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Activation of the Renal Erythropoietic Factor by a Cyclic AMP-Dependent Protein Kinase[1] (37982)

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The mechanism of action of adenosine 3′,5′-monophosphate (cyclic AMP) in most biological systems studied has been found to be associated with protein kinases (1), enzymes which are found in most tissues (2). Some investigators have suggested that cyclic AMP may phosphorylate a protein subsequent to stimulating a protein kinase (3). It has been demonstrated that the activated protein kinase catalyzes the transfer of the terminal phosphate of ATP to other important enzymes in events such as muscle glycogenolysis (4) and lipolysis (5). It has also been suggested that protein kinases may mediate the effect of cyclic AMP in steroid biogenesis (6). Other humoral events, such as erythropoietin production and erythroid cell differentiation, have not been well studied and the role of protein kinases in these processes has not been determined. Previous studies indicate that after treatment with cobalt (7) or exposure to hypoxia (8), increased kidney production of the hormone erythropoietin (ESF) results. We have previously proposed a possible mechanism for kidney ESF production after cobalt (9). Renal cyclic AMP levels were reported to be elevated as early as 45 min after treatment with cobaltous chloride hexahydrate and the renal erythropoietic enzyme, erythrogenin (renal erythropoietic factor, REF) was also found to be elevated after 6–9 hr. It was further shown that plasma levels of ESF were maximally elevated 12 hr after cobalt treatment. We postulated from these studies that a cyclic AMP-dependent mechanism involving a renal protein kinase was operative in the regulation of erythropoietin production by the kidney (9). The purpose of the present study was to purify and characterize a renal cyclic AMP-dependent protein kinase and to evaluate its role in the *in vitro* generation of ESF.

Materials and Methods. Materials. Male Sprague–Dawley rats (200–250 g) were used as the source of all tissues. The nucleotides and biochemicals used in these studies were obtained from the Sigma Chemical Company. Cyclic [^{3}H]AMP and $H_3{}^{32}PO_4$ for the preparation of [γ-^{32}P]ATP were purchased from New England Nuclear. The [γ-^{32}P]ATP was prepared according to the method of Glynn and Chappell (10). Radioactivity determinations were performed using a Packard Model 3320 Liquid Scintillation Counter. Protein kinase activity was determined using Whatman ET.31 chromatography paper. Data are expressed as the mean ± standard error and the statistical significance of our experimental data was determined by the use of Dunnett's test for comparing several treatments with a single control (11).

Methods. Enzyme characterization. A renal cyclic AMP-dependent protein kinase was prepared from rat kidneys (200 g) using the method of Miyamoto *et al.* (12) which included purification with DEAE–cellulose chromatography. Each fraction collected from the column contained approximately 7 ml of eluate. The enzyme was characterized by determining the amount of cyclic AMP binding activity and by esti-

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mating the ability of each fraction to phosphorylate histones. Binding of radioactive cyclic AMP was determined using the method of Gilman (13), and the ability of the enzyme to transfer ^{32}P from [γ-^{32}P]ATP to histones was measured using a previously described method (14). Estimation of protein was determined by the method of Lowry *et al.* (15).

Measurement of erythropoietic activity. Erythropoietic activity in the various samples was determined using the exhypoxic polycythemic mouse bioassay described by Cotes and Bangham (16) and modified in our laboratory (17). The renal erythropoietic factor was prepared and assayed via techniques previously described (9).

Results. Partially purified renal protein kinase was initially characterized by its ability to bind cyclic AMP. The lower panel of Fig. 1 depicts: (1) the protein elution curve following the addition of 300 m*M* KH_2PO_4 buffer, pH 7.0 to the DEAE–cellulose column and (2) the cyclic AMP binding activity. Cyclic AMP binding activity is expressed as pmoles cyclic [^{3}H]AMP bound/20 μl of sample. The results of the lower panel of Fig. 1 indicate a good correlation between cyclic AMP binding activity of a fraction and the protein concentration of that fraction.

