Biochimica et Biophysica Acta, 321 (1973) 489–495

BBA 67040

PHOSPHORYLATION OF MUSCLE PROTEINS BY CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM MUSCLE

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(Received May 1st, 1973)

SUMMARY

Tissue-specific substrates for a cyclic AMP-dependent protein kinase from bovine tracheal smooth muscle have been partially characterized. The substrates are phosphorylated by purified smooth muscle kinase or by kinases found in crude extracts of bovine smooth, skeletal, and cardiac muscle. The substrates derived from and phosphorylated by smooth muscle protein kinase have a molecular weight of approximately 70 000, as determined by disc gel electrophoresis in the absence of sodium dodecyl sulfate and of approx. 20 000–25 000 when electrophoresed in the presence of sodium dodecyl sulfate. It appears that there are a limited number of substrates for the smooth muscle cyclic AMP-dependent protein kinase.

INTRODUCTION

The widespread occurrence of cyclic adenosine monophosphate (cyclic AMP)-dependent protein kinases has lead to the hypothesis that many of the actions of cyclic AMP in mammalian systems are mediated by the activities of these enzymes[1]. In most *in vitro* studies of the protein kinase the phosphorylated substrates have been histones, casein or protamine. However, other physiologically important substrates have recently been identified[2–9]. In the course of studies designed to elucidate the mechanism by which cyclic AMP produces relaxation of tracheal smooth muscle we observed the presence of a cyclic AMP-dependent protein kinase in bovine tracheal smooth muscle[10]. During the purification and assay of this enzyme we also co-purified proteins which served as substrates for the cyclic AMP-stimulated protein kinase. This report describes the phosphorylation of these proteins from bovine tracheal smooth muscle as well as their partial characterization. Similar substrates were also found in bovine cardiac and skeletal muscle but not in bovine kidney or liver. The phosphorylation of certain muscle proteins was totally dependent on the presence of cyclic AMP.

METHODS

Cyclic AMP-dependent protein kinase and substrate proteins from smooth muscle were prepared as previously described[10] up to and including $(NH_4)_2SO_4$ fractionation. The precipitate obtained from the 0–50% $(NH_4)_2SO_4$ fraction was centrifuged and redissolved in 5 mM potassium phosphate, 2 mM EDTA, pH 7.0 (Buffer A) and dialyzed against Buffer A for 16 h. This preparation contained both cyclic AMP-dependent protein kinase activity and substrate proteins for the enzyme. Bovine liver, heart skeletal muscle, renal cortex and renal medulla were similarly treated.

4.8 mg of the above extract containing both protein kinase and its substrates were incubated in a reaction mixture of 1.5 ml (pH 6.5) containing: 50 mM sodium glycerol phosphate, 10 mM NaF, 2 mM theophylline, 3.3 mM ethyleneglycol-bis-(aminoethyl ether)-*N*,*N*′-tetraacetic acid, 10 mM $MgCl_2$, 6.6 μM [γ-^{32}P]ATP (containing $2 \cdot 10^7$–$4 \cdot 10^7$ cpm), with or without 10 μM cyclic AMP. After a 10-min incubation at 30 °C the reaction mixture was chilled in ice and then dialyzed against four 1-l changes of Buffer A or Buffer B (12.4 mM Tris, 9.6 mM glycine, pH 8.4) corresponding to the buffer used in the electrophoresis.

The procedures used for the assay of protein kinase and the preparation of partially purified protein kinase from bovine tracheal smooth muscle have been previously reported[10].

For electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, the method of Weber and Osborn[11] was used. Molecular weights were determined using hemoglobin, pyruvate kinase, myoglobin, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase serving as standards. For electrophoresis on gels without sodium dodecyl sulfate a modification of the methods of Ornstein[12] and Davis[13] was used. The running buffer system used was Buffer B. Molecular weight determinations were based on the method of Hedrick and Smith[14] using gels of 5, 6, 8 and 10% and hemoglobin, bovine serum albumin (monomer, dimer and trimer), glyceraldehyde-3-phosphate dehydrogenase, immuno gamma globulin and human gamma globulin as standards. All gels were stained and destained by the method of Fairbanks *et al.*[15], scanned with a Gilford linear gel scanner at 560 nm, sliced into 1-mm-thick pieces, and incorporation of ^{32}P determined by liquid scintillation techniques.

