

Inhibition of carbonic anhydrase by parathyroid hormone and cyclic AMP in rat renal cortex in vitro.

N Beck, K S Kim, M Wolak, B B Davis

J Clin Invest. 1975;**55**(1):149-156. <https://doi.org/10.1172/JCI107905>.

Research Article

It has been demonstrated that parathyroid hormone (PTH) inhibits the proximal tubular reabsorption of bicarbonate, and increases the urinary excretion of that ion. There is also a qualitative similarity between the alterations of the proximal tubular reabsorption of phosphate, sodium, and water after PTH administration and after acetazolamide administration. These findings suggest that the renal effect of PTH is possibly mediated through the inhibition of carbonic anhydrase in proximal tubules. Therefore, a possible inhibitory effect of PTH on carbonic anhydrase was evaluated in the homogenate of rat renal cortex by an indicator titration method. Incubation of cortical homogenates with PTH for 10 min at 37degreesC inhibited carbonic anhydrase activity. The inhibitory effect of PTH was ATP-, Mg^{++} -, and K^{+} -dependent and temperature-dependent; inactivation of PTH by heating at 100degreesC abolished the effect of PTH both to activate adenylate cyclase and to inhibit carbonic anhydrase. Calcium 5 mM also partially abolished effects of PTH to activate adenylate cyclase and to inhibit carbonic anhydrase. The inhibitory effect of PTH on carbonic anhydrase was specific to renal cortex. Cyclic AMP, the intracellular messenger substance for PTH, also inhibited carbonic anhydrase in renal cortex. The cyclic AMP-induced inhibition was also Mg^{++} dependent and temperature dependent, and required preincubation at 37degreesC. But 5'-AMP, a metabolic derivative of cyclic AMP without its biological effect, had no inhibitory [...]

Find the latest version:

<https://jci.me/107905/pdf>



Inhibition of Carbonic Anhydrase by Parathyroid Hormone and Cyclic AMP in Rat Renal Cortex In Vitro

NAMA BECK, KWANG SUP KIM, MARGARET WOLAK, and BERNARD B. DAVIS
with the technical assistance of DAVID H. DONALD

From the Department of Medicine, Veterans Administration Hospital, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15240

ABSTRACT It has been demonstrated that parathyroid hormone (PTH) inhibits the proximal tubular reabsorption of bicarbonate, and increases the urinary excretion of that ion. There is also a qualitative similarity between the alterations of the proximal tubular reabsorption of phosphate, sodium, and water after PTH administration and after acetazolamide administration. These findings suggest that the renal effect of PTH is possibly mediated through the inhibition of carbonic anhydrase in proximal tubules. Therefore, a possible inhibitory effect of PTH on carbonic anhydrase was evaluated in the homogenate of rat renal cortex by an indicator titration method.

Incubation of cortical homogenates with PTH for 10 min at 37°C inhibited carbonic anhydrase activity. The inhibitory effect of PTH was ATP-, Mg^{++} -, and K^+ -dependent and temperature-dependent; inactivation of PTH by heating at 100°C abolished the effect of PTH both to activate adenylate cyclase and to inhibit carbonic anhydrase. Calcium 5 mM also partially abolished effects of PTH to activate adenylate cyclase and to inhibit carbonic anhydrase. The inhibitory effect of PTH on carbonic anhydrase was specific to renal cortex.

Cyclic AMP, the intracellular messenger substance for PTH, also inhibited carbonic anhydrase in renal cortex. The cyclic AMP-induced inhibition was also Mg^{++} dependent and temperature dependent, and required preincubation at 37°C. But 5'-AMP, a metabolic derivative of cyclic AMP without its biological effect, had no inhibitory effect on carbonic anhydrase.

All the above results are consistent with the hypothesis that PTH inhibits proximal tubular reabsorption of bicarbonate and phosphate through the inhibition of

carbonic anhydrase, and that inhibitory effect is mediated through the cyclic AMP system.

INTRODUCTION

Hellman, Au, and Bartter (1) and other investigators (2-8) observed that parathyroid hormone (PTH)¹ increases the renal excretion of bicarbonate as well as phosphate, sodium, and water. Those investigators (1) postulated that the increased excretion of bicarbonate occurs via the inhibition of its reabsorption in the proximal tubule. This postulation is supported by the data of Agus and co-workers (6, 7) in micropuncture experiments that both PTH and its cellular messenger, cyclic AMP, inhibit proximal tubular reabsorption of phosphate, sodium, and water. Inhibition of carbonic anhydrase by pharmacological agents, such as acetazolamide, also inhibits proximal tubular reabsorption of phosphate, sodium, and water (8-11) as well as bicarbonate (12-17). The qualitative similarity between the inhibition of proximal tubular reabsorption of those ions after the PTH administration and that after the inhibition of carbonic anhydrase by acetazolamide suggests that at least a part of renal effect of PTH is mediated through the inhibition of carbonic anhydrase in proximal tubules.

Puschett and Goldberg (8) and Beck and Goldberg (17) proposed that inhibition of proximal tubular reabsorption of bicarbonate increases luminal pH, and decreases the ratio of $H_2PO_4^-$ vs. HPO_4^{--} . Since HPO_4^{--} is less diffusible across the tubular membrane than $H_2PO_4^-$, the increase of luminal pH consequently reduces the net reabsorption of phosphate. Then, it is reasonable to postulate that the phosphaturic effect of

Received for publication 14 December 1973 and in revised form 6 June 1974.

