Electroendosmotic Preparative Gel Electrophoresis and Peptide Mapping of Slow and Three Fast Myosin Heavy Chains

Corrado Rizzi and Ugo Carraro

C.N.R. Unit for Muscle Biology and Physiopathology, Institute of General Pathology, University of Padua, Italy.

Abstract

Myosin is an exapolypeptide constituted by two heavy chains and four light chains whose isoforms are segregated in different fiber types and/or during development. The isoforms of Myosin Heavy Chains (MHC) are polypeptides of MW of about 200 KDs which can be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). By analytical SDS 6% PAGE at least four bands are separable from adult rat muscles. We have applied this discriminating power to a new preparative method: Electroendosmotic Preparative Gel Electrophoresis (EPGE). By EPGE it is now possible to obtain single purified MHC isoforms for further analyses. We will here show peptide mapping of EPGE purified MHC. Taking advantage of these powerful preparative and analytical approaches we will show: 1) the similarity of the fourth rat MHC to MHC2A; 2) the structural variability of MHC1 from different strains of adult sheep and 3) its presence in electrostimulated muscle.

Key words: Isomyosin, Myosin Heavy Chains, SDS PAGE, Electroendosmotic Preparative Gel Electrophoresis, Peptide Mapping, Muscle Conditioning by Electrostimulation.

BAM 1 (1): 43-53, 1991

Myosin is an exapolypeptide formed by two heavy chains and four light chains, which isoforms are segregated in different fiber types and/or during development [26, 30, 31, 35, 39, 42, 47-49]. Myosin heavy chains are polypeptides of MW of about 200 KDs, which can be separated by SDS PAGE [11,16, 28, 41]. At gene level six sarcomeric MHC have been recognized: α-Cardiac, β-Cardiac or MHC1, MHC2A, MHC2B, MHCEmb, MHCNeo [6]. MHCEmb and MHCNeo are expressed in developing fibres during ontogeny and regeneration or in myofibers of the neuromuscular fuse. MHC1, MHC2A and MHC2B are expressed in distinct types of adult skeletal fibres. These fibres belong to motor units having distinct functional properties: type 1 corresponds to slow-contracting oxidative motor units, type 2A corresponds to fast-contracting, fatigue resistant, oxidative-glycolytic motor units, type 2B fibre corresponds to fast contracting, fatigue sensitive, glycolytic motor units. On the other hand three fast isomyosins characterized by peculiar MHC are known at protein level. The third component, in addition to MHC2A and MHC2B, is known as MHC2X according to Schiaffino et al. who described in rodents by monoclonal antibodies a fast fiber type belonging neither to 2A nor to 2B types [43, 44], or as MHC2d according to Bar and Pette who noted using SDS PAGE that the diaphragm is rich in this peculiar MHC [3]. We prefer, though we acknowledge that we will increase confusion in a confounding matter, a slight different nomenclature: MHC2B is the MHC peculiar of fast glycolitic fiber, MHC2A is the one peculiar to the majority of the non-2B fibres in EDL or diaphragm muscles and finally we name MHC2S the third fast MHC, since it is present in the soleus muscle as the only fast component and since the "S" character is reminiscent of Schiaffino [10, 14]. The subset of fast fibres containing the new MHC can be hardly distinguished by histochemical ATPase activity and staining pattern with anti-MHC monoclonal antibodies. MHC2S comigrates with MHC2A in SDS Polyacrylamide gel electrophoresis when SDS 6% PAGE is performed according to [28]. Slight changes of the running conditions of the SDS PAGE [3, 14, 34, 46] separate the MHC isoforms in four distinct bands which in SDS 6% PAGE run in the following order from the top of the gel slab: MHC2S, MHC2A, MHC2B and MHC1 [3, 10, 14].

The limit of MHC separation in gel slabs is that further analyses of this high molecular polypeptides are difficult. By Electroendosmotic Preparative Gel Electrophoresis (EPGE) [24, 25, 40] it's now possible to collect from complex mixtures of crude myosins or from tissue homogenates single isoforms of MHC in almost pure form. We will here confirm that three fast MHC bands appears in SDS 6% PAGE of fast myosins and describe the new results of EPGE separation of MHC and of peptide mapping of fast and slow MHC of adult rat and of MHC1 of normal and long-term electrostimulated sheep muscle.

(A preliminary short report of EPGE results have been presented [40]).

Materials and Methods

Myosin extraction

Myosin was extracted essentially according to [18] with the following modifications. All procedures were performed at 2-4° C. Muscle tissue homogenized with Polytron PT 10 OD using 50 mM KCI containing 10 mM EGTA, 200 mM pepstatin, 80 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM benzamidine (Sol A) to reduce effects of endogenous proteases. After centrifugation at 650 x g for 10 minutes, the pellet was resuspended and centrifuged in Sol A three or more times until the supernatant became clear. Myosin was then extracted for 20 minutes with 0.3 M KCI, 0.15 M potassium phosphate pH 6.5, 10 mM Mg acetate, 10 mM EGTA, 200 mM pepstatin, 80 mM PMSF, 1mM benzamidine (Sol B) to which 5 mM ATP was added before using. We used approximately 25 mL of Sol B for 1 g of fresh muscle. After 30 minutes samples were centrifuged at 20.000 x g for 20 minutes and then at 150.000 x g for two hours. The supernatant was dialyzed for 4-12 hours in 1 mM Tris HCI pH 7.2, 10 mM KCI, 0.1 mM dithiothreitol, 5 mM EGTA. Myosin was collected by centrifugation at low speed, dissolved in 0.5 M NaCI, 50% v:v Glycerol and stored at -20° C.

SDS PAGE

ONE DIMENSIONAL ANALYSIS OF MYOSIN LIGHT CHAINS (MLC)

Analysis of MLC was performed in SDS 12% PAGE essentially according to Laemmli [33].

ONE DIMENSIONAL ANALYSIS OF MHC.

Analytical SDS PAGE of MHC was performed on 6% polycrylamide slabs 0.75 mm x 130 mm x 130 mm. Slabs were prepared according to Laemmli [33], but 37.5% v:v glycerol was present in the separating and in the stacking gel [28]. Electrophoretic buffer was: 25 mM Tris, 192 mM glycine pH 8.3, 0,1% SDS. Overnight separation of MHC was achieved using constant current mode at amperage of about 6 mA per slab, but tuning the value to that which gave an initial voltage of 40 Volts. Usually gel electrophoresis started late in the afternoon and the front dye ran out of the slab during the night. After 16-18 hours of electrophoresis the voltage rose to 180-200 Volts and the run was stopped. When bands contained more than 0.2 μg of protein the slabs were stained with 0.1% Coomassie blue in 5% acetic acid, 40% methanol. If bands contained less than 0.1 μg of protein the slabs were stained with the silver method [37] modified according to [5].

EPGE

Aliquots of stored myosin were precipitated by 10-time dilution with ice cold H_2O and centrifugation at 650 x g for 10 minutes. Then the pellet was resuspended in 10% v:v glycerol, 2.3% SDS, 0.7 M 2-Mercaptoethanol, 62.5 mM Tris HCI pH 6.8 (Sol. C). For preparative separation of MHC1, MHC2A and MHC2B we used EPGE protocol 1. To separate MHC2S EPGE protocol 2 was used.

EPGE PROTOCOL 1

EPGE was performed as described in [24] using the EPGE system of Genofit, Geneva, Switzerland, imported in Italy by M-Medical Genenco, Florence. The cylindrical gel, 2 cm in diameter and 6 cm in height, was made of 6 mL of 7.5% polyacrylamide separating gel and 12 mL of 3.8% polyacrylamide stacking gel. One mg of myosin was loaded over the gel in a volume up to 2 mL of Sol C. A constant current of 10 mA was applied. After several hours the electrophoretic front was at the bottom end of the gel and we started collecting the effluent in fractions of 15 minutes. Four hours later MHC eluted from the gel.

