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Myosin light chain phosphorylation and isometric twitch potentiation in intact human muscle

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Abstract. The effects of a single voluntary contraction of the quadriceps muscle group on phosphate incorporation into the phosphorylatable light chains (P-light chains) of fast and slow myosin isolated from the vastus lateralis muscle and potentiation of the electrically stimulated twitch tension was studied in intact human muscle. Twitch potentiation was maximal 20 s after the voluntary contraction. Thereafter, twitch potentiation declined, but was still significantly higher than pre-contraction values 2 min after the voluntary contraction. Phosphate incorporation into the P-light chain of fast myosin followed a similar time course to twitch potentiation, but no phosphate was incorporated into slow myosin P-light chains. These observations suggest that myosin light chain kinase activity is mainly associated with fast-twitch muscle fibers and, in agreement with previous studies, suggests that twitch potentiation associated with P-light chain phosphorylation is confined to the fast-twitch fibers of human muscle.

Key words: Twitch potentiation of human skeletal muscle – P-light chain phosphorylation – Myosin light chain kinase – Myosin

Introduction

Skeletal muscle myosin contains a class of light chain subunits, designated P-light chains, that can be phosphorylated by a myosin light chain kinase in a Ca^{2+} dependent manner. The kinase is activated by Ca^{2+}-bound calmodulin, and activation occurs when the Ca^{2+} concentration is raised to a level found in contracting muscle (Blumenthal and Stull 1980). Dephosphorylation of P-light chains is catalyzed by a phosphatase which does not appear to be regulated (Morgan et al. 1976). Considerable attention has been directed to the physiological significance of myosin phosphorylation in skeletal muscle. Crow and Kushmerick (1982a) reported that an increase in myosin P-light chain phosphorylation was associated with a reduced rate of maximal muscle shortening velocity; however, Butler et al. (1983) subsequently showed that myosin phosphorylation did not affect myosin cross-bridge cycling rate. A consistent observation from a number of studies is that there is a correlation between the extent of P-light chain phosphorylation and potentiation of the isometric twitch tension following repetitive low frequency or tetanic stimulation in fast skeletal muscles (Manning and Stull 1979, 1982; Klug et al. 1982; Moore and Stull 1984).

In contrast to fast muscles, P-light chain phosphorylation and isometric twitch potentiation has not been observed in slow skeletal muscles from such species as the mouse (Crow and Kushmerick 1982b), rat (Moore and Stull 1984) and rabbit (Moore et al. unpublished observations). One explanation for this difference between slow and fast skeletal muscles is the relative proportion of myosin light chain kinase and phosphatase activities in these muscle types. Rat fast muscles contain more than three times as much kinase activity as slow muscles (DiSalvo et al. 1983; Moore and Stull 1984). Further, measurements of the rates of myosin dephosphorylation (Moore and Stull 1984) have demonstrated that myosin light chain phosphatase activity is greater in slow compared to fast skeletal muscles.

In this experiment, we have examined the effects of a single maximal voluntary contraction on electrically stimulated twitch tensions in human subjects. In addition, the relationship between P-light chain phosphate content in isolated fast and slow myosin and twitch potentiation was studied using an intact human muscle model.

Methods

Six healthy males, 19.7 ± 0.8 years (mean $\pm$ SEM) and 74.0 ± 4.2 kg participated in this experiment after being informed of the nature and risks of the procedures involved and signing consent forms approved by the University of Waterloo Committee for Human Research.

General protocol. During the experiment the subjects sat in a straight-backed chair with their legs at a knee angle of 90°. A restraining strap was positioned across their hips. The experiment was conducted over 3 consecutive days. On the first 2 days, the quadriceps muscle group of the right leg was electrically stimulated with single pulses using surface electrodes, and the isometric tension generated at the ankle was recorded. Three twitches separated by 30 s were elicited, and this was quickly followed by a single, maximal, voluntary knee extension of the right leg at $90° \cdot s^{-1}$ against the lever arm of a Cybex II isokinetic dynamometer. Three additional twitch contractions were then electrically generated at 20, 60 and 120 s following the maximal voluntary contraction. On the third day, four muscle biopsy samples were obtained from the vastus lateralis muscle of the right leg at the following times: when the subjects were seated and relaxed in the testing chair; immediately after the

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completion of a single, maximal, voluntary contraction of the right leg at $90° \cdot s^{-1}$; and 20 and 120 s after the voluntary contraction.

