Biochimica et Biophysica Acta, 462 (1977) 689–699

BBA 47401

PHOSPHORYLATION OF AN ACTIN · TROPOMYOSIN · TROPONIN COMPLEX FROM HUMAN SKELETAL MUSCLE

GARY BAILIN*

Department of Physiology and Biophysics and Department of Medicine, Mount Sinai School of Medicine, Fifth Avenue and 100th Street, New York, N.Y. 10029 (U.S.A.)

(Received May 5th, 1977)

SUMMARY

A human skeletal actin · tropomyosin · troponin complex was phosphorylated in the presence of [γ-^{32}P]ATP, Mg^{2+}, adenosine 3′:5′-monophosphate (cyclic AMP) and cyclic AMP-dependent protein kinase (protein kinase). Phosphorylation was not observed when the actin complex was incubated in the absence of protein kinase or 1 μM cyclic AMP. In the presence of 10^{-7} M Ca^{2+} and protein kinase 0.1 mole of [^{32}P]phosphate per 196 000 g of protein was incorporated. This was two-fold higher than the [^{32}P]phosphate content of a rabbit skeletal actin complex but two-fold lower than that of a bovine cardiac actin complex. At high Ca^{2+}, $5 \cdot 10^{-5}$ M, little change in the phosphorylation of a human skeletal actin complex occurred. Phosphoserine and phosphothreonine were identified in the [^{32}P]phosphorylated actin complex. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that 60 % of the label was associated with the tropomyosin binding component of troponin. The inhibitory component of troponin contained 16 % of the bound [^{32}P]phosphate. Increasing the Ca^{2+} concentration did not significantly decrease the [^{32}P]phosphate content of the phosphorylated proteins in the actin complex. No change in the distribution of phosphoserine or phosphothreonine was observed. Half maximal calcium activation of the ATPase activity of reconstituted human skeletal actomyosin made with the [^{32}P] phosphorylated human skeletal actin complex was the same as a reconstituted actomyosin made with an actin complex incubated in the absence of protein kinase at low or high Ca^{2+}.

INTRODUCTION

It is now fairly well established that the calcium mediated interaction of the

Abbreviations: cyclic AMP, adenosine 3′:5′-monophosphate; protein kinase, adenosine 3′:5′-monophosphate-dependent protein kinase; ATPase, ATP phosphohydrolase; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N′*-tetraacetic acid.

* Present address: Department of Biochemistry, N.J. School of Osteopathic Medicine, College of Medicine and Dentistry of New Jersey, Piscataway, N. J. 08854, U.S.A.

proteins of the thick and thin filaments in muscle is responsible for the contractile process [1–4]. In skeletal and cardiac muscle the proteins of the thin filament, namely actin, tropomyosin and the components of troponin, are responsible, in part, for the regulation of contraction [1–5]. In particular, the inhibitory and calcium binding components of troponin are two of the proteins that are involved in the regulatory process [1–6]. Recently it has been shown that the inhibitory component of troponin, termed Tn-I, from skeletal and cardiac muscle is a substrate for enzyme catalyzed phosphorylation [7–17]. Cardiac Tn-I phosphorylation is catalyzed by a cyclic AMP-dependent protein kinase [14–21]. Preliminary reports suggest that in cardiac muscle, at least, cyclic AMP-dependent protein kinase catalyzed phosphorylation of Tn-I may be linked is some manner to the overall regulatory mechanism [18–22]. An increase in the phosphorylation of cardiac Tn-I in native tropomyosin [19], natural actomyosin [20, 21] or in an actin complex [22] decreased the calcium sensitivity of an actomyosin containing the phosphorylated Tn-I.

This paper will show that cyclic AMP-dependent protein kinase phosphorylates the tropomyosin binding and inhibitory components of troponin in an actin · tropomyosin · troponin complex from human skeletal muscle. Evidence will also be presented to indicate that the phosphorylation, which more closely resembles that obtained in rabbit skeletal muscle, had little effect on the calcium sensitivity of a reconstituted human skeletal actomyosin.

METHODS AND MATERIALS

Human skeletal myosin was prepared by a method described recently [23]. A protein complex of actin, tropomyosin and troponin was isolated from acetone-dried human skeletal muscle powder at 37 °C by the method of Katz [24] with the following modification: the extracting solution contained 1.0 mM ATP, pH 7.0, 1.0 mM NaF and 1.0 mM dithiothreitol. The actin complex was allowed to polymerize overnight proir to centrifugation [23]. All the components were present as judged by gel electrophoresis in sodium dodecyl sulfate [23]. Actin complexes from bovine cardiac or rabbit skeletal muscle were prepared similarly [25].

