedure, we measured the intrinsic variability of MLC quantification (estimated by repeated measures of samples within and between gels). To ensure adequate material for repeatability measurements, samples consisted of pairs of large fibres from the semimembranosus muscle (isolated from freeze-dried muscle). To estimate the potential influence of fibre size on variability in MLC3/MLC1 in single fibres, we included two dilutions of the molar ratio of MLC1x/MLC1f was measured in single type 1, type 1-2 and type 2 fibres by quantitative densitometry of gels after SDS-PAGE. Linear regression showed that MLC1x/MLC1f ratio decreased in direct proportion to the percentage of type 1 MHC (slope=–0.003; r²=0.288; P=0.03).

Fig. 5. The MLC3/MLC1 ratio increased in proportion to the percentage of type 1 MHC in single fibres, but was highly variable within fibre types. (A) MLC3/MLC1 ratio and MHC isoforms were measured in single type 1, type 1-2 and type 2 fibres by quantitative densitometry of gels after SDS-PAGE. Fibres (N=100) were obtained from the anterior tibialis (AT), cruralis (CR), gluteal (GL), gracilis (GR), semimembranosus (SM) and sartorius (SA) muscles. Each symbol corresponds to a different muscle. Linear regression showed that the MLC3/MLC1 ratio increased in direct proportion to the percentage of type 1 MHC. There was substantial variability in MLC3/MLC1 ratio within each fibre type, and the highest values were all found in the GR muscle. (B) MLC3/MLC1 ratio for fibres that contained MLC1x (N=16; data are subset of A). The relationship between the MLC3/MLC1 ratio and percentage of type 1 MHC was similar to A, and the full range of fibre types was represented.

Fig. 6. MLC1x/MLC1f was higher in type 2 than type 1 fibres. The molar ratio of MLC1x/MLC1f was measured in single type 1, type 1-2 and type 2 fibres by quantitative densitometry of gels after SDS-PAGE. Linear regression showed that MLC1x/MLC1f ratio decreased in direct proportion to the percentage of type 1 MHC (slope=–0.003; r²=0.288; P=0.03).
The (MLC1+MLC3)/MLC2 ratio was 0.823 (±0.016; r = 0.98), which indicates a lack of saturation in the silver-stained gels.

To further characterize the accuracy of our quantification procedure for MLCs, we attempted to evaluate the molar ratio of ELCs to RLCs in single fibres. Because each MHC is known to be associated with one ELC and one RLC, the measured ratio of (MLC1+MLC3)/MLC2 should equal one. Unfortunately, because TnCf appears to comigrate with MLC2f, we could not explicitly calculate the (MLC1+MLC3)/MLC2 ratio. However, assuming that MLC2f and TnCf comigrate, the (MLC1+MLC3)/(MLC2f+TnCf) ratio was 0.823 (±0.016; N=100), which is reasonable considering that the lightly staining TnCf probably makes a small contribution to the density of the MLC2f-TnCf band. Also, linear regression analysis showed that the relationship between MLC2f and TnCf comigrate, the (MLC1+MLC3)/(MLC2f+TnCf) ratio was 0.823 (±0.016; N=100), which is reasonable considering that the lightly staining TnCf probably makes a small contribution to the density of the MLC2f-TnCf band. Also, linear regression analysis showed that the relationship between MLC2f and TnCf was highly correlated (r²=0.98), which indicates a lack of saturation in the silver-stained gels.

Variability in MHC and MLC isoforms along the length of single fibres

Having established that MLC3/MLC1 varied substantially within fibre types, we were interested in whether this parameter also varied along the length of fibres and whether its relationship to MHC isoforms was the same as that seen at the whole fibre level. MHC isoforms and MLC3/MLC1 ratio were quantified in 1 mm segments along the length of single fibres directly from gels after SDS-PAGE. As indicated in Fig. 7, both MHC isoform content and MLC3/MLC1 varied substantially along the length of single fibres (N=10 fibres). In the most extreme case, the percentage of type 1 MHC changed by 40.3 % between segments, while MLC3/MLC1 ranged from 2.43 to 1.03 in the same fibre (Fig. 7F). Non-parametric correlation analysis revealed no significant correlation between MLC3/MLC1 and the percentage of type 1 MHC in any individual fibre (P=0.17–0.93), but a significant correlation was found across all segments combined (N=64; P<0.001). Regression analysis of all segments showed that

