# Renal Adenylate Cyclase and the Interrelationship between Parathyroid Hormone and Vitamin D in the Regulation of Urinary Phosphate and Adenosine Cyclic 3′,5′-Monophosphate Excretion

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ABSTRACT This study examined the role of cyclic AMP in the phosphaturic response to parathyroid hormone in vitamin D-deficient rats. Infusion of purified bovine parathyroid hormone (13.3 μg/h) into control, D-fed, or D-deficient, thyroparathyroidectomized rats produced a sixfold increase in renal phosphate and cyclic AMP excretion in D-fed rats, but only a two- to threefold increase in both parameters in D-deficient animals. Intravenous injection of parathyroid hormone over the dosage range from 1-50 μg/kg resulted in a dose-dependent increase in phosphate and cyclic AMP excretion with both D-fed and D-deficient thyroparathyroidectomized rats. However, the D-deficient rats responded to these injections of parathyroid hormone with a two- to threefold increase in both renal phosphate and cyclic AMP excretion at the highest dose of 50 μg/kg, whereas the D-fed animals' response was 35-fold and 11-fold over control excretion levels of phosphate and cyclic AMP, respectively. To directly examine the role of the renal cortical adenylate cyclase system in the blunted phosphaturic and urinary cyclic AMP responses to parathyroid hormone in D-deficient rats, we prepared a plasma membrane fraction enriched in this enzyme activity from the renal cortex of D-fed and D-deficient thyroparathyroidectomized rats. The renal cortical adenylate cyclase of D-deficient rats showed significantly ($P < 0.001$) less activation by parathyroid hormone over the hormone concentration range from 0.3 to 7.0 μg/ml than was observed with the enzyme prepared from D-fed animals. Basal adenylate cyclase activity and the fluoride-stimulated enzyme activity were not altered by the state of D-deficiency. These experiments demonstrate that the blunted phosphaturic response to parathyroid hormone observed in D-deficient rats is associated with the reduced responsiveness of the renal cortical adenylate cyclase to the hormone. Moreover, the defect in the renal membrane adenylate cyclase system appears to be localized at the level of PTH binding to membrane receptors or, alternatively, at the level of transmission of the hormone-receptor binding signal to the catalytic moiety of this membrane enzyme.

## INTRODUCTION

A number of studies have demonstrated that the response to parathyroid hormone (PTH)[1] is impaired in vitamin D-deficient animals. In particular, the phosphaturic response to infused PTH (1-3) is markedly depressed in vitamin D-deficient rats, whereas the calcium mobilization response of bone may be severely impaired (4, 5) or absent (6, 7). Adenosine cyclic 3′,5′-monophosphate (cyclic AMP) has been shown to be involved as an intermediary in the expression of PTH action on kidney (8-11) and bone (12-14). It was of interest, therefore, to evaluate the role of the PTH-activated adenylate cyclase system of a target tissue, kidney, in the blunted response of this organ to PTH in vitamin D-deficient rats. Two parameters of PTH regulation of renal cell function were employed to

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[1] *Abbreviations used in this paper:* PTH, parathyroid hormone; TPTX, thyroparathyroidectomized.

evaluate whether or not the PTH-activated adenylate cyclase system of kidney cortex was defective in the vitamin D-deficient rat. These were the in vitro activation of rat kidney cortex adenylate cyclase by PTH and the in vivo increase in urinary cyclic AMP and phosphate excretion induced by PTH in thyroparathyroidectomized (TPTX) rats. We reasoned that correlation of the responsiveness of the renal adenylate cyclase to PTH in vitro and in vivo would provide information as to the participation of this component of the PTH-target cell regulatory system in the blunted response of the tissue to PTH in the vitamin D-deficient rat.

## METHODS

*Dietary procedure.* Male, 21-day-old weanling rats obtained from Holtzman Co., Madison, Wisc., were fed a vitamin D-deficient diet (0.47% calcium, 0.36% phosphorus, and 0.048% magnesium) and maintained in a room devoid of ultraviolet light (15). Control (D-fed) animals received the same diet and were pair-fed with the D-deficient rats, but also received 70 IU of vitamin $D_2$ (ergocalciferol, Winthrop Laboratories, New York) orally twice weekly. In some experiments a third set of rats was used; these animals were fed a vitamin D-deficient diet and received one oral dose of vitamin D (either 150 or 1,000 IU) 3 days before sacrifice. At the end of 3–5 wk the animals were surgically TPTX. Some of the rats were sacrificed by decapitation 3 h after TPTX and renal cortical plasma membranes were prepared for in vitro studies. The remaining TPTX rats were used for in vivo infusion experiments.

*Perfusion procedure.* A modification of the procedure developed by Cotlove (16) was used to infuse the conscious rat. With rats under ether anesthesia, surgical thyroparathyroidectomy was performed and a No. 240 polyethylene catheter was placed in the urinary bladder through an abdominal incision. The animals were transferred to stainless steel restraining cages and infused through a 25-gauge hypodermic needle inserted into a tail vein. A Harvard Apparatus infusion pump (Harvard Apparatus Co., Inc., Millis, Mass.) was used to infuse the animals with a solution containing 5 mM calcium chloride, 20 mM sodium chloride, 2.5 mM potassium chloride, and 0.22 M glucose at a rate of 4 ml/h. Urine samples were collected automatically at 0.5-h intervals through the course of the experiment with an LKB fraction collector (LKB Instruments, Inc., Rockville, Md.). The animals were infused for 16 h after surgery before measurements of urinary volume and determinations of electrolyte content were begun. Collections were then made during the control period of at least 5 h before PTH (purified PTH, 2,500 U/mg) was either added to the infusate or injected intravenously. Blood samples were obtained at the time of surgery and at the end of the infusion experiment by cardiac puncture. Urine and plasma calcium and magnesium were measured with a Perkin-Elmer atomic absorption spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Phosphate (17) and creatinine (18) were assayed with colorimetric assays adapted to the Technicon II autoanalyzer (Technicon Instruments Corp., Tarrytown, N. Y.).