EPGE PROTOCOL 2

MHC fractions which had been partially purified with EPGE Protocol 1 were concentrated by dialysis against polyethyleneglicol (PEG) and mixed with Sol C. Aliquots containing 50-100 μg of MHC were stratified on cylindrical gel 1 cm wide and 8.7 cm high. The cylindrical gel was composed by approixmately 1 mL of stacking gel and 6.5 mL of 7.5% polyacrylamide separating gel. This relatively high concentration of polyacrylamide gel and its height were needed to give mechanical resistance to gel cylinder and so avoid breakage or climbing of the polyacrylamide gel in the support. After about 8 hours of electrophoresis when the dye front reached the bottom end of the gel we started collecting the effluent.

Due to their low extinction coefficient MHC and their content was determined in EPGE fractions by SDS 6% PAGE and densitometric scanning by a GS 300 Transmission/Reflectance Scanning Densitometer (Hoefer Scientific Instrument, San Francisco) connected to a Macintosh SE (Apple Computer).

Peptide mapping by steady-state enzymatic proteolysis

Peptide mapping of EPGE purified MHC was performed essentially according to [47, 48].

S.V8 protease or α-Chymotrypsin were used as proteolytic agents. 0.1 or 0.2 μg of proteins to be mapped were mixed with equal volume of 1 M NaCI, 1 M Tris-HCI pH 6.8, 0.1 mM $MgCl_2$, 1% SDS, 20% glycerol, then S.V8 protease or α-Chymotrypsin at 0.5:1 or 1:1 (w:w) protease to protein ratio was added. After 10- and 20-minute incubations at 37° C, proteolytic reaction was stopped by adding 3% w/v SDS and 5% v/v 2-Mercaptoethanol and boiling for 2 minutes. Then the digests were analyzed in SDS 7.5% PAGE at a constant current of 6 mA overnight. Slabs were stained with the silver technique [5, 37].

Results

It is well known that in biological research it is often necessary to analyze pure molecules and that SDS PAGE is a simple approach that allows separation of myosin heavy chain isoforms. Figure 1A shows the MHC pattern obtained by SDS 6% PAGE of two different amounts of myosin isolated from rat muscles. In the adult rat soleus, myosin contains two MHC; they are from the top of the gel MHC2S and MHC1 in a 1:10 ratio (lanes 1 and 2). Tongue myosin contains three MHC: MHC2S, MHC2A and MHC2B, the second being the most abundant. Lanes 5 and 6 of Fig. 1A show the patterns obtained by SDS 6% PAGE of two different amounts of rat EDL myosin, while lanes 7 and 8 those of myosin from rat diaphragm. EDL myosin essentially contains MHC2B and MHC2A, but trace amounts of MHC1 and MHC2S are also present. In the diaphragm myosin four bands are present, the MHC2B being the minor component. Figure 1B shows SDS

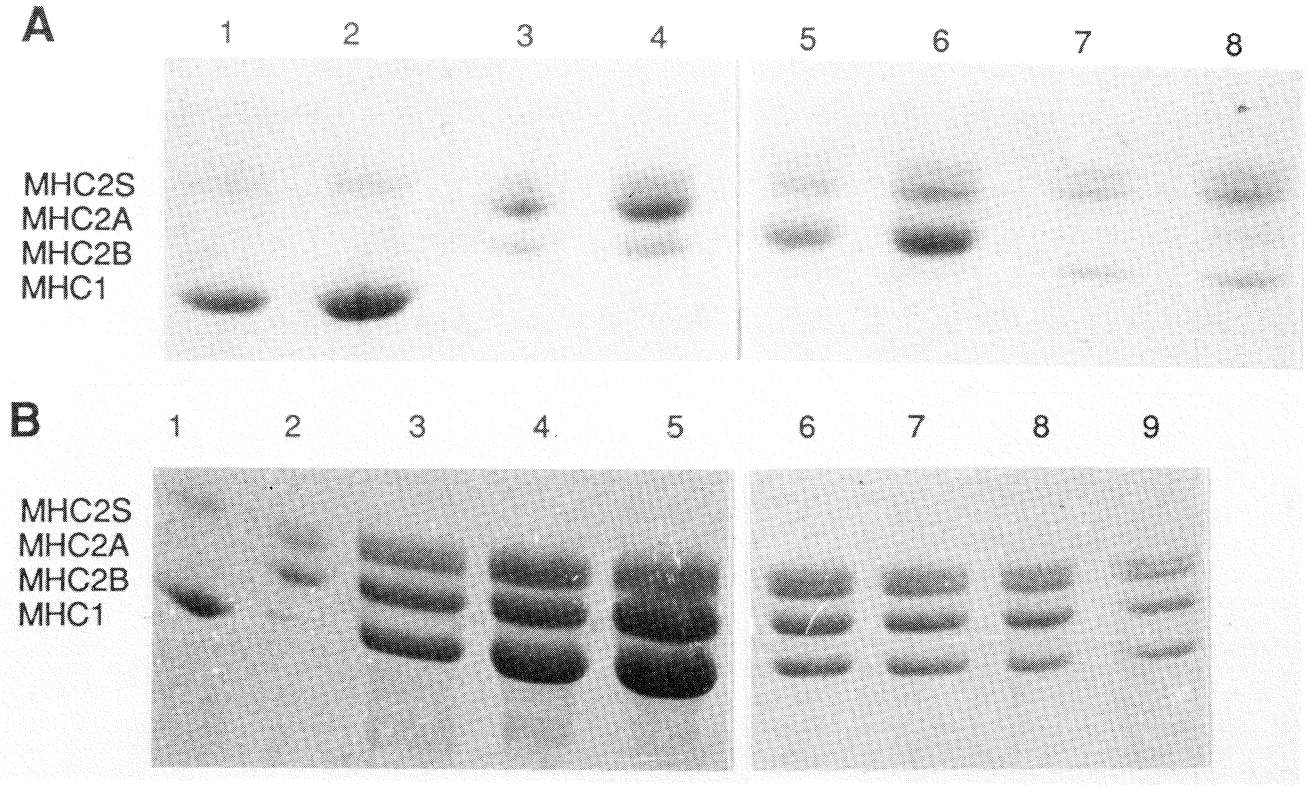

Figure 1. Myosin Heavy Chain of adult rat muscles. SDS 6% PAGE, Coomassie blue stain. A, MHC from: 1-2, soleus; 3-4, tongue; 5-6, EDL; 7-8, diaphragm, 0.25 and 0.5 μg of protein were loaded. B, MHC from: 1, soleus; 2, EDL; 3-9, EPGE fractions of a mixture of EDL and soleus myosins. MHC2S, MHC2A, MHC2B and MHC1: Myosin Heavy Chains of adult rat muscle.

6% PAGE of soleus (lane 1, 1 μg) and EDL (lane 2, 1 μg) myosin. In lanes 3-9 fractions of an EPGE of MHC from a mixture of EDL and soleus myosins are analyzed. Lane 3 shows a four MHC pattern while the overloaded lane 4 shows three bands only. The lanes 6-9 shows decreasing amounts of MHC1 in successive EPGE fractions. The four-band pattern became progressively better distinguishable in slots where decreasing amounts of protein were loaded. From the top of the gel the myosin heavy chains are: MHC2S, MHC2A, MHC2B and MHC1 [14].

Using Electroendosmotic Preparative Gel Electrophoresis, a new preparative approach, we have taken advantage of the discriminating power of SDS PAGE to purify individual MHC [40]. In EPGE a dialysis membrane is tightly held against the terminal end of a cylindrical polyacrylamide gel. Between gel and membrane there is a chamber with a near zero volume where a glass fiber is put in order to prevent plugging of the capillary outlet (situated in the middle of the gel cylinder) due to gel swelling. When an electrical field is applied between the electrodes the electroendosmotic flow of the buffer crosses the membrane rinsing the bottom surface of the gel. This flow elutes any molecular species emerging from the bottom end of the gel. This laminar flow generated underneath the gel by electroendosmosis is much more efficient for elution than conventional pumping, so that a high flow rate is not needed, allowing the recovery of purified samples in relatively small volumes [24, 25].