¹Abbreviation used in this paper: PTH, parathyroid hormone.

TABLE I
Effect of Various Study Substances on the Assay
System of Carbonic Anhydrase

Substances	n	Reaction time
		s
Control H ₂ O	6	110.8±0.7
0.1 mM ATP, 1.6 mM Mg ⁺⁺ , and 25 mM K ⁺	6	113.3±0.9
PTH 10 U/ml, ATP, Mg ⁺⁺ , and K ⁺	6	109.3±0.8
Cyclic AMP 0.4 mM, ATP, Mg ⁺⁺ , and K ⁺	6	110.8±0.8

Values are means and SE. $P > 0.05$ among four groups.

PTH might also be mediated through the inhibition of carbonic anhydrase and an alteration of luminal pH. The microperfusion data of Bank, Aynedjian, and Weinstein (18) are consistent with this postulate in showing that PTH inhibits H₂PO₄⁻ transport in proximal tubules, but that hormone has no demonstrable effect on HPO₄⁼ transport. All the above findings (1-18) support the hypothesis that PTH inhibits proximal tubular reabsorption of bicarbonate and H₂PO₄⁻ transport via the inhibition of carbonic anhydrase and the alteration of luminal pH. This hypothesis was evaluated in this series of experiments by measuring the effect of PTH and cyclic AMP, intracellular messenger of PTH, on carbonic anhydrase activity in renal cortex.

METHODS

Measurements of carbonic anhydrase activity. Maren's micro phenol red indicator method (19) was adapted for this experiment. The substrate buffer was 250 μ l/tube of a mixture containing 296 mM Na₂CO₃ and 204 mM NaHCO₃; the flow rate of 100% CO₂ was 400±SD 8 ml/min; the concentration of phenol red indicator was 1 μ g/ml-tube. This assay system yielded 120 s of noncatalytic reaction time. This reaction time produced greater precision and reproducibility in the experiments as compared to the 40 s noncatalytic reaction time used in Maren's original method. During the procedure, specimens were kept at 1.0±1.0°C by using an Aminco constant flow-through cold water bath (American Instrument Co., Inc., Silver Spring, Md.). The actual pH at the end point of phenol red indicator was measured by a Radiometer pH meter (Radiometer Co., Copenhagen, Denmark), and it was 7.2±SD 0.2. The enzyme activity which halves the noncatalytic reaction time under the specified conditions was defined as one unit of carbonic anhydrase activity as described by Maren, Ash, and Bailey (19, 20). To evaluate the effect of phenol red indicator on enzyme activity and the precision of the indicator method (21), the enzyme activity of the principle experiments was measured by both the indicator method and an electrometric method without indicator. The results of those two methods were similar, indicating no measurable effect of phenol red on the assay system. The various study substances did not induce any measurable differences in diffusability and solubility of CO₂ gas, and those substances did not induce any measurable effect on the carbonic anhydrase assay (Table I). A control group, enzyme without study

substance, was included in each series of experiments as an internal standard. The study mixture was composed of 250 μ l buffer, 500 μ l study substances, and 250 μ l carbonic anhydrase per tube.

Carbonic anhydrase inhibition. Sprague-Dawley rats weighing 200-250 g were anesthetized with 7 mg of sodium pentobarbital, and 60 ml of 150 mM NaCl was infused through the renal arteries in less than 15 s to remove the erythrocytes from the kidney tissue. Renal cortex was separated from the medulla and was homogenized in ice cold glass-distilled water using a Sorvall tissue grinder (Ivan Sorvall, Inc., Newtown, Conn.). The homogenate was then filtered through Whatman filter paper no. 2. Thorough removal of erythrocyte in the renal cortical homogenate was checked by the guaiac test. Hereafter, unless specified, carbonic anhydrase refers to the enzyme prepared from renal cortex. The carbonic anhydrase activity had a linear relationship with the enzyme concentration in the range of 0.05-1.5 U/tube. Therefore, in subsequent experiments, the basal activity of carbonic anhydrase was always maintained at about 1 U/tube, 70-100 μ g protein/tube. Carbonic anhydrase from gastric mucosa and erythrocytes was also suspended in glass-distilled water, and each of the enzyme preparations was titrated to yield enzyme activity comparable to that of renal cortex.

Carbonic anhydrase activities were assayed by the following procedure. Study substances, 500 μ l/tube, were mixed with 250 μ l of carbonic anhydrase preparation suspended in glass-distilled water. After variable lengths of incubation at variable temperatures, as described in the Results, the tubes were cooled to 1°C in the cold water bath. Then, 250 μ l of substrate buffer was added, and the sample was bubbled with CO₂ gas.

The methods to measure adenylate cyclase activity and phosphodiesterase activity have been described previously in detail (22). Protein concentration was measured by the method of Lowry, Rosebrough, Farr, and Randall (23).