<table>
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<th>Group</th>
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<th>P</th>
<th>r²</th>
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<tr>
<td>All fibres</td>
<td>0.007±0.001</td>
<td>0.454±0.096</td>
<td>&lt;0.0001</td>
<td>0.235</td>
<td>100</td>
</tr>
<tr>
<td>Non-MLC1 fibres</td>
<td>0.007±0.001</td>
<td>0.454±0.095</td>
<td>&lt;0.0001</td>
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<td>84</td>
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<tr>
<td>MLC1 fibres</td>
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<td>0.503±0.324</td>
<td>0.043</td>
<td>0.260</td>
<td>16</td>
</tr>
<tr>
<td>All except GR</td>
<td>0.004±0.001</td>
<td>0.540±0.066</td>
<td>0.001</td>
<td>0.139</td>
<td>75</td>
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<tr>
<td>AT only</td>
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<td>0.501±0.087</td>
<td>0.005</td>
<td>0.220</td>
<td>35</td>
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Values are for linear regression equations fitted to the data in Fig. 5. Values are means ± S.E.M.

AT, anterior tibialis; GR, gracilis muscle.
with frog MLCs and partial selectivity between MLCs. The (Figs 1, 2, Table 1). Although the antibodies used were raised MLCs: four types of MLC1, two of MLC2 and a single MLC3 isoform content, clearly delineated a total of seven unique antibodies. Western blots of single fibres, typed by MHC identified the MLCs in the full range of identification of a family of MLCs. In the present report we Although western analysis can potentially resolve these MLCs from uncommon fibre types limits this approach. of myosin degradation products and inadequate detection of of the myosin fraction by thin filament proteins, the presence myosin purified from whole muscle. However, contamination from soluble proteins. Two unique isoforms of TnI were identified; one in twitch fibres (TnIi) and one in tonic fibres (TnIr). A single TnC isoform was identified with anti-TnC in tonic fibres. A synopsis of the gel migration of all identified MLC, TnI and TnC isoforms in twitch and tonic fibres is shown in Fig. 4.

The MLC family in amphibian skeletal muscle has not been as well characterized as in other vertebrates. Only a single isoform of MLC1, MLC2 and MLC3 have been detected in myosin fractions purified from whole frog muscles (Kendrick-Jones et al., 1976; Giambalvo and Dreizen, 1978; Chanoine and Gallien, 1989) and from single fibres in *Rana esculenta* (Focant and Reznik, 1980). The fibre type expression pattern of MLCs in *Xenopus* has received the most critical scrutiny among amphibians (Lannergren, 1987). Single fibres were typed by native myosin bands on pyrophosphate gels and segments of the same fibres were analyzed by SDS-PAGE for MLC content (Lannergren, 1987). It was concluded that fast twitch type 1 and type 2 fibres of *Xenopus* contained MLC1*, MLC2* and MLC3, while slow twitch type 3 fibres expressed the slow isoform MLC1s, and did not contain MLC3. It was later reported that type 3, type 4 and type 5 (tonic) fibres all expressed slow isoforms MLC1* and MLC2* (Lannergren, 1992). However, it is difficult to justify these conclusions from the gels presented. Specifically, MLC2* and MLC2*, appear to comigrate, and an unidentified band in the MLC1 region complicates the identification of MLC1* and MLC1s (Lannergren, 1987). Further, no MLC gels were shown for type 4 or type 5 fibres. Thus, the fibre type expression pattern of MLCs in *Xenopus* remains unclear. However, if the interpretation of MLC expression patterns in *Xenopus* is correct, it differs significantly from the present findings in *R. pipiens*. There was no evidence of slow MLCs in any of the *R. pipiens* twitch fibres, whereas in *Xenopus* the fast twitch fibres (types 1 and 2) contained a different set of MLCs than the slow twitch fibres (type 3). Also, only two types of MLC1 were found in *Xenopus*, while four types of MLC1 were present in *R. pipiens*.