The results of a preparative electroendosmotic run of a mixture of rat EDL and soleus myosins are shown in Figure 2. We used SDS 12% PAGE to analyze the fractions of this preliminar experiment to comfirm that the buffer rinsing the bottom surface of the gel carries any emerging molecules present in the original mixture of fast and slow rat myosin preparations. The bands with the higher electrophoretic mobilities are MLC1S and MLC2S (MW of about 27 and 25 KDs respectively). At the middle of the gel there is the actin band (MW of about 46 KDs). Elution of MLC is documented in lane 5 in which is analyzed the fraction containing the elution tail of BBF dye which was collected 5 hours after starting the run. Lanes 5 and 6 show that, after BBF elution, actin and other minor proteins with MW of about 50 KDs coelute with the bulk of MLC. Both fast and slow MLC are present in these fractions. It is well knowh that MLC ca be individually separated only by SDS PAGE in which polyacrylamide concentration is higher than 10%: indeed we utilized for the preparation of EPGE a 6% polyacrylamide separating gel. Note that in lane 8 are concentrated 150 KDs proteins, which are very minor components in the original mixture. Lane 10 shows that a fraction collected 1 hours and 40 minutes after BBF elution contains MHC. Figure 2 confirms that using EPGE it is relatively easy to separate from complex mixtures proteins of different molecular weights. On the other hand in Figure 1 we showed that all the four MHC were present in each fraction. In this preliminar experiment separation of individual MHC isoforms was not obtained problably because the EPGE was overloaded.

Figure 3 shows that applying the EPGE protocol 1, that is, optimizing gel concentration and electric field, purification of MHC1 is easily achieved from soleus myosin (lanes 2-8). Compare lane 1 with lane 2 to recognize the sudden increase of pure MHC1 in the eluate. Lane 5 contains 0.1 μg of soleus myosin used as MHC marker and shows that in normal rat soleus the MHC1:MHC2S ratio is about 10:1. This is the most favourable

EPGE and peptide mapping of MHC

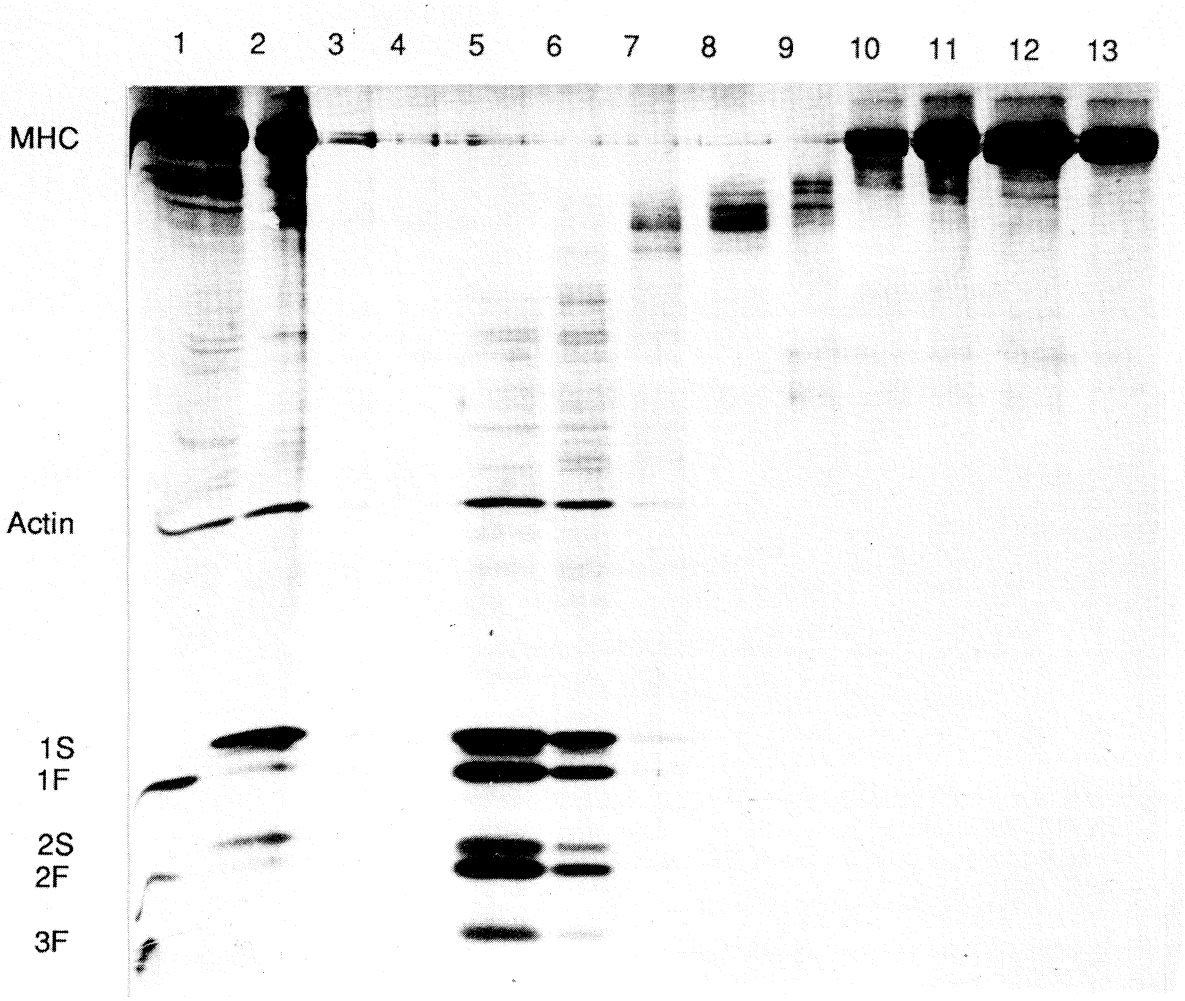

Figure 2: EPGE fractionation of rat myosin subunis. SDS 12% PAGE, silver stain. 1, EDL myosin 10 μg; 2, soleus myosin 10 μg; 3, EPGE fraction collected 4 hours after switch on of the electroendosmotic run; 4-13, twenty-minute fractions of electroendosmotic eluate. Progressive elution of MLC and actin (lanes 5-7), and of proteins with MW from 50 to about 100 KD (lanes 6-9) is shown. MHC are present in subsequent lanes.1S, 1F, 2S, 2F and 3F ,myosin light chains of fast and slow isomyosins. MHC, myosin heavy chains.

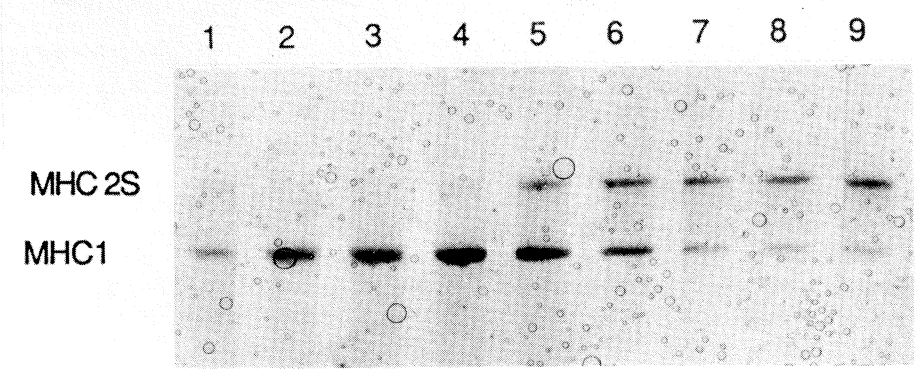

Figure 3: EPGE fractionation of MHC from rat soleus. SDS 6% PAGE, silver stain. 1, MHC elutes after 14 hours of EPGE; 2-4, successive EPGE fractions; 5, soleus myosin 0.1 μg; 6-9, EPGE fractions following that analyzed in lane 4. MHC1, MHC2S: Myosin Heavy Chains of adult rat soleus.