PTH and cyclic AMP solutions, and carbonic anhydrase mixture were adjusted to pH 4.5 and 7.0, respectively, using HCl and NaOH in order to evaluate the effect of pH on carbonic anhydrase. Since PTH and cyclic AMP solutions do not contain buffer, an extreme caution was needed in adjusting pH. Then, the mixture of carbonic anhydrase

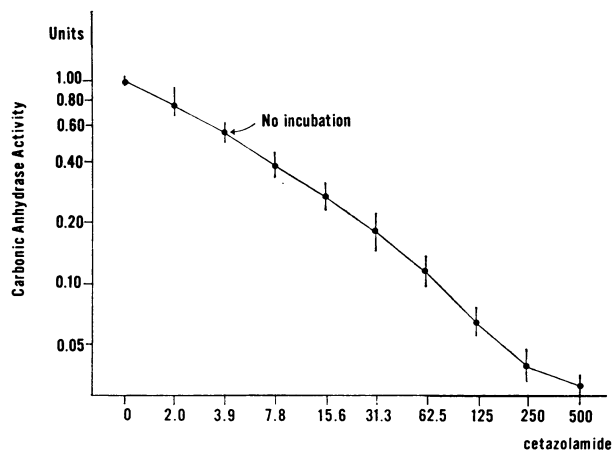


FIGURE 1 Inhibition of carbonic anhydrase by increasing concentrations of acetazolamide. Each value is mean and SE of six determinations.

alone (control), and the mixture with the addition of either PTH or cyclic AMP were preincubated for 10 min at 37°C as shown in Table V. Then carbonic anhydrase activity was determined.

To evaluate the reversibility of the inhibition of carbonic anhydrase by cyclic AMP, the carbonic anhydrase mixture was preincubated for 10 min at 37°C without cyclic AMP for the control group, and with 0.2 mM cyclic AMP for the cyclic AMP groups. After 24 h dialysis at 4°C against distilled water or against 0.2 mM cyclic AMP as shown in the Results, carbonic anhydrase activity was assayed.

Synthetic amino-terminal 1-34-PTH was obtained from the Beckman Instrument Inc. (Palo Alto, Calif.), and cyclic AMP, ATP, and anhydrous acetazolamide from the Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

There was a logarithmic relationship between the concentration of acetazolamide and its inhibitory effect on carbonic anhydrase activity (Fig. 1). 2 nM acetazolamide, the lowest concentration used in this experiment, inhibited carbonic anhydrase from $1.08 \pm \text{SE } 0.01$ U in the control to 0.80 ± 0.05 U, and 500 nM inhibited to 0.03 ± 0.03 U.

Incubation of the enzyme mixture with 7.8 nM acetazolamide for 10 min at 37°C did not measurably increase the inhibitory effect of acetazolamide: 0.42 ± 0.02 U without incubation vs. 0.35 ± 0.03 U after incubation, $P > 0.05$. However, the inhibitory effect of PTH 10 U/ml on carbonic anhydrase was demonstrable only after 10 min incubation at 37°C with 0.1 mM ATP, 1.6 mM MgCl₂, 25 mM KCl (Figure 2). The inhibitory effect of PTH on carbonic anhydrase was much less or not demonstrable: (a) without 10 min incubation, (b) with incubation at 1°C instead of 37°C for 10 min, (c) with PTH but without ATP, Mg⁺⁺, and K⁺, or (d) with PTH inactivated by heating in a boiling water bath for 3 min (Table II). There was a dose-

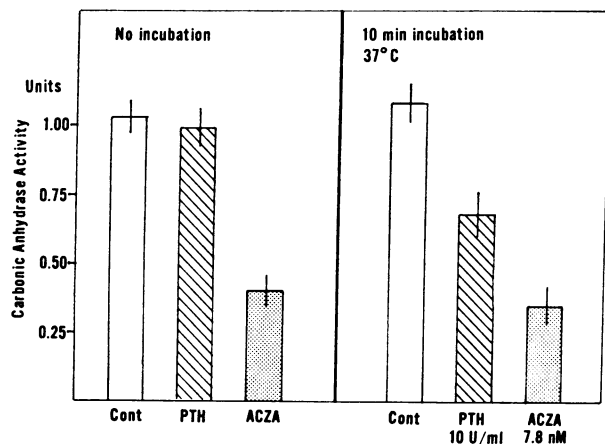


FIGURE 2 Comparison of the inhibitory effect of PTH 10 U/ml and acetazolamide (ACZA) 7.8 nM. Values are means and SE of eight determinations.

TABLE II
Effects of PTH on Carbonic Anhydrase and Adenylate Cyclase of Rat Renal Cortex

	Carbonic anhydrase	Adenylate cyclase
	U	pmol/mg protein-min
Control	1.01 ± 0.01	7.5 ± 0.1
PTH 10 U/ml, incubated for 10 min at 37°C	$0.70 \pm 0.01^*$	$19.5 \pm 0.3^*$
PTH 10 U/ml, without incubation	0.93 ± 0.02	
PTH 10 U/ml, incubated for 10 min at 1°C	0.95 ± 0.02	7.5 ± 0.2
PTH 10 U/ml, without ATP, Mg ⁺ , or K ⁺	0.94 ± 0.01	
PTH inactivated by heating	0.94 ± 0.01	$10.1 \pm 0.2^*$

Unless otherwise specified, incubation was with 0.1 mM ATP, 1.6 mM Mg⁺⁺, and 25 mM K⁺. Values are means and SE of eight determinations for carbonic anhydrase and 10 determinations for adenylate cyclase.

* $P < 0.01$ as compared to the corresponding control value.

inhibition relationship between PTH concentrations, 5-100 U/ml, and carbonic anhydrase activity (Fig. 3).

In a carbonic anhydrase mixture prepared identically to that used in the above experiments with PTH, the basal adenylate cyclase activity was augmented by PTH 10 U/ml in the presence of 0.1 mM ATP, 1.6 mM Mg⁺⁺, and 25 mM K⁺. Cyclic AMP concentration was increased from 10.0 ± 0.1 nM to 26.2 ± 0.4 , but there was no measurable augmentation of the enzyme activity with an incubation at 1°C, and much less augmentation by the inactivated PTH (Table II). In the same preparation, cyclic AMP-phosphodiesterase activity was 2.07 μmol hydrolysis/min in the presence of 0.2 mM cyclic AMP.

