PHOSPHORYLATION OF RABBIT MUSCLE TROPONIN AND ACTIN BY A 3′, 5′-c-AMP-DEPENDENT PROTEIN KINASE

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1. Introduction

Besides the well known effects of epinephrine on metabolism, the hormone apparently is directly involved in the control of skeletal muscle contraction. The early observations of Gruber [1, 2] showed that this catecholamine exerts a positive inotropic effect on fast contracting mammalian skeletal muscle, i.e. it increases the force of contraction. The hormone causes an accumulation of 3′, 5′-c-AMP [3] which activates one or several protein kinases [4]. These enzymes either directly phosphorylate glycogen synthetase (I to D conversion) or induce, by the intermediate phosphorylation of phosphorylase kinase, the interconversion of glycogen phosphorylase (*b* to *a* conversion) (for review see [5]). A twitch-potentiating action of epinephrine was observed even under anaerobic conditions with iodoacetate poisoned muscle fibers [6]. Thus, this activity of the hormone is probably unrelated to the above mentioned changes in metabolism. One may postulate that both effects of the hormone on metabolism and contractility are triggered by the same event, the phosphorylation of a protein by the 3′, 5′-c-AMP-dependent protein kinase.

The energy for muscle contraction originates from ATP hydrolysis catalyzed by the actomyosin ATPase. Troponin and tropomyosin are involved in the regulation of this ATPase activity. Saturation of troponin with Ca^{2+}, which is released from the sarcoplasmic reticulum upon nervous stimulation, leads to deinhibition of the myosin–actin interaction and thus ATP hydrolysis (for review see [7]). It is shown in this report that a 3′, 5′-c-AMP-dependent protein kinase phosphorylates specifically one subunit of troponin and also F actin.

* This work is part of a Ph.D. thesis of E. P.

2. Methods

Natural actomyosin (NAM), desensitized actomyosin (DAM) and the relaxing protein system (RPS) were prepared by the methods of Schaub et al. [8, 9]. Myofibrils were obtained from rabbit skeletal muscle as described by Perry et al. [10]. The relaxing protein system was the starting material for troponin and tropomyosin, prepared according to Hartshorne et al. [11]. Actin and myosin preparations were carried out according to Szent-Györgyi [12]. The protein kinase was purified as described by Krebs et al. [13]. The preparation of [γ ^{32}P]ATP was carried out according to Glynn et al.[14]. Protein was determined by the Lowry [15] method, using bovine serum albumin as standard.

2. 1. *Enzymic assays*

ATPase activity: The assays were carried out at 25° in a volume of 2 ml, containing 2.5 mM ATP; 10 mM glycerophosphate; 2.5 mM $MgCl_2$ and 1 mM EGTA, pH 6.9. The reaction was started by the addition of ATP. P_i liberated after 5 min of incubation was determined by the method of Fiske and Subba Row [16]. Lactate dehydrogenase was assayed as described by N. Bergmeyer [17]. The phosphorylase assays are described by Haschke et al. [18]; phosphorylase kinase and protein kinase activities were assayed according to Krebs et al. [13, 19]. Phosphorylation of muscle protein fractions (0.2 to 0.3 mg) was carried out at 30° in 0.04 mM 3′, 5′-c-AMP, 40 mM NaF,

4 mM $Mg(Ac)_2$, 0.5 mM [γ^{32}P]ATP (40 Ci/mole), 15 mM mercaptoethanol, 2 mM Tris/HCl pH 7.5 in a total volume of 0.25 ml containing 10 μg protein kinase. The reaction rates in the absence of added 3′, 5′-c-AMP were ca. 1/3 of those in its presence. The reaction was started by the addition of ATP and samples were removed and transferred to filter disks. The disks were washed with 10% trichloroacetic acid containing 1% pyrophosphate and 1% phosphate. Then they were rinsed several times with 10% trichloroacetic acid, dried and submerged in Bray's scintillation counting solution. Gel electrophoresis was carried out according to Weber et al. [20].

3. Results

According to Perry et al. [8–10] a mixture of troponin, tropomyosin and other proteins called relaxing protein system can be isolated from myofibrils or purified natural actomyosin. The distribution of several enzymes in different fractions obtained during the preparation of this system is shown in table 1. Typical sarcoplasmic enzymes, lactate dehydrogenase and phosphorylase kinase are not detectable in this relaxing protein fraction. Probably through adsorption of glycogen on the fibrils a small amount (0.02%) of the total phosphorylase remains in this preparation. In contrast, 0.2% of the total enzymatic activity of the protein kinase is found in the relaxing protein system showing a stronger adsorption of this enzyme to the myofibrillar structure than other cytoplasmic enzymes.

