The Phosphorylation of Troponin B by Phosphorylase b Kinase in Skeletal Muscle of Mice Carrying the Phosphorylase b Kinase Deficiency Gene

Stephen R. Gross and Steven E. Mayer

Division of Pharmacology
Department of Medicine
University of California, San Diego
La Jolla, Calif. 92037

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SUMMARY

In skeletal muscle of animals with the phosphorylase b kinase deficiency gene there is < 1% of the normal activity to convert phosphorylase b to a in the presence of Ca^{++}, Mg^{++}, and ATP (1). Correspondingly, there is < 1% of the normal activity to phosphorylate phosphorylase b. Nevertheless, under the same conditions, these extracts catalyze the phosphorylation of troponin at a rate 57% of normal. Phosphorylase b converting activity can be sedimented from skeletal muscle of control mice by centrifugation. This fraction isolated from I strain skeletal muscle extracts phosphorylates troponin at a rate 29-39% of the control. EGTA [1] (15 mM) inhibits troponin phosphorylation by 50-60% in this fraction from both strains. The EGTA inhibition is reversed by 15 mM Ca^{++}. Thus the phosphorylase b kinase in skeletal muscle of animals with the phosphorylase b kinase deficiency gene can phosphorylate troponin B, although it shows little or no activity with phosphorylase as a substrate. This observation is consistent with the normal muscle contractility of I strain animals.

INTRODUCTION

The phosphorylase b kinase deficiency gene (gene symbol k) is a single unit, sex-linked mutation (1) carried in I/LnJ strain mice that results in a deficiency of PBK activity. Skeletal muscle is affected to the greatest extent with less than 1% of the normal enzyme activity, as measured by the conversion of phosphorylase b to a (1-3). Nevertheless, this muscle contains protein that cross reacts with an antibody to purified rabbit PBK. Since the amount of cross reacting material is similar to that from control strain, Cohen and Cohen (4) conclude that the mutant PBK protein is present in concentrations similar to those found for the normal enzyme.

Purified PBK has recently been reported to phosphorylate troponin B (5).

[1] Abbrevations - EGTA, ethylene glycol bis (B-amino ethyl ether)-N,N'-tetra-acetic acid; PBK, phosphorylase b kinase

This discovery and the report that the PBK deficiency gene is a structural mutation (4) have prompted us to investigate the troponin phosphorylating activity in skeletal muscle extracts of I strain mice.

MATERIALS AND METHODS

The I/LnJ mice used in these experiments were from our colony which was started from breeding pairs of I/LnJ mice provided by Dr. Elizabeth Russell, The Jackson Laboratory. The I/LnJ mice are maintained in our colony by brother-sister matings. The control mice, C57Bl/St and Ha/ICR (a Swiss-Webster substrain) strain, were obtained from the L.C. Strong Research Foundation (La Jolla, Ca.).

Phosphorylase b (6), PBK (7), protein kinase (8), protein kinase inhibitor (9) and troponin (10) were prepared from rabbit skeletal muscle. Troponin B was separated from troponin A (11) and, when troponin B was electrophoresed on sodium dodecyl sulfate polyacrylamide gel (12), it showed three bands in proportions similar to those described by Stull et al. (5).

To prepare an antibody to phosphorylase kinase, guinea pigs were injected with 100 mg of purified rabbit PBK in Freund's complete adjuvant, 0.5 ml intramuscular, 0.5 ml intradermal. One week later another 100 mg of enzyme protein was injected in 0.9% NaCl.

RESULTS

The PBK activity of skeletal muscle extracts (10,000 x g, 10 min, supernatant fractions) assayed by the conversion of phosphorylase b to a was 4000 $\pm$ S.E. 200 units/g muscle in the control strain C57Bl as compared to 9 $\pm$ 1 for I strain. Crude homogenates of I strain skeletal muscle contained more phosphorylase b converting activity, up to 70 units/g, which is still less than 2% of normal activity. We have been unable, as have others (4), to repeat Huijing's observation that crude homogenates of skeletal muscle of mice homozygous for the PBK deficiency gene contain 16% of normal phosphorylase b converting activity (13).

PBK activity was also determined by the incorporation ^{32}P from (γ-^{32}P) ATP into phosphorylase. Extracts of skeletal muscle of I strain mice had less than 1% of the normal activity, but I strain extracts had 57% of the control troponin phosphorylating activity (Table I). A high concentration of

Table I

COMPARISON OF PHOSPHORYLASE b AND TROPONIN B AS SUBSTRATES FOR PHOSPHORYLASE b KINASE

	PHOSPHORYLASE b KINASE ACTIVITY (picomoles ^{32}P incorporated/min/g wet weight)	
	SUBSTRATES (0.7 mg/ml)	
	Phosphorylase b	Troponin B
I/LnJ (n=5)	7 ± 3	25 ± 3
C57Bl/St (n=5)	2670 ± 380	44 ± 3