Bovine cardiac protein kinase was prepared from frozen hearts and purified as described by Miyamoto et al. [26]. The protein kinase preparation (14 mg per ml) was stored at −20 °C. The specific activity of cardiac protein kinase was determined by following the incorporation of ^{32}P from [γ-^{32}P]ATP into histone [27, 28]. The amount of acid-precipitable phosphoprotein formed was determined by the method of Li and Felmly [28]. The cardiac cyclic AMP-dependent protein kinase specific activity of several preparations was in the range of 0.9–2.0 nmol of P per min per mg protein.

Phosphorylation of an actin · tropomyosin · troponin complex

A modified procedure essentially that of Kirchberger et al. [29], developed for the phosphorylation of bovine cardiac actin complex [22], was used.

Procedure A. The reaction mixture contained 50 mM histidine-HCl buffer (pH 6.8), 0.12 M KCl, 2.5 mM NaF, 1 μM cyclic AMP and bovine cardiac protein kinase (0.2 mg per ml). The actin complex (2 mg per ml) was added after equilibrating the partial reaction mixture for 5 min at 30 °C. The time of the actin complex addition was taken as zero time. The ratio of actin complex to protein kinase fraction was

chosen based on results obtained previously for the phosphorylation of a bovine cardiac actin complex [22]. Phosphorylation was initiated by the addition of equimolar amounts of $MgCl_2$ and [γ-^{32}P]ATP to final concentrations of 5.0 mM. The reaction mixture was incubated at 30 °C for 30 min in a final volume of 0.4 ml. A correction for the autophosphorylation of the protein kinase preparation was made: at an enzyme concentration of 0.2 mg per ml, less than 6 % of the total phosphorylation observed was attributable to a protein kinase preparation. In studies on the effect of Ca^{2+} on the phosphorylation of the actin complex, calcium-EGTA buffers were used to attain specific ionized Ca^{2+} concentrations ($CaCl_2$ = 0.15 mM and various amounts of EGTA). A Ca-EGTA binding constant of $4.4 \cdot 10^5$ at pH 6.8 [30] was used to calculate the ionized Ca^{2+} from the Ca-EGTA buffers. The Ca^{2+} concentrations were varied between 10^{-8} and 10^{-4} M; for Ca^{2+} concentrations above 10^{-5} M, a $CaCl_2$ solution was used instead of a Ca-EGTA buffer.

Reactions were terminated by the addition of cold 0.4 ml 10 % (w/v) trichloroacetic acid. The samples were centrifuged for 10 min at 1500 × *g*, 3 °C. The supernatant was aspirated and the pellets were suspended in 0.4 ml 5 % trichloroacetic acid, centrifuged and washed at least three times. The final pellets were dissolved in 0.5 ml of 0.5 N NaOH and aliquots were removed for liquid scintillation counting (0.1 ml) and protein determination (0.05 ml). Total phosphorylation is expressed as nanomoles of ^{32}P incorporated per mg of protein.

Procedure B. The actin complex was incubated as described above except that unlabeled ATP was used. The phosphorylated actin complex was centrifuged at 150 000 × *g*, homogenized and dialyzed against 0.1 M KCl, 0.5 mM dithiothreitol. Under these conditions the incorporation of phosphate was the same as that obtained for the trichloroacetic acid-precipitated actin complex. This was observed when [γ-^{32}P]ATP was substituted for unlabeled ATP in Procedure B. The actin complex was combined with human skeletal myosin to form the reconstituted actomyosin which was assayed for ATPase activity.

Identification of phosphoserine and phosphothreonine

The actin complex was phosphorylated under conditions as described in Procedure A. The pellet obtained after several washings with trichloroacetic acid was subjected to acid hydrolysis in 6 N HCl for 1.5 h at 110 °C in vacuo [31]. After removal of HCl, the hydrolyzate (0.05 ml) was applied to Whatman No. 3 MM paper (44 × 45 cm) and ascending chromatography was carried out for at least 18 hours in pyridine/methanol/water (4 : 80 : 20, v/v/v) [22]. Phosphoserine and phosphothreonine standards were located with 1 % ninhydrin in ethanol. The paper was cut into 1 × 4 cm strips and counted. Corrections were made for the breakdown of phosphoserine (31 %) and phosphothreonine (8 %) during partial acid hydrolysis [32].