A most remarkable feature of the MLC expression in *R. pipiens* was the ‘all or none’ expression pattern of MLC1s, which was either expressed in all fibres of a given frog or was completely absent (Fig. 2). MLC1s was always coexpressed along with the isoforms of MLC1 and MLC2 found in frogs that did not contain MLC1s. Interestingly, the quantitative relationship between MLC3/MLC1 ratio and percentage of type 1 MHC was maintained in frogs that had MLC1s (Fig. 5B). Thus, apart from the expression of MLC1s, the regulatory control of MLC content in fibre types was conserved.
in both groups of frogs. No differences were noted in any other myofibrillar proteins between frogs with MLC1x and non-MLC1x frogs.

Intraspecific variability in MLC1f expression has been documented in fish (Martinez et al., 1990; Crockford et al., 1991, 1995), and avian (Rushbrook and Somes, 1985) muscle. In fish, breeding experiments and genetic analysis clearly showed that intraspecific variability of two MLC1f isoforms was the result of allelic variation (Crockford et al., 1995). In agreement with our data from *R. pipiens*, the MLC3/MLC1 ratio was not affected by MLC1 isoform content and no other differences in other MLCs or other myofibrillar proteins were found.

The intraspecific variability in MLC1 isoforms in *R. pipiens* does not seem to be due to allelic variation. In 31 frogs examined, MLC1x was never the sole MLC1 isoform, yet 24 frogs did not have MLC1x, and seven coexperienced MLC1x with other isoforms of MLC1. If the MLC1 isoforms resulted from allelic variants, MLC1x homozygotes would have been observed in about 25% of the 31 frogs. Further, allelic variants are likely to be expressed with typical fibre type specificity, rather than across all fibre types. Finally, MLC1x expression did not appear to be related to environmental conditions, as both MLC1x and non-MLC1x frogs were found among frogs that were held in the laboratory for the same length of time (as long as 3 months) and were observed throughout the year. Together, these data suggest that the intraspecific polymorphism observed in MLC1 expression is probably due to an as yet undefined genetic polymorphism that is not allelic in nature. The genetic basis of MLC1 isoform expression not withstanding, the ubiquitous expression pattern of MLC1x across the full range of fibre types appears to be unprecedented for MLCs or any other myofibrillar protein. This ubiquitous fibre type expression pattern seems difficult to reconcile with a functional role for variation in MLC1 isoforms.

**Functional implications of MLC expression patterns in *R. pipiens***

There is substantial evidence from several experimental models that MLC isoforms are an important determinant of contractile kinetics in skeletal muscle, but the extent and nature of their influence remains controversial. Using an *in vitro* motility assay, Lowey and colleagues found that actin filament velocity was higher when myosin contained MLC3 rather than MLC1 (Lowey et al., 1993). Mechanical analysis of single fibres from controlled breeding populations of fishes with allelic variation in MLC1 isoforms showed that *V*\textsubscript{max} was significantly different between homozygotes for one or the other MLC1f isoform, while heterozygotes had intermediate properties (Crockford et al., 1995). Both of these models were uniquely powerful, as they allowed for experimental control of MLC content independent of other variables.

A correlation between MLC3 content and *V*\textsubscript{max} of single skinned fibres has been demonstrated in a variety of vertebrates (Moss et al., 1982; Sweeney et al., 1988; Greaser et al., 1988; Bottinelli et al., 1994a; Li and Larsson, 1996) (for reviews, see Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). In rodent muscle, MLC3/MLC2f content varied significantly among a population of fibres that expressed only the IIB MHC isoform and greater values of MLC3/MLC2f were associated with increased *V*\textsubscript{max} (Bottinelli et al., 1994a). Interestingly, MLC3/MLC2f did not appear to influence velocities at loads above zero, maximal power production or specific tension (Bottinelli et al., 1994b). In contrast, MLC3/MLC1 did not appear to influence *V*\textsubscript{max} in human skinned fibres (Larsson and Moss, 1993).