*Cyclic AMP assay.* Cyclic AMP of urine was assayed with a competitive protein binding assay similar to that reported by Gilman (19). The cyclic AMP binding protein was prepared from bovine skeletal muscle by the method of Miyamoto et al. (20). This binding protein was purified through the ammonium sulfate step that corresponds to step three of that procedure. The assay contained (50 $\mu$l): 0.16 M sodium acetate, pH 4.0, 10 $\mu$g binding protein, 0.5 pmol cyclic [$^3$H]AMP, and 0.25–20 pmol cyclic AMP in the standard curve, or 5–20 $\mu$l urine for experimental assay points. The incubation was for 60 min at 0°C and bound cyclic [$^3$H]AMP was isolated after the addition to each reaction tube of 2 ml 0.1 M potassium phosphate, pH 6.0, followed by immediate filtration of this mixture on nitrocellulose filters (Millipore Corp., Bedford, Mass., 0.45 $\mu$m). The filters were then washed successively with 3 and 4 ml of potassium phosphate buffer. Radioactivity on the filter was determined in a Beckman LS-100 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) with Bray's (21) scintillation medium. Serial dilutions of urine samples assayed for cyclic AMP by this method provided displacement of bound cyclic [$^3$H]AMP proportional to the displacement observed with authentic cyclic AMP. This indicated that the urine samples did not contain materials that interfered with the binding of cyclic [$^3$H]AMP.

*Plasma membrane preparation.* A subcellular fraction enriched in plasma membranes was prepared from the cortex of each pair of kidneys for the individual animals in the experimental groups by the procedure of Fitzpatrick et al. (22). Membranes were suspended in a solution containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4, at a protein concentration of about 5 mg/ml. Membranes were then frozen and stored at −20°C. Previous experiments showed that the PTH-activated adenylate cyclase of these plasma membranes was stable after storage under these conditions up to 72 h (23). Therefore, we assayed for adenylate cyclase within 72 h after the initial freezing. Samples of plasma membranes were thawed only once just before addition to the reaction tubes. Membrane protein was assayed by the method of Lowry et al. (24), with bovine serum albumin as the reference standard.

*Adenylate cyclase assay.* The method for assay of adenylate cyclase was essentially that of White and Zenser (25), as previously described, in application to rat kidney plasma membrane preparations (26). The reaction mixture (75 $\mu$l) contained: 50 mM Tris-HCl, pH 7.5, 6.7 mM $MgCl_2$, 12 mM creatine phosphate, 1 mM cyclic AMP, 16 mM caffeine, 1.2 mM [$\alpha$-$^{32}$P]ATP ($3–5 \times 10^{12}$ cpm/mol), 266 $\mu$g/ml of bovine serum albumin, 13.3 U/ml creatine phosphokinase, and 50–100 $\mu$g of membrane protein. After incubation at 30°C for either 10 or 20 min, the reaction was terminated by the addition of 20 $\mu$l of 0.1 M EDTA containing about 5,000 cpm of cyclic [$^3$H]AMP (for calculation of recovery of cyclic [$^{32}$P]AMP) and then heated for 2 min at 100°C. 1 ml of 50 mM Tris-HCl, pH 7.6, was added to this mixture and the denatured membranes were removed by centrifugation. Neutral alumina columns were used to separate cyclic [$^{32}$P]AMP from other $^{32}$P-labeled nucleotides and $^{32}$Pi (25). Recovery of cyclic AMP was 70–80%. Under these conditions the formation of the product, cyclic [$^{32}$P]-AMP, was linear for up to 20 min in the presence or absence of either PTH or NaF. The reaction blank $^{32}$P in 15 separate adenylate cyclase assays was 0.015±0.002% (mean±SEM) of the total $^{32}$P in the reaction. This was equivalent to 14±1 pmol "cyclic AMP", subtracted from the experimental values obtained in each experiment.

*Sodium-potassium-dependent ATPase.* Assay of renal membrane $Na^+K^+$-dependent ATPase activity was performed by colorimetric determination of $P_i$ release from ATP (27). The reaction medium (1.0 ml) contained: 40 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 10 mM KCl, 5 mM $MgCl_2$, 4 mM

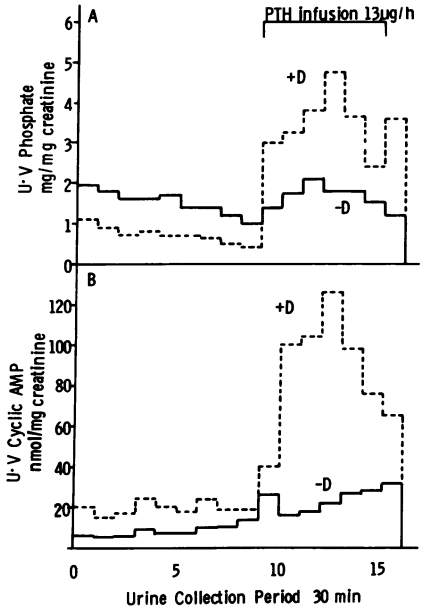


FIGURE 1 Effect of infusion of PTH on renal phosphate and cyclic AMP excretion in vitamin D-deficient and D-fed TPTX rats. PTH was infused intravenously as described in Methods at a rate of 13.3 μg/h after a control urine collection period. Panel A is urinary phosphate excretion for D-fed (----) and D-deficient (——) rats. Panel B is urinary cyclic AMP excretion for D-fed (----) and D-deficient (——) rats. The data are expressed as the means of three animals in each experimental group.

