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Research Article

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Pathogenic Role of Cyclic AMP in the Impairment of Urinary Concentrating Ability in Acute Hypercalcemia

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ABSTRACT A possible association between the impairment of urinary concentrating ability and an impairment of the vasopressin-dependent cyclic AMP system in hypercalcemia was investigated in rat kidneys both in vivo and in vitro. The increases of urinary osmolality and negative free water clearance and the increase of urinary cyclic AMP excretion by vasopressin injection were significantly less in the hypercalcemic rats than in the control rats. The increase of cyclic AMP concentration by vasopressin in renal medullary tissue was significantly less in the slices obtained from the hypercalcemic rats than in those obtained from the control rats. The activation of adenylate cyclase by vasopressin was significantly less in the group with an increased concentration of calcium in media than the control group, but phosphodiesterase activity was not affected by calcium concentration in the media. These data suggest that the impaired urinary concentrating ability in hypercalcemic kidneys is due at least in part to the direct inhibitory effect of calcium on the vasopressin-dependent cyclic AMP system at the level of adenylate cyclase in renal medulla.

INTRODUCTION

Impaired urinary concentrating ability during hypercalcemia has been demonstrated in several species, including man (1–5). With prolonged hypercalcemia, variable morphological changes are demonstrable in the kidney (1). These structural changes were though to be the mechanism for the impaired concentrating ability in hypercalcemia. But the concentrating defect was reversible with correction of hypercalcemia (2–4), suggesting that the lesion is functional rather than structural.

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Two major hypotheses have been proposed to explain the mechanism of the impaired urinary concentrating ability during hypercalcemia. The first suggests the impairment is due to an inability to maintain an adequate hypertonicity in the medullary interstitium. This could be due to either the reduced delivery of tubular fluid to (2, 3) or the impaired reabsorption in the ascending limb of Henle's loop (4, 5). The second hypothesis suggests the defect is an impairment of the hydro-osmotic response to vasopressin in collecting ducts (6, 7). This second hypothesis is supported by the data of Petersen and Edelman (8) and other investigators (6, 7, 9), that an increased calcium concentration in the media inhibits the response of water permeability to vasopressin in toad bladders. Handler, Butcher, Sutherland, and Orloff (10–12) and other investigators (13, 14) demonstrated that the hydro-osmotic effect of vasopressin is mediated through the cyclic AMP system. Therefore, it is reasonable to suspect that the impaired concentrating ability in hypercalcemia is due at least in part to the impairment of vasopressin-dependent cyclic AMP system in renal medulla. This possibility was indirectly supported by data of Marumo and Edelman (15), that high calcium concentration in the media inhibits the vasopressin-dependent adenylate cyclase of rat renal medulla. However, the effects of calcium on the cyclic AMP system in intact cells, or on the physiologic action of the hormone in vivo, cannot be extrapolated solely from that of adenylate cyclase in broken cell preparation (16). Therefore, both in vivo and in vitro systems were investigated for a possible interaction between the cyclic AMP system and an impaired concentrating ability during hypercalcemia. The results are compatible with the hypothesis that the urinary concentrating defect during hypercalcemia is at least in part due to the impairment of the vasopressin-dependent cyclic AMP system in renal medulla.

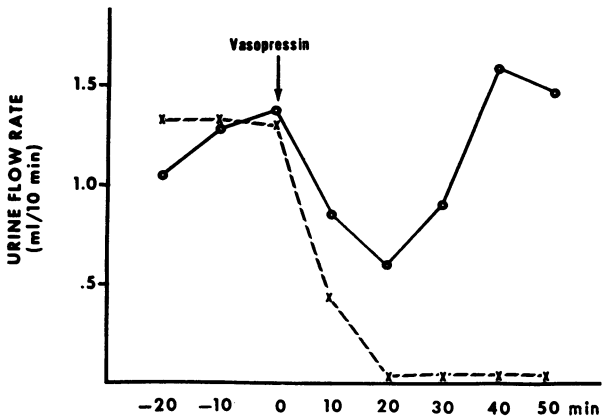

FIGURE 1 Change of urinary flow rates after the intravenous injection of arginine-vasopressin. Each point is mean of 10-min urine in three animals. Broken line is the change after 5 mU of vasopressin, and solid line is the change after 1 mU of vasopressin. Arrow mark is the time of vasopressin.

