Myosin phosphorylation regulates the ATPase activity of permeable skeletal muscle fibers

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1. INTRODUCTION

Reversible covalent phosphorylation is a mechanism which is widely used to regulate enzyme activity. The phosphorylation of one of the subunits of myosin, the P-light chain, has been found to occur in a variety of muscle and non-muscle cells (review [1]). The phosphorylation is catalyzed by a CA^{2+} activated kinase whose sole substrate appears to be the P-light chain of myosin. In skeletal muscle, myosin is phosphorylated in vivo during and after long tetanic contractions [2,3]. The time course of myosin phosphorylation is slower than the events of a muscle twitch and the maximum level of phosphorylation may occur several seconds after the contraction had ceased [4]. These findings suggest that myosin phosphorylation in skeletal muscle is not an obligatory event for the production of force, and thus any role which it does play must involve a modulation of the contractile interaction. In smooth muscle and in some non-muscle cells, myosin phosphorylation has been shown to regulate the actomyosin contractile interaction [5–8]. In contrast to these results phosphorylation of skeletal muscle myosin was found to have no effect on actomyosin ATPase [9,10]. In [11] myosin phosphorylation correlated with a decrease in the isometric ATPase of living fibers; however, this effect was not found in [12]. Here, we provide direct evidence that thiophosphorylation regulates the ATPase activity of isometric permeable skeletal fibers and that this effect occurs only in the organized array of the myofibril. A high degree of thiophosphorylation (50–90%) of the myosin light chain was achieved in situ in glycerinated rabbit psoas fibers by incubation with purified myosin light chain kinase. Thiophosphorylation decreased the ATPase activity of the fiber by a factor of ~2. Either thiophosphorylation or phosphorylation caused a similar effect in myofibrils only when they were prevented from shortening by light cross-linking with glutaraldehyde. We conclude that myosin phosphorylation in skeletal fibers is a mechanism which modulates the rate of ATP hydrolysis and that the expression of this modulation requires an intact filament array.

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether) – *N,N,N′,N′*-tetracetic acid; TES, 2-{[2-hydroxy-1, 1-bis (hydroxymethyl) ethyl]1-amino} ethanesulfonic acid; ATP-γs, ADP-(PO_2S); HMM, heavy meromyosin; MLCK, myosin light chain kinase

2. METHODS

2.1. *Fibers*

Rabbit psoas fibers were glycerinated by immersion in 50% glycerol, 50% Rigor Buffer (0.12 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM TES, pH 7.0) as in [13]. Thin bundles of fibers, 2 cm long with 3–6 fibers/bundle were mounted between 2 glass rods so that they were held isometrically and could be rapidly transferred from one vial to another. All fibers assayed has sarcomere lengths of 2.2–2.4 μm. Fibers were phosphorylated by immersion for 20 min at 25°C in 50 mM KCl, 10 mM $MgCl_2$, 50 mM

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Tris (pH 7.5), 0.1 mM $CaCl_2$, 0.1 mM TT and 5 mM ATP (or ATPγs) to which purified myosin light-chain kinase (50 nM) and calmodulin (2.5 μM) were added. Fibers were next washed by immersion in rigor buffer for 2 min and assayed for ATPase activity by transferring them to rigor buffer plus 1.1 mM $CaCl_2$ and 4 mM ATP. Total phosphate in the assay medium was determined as in [14]. After the assay the fibers were immediately clipped from the glass rack, dissolved in 8 M urea using sonication and total protein was assayed as in [15]. All fiber washes and assays were either stirred or gently shaken. Fiber tensions were measured by methods in [13]. The activity of myosin was determined assuming that the myosin comprised 43% of the total protein, a value obtained by densitometer scans of fibers electrophoresed on SDS gels [16].