ATP, and 0.3–0.4 mg of membrane protein. Incubation was for 10 min at 30°C.

*Cyclic nucleotide phosphodiesterase assay.* Phosphodiesterase activity of kidney subcellular fractions was assayed (26) with cyclic [$^{32}$P]AMP as substrate. The incubation medium (75 μl) contained 40 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, either 1 μM or 1 mM cyclic [$^{32}$P]AMP ($1–2 \times 10^5$ cpm/μmol) and approximately 100 μg of membrane protein or other subcellular fraction. Incubation was for 5 min at 30°C. Termination of the reaction, separation of nucleotides, and determination of recovery of cyclic [$^{32}$P]AMP was essentially the same as described for the adenylate cyclase assay.

*Materials.* Cyclic [$^3$H]AMP and cyclic [$^{32}$P]AMP was purchased from New England Nuclear, Boston, Mass. [α-$^{32}$P]ATP was obtained from ICN Corp., Chemicals and Radioisotopes Div., Irvine, Calif. All other reagents and chemicals were purchased from various suppliers and were of the best grade available.

## RESULTS

After 3 wk of the D-deficient diet, the plasma calcium of the D-deficient rats was 4.96±0.12 mg/dl ($n = 35$) as compared to 9.76±0.18 ($n = 65$) for the D-fed rats. Hypocalcemia was the primary criterion that we used for the establishment of a D-deficient state, but body weight was also monitored.

*Infusion of PTH into D-deficient and D-fed rats.* Fig. 1 shows the effect of PTH (13.3 μg/h) on renal phosphate and cyclic AMP excretion in vitamin D-deficient and vitamin D-fed animals. It can be seen that both the phosphaturic and the urinary cyclic AMP responses to PTH were blunted in D-deficient rats. PTH infusion increased urinary cyclic AMP excretion by about sixfold in D-fed rats, as compared to a two- to threefold increase in D-deficient animals. The phosphaturic responses in the D-deficient and D-fed animals were similar in magnitude to the level of cyclic AMP excretion after PTH infusion. These experiments suggested that the formation of cyclic AMP by renal PTH-target cells was reduced in the D-deficient rat.

*Assay of PTH-dependent adenylate cyclase of renal plasma membranes.* To obtain a direct measurement of the kidney adenylate cyclase, we prepared a plasma membrane fraction (22) from kidney cortex previously shown to be enriched in PTH-dependent adenylate cyclase activity (26). The renal membrane adenylate cyclase was assayed for basal enzyme activity, PTH responsiveness, and fluoride activation. Fig. 2A shows the results of these experiments. The basal adenylate cyclase activity of all the experimental groups was the same, whereas the PTH-dependent adenylate cyclase of membranes from D-deficient rats showed significantly less ($P < 0.001$) response to PTH in vitro than did the adenylate cyclase of the D-fed rats, the rats fed an ad lib rat chow diet (Purina Rat Chow, Ralston Purina Co., St. Louis, Mo.), or the D-deficient animals that received a single dose of vitamin-D (150 or 1,000 U) 3 days before sacrifice. In addition, it was observed in these experiments that the fluoride-stimulated (10 mM NaF, data not shown) adenylate cyclase activity of the renal cortical membranes was the same in all the experimental groups. The right panel (B) of Fig. 2 shows that the plasma calcium level of the D-deficient animals was approximately one-half that of the D-fed or D-repleted rats. Body weight was not significantly altered by the D-deficient diet when animals were pair-fed. These experiments showed that the catalytic moiety of the renal adenylate cyclase was functional but suggested a defect in the response to PTH in vitro in renal membranes from D-deficient rats. Furthermore, the responsiveness of the adenylate cyclase to PTH could be restored by administration of a single oral dose of vitamin D (150 or 1,000 U) to D-deficient rats 3 days before preparation of the renal plasma membranes.

To investigate the role that TPTX may play in the decreased responsiveness of the renal adenylate cyclase to PTH in vitro, we assayed the enzyme prepared from intact D-fed and both intact and TPTX D-deficient rat kidney cortex. These data are shown in Fig. 3. It can be seen that the responsiveness of the adenylate cyclase to PTH was much reduced with membranes prepared from renal cortex of D-deficient rats, whether the animals were TPTX or intact. These experiments also confirmed