METHODS

In vivo experiments. The experiments, both in vivo and in vitro, were designed as described previously (17), and the validity and specificity of those methods had been previously evaluated in detail (17, 18). Male Sprague-Dawley rats, weighing 200–250 g, were parathyroidectomized 2–3 days before the experiments to eliminate the effect of endogenous parathyroid hormone on urinary cyclic AMP excretion. After the parathyroidectomy, 1 g/100 ml calcium chloride was added to the drinking water. Neither parathyroid hormone nor vitamin D were supplemented because of the effect of these substances on kidney function (19, 20). For the induction of anesthesia and hydration, 15 ml of saline containing 7 mg pentobarbital with an osmolality of 180 mosmol/kg was administered intraperitoneally. Catheters were inserted into the femoral vein for the infusion of study substances, and into the urinary bladder through suprapubic incision for urine collection.

Hypercalcemia was induced in the experimental group by infusing 0.36 mmol of calcium chloride in 1.5 ml over 30 min, and the control group received an equal volume of isotonic NaCl instead of calcium chloride. Animals with plasma calcium concentration above 14.5 mg/100 ml in samples obtained 30 min after the vasopressin injection were excluded from the study group.

Both groups of rats were kept well hydrated by an infusion of hypotonic sodium chloride (180 mosmol/kg) at the rate of 0.1 ml/min. After an adequate water diuresis had been established, i.e. the urine flow rate was over 0.1 ml/min and the urine osmolality less than 180 mosmol/kg, three 10-min urine samples were collected for basal observations. If urine osmolality and flow rate were not stable or if the urine osmolality did not decrease below 180 mosmol/kg during the basal periods, the experiment in that animal was terminated. After the administration of 1 mU vasopressin, additional 10-min urines were obtained. Blood samples were obtained 30 min after vasopressin injection for determination of calcium and osmolality.

1 mU/rat vasopressin in vivo, and 2 mU/ml in the in vitro experiments are submaximal amounts (21). Therefore, these concentrations were used in the subsequent experiments.

The interaction of calcium and vasopressin on renal concentrating ability and cyclic AMP excretion was evaluated by comparing the means of three 10-min urine collections in basal periods before vasopressin injection versus the means of the three 10-min urines immediately after vasopressin injection (0–30 min), because the effects of vasopressin on renal concentrating ability and urinary cyclic AMP excretion were seen mainly during these periods (Fig. 1). The mean difference between pre- and post-vasopressin values in the control group and in the hypercalcemic group were then compared.

Plasma calcium concentration was most stable 60–150 min after the completion of calcium infusion, with changes of plasma calcium less than 0.5 mg/100 (17). Therefore, experiments were performed during this 60–150 min period.

Tissue cyclic AMP concentration. After water diuresis had been established as in the in vivo experiments to inhibit the secretion of endogenous vasopressin, one kidney was removed, and it served as control. Hypercalcemia was then induced by infusing the same amount of calcium chloride as used in the in vivo experiments, 0.36 mmol/250 g body weight over 30 min. 60 min were allowed after calcium infusion for the equilibration of the infused calcium. Then, the remaining kidney was removed, and it served as the hypercalcemic kidney. To a control group of animals, 0.36 mmol of NaCl were infused instead of calcium chloride; otherwise the animals were prepared similarly to the hypercalcemic rats. Immediately after removal of each kidney, medulla was separated from cortex and sliced to a thickness of less than 0.5 mm with a Stadie-Riggs microtome. Each slice was then divided into two: one half served as the basal and the other half as the vasopressin group. Each 35–60-mg slice was immediately incubated in Krebs-Ringer bicarbonate buffer containing 0.8 mM ionized calcium. To the vasopressin group, a submaximal concentration of vasopressin, 2 mU/ml (21), was added to the incubation media. To evaluate the effect of calcium in the incubation media, the medullary slices obtained from the control animals were incubated in the media with two different concentrations of calcium, 0 and 2.5 mM, and 2 mU/ml vasopressin in the vasopressin group. After 15 min incubation at 37°C in a Dubnoff metabolic shaker, the slices were homogenized in 0.5 ml of ice-cold distilled water with a glass tissue-grinder. The procedure of homogenization required less than 15 s for each slice. The tissue homogenate was then placed in a boiling water bath for 3 min and then centrifuged at 700 *g* for 15 min. Cyclic AMP concentration in the tissue slices progressively increased during the 15-min incubation with vasopressin. This time also corresponds to the time of maximal response to vasopressin in the in vivo experiments. Cyclic AMP in the supernate was then measured by Gilman's method (22) with modification (18). Calcium, vasopressin, or other substances in the specimens had no measurable effect on the cyclic AMP assay system (17). The validity of the assay system on the measurement of cyclic AMP in the supernate of the boiled homogenate has been previously evaluated (17, 18).

