Direct Inhibitory Effect of Hypercalcemia

on Renal Actions of Parathyroid Hormone

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A B S T R A C T   The effects of calcium on the renal actions of parathyroid hormone (PTH) were studied in vivo and in vitro. In parathyroidectomized rats, variable levels of blood calcium concentration were induced by intravenous infusion of calcium. The renal responses to the injected PTH, i.e. phosphate and cyclic AMP excretion, were compared in these animals. After PTH injection, the increases of both phosphate and cyclic AMP excretion were less in the calcium-infused animals than in the control group without calcium infusion. There was an inverse correlation between the renal responses to PTH and plasma calcium concentration of 4.2-13.5 mg/100 ml. But calcium had no effect on phosphate excretion induced by infusion of dibutyryl cyclic AMP. In the in vitro experiments, the increase of cyclic AMP concentration in response to PTH was less in renal cortical slices taken from the calcium-infused animals than in ones from the control group without calcium infusion. Calcium also inhibited the activation of renal cortical adenylate cyclase in response to PTH, but calcium had no effect on phosphodiesterase. The data indicate that calcium directly inhibits renal actions of PTH both in vivo and in vitro. Such inhibitory mechanism is probably at or before the step of PTH-dependent cyclic AMP generation in the kidney.

INTRODUCTION

Parathyroid hormone (PTH)[1] plays a major role in the regulation of the concentrations of calcium and phosphate in plasma. This appears to be accomplished primarily by a reciprocal relationship between the concentration of ionized calcium in plasma and the rate of secretion of PTH (1). The biological actions of PTH on kidney and bone seem to be mediated through cyclic AMP systems (2-7). Recent evidence suggests that in

addition to this effect on PTH secretion, calcium might influence the peripheral action of that hormone. This is suggested by the observation that PTH-induced increases in the activity of adenylate cyclase in bone and kidney are sensitive to the concentration of calicum in the incubation media. A smaller response to PTH was measured with increasing calcium concentration (5, 8). A possible effect of calcium on certain renal actions of PTH were therefore investigated in this series of experiments. Calcium was found to inhibit PTH-induced increases in excretion of both phosphate and cyclic AMP in vivo. Tissue slices obtained from the renal cortex of hypercalcemic animals were less responsive to PTH in terms of increasing tissue cyclic AMP concentration than were slices from hypocalcemic animal. In addition, calcium interfered with PTH activation of adenylate cyclase but not of phophodiesterase. The results of the studies indicate that calcium inhibits the renal action of PTH and that the mechanism of that inhibition is probably at the PTH-dependent cyclic AMP generation system in the kidney.

METHODS

In vivo studies. Male Sprague-Dawley rats weighing 200-250 g were used for the study. Parathyroid glands were removed surgically under a dissecting microscope to eliminate the effect of calcium on secretion of the endogenous PTH. The thyroid glands were left intact. After surgery, 4 days were allowed for the animals to stabilize. During that time, 1 g/100 ml calcium chloride was added to the drinking water. On the day of study, 10 mg of Nembutal (Abbott Laboratories, North Chicago, Ill.) in 12-15 ml of 75 mM NaCl, warmed to 37°C, was injected intraperitoneally for the purpose of an initial hydration and anesthesia. Catheters were inserted into a femoral vein for infusion of study substances and withdrawal of blood samples. Another catheter was inserted into the urinary bladder through a suprapublic incision. After the surgery, each rat received a solution of calcium gluconate in various doses from 0.1 to 0.36 mmol, and 0.1 μCi of [14C]inulin in 3 ml of 75 mM NaCl intravenously in 30 min. The control group received the same amount of NaCl and [14C]-inulin without calcium gluconate. After calcium infusion,
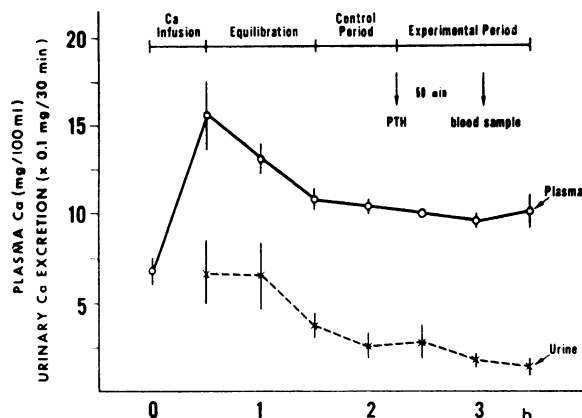
FIGURE 1 Change of plasma calcium concentration and urinary calcium excretion rate after 0.24 mmol of i.v. calcium gluconate infusion. Each point is mean and standard error of five animals, with duplicate determination for each specimen. The changes of plasma calcium were similar in the animals with 0.10 or 0.36 mmol of calcium infusion. Time table on the top is equivalent to the one in the subsequent experiments.

i.v. infusion was changed to a solution of 75 mM NaCl, 2.5 g glucose/100 ml, and 3.3 μCi [^{14}C]inulin/100 ml at the rate of 0.1 ml/min. This i.v. infusion was maintained at constant rate until the end of experiments. After calcium infusion, a 60 min period was allowed for the animals to equilibrate the infused calcium.

After three 20-min urine collections were obtained for control observations, 10 U (USP) of bovine PTH diluted in 0.25 ml of normal saline was injected intravenously. Six additional 20-min urines were collected after injection of PTH. Blood samples were obtained 50 min after PTH injection. Plasma calcium concentration at this period was chosen to represent each subject, because plasma calcium concentration was stable, and the maximal phosphaturic response to PTH was observed at this period (Figs. 1 and 5).

