Biochimica et Biophysica Acta, 445 (1976) 791—801

BBA 67912

CYCLIC AMP-STIMULATED PHOSPHORYLATION OF BOVINE TRACHEAL SMOOTH MUSCLE CONTRACTILE AND NON-CONTRACTILE PROTEINS

HOWARD SANDS, WILLIAM PENBERTHY, THEODORE A. MEYER, and RICHARD JORGENSEN

Division of Molecular and Cellular Biology, National Jewish Hospital and Research Center, 3800 E. Colfax Avenue, Denver, Colo. 80206 (U.S.A.)

(Received February 18th, 1976)

Summary

1. Various proteins isolated from bovine tracheal smooth muscle were examined as phosphate acceptor substrates for a cyclic AMP-dependent protein kinase isolated from the same tissue. A fraction prepared in a manner similar to that of skeletal muscle troponin was the best substrate of the presumptive contractile proteins isolate. Actomyosin and tropomyosin were relatively poor substrates.

2. An assay was developed for the rapid detection in a large number of samples of the muscle specific substrate for the protein kinase on which we reported previously.

3. Using this assay, the muscle specific substrate found in bovine tracheal smooth muscle was partially purified resulting in a preparation which when resolved by polyacrylamide gel electrophoresis showed a single peak of ^{32}P incorporated, and which could be further characterized.

4. Our findings suggest that the substrate contains a protein subunit of molecular weight 19 000, which can be phosphorylated at serine and threonine residues, in the presence of cyclic AMP and protein kinase. The phosphate is in a covalent ester linkage with these residues.

5. A phosphoprotein phosphatase was isolated from the bovine tracheal smooth muscle.

6. Bovine tracheal smooth muscle contains cyclic AMP dependent protein kinase and phosphoprotein phospahatase activity as well as the muscle specific substrate, suggesting that these elements may be part of a mechanism which regulates smooth muscle tone.

Introduction

The hypothesis that many, if not all, of the actions in mammalian systems of cyclic adenosine monophosphate (cyclic AMP) are mediated via activation of cyclic AMP-dependent protein kinases [1] has stimulated a search for physiologically significant substrates for the enzyme. While numerous substrate proteins have been reported the only substrates of proven physiological significance are [2,3] certain enzymes of glycogen and lipid metabolism. Phosphorylation of the contractile protein troponin, isolated from cardiac and skeletal muscle, has been reported [4,5]. The phosphorylation of the I subunit of cardiac troponin has recently been correlated with increased force of contraction [6]. It has also been suggested that the rate of calcium transport by the cardiac sarcoplasmic reticulum can be influenced by the degree of membrane phosphorylation [7,8].

Little is known about the substrates of the cyclic AMP-dependent protein kinase of smooth muscle. This report describes experiments designed to study the phosphorylation of the contractile proteins from bovine tracheal smooth muscle as well as the further purification and characterization of the muscle specific substrates for the protein kinase, found in this tissue, on which we previously reported [9].

Methods

Partially purified cyclic AMP-dependent protein kinase from bovine tracheal smooth muscle was prepared and assayed as previously described [10]. The $(NH_4)_2\ SO_4$ fraction [10] was the starting material for the further purification of the substrate. Bovine tracheal smooth muscle actomyosin was prepared by the method of Sands [11]. The method of Greaser and Gergely [12] was used for the preparation of an extract from tracheal smooth muscle with the extraction characteristics of skeletal muscle troponin and also for the preparation of tropomyosin.

