PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 150, 57–60 (1975)

Evidence Against Phosphorylation of a Cyclic AMP-Dependent Protein Kinase from Bovine Tracheobronchial Smooth Muscle (38973)

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Recent studies have demonstrated the autophosphorylation of the regulatory subunits of cyclic AMP[1]-dependent protein kinase purified from bovine cardiac muscle (1) and bovine brain synaptic membranes (2). The suggestion has been made that the phosphorylation of the protein may play a role in the regulation of kinase activity by facilitating the dissociation of the protein kinase into its regulatory and catalytic units (1). It would be interesting to know if autophosphorylation is a generalized phenomenon or limited to protein kinases from certain organs and/or species. We have performed experiments designed to detect the presence of autophosphorylation of partially purified protein kinase from a second type of bovine muscle, tracheobronchial smooth muscle. Our findings suggest that there is no autophosphorylation of either the regulatory or catalytic subunits of protein kinase from this organ.

Materials and Methods. Partially purified bovine tracheobronchial smooth muscle protein kinase was prepared by the method previously described (3). Phosphoprotein phosphatase was prepared from bovine tracheobronchial smooth muscle using the method described for the purification of this enzyme from rabbit skeletal muscle (4). The protein phosphatase was purified through the DE-cellulose procedure (4).

Proteins were resolved by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS according to the method of Weber and Osborn (5). All gels were stained and destained by the method of Fairbanks *et al.* (6), scanned with a Gilford linear gel scanner at 560 nm, and sliced into 1-mm thick pieces; incorporation of ^{32}P was determined by liquid scintillation techniques.

In order to phosphorylate calf thymus mixed histone (20 μg) and/or protein kinase (22 μg), the proteins were incubated for 30 min at 30° in a reaction mixture containing the following (Buffer A); 10 mM NaF, 2 mM theophylline, 3.3 mM EGTA, 10 mM MgCl, 3.3 μM [^{32}P]-ATP containing (1000–2000 cpm/pmole), and, when present, cyclic AMP at 10^{-6} M. The reaction was stopped by the addition of SDS (final concentration 0.13%) and the mixture resolved on polyacrylamide gels. Each slice of the gel contained 69.6 $\pm$ 3.1 (SE) cpm if no protein kinase was added to the reaction mixture.

For the protein phosphatase experiments 500 μg of calf thymus mixed histone were phosphorylated by incubation at 30° in Buffer A containing purified protein kinase. After 30 min of incubation the reaction mixture (0.5 ml) was dialyzed overnight against 4 liters of 50 mM Tris pH 7.4, 5 mM $MnCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol (Buffer B) at 4°. Two hundred micrograms of this phosphorylated histone were then incubated in Buffer B with 75 μg of the protein phosphatase. After 2 hr at 30° the reaction mixture (0.42 ml) was dialyzed overnight at 4° against 4 liters of 5 mM sodium glycerol phosphate pH 6.5, 10 mM NaF. Ninety-five micrograms of the dephosphorylated histone were then rephosphorylated by incubation with 44 μg of protein kinase in Buffer A. After 30 min of incubation at 30° the reaction was slowed by chilling the tube to 4°. Aliquots of the phosphorylated, dephosphorylated, and rephosphorylated histone containing approximately 20 μg of protein were precipitated with 30% TCA. The precipitate was collected on 0.45 nm Millipore filters and washed with 50 ml of 5% TCA. The amount

[1] The abbreviations used are: cyclic AMP, cyclic adenosine 3′:5′ monophosphate; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol-bis-(β aminoethyl), N,N'-tetraacetic acid; TCA, trichloroacetic acid.

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of radioactivity on the filter was determined in a liquid scintillation spectrometer.

The protein kinase (440 μg) was incubated with 75 μg of protein phosphatase as described above and "rephosphorylated" (191 μg of protein kinase) by incubation for 30 min at 30° with 44 μg of additional protein kinase. The reaction was stopped by the addition of SDS and an aliquot resolved on polyacrylamide gels.

Protein was determined by the method of Lowry *et al.* (7). γ-^{32}P ATP was purchased from ICN and calf thymus mixed histone was purchased from the Sigma Chemical Co.