In another series of experiments, both control and hypercalcemic animals were prepared in the same manner as the above studies. In that series, dibutyryl cyclic AMP was infused at the rate of 50 μg/min instead of PTH.

Variable concentrations of plasma calcium were obtained by infusing 0.1–0.36 mmol of calcium gluconate per rat. The animals with plasma calcium concentration over 14.5 mg/100 ml in the specimens obtained 50 min after PTH injection were excluded because of questionable physiological significance of such a high plasma calcium concentration.

To evaluate the temporal changes of plasma calcium concentration and urinary calcium excretion, a separate series of animals was prepared in the same way, with an infusion 0.1–0.36 mmol of calcium gluconate to each animal. After calcium infusion, multiple blood and urine samples were obtained every 30 min for 3½ h (Fig. 1).

Cyclic AMP concentration in renal cortical tissue. The animals were prepared like those in the in vivo experiments except that one kidney was removed before the calcium infusion and served as a control. Then 0.36 mmol of calcium was infused intravenously, and 60 min were allowed for equilibration, after which the remaining kidney was removed.

Immediately after the removal, the cortex was separated from the medulla, and sliced to a thickness of less than 0.5 mm with a Stadie-Riggs microtome. The slices, weighing 60–70 mg, were then incubated at 37°C for 15 min in a Krebs-Ringer bicarbonate buffer, containing 10 mM theophylline, 1 g bovine albumin/100 ml, and 0.84 mM ionized calcium. 5 U (USP) PTH/ml of media was added to the PTH group. After a 15-min incubation, the slices were quickly transferred to a glass tissue-grinder containing 0.5 ml of glass distilled water. After grinding of tissue slices, the homogenate was boiled in water bath for 3 min to terminate the reaction. The procedures of transfering the tissue slices, grinding, and placing of specimens in a boiling water-bath took less than 40 s. The boiled homogenates were then centrifuged at 700g for 20 min, and cyclic AMP in the supernate was measured by Gilman's method (9) with modification as described previously (10).

Adenylate cyclase activity in the renal cortex. Adenylate cyclase enzyme was prepared as described by Marcus and Aurbach (11). Sprague-Dawley rats were sacrificed by decapitation, and their kidneys were removed rapidly. The renal cortex was separated and homogenized in 0.05 M Tris-HCl, pH 7.4, 4°C, in the proportion of 20 ml Tris/g wet tissue. The homogenate was filtered through six layers of surgical gauze, and the filtrate was centrifuged at 2,000 g for 20 min. The precipitate was resuspended in 0.05 M Tris-HCl, pH 7.4. The protein concentration of the final suspension was 10.4 mg protein/ml. This mixture is hence referred to as adenylate cyclase enzyme. The enzyme activity was studied on the same day as the enzyme preparation. The optimal amount of adenylate cyclase enzyme was 0.3 ml in the preliminary determination. This amount of enzyme was mixed with 0.3 ml of 0.5 M Tris-HCl, pH 7.4, 2 mM ATP for substrate, 20 mM theophylline, 25 mM KCl, 1.65 mM MgCl$_2$, and 2 g bovine albumin/100 ml, and ionized calcium and PTH as indicated in the results. After a 15-min incubation at 37°C in a metabolic shaker, the reaction was terminated by boiling the specimens in a water bath for 3 min, and centrifuged at 700g for 20 min. Cyclic AMP concentration in the supernate was then measured by Gilman's method (9) with modification (10). Adenylate cyclase activity was linear up to 25 min, and ATP 2 mM was not a rate-limiting factor without an ATP regeneration system.

Boiled adenylate cyclase enzyme served as a blank. This blank value, which represents the cyclic AMP pre-existing in the enzyme preparation, was subtracted from each experimental value. Adenylate cyclase activity was expressed as picomoles of cyclic AMP formation per milligram protein of adenylate cyclase enzyme per minute.

Cyclic AMP-phosphodiesterase activity. Phosphodiesterase of rat renal cortex was prepared as described by Cheung (12), by homogenizing the cortex in distilled water and centrifuging at 30,000g. The proper amount of phosphodiesterase enzyme that hydrolyzes 30–40% of substrate (1 μmol of cyclic AMP) in 15 min was determined in preliminary assay. This amount of enzyme was then mixed with 0.05 M Tris-HCl, pH 7.4, 25 mM KCl, 1.65 mM MgCl$_2$, 1 μmol of cyclic AMP per tube, a tracer amount of [^{3}H]cyclic AMP, and the study substances (calcium and PTH), as indicated in the results. This mixture was incubated at 37°C for 15 min in the metabolic shaker. The reaction was terminated by boiling for 3 min.

Phosphodiesterase activity was evaluated by measuring the remaining cyclic AMP in the mixture after a 15-min incubation. Cyclic AMP in the mixture was extracted with ZnSO$_4$ and BaOH, and AG 50W-X4 cation exchange resin

chromatograph, as described by Krishna, Weiss, and Brodie (13), vide infra. After extraction, [³H]radioactivity in the cyclic AMP fraction of the chromatograph was counted in a beta liquid scintillation spectrometer. Phosphodiesterase activity was then expressed as nanomoles of cyclic AMP hydrolyzed per milligram protein of phosphodiesterase enzyme per minute. The loss of cyclic AMP by the extraction procedure itself was corrected by using [¹⁴C]cyclic AMP as an internal standard.

Cyclic AMP assay method. Cyclic AMP binding protein was prepared from bovine myocardium as described by Miyamoto, Kuo, and Greengard (14), up to the steps of ammonium sulfate precipitation and DEAE cellulose chromatography (9). Protein kinase inhibitor was prepared with skeletal muscle as Appleman, Birnbaumer, and Torres described (9, 15).