Due to the presence of this protein kinase, a slow 3′, 5′-c-AMP-dependent phosphorylation of proteins of the relaxing protein system occurs. Using labeled [γ^{32}P]ATP as phosphorylating agent, radioactivity is incorporated into the protein in a trichloroacetic acid precipitable form. The rate of phosphate uptake is enhanced by the addition of purified protein kinase. Control experiments show that neglectible amounts of radioactivity become incorporated into the added enzyme (not shown). The proteins present in the relaxing protein system are characterized by sodium dodecyl sulphate (SDS) gel electrophoresis (fig. 1). The pattern of gel C is obtained from the relaxing protein system. The material of band I (M.W. 45,000) is identical to purified actin (gel A). Band III containing protein of M.W. 36,000 shows the same mobility as the main component of purified tropomyosin (gel B). The tropomyosin preparation is contaminated with material of M.W. 39,000 which was reported previously [20]. Purified troponin (gel D) contains proteins of M.W. 39,000 (band II), M.W. 28,000 (band IV), M.W. 24,000 (band V) and M.W. 18,000 (band VI) prepared from the relaxing protein system as starting material. Graeser et al. [21] found a similar composition in SDS gel electrophoresis by purify-

Table 1

Distribution of phosphorylase, phosphorylase kinase, lactate dehydrogenase and protein kinase among the various fractions during preparation of the relaxing protein system.

Fraction	Volume	Protein		Phosphorylase		Phosphorylase Kinase		Lactate Dehydrogenase		Protein Kinase	
	ml	g	%	U	%	U	%	U	%	U	%
Crude Extract	1800	46	78	268000	91	38200	72	398000	95	2095	84
1 Wash	1000	4.2	7.1	15700	6.4	9290	18	16800	4	132	5.3
2 "	800	2.6	4.4	6140	2.5	1274	2.4	4140	1	83	3.6
3 "	800	1.0	1.7	2180	0.7	955	1.8	1510	0.4	65	3.0
4 "	800	0.6	0.9	362	0.1	636	1.2	942	0.2	47	2.0
5 "	800	0.5	0.6	392	0.1	318	0.7	329	0.08	43	1.7
6 "	800	0.2	0.2	138	0.05	–	–	145	0.03	9	0.4
7 "	800	0.1	0.1	116	0.04	–	–	76	0.02	5	0.2
8 "	800	0.1	0.1	72	0.02	–	–	25	0.006	2	0.1
NAM Sup	13700	0.6	1.1	1205	0.4	2180	4.1	118	0.3	13	0.5
RPS	560	3.3	5.6	73	0.02	–	–	–	–	6	0.2
		59	100	292577	100	52853	100	422085	100	2500	100

This system was prepared according to [8]. Enzymes were assayed as described in Methods. (–) indicates unmeasurably low activities.

Fig. 1. SDS gel electrophoresis of muscle protein fractions. To each gel ca. 5 μg protein was applied. (A) actin, (B) tropomyosin, (C) relaxing protein system and (D) troponin. Electrophoresis was carried out in gels of 10% acrylamide, 0.3% methylenebisacrylamide containing 0.1% SDS. The origin is at the top and the anode at the bottom. A constant current of 8 mA/tube was used.

ing this protein directly from myofibrils. The amount of protein of M.W. 28,000 and 18,000 varies considerably from preparation to preparation in both the relaxing protein system and the purified troponin fraction. As was observed by Ebashi et al. [22] the materials of M.W. 39,000 and 24,000 always represent the two major components.

Phosphorylation of proteins of the relaxing protein system does not change their pattern in SDS gel electrophoresis. From fig.2 can be seen that mainly two fractions, actin and one component of the troponin (M.W. 39,000) are labeled. Only a minor amount of radioactivity is found in one other troponin fraction. Tropomyosin did not have any detectable phosphate incorporated.

As demonstrated by Perry et al. [8–10] readdition of the relaxing protein system to desensitized actomyosin (this material does not respond to concentrations of 10^{-7} to 10^{-6} M Ca^{2+}) produces a sensitization to Ca^{2+} of the actomyosin ATPase. Fig. 3 shows that the same amount of phosphorylated or nonphosphorylated relaxing protein system causes sensitization of the myosin–actin ATPase to Ca^{2+}. Using the purified proteins, actin, troponin and tropomyosin as subtrates for the protein kinase only actin and troponin can be phosphorylated (fig. 4). In the troponin fraction the component of M.W. 39,000 is almost exclusively labeled. This can be seen from the distribution of radioactivity among the troponin subunits in SDS gel electrophoresis (not shown).

A definite estimate of the molar stoichiometry of phosphate incorporation will require not only proof of the purity of the protein fractions but more important must exclude proteolytic fragmentation known to occur in these proteins [22].

4. Discussion

The experiments show that mainly two proteins of the myofibrillar system (actin and troponin) can be phosphorylated by a 3′, 5′-c-AMP-dependent protein kinase. The troponin is directly involved in the control of muscle contraction. This was shown *in vitro* by its effect on the myosin–actin ATPase [7].