Results and Discussion. The pattern of staining and ^{32}P incorporation shown in Fig. 1A and B clearly demonstrates that the enzyme studied is a cyclic AMP-dependent protein kinase and that the preparation is not significantly contaminated with either a protein phosphatase or kinase inhibitor. The identification of the protein kinase subunits was based on the previously published molecular weights (3). Figure 1C and D represent the corresponding data for the "autophosphorylation" of the smooth muscle protein kinase. The predicted incorporation of ^{32}P into the regulatory unit of the smooth muscle protein kinase is indicated in Fig. 1D. This predicted value is based on the following calculation: twenty-two micrograms of protein were placed on each gel. If we assume (based on the protein staining of the gels) that the protein kinase is 50% pure, then in fact 11 μg of protein kinase were placed on each gel. Based on published molecular weights of the protein kinase subunits (3) we can assume that 7 μg of the 11 μg correspond to regulatory subunits. If each regulatory subunit incorporated two phosphate groups [as reported for the bovine cardiac muscle protein kinase (1)], a total of 146 pmoles of ^{32}P would be incorporated. If only 25% of the regulatory units were phosphorylated, 37 pmoles of ^{32}P or 37,000 cpm would be incorporated into the region of the

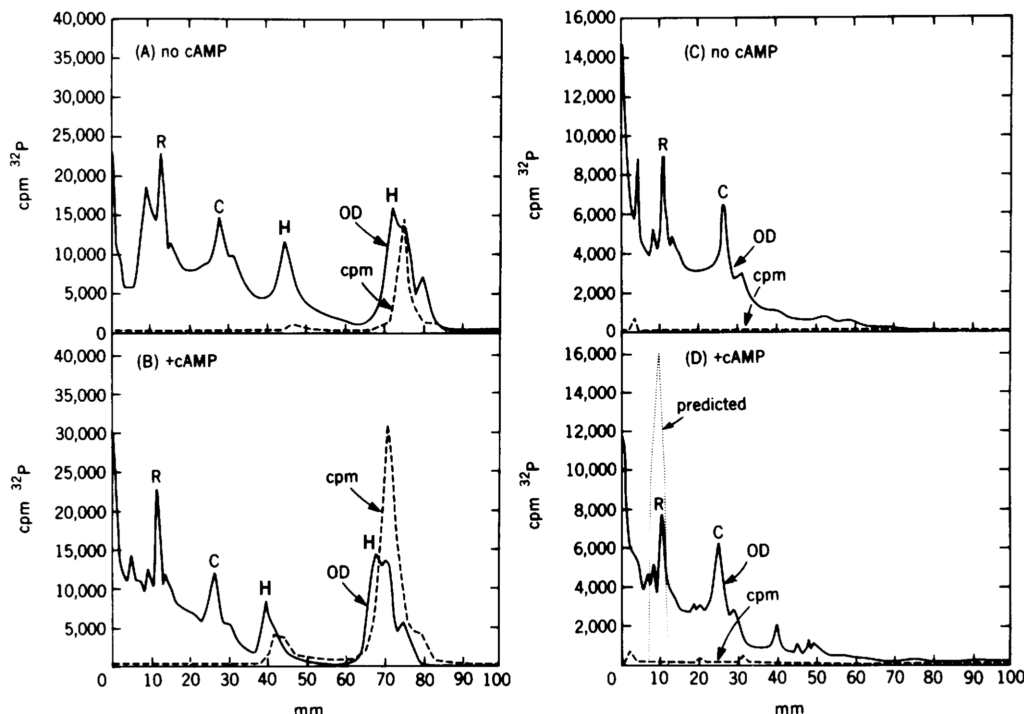

FIG. 1A, B. Resolution on polyacrylamide gels of 20 μg of calf thymus histone phosphorylated by incubation with 22 μg of smooth muscle protein kinase in the absence (A) and presence (B) of 10^{-6} *M* cyclic AMP. R = regulatory subunit. C = catalytic subunit. H = histone protein. C, D. Resolution on polyacrylamide gels of 22 μg of "autophosphorylated" smooth muscle protein kinase in the absence (C) and presence (D) of 10^{-6} *M* cyclic AMP. R = regulatory subunit. C = catalytic subunit.