Muscle stimulation. To stimulate the quadriceps muscle group, two large brass electrodes (13 × 13 cm) covered in gauze and soaked in saline were placed proximally and distally on the antero-lateral portion of the thigh (Hultman et al. 1981). Twitch responses were elicited by single square pulses, 50 μs in duration from a Grass Stimulator (Modell S-44). The voltage necessary to elicit a maximal isometric twitch contraction from each subject was determined by starting at an initial intensity of 90 V, and increasing this by 5 V until three successive voltages produced similar twitch tensions. The highest of these voltages was employed for all subsequent twitch measures for each subject.

Twitch tensions. To measure the isometric twitch tension of the quadriceps muscle group, a cuff was placed around the lower leg just above the malleous. This was attached to a rapid-release metal coupler and cable which, in turn, was connected to a linear variable displacement transducer. The isometric twitch tension in Newtons was recorded on a Hewlett Packard chart recorder. Twitch tensions were expressed as ratios (P*/P) where P represents the initial twitch tension before the voluntary contraction and P* represents all subsequently measured twitch tensions. To perform the single dynamic contractions, the coupler linking the force transducer to the leg cuff was released and the leg of the subject was quickly attached to the lever arm of the Cybex II isokinetic dynamometer. This procedure was reversed to obtain twitch tensions after the voluntary contraction.

Muscle samples. At least 30 min prior to the experiment, four closely spaced incisions were made through locally anesthetized skin and fascia overlying the right vastus lateralis muscle of each subject. Later, with the subject seated in the testing chair, two resting biopsy samples were obtained from the same incision using the needle technique (Bergström 1962). The first biopsy sample was quickly placed in liquid nitrogen. The second muscle sample was mounted on a freezing chuck in embedding medium, frozen in isopentane cooled in liquid nitrogen and stored at −80° C for subsequent histochemical analysis. Three additional biopsy samples were obtained, immediately upon completion of the maximal voluntary contraction (0 s) and 20 and 120 s later. For the latter three biopsies, the needle was immediately placed in liquid nitrogen. With all biopsies, care was taken to ensure that the samples obtained were close to the fascia overlying the vastus lateralis. The muscle samples for biochemical analyses were removed from the needle under liquid nitrogen and subsequently stored at −80° C.

Muscle analysis. For histochemical analysis, cross sections (10 μm) were cut in a cryostat maintained at −20° C. After air drying these sections were stained for myofibrillar adenosine triphosphatase (ATPase) activity at pH 9.4 subsequent to alkaline (pH 10.3) preincubation (Padykula and Herman 1967). Later, the percentage of dark staining (type II) and light staining (type I) muscle fibers was determined in the sections stained for ATPase activity.

The P-light chain phosphate content of isolated fast and slow myosin was assessed using the procedure of Silver and Stull (1982), as recently modified by Moore and Stull (1984).

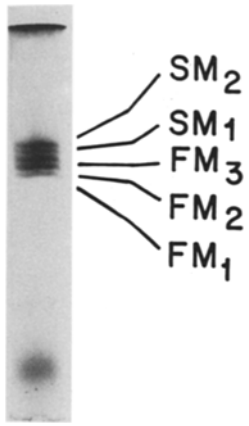

Fig. 1. Pyrophosphate gel electrophoresis of a homogenate from human vastus lateralis muscle showing two slower migrating slow myosin isozymes (SM_2 and SM_1) and three faster migrating fast myosin isozymes (FM_3, FM_2, FM_1), identified according to the terminology of Fitzsimons and Hoh (1981). Gel slices containing SM_2 and SM_1 or FM_3, F_2 and F_1 were excised and subsequently isoelectrofocused