Gel electrophoresis

Polyacrylamide gel electrophoresis was performed with 10 % gels in 12.5 cm tubes at a current of 4 mA per tube for 18 h. A 0.1 M phosphate buffer (pH 7.1) containing 0.1 % sodium dodecyl sulfate [33] was used as described recently [34]. Molecular weights were estimated by co-electrophoresis of a mixture of standard proteins such as: cytochrome *c* (12 400); myoglobin (17 800); chymotrypsinogen

(25 000); ovalbumin (45 000); bovine serum albumin (67 000); and phosphorylase *a* (90 000). The gels were cut and counted as described in the legend to Fig. 3.

Actomyosin ATPase activity

The ATPase activity of a reconstituted actomyosin made from human skeletal myosin and an actin-tropomyosin-troponin complex in a ratio of 3 : 1 (w/w) was determined at 25 °C in 0.08 M KCl, 5 mM $MgCl_2$, 25 mM histidine, 2 mM ATP at pH 6.8. The assay medium also contained 0.15 mM $CaCl_2$ and various amounts of EGTA to obtain specific ionized Ca^{2+} concentrations as described in Procedure A. The myosin concentration was 0.24 mg per ml and that of the actin · tropomyosin · troponin complex was 0.08 mg per ml. The reaction was initiated with ATP and the liberation of inorganic phosphate was measured [25, 35].

Total phosphate was determined by the method of Bartlett [36].

Protein concentration was determined by the Lowry method using bovine serum albumin as a standard [37].

Materials

[γ-^{32}P]ATP tetra (triethylammonium) salt was obtained from International Chemical and Nuclear Corporation, with a specific activity of 4 to 11 mCi per μmol. Aliquots (10–35 μl) were removed and added to a stock solution of 5 mM $MgATP^{2-}$. This solution had a specific radioactivity in the range of 0.6 to 1.5 · 10^7 cpm per μmol.

RESULTS

Phosphorylation of a human skeletal actin · tropomyosin · troponin complex

Incubation of an actin · tropomyosin · troponin complex with $MgCl_2$ and ATP in the absence of cyclic AMP or protein kinase resulted in little or no phosphorylation of the protein complex (Fig. 1). In the presence of cyclic AMP and protein kinase in a ratio of 10 : 1 (by weight, actin complex to protein kinase fraction) the

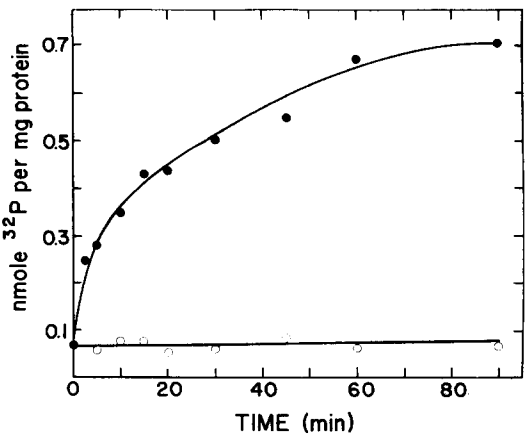

Fig. 1. Time course of phosphorylation of a human skeletal actin complex. A human skeletal actin complex (2 mg/ml) was incubated with and without protein kinase as described in Procedure A (see Methods and Materials). (○), without protein kinase; (●), with protein kinase.

extent of phosphorylation increased about 10-fold. The incorporation of [^{32}P]phosphate was studied after a 30 min incubation period. Under these conditions, more than 70 % of the maximum [^{32}P]phosphate incorporated was found (Fig. 1). Thus, 0.5 nmol P per mg protein was found after 30 min incubation compared to 0.70 nmol P per mg protein obtained after 90 min. Phosphorylation of the actin complex for periods of time in excess of 90 min did not significantly increase the [^{32}P]phosphate content of the actin complex. The addition of NaF to the reaction medium insured maximum recovery of phosphoprotein since there was a possibility that phosphatases might be present.

Phosphate content of a human skeletal actin · tropomyosin · troponin complex

To determine if non-enzymic or enzyme-catalyzed phosphorylation of the actin complex had occurred during the extraction of the acetone-dried human skeletal muscle powder, the actin complex was extracted with a solution containing 0.5 mM [γ-^{32}P]ATP and 1.0 mM dithiothreitol [22]. The actin complex, obtained after overnight polymerization and centrifugation, contained no radioactivity. Although it appeared that native endogenous (protein) kinase activity was absent in the actin complex preparation, there was a possibility that phosphorylation might have occurred before the preparation of the acetone-dried muscle powder as reported recently for a bovine cardiac actin complex [22]. Extraction of the acetone-dried powder with 1.0 mM dithiothreitol followed by the isolation of the actin complex showed that it contained 0.48 mol P bound per 196 000 g of actin complex.