MATERIALS

[γ-^{32}P]ATP was obtained from ICN or prepared from carrier-free ^{32}P using the method of Glynn and Chappell[16].

RESULTS

The activity of the cyclic AMP-dependent protein kinase, obtained by the $(NH_4)_2SO_4$ fractionation of various tissues, is shown in Table I. The addition of 10 μM cyclic AMP to the incubation mixture resulted in an increase in the phosphorylation of the co-purified proteins of bovine cardiac, skeletal and smooth muscle. Phosphorylation of the co-purified proteins obtained from tissues other than muscle either

TABLE I

PHOSPHORYLATION OF CO-PURIFIED PROTEINS AND EXOGENOUS HISTONES BY MUSCLE AND NON-MUSCLE BOVINE TISSUE EXTRACTS

Tissue	*Co-purified phosphorylation**			*Histone phosphorylation***		
	− cyclic AMP	*+ cyclic AMP (10 μM)*	*Fold increase*	*− cyclic AMP*	*+ cyclic AMP (10 μM)*	*Fold increase*
Smooth muscle	10.3	33.5	3.3	55.8	237.5	4.3
Skeletal muscle	11.5	21.2	1.8	11.2	30.8	2.8
Cardiac muscle	33.5	61.8	1.8	14.3	320.0	22.4
Liver	8.9	9.7	1.1	2.1	15.2	7.2
Renal cortex	34.8	27.6	—	0.0	65.1	∞
Renal medulla	10.5	16.3	1.6	5.3	32.3	6.1

* pMoles of ^{32}P incorporated per mg of protein in 7 min.
** pMoles of ^{32}P incorporated per mg of protein in 7 min, corrected by subtraction of co-purified phosphorylation.

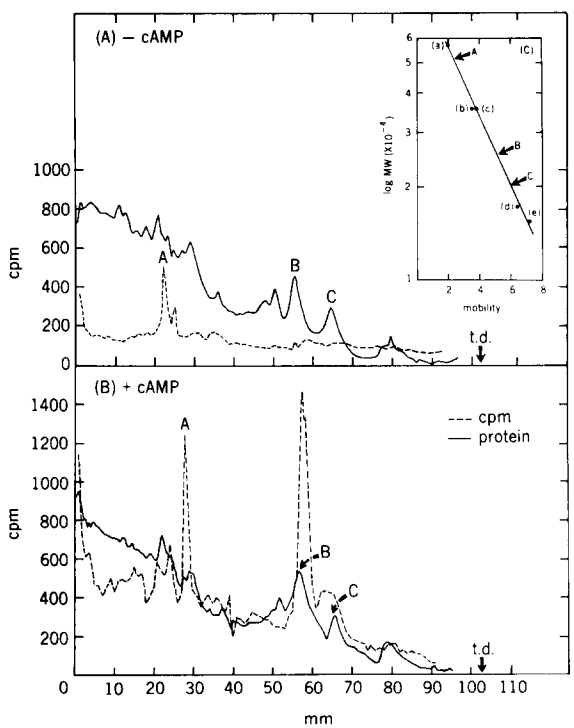

Fig. 1. Electrophoresis of 130 μg of smooth muscle protein on 0.1% sodium dodecyl sulfate, 10% polyacrylamide gels (TD, tracking dye). A, no cyclic AMP in the incubation mixture; B, 10 μM cyclic AMP in the incubation mixture; C, molecular weight determination. a, pyruvate kinase; b, glyceraldehyde-3-phosphate dehydrogenase; c, lactate dehydrogenase; d, myoglobin; e, hemoglobin.

was considerably less than that of the muscle proteins or was not stimulated by the addition of 10 μM cyclic AMP to the incubation mixture.

Figs 1A and 1B show the distribution of phosphorylated proteins of tracheal smooth muscle in an 0.1% sodium dodecyl sulfate, 10% polyacrylamide gel. In the absence of cyclic AMP only proteins of a molecular weight of approx. 50 000 were phosphorylated (Region A). With the addition of 10 μM cyclic AMP to the incubation mixture (Fig. 1B) there was slight stimulation of the phosphorylation of many of the proteins. The major effect of the cyclic nucleotide, however, was the increased phosphorylation of a few proteins (Regions A, B and C). In the presence of cyclic AMP there was phosphorylation of protein in two regions corresponding to molecular weights 25 000 (Region B) and 20 000 (Region C). Phosphorylation of these lower molecular weight proteins was totally dependent on the presence of cyclic AMP in the incubation mixture and represented 24% of the total ^{32}P found in the gel.