The proper amount of cyclic AMP-binding protein and protein kinase inhibitor for cyclic AMP assay was determined by titration of each batch of binding protein preparation. 20–25% binding affinity of [³H]cyclic AMP was optimal for the assay. The proper amount of binding protein and protein kinase inhibitor in 20 mM potassium phosphate buffer, pH 4.0, was mixed with a constant known amount of [³H]cyclic AMP (5,000 cpm/tube) and the study specimens. After 60 min incubation at 0°C, the protein-bound cyclic AMP was separated by adsorbing unbound cyclic AMP to dextran-albumin–coated charcoal, and centrifuging at 700g for 20 min at 0°C. The supernate, containing the protein-bound cyclic AMP, was separated and counted in a beta liquid scintillation spectrometer.

Dextran-albumin–coated charcoal was prepared by mixing 1.8 g dextran (mol wt 7,500), 1.5 g bovine albumin, and 2.5 g neutral activated charcoal in 100 ml of 20 mM potassium phosphate buffer, pH 7.0. The solution was thoroughly mixed with a magnetic stirrer for more than 2 h at 0°C before use. Dextran-albumin–coated charcoal was always prepared on the day of experiment. The method using dextran-albumin–coated charcoal was compared with Gilman's original method, using Millipore filter paper (9). The charcoal method yielded better reproduction in both inter- and intra-serial determination than Millipore filter paper.

To evaluate validity of cyclic AMP assay method, rat renal cortex was homogenized, boiled, and centrifuged at 700g for 20 min. The supernate was divided into three groups. One group was incubated with cyclic AMP-phosphodiesterase at 37°C for 15 min. Another group of specimens was treated in the same way except the phosphodiesterase was boiled before the incubation. A third group was incubated like the previous two but without phosphodiesterase. The specimens containing 25 pmol of cyclic AMP/tube instead of renal cortex were also divided into three groups, and treated in an identical way with either active or boiled phosphodiesterase or no phosphodiesterase. Cyclic AMP in each specimen was then measured by Gilman's method (9) with modification (10). As shown in Fig. 2, the measured cyclic AMP concentrations were not different between the groups with boiled phosphodiesterase and the ones without phosphodiesterase. But the group of specimens treated with active phosphodiesterase showed no measurable cyclic AMP content by Gilman's assay method.

To evaluate the effect of cyclic AMP extraction procedures, both urine and renal cortical tissues specimens were divided into two groups. One group of specimens was extracted as described by Aurbach and Houston (16) and others (13, 17). To 0.5 ml of urine or tissue specimens, 0.2 ml each of 5% ZnSO₄ and 0.3 N BaOH were added and
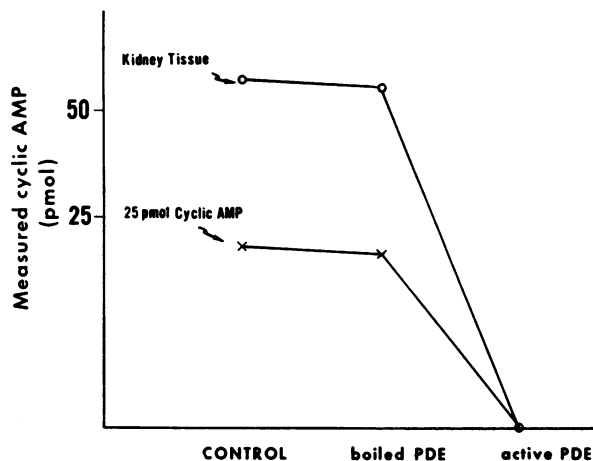
FIGURE 2 Specificity of cyclic AMP assay method. Control = untreated specimens were assayed for cyclic AMP with Gilman's method. Boiled PDE = specimens were treated with phosphodiesterase that had been inactivated by boiling, and then assayed for cyclic AMP. PDE = specimens were treated with active phosphodiesterase for 15 min at 37°C. Then cyclic AMP in the specimens were assayed. Each point represents mean of six determinations.

centrifuged at 700g for 20 min. ZnSO₄-BaOH precipitation was repeated twice. Then the supernate was chromatographed through a 0.3 × 7.0-cm column of AG 50W-X4 cation exchange resin, 200–400 mesh, which had been rinsed throughly with glass distilled water. The column was eluted with distilled water, and the eluate of 2.5–5.5 ml fraction was collected. In the preliminary study, cyclic AMP was found in this fraction. Samples were lyophilized overnight, and resuspended in 0.05 M Tris-HCl, pH 7.4. The loss of cyclic AMP during extraction procedures was measured by calculating the recovery rate of [¹⁴C]cyclic AMP that was added before the extraction. In a separate series of experiments, [¹⁴C]ADP or [³H]5' AMP, instead of [³H]cyclic AMP was added and treated in an identical manner. The removal of [¹⁴C]ATP, [¹⁴C]ADP or [³H]5' AMP with this procedure was more than 99.99%. These values are similar to the results of other investigators (16, 17). Both the extracted specimens and the ones without extraction were assayed for cyclic AMP by Gilman's method (9) with modification (10). The final results of cyclic AMP with and without extraction procedures were similar 1.50 ± 0.09 nmol cyclic AMP/g of tissue with extraction vs. 1.61 ± 0.09 without extraction; and 1.2 ± 0.04 nmol cyclic AMP/ml of urine vs. 1.11 ± 0.02, $P > 0.05$. Therefore, the specimens in the main series of experiments were assayed for cyclic AMP without extraction.