A small portion of frozen tissue (5−10 mg) was homogenized and diluted according to the detailed procedure described by Moore and Stull (1984). An aliquot of the diluted homogenate containing approximately 10−20 μg of myosin was layered on pyrophosphate gels containing 4.5% (w/v) total acrylamide monomers and 3% (w/v) N,N′-methylenebisacrylamide. Conditions for pyrophosphate gel electrophoresis were similar to those described by Silver and Stull (1982) with the exception that electrophoresis was carried out for 20 h at 60 V and the electrophoresis buffer contained 2 mM cysteine. Following brief staining and destaining (Moore and Stull 1984), gel slices representing the two slow isomyosins and the three fast myosin isozymes (see Fig. 1) were placed in 100 μl of denaturing buffer (8 M urea, 2% Isolab isolytes pH 4−6, 15 mM 2-mercaptoethanol). After 1 h at room temperature, the gel slices and denaturing buffer were layered onto 1.5 mm slab gels as described previously (Moore and Stull 1984). Isoelectric focusing was carried out for approximately 4 h at 15° C, following which the gels were fixed and stained according to Silver and Stull (1982). Gels were scanned with an LKB 2202 ultroscan laser densitometer and the relative proportion of the phosphorylated and unphosphorylated P-light chains was assessed with a LKB 2200 recording integrater. Actomyosin from rabbit soleus muscle was incubated with [γ-^{32}P]ATP, calmodulin, calcium and rabbit skeletal myosin light chain kinase as described previously (Silver and Stull 1982). Aliquots representing various incubation times were applied to pyrophosphate gels and the myosin was isolated and subsequently isoelectric focused as described above. Following silver staining, bands representing the slow myosin light chains were isolated and ^{32}P incorporation measured to identify the phosphorylated light chains (Silver and Stull 1982). Human slow myosin light chains were identified by comparison with the light chains from rabbit soleus muscle.

For the determination of myosin light chain kinase activity, frozen tissue was homogenized as described by Moore and Stull (1984), and the kinase activity was assessed in supernatant fractions according to Blumenthal and Stull (1980). The subunit molecular weight of the human kinase

was assessed by the Western blot procedure with antibodies raised in a goat to rabbit skeletal muscle myosin light chain kinase (Nunnally and Stull 1984). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analyses. Paired twitch tensions for similar time periods on day 1 and day 2 were tested for differences using a two-tailed paired *t*-test. Since differences were not observed ($P > 0.50$), mean values for each subject at each measuring time were determined. Analysis of variance was used to assess differences in twitch tensions and P-light chain phosphorylation for the various sampling times. A P value of less than 0.05 was accepted to reveal a significant difference.

Results

Myosin light chain kinase activity and the percentage of type II fibers in muscle samples obtained from the vastus lateralis of the subjects are shown in Table 1. The mean myosin light chain kinase activity for the six subjects was 6.7 ± 0.9 nmol phosphate incorporated $\cdot$ mg^{-1} protein $\cdot$ min^{-1}. With the narrow range of fiber types for the six subjects, it was not possible to correlate the percentage of type II fibers and myosin light chain kinase activity as previously described (Moore and Stull 1984). The myosin light chain kinase activity was determined to be Ca^{2+}-calmodulin dependent since inclusion of 5 mM ethyleneglycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) in the reaction solution inhibited the activity of the enzyme (Blumenthal and Stull 1980). A single polypeptide with an apparent melecular weight of $68{,}000 \pm 2{,}000$ was identified in human muscle extracts with an antiserum to rabbit skeletal muscle myosin light chain kinase and Western blot analysis (data not shown). The subunit molecular weight of human skeletal muscle myosin light chain kinase is lower than has been reported for such species as mouse, rat, guinea pig, rabbit, dog and steer (range $75{,}000 - 108{,}000$; Nunnally and Stull 1984). The existence of a single polypeptide in the human vastus lateralis muscle samples is consistent with current evidence showing that the same kinase exists in slow and fast muscles of the same animal species (Nunnally and Stull 1984).