Adenylate cyclase activity. Adenylate cyclase of rat renal medulla was prepared as described by Marcus and Aurbach (23). Animals were sacrificed by decapitation, and the medullary portion of the kidney was separated from the cortex. Tissue was homogenized in 0.05 M Tris-HCl, pH 7.4, and centrifuged at 2,000 *g* for 15 min. The precipitate

was resuspended in 0.05 M Tris-HCl, pH 7.4. This mixture is hence referred to as adenylate cyclase enzyme. The enzyme activity was measured as described previously in detail (17, 18) on the same day of the enzyme preparation. In summary, the enzyme preparation was incubated with the reaction mixture, containing 10 mM theophylline, 25 mM KCl, 1.65 mM $MgCl_2$, and the study substances in a total volume of 0.6 ml/tube. Without a chelating agent, the lowest calcium concentration of the study mixture was 0.034 mM ionized calcium. The mixtures were then incubated in a Dubnoff metabolic shaker at 37°C for 15 min. The reaction was terminated by placing the tubes in a boiling water bath for 3 min, and the samples were centrifuged at 700 *g* for 15 min. Adenylate cyclase activity was determined by measuring cyclic AMP in 25 μl aliquots from the supernate in triplicate by Gilman's method (22) with modification (17, 18). The enzyme preparation, boiled without incubation at 37°C, served as the blank, representing the amount of preexisting cyclic AMP in the enzyme preparation. The blank values were less than 7% of the experimental values, and these blank values were then subtracted from the experimental values, and the difference was counted as cyclic AMP formed during a 15-min incubation. The results were expressed as picomoles of cyclic AMP per milligram protein-minute.

Enzyme concentration had been titrated in advance to yield a linear enzyme activity with time during the 15-min incubation. Study substances and ATP at the concentrations comparable to those remaining in the mixture after 15 min incubation had no measurable effect on the assay system, and the validity of the methods had been carefully evaluated (17, 18).

Cyclic AMP-phosphodiesterase activity. Phosphodiesterase enzyme was prepared as described by Cheung (24), by homogenizing rat renal medulla in distilled water and centrifuging at 30,000 *g* for 30 min. The reaction mixtures were composed of 50 mM Tris-HCl, pH 7.4, 1.65 mM $MgCl_2$, a tracer amount of [^{3}H]cyclic AMP (0.5 pmol/tube), the study substances as described in the results, 10^{-7} M or 10^{-4} M cyclic AMP-substrate, and the amount of the enzyme titrated to yield about 20% hydrolysis of the substrate. After 10 min incubation at 37°C, the reaction was terminated by placing the tubes in a boiling water bath for 3 min. 5′ AMP, hydrolyzed by phosphodiesterase, was assayed as described by Thompson and Appleman (25) and other investigators (26). The specimens were reincubated with *Ophiophagus hannah* venom 0.1 mg/tube for 10 min at 37°C. Then 1 ml of the suspension of anion exchange resin, AG 1—X2, 200–400 mesh chloride form, were added to each tube, and centrifuged at 700 *g* for 15 min at 4°C. [^{3}H]adenosine in the supernate was counted by a liquid scintillation spectrometer. The enzyme activity was linear with time for 40 min, and 2.5 mM calcium in the media had no measurable effect on the phosphodiesterase assay system.

Osmolality was measured by freezing-point determination with a Fiske osmometer (Fiske Associates, Inc., Uxbridge, Mass.); protein, by Lowry, Rosebrough, Farr, and Randall's method (27); ^{3}H-radioactivity, by a beta liquid scintillation spectrometer; calcium, by an atomic absorption spectrometer; and ionized calcium by a calcium electrode (Orion Research, Inc., Cambridge, Mass.) with a flow-through system. Free water clearance was calculated in the standard fashion.

Arginine-vasopressin was obtained from Parke, Davis & Company (Detroit, Mich.), and the same batch of hormone was used for the entire experiment. [^{3}H]cyclic AMP was obtained from New England Nuclear (Boston, Mass.), and cyclic AMP from Sigma Chemical Co. (St. Louis, Mo.). Specific activity of [^{3}H]cyclic AMP was 37.4 Ci/mmol.