Studies of the phosphorylation of various proteins were carried out by incubating the protein in a reaction mixture containing: 50 mM sodium glycerol phosphate, 10 mM NaF, 2 mM theophylline, 3.3 mM EGTA, 10 mM $MgCl_2$, 6.6 μM [γ^{32}P]ATP (containing 2000—4000 cpm per pmol) with or without 1.0 μM cyclic AMP. Exogenous partially purified enzyme was added to fractions which did not contain endogenous protein kinase. All incubations were for 10 min unless otherwise stated and at a temperature of 30°C. The reaction mixture was chilled in ice and then dialyzed against four 1-l changes of 5 mM potassium phosphate, 2 mM EDTA, pH 7.0 (Buffer A). In the case of the contractile proteins the final dialysis was against 0.1% sodium dodecyl sulfate (SDS), 0.1% β-mercaptoethanol, 0.01 M sodium phosphate buffer, pH 7.0. Portions were then analyzed by electrophoresis as previously reported [9]. Phosphoprotein phospatase was prepared from bovine tracheal smooth muscle using the method described for the purification of this enzyme from rabbit skeletal muscle [13].

The sites of phosphorylation of the muscle specific substrate were determined by high voltage electrophoresis. Phosphorylated substrate was partially

hydrolyzed by 6 M HCl at 90°C in a sealed evacuated ampule for 90 min. The samples were dried in vacuo and dissolved in a small aliquot of water. The components were resolved on Whatman No. 3 MM paper by electrophoresis in 7% formic acid, at 3000 V for 2 h. Phosphoserine, phosphothreonine, ortho[^{32}P]-phosphate, and [^{32}P]ATP were also analyzed. The paper was dried in an oven at 70°C and then stained for amino acids with 1% pyridine, 0.2% ninhydrin, in acetone. ^{32}P was located by autoradiography.

Protein was determined by the method of Lowry et al., using bovine serum albumin as standard [14].

Materials

[γ-^{32}P]ATP was obtained from ICN, Irvine, California.

Results

Phosphorylation of smooth muscle contractile proteins

The data presented in Table I are the amounts of ^{32}P incorporated into acid precipitable components of various tracheal smooth muscle contractile protein extracts. The phosphorylation of calf thymus mixed histone is shown for comparison. The data are presented as pmoles of ^{32}P incorporated per mg of substrate protein in the presence of the same amount of partially purified tracheal smooth muscle protein kinase. Of the smooth muscle proteins tested the smooth muscle troponin-like extract appeared to be the most effective substrate. The term smooth muscle troponin-like extract is employed since there have been several reports that troponin does not exist in smooth muscle (including tracheal smooth muscle) [11,15—17]. To determine whether specific protein components were phosphorylated, aliquots of the phosphorylated smooth muscle troponin-like extract were resolved on polyacrylamide gels (data not shown). Several distinct protein peaks were evident; however, no distinct peaks of ^{32}P were seen.

TABLE I

ACID-PRECIPITABLE ^{32}P INCORPORATED INTO THE CONTRACTILE PROTEINS OF BOVINE TRACHEAL SMOOTH MUSCLE

40—160 μg of each protein preparation were incubated with 16 μg of partially purified protein kinase as described under Methods. The reaction was terminated after 7 min at 30°C with the addition of trichloroacetic acid to a final concentration of 12%. The precipitate was collected and washed on Millipore (HA) filters.

Protein	No. of observations	10^{-6} M Cyclic AMP	pmol ^{32}P incorporated/mg substrate protein	S.E.
Histone	6	—	57.9	±12.9
	6	+	330.7	±28.0
Smooth muscle troponin-like extract	6	—	5.5	± 4.0
	6	+	53.5	± 8.5
Tropomyosin	4	—	3.8	± 1.9
	4	+	1.0	± 0.8
Actomyosin	3	—	2.1	± 1.1
	3	+	7.1	± 1.0