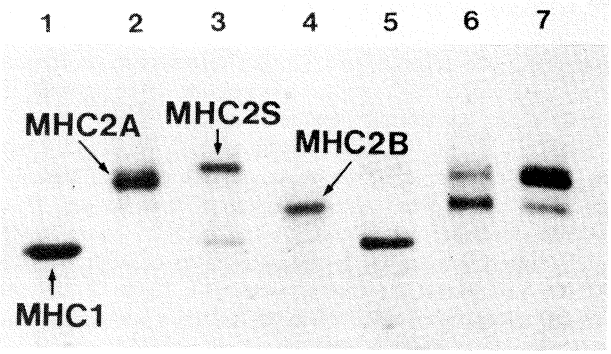

Figure 4: EPGE fractionation of rat MHC.SDS 6% PAGE, silver stain. 1, MHC1 in 1.8 µl of an EPGE fraction of soleus; MHC2A in 1.2 µl of an EPGE fraction of tongue; 3, MHC2S in 2 µl of a fraction of an EPGE protocol 1 of soleus; 4, MHC2B in 2.6 µl of an EPGE fraction of EDL; soleus 0.2 µg; 6, EDL 0.2 µg; 7, tongue 0.2 µg.

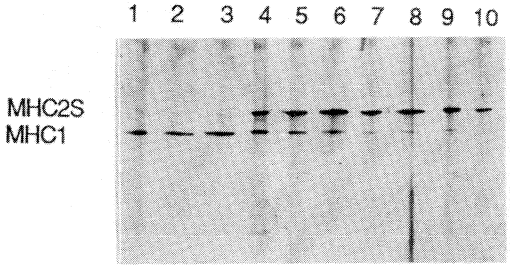

Figure 5: EPGE fractionation of MHC from rat soleus. SDS 6% PAGE, silver stain. 1, fraction number 48 collected at 19-hour electroendosmotic run; 2-10, 15-minute EPGE fractions 51-59. A better resolution of adult MHC2S is achieved with two successive EPGE (protocol 2).

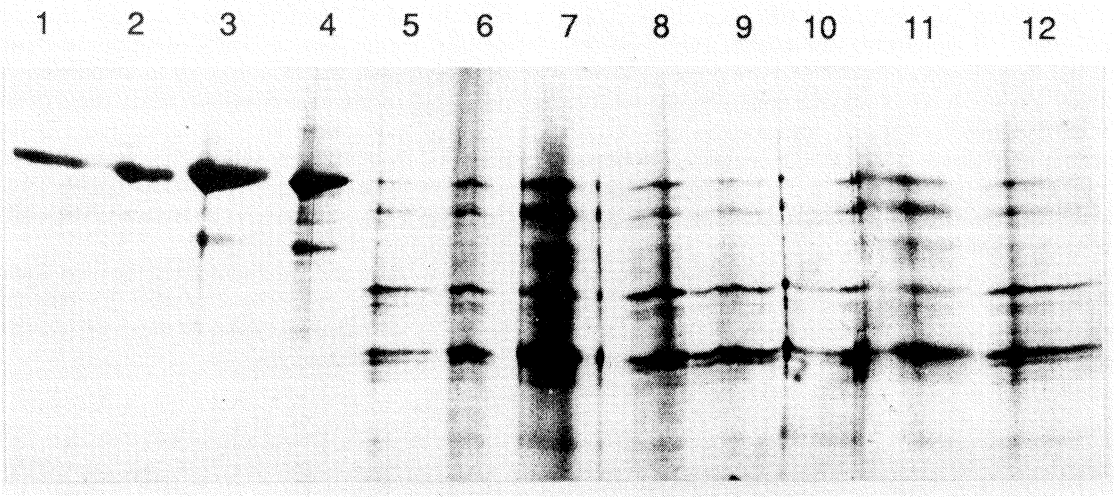

Figure 6: α-Chymotryptic Steady-State Peptide Mapping of MHC1 from rat soleus. SDS 7.5% PAGE, silver stain. Comparison of MHC1 obtained from several different EPGE of two different myosin extractions. 1, 5 and 9, MHC1 purified on 01.24.90 (Exp 1) from myosin extracted on 12.05.89 (Ext. 2); 2, 6 and 10, MHC1 purified on 01.22.90 (Ept 2) from myosin extracted on 12.05.89 (Ext 2); 3, 7 and 11, MHC1 purified on 05.23.90 (Exp 3) from myosin extracted on 02.01.89 (Ext 1); 4, 8 and 12, MHC1 purified on 04.17.89 (Exp 4) from myosin extracted on 02.01.89 (Ext 1). Peptide maps of MHC1 from myosin or purified MHC stored in glycerol at -20°C from 1 to 15 months.

natural mixture we found to separate MHC1 by EPGE. Using EDL, tongue and diaphragm myosins and EPGE protocol 1 we purified MHC2A and MHC2B in almost pure molecular form (see Figure 4). To fully purify MHC2S we were obliged to perform two successive EPGE runs (see EPGE protocol 2). Figure 5 shows the results of a second EPGE on fractions concentrated by dialysis against PEG to increase the amount of MHC isoforms loaded onto the second EPGE. To obtain protein concentration useful to further analyses, it is necessary to use an EPGE cylinder of 1 cm in diameter which realizes a smaller electroendosmotic flow than that of 2 cm. Due to low absolute amount of MHC2S in rat muscles to load 50-100 µg of protein it is necessary to concentrate five-ten times the eluate of the first EPGE. Figure 5 shows the MHC pattern of the fractions from such an EPGE protocol 2 run. Lane 1 of Fig. 5 shows a fraction collected 19 hours after the starting of the EPGE run: it contains MHC1 in pure molecular form. Lanes 2-10 show the following 15-minute fractions, which also contain pure MHC1. In lane 10 MHC2S is present in virtually pure molecular form. If necessary, a second protein concentration step by dialysis against PEG can be performed.

Steady-State Peptide Mapping (SSPM) of MHC.

The availability of pure MHC, though not full satisfactory for MHC2S, allows comparison by peptide mapping of these isoforms. Figure 6 shows the SSPM of rat soleus MHC1 purified

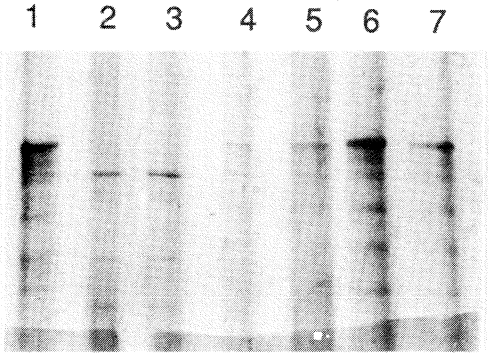

Figure 7: α-Chymotryptic Steady-State Peptide mapping of rat MHC2S, MHC2A, MHC2B and MHC1. SDS 7.5% PAGE, silver stain. Peptide patterns of: 1, MHC2B; 2 and 3, MHC1; 4 and 5, MHC2S; 6 and 7, MHC2A. Peptide patterns of MHC1, MHC2A and MHC2B are very different, on the other hand the patterns of MHC2A and MHC2S could be virtually superimpos ed.

by EPGE from two independent myosin preparations. MHC1 was incubated with a-Chymotrypsin at protein:protease ratio of 1:1 at 37° for 0, 10 and 20 minutes. In lanes 2 and 3 ahead of the MHC1 band few additional bands are present. These polypeptides are products of spontaneous degradation of MHC during freezing, thawing and storage at low concentration, since they were absent in SDS PAGE analyses performed just after purification (see Figures 3, 4, 5). These minor bands do not interfere with the chymotryptic patterning, since they disappear in the digested samples.