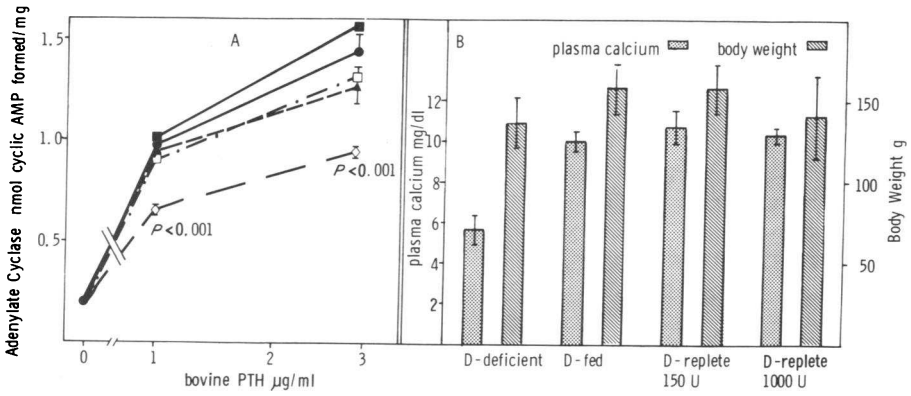


FIGURE 2 Renal plasma membrane adenylate cyclase activity, body weight, and plasma calcium levels of vitamin D-fed, D-deficient, ad lib rat chow diet and D-replete TPTX rats. Male weanling rats were placed on a D-deficient diet, as described in Methods. Some of the animals received vitamin D weekly (140 U/wk) (D-fed) and received the indicated dosage of vitamin D 3 days before sacrifice (D-replete). One group of animals were fed the standard rat chow diet on an ad lib basis. All animals were TPTX 3 h before sacrifice. The data are expressed as the mean±SEM of eight rats in the D-fed, regular rat chow (ad lib), and D-deficient groups, and four rats in the D-replete groups. Renal membrane adenylate cyclase was assayed in duplicate for each incubation condition. Panel A is the basal and PTH-activated adenylate cyclase activity of membranes prepared from the renal cortex of D-fed (pair-fed with D-deficient group (○——○), D-deficient (◇----◇), regular rat chow (ad lib) (■——■), D-replete (150 U) (▲----▲), and D-replete (1,000 U) (□-·-·-·-□). Panel B is the data from body weight and plasma calcium measurements for the experimental groups of rats (with the regular rat chow, ad lib group omitted).

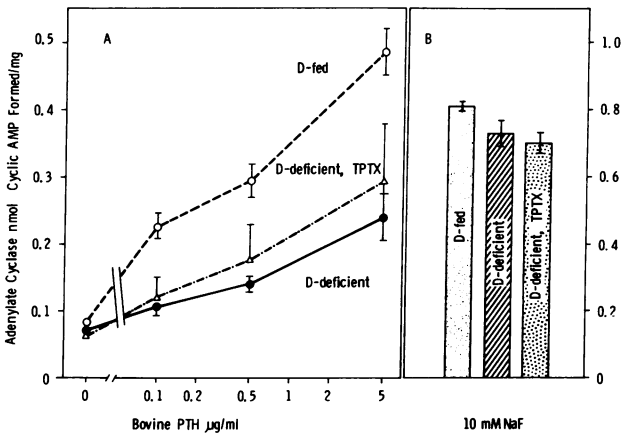


FIGURE 3 Activation of renal cortical adenylate cyclase by PTH and sodium fluoride in membranes from intact and TPTX rats. The animals were fed vitamin D-deficient diets as described in Methods. After 3 wk, part of the D-deficient rats were TPTX 3 h before sacrifice, whereas the remaining D-deficient rats and D-fed rats were killed with glands intact. Renal cortical adenylate cyclase of the membrane preparation for each animal was assayed in duplicate for each experimental point. The data are expressed as enzyme specific activity (per 10-min assay) and are mean±SEM of six animals in the intact groups and four rats in the TPTX group. Panel A is the PTH-dependent adenylate cyclase activity of membranes from D-fed intact (○----○), D-deficient intact (●——●), and D-deficient TPTX (△—·—△). Panel B is fluoride-stimulated adenylate cyclase activity of the same experimental groups.

the previous results (Fig. 2), which demonstrated that the basal and fluoride-stimulated adenylate cyclase activity was not significantly changed in D-deficient rats.

Since vasopressin has been shown to predominately activate the renal medulla plasma membrane adenylate cyclase, whereas the PTH-dependent adenylate cyclase is primarily localized in the renal cortex (10), it was of interest to examine the effect of vitamin-D deficiency on the medullary enzyme. The data from the assay of renal medulla adenylate cyclase are given in Table I. It can be seen that D deficiency did not reduce the responsiveness of the medullary adenylate cyclase to either vasopressin or sodium fluoride. In this experiment the adenylate cyclase activity of membranes from D-deficient rats was slightly higher than the control (D-fed) activity. These data suggest that the state of vitamin-D deficiency produces a specific action on the renal cortical PTH-dependent adenylate cyclase.

To examine further the effect of vitamin-D deficiency on the renal adenylate cyclase, we examined the PTH-dependent and fluoride-activated adenylate cyclase of kidney cortex homogenates. This was done so that total units of cortical adenylate cyclase could be determined. These data are shown in Table II. This experiment demonstrated that the total of PTH-dependent adenylate cyclase in renal cortical homogenates prepared from D-deficient rats is significantly less than found with D-fed animals. Moreover, the total fluoride-stimulated

TABLE I

*Activation of Renal Medulla Adenylate Cyclase by Vasopressin and Sodium Fluoride*

| Addition | Adenylate cyclase activity | |
|---|---|---|
| | D-fed | D-deficient |
| | *nmol cyclic AMP/mg* | |
| None | 0.15±0.01 | 0.14±0.03 |
| Vasopressin 0.7 U/ml | 0.82±0.01 | 0.93±0.01 |
| Vasopressin 7.0 U/ml | 0.99±0.09 | 1.14±0.09 |
| Vasopressin 13.0 U/ml | 1.05±0.01 | 1.24±0.02 |
| Sodium fluoride 10 mM | 1.41±0.14 | 1.87±0.15 |

Male, weanling rats were placed on the standard vitamin D-deficient diet for 3 wk. Half the animals were treated with vitamin D (see Methods) and pair-fed with the D-deficient group. The animals were TPTX and sacrificed 3 h later. Kidney medulla was dissected free of cortex and the medullary tissue (including papilla) from three rats was pooled. Plasma membranes from the pooled medulla were prepared and assayed for adenylate cyclase activity. The enzyme incubation contained 58 μg and 65 μg membrane protein for the D-fed and D-deficient preparations, respectively. Data are expressed as the mean±SD of duplicate assays for each pooled membrane preparation.