Effect of calcium

The protein kinase catalyzed phosphorylation of an actin · tropomyosin · troponin complex from human skeletal muscle was not affected by Ca^{2+} concentrations in the range of 10^{-8} to 10^{-4} M (Fig. 2). For instance, there was only a 12 %

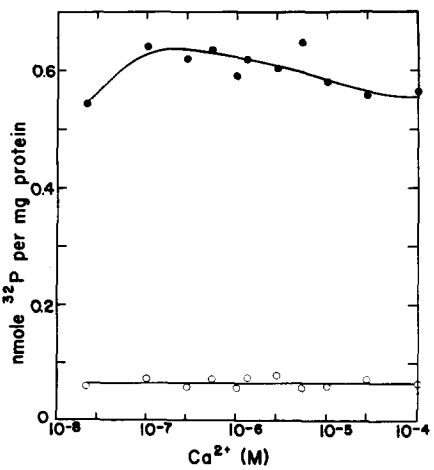

Fig. 2. Effect of calcium on protein kinase catalyzed phosphorylation of a human skeletal actin complex. A human skeletal actin complex was incubated for 30 min in the absence (○) and presence (●) of protein kinase (0.2 mg/ml). The calcium concentrations represent ionized calcium obtained from calcium-EGTA buffers as described in Methods and Materials.

TABLE I

COMPARISON OF CYCLIC AMP-DEPENDENT PROTEIN KINASE CATALYZED PHOSPHORYLATION OF VARIOUS ACTIN COMPLEXES

The actin complexes from bovine cardiac, human skeletal, and rabbit skeletal muscle at 2 mg/ml were phosphorylated under conditions described in Procedure A (see Methods and Materials). Results given in nmol ^{32}P per mg protein.

Actin complex	In the presence of	
	10^{-7} M Ca^{2+}	$5 \cdot 10^{-5}$ M Ca^{2+}
Bovine cardiac	1.15	0.83
Human skeletal	0.48	0.42
Rabbit skeletal	0.23	0.21

decrease in the [^{32}P]phosphate content of the actin complex at $5 \cdot 10^{-5}$ M Ca^{2+} compared to that obtained at 10^{-7} M Ca^{2+} concentration (Fig. 2). Similar results were obtained when a rabbit skeletal actin · tropomyosin · troponin complex was used as a substrate for the protein kinase catalyzed phosphorylation reaction (Table I). In this case, the ^{32}P content of the rabbit skeletal actin complex was approximately half that of the human skeletal actin complex. There was little change in the incorporation values at low or high Ca^{2+} (Table I). In contrast, the extent of phosphorylation of a bovine cardiac actin complex by protein kinase was more than twofold greater than that of a human skeletal actin complex (Table I). In addition, there was a 30 % difference in the [^{32}P]phosphate content of the actin complex at low and high Ca^{2+} concentrations (Table I). Increasing the Ca^{2+} concentration did not affect the protein kinase catalyzed phosphorylation of histone [22] or cardiac microsomes [29].

Distribution of phosphoserine and phosphothreonine in the phosphorylated human skeletal actin complex

When the human skeletal actin complex was incubated with cyclic AMP and protein kinase in the presence of 10^{-7} M Ca^{2+} (corresponding to the incorporation of 0.52 nmol P per mg of protein) 46 % of the total radioactivity recovered was associated with phosphoserine and 30 % of the radioactivity was associated with phosphothreonine. Of the total radioactivity applied to the chromatogram 15 % remained at or near the origin. The percentage distribution values for phosphoserine and phosphothreonine have been corrected for those obtained for the autophosphorylation of protein kinase in the absence of the actin complex. There was little difference in these values at low or high Ca^{2+} concentrations. Phosphorylation of the actin complex at $5 \cdot 10^{-5}$ M Ca^{2+}, corresponding to the incorporation of 0.45 nmol P per mg of protein, showed a slight change in the distribution of [^{32}P]phosphate in phosphoserine and phosphothreonine, but it was not significant.

Phosphorylation of troponin in the actin complex by protein kinase

A human skeletal actin-tropomyosin-troponin complex, phosphorylated by cyclic AMP and protein kinase in the presence of 10^{-7} M Ca^{2+}, showed a peak (peak II) with a major part of the radioactivity when the ^{32}P-labelled actin complex was subjected to gel electrophoresis and the radioactivity of sliced gels was measured (Fig.