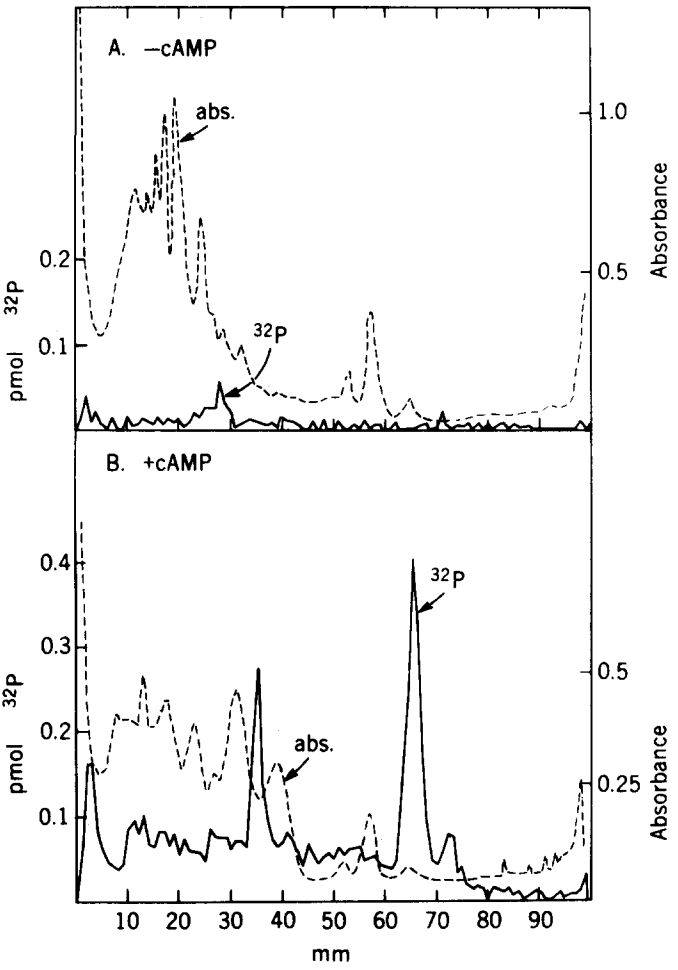

Fig. 1. Electrophoresis of 140 μg of the phosphorylated ammonium sulfate fraction on 0.1% SDS, 10% polyacrylamide gel. A, no cyclic AMP in the incubation mixture; B, 1.0 μM cyclic AMP in the incubation mixture.

Further purification of the muscle-specific substrate from tracheal smooth muscle

Experiments were designed to continue the purification and characterization of the muscle specific substrates of the protein kinase found in bovine tracheal smooth muscle, on which we previously reported [9]. Purification of the substrate started with the 0—50% $(NH_4)_2SO_4$ fraction [9]. Figs. 1a and b show the distribution on polyacrylamide gels of the phosphorylated proteins of the $(NH_4)_2SO_4$ fraction from tracheal smooth muscle. The total amounts of ^{32}P distributed on the gels were 0.65 pmol when incubated in the absence of cyclic AMP and 5.01 pmol when incubated in the presence of 10^{-6} M cyclic AMP. The area previously defined as the muscle specific substrate protein (the region approximately 60—75 mm into the gel) contained 25.5% of the ^{32}P incorpo-

rated into the protein in the presence of cyclic AMP. Essentially no ^{32}P was incorporated into this region in the absence of cyclic AMP.

Fractionation with calcium phosphate gel was used to separate the substrate protein from the protein kinase. The $(NH_4)_2SO_4$ fraction was dialyzed against Buffer A and then added to 20 g (wet weight) of calcium phosphate gel equilibrated with the same buffer. The suspension was stirred continuously for 30 min (at 4°C) then centrifuged at 5000 × *g* for 15 min (at 4°C). The supernatant was then dialyzed against Buffer A. Essentially all of the protein kinase (99.3%) is adsorbed onto the gel while much of the substrate is not. This enzyme can be recovered by washing the gel with 0.1 M and 0.2 M phosphate buffer [10]. These fractions contain little if any substrate protein. While this purification step does not result in a substantial change in the amount of ^{32}P on the gel which is in the substrate region it does remove all of the protein kinase from the preparation. Thus, the amount of enzyme present during further assays is controlled by the addition of exogenous protein kinase.