To evaluate the effect of high calcium concentration on cyclic AMP assay method itself, specimens were divided into three groups: 0, 1, and 20 mM calcium chloride. Each group was composed of two sets of tubes: one containing no cyclic AMP, and the other set of tubes containing 25 pmol of cyclic AMP. Then the cyclic AMP in each specimen was assayed by Gilman's method (9) with modification (10). As shown in Fig. 3, neither 1 mM calcium nor 20 mM calcium had any measurable effect on the cyclic AMP assay method.

PTH preparation. Synthetic amino-terminal 1–34 PTH, Lot 15012, claimed potency of 3,000 USP U/mg, was ob-

tained from Beckman Instrument, Inc. (Palo Alto, Calif.). The biological potency of the synthetic PTH was rechecked by measuring its power to activate adenylate cyclase of rat renal cortex as described by Marcus and Aurbach (11), and compared to bovine parathyroid extract of Eli Lilly & Co. (Indianapolis, Ind.), batch 6TN67A, 100 USP U/ml. Their potencies were close: 38 pmol of cyclic AMP formation/mg protein per min by synthetic 1-34 PTH equivalent to 1 USP U, and 41 pmol by 1 USP U of bovine parathyroid extract of Eli Lilly Co. To further ensure uniformity, the same batch of each PTH preparation was used for entire series of experiments.

To test possible nonspecific factor(s) in the PTH preparation on either cyclic AMP or its assay method, PTH was inactivated by oxidation with chloramine T and then reduction with sodium metabisulfite, or by adsorption to Quso 32 (Philadelphia Quartz Co., Philadelphia, Pa.), and removal by centrifugation. Both the biologically active PTH and the inactive hormone preparation were tested on adenylate cyclase of rat renal cortex as described above. The control value of 5.9 ± 0.8 pmol cyclic AMP formation/mg protein per minute was increased to 34.5 ± 1.5 by 10 USP U untreated PTH/ml. But the same amounts of either oxidized or Quso 32-treated PTH did not activate adenylate cyclase, and the values were not different from the control value: 5.7 ± 0.06 and 5.3 ± 0.7, respectively. This result indicates that PTH preparation used in this experiment did not show any nonspecific effects on either cyclic AMP study or its assay method. A dose-response study of PTH on renal cortical adenylate cyclase showed that 5 U/ml was a submaximal dose: $367 \pm 32\%$ increase by 5 U and $420 \pm 48\%$ by 10 U of PTH as compared to the basal activity without PTH. These results are similar
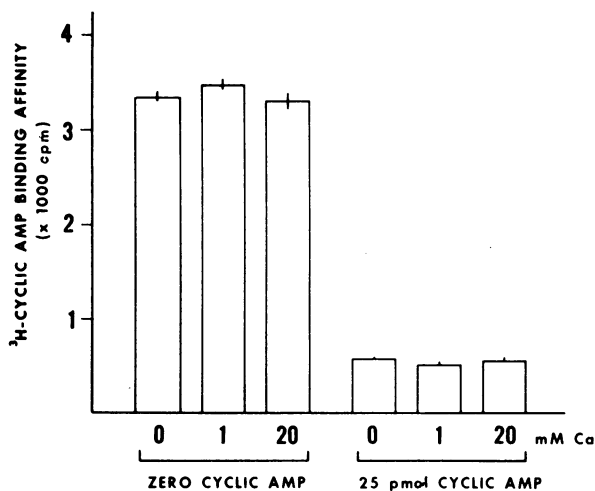

FIGURE 4 Phosphate excretion rate. Ca 6.8 ± 0.9 mg/100 ml in Control; 13.5 ± 1.0 in high Ca group. Solid line is means and standard errors of eight control subjects without calcium infusion. Dotted line is means and standard errors of nine subjects with calcium infusion.

to the previous findings (18). Therefore 5 U of PTH/ml were used in the subsequent studies.

Inulin clearance. Inulin clearance was measured as described by Andreucci, Herrera-Acosta, Rector, and Seldin (19). [^{14}C]inulin, 0.1 μCi, was infused intravenously at the beginning for priming, followed by a constant infusion of 3.3 nCi/min per rat throughout the entire experimental periods. The radioactivity in the blood and urine specimens was then counted by beta liquid scintillation spectrometer. The quenching effect of the specimens on the counting of radioactivity was checked by measuring the ratio of automatic equalization standard of each specimen and appropriate corrections were made when it is indicated. Inulin clearance was then calculated by dividing urinary excretion rate of radioactivity by plasma radioactivity (C_{in} = urine ^{14}C per minute/plasma ^{14}C per ml). This method was reproducible with less than 5% deviation. Both blood and urine concentrations of ^{14}C radioactivity and the calculated glomerular filtration rate (GFR) became stable in 30 min after the priming, and they remained stable during the experimental periods. The value obtained by this method was close to the values obtained by the conventional method (20)

Ionized calcium was measured by flow-through calcium electrode of Orion Research, Inc. (Cambridge, Mass.) Model 99-20, and Orion digital pH meter, Model 801, at 25°C, as Moore described (21).

Protein was measured by Lowry, Rosebrough, Farr, and Randall's method (22), calcium by atomic absorption spectrometer, phosphate by the method of Chen, Toribara, and Warner (23), ^{14}C and ^{3}H radioactivity by liquid scintillation spectrometer.

