PHOSPHORYLATION OF SKELETAL MUSCLE CONTRACTILE PROTEINS IN VIVO

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SUMMARY

The phosphorylation of troponin·tropomyosin and the 18,000 dalton light chain of myosin was investigated in white gracilis muscles of rabbits in vivo. Troponin·tropomyosin contained 1 mol phosphate per mol protein in resting muscle. The phosphate content was not altered by the intra-arterial injection of 1 nmol isoproterenol which increased cyclic AMP and phosphorylase a formation. Also tetanic electrical stimulation produced no change in the phosphate content of troponin·tropomyosin. The light chain of myosin contained 0.50 mol phosphate per mol protein in control muscles which increased to 0.90 with tetanic electrical stimulation. These results demonstrate that phosphorylation of myosin, but not troponin can be stimulated in skeletal muscle in vivo in response to contractile activity.

INTRODUCTION

The original observations that both myosin (1) and troponin (2,3) from rabbit skeletal muscle are phosphoproteins stimulated interest in the possible regulatory role of protein phosphorylation in myofibrils. It has been shown that cyclic AMP-dependent protein kinase (2,4,5) and phosphorylase kinase (2,4,5) catalyze the phosphorylation of purified troponin subunits, but a role for these phosphorylation reactions in regulating the biochemical activity of troponin has been lacking.

The 18,000 dalton light chain of myosin (LC-2) was phosphorylated by a specific Ca^{2+}-dependent light chain kinase (6). The existence of endogenous phosphate with purified troponin and myosin and the Ca^{2+}-dependency of two kinases, phosphorylase kinase and myosin light chain kinase indicated that the Ca^{2+} mobilized to the myofibrils during contraction may stimulate the phosphorylation of troponin and myosin in vivo. A physiological role for

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these phosphorylation reactions in vivo may be suggested if the phosphorylation occurred in association with contractile activity.

MATERIALS AND METHODS

The gracilis muscle of anesthetized rabbits was surgically isolated (7) and frozen in situ (8) after the indicated interventions and powdered by percussion. For purification of troponin·tropomyosin 330 mg of frozen muscle powder was homogenized in 10 ml 60% glycerol, 2 mM EDTA, 15 mM mercaptoethanol, 100 mM $NaHPO_4$, 50 mM NaF at pH 7.2 for 15 seconds at -20° and another 15 seconds at 0°. After centrigugation at 20,000 x g for 1.5 minutes, the supernatant fraction was discarded and the pellet was homogenized in 8 ml 95% ethanol. The precipitated protein was washed 3 times in 95% ethanol, twice in diethyl ether, and dried under vacuum. The maximal amount of time from the initial homogenization to the addition of ethanol was 3 minutes for any one sample. Troponin·tropomyosin was purified from the residue essentially as described (9). Quantitation of the protein components of the troponin·tropomyosin complex (10) after polyacrylamide gel electrophoresis in SDS demonstrated a purity of 95 ± 0.6% with a molar ratio of troponin to tropomyosin equal to 1.0. Protein bound phosphate was measured as described (11). For analyzing the phosphorylation of the 18,000 dalton light chain, 1 g frozen muscle was homogenized directly in 6 M guanidine·HCL and purified as described (12). The phosphate content of the 18,000 dalton light chain was determined by measuring the relative amounts (10) of the nonphosphorylated and phosphorylated forms separated by polyacrylamide gel electrophoresis in 8 M urea (12).

RESULTS AND DISCUSSION

The primary concern in determining the phosphorylated state of proteins in vivo is the possible dephosphorylation or phosphorylation of these proteins during purification. The techniques employed here appear to eliminate these problems. The addition of various phosphoproteins to a homogenate or any subsequent steps in the purification of troponin·tropomyosin did not result in any significant dephosphorylation. This included incubations of the protein mixtures at 30° for as long as 6 hours with ^{32}P-phosphorylase a, ^{32}P-troponin, ^{32}P-casein or ^{32}P-histone. Likewise, the addition of the nonphosphorylated proteins and 3 mM [γ-^{32}P]ATP produced no phosphorylation. Similar results were obtained in the 6 M guanidine·HCl used to purify the myosin light chain subunit. These results indicate that the protein bound phosphate in troponin·tropomyosin and the 18,000 dalton light chain are reasonable measurements of phosphorylated states in vivo.

TABLE I

PHOSPHORYLATION OF RABBIT SKELETAL MUSCLE MYOFIBRILLAR PROTEINS *IN VIVO*

Condition	Cyclic AMP	Phosphorylase Activity	Troponin Tropomyosin	Myosin
	μmol/kg	-AMP/+AMP	mol phosphate/mol protein	
Control	0.18 ± 0.05	0.06 ± 0.01	1.02 ± 0.07	0.50 ± 0.03
Isoproterenol	0.61 ± 0.05	0.38 ± 0.06	0.99 ± 0.08	-
Electrical Stimulation	0.21 ± 0.06	0.41 ± 0.06	1.00 ± 0.07	0.87 ± 0.04

The values are means ± S.E.M. for 6 experiments. Biopsy samples were obtained 30 seconds after the intra-arterial injection of 1 nmol isoproterenol or after 15 seconds of tetany produced by direct electrical stimulation. Cyclic AMP and phosphorylase *a* formation were measured as described (16).