The carbonic anhydrase activity, 1.02 ± 0.01 U, was not affected by 5 mM calcium alone, 1.04 ± 0.01 U, $P > 0.05$. But the inhibition of carbonic anhydrase to 0.70 ± 0.01 U by PTH 10 U/ml was partially abolished to 0.93 ± 0.01 by 5 mM calcium, $P < 0.05$ (Figure 4).

Other hormones which act on the kidney through the cyclic AMP system, i.e., vasopressin, isoproterenol, or prostaglandin E₁, had no measurable effect on carbonic anhydrase prepared from renal cortex (Table III). The inhibitory effect of PTH 10 U/ml on carbonic anhydrase was demonstrable only in renal cortical homogenate, but not in homogenates of renal medulla, erythrocytes, or gastric mucosa (Table IV).

The incubation with cyclic AMP at 37°C inhibited carbonic anhydrase in a dose-inhibition (Fig. 5) and in a progressive time-dependent fashion (Fig. 6) from the basal activity of 1.06 ± 0.04 U to 0.24 ± 0.06 U after 20 min incubation. However, the inhibitory effect of cyclic AMP was not demonstrable in the absence of Mg⁺⁺, 0.99 ± 0.02 ; without incubation, 0.99 ± 0.03 U; or with 20 min incubation at 1°C instead of 37°C, 1.00 ± 0.01 , $P > 0.05$. In contrast to the anatomical speci-

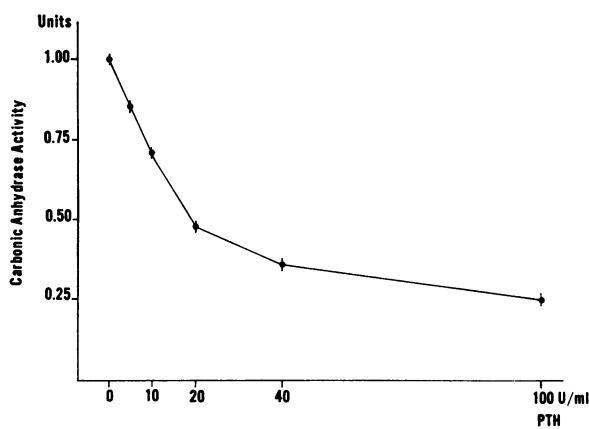


FIGURE 3 Dose-inhibition of carbonic anhydrase by increasing concentrations of PTH. Each value is mean and SE of eight determinations. The enzyme mixture containing 0.1 mM ATP, 1.6 mM Mg^{++} , and 25 mM K^+ were incubated for 10 min at 37°C.

ficity of PTH to renal cortex, 0.2 mM cyclic AMP inhibited carbonic anhydrase in renal medulla and erythrocytes, as well as that in renal cortex (Table IV).

pH of the homogenate of renal cortex was 4.8 ± 0.1 in the presence of PTH 10 U/ml, and pH 4.5–5.2 with cyclic AMP. When pH of cortical homogenates had been adjusted to 4.5 or 7 by HCl or NaOH, and then incubated for 10 min at 37°C, there was no measurable difference in carbonic anhydrase activities between the pH 4.5 group and the pH 7.0 group, $P > 0.05$ (Table V). PTH and cyclic AMP solutions of both pH 4.5 and 7.0 significantly inhibited carbonic anhydrase, $P < 0.01$ (Table V). However, the inhibitory effect of cyclic AMP on carbonic anhydrase was significantly less in the pH 7.0 group than in the pH 4.5 group, $P < 0.01$.

In the control group, dialysis alone against 0.2 mM cyclic AMP at 4°C in the absence of magnesium in the dialysate, had very little effect on carbonic anhydrase activity as compared to dialysis against distilled water (Table VI). However, after carbonic anhydrase had been inhibited by preincubation at 37°C with 0.2 mM cyclic AMP and 1.6 mM magnesium, the enzyme activity was much greater in the group dialyzed against distilled water than in the group dialyzed against cyclic AMP, $P < 0.01$, suggesting a partial restoration of the enzyme activity by the dialysis against water.

DISCUSSION

There are several methods to measure carbonic anhydrase activity, each subject to certain criticisms (21). Because of its simplicity, indicator methods have been widely used. In the present experiments, the principle

of Maren's method was used with some modifications to improve the precision and reproducibility. The validity of this assay system (21) was carefully evaluated as described in the Methods section.

The dose-inhibition relationship between the concentration of acetazolamide and the inhibition of carbonic anhydrase in the present assay system shown in Fig. 1 is comparable to the data of other investigators (19, 20, 24–26).