[^{14}C]inulin was obtained from Amersham/Searle (Arlington Heights, Ill.), [^{3}H]cyclic AMP from New England Nuclear (Boston, Mass.), [^{14}C]cyclic AMP, [^{14}C]ADP, [^{14}C]ATP, and [^{3}H]5′ AMP from Schwartz/Mann (Orangeburg, N. Y.), cyclic AMP and bovine albumin from Sigma Chemical Co. (St. Louis, Mo.), Dextran 75 from Travenol Laboratories (Morton Grove, Ill.) and AG 50W-X4 cation exchange resin, 200-400 mesh, from Bio-Rad Laboratories (Richmond, Calif.).

FIGURE 3 Effect of calcium on cyclic AMP assay. Zero, 1, and 20 mM calcium in the specimens. cpm in ordinate = [^{3}H]cyclic AMP bound to the protein in the supernate. Zero cyclic AMP represents total binding affinity of [^{3}H]cyclic AMP to the protein without displacement by nonradioactive cyclic AMP. 25 pmol AMP represents the ability of 25 pmol of nonradioactive cyclic AMP to displace the [^{3}H]cyclic AMP binding to the protein. Each point represents mean of six determinations. Values between 0,1, and 20 mM calcium are statistically not different, $P > 0.05$.
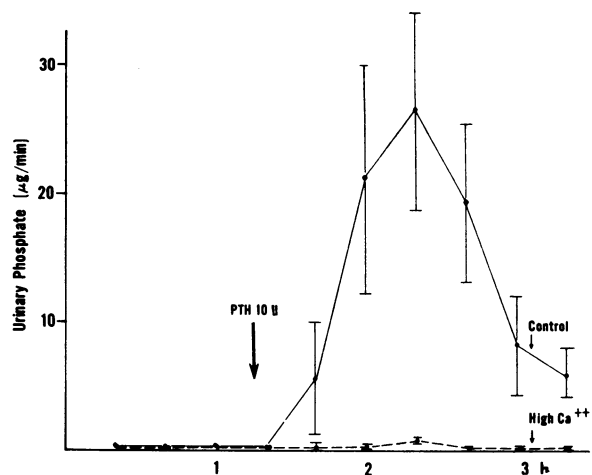
TABLE I

Effects of Calcium Infusion in Rats

		−60 ∼ 0 min before PTH	40 ∼ 80 min after PTH
Control group, without calcium infusion: (12 rats)	plasma calcium, *mg/100 ml*	7.4±1.0	6.8±0.9*
	plasma phosphate, *mg/100 ml*	11.1±1.7	14.4±2.0*
	GFR, *ml/min*	1.72±0.19	1.71±0.18*
	phosphate excretion, *μg/min*	0.095±0.023	26.43±7.79
	TRP‡, %	99.95±0.01	89.25±3.17
Calcium-infused rats: (13 rats)	plasma calcium	13.9±1.2	13.5±1.0*
	plasma phosphate	10.5±0.9	11.9±1.0*
	GFR	1.51±0.20	1.72±0.25*
	phosphate excretion	0.041±0.017	0.538±0.495*
	TRP	99.97±0.01	99.76±0.90*

Values are means±standard error.
*$P > 0.05$ when the value of "before PTH" is compared with the one "after PTH."
‡TRP, tubular reabsorption rate of phosphate.

RESULTS

Conditions of in vivo experiments. The change of plasma calcium concentration after calcium infusion is shown in Fig. 1. Immediately after calcium infusion, plasma calcium increased from 6.2 to 15.4 mg/100 ml. But during 30 min of equilibrium, it dropped to 13.2. Thereafter, plasma calcium became stable. Particularly, the values during the periods of 2–4 h were stable, 11.0–10.4, and they were not measurably different, $P > 0.05$. Animals with different amounts of calcium infusion showed similar changes of plasma calcium. Therefore, the subsequent experiments were performed during this 2–4-h period after calcium infusion.

The mean plasma calcium concentration was 13.5±SE 1.0 mg/100 ml in the calcium-infused animals, and 6.8±0.9 in the control animals without calcium infusion, $P < 0.01$. Plasma phosphate concentration or inulin clearance had no correlation with plasma calcium concentration. PTH injection during the experiments also did not induce any measurable change of GFR or plasma phosphate concentration (Table I).

Phosphate excretion. Intravenous injection of 10 U (USP) of PTH increased phosphate excretion of the control subjects without calcium infusion as shown in Fig. 4 (solid line). The maximal responses were observed at 60-min periods. But calcium-infused animals showed a marked diminution of phosphaturic response to PTH injection (dotted line in Fig. 4).

The phosphaturic response to PTH was expressed by the ratio of [mean of phosphate excretion in five experimental periods] after PTH injection over [mean of phosphate excretion in three control periods] before PTH injection. The phosphaturic responses to PTH and plasma calcium concentrations showed an inverse logarithmic correlation (Fig. 5). Regression coefficient

was: log [phosphaturic response] = 85.63 − 1.46 [plasma calcium]. Expected standard deviation of regression coefficient (Sx.y.) was 4.18; and the significance of the regression coefficient was at the level of $P < 0.001$.

Urinary cyclic AMP excretion. The response of urinary cyclic AMP excretion to PTH occurred sooner than the phosphaturic response. The maximal response was observed at 20 min, as compared to 60 min for phosphaturic response (Fig. 6). But the effect of hypercalcemia on urinary cyclic AMP excretion was similar to its effect on phosphate excretion, (Fig. 4). The control group without calcium infusion increased urinary cyclic AMP excretion sevenfold in response to PTH, but the calcium-infused animals had significantly less increase, $P < 0.01$.

The response of urinary cyclic AMP excretion to PTH was expressed by the ratio of [mean of two experimental periods] after PTH injection over [mean of three control periods] before PTH injection. Plasma calcium concentrations and the responses of urinary cyclic AMP excretion had an inverse logarithmic correlation (Fig. 7). Log [urinary cyclic AMP response] = 41.64 − 0.238 [plasma calcium]. The expected standard deviation of the regression coefficient was 0.60; and it was significant with $P < 0.001$.