2.2. *Myofibrils*

Myofibrils were prepared from rabbit back and leg muscles and washed by repeated sedimentations [17]. Phosphorylation of the myofibrils (2–3 mg/ml) was carried out in the solutions described above for fibers. The myofibrils were washed free of kinase and calmodulin by sedimentation and the ATPase activities of the washed myofibrils were measured by addition of ~75 μg protein to 1.5 ml rigor buffer plus 1.1 mM $CaCl_2$ and 4 mM ATP. Assay mixtures were also gently shaken.

2.3. *Proteins*

Myosin light chain kinase and calmodulin were purified by standard methods [18].

3. RESULTS

High levels of P-light chain phosphorylation were difficult to achieve in fibers. This difficulty may be due to endogenous levels of phosphatase not solved during the glycerination procedure, to a slower diffusion of kinase into fibers, or to some inaccessibility of the light chain in the filament array. This problem was removed by use of the ATP analog, ATP-γs, which thiophosphorylates fibers and myofibrils. Myosin thiophosphorylation is resistant to attack by phosphatase [7]; we have found that the extent of thiophosphorylation achieved using ATP-γs was much higher than the extent of phosphorylation obtained with ATP. A second reason why ATPγs is required for the fiber studies is that it is a poor substrate for the actomyosin interaction so that fibers did not undergo a lengthy period of maximum contraction during the phosphorylation procedure. Studies with both smooth muscle myosin and phosphorylase have found that phosphorylation and thiophosphorylation have similar effects on enzyme function [7,8,20]. The extent of thiophosphorylation of myosin in fibers was assessed by isoelectric focusing. The P-light chain of the fibers could be thiophosphorylated to an extent which varied from ~50–80% (fig.1). The majority of the protein bound ^{32}P is incorporated into the P-light chain of myosin (fig.2).

Myosin phosphorylation has a clear effect on the ATPase activity of glycerinated fibers (table 1). The ATPase acitivity of the thiophosphorylation fiber is ~1/2 that of control. Thiophosphorylation is only achieved when the fiber is preincubated in all 3 components of the thiophosphorylation reaction. Ommision of any one of the 3 results in a low level of thiophosphorylation and in an ATPase activity that is close to that of control fibers. It thus appears that the decrease in the ATPase activity is due specifically to the thiophosphorylation, and not to some other action of the phosphorylation system such as proteolysis or side effects caused by incubation in ATP-γs. The state of thiophosphorylation of the fibers was also examined following the ATPase reaction, and no changes in thiophosphorylation occured during the assay. The amount of thiophosphorylation achieved in the fibers varied from 50–80%. The ATPase data represents an average of all these samples, and a more extensive study will be required to determine the exact dependence of decreased ATPase activity on the extent of phosphorylation. The isometric tension generated by the fibers (~2±0.2 kg/cm^2) was not altered by thiophosphorylation.

In [9,10] phosphorylation of purified heavy meromyosin or of myofibrils had no effect on their ATPase activities. One report that phosphorylation elevates actomyosin ATPase [21] has not been reproduced in our laboratory. It is possible that preservation of an intact filament array is required for the expression of the effects of phosphorylation; a well-ordered filament array is of course not achieved with purified proteins but is also not seen in myofibrils after addition of ATP due to their extensive contraction. To examine this possibility we attempted to inhibit the contraction of the myofi-