Fig. 2 is a composite of the distribution patterns of radioactivity found after the phosphorylation of the proteins of cardiac, skeletal and smooth muscle. The protein staining pattern (not shown) of skeletal muscle was similar to that of smooth muscle shown in Fig. 1A and 1B. Two distinct protein bands, corresponding to molecular weights of 20 000–25 000, were seen. The staining pattern of the cardiac proteins differed in that there was protein distributed throughout the gel and no distinct protein bands corresponding to molecular weights of 20 000–25 000 (not

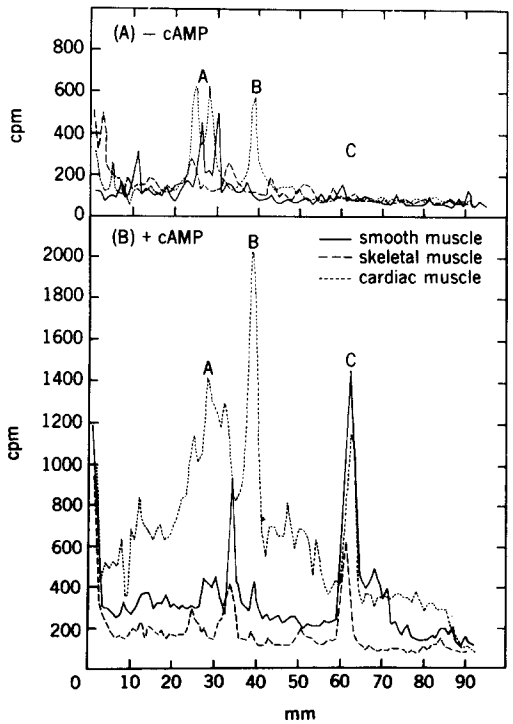

Fig. 2. Electrophoresis of 130 μg of smooth, cardiac and skeletal muscle proteins on 0.1% sodium dodecyl sulfate, 10% polyacrylamide gels. A, no cyclic AMP in the incubation mixture; B, 10 μM cyclic AMP in the incubation mixture. Molecular weights: A, 50 000; B, 35 000; C, 20 000–25 000.

shown). All three tissues contained proteins of molecular weights of approx. 50 000 (Region A) which were phosphorylated in the absence of cyclic AMP. The cardiac extract contained an additional protein of approx. 35.000 mol. wt (Region B) which was phosphorylated in the absence of cyclic AMP.

The addition of 10 μM cyclic AMP to the incubation mixtures resulted in some stimulation of the phosphorylation of most of the proteins present. There was a large increase in the ^{32}P incorporated into the region corresponding to a molecular weight of 50 000 in all three tissues. The phosphorylation of the 35 000-dalton protein of cardiac muscle was also markedly stimulated. The most dramatic effect of cyclic AMP was on the phosphorylation of the proteins of molecular weights of 20 000–25 000 (Region C), which was totally dependent on the presence of the cyclic nucleotide. This cyclic AMP-dependent phosphorylation was found in the extracts of all three muscle types. Addition of partially purified smooth muscle protein kinase to the incubation mixtures (with and without 10 μM cyclic AMP) increased the amount of ^{32}P incorporated but did not alter the pattern of the incorporation (data not shown).

To determine if the proteins which were phosphorylated were found only in muscle extracts, similar experiments were performed using extracts prepared from bovine renal cortex, renal medulla, and liver (data not shown). Preparative procedures were the same as previously described for muscle. In none of these three extracts was there an indication for the occurrence of substrates for protein kinase