In order to continue the purification and characterization of the substrate it was necessary to develop the following method for the rapid assay of the substrate in a large number of samples. Each sample was incubated at 30°C in: the protein kinase buffer described in Methods; added exogenous partially purified protein kinase; 6.6 μM[γ-^{32}P]ATP (2000—3000 cpm/pmol) and $2 \cdot 10^{-6}$ M cyclic AMP. The final volume was 0.15 ml. The reaction was stopped by the addition of SDS to a final concentration of 0.13%. The entire reaction mixture was then resolved on polyacrylamide gels. After the tracking dye reached the end of the gel the electrophoresis was stopped and the region 55—70 mm from the origin of the unstained gel was removed, dried, and the ^{32}P incorporated determined. Fig. 2 illustrates the time dependence of the reaction using partially purified substrate. In routine assays the reaction was stopped after 30 min. This assay was used to locate the substrate in the eluent from the G-100 column as described below.

Fig. 3 illustrates the elution pattern of the substrate (using the assay as described above) when the calcium phosphate fraction was applied to a G-100

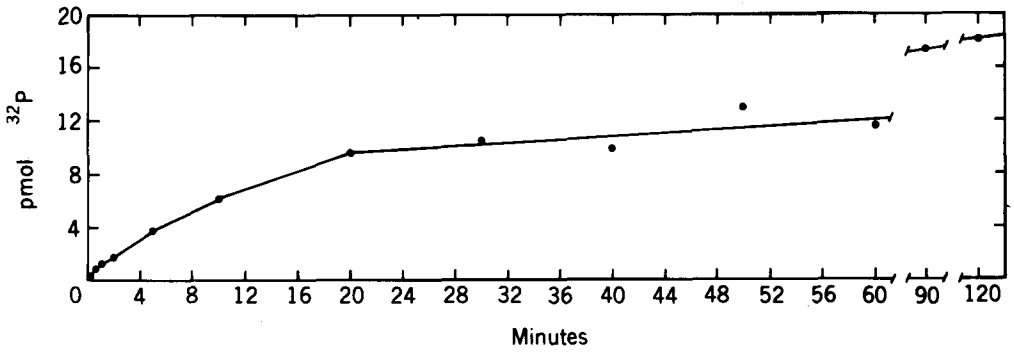

Fig. 2. Time course of the phosphorylation of the muscle-specific substrate. 11.6 μg of the partially purified substrate were incubated with 16 μg of the partially purified smooth muscle protein kinase for the time indicated, as described in the text. The reaction was stopped by the addition of SDS to a final concentration of 0.13%. The entire reaction mixture was then resolved on polyacrylamide gels and the region 55—70 mm from the origin of the unstained gel was removed, dried and the ^{32}P incorporated determined.