The peptide maps of MHC1 obtained from myosin preparations isolated from different groups of rats are superimposable, demonstrating that there are no modifications of the analyzed isoforms during EPGE and that the effects of the protease in the conditions used are reproducible. Note that the peptide patterns are independent from the absolute amount of substrate and the digestion time, since the bands represent the initial products of proteolysis. This is a necessary control before comparison of different proteins by peptide mapping could be made in non-equilibrium peptide mapping.

Figure 7 shows the chymotryptic pattern of the different MHC isoforms of adult rat. While peptide maps of MHC2A and MHC2B are very different from each other and from MHC1, the patterns of MHC2A and MHC2S could be virtually superimposed. While the differences between MHC1, MHC2A and MHC2B were expected [11, 13, 18, 21, 27, 39, 42, 48], similarity of MHC2A to MHC2S is a new result. The electrophoretic mobility of these two isoforms is almost identical (see introduction), but their structural identity or non-identity could be only inferred. Up to date a MHC2S gene has not been identified.

Figure 8 shows comparison of the steady state peptide maps of MHC1 from rat and sheep slow myosins. Incubation was at 37^0 C for 10 and 20 minutes with α-Chymotrypsin. In this analysis we used MHC1 purified with EPGE from two normal and two electrostimulated sheep *Latissimus Dorsi* (LD) and from rat soleus. As expected, remarkable differences between MHC1 from rat and sheep are demonstrated. Differences between MHC1 from myosins of normal and stimulated LD are also present. This surprising result can be explained either suggesting that the slow myosin in stimulated muscle is a different isoform of slow myosin heavy chain or stressing the fact that the normal and the stimulated LD myosin were prepared from different sheep. This last interpretation is sustained by the observation that the two normal LD prepared from two additional different animals also show different peptide patterns one from the other.

Since we have above demonstrated the remarkable reproducibility of our α-chymotriptic peptide mapping method (see Figure 6), we relate variability of sheep MHC1 to their origin from different individuals.

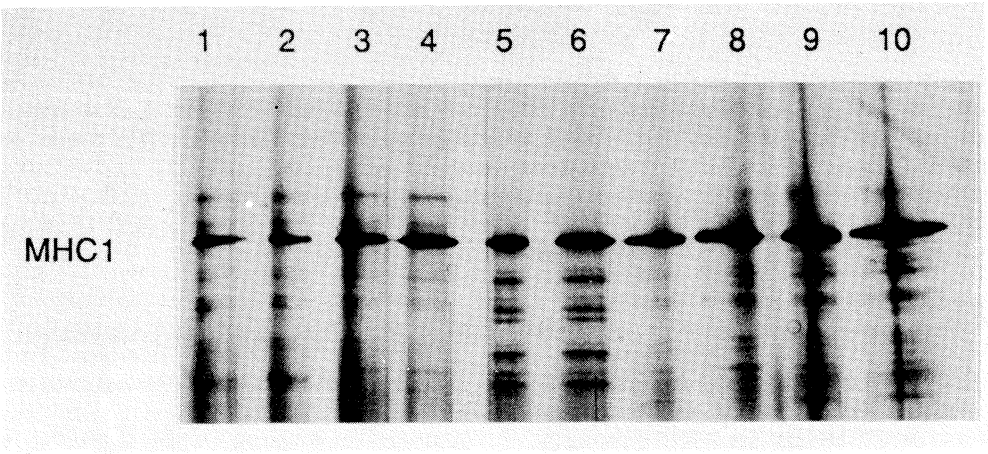

Figure 8: α-Chymotryptic Steady-State Peptide Mapping of MHC1 of rat and sheep. SDS 7.5% PAGE, silver stain. MHC1 from two normal and two electrostimulated LD of four different sheep and MHC1 from rat soleus are compared. Peptide patterns of MHC1 from: 1-2, normal LD of sheep 201; 3-4, normal LD of sheep 202; 5-6, electrostimulated LD of sheep Bo8; electrostimulated LD of sheep Bo7; 9-10, rat soleus. While duplicates are superimposable a remarkable difference between MHC1 of rat and sheep is shown. Differences exist not only between MHC1 of normal and stimulated LD, but also among the two different normal muscles.

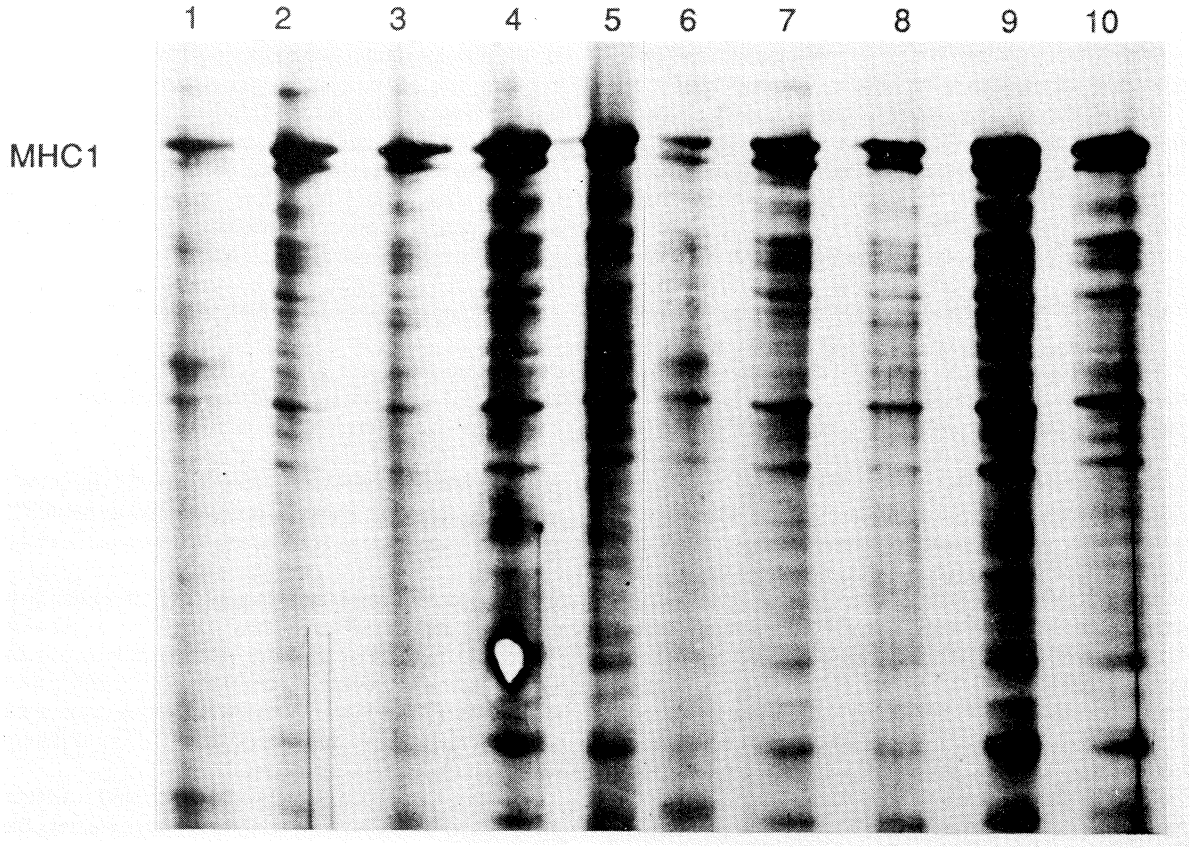

Figure 9: S.V8 Steady-State Peptide Mapping of MHC1 from rat and sheep. SDS 7.5% PAGE, silver stain. Peptide patterns of MHC1 from: 1 and 6, normal LD of sheep 201; Lanes 2 and 7, normal LD of sheep 202; 3 and 8, electrostimulated LD of sheep Bo 8; 4 and 9, electrostimulated LD of sheep Bo 7; 5 and 10, rat soleus. SV8 patterns of MHC1 of rat soleus and of normal and electrostimulated sheep LD are virtually identical.