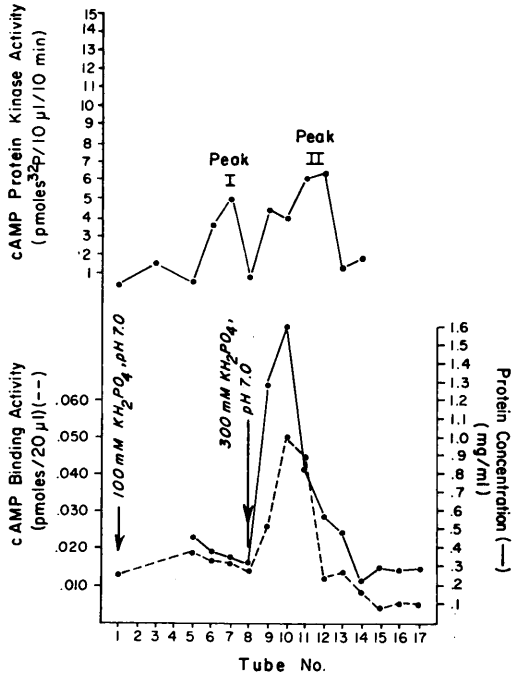

FIG. 1. Characterization of rat renal cyclic AMP-dependent protein kinase on DEAE–cellulose column. Fractions of 7 ml volume were collected. Each fraction was assayed for cyclic AMP binding (lower panel) and protein kinase activity (upper panel). Experimental details are given under Methods.

The ability of the fractions to stimulate the incorporation of ^{32}P from [γ-^{32}P]ATP into histones, with and without cyclic AMP (3×10^{-6} *M*), was also studied and the results are shown in the upper panel of Fig. 1. Cyclic AMP-dependent protein kinase activity was eluted in two peaks, the first peak was eluted with 100 m*M* KH_2PO_4 buffer at pH 7.0 and the second with 300 m*M* KH_2PO_4 buffer at pH 7.0. Peak II was associated with a greater amount of both cyclic AMP binding activity and protein kinase activity and was chosen for use in later studies. The kinase activity of each fraction was determined using a filter paper method (14) and was expressed as pmoles ^{32}P incorporation into histones/10 μl sample/10 min. Each point in the upper panel (Fig. 1) represents the net ^{32}P incorporation into histones after addition of the various fractions.

In order to determine more directly the role of a renal protein kinase in the production of ESF via activation of REF, experiments were performed to evaluate the possible action of the purified protein kinase on the *in vitro* generation of ESF. As indicated in Table I, an active fraction of Peak II was incubated with REF obtained from normal rat kidneys (9) for 30 min at 37°C, with and without cyclic AMP (3×10^{-6} *M*). After this incubation, an equal volume of normal dialyzed rat plasma (9) was added to REF–protein kinase mixture and a second 30-min incubation performed. All incubation mixtures contained 2×10^{-4} *M* ATP, which is required for kinase reactions. These mixtures, together with appropriate control reactions, were tested for erythropoietic activity in the polycythemic mouse bioassay. Erythropoietic activity (Table I) derived from the percentage

TABLE I. Effects of a Cyclic AMP-Dependent Protein Kinase (PK) on Renal Erythropoietic Factor (REF) Activity and Erythropoietin (ESF) Production.[a]

Incubation mixture	IRP-ESF in mUnits
Plasma	49 ± 6
REF	Not detectable
PK	Not detectable
Plasma + PK	57 ± 32
REF + plasma	116 ± 32
(REF + cyclic AMP) + plasma	80 ± 39
(REF + PK) + plasma	51 ± 25
Cyclic AMP ($3 \times 10^{-6} M$)	Not detectable
(REF + PK + cyclic AMP) + plasma	183 ± 14*

[a] The data are expressed as the mean ± standard error of four experiments (seven mice in each group per experiment). Control values for saline and 0.1 units ESF averaged 3.05 and 9.5% ^{59}Fe incorporation, respectively. Substances in parentheses were preincubated together for 30 min before further incubation with plasma. An asterisk denotes the experimental value which is significantly different from all other values ($P < 0.05$).

of ^{59}Fe incorporation into red cells is expressed as milliunits (mU), based on a standard International Reference Preparation (IRP) dose-response curve of ESF. At a concentration of $3 \times 10^{-6} M$, cyclic AMP had no erythropoietic effect. In addition, neither normal dialyzed plasma nor REF alone had significant erythropoietic activity. The combination of REF and plasma when incubated together at 37°C for 30 min was found to possess significant ($P < 0.05$) erythropoietic activity when assayed in polycythemic mice. When the REF was preincubated with cyclic AMP ($3 \times 10^{-6} M$) prior to incubation with plasma, no further change in erythropoietic activity was seen. Similarly, when the protein kinase (250 μl of fraction 11 from Peak II) was preincubated with REF before incubation with plasma, a slight but not significant decrease in erythropoietic activity was seen. However, when both the protein kinase (250 μl) and cyclic AMP ($3 \times 10^{-6} M$) were preincubated with REF, the resulting incubation with plasma engendered a significant ($P < 0.05$) increase in erythropoietic activity. This value (183 mUnits) is significantly different from all control values and represents a 3-fold increase in erythropoietic activity over the appropriate control [(REF + PK) + Plasma]. The standard errors are large when considered as a percentage of the individual mean values representing around 25–30 mUnits of activity but are reasonable since the bioassay system has a lower sensitivity limit of about 50 mUnits. Regardless of the magnitude of the standard errors, a significant difference exists ($P < 0.05$) between the experimental value and its respective controls. These data indicate that cyclic AMP activates the renal erythropoietic factor (via a protein kinase) resulting in increased production of ESF.