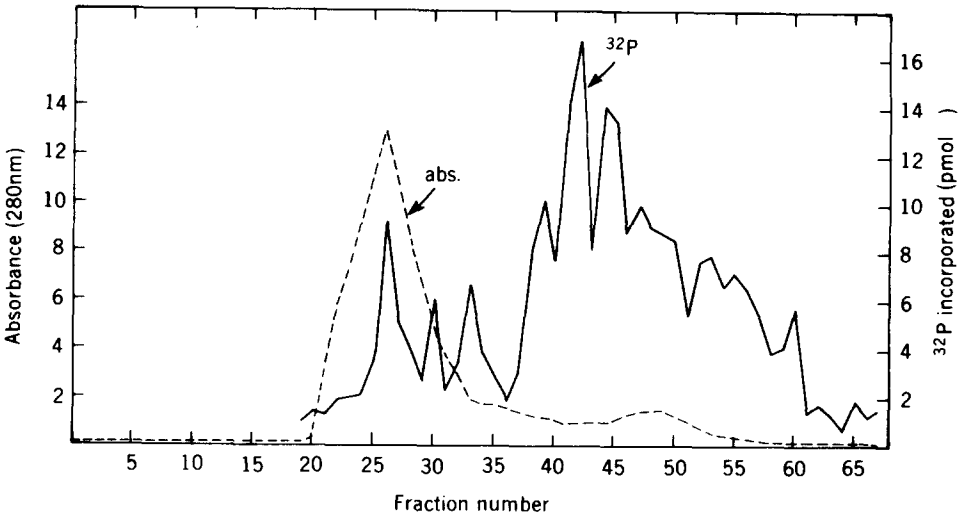

Fig. 3. Chromatography of the muscle specific substrate on the G-100 column. Approximately 662 mg from the calcium phosphate gel purification step were applied to a column of G-100 (2.5 X 102 cm) which had been equilibrated with 5 mM potassium phosphate and 2 mM EDTA, pH 7.0. Substrate activity was determined by incubating 0.06-ml aliquots of each fraction (5.5 ml) with 39 μg of partially purified protein kinase as described in the text. After 30 min the reaction was stopped by the addition of SDS and the incubation mixture resolved on polyacrylamide gels. After electrophoresis the region 55—70 mm from the origin was removed, dried, and the ^{32}P incorporated determined. Fractions 37—50 were pooled and used for the further characterization of the muscle-specific substrate.

TABLE II

STABILITY OF THE MUSCLE-SPECIFIC SUBSTRATE TO VARIOUS TREATMENTS

For control in each treatment the active agent was substituted with buffer and the procedure carried out as outlined. Data are presented as the percentage of ^{32}P remaining in the 55—70 mm section of unstained gels after each treatment as compared with the respective controls. Each determination is the mean of four gels.

Treatment	% of ^{32}P remaining
Pronase [a]	21.3
Trichloroacetic acid [b]	1.8 (after precipitation)
RNAase [c]	102.9
DNAase [d]	110.7
NH_2OH [e]	85.2
KOH [f]	10.5
HCl [g]	105.4

[a] The substrate was labeled and dialyzed. Then 443 μg of substrate were incubated with 10 μg of pronase for 30 min at 37°C and 81 μg of substrate were placed on each gel.

[b] Trichloroacetic acid (final concentration, 12%) was added to 443 μg of labeled substrate and the mixture incubated for 30 min at 2°C. After centrifugation the supernatant was dialyzed and an aliquot placed on each gel.

[c] RNAase (100 μg) was added to 443 μg of labeled substrate. The mixture was incubated for 30 min at 37°C in 50 mM Tris pH 7.0. To each gel 82 μg of substrate were added.

[d] The procedure outlined in footnote c was followed except 100 μg of DNAase was added and the buffer contained 30 mM $MgSO_4$.

[e] Labeled substrate was incubated with 1 M hydroxylamine in 0.1 M sodium acetate buffer, pH 5.6, for 30 min at 30°C. The substrate was then dialyzed and 82 μg placed on each gel.

[f] Labeled substrate was incubated in 0.25 N KOH at 22°C for 24 h. The substrate was then dialyzed and 82 μg placed on each gel.

[g] Same procedure as described in footnote f except the incubation medium was 0.25 M HCl.

