Abstract—Cyclic AMP-dependent protein kinase activity and endogenous protein phosphorylating activity are reported for 6 cortical segments of the rabbit nephron which were microdissected and collected according to their morphology. The 6 cortical nephron segments, namely the glomerulus (Glm), proximal convoluted tubule (PCT), proximal straight tubule (PST), cortical ascending limb of Henle’s loop (CAL), distal convoluted tubule (DCT), and cortical collecting tubule (CCT), showed protein kinase activities which were increased 1.8–4.9 fold by $10^{-6}$ M cyclic AMP in the presence of histone IIA, histone f2b or histone f3 as a protein substrate. However, all these segments showed little or no cyclic AMP dependent increase of activity with either protamine or $\alpha$-casein as a protein substrate. Cyclic AMP increased the endogenous protein phosphorylation of Glm ($10^{-6}$ M cyclic AMP), of CAL ($10^{-7}$ and $10^{-8}$ M cyclic AMP), of DCT ($10^{-6}$ M cyclic AMP) and of CCT ($10^{-8}$, $10^{-7}$ and $10^{-6}$ M cyclic AMP). In contrast, PCT showed decreased endogenous protein phosphorylation in the presence of $10^{-7}$, $10^{-5}$ and $10^{-4}$ M cyclic AMP.

It has been established that cyclic AMP mediates the actions of the several hormones that have an affect on the kidney cortex such as parathyroid hormone (1–4), vasopressin (5), and catecholamines (6–9). Studies on the role of cyclic AMP as a “second messenger” for hormone action in many tissues have led to the theory that the final effects of cyclic AMP may be a consequence of activation of protein kinases (10–14). Cyclic AMP-dependent protein kinase and endogenous phosphorylation of the renal cortex have been studied in the cortical cytosol and membrane fractions (15–17).

**MATERIALS AND METHODS**

Preparation of isolated cortical segments of rabbit nephron: The cortical segments of
rabbit nephron were isolated and collected according to the method of Imbert et al. (23). Male New Zealand white rabbits weighing 1.9–2.4 kg were used. The left kidney was perfused via the left renal artery with an ice-cold collagenase solution until the kidney swelled. The collagenase solution contained 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.0 mM MgCl₂, 1.0 mM CaCl₂, 10 mM Tris HCl (pH 7.4), 0.1% bovine serum albumin and 0.1% collagenase. The kidney was cut into 1–2 mm thick slices along the cortico-medullary axis, and these slices were incubated for 50 min at 37°C with the collagenase solution bubbled with 95% O₂ + 5% CO₂. At the end of the incubation, the slices were rapidly washed and placed in an ice-cold microdissection solution which contained 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.0 mM MgCl₂, 0.25 mM CaCl₂ and 10 mM Tris HCl (pH 7.4); and the nephron segments were microdissected under a stereomicroscope. After the structure of the nephron segments were examined further under a phase contrast microscope, they were transferred into the wells of a microtiter plate and centrifuged at 2,000 rpm for 10 min in order to remove the microdissection solution. Sucrose solution (0.25 M) containing 1 mM EDTA and 10 mM Tris HCl (pH 7.5) was then added to the segments and they were kept at -80°C until use. Under these conditions of storage, the protein kinase activity and the endogenous protein phosphorylating activity did not change within a 2 month period. The nephron segments from 3–4 rabbits were pooled and used as samples for the enzyme assay. The protein content of the homogenate was determined by the method of Lowry et al. (24) in a total reaction volume of 65 μl using bovine serum albumin as a standard. This method was sensitive enough to detect 0.1 μg of bovine serum albumin.

Measurement of protein kinase activity and endogenous protein phosphorylation: Protein kinase activity was assayed according to the method of George et al. (21). The reaction mixture contained 62.5 mM MES (2[N-morpholino]ethane sulfonic acid) buffer (pH 6.0), 10 mM MgCl₂, 10 mM KF, 0.5 mM [γ-³²P]Tris ATP (approx. 2,000 cpm/pmol), 5 mM theophylline, 1 mM EGTA, 0.25 mM EDTA, 1 mM ouabain (strophanthin-g) 12.5 mM DL-dithiothreitol, and 2 mg/ml exogenous protein substrate in the presence or absence of 10⁻⁶ M cyclic AMP in a total volume of 20 μl. To determine endogenous protein phosphorylation, the exogenous protein substrate was omitted. The reaction was started by adding the homogenate of the nephron segment. Incubation was carried out in a shaking water bath at 30°C for 3 min and reaction was terminated by adding 1 ml of a stopping solution containing 10% (w/v) trichloroacetic acid, 6.25 mM ATP, and 6.25 mM NaH₂PO₄. Bovine serum albumin (630 μg) was added to facilitate recovery. After letting the mixture stand for 15 min, it was centrifuged at 27,000×g for 15 min, and the supernatant was discarded. The pellet was dissolved in 0.2 ml of 1 N NaOH and reprecipitated with the stopping solution. This procedure was repeated, and the final precipitate was collected on a Whatman GF/C glass fiber filter. The filter was dried, and radioactivity was counted in Aquasol-2 using a Packard Tri-Carb liquid scintillation counter, model 3320. Under these assay conditions, protein kinase activity and endogenous protein phosphorylating activity of the cortical nephron segments were linear with time up to 3 min and with protein amounts up to 2.5 μg.
Table 1. Protein kinase activity of the 6 cortical nephron segments and the effect of cyclic AMP. The homogenate of the segment was incubated with [γ-32P]ATP and the exogenous protein substrate in the presence or absence of 1 μM cyclic AMP. The time of the incubation was 3 min and the temperature was 30°C. Details are given in the Methods section.