Fast (FM_1, FM_2 and FM_3) and slow (SM_1 and SM_2) human myosin isozymes were isolated from pyrophosphate gels (Fig. 1), and the light chain subunits were separated on isoelectric focusing gels. Samples representative of rested muscle and muscle obtained 20 s following a single dynamic contraction are shown for the human myosin (Fig. 2). In addition, light chains of myosin from rested and electrically stimulated rabbit fast (tibialis anterior) and slow (soleus) muscles are shown in Fig. 2 for comparison. The light chain pattern of human and rabbit fast myosin is similar, consisting of LC1F and LC3F along with the P-light chain LC2F and its phosphorylated derivative LC2F-P. For both animal species, muscle contraction was associated with a relative increase in the proportion of LC2F-P.

Several notable species differences can be observed for the light chain components in slow myosins isolated from human vastus lateralis and rabbit soleus (Fig. 2). Human slow myosin had a single 1S component, whereas two light chains, 1Sa and 1Sb, were found in the rabbit soleus. Two forms of the P-light chain with similar pI values were observed for both animal species. These were identified as LC2S and LC2S′, and corresponded to the P1 and P2 light chains reported by Westwood et al. (1984) in rabbit soleus myosin. No evidence of phosphorylated forms of either LC2S or LC2S′ was found in any of the human muscle slow myosin samples analyzed. However, in both rested and stimulated rabbit myosin samples, LC2S′-P and LC2S-P were present, although the latter was difficult to measure because its pI value was almost identical to that of LC2S′.

The P-light chain phosphate content in fast myosin isolated from the vastus lateralis muscle along with the electrically stimulated isometric twitch potentiation ratio (P*/P) are illustrated in Fig. 3 at time periods before and following a single voluntary contraction of the quadriceps muscle group.

Table 1. Muscle fiber composition and myosin light chain kinase activity in the vastus lateralis muscle of the subjects. Myosin light chain kinase activities for each subject represent mean values for assays determined on two muscle samples

Subject	Fiber composition type II (%)	Myosin light chain kinase activity (nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein)
1	56.4	8.2
2	52.7	6.3
3	43.7	6.5
4	52.9	6.2
5	47.5	3.3
6	67.0	9.8

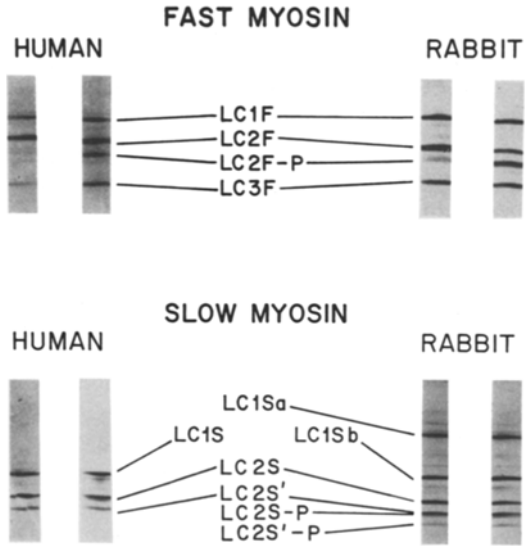

Fig. 2. Isoelectric focusing gels showing the light chain components of fast and slow myosins from human and rabbit skeletal muscles. For each species and each myosin type, the lane to the left shows the light chain pattern in rested muscle; the lane to the right shows biopsies obtained 20 s after a 1 s voluntary contraction (human) or 25 s following electrical stimulation at 5 Hz (rabbit). No phosphorylated forms of the P-light chains (LC2S-P and LC2S′-P) were observed in human slow myosin, but both forms were observed in rested and stimulated rabbit soleus myosin. Because the isoelectric point of rabbit LC2S′-P is only slightly more acidic than LC2S′, these two bands appear as a closely spaced doublet