PTH inhibited carbonic anhydrase only after 10 min preincubation at 37°C in the presence of ATP, Mg^{++} , and K^+ , with a dose-inhibition relationship between PTH 5–100 U/ml and carbonic anhydrase activity (Fig. 3). However, with a submaximal concentration of 7.8 nM acetazolamide, which inhibits 60% of carbonic anhydrase activity, 10 min preincubation at 37°C did not augment the inhibitory effect of acetazolamide. The Mg^{++} dependency and the need for incubation to inhibit carbonic anhydrase suggest that PTH is probably not a direct inhibitor of carbonic anhydrase, but rather that PTH initiates certain biochemical reactions, and those reactions may subsequently inhibit carbonic anhydrase. Since the renal effects of PTH (22, 27, 28) are mediated through the cyclic AMP system, it is reasonable to postulate that the inhibitory effect of PTH on carbonic anhydrase may also be mediated through the cyclic AMP system. This postulation is supported by the following findings: (a) in the homogenate of renal cortex, PTH inhibits carbonic anhydrase, and the same hormone activates adenylate cyclase (Table II); (b) both the inhibition of carbonic anhydrase and the activation of adenylate cyclase induced by PTH were time and temperature dependent; (c) ATP,

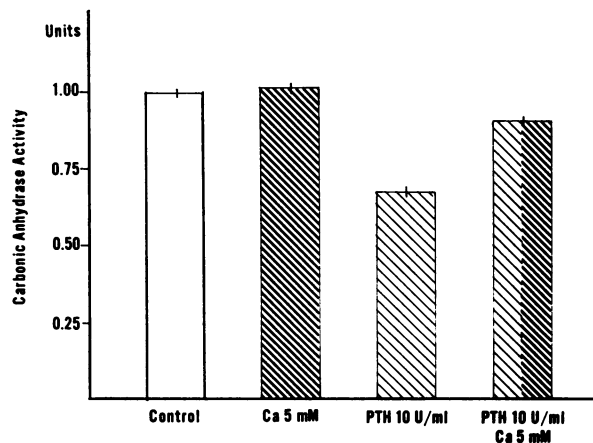


FIGURE 4 Interaction between calcium and PTH. The enzyme mixtures with 0.1 mM ATP, 1.6 mM Mg^{++} , and 25 mM K^+ , and the study substances as shown in the abscissa were incubated for 10 min at 37°C. Each bar is mean and SE of eight determinations.

Mg⁺⁺, and K⁺ were necessary for both the inhibition of carbonic anhydrase and the activation of adenylate cyclase by PTH (22); (d) heating of PTH to 100°C abolished the effect of the hormone both to inhibit carbonic anhydrase and to activate adenylate cyclase; (e) high calcium concentration, which inhibits the phosphaturic effect of PTH through the inhibition of PTH-dependent adenylate cyclase in renal cortex (22), also diminished the inhibitory effect of PTH on carbonic anhydrase; (f) the inhibitory effect on carbonic anhydrase was hormonally specific to PTH. Other hormones acting in the kidney, such as vasopressin (29), catecholamine (30), or prostaglandin E₁ (29), had no measurable inhibitory effect on carbonic anhydrase; (g) the inhibitory effect of PTH on carbonic anhydrase was also specific to renal cortex, and PTH had no measurable effect on carbonic anhydrase prepared from renal medulla, gastric mucosa, or erythrocyte. The hormonal and tissue specificities of the carbonic anhydrase system are similar to those of the PTH-dependent cyclic AMP system in the kidney. However, cyclic AMP, the intracellular messenger of PTH and of many other hormones (27), did not demonstrate the anatomical specificity. Even though these experiments were in the in vitro system, all the above findings are consistent with the hypothesis that PTH inhibits carbonic anhydrase in renal cortex through the cyclic AMP system, and suggest that the inhibition of carbonic anhydrase is due to a physiological effect rather than a nonspecific toxic effect of PTH.

Cyclic AMP per se does not appear to be a direct inhibitor of carbonic anhydrase, since that nucleotide also requires Mg⁺⁺ and an incubation at 37°C to inhibit the enzyme activity. The lack of inhibition with

TABLE III
Effects of Various Hormones on Renal Cortical Carbonic Anhydrase

Substances	n	Carbonic anhydrase activity
		U
Control	8	1.14±0.01
PTH 10 U/ml	8	0.78±0.03*
Vasopressin 10 mU/ml	8	1.11±0.03
Isoproterenol 1 μM	8	1.16±0.02
Prostaglandin E ₁ 1 μM	8	1.12±0.02

The enzyme preparations were incubated with the study substances for 10 min at 37°C, and the enzyme activity was measured after cooling the specimen to 1°C. Values are means and SE.

* *P* < 0.01 as compared to the control value.

TABLE IV

Effect of PTH and Cyclic AMP on Carbonic Anhydrase in Various Tissues

	n	Basal	PTH 10 U/ml	Cyclic AMP 0.2 mM
		U	U	U
Renal cortex	8	1.01±0.01	0.70±0.01*	0.24±0.06*
Renal medulla	6	1.02±0.02	0.98±0.01	0.69±0.01*
Erythrocytes	6	0.95±0.03	0.91±0.03	0.75±0.01*

Carbonic anhydrase preparations were preincubated for 10 min at 37°C without study substance (basal), PTH 10 U/ml, or cyclic AMP 0.02 mM. Values are means and SE. * *P* < 0.01 as compared to the corresponding basal value.

5'-AMP, which is a metabolic derivative of cyclic AMP without its biological effect (22), further suggests that the inhibitory effect of cyclic AMP on carbonic anhydrase is related to the biological effect of the nucleotide.

The minimal concentration of PTH to inhibit carbonic anhydrase was 5 U/ml, and 50% inhibition of the activity was seen with 20 U/ml. This may appear to be a high concentration of PTH as compared to the plasma concentration of PTH (31). However, an optimal concentration of the hormone in an in vitro system is not directly comparable to that of in vivo. In the broken-cell preparation of renal cortex, Marcus and Aurbach (32) reported a minimal activation of adenylate cyclase with PTH 4 U/ml, and a maximal activation with PTH 100 U/ml, a range of concentration similar to our results (Fig. 3).