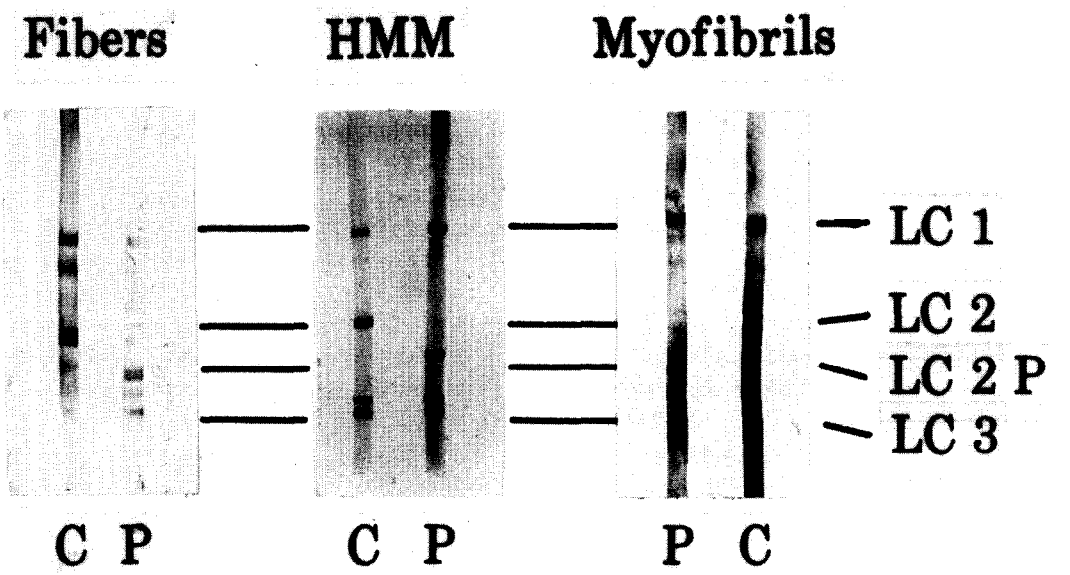

Fig.1. Patterns of HMM, myofibrils and fibers electrofocussed in 50% polyacrylamide gels in the presence of 8 M urea with gradients from pH 4–8. The myosin from fibers and myofibrils was partially purified by electrophoresis prior to electrofocussing as in [19]. Samples marked 'P' were incubated with myosin light chain kinase calmodulin, and ATP (HMM) or ATP-γs (fibers and myofibrils) while those marked 'C' were incubated in buffer alone. The phosphorylation of purified heavy meromyosin is shown for comparison. LC1, LC2, and LC3 identify the 3 light chains of myosin; LC2 and LC2-P are the unphosphorylated and phosphorylated P-light chains.

brils by lightly crosslinking them with an agent which would form a few covalent bonds that would prevent the sliding of filaments. We reasoned that the formation of a few such bonds in each sarcomere would prevent the sarcomere from shortening but would leave the vast majority of the crossbridges free to function normally as they would in an isometric contraction. Crosslinking with 0.01% glutaraldehyde for 10–15 min at 0°C was found to be sufficient to maintain a constant sarcomere length of the myofibrils in a contracting medium, and the ATPase activity of these crosslinked myofibrils was identical to that of control myofibrils (see table 1). When myofibrils that had been crosslinked in 0.01%

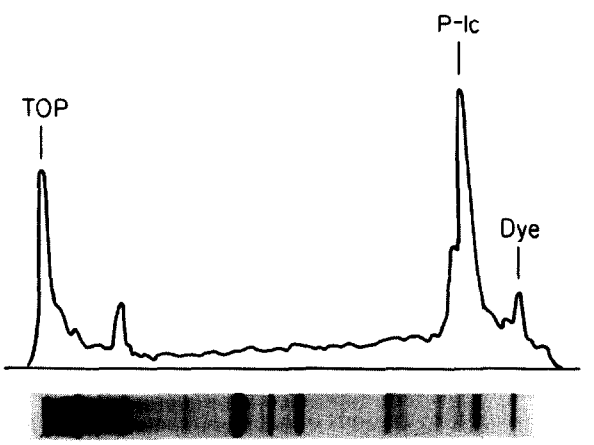

Fig.2. A densitometric scan of the autoradiogram of the gel shown below. Fibers were incubated with myosin light chain kinase, calmodulin and [^{32}P]ATP as in section 2. The fibers were dissolved in 8 M urea and the protein electrophoresed on 12% polyacrylamide –SDS gels. The amount ^{32}P incorporated into the fiber protein using ATP was found to be low, corresponding to ~5–10% phosphorylation of the myosin P-light chain. The radioactivity seen in the band near the top of the gel was not always present. Some ^{32}P was incorporated into material which does not enter the gel. This material could be eliminated by sedimentation prior to electrophoresis and probably represents protein not solubilized during the preparation of the sample.