RESULTS

In vivo experiments. During the basal periods before vasopressin injection, urine osmolality, urine flow rates, osmolar clearance, and free water clearances were not measurably different between the control and the hypercalcemic groups ($P > 0.05$, Table I). However, the increase of urine osmolality and the decreases of urine flow rates and free water clearance after 1 mU vasopressin injection was significantly less in the hypercalcemic group than in the control group ($P < 0.05$). But osmolar clearances were not measurably different between the two groups. Plasma osmolality in the specimens obtained 30 min after vasopressin injection was significantly higher in the hypercalcemic animals than in the control animals ($P < 0.05$).

Cyclic AMP concentration in plasma water was not measurably different between the specimens obtained before vasopressin injection and those obtained 15 min after vasopressin injection (Table I). However, urinary cyclic AMP excretion was increased after vasopressin injection, and that increase was significantly less in the hypercalcemic group than in the control group animals ($P < 0.05$).

In a separate series of animals with plasma calcium concentrations of 10.2±0.3 mg/100 ml, the changes of both free water clearance (Δ − 85±4 μl/min) and urinary cyclic AMP excretion (Δ 31.8±7.1 pmol/min) by vasopressin were significantly greater than those of the hypercalcemic animals ($P < 0.05$); but they were statistically not different from those of the control animals with mean plasma calcium 6.9 mg/100 ml ($P > 0.05$).

Tissue cyclic AMP concentration. The basal (without vasopressin) cyclic AMP concentrations in renal medullary slices were not different between the control and the hypercalcemic groups: 2.3±0.2 pmol/mg of wet tissue in the slices otbained from the control kidney versus 2.3±0.2 in the slices obtained from the calcium infused kidney ($P > 0.05$, Fig. 2). But the increase of tissue cyclic AMP concentration by 2 mU/ml vasopressin was significantly less in the slices obtained from the hypercalcemic kidney: the increase of Δ 1.1±0.2 (to 3.4±0.3) by vasopressin in the control group versus Δ 0.2±0.2 (to 2.5±0.2) in the slices obtained from the hypercalcemic kidney ($P < 0.01$, Fig. 2).

The changes of cyclic AMP concentration by vasopressin in NaCl-infused kidneys, from 2.4±0.3 pmol/mg wet tissue to 3.8±0.6 (Δ 1.4±0.3), were not different from those of the control kidney obtained before calcium infusion, Δ 1.1±0.2 ($P > 0.05$). When the slices from the control group animals were incubated in the media

TABLE I

Effects of Hypercalcemia on Urinary Concentrating Ability in Rats

	Control group		Hypercalcemic group		
	Before VP	After VP	Before VP	After VP	*P* values
Number of experiments		11		9	
Plasma total calcium, *mg/100 ml*		6.9±0.1		12.4±0.7	<0.01
Plasma osmolality, *mosmol/kg*		275±1		288±2	<0.01
Urine osmolality, *mosmol/kg*	133±8	371±40	145±16	271±24	<0.05
Urine flow rate, *μl/min*	139±15	44±5	136±10	72±9	<0.05
Changes of urine flow rate by VP		−95±5		−64±9	<0.05
Glomerular filtration rate, *ml/min*	1.72±0.19	1.71±0.17	1.71±0.18	1.70±0.19	>0.05
Osmolar clearance, *μl/min*	63±7	64±5	70±3	75±7	>0.05
Free water clearance, *μl/min*	75±10	−23±5	70±9	−7±6	<0.05
Changes of C_{H_2O} by VP		−98±5		−77±6	<0.05
Plasma cyclic AMP, *nM*	4.29±0.35	4.30±0.69			
Urinary cyclic AMP, *pmol/min*	57.8±8.6	91.9±13.4	61.8±7.5	65.6±8.4	<0.05
Changes of urinary cyclic AMP by VP, *pmol/min*		Δ34.1±13.6		Δ3.8±4.5	<0.05

Values are means±standard errors. Before VP, mean of three 10-min samples before vasopressin injection. After VP, mean of three 10-min samples immediately after vasopressin injection. *P* values are for comparison between control and hypercalcemic groups of "after VP" values. VP = 1 mU vasopressin i.v. per rat. Changes of urine flow, free water clearance, and urine cyclic AMP are means of [after VP-before VP] in paired analysis.

with 2 mU/ml vasopressin and two different concentrations of calcium (0 and 2.5 mM), cyclic AMP concentrations were not measurably different between the two groups: 3.2±0.2 with no calcium versus 3.5±0.2 with 2.5 mM calcium in the media ($P > 0.05$).