TABLE II

*Units and Specific Activity of Renal Cortical Homogenate Adenylate Cyclase from D-Fed and D-Deficient TPTX Rats*

| Addition to incubation | *n* | Adenylate cyclase | | *P* |
|---|---|---|---|---|
| | | D-fed | D-deficient | |
| | | *sp act* | *pmol/mg/10 min* | |
| None | 3 | 64±4 | 84±5 | <0.05 |
| PTH, 6.7 μg/ml | 3 | 317±12 | 244±4 | <0.01 |
| NaF, 10 mM | 3 | 488±29 | 517±38 | NS |
| | | *enzyme U/pair kidneys* | | |
| None | 3 | 11.1±0.9 | 14.7±1.5 | NS |
| PTH, 6.7 μg/ml | 3 | 55.7±3.7 | 43.5±4.6 | <0.01 |
| NaF, 10 mM | 3 | 85.1±3.6 | 90.3±6.6 | NS |

Male, weanling rats were placed on the vitamin D-deficient diet for 3 wk. Half of the animals received vitamin $D_2$ (140 U/wk, D-fed) orally and were pair-fed with D-deficient rats. At the end of this period, the rats were TPTX and then sacrificed 3 h later. Kidney cortex homogenates were prepared (22) and assayed for adenylate cyclase activity with the above additions to the incubation and also for total protein. The data are expressed as total units of adenylate cyclase in nanomoles cyclic [$^{32}$P]AMP formed per pair kidneys (corrected for differences in total homogenate protein, i.e. kidney weight) and are mean±SEM of three homogenates (three rats) for the experimental groups, each assayed in duplicate.

enzyme activity was not altered by D-deficiency, which suggests that the catalytic component of the adenylate cyclase is functionally intact in kidney cortex of D-deficient rats. In these experiments, the basal adenylate cyclase specific activity of homogenates prepared from the renal cortex of D-deficient rats was slightly greater than the enzyme activity from D-fed rats. The total units of cortical adenylate cyclase were not significantly different between the two experimental groups when the enzyme was assayed in the basal state.

The reduced urinary cyclic AMP response to PTH infusion in D-deficient rats and the apparent reduction in the response of the isolated renal adenylate cyclase to PTH could be explained by enhanced metabolism of cyclic AMP rather than by reduced formation of cyclic AMP under the above experimental conditions. To examine this possibility, we assayed the cyclic nucleotide phosphodiesterase activity of kidneys from D-deficient and D-fed animals. The data in Table III demonstrated that the renal cortical phosphodiesterase activity was not altered by the state of D-deficiency. This was evident at both 1 μM and 1 mM levels of substrate (cyclic AMP). We found no alteration of either the membrane-bound or soluble phosphodiesterase activities in these experiments.

Another possible explanation for the reduced response of the membrane adenylate cyclase to PTH in vitro is that D-deficiency might decrease the yield of plasma membranes in the subcellular fraction employed for the adenylate cyclase assay. To investigate this possibility, we assayed a separate plasma membrane marker enzyme, the $Na^+K^+$-dependent ATPase. As shown in Table III, the activity of this enzyme was essentially the same in membrane preparations from renal cortex of both D-deficient and D-fed rats. These data, plus the observation that both the basal and fluoride-stimulated adenylate cyclase activities of renal membrane preparations was not altered by D-deficiency, indicated that D-deficiency did not reduce the yield of plasma membranes in the subcellular fraction employed for assay of PTH-dependent adenylate cyclase.

*Examination of the PTH dose-response relationship in vitro and in vivo.* The dose-response relationship for PTH activation of renal adenylate cyclase in vivo was compared with the in vitro response to PTH. Both urinary cyclic AMP and phosphaturic responses were measured in D-deficient and D-fed rats by the infusion procedure previously described (see Methods). After a 12-h equilibration and initial 5-h control urine collection, PTH was administered intravenously (over 5-min) at doses of 1, 5, 10, and 50 μg PTH/kg body wt. The indicated time intervals between PTH injections in Fig. 4 were chosen to avoid overlap between the urinary cyclic AMP and phosphate responses. Results of these experiments are shown as representative renal excretion

TABLE III

*Activities of Cyclic Nucleotide Phosphodiesterase and Sodium-Potassium ATPase in Kidney Subcellular Fractions*

| Enzyme assay | Phosphodiesterase | |
|---|---|---|
| | D-fed | D-deficient |
| | *nmol/mg/min* | |
| Plasma membrane | | |
| Cyclic AMP 1 μM | 0.18±0.01 | 0.20±0.01 |
| Soluble cytoplasmic | | |
| Cyclic AMP 1 μM | 0.12±0.01 | 0.12±0.01 |
| Cyclic AMP 1 mM | 6.6±0.4 | 6.3±0.20 |
| Sodium-potassium ATPase | 0.46±0.02 | 0.47±0.04 |

Renal cortical plasma membrane and post-membrane supernate (600 *g* fraction) preparations were isolated for assay of cyclic nucleotide phosphodiesterase and Na-K-dependent ATPase activities (for details see Methods). Eight rats in each of two groups, D-fed and D-deficient, were TPTX 3 h before sacrifice and these subcellular fractions were isolated from renal cortical homogenates. The data are expressed as enzyme specific activity with substrate concentrations of 1 μM and 1 mM cyclic [$^{32}$P]AMP for the phosphodiesterase and 4 mM ATP for the ATPase. Both membranes and the post-membrane supernate were assayed for phosphodiesterase activity since this enzyme is found in both particulate and soluble forms. The data are the mean±SEM of eight experiments, each assayed in duplicate.

experiments in Fig. 4, and the cumulative data for all the animals in the D-deficient and D-fed groups are expressed as PTH dose-response curves for both the phosphaturic and cyclic AMP responses in Fig. 5. Over the PTH dosage range of 1-50 μg/kg, we found that both the D-fed and D-deficient rats demonstrated a dose-dependent increase in urine phosphate and cyclic AMP excretion. However, the D-deficient animals exhibited a marked reduction in their responsiveness to PTH for both phosphate and cyclic AMP parameters when compared to the D-fed rats' responses. At the highest PTH dosage employed (50 μg/kg), the D-fed animals responded with 11-fold and 35-fold increases over the control levels in urine cyclic AMP and phosphate excretion (Fig. 5). These data further demonstrated that the D-deficient rat exhibits both a blunted phosphaturic and urine cyclic AMP response to PTH in vivo.