Tabel 1

The effect of myosin phosphorylation on the ATPase activity of fibers and myofibrils

	Preincubation[a]	% Phosphorylation	ATPase[b]
Fibers	Control	< 5	0.82 ± 0.1
	MLCK + calmodulin	< 5	0.83 ± 0.1
	ATP-γs	5–10	0.80 ± 0.1
	MLCK + calmodulin + ATP-γs	50–80	0.44 ± 0.04
Myofibrils	Control	5	0.97 ± 0.1
	MLCK + calmodulin + ATP	80 ± 20	1.0 ± 0.1
Crosslinked myofibrils	Control	< 5	1.0 ± 0.15
	MLCK + calmodulin	< 5	0.9 ± 0.15
	MLCK + calmodulin + ATP-γs	80 ± 20	0.46 ± 0.06
	MLCK + calmodulin + ATP	80 ± 20	0.50 ± 0.1

[a] Fibers or myofibrils were incubated in a buffered solution which contained the following additions; MLCK refers to myosin light chain kinase

[b] ATPase is given in s^{-1}/myosin head; assays were performed in rigor buffer plus 1.1 mM $CaCl_2$ and 5 mM ATP at 35°C

[c] Errors given for ATPase activities represent the standard error of the mean of ≥ 10 determinations, errors given for the extent of phosphorylation represent the range of values found in different experiments

glutaraldehyde were electrophoresed on 12% polyacrylamide gels in the presence of urea or SDS, the pattern of protein bands was virtually unchanged from that of controls. This shows that few proteins have been crosslinked by this procedure, and thus the lightly crosslinked myofibrils present a system in which the great majority of the myosin heads are functioning as they would during an isometric contraction. However, these myofibrils are a more simple system to explore than are fibers. Due to their small size ATP can diffuse freely through them eliminating possible artifacts arising from insufficient ATP supply. A greater extent of phosphorylation of the endogenous myosin could be achieved with ATP than could be achieved in fibers. Furthermore, they avoid other potential problems such as those which arise from the heterogeneity of sarcomere lengths that develops during the contraction of fibers. As shown in table 1 the ATPase activity of the crosslinked myofibrils can be modulated by myosin thiophosphorylation in a manner identical to fibers. This effect is not seen in the uncrosslinked myofibrils. Phosphorylation has an effect that is similar to that of thiophosphorylation, a result which indicates that our conclusions drawn from the thiophosphorylation of fibers can be applicable to the living system.

4. DISCUSSION

Following the initial observation that the 20 000-M_r light chain of myosin could be phosphorylated [22], considerable effort has been devoted to understanding the role of the phosphorylation. In skeletal muscle this role has been elusive. These results show that myosin phosphorylation is a mechanism which decreases the rate of energy expenditure in skeletal muscle fibers. Myosin phosphorylation is a slow step which occurs over a period of at least several seconds, thus the level of myosin phosphorylation only becomes appreciable during relatively long periods of contractile activity in skel-

etal muscle [4]. A number of studies of the energetics of living muscle have demonstrated that during long tetanic contractions, at a time when myosin is becoming phosphorylated, the rate at which the muscle uses energy decreases by a factor ~2–3 [23,24]. The level of myosin phosphorylation correlates well with the decrease in energy utilization [11]; however, this correlation was not found in [12]. These results show directly that myosin phosphorylation does indeed decrease the ATPase activity of the fibers in agreement with the conclusions drawn from the living fibers [11]. The utility of myosin phosphorylation in skeletal muscle appears obvious. When the fast muscle is used in a prolonged contraction, myosin phosphorylation decreases its rate of energy expenditure. The possible relationship between this decrease in ATPase activity and potentiation of isometric twitch tension in living skeletal muscle [4,25,26] is not known, and further investigations are needed.

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