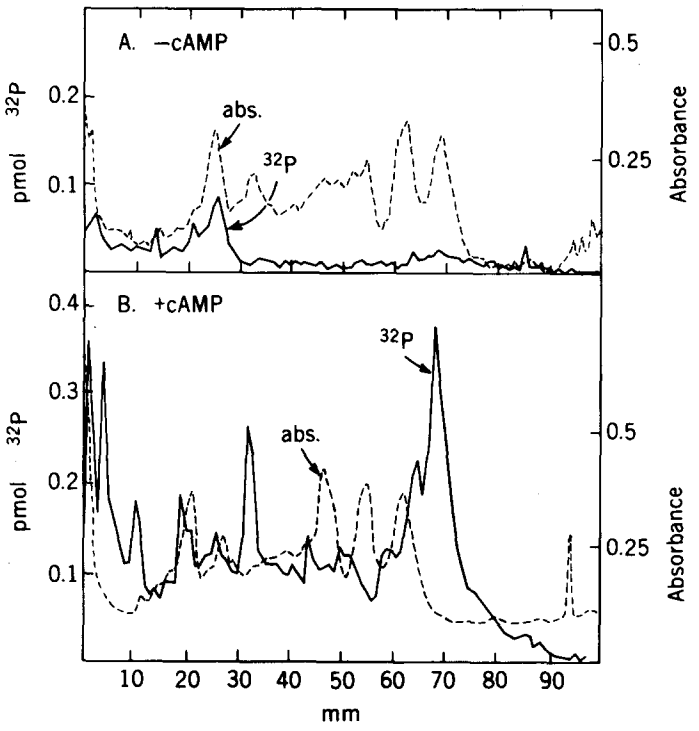

Fig. 4. Electrophoresis of 132 μg of the phosphorylated pooled G-100 fraction of 0.1% SDS, 10% polyacrylamide gels. A, in the absence of cyclic AMP; B, in the presence of 10^{-6} M cyclic AMP. The gels were stained and destained.

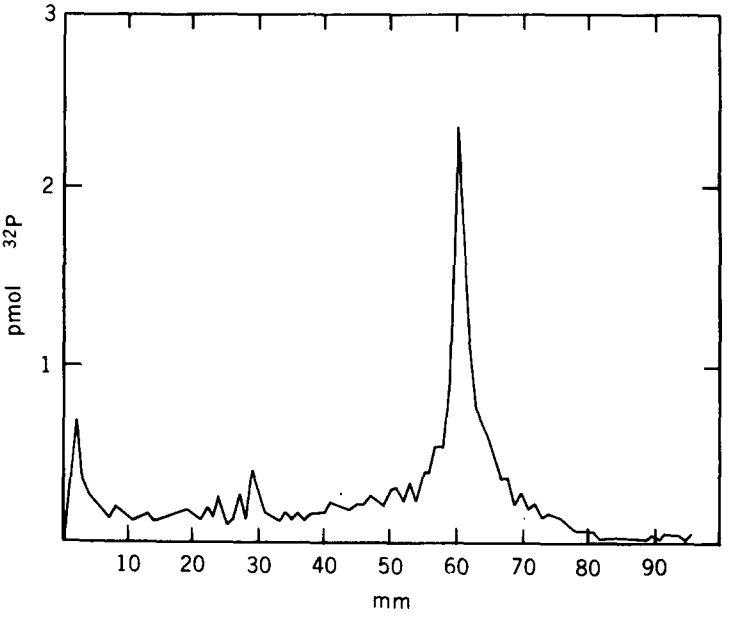

Fig. 5. Same as Figure 4B except gel was not stained and destained.

column equilibrated and eluted with 5 mM phosphate buffer pH 7.0 containing 2 mM EDTA. The fractions containing the substrate were retarded on the column and were separated from the main protein peak as determined by absorption at 280 nm. Fractions 37—50 were pooled and used for the further characterization of the muscle-specific substrate.

Figs. 4a and b show the distribution on polyacrylamide gels of the phosphorylated substrate of the pooled G-100 fraction. The total amount of ^{32}P distributed on the gels was 1.71 pmol when incubated in the absence of cyclic AMP and 11.09 pmol when incubated in the presence of 10^{-6} M cyclic AMP. The area corresponding to the muscle-specific substrate contained 2.67 pmol or 24.1% of the total ^{32}P incorporated into protein in the presence of cyclic AMP. However, the data shown in Figs. 4a and b are misleading since at this stage of the purification a great amount of ^{32}P is lost from the gel during the staining and destaining procedure. Because of this loss, all further characterization experiments (with the exception of the molecular weight determination) were done by measuring the ^{32}P incorporated into the 55—70 mm region of the unstained gel. The pattern shown in Fig. 5 is seen when an unstained gel is sliced, and the ^{32}P incorporated (in the presence of cyclic AMP) into the G-100 frac-