Only one study has attempted to determine the influence of MLC isoforms on contractile kinetics in intact fibres (Lannergren, 1987). In that study of *Xenopus laevis* fibre types, MLC3/MLC1f did not appear to influence shortening velocity at half isometric tension, but complications in the identity of MLCs may weaken this conclusion (see above) (Lutz and Lieber, 2000). In accordance with this conclusion, preliminary analysis of intact *R. pipiens* single fibres revealed no differences in *V*\textsubscript{max} among fibres that had substantial variability in MLC3/MLC1 but nearly constant MHC isoform content (G. J. Lutz and R. L. Lieber; unpublished data). Unfortunately, most studies have focused on the influence of MLCs on *V*\textsubscript{max}, while ignoring the more physiologically relevant parts of the force–velocity and power–velocity relationships (Moss et al., 1995; Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000). However, the preponderance of evidence to date suggests that MHCs have a greater influence on shortening velocity than MLCs, especially at velocities below *V*\textsubscript{max}.

The variability in MLC3/MLC1 ratio within *R. pipiens* type 1, type 1-2 and type 2 fibre types and absolute levels (range 0.2–2.9; Fig. 5) were larger overall than previously reported for avian and mammalian fibres (Sweeney et al., 1986, 1988; Salvietti et al., 1982; Greaser et al., 1988; Wada and Pette, 1993; Bottinelli et al., 1994a). For example, in rodent IIB fibres, MLC3 ranged between 0 and 0.5 of the total ELC content (Bottinelli et al., 1994a). Despite the relatively low magnitude and low variability in MLC3 molar content, a large range was observed in *V*\textsubscript{max} among the same fibres. It could be argued that the comparatively high magnitude of MLC3/MLC1 in *R. pipiens* will produce an even larger influence on mechanical function than in mammalian muscle. Alternatively, the weak correlation between MLC3/MLC1 ratio and type 1 MHC, and the substantial variability in MLC3/MLC1 ratio within a given fibre type, may indicate a lack of tight regulation and hence a lack of functional significance.

**Subcellular variability in myosin isoform composition**

We quantified MHC and MLC isoform content in discrete segments along the full length of single fibres and found that both MHC isoform and MLC3/MLC1 ratio varied significantly between segments (Fig. 7). The magnitude of the differences among segments was as high as 40% for MHC isoforms and even larger for the MLC3/MLC1 ratio. The relationship...
between MLC3/MLC1 ratio and MHC isoform content was the same whether compared between segments or whole fibres. Previously, coexpression of two MHC isoforms in single fibres has been shown to occur in a significant portion and even a majority of the fibres in a given muscle (Larsson and Moss, 1993; DeNardi et al., 1993; Peuker and Pette, 1997; Lutz et al., 1998b). It is apparent that this coexpression is a common feature of adult muscle in steady-state conditions, and not simply present during fibre type transitions (Peuker and Pette, 1997). The variability in myosin isoform content along the length of fibres also appears to be a feature of adult muscle under steady-state conditions. Only one previous study has documented changes in MHC isoform content along the length of single fibres using direct methods, but quantitative differences were not reported (Peuker and Pette, 1997). Single cell myosin-ATPase and immunohistochemistry suggest that myosin isoform composition may change along the length of *Rana temporaria* single fibres (Edman et al., 1988), but this assay for myosin isoform content was indirect and only semi-quantitative. There appear to be no previous studies of variability in MLC isoforms along the length of fibres. Thus, to our knowledge, this is the first study to quantify directly the MHC and MLC isoforms along the length of the same individual cells.