Phosphate excretion induced by dibutyryl cyclic AMP. The infusion of dibutyryl cyclic AMP increased urinary phosphate excretion from 5.0±2.1 μg/min to 43.13±7.6 in controls, and in the hypercalcemic rats from 1.1±0.8 to 40.7±7.8. Values are statistically not different between two groups (Fig. 8).

Cyclic AMP concentration in renal cortical tissue. Renal cortical slices taken from the control animals without calcium infusion contained 2.1±0.4 nmol cyclic AMP/g wet tissue (Fig. 9). 5 USP U of PTH/ml of
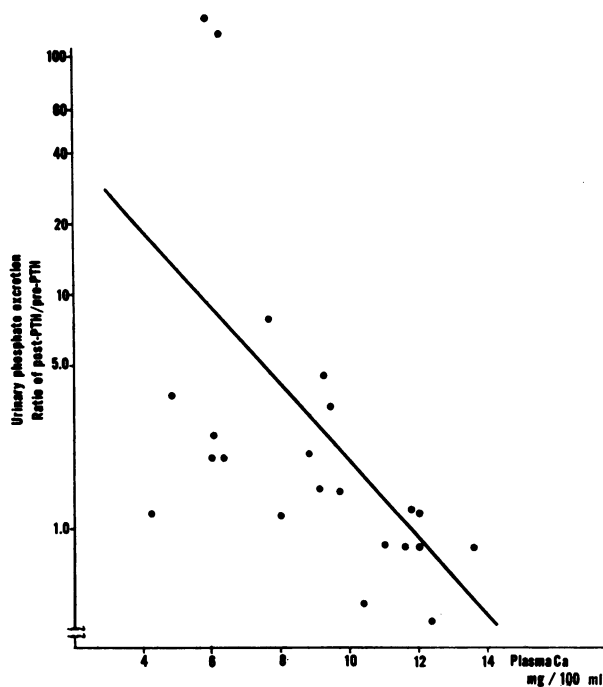
FIGURE 5 Correlation between the increase of phosphaturia by PTH and plasma calcium. Each point presents the ratio of [mean phosphate excretion in five experiment periods] after PTH injection over [mean phosphate excretion of three control periods] before PTH injection versus plasma calcium measured 50 min after PTH injection. Each point indicates one animal study. Solid line represents regression coefficient: Log [phosphaturic response] = 85.63–1.46 [plasma calcium]. Sx.y. = standard deviation of estimate is 4.18. The significance of regression coefficient is $P < 0.001$.

FIGURE 7 Correlation between plasma calcium and urinary cyclic AMP excretion. Increase of urinary cyclic AMP in ordinate: the ratio of [mean urinary cyclic AMP in two experimental periods] after 10 U of PTH injections over [mean urinary cyclic AMP in three control periods] before PTH injection. Each point represents each animal studied. Abscissa represents plasma calcium concentration measured 50 min after PTH injection. Solid line represents regression coefficient: Log [response of urinary cyclic AMP] = 41.64–0.238 [plasma calcium]. Sx.y. = standard deviation of estimates is 0.60. Significance of regression coefficient is $P < 0.001$.
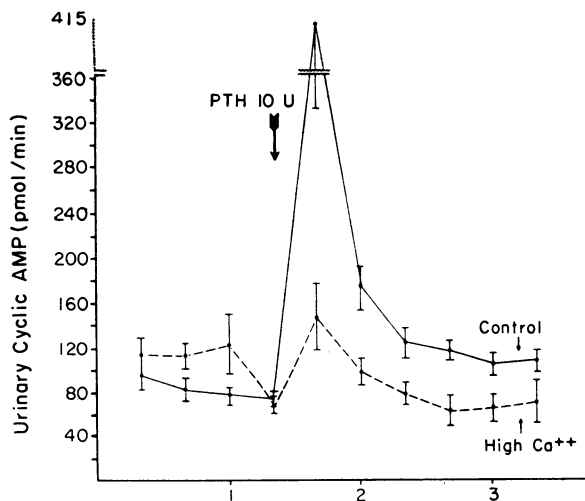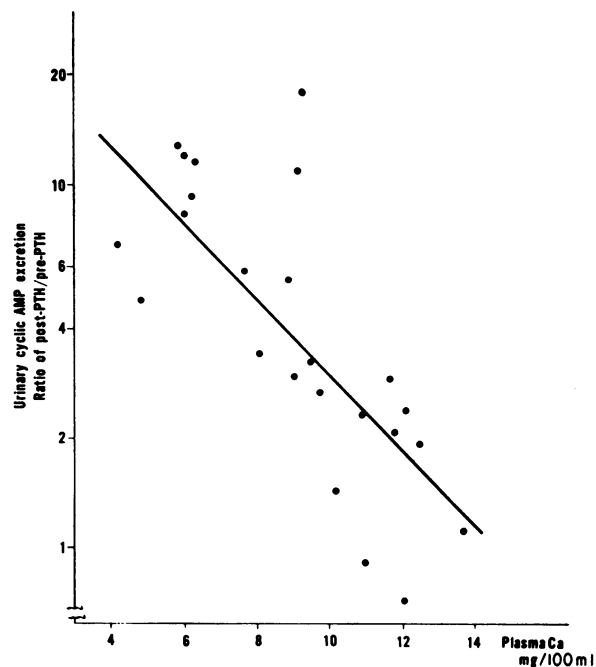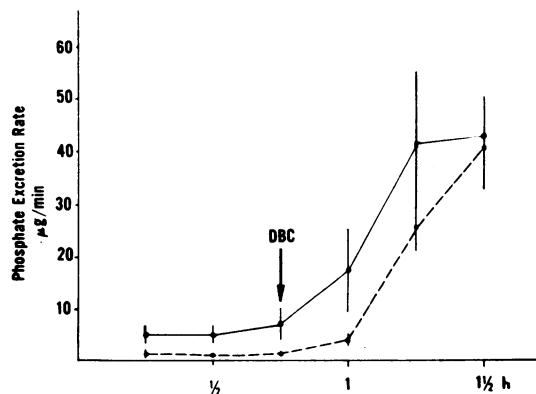