The minimal concentration of cyclic AMP to inhibit carbonic anhydrase was 0.2 mM in the present in vitro system. This value is higher than the measured cyclic AMP concentration, 0.05 mM, in the isolated renal tubular cells (33), and the measured value, 26.2 nM, in the mixture of carbonic anhydrase after 10 min incubation with PTH in the present experiments. Here again, it should be emphasized that the optimal concentration of cyclic AMP in an in vitro system is not directly comparable to that in vivo. Furthermore, the measured concentration of cyclic AMP in the mixture of carbonic anhydrase after the incubation with PTH and the concentration of an exogenous cyclic AMP cannot be quantitatively compared for their respective physiological effects. The measured cyclic AMP concentration in the PTH group is the one remaining after the incubation and after the hydrolysis by cyclic AMP-phosphodiesterase in the enzyme mixture. But the concentration of cyclic AMP in the exogenous cyclic AMP group is the initial concentration added, and does not

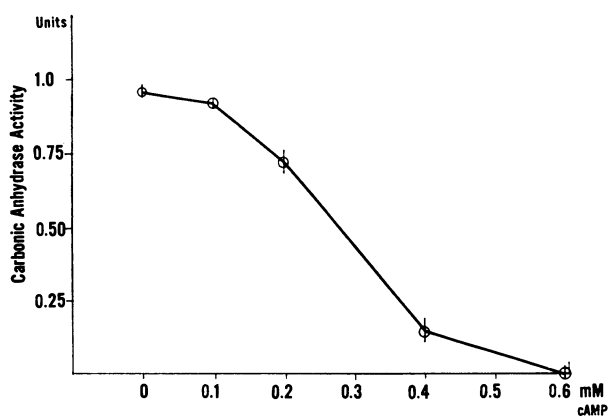


FIGURE 5 Dose-inhibition relationship between cyclic AMP (cAMP) concentrations and carbonic anhydrase activity. Renal cortical homogenate contained 1.6 mM Mg^{++} and 25 mM K^+ , and incubated for 2 min at 37°C. Values are means and SE of eight determinations.

take into account the amount of cyclic AMP hydrolyzed during 10 min incubation. However, the lower concentration of cyclic AMP in the PTH group may in part explain a lesser inhibition of carbonic anhydrase by PTH than that by exogenous cyclic AMP.

Since cyclic AMP is a phosphoric acid, and since there is no buffer in the assay mixture of carbonic anhydrase, an addition of cyclic AMP or PTH lowered the pH of the carbonic anhydrase mixture to the range of 4.5 to 5.2. The acidic pH, per se, may inhibit carbonic anhydrase. Therefore, the effect of variable pH, per se, on carbonic anhydrase activity was investigated as shown in Table V. Lowering of pH from 7.0 to 4.5 had no measurable effect on the carbonic anhydrase activity, and furthermore, both PTH and cyclic AMP had an inhibitory effect on carbonic anhydrase both at pH 4.5 and 7.0. These results suggest that the inhibitory effect of PTH or cyclic AMP on carbonic anhydrase is not due to the alteration of pH induced by those substances. The inhibitory effect of cyclic AMP on carbonic anhydrase was significantly less in the solution with pH 7.0 than in that with pH 4.5. Since cyclic AMP is a phosphoric acid, and since pH of cyclic AMP solution was adjusted in the absence of buffer, it is very conceivable that a part of cyclic AMP was chemically denatured by alkaline pH during the adjustment of pH. Whatever mechanism is involved for the difference in the inhibitory effect of cyclic AMP between pH 4.5 and pH 7.0, the data in Table V clearly demonstrate that cyclic AMP inhibits carbonic anhydrase at pH 7.0.

The findings that dialysis against distilled water partially restores carbonic anhydrase activity which had been inhibited by cyclic AMP suggest that at least a part of the inhibitory mechanism of cyclic AMP on

carbonic anhydrase was reversible, and that reversibility further suggests that the inhibition was not due to denature of the enzyme by a nonspecific toxic effect of cyclic AMP.

Both PTH and cyclic AMP are natural physiological substances, and their inhibitory effect on carbonic anhydrase was Mg^{++} , K^+ , and ATP-dependent, and temperature-dependent, and the biologically inactive derivatives, i.e. heated PTH and 5'AMP, did not inhibit the enzyme activity. Furthermore, there is no evidence in the system tested to indicate that the inhibitory effect of those substances on carbonic anhydrase is due to a nonspecific toxic effect. Those findings suggest that the inhibitory effect of PTH and cyclic AMP is probably a biological function of PTH and cyclic AMP.

Based on calculation of the inhibitory kinetics of acetazolamide in homogenates in vitro and the concentration of acetazolamide in the homogenate of renal cortex, Maren (34) postulated that more than 90% of carbonic anhydrase in the kidney (35) should be inhibited to induce a measurable increase of bicarbonate excretion. PTH is a natural physiological hormone; it induces multiple cascade reactions including the activation of the cyclic AMP system, and a final reaction of those intermediate reactions possibly involves inhibition of carbonic anhydrase. In contrast, acetazolamide is a pharmacological agent with a direct inhibitory effect on carbonic anhydrase. Therefore, the effect of PTH on carbonic anhydrase cannot be quantitatively compared to that of acetazolamide. However, cyclic AMP, which is the intracellular messenger for PTH, depends on less intermediate reactions than PTH, and that can cause more than 90% inhibition of carbonic anhydrase. These findings are compatible with Maren's hypothesis (34).