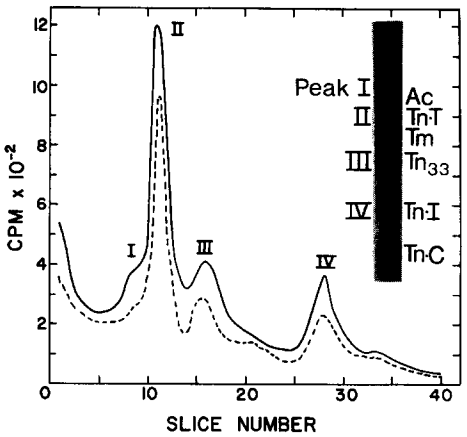

Fig. 3. Distribution of radioactivity in a ^{32}P-labelled actin complex. A human skeletal actin complex was incubated with protein kinase as described in Procedure A (see Methods and Materials). Samples (0.2 mg) were removed at specified intervals and the reaction was terminated by the addition of an equal volume of 10 % trichloroacetic acid. The pellet obtained after centrifugation was solubilized in 0.1 M phosphate buffer (pH 7.1) containing 1 % sodium dodecyl sulfate. The protein solutions were dialyzed against the electrophoresis buffer solution (containing 0.5 mM dithiothreitol). Subsequently, 150 μg were applied to the gels. Following electrophoresis (see Methods and Materials) the gels were fixed in 10 % trichloroacetic acid, cut in 2 mm slices for liquid scintillation counting. Solid line, in the presence of 10^{-7} M Ca^{2+}; broken line, in the presence of $5 \cdot 10^{-5}$ M Ca^{2-}. Insert, gel pattern of the human skeletal actin complex. Ac, actin; Tm, tropomyosin; Tn-T, Tn_{33}, Tn-I and Tn-C, components of troponin. Only that portion of the gel which resolved the proteins of the complex is shown. The top and bottom of the gel were cut off.

3.) The recovery of radioactivity from the gel was 70–80 %. The major protein band phosphorylated, peak II, contained 41 % of the radioactivity recovered. The migration of the ^{32}P-labelled protein band corresponded to the tropomyosin binding component of human skeletal troponin, Tn-T, of molecular weight 37 000 [23] in the human skeletal actin complex (compare peak II of Fig. 3 and Tn-T protein band of gel insert). The remaining radioactivity was located in peaks I, III, and IV and at the origin. The [^{32}P]phosphate content of peak I was 5 %; the migration of the protein band corresponding to peak I resembled that of a component of protein kinase phosphorylated in the absence of the actin with an estimated molecular weight of 50 000. There was no phosphorylation of purified F-actin. Peak III contained 19 % of the radioactivity and this protein had an estimated molecular weight of 33 000 (compare peak III in Fig. 3 with Tn_{33} protein band of gel insert). Peak IV, resembling the inhibitory component of human skeletal troponin, Tn-I, of molecular weight 22 000 [23] contained only 16 % of the radioactivity recovered. The [^{32}P]phosphate content of peaks II, III, and IV, corresponding to Tn-T, Tn_{33} and Tn-I, components of troponin, was 76 % of the total radioactivity recovered. The origin contained 20 % of the radioactivity. When the Ca^{2+} concentration was increased to $5 \cdot 10^{-5}$ M, there was a general decrease in the radioactivity under peaks I through IV (Fig. 3). For example, a 14 % and 20 % decrease was found under peaks II and IV, corresponding to protein bands Tn-T and Tn-I, respectively. The effect of Ca^{2+} does not appear to be associated with any one component of troponin.

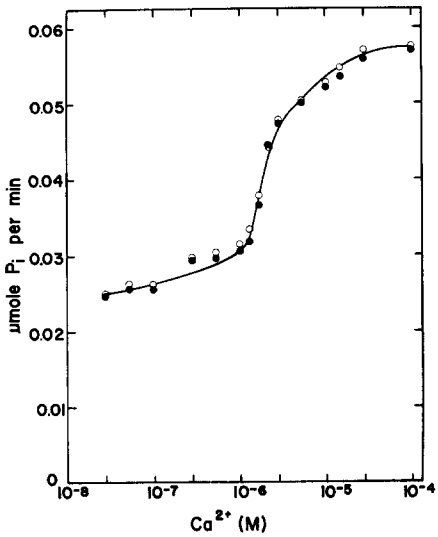

Fig. 4. Effect of Ca^{2+} on the ATPase activity of reconstituted human skeletal actomyosin. The assay was carried out in the presence of various ionized Ca^{2+} concentrations as described in Methods and Materials. Reconstituted human skeletal actomyosin was made from human skeletal myosin and an actin complex incubated for 30 min according to Procedure B (see Methods) in the absence (○) and presence (●) of 1 μM cyclic AMP and protein kinase.