The renal excretion of creatinine was measured in the experiments shown in Figs. 4 and 5 and the urinary excretion levels for phosphate and cyclic AMP were adjusted for the quantity of creatinine excreted during each 30-min collection period. It is unlikely that variations in glomerular filtration rate produced these marked differences in renal phosphate and cyclic AMP excretion between the experimental groups, since both the excretion of creatinine and urinary volume did not exhibit marked variance during the infusion period. The coefficient of variance for D-fed rats was (mean±SEM) 0.26±0.03 for urine volume per period and 0.17±0.02 for creatinine, and for D-deficient rats, 0.22±0.03 for urine volume per period and 0.16±0.02 for creatinine. Previous studies have demonstrated that under similar experimental conditions, the urinary excretion of inulin was not altered by the intravenous administration of purified PTH (1). Moreover, the results of the in vitro studies that demonstrate a blunted response of the adenylate cyclase to PTH in D-deficient rat kidney are consistent with the results of the in vivo renal excretion experiments.

The animals from the same experimental groups used in the above in vivo PTH dose-response experiments (Figs. 4 and 5) were first TPTX and then sacrificed 3 h later to prepare the renal cortical plasma membrane subcellular fraction for adenylate cyclase assay. Fig. 6 shows the dose-response relationship for the in vitro stimulation of cyclic AMP formation by PTH (panel A) and also for maximal activation of the enzyme by 10 mM NaF (panel B). These experiments show a decrease in hormonal responsiveness of the renal adenylate cyclase in membrane preparations from D-deficient rats when compared to the response observed with membranes from the renal cortex of D-fed rats. However, the difference in response between these groups in vitro is about 50%, whereas the difference seen in the in vivo experiments was about eightfold. The PTH dose-response relationship in vitro shows a divergence in responsiveness of the renal adenylate cyclase at the higher levels of PTH. The curve for cyclic AMP formation with membranes from D-deficient rats reaches maximum at a PTH concentration considerably less than that for the D-fed group. This quantitative difference in the PTH dose-response curves was also seen in vivo. As previously shown (Figs. 2 and 3), the basal enzyme activity and fluoride-activated adenylate cyclase activity was the same in kidney membranes from both D-deficient and D-fed rats. These experiments demonstrate that the formation of cyclic AMP by kidney adenylate cyclase as measured by direct assay in vitro and indirectly through urine excretion of cyclic AMP in vivo is markedly depressed in the D-deficient rat. Furthermore, the blunted phosphaturic response to PTH in vivo in D-deficient rats is well correlated with the defective renal adenylate cyclase.

## DISCUSSION

This study confirms the previous reports that the phosphaturic response to PTH is blunted in the D-deficient rat (1-3). In addition, our experiments showed that the

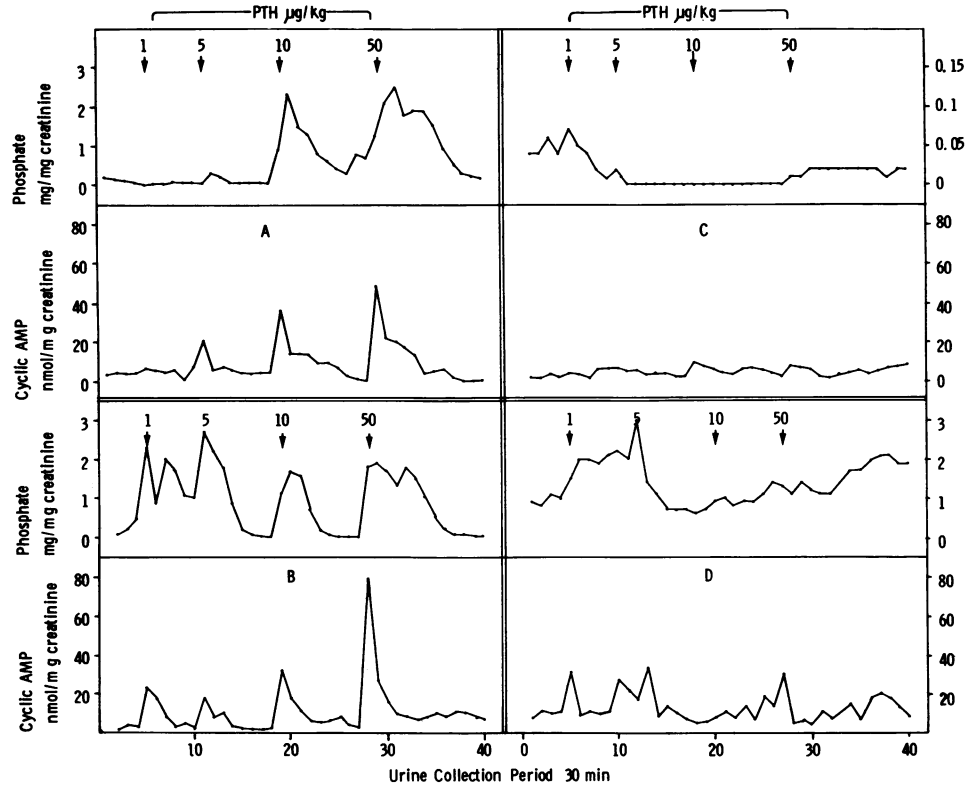


FIGURE 4 Effect of various doses of PTH on renal phosphate and cyclic AMP excretion in vitamin D-deficient and D-fed TPTX rats. The renal excretion format is described in detail in Methods. PTH was injected intravenously at the indicated periods and dosages depicted in the figure. Panels A and B are representative experiments with D-fed TPTX rats and panels C and D are representative experiments with D-deficient TPTX rats. The arrows indicate the first 30-min collection period immediately after the administration of the dose of PTH.

reduced phosphaturic response to PTH was associated with a defect in the renal adenylate cyclase system.