| Neophron segment | Protein Kinase activity (pmoles 32P-incorporated/min/mg) |
|------------------|--------------------------------------------------------|
|                  | Histone II A  | Histone VII-S (f2b) | Histone VIII (f3) | Protamine | α-Casein |
| Glm              | -cAMP<sup>1</sup> | 404±56 | 447±60 | 333±71 | 1035±89 | 524±20 |
|                  | + cAMP<sup>1</sup> | 1526±117 | 1232±65 | 554±76 | 1178±26 | 534±72 |
| PCT              | -cAMP         | 152±8  | 132±4  | 150±10 | 112±16 | 168±6  |
|                  | + cAMP        | 439±25 | 386±32 | 179±26 | 152±9  | 171±7  |
| PST              | -cAMP         | 168±6  | 124±16 | 141±11 | 81±9   | 126±10 |
|                  | + cAMP        | 302±19 | 256±18 | 161±21 | 108±16 | 124±3  |
| CAL              | -cAMP         | 150±21 | 231±20 | 111±8  | 220±25 | 217±24 |
|                  | + cAMP        | 562±55 | 548±33 | 200±15 | 266±34 | 212±9  |
| DCT              | -cAMP         | 144±18 | 161±32 | 101±21 | 114±30 | 142±6  |
|                  | + cAMP        | 708±56 | 448±50 | 126±13 | 88±17  | 162±15 |
| CCT              | -cAMP         | 220±28 | 245±31 | 132±19 | 324±37 | 311±30 |
|                  | + cAMP        | 793±89 | 638±42 | 239±55 | 421±31 | 274±25 |

<sup>1</sup> without cyclic AMP
<sup>2</sup> in the presence of 1 μM cyclic AMP
All values are the mean±S.D. of three experiments. No correction was made as to the amount of endogenous protein phosphorylation.

Chemicals: Collagenase type I from C. histolyticum, tris ATP, histone II A histone VII-S (f2b), histone VIII (f3), protamine sulfate, α-casein, theophylline, and ouabain were obtained from Sigma; adenosine 5'-[γ-32P]triphosphate (3,000 Ci/mmole) was obtained from the Radiochemical Centre (Amersham); cyclic AMP was obtained from Boehringer; and all other chemicals were of the highest quality available from standard suppliers.

RESULTS

Table 1 shows the protein kinase activities of the 6 cortical nephron segments with exogenous protein substrates. All of the segments showed cyclic AMP dependent increase of protein kinase activity with histone II A, histone f2b and histone f3. The highest activity was observed in Glm*, then CCT and PST, PCT, CAL and DCT in decreasing order of kinase activity. Cyclic AMP (10⁻⁶ M) stimulated the phosphorylation 1.8–4.9 fold with histone II A and histone f2b as protein substrates. With histone f3 as a protein substrate, a low level of cyclic AMP dependent stimulation of phosphorylation was observed. Protamine and α-casein were poor substrates for the cyclic AMP dependent protein kinases of the cortical nephron segments, and the kinases showed little or no cyclic AMP dependent increase in activity for these substrates. These observations are consistent with the reported protein kinase activity of the kidney cortex (15, 17). The substrate specificity of the protein kinases of the 6 cortical nephron segments were nearly identical. Therefore, differences in the physiological function of
the nephron segments in the cortex may not be attributed to the specificity of the protein kinases. Recently, Edwards and coworkers have demonstrated using histone f2b as substrate that vasopressin activated both the rat medulla cyclic AMP-dependent kinases of the medullary collecting tubule and the medullary thick ascending limb of Henle's loop (25).