Discussion. We have reported previous studies which have suggested a role of cyclic AMP in the renal production and/or release of erythropoietin after the administration of erythropoietic stimulants such as cobalt and prostaglandins (9, 18). In these studies, the renal levels of cyclic AMP were found to be elevated prior to an increase in REF and plasma ESF after the administration of cobalt or prostaglandin E_1. The time sequence for the increases in renal cyclic AMP, REF, and plasma ESF suggested a correlation between cyclic AMP and REF activation. However, the precise mechanism through which cyclic AMP might activate REF and mediate the production of ESF has not been elucidated. One possible mechanism is the activation of a cyclic AMP-dependent protein kinase, the enzyme which has been demonstrated to be intimately involved in the mediation of other

cyclic AMP regulated events. Since cyclic AMP has been shown to mediate the effects of several other hormones (19) and in that many of the hormonal effects involve protein phosphorylation (3), it has been generalized that cyclic AMP effects are mediated by such protein kinases. Protein kinases catalyze the transfer of the terminal phosphoryl group of nucleoside triphosphates (in this case, ATP) to amino acid residues in protein substrates such as serine or threonine (20). Cyclic AMP-dependent protein kinases are enzymes which require cyclic AMP for full enzymatic activity. In our studies with cobalt, increased renal cyclic AMP levels (9) would be available for interaction with a renal cyclic AMP-dependent protein kinase. Recent studies have indicated that this interaction of cyclic AMP with a tissue protein kinase involves the binding of the nucleotide to a regulatory subunit, resulting in the dissociation of the enzyme into both catalytic and regulatory subunits. This dissociation leads to an increased catalytic efficiency, since in the presence of the regulatory subunit, the kinase may be viewed as being inactive or inhibited (21). In the present studies, the characterization of a renal protein kinase (Fig. 1) and its effects on REF (Table I) suggest that (1) cyclic AMP is involved in renal ESF production and (2) the mechanism of action of cyclic AMP in the generation of ESF by the kidney is similar to the mechanism of action of cyclic AMP in other tissues. Another similarity between the effects of this renal protein kinase on REF activity and the effects of protein kinases in general is the observation that without cyclic AMP, REF is inhibited by the protein kinase (Table I). An inhibition of lipase enzymes by a protein kinase alone (no cyclic AMP present) has been previously reported (22). However, when cyclic AMP is present, marked activation of REF occurs (Table I) suggesting a cyclic AMP–protein kinase mechanism similar to that shown for many other enzyme systems (23).

Since this protein kinase is found in the kidney, it is assumed that this kinase is available for interaction with REF during erythropoietic stimulation. Prostaglandins and cobalt have been reported to exert their erythropoietic effects via cyclic AMP (9, 18). Additional studies in our laboratory have also demonstrated significant increases in renal cyclic AMP after hypoxic stimulation (24). After erythropoietic stimulation, renal cyclic AMP should be available for the activation of the cyclic AMP-dependent protein kinase which subsequently should enhance the activity of REF resulting in increased levels of ESF. Thus, renal cyclic AMP-dependent protein kinase may be a common intermediate, linking erythropoietically induced cyclic AMP with the erythropoietic enzyme, REF.

Summary. A protein kinase, which is one of the key components of a renal cyclic AMP mechanism, has been isolated, characterized, and studied with respect to its ability to generate erythropoietin (ESF) in an *in vitro* system. This enzyme was found to activate the renal erythropoietic factor (erythrogenin) in the presence of cyclic AMP which led to an increase in the generation of ESF from plasma. These data suggest that this renal cyclic AMP-dependent protein kinase which is present in the normal kidney plays an important role in kidney ESF production after erythropoietic stimulation.

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