Mice from either strain were anesthetized with chloral hydrate (600 mg/kg, i.p.). The gastrocnemius muscle was removed, weighed, and homogenized in 8 volumes of 10 mM Tris-HCl, 2 mM EDTA pH 7.9. The 1,200 x g (10 min.) supernatant fraction was assayed for phosphorylase kinase activity by measuring ^{32}P incorporation from (γ-^{32}P)ATP into acid precipitable material. The assay mixture (60 μl) contained 85 mM Tris (pH 8.2), 3 mM (γ-^{32}P)ATP, 18 mM Mg^{++}, 0.7 mg/ml substrate, and the muscle extract. Purified rabbit phosphorylase a (4 mg/ml) was added to inhibit phosphorylase phosphatase. The reaction was started by the addition of the Mg^{++} + ATP and terminated by the addition of 75 μl of 100 mM EDTA, 100 mM KF, and 50 μg/ml bovine albumin. 100 μl of the sample was applied to a filter paper disc, which was washed three times in 5% perchloric acid and then counted (8). The enzyme reaction was run for 6 minutes and the reaction rate was linear for this interval. The results are expressed as a mean ± the standard error of the mean.

phosphorylase a (4 mg/ml) was used to inhibit phosphorylase phosphatase mediated dephosphorylation of phosphotroponin B (14), because F^- (which is normally used to inhibit this phosphatase) inhibited phosphorylase kinase in mouse muscle extracts.

The possibility was investigated that cyclic AMP dependent protein kinase phosphorylated troponin B (15). The PBK protein can be sedimented from extracts of rabbit skeletal muscle by centrifugation for three hours at 105,000 x g (40 P fraction)(16). Of the recovered phosphorylase b converting activity of Swiss-Webster (control) skeletal muscle extracts 47% was found in the 40 P

fraction, and the phosphorylase b converting activity of I strain 40 P fraction was < 0.1% of that recovered from an equal weight of control mouse muscle (Table II). Correspondingly, the I strain 40 P fraction catalyzed no

Table II

DISTRIBUTION OF PHOSPHORYLASE b CONVERTING ACTIVITY WITH DIFFERENTIAL CENTRIFUGATION OF MOUSE MUSCLE EXTRACTS

Fraction		Phosphorylase b Converting Activity (Units) Swiss-Webster	I/LnJ
Extract	(10,000 x g, 20')	186,700	270
30 Supernatant	(78,000 x g, 90')	133,000	60
30 Precipitate	(78,000 x g, 90')	29,000	90
40 Supernatant	(105,000 x g, 180')	21,000	40
40 Precipitate	(105,000 x g, 180')	44,000	10

Hind-limb and back muscles (33 g) were homogenized in 2.5 volumes ice cold 4 mM EDTA, pH 7.4. The centrifugal fractionation was carried out by the direct centrifugation method of Meyer et al. (16). Phosphorylase kinase was assayed as previously described (21). One unit of phosphorylase kinase activity = 1 unit of phosphorylase a formed per min. One unit of phosphorylase activity = 1 μmole glucose-1-P min^{-1} from glycogen and Pi.

significant phosphorylation of rabbit phosphorylase b (Figure 1). Nevertheless, this fraction catalyzed the phosphorylation of troponin B at a rate 29-34% of that measured in the control (Figures 1 and 2). EGTA (15 mM), a specific chelator of calcium ions, inhibited troponin phosphorylation by I strain 40 P fraction 50% and 60% in the control 40 P. This inhibition was reversed by the addition of an equivalent concentration of Ca^{++} (Figure 2). Lower EGTA concentrations inhibited troponin phosphorylating activity to a lesser, but similar, extent in control and I strain 40 P fractions. EGTA did not inhibit troponin B phosphorylation catalyzed by cyclic-AMP dependent protein kinase purified from rabbit skeletal muscle. Protein kinase inhibitor did not inhibit troponin phosphorylation catalyzed by the 40 P fraction from either strain under conditions in which it inhibited by 86% cyclic-AMP dependent protein kinase catalyzed phosphorylation of troponin B.

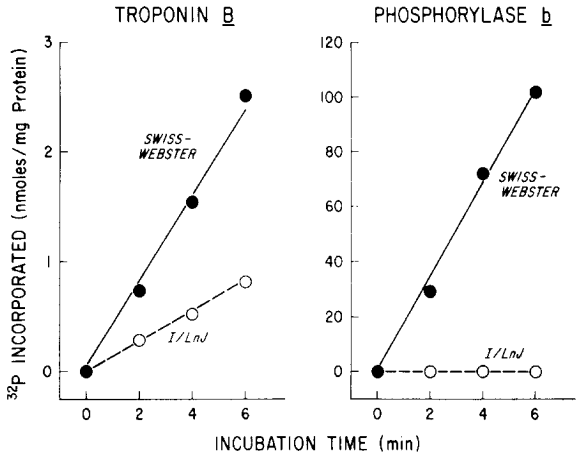

Figure 1. Phosphorylation of Phosphorylase b and Troponin B by 40 P Fraction from Mouse Skeletal Muscle.