Cyclic AMP-dependent protein kinase phosphorylates not only exogenous protein substrates, but also endogenous proteins within the cell. It is postulated that the luminal membrane of the renal cell is phosphorylated. The alteration of reabsorption is supposed to take place at that time. Figures 1–5 show the endogenous protein phosphorylation of the 6 cortical segments as a function of cyclic AMP concentration. The Glm showed an increased phosphorylation at a concentration of 10^{-6} M cyclic AMP (Fig. 1). The PCT, where the parathyroid hormone-sensitive adenylate cyclase is most abundantly distributed (4), showed decreased phosphorylation at concentrations of 10^{-7}, 10^{-5} and 10^{-4} M cyclic AMP (Fig. 2), although the protein kinase activity of the PCT was several fold increased by 10^{-6} M cyclic AMP with the exogenous protein substrates (Table 1). It could be postulated that although the protein kinase of the PCT was activated by cyclic AMP, an endogenous protein which is the natural substrate in the PCT had undergone such a change by cyclic AMP that the amount of its phosphorylation was decreased in the presence of cyclic AMP. No significant cyclic AMP dependent increase or decrease of phosphorylation was observed in the PST. Cyclic AMP increased the endogenous protein phosphorylation of the CAL (Fig. 3, 10^{-7} and 10^{-6} M cyclic AMP), of the DCT (Fig. 4, 10^{-6} M cyclic AMP) and of the CCT (Fig. 5, 10^{-8}, 10^{-7} and 10^{-6} M cyclic AMP), where the vasopressin-sensitive adenylate cyclase and catecholamine-sensitive adenylate cyclase are most abundantly distributed (5, 26). The amount

**Fig. 1.** Effect of cyclic AMP concentration on the endogenous phosphorylation of the Glm. The homogenate of the Glm was incubated for 3 min without exogenous protein substrate. Each point represents the mean of three experiments, and standard deviations are shown as vertical bars. *Significantly increased from the control (P<0.05, Student's t-test)

**Fig. 2.** Effect of cyclic AMP concentration on the endogenous phosphorylation of the PCT and PST. The homogenates of the PCT and the PST were incubated for 3 min without exogenous protein substrate. Open circles: PCT, closed circle: PST. Each point represents the mean of four experiments, and standard deviations are shown as vertical bars. *Significantly decreased from the control (P<0.05, Student's t-test)
of endogenous phosphorylation of Gm, CAL, DCT and CCT returned to the basal level at cyclic AMP concentrations of more than \(10^{-5}\) M. This could possibly be due to either co-existence of phosphatases in the homogenate which could not be blocked completely by 10 mM KF (21), or due to the inhibitory effect of non-physiological cyclic AMP concentration (21).

**DISCUSSION**

Ausiello et al. (22) have reported that the endogenous protein phosphorylation of the cortical isolated tubules is increased by parathyroid hormone. However, parathyroid hormone has an effect not only on the PCT and the PST, but also on the CAL, the DCT and the CCT, since the parathyroid hormone-sensitive adenylate cyclase is present not only in the PCT and the PST, but also in the CAL, the DCT and the CCT (4). In addition to this, the PCT and the PST are more susceptible to mechanical stresses than the CAL, the DCT and the CCT (our unpublished observation). The proximal tubules might have been damaged more than the other tubules during the preparation of their isolated tubules.

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**Fig. 3.** Effect of cyclic AMP concentration on the endogenous phosphorylation of the CAL. The homogenate of the CAL was incubated for 3 min without exogenous protein substrate. Each point represents the mean of three experiments, and standard deviations are shown as vertical bars. *Significantly increased from the control (P<0.05, Student's t-test)

**Fig. 4.** Effect of cyclic AMP concentration on the endogenous phosphorylation of the DCT. The homogenate of the DCT was incubated for 3 min without exogenous protein substrate. Each point represents the mean of three experiments, and standard deviations are shown as vertical bars. *Significantly increased from the control (P<0.05, Student's t-test)

**Fig. 5.** Effect of cyclic AMP concentration on the endogenous phosphorylation of the CCT. The homogenate of the CCT was incubated for 3 min without exogenous protein substrate. Each point represents the mean of three experiments, and standard deviations are shown as vertical bars. *Significantly increased from the control (P<0.05, Student's t-test)
tubules. Therefore, their observation might not represent exactly the effect of parathyroid hormone on the proximal tubules.

It is already established that vasopressin and cyclic AMP enhance the water reabsorption of the CCT and that the parathyroid hormone and cyclic AMP decrease the reabsorption of inorganic phosphate of the proximal tubules. Our experiments on the endogenous protein phosphorylation of the cortical nephron segments showed a possible parallel relationship between the phosphorylation and the reabsorption. However, it is not known at present whether the decrease of endogenous phosphorylation of the PCT by cyclic AMP may represent the dephosphorylation of the regulatory subunit of the protein kinase or not (16, 17).

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