The cellular and molecular mechanisms that regulate the distribution of myosin isoforms along the length of multinucleated muscle cells are not known. Since frog muscle fibres often contain more than one motor endplate, the observed variability in myosin isoforms along fibres could be associated with the position of motor endplates. In *R. pipiens*, the sartorius muscle averaged 2.5–3 endplates per fibre (Weakly, 1980). The anterior tibialis (AT) muscle has similar fibre type composition to the sartorius, but the fibres are two- to fourfold shorter. This indicates the AT fibres will average at most 2–3 endplates per fibre. In our study, we measured MHC and MLC isoforms in 6–7 contiguous segments along the full length of fibres (Fig. 7). In some cases the variability in MHC and MLC isoform content was random along the fibre length, while in other cases there was a clear gradient from one end to the other. Also, in some fibres MLC3/MLC1 was independent of MHC isoform content, while in other fibres they were more closely correlated. It seems unlikely that these patterns of variability in myosin isoform expression in AT fibres were correlated with the location of endplates.

Interestingly, it has previously been shown in single intact amphibian fibres that mechanical properties are not uniform along the length of single fibres (Edman et al., 1985). Whether this non-uniformity in mechanical behavior is related to differences in myosin isoforms remains to be determined. Edman and colleagues reported a weak correlation along the length of individual frog muscle fibres between $V_{\text{max}}$ and indirect measures of myosin isoform composition (myosinATPase staining and MHC antibody reactivity) (Edman et al., 1988). They found that variability in $V_{\text{max}}$ along the length of fibres was weakly correlated with changes in apparent MHC composition. These studies were limited, however, in that MHC isoforms were not identified and MLCs were not included in the analysis.

**Comparison of frog MLC expression with mammals**

The fibre type expression pattern of MLCs in *R. pipiens* showed a striking similarity to those of mammals. In *R. pipiens*, the MHCs from twitch fibres (MHC1, MHC2 and MHC3) were associated with a different set of MLCs compared to the MHC in tonic fibres (MHCT). These four MHCs in *R. pipiens* were cloned and their evolutionary relationships were established (Lutz et al., 1998a, 2000; Lutz and Lieber, 2000). Homology analysis showed MHC1, MHC2 and MHC3 were all much more similar to each other than to MHCT. Thus, the MLC isoform expression pattern mirrored the evolutionary relatedness between MHCs. A similar relationship occurs in mammalian muscle, where fast and slow MLCs are associated with the most evolutionarily divergent MHCs.

There was a notable difference in the MLC expression pattern between frogs and mammals that may have functional consequences. Twitch fibres in *R. pipiens*, which make up over 95 % of the muscle volume in the hindlimb (Lutz et al., 1998b), did not contain a slow MLC isoform. In contrast, slow isoforms of MLC1 and MLC2 in mammals are expressed in twitch fibres that make up a much larger fraction of some muscles (Salviati et al., 1982). The lack of a slow MLC in *R. pipiens* twitch fibres is consistent with the lack of a type 4 fibre such as is found in *X. laevis* muscle, indicating that the expression patterns may be different within anurans.

**Conclusions**

In this study, the MLC family in *R. pipiens* skeletal muscle was defined using western blot analysis of single fibre proteins. This approach yielded a more complete definition of MLCs in the full range of mechanically divergent fibre types than was possible using traditional purification methods. A total of seven unique MLCs were identified, including four types of MLC1, two types of MLC2 and a single MLC3. Our analysis included the first ever quantitative measurements of MHC and MLC isoform content along the length of fibres. Both MHC isoform and MLC3/MLC1 ratio varied substantially along the length of cells. MLC3/MLC1 was also highly variable within and between fibre types, and was not tightly coupled to MHC isoform expression. This study also provided the first example of the all-or-none expression of an MLC isoform across all fibre types, and it was shown that this MLC is subject to intraspecific variability. The precise definition of MLCs in this study provides the foundation for establishing the influence of MHC and MLC isoforms on mechanical properties of intact ‘living’ single fibres.

We are grateful to Ana Valiere, Dustin Robinson and Sarah Shepard-Palmer for excellent technical assistance. We also thank Dr Frank Stockdale for his generous gift of MLC monoclonal antibodies. Supported by NIH grants AR40050, AR45631 and AR46469 and a grant from the Department of Veterans Affairs.