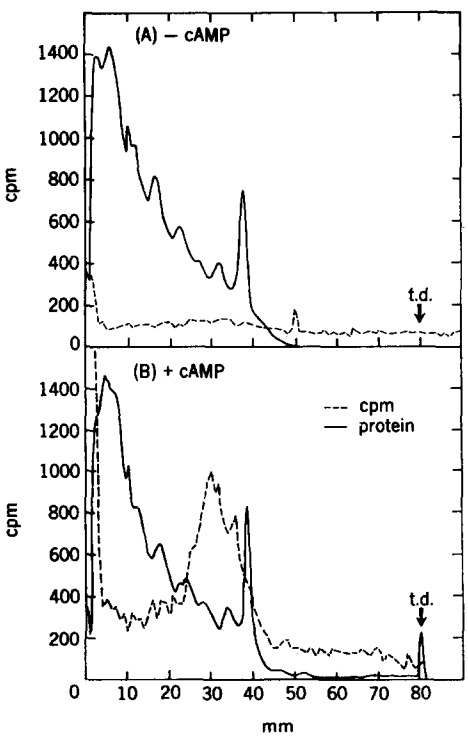

Fig. 3. Electrophoresis of 130 μg of smooth muscle protein on 10% polyacrylamide gels containing no sodium dodecyl sulfate (TD, tracking dye). A, No cyclic AMP in the incubation mixture; B, 10 μM cyclic AMP in the incubation mixture.

when tested in either the presence or absence of cyclic AMP. Furthermore, there was no suggestion of the occurrence of distinct protein bands in the region corresponding to 20 000–25 000 daltons. The addition of partially purified tracheal smooth muscle protein kinase to the incubation mixtures (with and without cyclic AMP) had no effect on phosphorylation. All three extracts did contain measurable amounts of cyclic AMP-dependent protein kinase activity when histones were used as the substrate (Table I). These data suggest that the three muscle extracts differ from extracts obtained from liver, renal cortex and renal medulla, respectively, in the occurrence of substrates for the cyclic AMP-dependent protein kinase and not in the occurrence of the protein kinase. It should be emphasized that we used partially purified extracts, not crude extracts, and that substrates may have been eliminated.

Polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate was used to determine the molecular weights of the native proteins from smooth muscle which were phosphorylated in the presence of cyclic AMP (Figs 3A and 3B). A broad peak of ^{32}P incorporation was seen in the presence of cyclic AMP. The proteins in this region of the gel had molecular weights of approx. 70 000. Incorporation of ^{32}P in this region was totally dependent on the presence of cyclic AMP in the incubation mixture.

DISCUSSION

The data presented demonstrate pronounced heterogeneity of muscle proteins with respect to their ability to serve as substrates for a co-purified cyclic AMP-dependent protein kinase. Sodium dodecyl sulfate polyacrylamide gel electrophoresis resolved these substrates into two distinct types: (a) a group of proteins with molecular weights of around 50 000 whose phosphorylation was stimulated by, but not entirely dependent on, cyclic AMP; and (b) a second group with molecular weights of 20 000–25 000 whose phosphorylation was totally dependent on cyclic AMP. This latter group of proteins was present in the extracts of the three types of muscle, but was not found in extracts derived from renal cortex, renal medulla and liver.

Stull *et al.*[17] have reported the phosphorylation of the inhibitory component of skeletal muscle troponin by the enzyme phosphorylase kinase. The troponin inhibitory component has a molecular weight of approx. 24 000; this is the molecular weight reported here for one of the proteins whose phosphorylation is entirely dependent on the presence of cyclic AMP. Stull *et al.*[17] and Bailey and Villar-Palasi[9] also report the phosphorylation of the troponin inhibitory component by the enzyme protein kinase. Pratje and Heilmeyer[8] have reported the phosphorylation of another component of troponin (molecular weight of approx. 36 000) by purified skeletal muscle protein kinase; no phosphorylation of the troponin inhibitory component was seen. It is unlikely that the phosphorylated proteins described here are proteins of the contractile elements, since the initial homogenization was in 4 mM EDTA, an ionic concentration at which known contractile proteins are insoluble. It is also unlikely that the phosphorylated proteins are enzymes of glycogen metabolism (enzymes which have been shown to be activated or inactivated by the process of phosphorylation); such enzymes would be removed by precipitation at pH 4.8, furthermore they were not found in our liver and kidney cortex extracts (presumably a rich source of the enzymes). The data suggest that the substrates of the protein

kinase are found only in muscle tissue. We are currently purifying the muscle-specific substrates of the cyclic AMP-dependent protein kinase in order to characterize their physical properties and physiological role in muscle metabolism and function.

ACKNOWLEDGMENTS

The authors would like to thank the Pepper Packing Company of Denver for their generosity and cooperation in supplying animal tissue. This work was supported by the National Institutes of Health, United States Public Health Service grants HL-14964 and RR-05474.

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