Adenylate cyclase activity. As shown in Fig. 3, an increase of calcium concentration in the incubation media inhibited adenylate cyclase activity progressively, both in the absence and presence of vasopressin. In the lowest calcium concentration tested, 0.034 mM, adenylate cyclase activities were maximal: the basal activity was 2.31±0.15 pmol/mg protein-min, and 4.24±0.25 with vasopressin.

Cyclic AMP-phosphodiesterase. The supernate of 30,000 *g* retained most of the cyclic AMP-phosphodies-

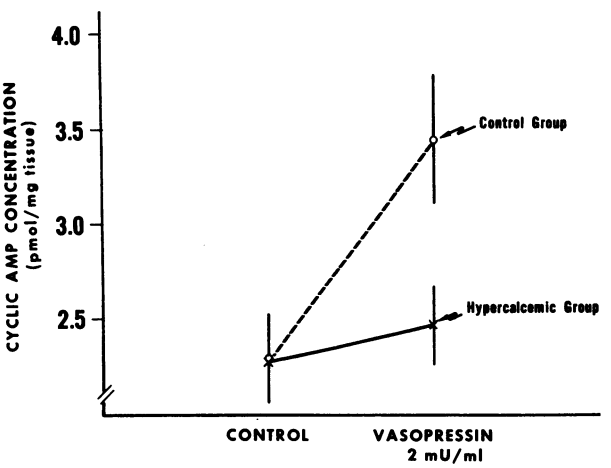

FIGURE 2 Cyclic AMP concentration in renal medullary tissue. Control group: renal medulla obtained from the animal before calcium infusion. Hypercalcemic group: renal medulla obtained from the hypercalcemic animal, induced by intravenous calcium chloride infusion. Ionized calcium concentration in incubation media was 0.8 mM in both groups. The slices were incubated for 15 min at 37°C. Each point is mean of 15 experiments, each done in triplicate with standard errors.

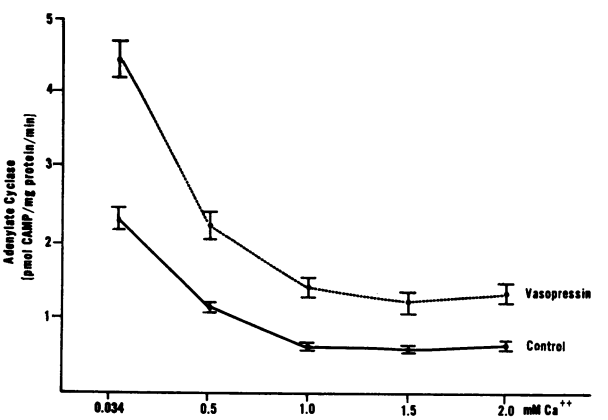

FIGURE 3 The solid line represents basal adenylate cyclase activities of renal medullary tissue and the dotted line represents the enzyme activity in response to 1 mU/ml of vasopressin in the incubation media with variable concentrations of calcium. Calcium concentrations are expressed by ionized calcium. Each point is mean of 10 experiments with triplicate determination for each experiment, and standard error of mean.

TABLE II

Effects of Calcium and Vasopressin on Phosphodiesterase Activities in Renal Medulla

Cyclic AMP (substrate)	Ca^{++}		Vasopressin 1 mU/ml and Ca^{++}	
	0.17 mM	2.76 mM	0.17 mM	2.76 mM
10^{-7} M, *pmol/mg protein-min*	63.7±0.7	64.1±0.8	64.1±0.9	64.1±1.9
10^{-4} M, *nmol/mg protein-min*	10.14±0.18	10.56±0.18	10.07±0.17	10.58±0.27

Values are means and standard errors of 10 determinations in each group. Enzyme mixtures were incubated for 10 min at 37°C.

terase in renal medulla, 12.2±0.3 nmol hydrolysis/mg protein min; and negligible enzyme activity was found in the precipitate fraction, 0.7±0.4. In both high K_m (10^{-4} M) and low K_m (10^{-7} M) systems, neither 2.5 mM calcium nor 1 mU/ml vasopressin had any measurable effect on cyclic AMP-phosphodiesterase activities (Table II).

DISCUSSION

In the in vivo experiments, the acute induction of hypercalcemia (12.4 mg/100 ml) impaired both urinary concentrating ability, measured by urine flow rate, urine osmolality, and free water clearance, and the increase of urinary cyclic AMP excretion in response to vasopressin. These results suggest that the impairment of urinary concentrating ability and the impairment of vasopressin-induced increase of urinary cyclic AMP excretion in the hypercalcemic animals may have a cause-effect relationship.