Effect of phosphorylation on the Ca^{2+}-activated ATPase activity of reconstituted actomyosins containing the phosphorylated actin · tropomyosin · troponin complex.

The ATPase activity of reconstituted human skeletal actomyosin made from human skeletal myosin and a human skeletal actin · tropomyosin · troponin complex treated in the absence of protein kinase and cyclic AMP, was activated by Ca^{2+} (Fig. 4). The half maximal activation occurred at 3.11 ± 0.13 (S.E.M., $n = 3$) μM Ca^{2+}. A reconstituted actomyosin made with human skeletal myosin and an actin complex that had been phosphorylated in the presence of protein kinase, cyclic AMP and 10^{-7} M or $5 \cdot 10^{-5}$ M Ca^{2+} also showed sigmoidal activation of ATPase activity by Ca^{2+} (Fig. 4). Compared to a control reconstituted actomyosin, however, the reconstituted actomyosin made with the [^{32}P]phosphorylated actin complex showed no differences in the half maximal activation value or in the ATPase activity at 10^{-4} M Ca^{2+} (Fig. 4).

DISCUSSION

Phosphorylation of a human skeletal actin·tropomyosin·troponin complex by cyclic AMP-dependent protein kinase

The present data show that [^{32}P]phosphate from [γ-^{32}P]ATP was incorporated into an actin · tropomyosin · troponin complex in the presence of cyclic AMP and protein kinase (Fig. 1, Table I). The incorporation of 0.5 nmol P per mg protein corresponds to a value of 0.10 mol P per mol of actin complex (1 mol of actin complex = 196 000 g of protein). This was two-fold higher than the [^{32}P]phosphate content of a rabbit skeletal actin complex but two-fold lower than that of a bovine cardiac actin

complex (Table I). The reactivity of the protein kinase appears to be of the order: bovine cardiac > human skeletal > rabbit skeletal actin complex. The low value obtained for the [^{32}P]phosphate content of the human actin complex could be due to the use of a protein kinase from a different tissue source, i.e. bovine cardiac muscle. Preliminary studies, however, with a human skeletal cyclic AMP-dependent protein kinase preparation, show that 0.4 nmol P per mg human actin complex were incorporated. This is close to the value obtained when the bovine cardiac protein kinase was used (Table I).

Although native endogenous protein kinase activity was not found in the isolated actin complex, covalently bound phosphate was present. A phosphate content of 0.48 mol P bound per 196 000 g of actin complex suggests that in vivo, there is endogenous kinase (protein kinase or phosphorylase kinase) activity. The presence of endogenous kinase activity in cardiac or skeletal troponin [13] and cardiac native tropomyosin [7] has been reported.

The stability of the acid-precipitable [^{32}P]phosphate in hydroxylamine and the rapid loss of [^{32}P]phosphate from the actin complex in the presence of alkali indicated the presence of a phosphoester bond. Phosphorylation of serine (46 %) occurred to a greater extent than that of threonine (30 %). Since partial acid hydrolysis occurred (see Results) then 91 % of the label may be identified with phosphoserine and phosphothreonine. Both of these phosphoamino acids were found in phosphorylated rabbit skeletal troponin [10, 11] and in a bovine cardiac actin complex [22].

Phosphorylation of troponin in the actin complex

The Tn-T component of troponin in the ^{32}P-labeled human skeletal actin complex contained 41 % of the radioactivity (peak II in Fig. 3). Based on this finding, the incorporation of 0.5 nmol of P per mg of actin complex corresponds to a value of 0.3 mol of phosphate per mol of Tn-T. There was, however, a protein component of molecular weight 33 000, Tn_{33} (peak III, Fig. 3), that contained 19 % of the radioactivity. This component resembled a proteolytic digestion product of rabbit skeletal Tn-T [23, 25, 38]. Thus, 60 % of the total radioactivity or 0.4 mol of phosphate per mol of protein could be associated with the Tn-T component. Peak I, of molecular weight of 50 000, corresponds to the autophosphorylated cyclic AMP binding subunit of bovine cardiac protein kinase [39]. Peak IV, resembling Tn-I of human skeletal troponin [23], contained only 16 % of the radioactivity. This corresponds to 0.1 mol of phosphate per mole of Tn-I, one fourth of the Tn-T value. This low value may be a consequence of the large amount of endogenous phosphate found in the human skeletal actin complex. It is possible that some or all of the endogenous phosphate was bound to the Tn-I component thereby making fewer sites available on Tn-I during the in vitro phosphorylation.