The present study tends to rule out the acute participation of thyrocalcitonin in the blunted phosphaturic and cyclic AMP responses to PTH observed in the D-deficient rat, since we TPTX all of the animals before both in vivo and in vitro experiments. In prior studies (1) the blunted phosphaturic responses of vitamin D-deficient rats were similar whether the animals were parathyroidectomized or TPTX. Our present data indicates that removal of the parathyroid glands is not necessary for demonstration of the decrease in response of the adenylate cyclase to PTH with renal cortical membranes prepared from vitamin D-deficient rats. Previous studies demonstrated that the blunted phosphaturic response to PTH in vivo was enhanced if the rats were not parathyroidectomized before the infusion of PTH into D-deficient rats (1). This was presumably due to high circulating levels of PTH in the hypocalcemic, D-deficient animals.

Examination of the action of PTH on formation of cyclic AMP both directly by assay of the renal cortical adenylate cyclase in vitro and indirectly through analysis of urine cyclic AMP excretion in vivo effectively demonstrated that the renal PTH-dependent adenylate cyclase system is impaired in the D-deficient rat. Furthermore, since the basal and fluoride-stimulated adenylate cyclase activity of renal cortical membranes was unchanged in D deficiency, it may be reasoned that the underlying cellular defect is at the PTH-receptor level. This suggests that either the PTH-receptor interaction may be defective or, alternatively, the transmission of the hormone-receptor interaction signal to the catalytic moiety of the adenylate cyclase system may be impaired. Since the renal cyclic nucleotide phosphodiesterase activity is not altered by D-deficiency, it is unlikely that this enzyme plays a significant role in influencing the apparent level of formation of cyclic AMP either in vivo or in vitro. Our experiments are at variance with those of Nagata and Rasmussen (9), who found that

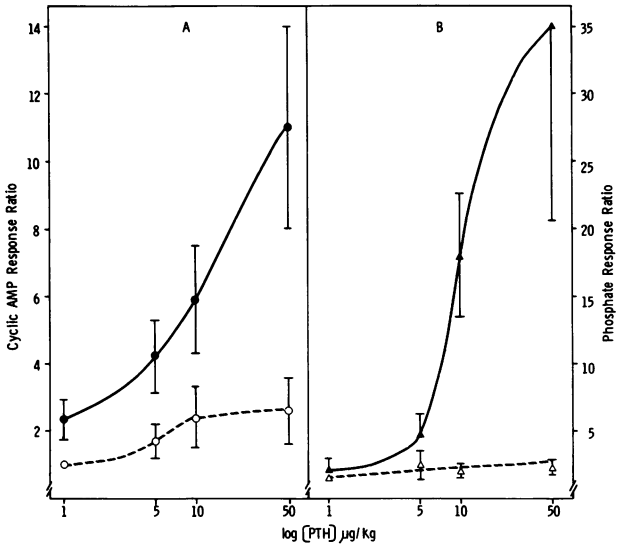


FIGURE 5 PTH dose-response relationship for renal phosphate and cyclic AMP excretion in vitamin D-fed and D-deficient TPTX rats. The data obtained from the renal excretion experiments shown in Fig. 4 for eight D-fed and six D-deficient rats was converted to a dose-response format. The urinary cyclic AMP and phosphate response ratios are the ratios of the mean responses after PTH injection to mean control values immediately before the PTH injection. Three control periods were utilized for the base-line mean value and the mean PTH responses value was calculated from the cyclic AMP or phosphate response values above base line. Data are expressed as mean ±SEM for the urinary cyclic AMP response to PTH (panel A) of D-fed (●——●) and D-deficient (○- - - -○) groups and phosphaturic response to PTH (panel B) of D-fed (▲——▲) and D-deficient (△- - - -△) groups. Plasma phosphate levels for these groups (mean±SEM) were: D-fed, 12.0±0.5 mg/dl; and D-deficient, 11.7±1.0 mg/dl.