FIGURE 6 Urinary cyclic AMP excretion. Solid line is means and standard errors of urinary cyclic AMP excretion in eight subjects without calcium infusion. Dotted line is means and standard errors of urinary cyclic AMP excretion in nine subjects with calcium infusion.

FIGURE 8 Phosphate excretion induced by the infusion of dibutyryl cyclic AMP (DBC) 50 μg/min. Solid line is means and standard errors of five control subjects without calcium infusion. Dotted line is means and standard errors of four subjects with calcium infusion. Values between the control and calcium infused rats are statistically not different, $P > 0.05$.

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incubation media increased cyclic AMP concentration to 5.8±0.4, $P < 0.001$. Cyclic AMP concentration in renal cortical slices obtained from the calcium-infused animals was not significantly different from the control group in the absence of PTH: 1.9±0.1 vs 2.1±0.4, $P > 0.05$. But the response to PTH was significantly less in the slices from the calcium-infused animals, 4.1±0.3, than the control group, 5.8±0.4, $P < 0.01$ (Fig. 9).

In vitro adenylate cyclase. The inhibitory effect of hypercalcemia on renal cyclic AMP system was further evaluated at subcellular level by studying the effect of that ion on adenylate cyclase. In the presence of 0.24 mM ionized calcium, the enzyme activity increased from 12.1±1.2 pmol cyclic AMP formation/mg protein per minute to 29.0±1.3 with 5 USP U of PTH/ml in the incubation media, $P < 0.001$ (Fig. 10). Increasing the calcium concentration to 0.78 mM lowered the basal enzyme activity slightly, 9.1±1.1 ($P < 0.05$ as compared to the 0.24-mM calcium group). But the response to PTH was markedly inhibited from 29.0±1.3 to 15.7±1.3, $P < 0.01$. Further increase of ionized calcium concentration to 1.25 and 1.85 mM lowered the values further. The activation of adenylate cyclase by 10 mM NaF in the presence of 0.24 mM calcium, 43.2±4.5, was also inhibited to 27.4±2.4 by 1 mM calcium, $P < 0.01$.

Cyclic AMP phosphodiesterase. The effect of calcium concentration and PTH on cyclic AMP-phosphodiesterase was evaluated to test the possibility that the lowered renal tissue cyclic AMP concentration in hypercalcemia could be due in part to the increased catabolism

FIGURE 10 Effect of calcium on PTH-dependent adenylate cyclase of rat renal cortex. Each point represents mean and standard error of six determinations with triplicate measurement for each specimen. Abscissa is "ionized" calcium.

of cyclic AMP, i.e., activation of phosphodiesterase. The control value of phosphodiesterase 1.26±0.03 nmol cyclic AMP hydrolysis/mg protein per minute was not significantly different from 1.29±0.02 with PTH 2 USP U/ml, or 1.29±0.03 with both calcium 10 mM and PTH 2 USP U/ml, or 1.27±0.02 with 10 mM calcium alone.

DISCUSSION

The biological effects of PTH on the kidney are believed to be mediated through cyclic AMP (2, 4, 6, 7). Streeto (8) and Chase, Fedak, and Aurbach (5) demonstrated that a high calcium concentration inhibits the PTH-dependent adenylate cyclase activation in both renal cortex and bone. But the in vitro data cannot be applied directly to the physiological mechanisms in vivo. For example, Nagata and Rasmussen (24) demonstrated that although 2.5 mM calcium inhibits PTH-dependent adenylate cyclase activity in broken cell preparations (5, 8), the same concentration of calcium in the media had no measurable effect on the cyclic AMP concentration in the isolated intact tubular cells (24). The discrepancy of the effects of calcium on the cyclic AMP systems between broken cell preparation and in intact cells has also been noted in several tissues and other hormones (25, 26). Some biochemical reactions related to PTH in the kidney are augmented by high calcium (24) rather than inhibited as seen in the cyclic AMP system (5, 8). These findings (5, 8, 24–28) suggest that the effect of calcium noted on the adenylate cyclase in broken cell preparation (5, 8) does not necessarily correlate with the change of cyclic AMP in intact cells, or the physiological effects of the hormone in vivo. Therefore, a possible interaction between the biological effect of PTH and cal-
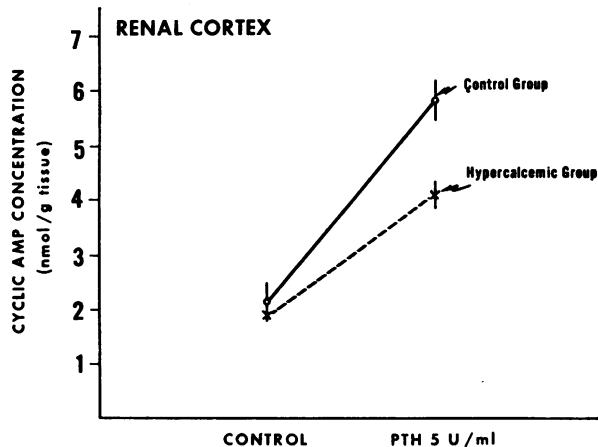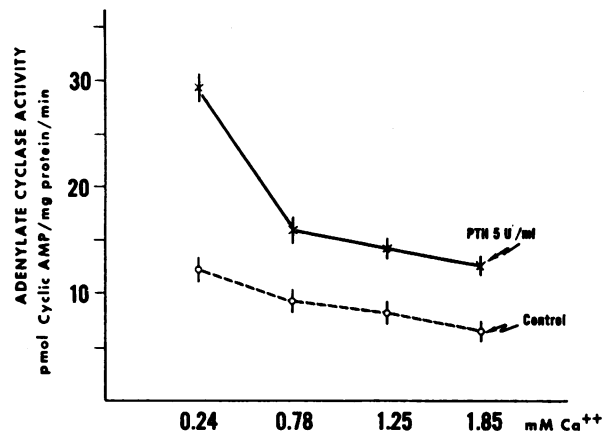