Though MHC1 from different mammals have conserved SV8 peptide mapping [12, 18], fragmentation with α-Chymotrypsin, which has a different cleavage specificity [4, 29], reveals the expected sequence differences among rat and sheep. Since myosin was purified from muscle of different sheep, the variability reflects the genetic drift between strains or individual differences between sheep of wild strains. It is interesting to remember that all the analyzed MHC1 have the same electrophoretic mobility in SDS 6% PAGE so that the structural differences do not appear to coarsely modify their molecular weight, shape and flexibility [45]. Figure 9 shows the proteolitic maps obtained by 10 and 20 minutes digestion with S.V8 protease of the MHC1 purified from rat and sheep slow myosins. These peptide maps confirm our previous results, i.e. that rat and sheep MHC1 gave very similar SV8 patterns, and originally demonstrate that under such conditions MHC1 from normal and chronically electrostimulated LD are virtually identical. Thus we deduce that during phylogeny there were not genetic modifications on the dicarboxylic amino acids which are the specific sites of clevalage of SV8 protease [4, 29].

Discussion

Many muscle proteins exist as multiple isoforms, characteristic of different muscle types, and indeed frequently present in non-muscle tissue too [6, 31]. This is particularly striking for the contractile proteins of both sarcomeric and non-sarcomeric muscles. Actin, the major structural protein of the thin filaments of sarcomeres, for example, is present in at least six different isoforms in higher vertebrates. The extent of gene diversification is different for different proteins: cardiac and skeletal actins are remarkably conserved, with only 4 substitutions in 376 aminoacid residues. Much larger gene diversions are known for myosin molecule, the constitutive protein of the thick filament, since not only are different isoforms present in different adult muscle types, and in non-muscle cells, but there are also distinct developmental myosins present in fetal and neonatal muscles [8]. How such a diversity is generated is an important consideration in any discussion on regulatory factors and mechanisms of muscle plasticity. Essentially four strategies can be distinguished: i) Muscle genes are organized into multigene families where distinct genes encode each isoform (e.g. isoactins); ii) The same gene encodes two or more isoforms which are generated by different combinations of exons during splicing of the pre-messanger RNA in the nucleus (e.g., myosin alkali light chains and α-tropomyosins); iii) Different isoforms of multisubunit proteins are obtained combining different subsets of two of more gene families (e.g., fast isomyosins); iiii) Post-translational modifications (phosphorylation, methylation, acetylation, glycosylation or myristylation of aminoacid residues) produce structural

changes which, being distinguishable by immunochemical (epitopes) or chemical analyses, allow further subgrouping of contractile protein isoform (e.g., myosin light and heavy chains). Different combinations of the previous mechanisms add to contractile protein diversification, making the number of different types of sarcomeres in the animal kingdom almost infinite [39].

In sarcomeric muscles of high vertebrates myosin is an exapolypeptide made of two heavy and four light subunits. Gel electrophoresis in non denaturing conditions separates the slower running band of slow isomyosin from the faster running bands of fast isomyosins. Embryonic and neonatal isomyosins run with or ahead of the fast isomyosins.

Six sarcomeric myosin heavy chain genes had been recognized: α-cardiac MHC, β-cardiac MHC or MHC1, MHC2A, MHC2B, MHCEmb, and MHCNeo. MHC1, MHC2A and MHC2B are usually segregated in distinct types of adult skeletal fibres, that is, each of them is present alone in a single myofiber. MHCemb and MHCneo are expressed in developing fibres during both ontogenesis and regeneration. A fourth isomyosin, characterized by a fast type MHC, has been identified at protein level. MHC2B is the heavy chain peculiar of the fast glycolytic fiber, MHC 2A is that of the majority of the oxidateve-glycolytic (that is, non-2B fibers) in fast or mixed muscle (e.g., EDL, *tibialis anterior* or diaphragm) and finally we name MHC2S the third fast MHC since it is the only fast MHC which is present in rat soleus, a typical fatigue-resistant slow muscle which contains a minor proportion of oxidative-glycolytic fibers. MHC2S usually comigrates with MHC2A in SDS 6% PAGE. We here confirm that slight but critical changes of the electrophoretic conditions allow the separation of the MHC isoforms of adult muscles in four distinct bands which run from the top of the gel in the following order: MHC2S, MHC2A, MHC2B and MHC1. The MHCemb and MHCneo run in between MHC2A and MHC2B, but resolution is really poor in complex mixtures of MHC [9,17]. While the electrophoretic mobility of the fast MHC slightly changes in different mammals making the correspondence very difficult, the MHC1 seems to be a very conserved gene: all the different species MHC1 so far analyzed comigrate in SDS 6% PAGE [10]. MHC1 is indeed the most reliable marker of fatigue resistant muscle.

Taking advantage of these powerful analytical methods we have previously demonstrated that: i) denervated muscle preferentially loses slow myosin [7, 14, 19, 21]; ii) aneural regenerating muscle accumulates fast isomyosins [14, 19, 38]; iii) slow myosin accumulates in denervated fast muscle subjected to long-term continuous [7, 13, 15] or cyclic [10, 20] functional electrostimulation.

In this paper we tried to determine by peptide mapping of EPGE purified MHC if MHC2S belong to the MHC2A gene family and if inter- and intra-specific polymorphisms of MHC1 exist.

Electroendosmotic preparative gel electrophoresis is a new preparative method to purify high molecular weight proteins from complex mixtures. This method takes advantage of the discriminating power of SDS PAGE and of a flow of buffer generated by the electric field through a capillary inserted in a polyacrylamide gel cylinder whose extremity is closed with a dialysis membrane. The flow carries the proteins eluting from the bottom surface of the gel, so that they can be independently collected as purified fractions. Purification of proteins depends on the absolute and relative amounts in complex mixtures and on their relative mobility in gel electrophoresis. Using this approach we virtually purified all the adult type MHC.

As for any chromatographic method a limit is the superimposition of the gaussian contour lines that indicate the protein concentration in the EPGE flow. To obviate this problem we utilized natural mixtures of MHC with favourable ratio regarding their relative concentration and electrophoretic mobility in SDS PAGE. In spite of that the concentration of MHC2S in any tested mixture was useful high enough to obtain a one step amounts of pure material by EPGE. By a two-step EPGE purification (Protocol 2) we collected pure MHC2S in an amount just sufficient to perform peptide mapping.

Using silver staining it has been possible to compare peptide mapps of MHC2S with those of other MHC. We used two proteolytic enzymes: S.V8 protease and α-Chymotrypsin.

S.V8 protease is a bacterial protease that at pH 6.8 cleaves specifically the peptide bonds on the carboxyl-terminal side of either aspartate or glutamate residues. α-Chymotrypsin is a less specific protease which cleaves peptide bonds after larger hydrophobic chains.

The reliability of steady-state peptide mapping is soundly stated on the following observations: i) peptide patterns of independent proteolysis of each MHC preparation are identical; ii) α-chymotrypic patterns of MHC1 from different individuals of inbred rats are identical.

Applying these powerful preparatives and analytical approaches to MHC isoform analysis we here showed the high resemblance of MHC2S and MHC2A peptide patterns. The different electrophoretic mobility of MHC2S and MHC2A could be the result of differences in the primary nucleotide sequence of two genes, of differential DNA splicing of an unique gene or of different transcriptional modifications of an unique gene product [14, 43]. Peptide pattern identity suggest that MHC2S and MHC2A are products of an unique gene. In any case modulation of MHC2A and MHC2S plays a physiological role since they are differentially expressed in different fiber types and in denervated and chronically electrostimulated muscles [2, 14, 43].

By peptide mapping we have here also compared MHC1 purified by EPGE from rat and sheep muscles and from normal and electrostimulated sheep skeletal muscles.

S.V8 proteolytic patterns of MHC1 show remarkable similarity in animals of different species, i.e. rat and sheep. Therefore we deduce that during phylogeny there have been not major modifications on aspartate and/or glutamate residues. By SV8 mapping we have here also comfirmed that the MHC1 accumulated in chronically electrostimulated sheep muscles has the SV8 peptide pattern of sheep MHC1.