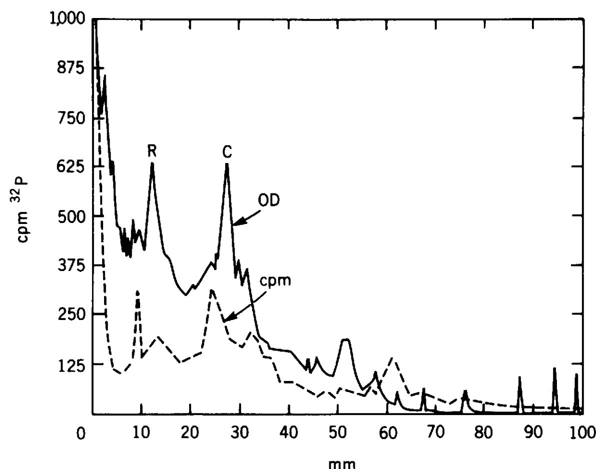

FIG. 2. Resolution on polyacrylamide gels of 36 μg of "rephosphorylated" smooth muscle protein kinase. R = regulatory subunit. C = catalytic subunit.

regulatory unit on the gel. Figure 1D demonstrates that the incorporation of ^{32}P was essentially nil. The results were similar to that shown in Fig. 2 where the scale was not compressed. Even if the assumptions made in the above calculation were off by a factor of ten the predicted incorporation of ^{32}P would be severalfold higher than shown in Fig. 2.

It is possible that the smooth muscle protein kinase did not incorporate ^{32}P into its regulatory subunit because it was already fully phosphorylated. To explore this possibility, the smooth muscle protein kinase was incubated with phosphoprotein phosphatase prepared from bovine tracheobronchial smooth muscle. Erlichman *et al.* (1) have shown that this enzyme, isolated from rabbit skeletal muscle, is capable of dephosphorylating the regulatory subunit of the bovine cardiac muscle protein kinase. Table I shows the result of incubating the protein phosphatase with phosphorylated histone for 2 hr. Most of the ^{32}P previously incorporated was removed. By incubating the dephosphorylated histone with the protein kinase the ^{32}P was reincorporated. This series of experiments showed that the protein phosphatase was active and that the incubation and dialysis conditions used were sufficient to inhibit the actions of the phosphatase during the rephosphorylation procedure.

The gel profile shown in Fig. 2 resulted from treating the purified protein kinase with the phosphoprotein phosphatase under similar conditions to those used to dephosphorylate histone and then incubating it with additional protein kinase, $\gamma^{32}P$-ATP, and cyclic AMP: essentially no ^{32}P was incorporated into either subunit of the protein kinase; the profile resembles that presented in Fig. 1D.

The results presented here are evidence against the autophosphorylation of the regulatory subunit of the bovine tracheobronchial smooth muscle protein kinase. This result contrasts with the ability of the bovine cardiac protein kinase to phosphorylate itself. While the two enzymes are similar in many respects they differ in the size of their regulatory subunits. The regulatory subunit of the cardiac muscle kinase has been reported to be a dimer of two units with a molecular weight of 49,000 each (8). The molecular weight of the smooth muscle kinase has been reported to be 88,500 (3); at this time there is no evidence from gel electrophoresis, that this subunit is itself a dimer. The difference in the ability of the enzyme to phosphorylate its own regulatory

TABLE I. DEPHOSPHORYLATION AND REPHOSPHORYLATION OF HISTONE.

Phosphorylated histone	Dephosphorylated histone	Rephosphorylated histone
	cpm/μg protein	
675	6	1283

unit may be related to these differences in the size and composition of the regulatory subunits, and may indicate a fundamental difference in regulation of kinase activity *in vivo*.

Summary. The data presented in this report are evidence against the autophosphorylation of the cyclic AMP-dependent protein kinase isolated from bovine tracheobronchial smooth muscle. This suggests that there may be a fundamental difference in the regulation *in vivo* of the protein kinases from bovine heart and tracheobronchial smooth muscle.

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

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Received March 24, 1975. P.S.E.B.M., 1975, Vol. 150