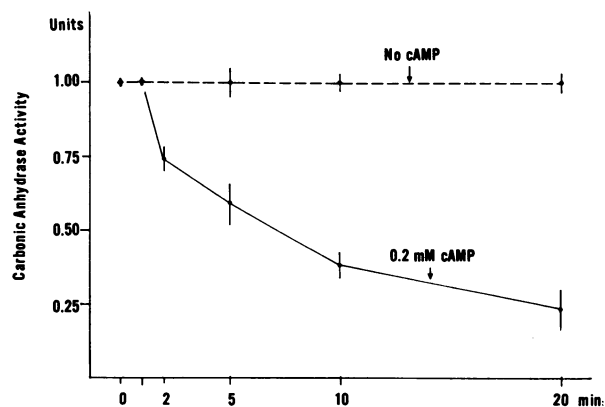


FIGURE 6 The relationship between the duration of incubation and the inhibition of carbonic anhydrase. The homogenate was incubated with 0.2 mM cyclic AMP (cAMP), 1.6 mM Mg^{++} , and 25 mM K^+ , and incubated at 37°C. Values are means and SE of eight experiments.

TABLE V

Effect of pH on the Inhibitory Effect of PTH and Cyclic AMP

	n	pH 4.5	pH 7.0
		U	U
Control	10	1.03±0.04	0.95±0.04
PTH 10 U/ml	5	0.70±0.03*	0.64±0.01*
Cyclic AMP 0.6 mM	5	0.09±0.01*	0.25±0.01*

The carbonic anhydrase activity after 10 min incubation at 37°C. Values are means and SE.

* $P < 0.01$ as compared to the corresponding control values.

The attempts by Puschett and Goldberg (8) and other investigators (17) to demonstrate a direct inhibitory effect of PTH and cyclic AMP on carbonic anhydrase have not been successful. In their publications it is not clear whether they used the carbonic anhydrase prepared from renal cortex or erythrocytes. However, their observation is not inconsistent with the data in the present experiments: neither PTH nor cyclic AMP demonstrated any direct inhibitory effect on carbonic anhydrase unless the enzyme had been incubated with PTH or cyclic AMP and Mg^{++} at 37°C. Furthermore, since the inhibitory effect of PTH and cyclic AMP on carbonic anhydrase probably occurs through an induction of intermediate reactions during the incubation, those intermediate reactions may not occur in a purified preparation of carbonic anhydrase (8, 17).

The results in this experiment are consistent with the hypothesis that: (a) PTH inhibits carbonic anhydrase specifically in renal cortex in vitro, (b) this inhibition is probably through the cyclic AMP system, (c) and the inhibition of proximal tubular reabsorption of bicarbonate, phosphate, sodium, and water after the administration of PTH or cyclic AMP (1-8) might be at least in part mediated through the inhibition of carbonic anhydrase.

TABLE VI

Effect of Dialysis on the Inhibition of Carbonic Anhydrase by Cyclic AMP

Preincubation for 10 min at 37°C	n	Dialysis for 24 h at 4°C against	
		Water	0.2 mM cyclic AMP
		U	U
Control	6	1.14±0.02	1.01±0.02
Cyclic AMP 0.2 mM	6	0.60±0.02*	0.28±0.01*

Values are means and SE.

* $P < 0.01$ between the two values.

ACKNOWLEDGMENTS

The authors thank Wono Lee for his editorial advice, and Ruth Greenlee and Marilyn Schuster for their assistance on preparation of this manuscript.

REFERENCES

- Hellman, D. E., W. Y. W. Au, and F. C. Bartter. 1965. Evidence for a direct effect of parathyroid hormone on urinary acidification. *Am. J. Physiol.* **209**: 643-650.
- Ellsworth, R., and W. M. Nicholson. 1935. Further observations upon the changes in the electrolytes of the urine following injection of parathyroid extract. *J. Clin. Invest.* **14**: 823-827.
- Kleeman, C. R., and R. E. Cooke. 1951. The acute effects of parathyroid hormone on the metabolism of endogenous phosphate. *J. Lab. Clin. Med.* **38**: 112-127.
- Nordin, B. E. C. 1960. The effect of intravenous parathyroid extract on urinary pH, bicarbonate and electrolyte excretion. *Clin. Sci. (Oxf.)* **19**: 311-319.
- Fourman, P., B. McConkey, and J. W. G. Smith. 1960. Defects of water reabsorption and of hydrogen-ion excretion by the renal tubules in hyperparathyroidism. *Lancet* **1**: 619-623.
- Agus, Z. S., L. B. Gardner, L. H. Beck, and M. Goldberg. 1973. Effects of parathyroid hormone on renal tubular reabsorption of calcium, sodium and phosphate. *Am. J. Physiol.* **224**: 1143-1148.
- Agus, Z. S., J. B. Puschett, D. Senesky, and M. Goldberg. 1971. Mode of action of parathyroid hormone and cyclic adenosine 3',5'-monophosphate on renal tubular phosphate reabsorption in the dog. *J. Clin. Invest.* **50**: 617-626.
- Puschett, J. B., and M. Goldberg. 1969. The relationship between the renal handling of phosphate and bicarbonate in man. *J. Lab. Clin. Med.* **73**: 956-969.
- Counihan, T. B., B. M. Evans, and M. D. Milne. 1954. Observations on the pharmacology of the carbonic anhydrase inhibitor "Diamox." *Clin. Sci. (Oxf.)* **13**: 583-598.
- Fulop, M., and P. Brazeau. 1968. The phosphaturic effect of sodium bicarbonate and acetazolamide in dogs. *J. Clin. Invest.* **47**: 983-991.
- Malvin, R. L., and W. D. Lotspeich. 1956. Relation between tubular transport of inorganic phosphate and bicarbonate in the dog. *Am. J. Physiol.* **187**: 51-56.
- Rector, F. C., Jr., D. W. Seldin, A. D. Robert, Jr., and J. S. Smith. 1960. The role of plasma CO_2 tension and carbonic anhydrase activity in the renal reabsorption of bicarbonate. *J. Clin. Invest.* **39**: 1706-1721.
- Rector, F. C., Jr., N. W. Carter, and D. W. Seldin. 1965. The mechanism of bicarbonate reabsorption in the proximal and distal tubules of the kidney. *J. Clin. Invest.* **44**: 278-290.
- Bernstein, B. A., and J. R. Clapp. 1968. Micropuncture study of bicarbonate reabsorption by the dog nephron. *Am. J. Physiol.* **214**: 251-257.
- Clapp, J. R., J. F. Watson, and R. W. Berliner. 1963. Effect of carbonic anhydrase inhibition on proximal tubular bicarbonate reabsorption. *Am. J. Physiol.* **205**: 693-696.
- Kunau, R. T., Jr. 1972. The influence of the carbonic anhydrase inhibitor, benzolamide (CL-11,366), on the reabsorption of chloride, sodium and bicarbonate in the proximal tubules of the rat. *J. Clin. Invest.* **51**: 294-306.