40 P fractions were prepared as described in Table II. Phosphorylation was measured as described in Table I except that phosphorylase a was not used to inhibit phosphorylase phosphatase. The substrate concentration was 0.7 mg/ml for rabbit phosphorylase b and 2.5 mg/ml for rabbit troponin B, a saturating concentration. ●——● , Swiss-Webster; O——O , I/LnJ.

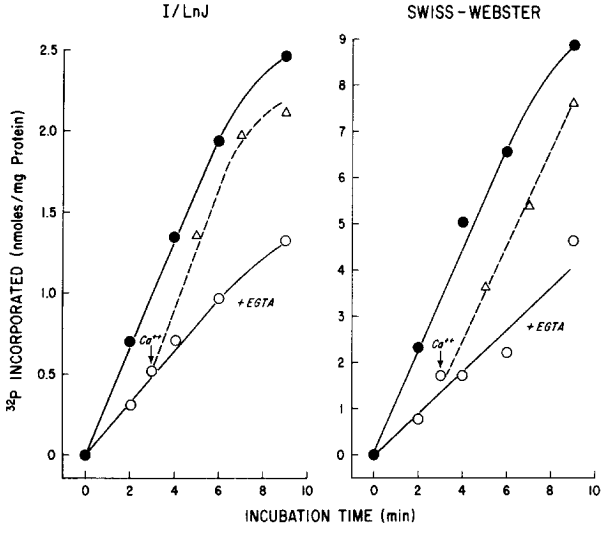

Figure 2. Inhibition of Troponin B Phosphorylation by 40 P Fraction from Mouse Skeletal Muscle.

The samples were prepared and the assays run as described in the legend to Figure 1. Calcium chloride was added to one-half of the tubes containing EGTA after three minutes of incubation. ●——● , control; O——O , +15 mM EGTA; Δ--Δ , 15 mM EGTA and +15 mM Ca^{++}.

Further confirmation that PBK was the enzyme in the 40 P fraction phosphorylating troponin B was obtained by demonstration that an antiserum against this enzyme inhibited by > 95% troponin phosphorylating activity in Swiss-Webster 40 P fraction. Unlike the antiserum prepared in the hen (4), the antiserum to rabbit PBK prepared in the guinea pig did not cross react with the I strain mutant enzyme determined by either the Ouchterlony double diffusion technique or quantitative precipitation (17).

The ratio of troponin phosphorylating activity to phosphorylase phosphorylating activity was consistently low, approximately 1:50, for the normal mouse PBK preparations. This compares to a ratio of 1:7 for purified rabbit PBK assayed under similar conditions. The 1:7 ratio is consistent with the kinetic analysis of Stull et al. (5). For unknown reasons rabbit troponin was a better substrate for rabbit phosphorylase kinase than it was for mouse (either Swiss-Webster or C57Bl) phosphorylase kinase. Whether mouse troponin B is a better substrate for mouse phosphorylase kinase than is rabbit troponin B is currently under investigation.

DISCUSSION

Stull et al. (5) recently demonstrated that purified rabbit muscle PBK phosphorylated troponin B and proposed that this played a role in muscle contraction. Since I strain mice have no apparent deficiency in their skeletal muscle contractile processes, we reasoned that either PBK-mediated troponin phosphorylating activity has no important physiological role or I strain mice would have substantial troponin phosphorylating activity. The troponin phosphorylating activity seen in the 40 P fraction from I strain mice is not due to cyclic AMP dependent protein kinase, because this is a soluble enzyme found primarily in the 40 S (not 40 P) fraction and is not Ca^{2+} dependent. Troponin phosphorylating activity in 40 P fractions from skeletal muscle of I strain mice is Ca^{2+} dependent.

The relatively greater deficiency in phosphorylase phosphorylating activ-

ity than in troponin phosphorylating activity in I strain muscle is analogous to heriditary fructose intolerance in which liver aldolase is the deficient enzyme. The reduction in aldolase activity is more pronounced when fructose-1-phosphate is used as a substrate (96%) than when fructose-1,6-diphosphate is used (75%)(18).

Conversion of phosphorylase b to a during tetanic contraction accounts for the activation of glycogenolysis synchronous with contraction (19). These processes are probably largely controlled by Ca^{++} (20,21). Since troponin phosphorylating activity is also controlled by Ca^{++} dependent PBK, then if this process is important in muscle contraction, mutations abolishing this activity should result in dysfunction of the skeletal muscle contractile apparatus. The demonstration of troponin phosphorylation in I strain of 29-57% of that observed in controls despite less than 1% of normal phosphorylase b to a converting activity suggests that sufficient troponin phosphorylation could be present in I strain skeletal muscle to maintain normal function. Since Duchenne muscular dystrophy like the PBK deficiency is a sex-linked condition affecting skeletal muscle it is tempting to speculate that the human myopathy is the result of a severe deficiency of troponin phosphorylating activity.

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