The findings are consistent with studies on the cyclic AMP-dependent phosphorylation of rabbit skeletal troponin [8, 12]. Phosphorylation was limited to the Tn-T and Tn-I components [8]. These components were also phosphorylated in a bovine cardiac actin complex [22]. It is noteworthy that rabbit skeletal Tn-T is the major protein phosphorylated by phosphorylase kinase [8, 13, 40], whereas Tn-I is mainly phosphorylated by cyclic AMP-dependent protein kinase [8, 13, 40]. This indicates that the association of Tn-T with the other components of troponin in the actin complex may have a significant effect on its conformation [8].

Effect of calcium

The phosphorylation of Tn-T and Tn-I in the human actin complex was not inhibited significantly when the Ca^{2+} concentration was increased from 10^{-7} M to $5 \cdot 10^{-5}$ M (Figs. 2 and 3). On the other hand, phosphorylation of rabbit skeletal Tn-I by protein kinase or phosphorylase kinase was inhibited by 10^{-5} M Ca^{2+} in the presence of Tn-C [8]. Phosphorylation of Tn-I in a bovine cardiac actin complex by protein kinase was also inhibited at high Ca^{2+} [22]. It is not clear why these differences exist. The distribution of phosphoserine and phosphothreonine in the actin complex also remained the same at low or high Ca^{2+} concentrations. Possibly other serine and threonine residues of Tn-T (or Tn-I) become available for phosphorylation depending upon the Ca^{2+} concentration. This would suggest a more subtle change in the conformation of Tn-T in the actin complex. More than likely the small but broad inhibitory effect of Ca^{2+} is non-specific (Fig. 3).

ATPase activity of reconstituted actomyosin containing the phosphorylated actin complex

Hydrolysis of ATP by reconstituted human skeletal actomyosin made from human skeletal myosin and an actin complex incubated in the absence of added protein kinase was activated by Ca^{2+} and the half maximal activation occurred at 3.1 μM Ca^{2+} (Fig. 4). The sigmoidal response and ATPase activity (0.058 μmol of P_i per min per mg of protein) measured at saturating Ca^{2+} concentrations are similar to those values obtained for human skeletal natural or reconstituted actomyosins (ref. 23, and references therein). A reconstituted actomyosin made with a phosphorylated human skeletal actin complex (containing 0.5 nmol of [^{32}P]phosphate per mg of protein) did not shift the half maximal activation value to lower or higher Ca^{2+} concentration (Fig. 4). There was no change in Ca^{2+} sensitivity for reconstituted actomyosins made with actin complexes that had been phosphorylated in the presence of 10^{-7} M or $5 \cdot 10^{-5}$ M Ca^{2+}. The absence of any effect could be due to the possibility that the Tn-I component of the actin complex contained large amounts of endogenous phosphate thus minimizing the effects of the in vitro phosphorylation. Similar effects were reported for reconstituted rabbit skeletal actomyosin made with phosphorylated rabbit skeletal troponin [8, 9, 12, 40]. In contrast, protein kinase catalyzed phosphorylation of bovine cardiac Tn-I decreased the Ca^{2+} sensitivity of an actomyosin made with the phosphorylated Tn-I [19–22].

In conclusion, cyclic AMP-dependent protein kinase catalyzed phosphorylation, which appears to be associated with the regulation of myocardial contractility [14—22, 29], may be of lesser consequence in skeletal muscle. Phosphorylation of troponin, however, in human or rabbit skeletal muscle could be of importance as rabbit skeletal troponin [8, 13] and a human skeletal actin · tropomyosin · troponin complex contained endogenous protein-bound phosphate. Moreover, other kinases and phosphatases that may catalyze the phosphorylation and dephosphorylation in vivo have been identified in rabbit skeletal muscle [8, 13].

ACKNOWLEDGEMENTS

This work was supported by a grant from the Muscular Dystrophy Association. The indefatigable and careful work of Mu-Ju Shen is greatly appreciated.