17. Beck, L. H., and M. Goldberg. 1973. Effects of acetazolamide and parathyroidectomy on renal transport of sodium, calcium, and phosphate. *Am. J. Physiol.* 224: 1136-1142.
18. Bank, N., H. S. Aynedjian, and S. W. Weinstein. 1974. A microperfusion study of phosphate reabsorption by the rat proximal tubule. *J. Clin. Invest.* 54: 1040-1048.
19. Maren, T. H. 1960. A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. *J. Pharmacol. Exp. Ther.* 130: 26-29.
20. Maren, T. H., V. I. Ash, and E. M. Bailey, Jr. 1954. Carbonic anhydrase inhibition. II. A method for determination of carbonic anhydrase inhibitors, particularly of Diamox. *Bull. Johns Hopkins Hosp.* 95: 244-255.
21. Davis, R. P. 1963. The measurement of carbonic anhydrase activity. In *Methods of Biochemical Analysis*. D. Glick, editor. Interscience Pubs, Inc., John Wiley and Sons, Inc., New York. 2: 307-327.
22. Beck, N., H. Singh, S. W. Reed, and B. B. Davis. 1974. Direct inhibitory effect of hypercalcemia on renal action of parathyroid hormone. *J. Clin. Invest.* 53: 717-725.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
24. Maren, T. H. 1967. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol. Rev.* 47: 595-781.
25. Kernohan, J. C. 1966. A method for studying the kinetics of the inhibition of carbonic anhydrase by sulfonamides. *Biochim. Biophys. Acta.* 118: 405-412.
26. Maren, T. H., A. L. Parcell, and M. N. Malik. 1960. A kinetic analysis of carbonic anhydrase inhibition. *J. Pharmacol. Exp. Ther.* 130: 389-400.
27. Chase, L. R., and G. D. Aurbach. 1967. Parathyroid function and the renal excretion of 3',5'-adenylic acid. *Proc. Natl. Acad. Sci. (U. S. A.)*. 58: 518-525.
28. Kurtzman, N. A., M. L. Karlinsky, and D. S. Sager. 1973. Effects of infusion of cyclic AMP and parathyroid hormone on renal bicarbonate reabsorption. *Clin. Res.* 21: 283. (Abstr.)
29. Beck, N. P., T. Kaneko, U. Zor. J. B. Field, and B. B. Davis. 1971. Effects of vasopressin and prostaglandin E_1 on the adenylyl cyclase-cyclic 3',5'-adenosine monophosphate system of the renal medulla of the rat. *J. Clin. Invest.* 50: 2461-2465.
30. Beck, N. P., S. W. Reed, H. V. Murdaugh, and B. B. Davis. 1972. Effects of catecholamines and their interaction with other hormones on cyclic 3',5'-adenosine monophosphate of the kidney. *J. Clin. Invest.* 51: 939-944.
31. Arnaud, C. D., H. S. Tasao, and T. Littledike. 1971. Radioimmunoassay of human parathyroid hormone in serum. *J. Clin. Invest.* 50: 21-34.
32. Marcus, R., and J. D. Aurbach. 1969. Bioassay of parathyroid hormone *in vitro* with a stable preparation of adenylyl cyclase from rat kidney. *Endocrinology*. 85: 801-810.
33. Michelakis, A. M. 1970. Hormonal effects on cyclic AMP in a renal-cell suspension system. *Proc. Soc. Exp. Biol. Med.* 135: 13-16.
34. Maren, T. H. 1963. The relation between enzyme inhibition and physiological response in the carbonic anhydrase system. *J. Pharmacol. Exp. Ther.* 139: 140-153.
35. Datta, P. K., and T. H. Shepard, II. 1959. Intracellular localization of carbonic anhydrase in rat liver and kidney tissues. *Arch. Biochem. Biophys.* 81: 124-129.