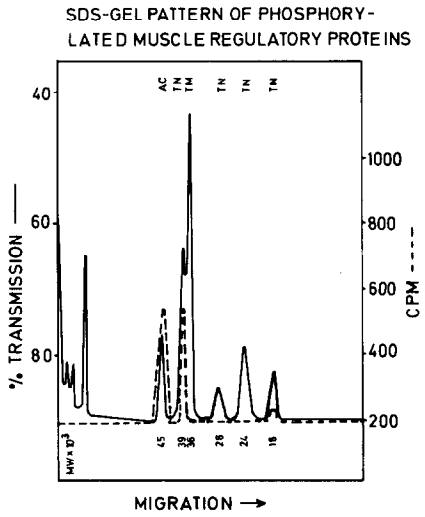

Fig. 2. SDS gel electrophoresis was carried out as described in the legend to fig. 1. Phosphorylation of the relaxing protein system was carried out as described in Methods. The optical density was recorded on a Gilford automatic densitometer at 550 nm. Molecular weights were obtained from a plot of the logarithm of molecular weight versus mobility using albumin, chymotrypsinogen and cytochrome *c* as standards. The gel was cut in 1 mm slices which were dissolved in 30% H_2O_2, 0.2% NH_3 at 50° and radioactivity was determined in a Packard scintillation counter using Bray's fluid. Abbreviations: (Ac) actin, (TN) troponin, (TM) tropomyosin.

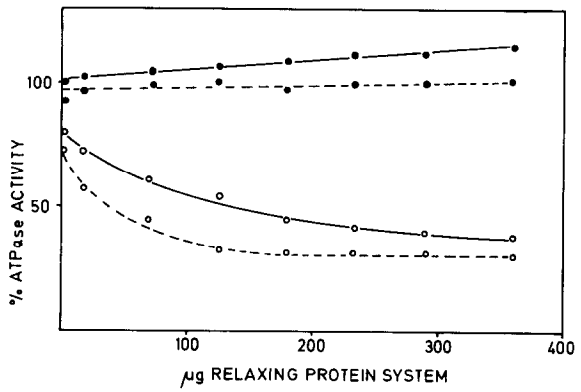

Fig. 3. Calcium sensitization of actomyosin by the relaxing protein system. To desensitized actomyosin (1 mg) the fraction containing the relaxing protein system (- - - - - - -) or the phosphorylate relaxing protein system (———) was added in amounts as indicated. Phosphorylation of the proteins was carried out and ATPase activity was measured as described in Methods. (•) In presence of 1 mM EGTA; (o) in presence of 10^{-5} M free Ca^{2+}.

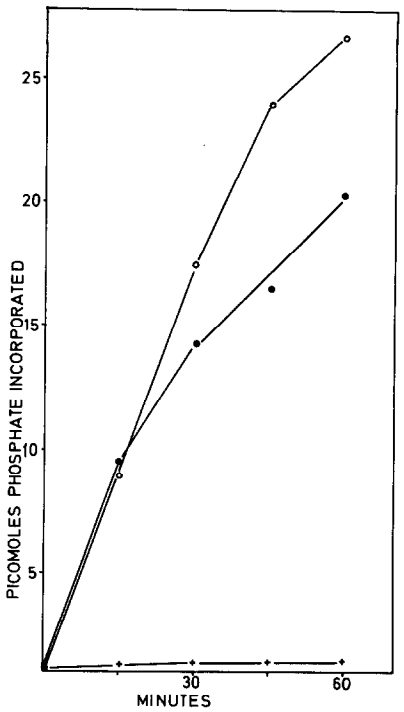

Fig. 4. Phosphorylation of purified actin, troponin and tropomyosin. Phosphorylation of 0.2 mg actin (•–•–•), of 0.2 mg troponin (o–o–o) or 0.2 mg tropomyosin (+–+–+) was carried out as described in Methods.

The protein kinases are unusual enzymes since their specificity towards different protein substrates appears rather low [4]. Nevertheless, from the muscle proteins which we have studied, only troponin and actin serve as substrates for these enzymes. Tropomyosin and myosin (not shown here also) took up no detectable acid stable phosphate. Furthermore, only one component of the troponin (M.W. 39,000) accepted almost exclusively phosphate, suggesting specificity of the protein kinase at least in respect to the different subunits of this protein. It seems that this particular troponin fraction mediates the formation of the troponin–tropomyosin complex, whereas the smaller component (M.W. 24,000) probably acts as an inhibitor for the myosin-actin ATPase and the smallest subunit (M.W. 18,000) seems to bind Ca^{2+} [21]. Following the idea of Szent-Györgyi [23] one might speculate that phosphorylation of one of the troponin subunits influences the shuttle of this protein between myosin, to which this regulatory protein may be bound in the Ca^{2+} free form, and the tropomyosin, to which the Ca^{2+} containing troponin may be bound. This could change the myosin-actin ATPase activity.

Krebs et al. [13] have shown that 3′, 5′-c-AMP-dependent protein kinase occurs in muscle in multiple forms (at least three) which are not interconvertible *in vitro*. As shown here a protein kinase is adsorbed on the fibrils more tightly than other typical cytoplasmic enzymes. A protein kinase closely associated with the myofibrillar system would seem especially suited to serve as a transmitter for the hormonal signal to the contractile machinery.

At the present not much would be gained by further speculation on the possible biological significance of the covalent modification of contractile proteins and their regulatory components. Additional experiments with both *in vivo* and *in vitro* systems are required to clarify the role of phosphorylation of these proteins and to find conditions where reversibility (i.e. dephosphorylation) can be demonstrated.

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