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DIURETICS AND THE RENAL ADENYLATE CYCLASE SYSTEM

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1 The relationship between the diuretic effectiveness and the effect on the renal adenylate cyclase of three diuretics, acetazolamide, frusemide and ethacrynic acid, was examined. The hypothesis that acetazolamide and parathyroid hormone (PTH), inhibit renal carbonic anhydrase by a cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-dependent mechanism was also tested.

2 *In vitro*, acetazolamide, frusemide and ethacrynic acid at high concentrations (10^{-3}M) all produced some inhibition of basal and stimulated rat kidney plasma membrane adenylate cyclase. The effect of acetazolamide was much less than that of frusemide and ethacrynic acid. These plasma membrane effects were reproduced in studies of cyclic AMP formation in isolated kidney tubules of rats.

3 Intravenous injections of acetazolamide did not change the total cyclic AMP content of the kidneys of rats killed by microwave irradiation.

4 Acetazolamide produced a diuresis in the rat and a slight inhibition of the antidiuretic effect of Pitressin. Frusemide produced a diuresis and greatly reduced the antidiuretic response to Pitressin. Ethacrynic acid was ineffective as a diuretic in the rat and actually enhanced the antidiuretic response to Pitressin.

5 In investigating the possible influence of diuretics and PTH on the activity and state of phosphorylation of carbonic anhydrase it was found that: there was no correlation between the ability of diuretics to inhibit carbonic anhydrase activity and to inhibit carbonic anhydrase phosphorylation; neither PTH nor cyclic AMP (in the presence of adenosine triphosphate, Mg^{2+}, K^+ and incubation at 37°C) inhibited rat cortex homogenate carbonic anhydrase activity.

6 It seems unlikely that any of the tested diuretics exerts its pharmacological effect by means of changes in kidney cyclic AMP metabolism.

Introduction

Several studies have suggested that certain diuretics, notably frusemide and ethacrynic acid, may produce some of their renal effects by inhibiting renal adenylate cyclase activity. (Ferguson & Twite, 1973; Ebel, 1974; Ebel & Sharp, 1975; Barnes, Hui & Dousa, 1975). The situation with acetazolamide is more complicated. Rodriguez, Walls, Yates & Klahr (1974) showed similar effects of acetazolamide and parathyroid hormone (PTH) on urinary cyclic adenosine 3′,5′-monophosphate (cyclic AMP) and phosphate and found that, like PTH, acetazolamide stimulated the adenylate cyclase of the rat renal cortex. Beck, Kim, Wolak & Davis (1975), investigating the similar effects of acetazolamide and PTH on bicarbonate excretion, found that both PTH and cyclic AMP inhibited the activity of rat kidney carbonic anhydrase *in vitro*. Since acetazolamide is a potent inhibitor of carbonic anhydrase, this report together with that of Rodriguez *et al.* (1974) could be interpreted as suggesting that acetazolamide and PTH both increase cyclic AMP production by stimulating the renal adenylate cyclase (Jacobsen & Kokko, 1976). The cyclic AMP generated, acting by a protein kinase, could then inhibit renal carbonic anhydrase, to produce the observed effects on phosphate and bicarbonate excretion.

The present study was designed to evaluate the effects of acetazolamide, frusemide and ethacrynic acid on renal adenylate cyclase activity *in vitro* and to relate these to pharmacological effects of the drugs *in vivo*. The effects of PTH and cyclic AMP on carbonic anhydrase activity were also studied.

Methods

Drugs and chemicals

(α–^{32}P]-adenosine triphosohate (0.5–30 Ci/mmol) and [8–^{3}H]-cyclic AMP (20–30 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Cyclic AMP (crystallized free acid) and the sodium

salt of adenosine triphosphate (ATP) were obtained from Boehringer Mannheim, London.

Acetazolamide (Crystalline) was obtained from Sigma Chemical Co. and the sodium salt of acetazolamide (Diamox), from Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., frusemide (20 mg ampoules for injection) from Heochst Pharmaceuticals and sodium ethacrynate (Edecrin) from Merck, Sharp and Dohme Ltd. All other chemicals were of A.R. grade.

Hormone preparations

Parathyroid hormone (PTH) was a bovine preparation, (code name 69TP; Moseley, Martin, Robinson, Reit & Tregear (1975), of 1,000 iu/mg potency; Nahorski, Hunt, Rogers, Jones & Martin (1976)). Synthetic salmon calcitonin (CT), 4,700 u/mg was obtained from Dr J.W. Bastian, Armour Pharmaceuticals, Kankakee, Illinois. Pitressin, 20 u/ml, was obtained from Parke-Davis & Co.

Water-loaded, alcohol-anaesthetized rat preparations

Male CFY, Remote Sprague-Dawley rats (120–150g) East Anglia Laboratory Animals Ltd., were used in these experiments. In this preparation the animal is anaesthetized with alcohol and water-loaded to produce a state of constant water diuresis which is very responsive to exogenous ADH. Surgery was carried out as described by Harris & Jenner (1972). Animals were infused at 0.2ml/min with a modified Czaczkes solution (Czaczkes, Kleeman & Koenig, 1964) containing 51.2 mM NaCl, 92.7 mM glucose and 2.5% v/v ethanol. The diuretics under study were added to this infusion.