Latissimus Dorsi (LD) is one of the muscles used in experimental development of muscle-powered cardiac assistance [1, 22, 32, 36]. Prolonged electrical stimulation is used to change this fast-fatiguable muscle into being virtually fatigue-resistant. This requires a change in metabolism from glycolytic to oxidative and in myosin isoforms from fast to slow [39]. Fiber type transformation has been demonstrated by histochemical an biochemical analyses. However in goat Chachques et al. [23] have shown that the myosin present in the LD after long-term conditioning by a cyclic 30 Hz pattern is very similar but not identical to the slow isomyosin: indeed myosin shows a slight but different behaviour in polyacrylamide gel electrophoresis

in non denaturing conditions with respect to both slow-skeletal and ventricular myosins.

In our experimental sheep the gel electrophoretic analysis of the native myosin molecule shows that LD of normal adult sheep is a mixed muscle in which fast isomyosins largely prevail. After few weeks of electrostimulation there is a substantial increase in the proportion of slow isomyosin (up to 100%), compared to the 10-20% in the control pre-stimulated LD. However, while SDS PAGE and SDS OPM confirm the adult slow nature of the myosin heavy chains, in preliminary experiments the myosin light chain analyses did not show the pattern peculiar to adult slow fibers [15]. Therefore we further investigated by 2D gel electrophoresis and HPLC the possibility that the cyclic, cardiac-like pattern of electrostimulation may induce the expression of some of the cardiac myosin subunits in the conditioned muscle [20]. Furthermore we compared the effects of the cyclic electrostimulation protocol with those of a continuous 2 Hz frequency pattern. The effects of the continuous 2 Hz pattern (24 hs per day, i.e. 165.600 twitches per day) or of cyclic but uninterrupted protocol (24 hs per day of 125-msec-long 30 Hz trains followed by 1 sec rest, i.e. 230.000 0.2-msec-long electric currents, producing 76.800 fused tetani per day) are comparable. In both cases the analyses of native myosin and of its light and heavy subunits reveal that the stimulated LD is turned into a typical slow type skeletal muscle, i.e. it expresses the tissue specific 1'S myosin light chain [9, 10, 17, 20].

On the other hand we have here shown that chymotryptic patterns of MHC1 accumulated in electrostimulated muscles are different from those of MHC1 from normal muscle.

However this difference is related to population polymorphism and therefore probably localized in portions not essential for protein functions. In fact chymotryptic patterns of MHC1 isolated from normal LD of two additional sheep also are different one to the other. Chymotryptic pattern identity of MHC1 from different rat preparations are due to the inbred origin of the rat strain we used. The variability of the patterns of sheep MHC1 results from the use of animals different in strain, sex and age.

Fast to slow isomyosin transition in conditioned muscle has been soundly demonstrated. On the other hand recently it has been claimed that a "cardiac" myosin is expressed during early myogenesis [49]. This finding reopened the possibility that eventual accumulation of ventricle myosin in conditioned LD could be interpreted as demonstration of muscle regeneration during muscle transformation. Our results showing that the conditioned muscle express a typical skeletal slow myosin negate this interpretation and confirm that muscle damage is not a necessary step of muscle conditioning to fatigue resistance. Furthermore during all this experimentation we had further evidence that a muscle become able to sustain continuous (24 hs per day) tetanic/cardiac-like work during the second week of muscle conditioning. It can be easily predicted that early after surgery with an excalating cycling protocol it will be possible to obtain a low, but significant amount of work, which could be used for cardiac support and meanwhile to drive changes of muscle characteristics. In conclusion, on the sound ground of our own experimental knowledge, we believe that skeletal muscle can be made tireless and used in biological activated circulation support devices and that such devices will have in the near future an increasing role in reducing disability and death from heart diseases.

Acknowledgments

Supported by funds from the Italian C. N. R. to the Unit for Muscle Biology and Physiopathology, to the Progetto Finalizzato: Tecnologie Biomediche e Sanitarie (TBMS) and from the Italian Ministero della Pubblica Istruzione 60% to Prof. U. Carraro.

Address correspondence to:

U. Carraro, Institute of General Pathology, Via Trieste 75, I-35121 Padova Italy; FAX INT 39 49 8286576.

References

[1] Acker MA, Hammond RL, Mannion JD, Salmons S, Stephenson LW: Skeletal muscle as the potential power source for a cardiovascular pump: assessment in vivo. *Science* 1987; 236:324-327.

[2] Ausoni S, Gorza L, Schiaffino S, Goundersen K, Lømo T: Expression of myosin heavy chain isoforms in stimulated fast and slow rat muscles. *J Neurosci* 1990; 10: 153-160.

[3] Bar A, Pette D: Three fast myosin heavy chain in adult rat skeletal muscle. *FEBS lett* 1988; 235: 153-155.

[4] Bennet CJ: Denaturation of polypeptide substrates. *Methods in Enzymology* 1967; XI: 211-231.

[5] Betto R: Rivelazione delle proteine con Argento-Silver Staining, in Carraro U e Dalla Libera L (eds): *Elettroforesi ed HPLC di biopolimeri e loro frammenti.* Padova, Unipress Padova 1989, pp124-125.

[6] Buckingham ME: Actin and myosin multigenes families: their expression during formation of skeletal muscle. *Essays Biochem* 1985; 20: 77-109.

[7] Carraro U: The denervated muscle: a standby engine, in Stagnaro E (ed): *Towards the Artificial Heart.* Padova, Imprimitur, 1988, pp 179-188.

[8] Carraro U (ed): *Sarcomeric and Non-Sarcomeric Muscles: Basic and Applied Research Prospect for the 90's.* Padova, Unipress Padova, 1988, pp 1-777.

[9] Carraro U: Gel elettroforesi bidimensionale sec. O'Farrell, in Carraro U e Dalla Libera L (Eds): *Elettroforesi e HPLC di Biopolimeri e loro Frammenti.* Padova, Unipress Padova 1989, pp 104-108.

[10] Carraro U: Contractile proteins of fatigue resistant muscle. *Sem. Thor Card Surg* 1991; 3:1-5.

[11] Carraro U, Catani C: A sensitive SDS-PAGE method separating myosin haevy chain isoforms of rat skeletal muscles reveals the heterogeneous nature of the embryonic myosin. *Biochem Biophsy Res Commun* 1983; 116: 793-802.

[12] Carraro U, Catani C: Myosin haevy chains analyses as a tool for the study of electrostimulated muscle. Proceedings of the *2nd Vienna Int Workshop on Functional Electrostimulation.. 1986; 51-54.*

[13] Carraro U, Catani C, Belluco S, Cantini M, Marchioro L: Slow-like electrostimulation switches on slow

moysin in denervated fast muscles. *Exp Neurol* 1986; 94: 573-553.

[14] Carraro U, Catani C, Rizzi C: Myosin expression in denervated fast and slow twitch muscles: Fiber modulation and substitution, in Pette D (ed): *The Dynamic State of Muscle Fibers*. Berlin-New York, Walter de Gruyter, 1990, pp. 247-262.

[15] Carraro U, Catani C, Dell'Antone P, Danieli Betto D, Arpesella G, Parlapiano M, Cirillo M, Albanese S, Pierangeli A: An experimental pumping chamber made in situ with sheep latissimus dorsi: light microscopy and isomyosin , in Carraro U (ed): *Sarcomeric and Non-Sarcomeric Muscles: Basic and Applied Research Prospect for the 90's.* Padova, Unipress Padova, 1988, pp. 459-470.

[16] Carraro U, Catani C, Kessler Icekson G, Schlesinger H, Betto R, Danieli Betto D, Biral D: Gel electrophoretic analyses of myosin haevy chain isoforms by SDS PAGE and SDS OPM, in Carraro U (ed): *Sarcomeric and Non Sarcomeric Muscles: Basic and Applied Research Prospects for the 90's.* Padova, Unipress Padova, 1988, pp. 81-92.