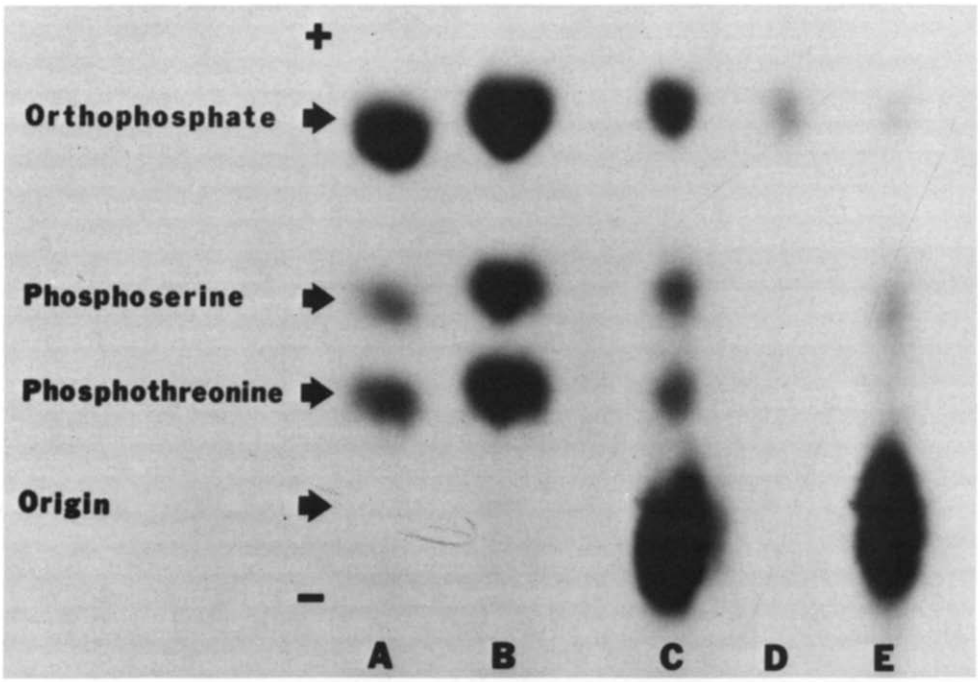

Fig. 6. Autoradiograph of the partial acid hydrolysis of the phosphorylated G-100 fraction. 700 μg of the G-100 fraction were phosphorylated by partially purified protein kinase in the presence or absence of cyclic AMP (10^{-6} M). After hydrolysis approximately 70 μg of protein mixture, plus marker phosphoserine and phosphothreonine were spotted on Whatman 3 MM paper and the amino acids resolved by high voltage electrophoresis. ^{32}P was located by autoradiography. A, Partially hydrolyzed muscle specific protein after phosphorylation in the absence of cyclic AMP. B, Partially hydrolyzed muscle specific protein after phosphorylation in the presence of 10^{-6} M cyclic AMP. C, Partially hydrolyzed muscle specific protein after phosphorylation + [^{32}P]orthophosphate + [^{32}P]ATP. D, [^{32}P]orthophosphate. E, [^{32}P]-ATP.

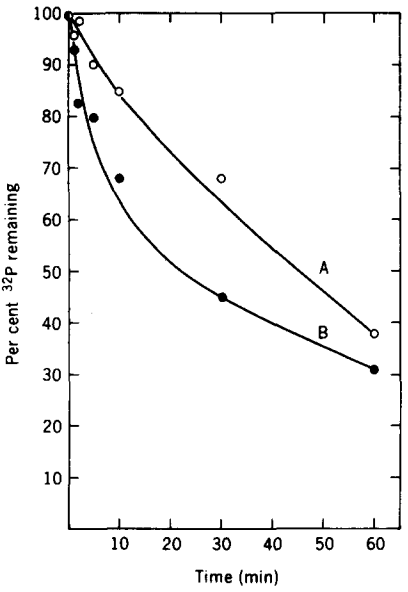

Fig. 7. Effect of phosphoprotein phosphatase. 33 μg of phosphorylated histone (A), or 89 μg of phosphorylated pooled G-100 fraction (B) were incubated with 28 μg of tracheal smooth mucle phosphoprotein phosphatase for the time indicated. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 12%. The precipitate was collected and washed on Millipore HA filters and the ^{32}P remaining determined.