FIGURE 9 Effect of calcium on cyclic AMP concentration in renal cortex. Hypercalcemic groups = renal cortical slices obtained from hypercalcemic animal. Control = renal cortical slices obtained from the animal without calcium infusion. PTH = 5 U/ml of PTH added to the incubation media in vitro. Each point represents mean and standard error of 15 slices with triplicate measurement for each slice. $P < 0.01$ between hypercalcemic and control slices with PTH.

cium concentration was evaluated both in vivo and in vitro.

In the absence of PTH, plasma phosphate concentration, phosphate excretion rates, and GFR of calcium-infused rats were not different from those of control animals without calcium infusion. But the phosphaturic response to the exogenous PTH differed markedly between the control and the calcium-infused animals. Furthermore, the inhibition of the phosphaturic response to PTH showed a significant logarithmic regression coefficient with plasma calcium concentrations. But plasma phosphate concentration and GFR were affected by neither PTH nor hypercalcemia. One of the well-known roles of the calcium ion is inhibition of PTH secretion (1). Our data indicate that high calcium concentration also has a direct inhibitory effect on PTH-induced phosphaturia, unrelated to the mechanism of PTH secretion. These results indicate a dual feedback mechanism of calcium ion on PTH: inhibition of PTH-secretion centrally, and inhibition of PTH action at end-organ, distally.

The inhibitory mechanism of hypercalcemia on PTH activity in the kidney was further evaluated in the renal cyclic AMP system. The increase in the rate of urinary excretion of cyclic AMP in response to PTH was markedly inhibited by hypercalcemia. Such inhibitory effect of hypercalcemia on PTH-induced urinary cyclic AMP excretion had an inverse logarithmic relationship. Hypercalcemia also inhibited the effect of PTH to increase the concentration of cyclic AMP in slices of rat renal cortex. Both of these results suggest that the inhibitory effect of calcium on the phosphaturic response to PTH is associated with the change of PTH-dependent cyclic AMP in the renal cortex.

A decrease in tissue cyclic AMP concentration is potentially caused by two factors: decreased synthesis of cyclic AMP by inhibition of adenylate cyclase activity, and increased catabolism of cyclic AMP by activation of phosphodiesterase (13). Therefore, the effect of calcium on the cyclic AMP system was evaluated with respect to both adenylate cyclase and phosphodiesterase. High calcium concentration in the media inhibited the activation of adenylate cyclase in response to PTH, but had no measurable effect on the activity of phosphodiesterase. The data, therefore, indicate that the decreased cyclic AMP production is the cause of the decrease in urinary and tissue cyclic AMP.

In the present experiments, the activation of adenylate cyclase in response to PTH was markedly inhibited as compared to a minimal inhibition of the enzyme activity in the absence of PTH. It suggests that the inhibitory effect of calcium on PTH-dependent adenylate cyclase in the renal cortex is probably more than a nonspecific effect. In the broken cell preparation of adenylate cyclase, the major inhibition was seen with 0.78 mM ionized

calcium. But in the intact cell, a much higher calcium concentration, 2.5 mM, in the media had no effect on PTH-dependent cyclic AMP generation. (24).[2] These findings suggest that calcium concentration in a compartment other than the plasma compartment might be important to the adenylate cyclase system.

In addition to the inhibitory effect of calcium on the PTH-dependent cyclic AMP system in renal cortex, calcium may also affect the biochemical reaction(s) associated with PTH but after the generation of cyclic AMP (24). This possibility was evaluated by measuring the effect of calcium on the phosphaturia induced by the infusion of dibutyryl cyclic AMP. As shown in Fig. 8, the phosphate excretion rate in the hypercalcemic rats was not measurably different from the control animals. The latter results are consistent with the hypothesis that hypercalcemia inhibits the renal action of PTH by blunting the PTH-dependent cyclic AMP generation but not the processes subsequent to the cyclic AMP system. However, dibutyryl cyclic AMP mimics the biological actions of many intra- and extra-renal hormones (29). Such effects of dibutyryl cyclic AMP may also affect phosphate excretion directly or indirectly. Therefore, these findings with dibutyryl cyclic AMP infusion should be interpreted with extreme caution.

All the above findings are consistent with the hypothesis that the calcium ion has a dual feed-back control mechanism on PTH: a central control of PTH secretion, and a distal control of PTH action in the kidney. The latter effect of the calcium ion is on the PTH-dependent cyclic AMP system in renal cortex, particularly on adenylate cyclase.

ACKNOWLEDGMENTS

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