[17] Carraro U, Catani C, Rizzi C, Danieli Betto D, Arpesella G, Mikus PM, Cirillo M, Parlapiano M, Senni M, Pierangeli A: Muscle conditioning for circulatory assistance: effective protocol by using continuous or cyclic stimulation, in Salmons S (ed): *Expert Meeting* HEART. *Muscle Conditioning for Circulatory Assistance.* Proceedings of the topical workshop held in Liverpool, 1990, 9, pp. 1-5.

[18] Carraro U, Catani C, Saggin L, Zrunek M, Szabolcs M, Gruber H, Streinzer W, Mayr W, Thoma H: Isomyosin changes after functional electrostimulation of denervated sheep muscle. *Muscle Nerve* 1988; 11: 1016-1028.

[19] Carraro U, Dalla Libera L, Catani C: Myosin light and heavy chains in muscles regenerating in absence of the nerve: transient appearance of the embryonic light chain. *Exp Neurol* 1983; 79: 106-117.

[20] Carraro U, Dalla Libera L, Catani C, Rizzi C: Myosin light chain composition of electrostimulated sheep muscle, in: Freilinger E (ed): *3rd Vienna Muscle Symposium.* Vienna, Facultas, in press.

[21] Carraro U, Morale D, Mussini I, Lucke S, Cantini M, Betto R, Catani C, Dalla Libera L, Danieli-Betto D, Noventa D: Chronic denervation of rat diaphragm: maintenance of fiber heterogeneity with associated increasing uniformity of myosin isoforms. *J Cell Biol* 1985; 100: 161-174.

[22] Carpentier A, Chachques J C: Myocardial substitution with a stimulated skeletal muscle: First succesful clinical case. *Lancet* 1985; 8440: 1267.

[23] Chachques JC, Grandjean PA, Schwartz K, Pfeffer TA, Swynghedauw B, Fardeau M, Sebille A, Tommasi JJ, Romero N, Carbrera-Fiscer EI, Nataf P, Carpentier A: Chronically stimulated skeletal muscle: enhancement of cardiac performance, in Carraro U (ed): *Sarcomeric and Non-Sarcomeric Muscles: Basic and Applied Research Prospects for the 90's:* Padova, Unipress Padova, 1988, pp. 429-447.

[24] Curioni A, Dal Belin Peruffo A: Electroforesi preparativa con eluizione delle proteine separate mediante elettroendosmosi. Carraro U and Dalla Libera L (eds): *Elettroforesi ed HPLC di biopolimeri e loro frammenti.* Padova, Unipress Padova, 1989; pp. 92-95.

[25] Curioni A, Dal Belin Peruffo A, Nuti MP: Purification of cellulases from Streptomices strain A20 by electroendosmotic preparative electrophoresis. *Electrophoresis* 1988; 9: 327-330.

[26] D'Albis A, Couteaux R, Jammot C, Mira JC: Myosin isoform transitions in regeneration of fast and slow muscles during postnatal development of the rat. *Devel Biol* 1989; 135: 320-325.

[27] Dalla Libera L, Sartore S, Pierobon-Bormioli S, Schiaffino S: Fast-white and fast-red isomyosin in guinea pig muscles. *Biochem Biophis Res Commun*1980; 96: 1652-1660.

[28] Danieli-Betto D, Zerbato E, Betto R,: Type 1, 2A, 2B myosin heavy chain electrophoretic analysis of rat muscle fibers. *Biochem Biophis Res Commun*1986; 138: 981-987.

[29] Drapeau RC: Cleavage at glutamic acid with staphylococcal protease. *Methods in Enzymology.* 1977; XLVII: 189-191.

[30] Gautier GF, Lowey S 1979: Distribution of myosin isoenzymes among skeletal muscle fiber types. *J Cell Biol* 1979; 81: 10-25.

[31] Giometti CS: Muscle protein analysis by two-dimensional gel electrophoresis CRC *Crit Rev Clin Lab Sci* 1981; 18: 79-109.

[32] Gruber H, Frey M, Happak WW, Burgasser G, Mayr W, Losert U, Deutinger M, Winberger M, Thoma H: Cardiomyoplasty: morphological changes in the sheep latissimus dorsi muscle. In Carraro U (ed): *Sarcomeric and Non-Sarcomeric Muscles: Basic and Applied Research Prospects for the 90's.* Padova, Unipress Padova, 1988, pp. 471-476.

[33] Laemmli UK,: Cleavage of structural proteins during assembly of the head of bacteriophage. *Nature* 1970; 227: 680-685.

[34] LaFramboise WA, Daood MJ, Guthrie RD, Moretti O, Schiaffino S, Ontell M: Electrophoretic separation and immunological identification of type 2X myosin heavy chain in rat skeletal muslce. *Biochim Biophys Acta* 1990; 1035: 109-112.

[35] Lompre' AM, Nadal-Ginard B, Mahdavi V: Expression of the cardiac venticular α-and β-myosin heavy chain genes is developmentally and hormonally regulated. *J Biol Chem* 1984; 259: 6437-6446.

[36] Magovern GJ, Heckler FR, Park SB, Christlieb IY, Lieber GA, Burkholder JA, Maher TD, Beneckart DH, Magovern GJ, Kao RL: Paced skeletal muscle for

diynamic cardiomyoplasty. *Ann Thorac Surg* 1988; 45: 614-619.

[37] Merril CR, Goldman D, Sedman SA, Ebert MH: Ultra sensitive strain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 1981; 211: 1437-1438.

[38] Mussini I, Favaro I, Carraro U: Maturation, dystrophic changes and the continuous production of fibers in skeletal muscle regenerating in the absence of nerve. *Neuropath Exp Neurol* 1987; 46: 315-331.

[39] Pette D, Vrbova G: Neural control of phenotypic expression in mammalian muscle fibers. *Muscle Nerve* 1985; 8: 676-689.

[40] Rizzi C, Catani C, Carraro U: Electroendosmotic preparative electrophoresis (EPE), the final answer to protein subunit fractionation: Myosin heavy chain isoform. *Europ J Cell Biol* 1989; Supplement 28 (Vol. 49): 46.

[41] Rushbrook Jl, Stracher A: Comparison of adult, embryonic and dystrophic myosin heavy chain from chicken muscles by SDS-Polyacrylammide gel electrophoresis and peptide mapping. *Proc Natl Acad Sci* USA 1979; 76: 4331-4334.

[42] Salmons S, Henriksson J: The adaptive response of skeletal muscle to increased use. *Muscle Nerve* 1981; 4: 94-105.

[43] Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lomo T: Three myosin heavy chain isoforms in type 2 muscle fibres. *J Muscle Res Cell Motil* 1989; 10: 197-205.

[44] Schiaffino S, Saggin L, Viel A, Gorza L: Differentiation of fibre types in rat skeletal muscle visualized with monoclonal antibodies. *J Muscle Res Cell Motil* 1985; 6, 60-61

[45] Smisek LD, Hoagland AD: Electrophoresis of flexible macromolecules: Evidence for a new mode of transport in gels. *Science* 1990; 248: 1221-1223.

[46] Sugiura T, Murakami N: Separation of Myosin Heavy Chain isoforms in rat skeletal muscles by gradient Sodium Dodecil Sulfate-Polyacrylamide Gel Electrophoresis. *Biomed Res* 1990; 11: 87-91.

[47] Whalen R G, Schwartz K, Bouveret P, Sell S M, Gros F: Contractile protein isozymes in muscle development: Identification of an embryonic form of myosin heavy chain. *Proc Nat Acad Sci* USA 1979; 76: 5197-5201.

[48] Whalen R G, Sell S M, Bulter-Browne G S, Schwartz K, Bouveret P, Pinset-Harstrøm I: Three myosin heavy chain isoenzymes appaer sequentially in rat muscle development. *Nature* 1981; 292: 805-809.

[49] Wick M, Bandman E: Differentiating chicken skeletal muscles cells express a ventricular myosin heavy chain. *J Cell Biol* 1989; 109 [4] part 2: 262a.