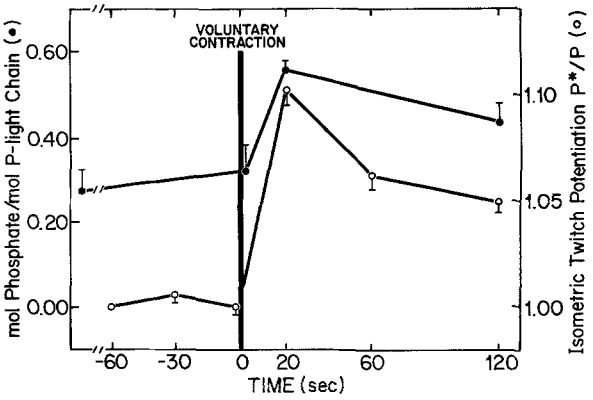

Fig. 3. The relationship between fast myosin P-light chain phosphorylation and isometric twitch potentiation in intact human muscle. Electrically stimulated twitch tensions were recorded from the quadriceps muscle group at designated times before and after a single maximal dynamic contraction of the quadriceps. Twitch potentiation is expressed as a ratio (P*/P) where P represents the initial twitch tension and P* each subsequent twitch tension. Muscle biopsy samples were obtained from the vastus lateralis muscle at designated times before and after the maximal dynamic contraction. Fast myosin was isolated by pyrophosphate gel electrophoresis and the P-light chain phosphate content was determined from isoelectric focusing gels. The data shown represent mean ± SEM for six male subjects

In rested muscle, P-light chain phosphate content averaged 0.28 mol phosphate/mol of P-light chain. Immediately following the voluntary contraction, P-light chain phosphate content was 0.32 mol phosphate/mol P-light chain. This value was increased to 0.56 ($P < 0.05$) at 20 s after the contraction. Over the next 100 s, P-light chain content decreased by 23% ($P < 0.05$), but was still significantly elevated over the value measured before the voluntary contraction. The peak isometric twitch potentiation ratio (P*/P) measured at 20 s after the voluntary contraction was 1.10, coinciding with the peak in P-light chain phosphate content. Thereafter, twitch potentiation decreased to a value of 1.05 ($P < 0.05$), 120 s after the voluntary contraction which was significantly greater than pre-contraction values. There was a significant correlation ($r = 0.85$, $P < 0.05$) between individual increases in the twitch potentiation ratio (P*/P) and the increases in phosphate incorporation in the P-light chain of fast myosin 20 s after the voluntary contraction.

Discussion

The results from this study reveal a temporal association between the P-light chain phosphate content in fast myosin and potentiation of the electrically stimulated isometric twitch in intact human muscle following brief, intense muscle activity. On the other hand, slow myosin was not phosphorylated as a result of the contractile activity. These results are consistent with previous experiments that have shown a correlation between isometric twitch potentiation and P-light chain phosphate content in fast, but not slow, rat and rabbit muscles (Manning and Stull 1982; Moore and Stull 1984; Moore et al. unpublished observations).

The extent of P-light chain phosphate content in muscle is determined by the opposing effects of Ca^{2+}-regulated myosin light chain kinase and unregulated myosin light chain phosphatase activities. Myosin light chain kinase becomes activated in the presence of calmodulin when free sarcoplasmic Ca^{2+} concentrations are raised to a level occurring in contracting muscle (Blumenthal and Stull 1980). Recently, Moore and Stull (1984) developed a model to account for the stimulation frequency and time dependence of myosin phosphorylation and dephosphorylation, taking into account the known characteristics of activation and inactivation of myosin light chain kinase in response to alterations in sarcoplasmic Ca^{2+} concentrations and myosin light chain phosphatase activity. This model could reasonably account for the P-light chain phosphate content observed in rat fast and slow muscles with various frequencies of stimulation if the muscles were not stimulated to fatigue (Moore and Stull 1984). The model of Moore and Stull (1984) could not be satisfactorily employed to account for the P-light chain phosphate content observed in the fast myosin samples in the present study. To do so requires a reasonable estimation of the relative myosin light chain kinase and phosphatase activities in the human fast twitch muscle fibers. Myosin light chain phosphatase activity was not assessed in the small muscle samples, while the kinase activity was measured in samples containing a mixture of slow and fast twitch fibers.