There was no measurable change in cyclic AMP concentration in plasma water or in glomerular filtration rate after vasopressin injection. Furthermore, these values were not measurably different between the two groups of animals. Therefore, it is unlikely that the lesser increase of urinary cyclic AMP excretion by vasopressin in the hypercalcemic animals is due to a change in the glomerular filtered load of cyclic AMP. It is more likely that there was a difference in renal production of cyclic AMP. The dissociation between changes in excretion rate and plasma concentration of cyclic AMP after vasopressin injection is similar to that after parathyroid hormone injection (28, 29).

Although parathyroid glands had been removed before the experiments to eliminate PTH effect on urinary cyclic AMP excretion (29, 30), many other hormones, such as catecholamines (18), prostaglandin E_1 (21), or glucagon (28), could still affect urinary cyclic AMP excretion. Some unknown factor associated with the induction of hypercalcemia may also indirectly affect the urinary cyclic AMP excretion. Therefore, a direct effect of calcium on the specific vasopressin-dependent cyclic AMP system was further investigated in vitro. The increase of cyclic AMP concentration by vasopressin was significantly less in the slices obtained from the hypercalcemic animals than in those from the control animals. These findings indicate that hypercalcemia has a direct inhibitory effect on the vasopressin-dependent cyclic AMP system in renal medulla. These results suggest that in the hypercalcemic rats, the lesser increase of urinary cyclic AMP excretion after vasopressin injection is due to a direct inhibition of the vasopressin-dependent cyclic AMP system by high calcium concentration.

Even though each kidney slice was studied immediately after nephrectomy, certain unknown factors associated with the induction of hypercalcemia, e.g. effect of unilateral nephrectomy, anesthesia, or status of hydration, may indirectly affect the cyclic AMP concentration. This possibility was evaluated in the NaCl-infused animals. The increases of cyclic AMP concentration by vasopressin were not different between the medullary slices obtained from the first kidney, removed before NaCl infusion, and those from the second kidney, removed after the infusion.

To evaluate differentially the effect of calcium concentration in extracellular space, the slices obtained from the control animals were incubated in the media with two different concentrations of calcium (0 and 2.5 mM). The increases of cyclic AMP concentration by vasopressin were not different between the two groups, indicating that a change in calcium concentration in the incubation media alone does not affect the cyclic AMP concentration in medullary slices.

Cyclic AMP concentration in renal medullary slices can be affected by either adenylate cyclase (cyclic AMP synthesis) or phosphodiesterase (cyclic AMP catabolism). Therefore, the effect of hypercalcemia on the vasopressin-dependent cyclic AMP system was further investigated differentially on adenylate cyclase and phosphodiesterase.

Renal medullary tissue is the mixture of three compartments, i.e., intracellular, interstitial, and luminal, in addition to the difference in calcium concentration at different levels of nephron. Furthermore, the intracellular ionized calcium could not be measured. However, in other tissue (31), the intracellular calcium

concentration is estimated to be lower than plasma concentration. Therefore, the in vitro effects of calcium in the enzyme activities were evaluated in the range from 0.034 mM, the lowest calcium concentration available in the enzyme preparation without a chelating agent, to 2.5 mM.

Neither vasopressin nor 2.5 mM calcium had any demonstrable effect on soluble cyclic AMP-phosphodiesterase in both high and low K_m systems. But adenylate cyclase activity was inhibited by increased concentration of calcium in the media. Calcium has a nonspecific effect on many enzyme systems, including adenylate cyclase (16, 32). Therefore, depression of basal activity of adenylate cyclase by calcium could be a reflection of such a nonspecific effect. However, the activation of adenylate cyclase by vasopressin specifically represents the vasopressin-dependent adenylate cyclase, and that enzyme activation was inhibited at high calcium concentration. The results of adenylate cyclase at least suggest that the inhibition of vasopressin-dependent cyclic AMP in urine in vivo and in tissue slices in vitro are due to the inhibition of cyclic AMP synthesis rather than to the augmentation of cyclic AMP catabolism in the systems tested.

All the above findings are consistent with the hypothesis that the urinary concentrating defect in hypercalcemia is due at least in part to the impairment of the vasopressin-dependent cyclic AMP system in renal medulla at the level of adenylate cyclase. These results neither exclude nor support other possible effects of calcium on the concentrating process, as suggested by other investigators (3, 4), or on other steps in the cyclic AMP system.

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