tion is determined. Almost 25 pmol are now found distributed on the gel. Almost 12 pmol (48%) of the ^{32}P is found in the region of the muscle specific protein, and represents the only major ^{32}P peak on the gel. Cyclic AMP increased the total incorporation of ^{32}P found in the unstained gels 7.2-fold.

The G-100 fraction of muscle specific substrate was characterized by using the assay described above to determine the percentage of ^{32}P remaining in the region corresponding to the muscle-specific proteins of the unstained gels after various treatments. The incorporated ^{32}P was unaffected by DNAase, RNAase, hydroxylamine, or HCl but reduced significantly by pronase, KOH, and after trichloroacetic acid precipitation (Table II). These porperties are consistent with the incorporation of the phosphate group into the threonine and serine residues of a protein. The phosphorylation of serine and threonine was confirmed by resolving the components of the substrate (after partial acid hydrolysis) using high-voltage paper electrophoresis. ^{32}P incorporation was determined by autoradiography (Fig. 6). Molecular weight determination, using stained SDS polyacrylamide gel electrophoresis of the G-100 fraction, indicated a value of 19 000.

The fact that bovine tracheal smooth muscle contains an enzyme which will remove the ^{32}P incorporated into the muscle specific substrate is shown in Fig. 7. Phosphoprotein phosphatase prepared from tracheal smooth muscle removed ^{32}P from the trichloroacetic acid precipitable component of both phosphorylated histone and partially purified phosphorylated muscle specific protein.

Discussion

A search for substrates of the cyclic AMP-dependent protein kinase conducted in a number of laboratories has resulted in several reports of substrate proteins [2,3]; some are soluble, others particulate, some have enzymatic activity, others structural. However, little attention has been paid to smooth muscle. The data presented in this report suggest that the relative order of susceptibility of phosphorylation of the contractile proteins found in bovine tracheal smooth muscle is the same as that found in rabbit skeletal muscle [18]. However, interpretation of the data is made difficult because of the lack of information concerning the nature of the contractile proteins of smooth muscle. Recently, the existence of troponin in smooth muscle has been questioned [11,15—17].

An assay was developed which was utilized to further purify and characterize the muscle specific substrate protein previously reported [9]. The data presented suggest that the substrate contains a protein subunit of molecular weight 19 000, which can be phosphorylated at serine and threonine residues in the presence of cyclic AMP. The phosphate is a covalent ester linkage with the residue. The enzymes necessary to phosphorylate and dephosphorylate this protein are both found in bovine tracheal smooth muscle. The purification scheme presented results in a preparation which when resolved by polyacrylamide gel electrophoresis showed: on the unstained gels only one peak of ^{32}P incorporated (representing almost 50% of the ^{32}P seen on the gel); and four major protein bands on the stained gel. The reason for the loss of ^{32}P from SDS polyacrylamide gels is not known.

The fact that bovine tracheal smooth muscle contains protein kinase, phosphoprotein phosphatase and the muscle specific substrate suggests that these proteins may be part of the mechanism by which β-adrenergic agents regulate smooth muscle tone. The function of the muscle specific protein is unknown. Its molecular weight is close to that of the cardiac sarcoplasmic reticulum fraction "phospholamban" reported to be phosphorylated by the cardiac cyclic AMP-dependent protein kinase [19]. The relationship between "phospholamban" and our muscle-specific protein is not known. Studies to determine the physiological function of the protein are being undertaken.

Acknowledgments

This work was supported by Research Grant HL-14964 from the National Institutes of Health, United States Public Health Service.

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