At rest and following the contractile activity, the quadriceps muscles of the human subjects could not be maintained as quiescent as animal muscles used for in situ stimulation preparations. Moreover, the entry of the biopsy needle into the vastus lateralis muscle likely activated to some extent the adjacent tissue, resulting in Ca^{2+} transients that would activate myosin light chain kinase. The significance of these latter points is best illustrated by the fact that P-light chain phosphate content in the rested muscle of the human subjects was 0.28 compared to 0.12 mol phosphate/mol P-light chain for quiescent rat fast muscle (Moore and Stull 1984).

In the present experiment, no evidence of phosphorylated forms of LC2S and LC2S′ was observed either before or after the 1 s voluntary contraction. Slow myosin from rat soleus muscle was essentially unphosphorylated at rest, and even stimulation for 10 s at 5 Hz did not appreciably increase P-light chain phosphate incorporation (Moore and Stull 1984). Thus, the lack of phosphorylation of P-light chain in slow muscles may be explained by the fact that in slow muscles myosin light chain kinase activity is lower while myosin light chain phosphatase activity is higher than the activities measured in corresponding fast muscles from the same species (DiSalvo et al. 1983; Moore and Stull 1984). Since only the fast myosin from human muscle was phosphorylated, it seems likely that the preponderance of myosin light chain kinase activity is in fast twitch human fibers.

In several previous studies (Volpe et al. 1981; Pons et al. 1983), results from two-dimensional gel electrophoresis of myosin isolated from human muscles were interpreted as showing a phosphorylated and dephosphorylated form of LC2S. These interpretations were based in part on similarities in the electrophoretic pattern of LC2S components from the human myosin with those from myosin isolated from the rabbit soleus. Recently, Westwood et al. (1984) showed that there are two types of P-light chains in rabbit soleus muscle which they identified as P1 and P2. Both of these P-light chains were shown to exist in phosphorylated

forms, identified as P_3 and P_4, respectively, when endogenous protein phosphatase activity was destroyed by homogenization in trichloroacetic acid at 0° C (Westwood et al. 1984). Indeed, these authors suggested that P-light chain polymorphism may be characteristic of slow myosin. Accordingly, the phosphorylated form of LC2S (LC2SP) identified by Volpe et al. (1981) and Pons et al. (1983) is more likely the other unphosphorylated polymorphic form of LC2S, which we have identified as LC2S′ and Westwood et al. (1984) described as P2.

We have shown both dephosphorylated (LC2S and LC2S′) and phosphorylated (LC2S-P and LC2S′-P) P-light chains in rabbit soleus muscle (Fig. 2) when phosphatase inhibitors are included in the homogenization medium. However, while both rabbit P-light chains could be phosphorylated equally well upon incubation with myosin light chain kinase, stimulation of rabbit soleus muscle in situ for 15 s at 100 Hz was associated with a total P-light chain phosphate content of 0.16 compared to 0.14 mol phosphate/mol P-light chain for rested soleus muscle (Moore et al. unpublished observations). On the other hand, phosphorylated P-light chains were not observed either in rested human muscle or following a maximal voluntary contraction which is similar to results obtained with rat and mouse soleus muscles (Crow and Kushmerick 1982a, b; Moore and Stull 1984). Potentiation of the isometric twitch is a characteristic property of adult fast, but not slow, muscle (Close 1972), and as discussed earlier, twitch potentiation has been shown to be positively correlated with P-light chain phosphorylation in fast, but not slow, animal muscles. In this study, twitch potentiation of the quadriceps muscle group was associated with phosphorylation of only fast myosin in the vastus lateralis, a muscle which in the present subjects was composed of a nearly equal mix of slow and fast twitch muscle fibers. These results are consistent with the concept that myosin P-light chain phosphorylation is related to twitch potentiation in fast twitch muscle fibers.

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