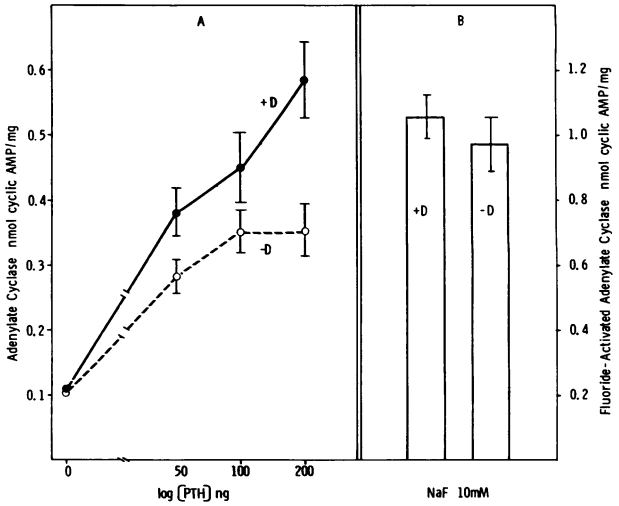


FIGURE 6 Activation of renal cortical adenylate cyclase by parathyroid hormone and sodium fluoride. Male weanling rats were fed a vitamin D-deficient diet for 3 wk, as described in Methods. Half of the animals were given 140 U vitamin D/wk (D-fed) and were pair-fed with D-deficient rats. These animals were TPTX 3 h before sacrifice. Renal cortical membranes were prepared from the kidneys of each rat and then assayed for adenylate cyclase activity under the conditions shown in the figure. Panel A shows the in vitro activation of adenylate cyclase by PTH, and panel B the activation by fluoride. The PTH dose-response curve shown as D-fed (+D) (●——●) and D-deficient (−D) (○- - - -○). The data are expressed as the mean±SEM of eight animals (i.e., eight membrane preparations) in each experimental group. Each experimental point was obtained by duplicate adenylate cyclase assays. Incubation was for 10 min at 30°C as described in Methods. The plasma calcium levels of these rats at the time of sacrifice were (mean±SEM): D-fed, 9.09±0.26 mg/dl; and D-deficient, 4.56±0.13 mg/dl, whereas plasma phosphate levels were: D-fed, 12±1 mg/dl, and D-deficient, 14.1±0.5 mg/dl. The concentration of PTH is ng/75 μl incubation volume.

PTH infusion into D-deficient, TPTX rats resulted in an increase in whole kidney cyclic AMP levels of the same magnitude as found with D-fed animals. We presently have no explanation for this discrepancy.

Previous studies have shown that the phosphaturic action of PTH in TPTX rats can be produced through infusion of either dibutyryl cyclic AMP (2, 28-30) or theophylline (28). In addition, it has been reported that the phosphaturic response to both PTH and dibutyryl cyclic AMP is blunted in the D-deficient rat (2). One interpretation of these results is that the cellular defect in D-deficiency is at a site beyond the renal adenylate cyclase step. However, it has recently been shown that dibutyryl cyclic AMP is relatively ineffective as a cyclic AMP agonist with respect to the capacity of dibutyryl cyclic AMP to activate a renal cortical cyclic AMP-dependent protein kinase (31). This lack of efficacy of dibutyryl cyclic AMP as a cyclic AMP agonist has also been demonstrated with cyclic AMP-dependent protein kinase systems of other tissues (32, 33). These experiments suggest that dibutyryl cyclic AMP may be acting in vivo through an alternate mechanism to produce a phosphaturic response. It has been proposed that dibutyryl cyclic AMP mimics the PTH action on bone through an inhibitory action of the nucleotide on the bone cyclic nucleotide phosphodiesterase enzyme (34). We have found that dibutyryl cyclic AMP is as effective an inhibitor of the renal cortical phosphodiesterase in vitro as theophylline (data not reported). If dibutyryl cyclic AMP produces its phosphaturic action through a theophylline-like mechanism, then it is conceivable that the underlying cellular defect in D-deficient rats is manifest predominately at the level of the cyclic AMP-forming enzyme, the membrane adenylate cyclase. Even though we found no change from control in the basal adenylate cyclase activity of renal cortical membranes prepared from D-deficient TPTX rats, it is conceivable

that the formation of cyclic AMP by PTH-target cells in vivo may be impaired in the TPTX, D-deficient rat. Under such a condition, the pharmacologic action of either dibutyryl cyclic AMP or theophylline on renal phosphate excretion would depend on the ability of the cell to generate cyclic AMP. An alternate explanation for these experimental observations is that the renal cortical PTH-dependent adenylate cyclase and other regulatory components, such as the renal cyclic AMP-dependent protein kinase system, may be impaired in the D-deficient rat. This dual defect in the D-deficient animal would be more strongly supported if it could be shown that renal cortical cells can convert dibutyryl cyclic AMP to *N*-6-monobutyryl cyclic AMP, an effective activator of renal cortical cyclic AMP-dependent protein kinase (31). Therefore, it is of extreme importance to determine the actual cellular mechanism of action of dibutyryl cyclic AMP in vivo before adequate interpretation of previous experiments with this cyclic nucleotide can be accomplished.

Since our vitamin-D deficient diet resulted in a significant reduction in plasma calcium after 2 wk and a marked (50%) reduction at the end of the routine 3-wk experimental period, it is conceivable that chronic hypocalcemia rather than D-deficiency per se mediates the reduced responsiveness of the renal adenylate cyclase to PTH. This degree of hypocalcemia would stimulate the secretion of PTH from the parathyroid gland and probably induce a state of hyperparathyroidism in the D-deficient animals. The chronic high level of plasma PTH may decrease the number of PTH receptors associated with the plasma membrane of target cells and therefore less end-organ response would be observed both in vivo and in vitro with respect to renal cyclic AMP formation. Such a situation has been described in vivo in states of hyperinsulinemia and target cell resistance to insulin in the obese hyperglycemic mouse (35). In this experimental animal, the number of insulin receptors associated with membranes of target tissues, such as the hepatocyte and adipocyte, have been shown to decrease in proportion to the elevated plasma insulin levels. In vitro experiments with cultured human lymphocytes have also shown that the quantity of insulin receptors per cell decreased after exposure of the cells to $1 \times 10^{-8}$ M insulin for longer than 2 h (36). Alteration in renal PTH receptor populations in response to hypocalcemia and the resultant secondary hyperparathyroidism may explain the experimental observation of Arnaud et al. (1) and Au and Raiz (5), who found that maintenance of relatively normal plasma calcium levels would prevent the blunted end-organ response to PTH observed in those studies with the vitamin D-deficient rat. This would suggest that prevention of the blunted target-tissue response to PTH may be due to a relatively normal level of circulating PTH in the face of normocalcemia and vitamin D-deficiency, resulting in no alteration of renal PTH receptors. This interesting speculation is at present difficult to analyze experimentally since the methodology both for measuring rat immunoreactive PTH and assaying the binding of a biologically active PTH to cell receptors has not been adequately developed at the present time.

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