The troponin·tropomyosin complex contains 1 mol phosphate per mol protein under resting conditions (Table I). The intra-arterial injection of isoproterenol stimulates cyclic AMP and phosphorylase *a* formation, but does not alter the phosphate content in troponin·tropomyosin. Likewise, tetanic contractions produced by direct electrical stimulation failed to alter the phosphate content in these proteins. Thus, under conditions of activation of cyclic AMP dependent protein kinase and phosphorylase kinase *in vivo* troponin was not phosphorylated. The endogenous phosphate of 1 mol per mol protein presumably was in the tropomyosin binding subunit (5, 13). The lack of phosphorylation associated with electrical stimulation is similar to the results obtained with measurements of phosphate content in the inhibitory subunit of troponin from frog skeletal muscle (14). Although tropomyosin from frog skeletal muscle may contain some protein bound phosphate (15), the results obtained with rabbit skeletal muscle

POLYACRYLAMIDE GEL ELECTROPHORESIS OF MYOSIN LIGHT CHAINS FROM WHITE SKELETAL MUSCLE

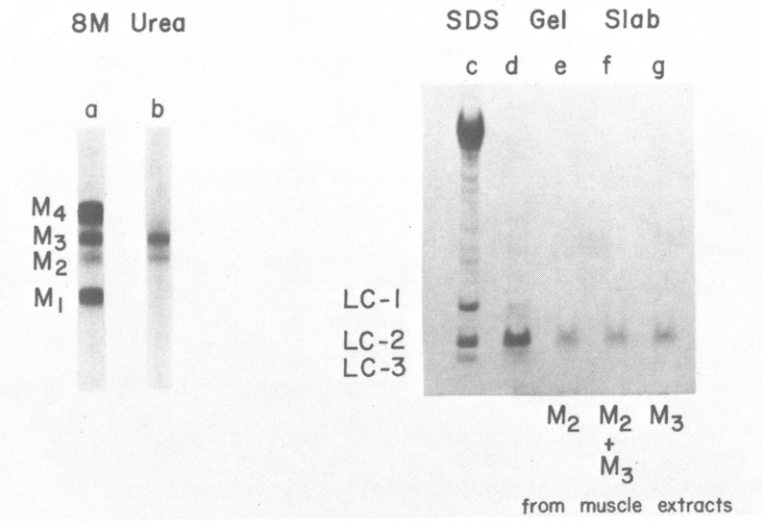

Figure 1. Polyacrylamide gel electrophoresis of myosin light chains from rabbit white skeletal muscle. The two gels on the left were done in 8 M urea and represent a light chain fraction containing all three light chains (a) and the purified 18,000 dalton light chain (b). The specific light chains have been characterized (12) to show that M_2 and M_3 represent the phosphorylated and nonphosphorylated forms of the 18,000 dalton light chain respectively. A light chain fraction obtained from muscle homogenized directly in 6 M guanidine·HCl was also subjected to gel electrophoresis in 8 M urea. The stained proteins corresponding to M_2 and M_3 were isolated and analyzed by polyacrylamide gel electrophoresis in SDS (right). These proteins separately (e,g) or in combination (f) comigrated with the purified 18,000 dalton light chain (d) and the 18,000 dalton light chain in myosin (c).

would not suggest any phosphorylation of this protein associated with contractile acitvity.

The phosphorylated and nonphosphorylated forms of the 18,000 dalton light chain were separated by polyacrylamide gel electrophoresis in 8 M urea (Fig. 1). The two forms separated from muscle extracts were isolated from the urea polyacrylamide gels and shown to comigrate with the purified light chain after polyacrylamide electrophoresis in the

presence of SDS (Fig. 1). Thus, the nonphosphorylated and phosphorylated forms of the 18,000 dalton light chain can be measured after extraction of muscle directly in 6 M guanidine·HCl and separation by polyacrylamide gel electrophoresis in 8 M urea.

Quantitation of the two forms obtained from resting muscles indicated that about 50% of the light chain was phosphorylated (Table 1). This is in contrast to previous results which suggested that the light chain was fully phosphorylated (12) and may be related to the lack of quick freezing in situ in that report. Adequate freezing procedures are critical for assessing enzymic reactions associated with physiological processes since the trauma of muscle removal may stimulate these reactions (8). Tetanic contractions increased the amount of the phosphorylated light chain (Table I), presumably by Ca^{2+} activation of the Ca^{2+}-dependent light chain kinase. This phosphorylation was associated with an increase in phosphorylase a formation probably due to stimulation of the catalytic activity of nonactivated phosphorylase kinase by Ca^{2+} (16). Although these results clearly show that the 18,000 dalton light chain of rabbit skeletal muscle myosin may be phosphorylated in vivo in association with contractile activity, a more detailed analysis is needed between the relationship of phosphorylation and contractile state. Such information may provide clues to the role of phosphorylation of skeletal muscle myosin.

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