REFERENCES

1 Fuchs, R. and Briggs, F. N. (1968) J. Gen. Physiol. 51, 655–676
2 Hartshorne, D. J. and Pyun, H. Y. (1971) Biochim. Biophys. Acta 229, 698–711
3 Weber, A. and Murray, J. M. (1973) Physiol Rev. 53, 612–673
4 Ebashi, S., Ohnishi, S., Abe, S. and Maruyama, K. (1974) in Calcium Binding Proteins (Drabikowski, W., Strzelecka-Gołaszewska, H. and Carafoli, E., eds.), pp. 179–196, Elsevier, Amsterdam
5 Potter, J. D. Seidel, J. C., Leavis, P., Lehrer, S. and Gergely, J. (1974) in Calcium Binding Proteins (Drabikowski, W., Strzelecka-Gołazewska, H. and Carafoli, E. eds.), pp. 129–152, Elsevier, Amsterdam
6 Bailey, C. and Villar-Palasi, C. (1971) Fed. Proc. 30, 1147a
7 Reddy, Y. S., Ballard, D., Giri, N.Y. and Schwartz, A. (1973) J. Mol. Cell Cardiol. 5, 461–471
8 Krebs, E. G., Beavo, J. A., Bechtel, P. J., England, P. J., Huang, T.-S. and Stull, J. T. (1974) in Exploratory Concepts in Muscular Dystrophy Volume II (Milhorat,. T., ed.) p. 445, Excerpta Medica, Amsterdam
9 Perry, S. V. and Cole, H. A. (1974) Biochem. J. 141, 733–743
10 Huang, T.-S., Bylund, D. B., Stull, J. T. and Krebs, E. G. (1974) FEBS Lett. 42, 249–252
11 Moir, A. J. G., Wilkinson, J. M. and Perry, S. V. (1974) FEBS Lett. 42, 253–256
12 Horl, W. H., Jennissen, H. P., Gröschel-Stewart, U. and Heilmeyer, L. M. G. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, E., Clementi, F., Drabikowski, W. and Margreth, A., eds.), pp. 535–546, North Holland Publishing Company, Amsterdam
13 Perry, S. V., Cole, H. A., Morgan, M., Moir, A. J. G. and Pires, E. (1975) FEBS, 9th Meet., pp. 163–176
14 Rubio, R., Bailey, C. and Villar-Palasi. C. (1975) J. Cyclic Nucleotide Res. 1, 143–150
15 Cole, H. A. and Perry, S. V. (1975) Biochem. J. 149, 525–533
16 Stull, J. T. and Buss, J. E. (1977) J. Biol. Chem. 252, 851–857
17 Reddy, Y. S. (1976) Am. J. Physiol. 231, 1330–1336
18 England, P. J. (1976) Biochem. J. 160 295–304
19 Ray, K. P. and England, P. J. (1976) FEBS Lett. 70, 11–16
20 Reddy, Y. S. and Wyberny, L. E. (1976) Biochem. Biophys. Res. Commun. 73, 703–709
21 Solaro, R. J., Moir, A. J. G. and Perry, S. V. (1976) Nature 262, 615–616
22 Bailin, G. (1977) Biophys. J. 17, 159a
23 Bailin, G. (1976) Biochim. Biophys. Acta 449, 310–326
24 Katz, A. M. (1966) J. Biol. Chem. 241, 1522–1529
25 Bailin, G., Shen, M.-J. and Katz, A. M. (1977) Biochim. Biophys. Acta 480, 469–478
26 Miyamoto, E., Kuo, J. F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395–6402
27 Kuo, J. F. and Greengard, P. (1970) J. Biol. Chem. 245, 4067–4073
28 Li, H.-C. and Felmly, D. A. (1973) Anal. Biochem. 52, 300–304
29 Kirchberger, M. A., Tada, M. and Katz, A. M. (1974) J. Biol. Chem. 249, 6166–6173
30 Ogawa, Y. (1968) J. Biochem. (Tokyo) 64, 255–263
31 Allerton, S. E. and Perlman, G. E. (1965) J. Biol. Chem. 240, 3892–3898
32 Bylund, D. B. and Huang, T.-S. (1976) Anal. Biochem. 73, 477–485
33 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
34 Bailin, G. (1975) Biochim. Biophys. Acta 400, 167–177
35 Bárány, M., Gaetjens, E., Bárány, K. and Karp, E. (1964) Arch. Biochem. Biophys. 106, 280–293
36 Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
37 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
38 Dabrowska, R., Barylko, F., Nowak, E. and Drabikowski, W. (1973) FEBS Lett. 29, 239–242
39 Erlichman, J., Rosenfeld, R. and Rosen, O. M. (1974) J. Biol. Chem. 249, 5000–5003
40 Lallemant, C., Serydarian, K., Mommaerts, W. F. H. M. and Suh, M. (1975) Arch. Biochem. Biophys. 169, 367–371