The bladder cannula was connected to a drop counter with a digital display. The antidiuretic response to intravenous injections of Pitressin was calculated as the percentage reduction in urine flow in the 10 min following injection compared to the 10 min before injection. The same calculation was used to measure the effect of single intravenous injections of diuretics on urine output. The effects of sustained infusion of the diuretics on the response to Pitressin were also examined. Injections of 40, 80 and 120μu Pitressin were given at 20 min intervals during control periods and during infusion of diuretics.

Administration of diuretics to intact animals

Injections of 0.4 ml 0.9% w/v NaCl solution (saline) or of saline containing 0.4 mg frusemide or sodium acetazolamide (Diamox) were given via the tail vein to male Wistar rats of 40 g weight, which were killed by microwave irradiation (Nahorski *et al.*, 1976). Kidneys were removed and stored at −20°C for total cyclic AMP determinations.

Kidney tubule preparation

Rat kidney cortex tubules were made by the method of Larkins, MacAuley, Rappoport, Martin, Tulloch, Byfield, Matthews & MacIntyre, (1974) resuspended in the required volume of oxygenated buffer (Larkins *et al.* 1974) containing 10 mM theophylline and 0.13% bovine serum albumin (BSA) and incubated for 20 min at 37°C.

The accumulation of unlabelled cyclic AMP was measured in incubations of final volume 2ml containing 10mM theophylline, 0.13% BSA and the test substances. The incubations were started by the addition of 500μl cell suspension and after 20 min at 37°C were terminated by placing the glass vials in a boiling water bath for 2 to 3 minutes. Supernatants from these incubations were stored for cyclic AMP determinations.

Cyclic AMP determinations

Kidneys from animals killed by microwave irradiation were homogenized in 2ml/100mg w/w of 4:1, ethanol:water, using a Polytron model PCU-2 speed 4, 30 seconds. After centrifugation (5,000*g* for 10 min) the pellet was re-extracted as before. The combined supernatants were dried in air and resuspended in assay buffer.

The cyclic AMP content of rat kidneys and rat tubule supernatant was assayed by the protein binding method of Brown, Albano, Elkins & Sgherzi (1971). Acetazolamide, frusemide and ethacrynic acid at concentrations from 10^{-5}M to 10^{-3}M did not affect the binding of cyclic AMP to the binding protein.

Adenylate cyclase determinations

Kidneys removed for assay of adenylate cyclase were cut longitudinally and the medulla and papilla removed from the cortex. Plasma membrane preparations were then made of the cortex and medulla. The tissue was homogenized in cold 25mM Tris-HCl (pH 7.4) containing 0.25M sucrose and 1mM tetrasodium edetate, using 3 strokes of a loose and 3 strokes of a tight Dounce pestle. The homogenate was then spun to 2200 *g* (Sorvall RC2-B at 4°C) and stopped immediately. The supernatant was poured off and then spun at 2200 *g* for 10 min and the resultant pellet resuspended in 25 mM Tris-HCl (pH 7.8) buffer containing 0.013% (w/v) BSA, 30 mM KCl and 4.5 mM $MgCl_2$.

Adenylate cyclase reaction mixtures contained, in a volume of 100μl, 25 mM Tris-HCl (pH 7.8); 1 mM [α^{32}P]-ATP (10 ct min^{-1} $pmol^{-1}$); 1 mM cyclic AMP; 2.5 mM phosphoenolpyruvate; 1.5 iu pyruvate kinase; 0.013% (w/v) BSA; 30 mM KCl; 4.5 mM $MgCl_2$; and the appropriate hormone and/or diuretics. The reaction was started by addition of 50μl of the plasma membrane suspension containing 100 to 300mg pro-

Table 1 Effects of diuretics on renal plasma membrane adenylate cyclase

		Cyclic AMP (pmol mg^{-1} protein $10min^{-1}$)					
		Cortex			*Medulla*		
Diuretic	*(M)*	*Basal*	*PTH*	*NaF*	*Basal*	*ADH*	*NaF*
Control	0	38.7 ± 3.2	99.1 ± 1.9	165.9 ± 25.3	43.5 ± 4.9	105.5 ± 7.5	115.2 ± 2.2
Acetazolamide	10^{-6}	37.2 ± 4.2	100.6* ± 5.4	162.5 ± 4.9	43.5	104.4 ± 7.4	100.9 ± 8.4
	10^{-5}	36.4 ± 4.2	119.0 ± 8.5	138.7 ± 9.3	46.1 ± 2.9	113.1 ± 6.5	94.9 ± 4.3
	10^{-4}	37.9 ± 1.9	100.4 ± 4.6	145.1 ± 27.2	41.3 ± 2.9	111.8 ± 6.5	100.4 ± 18.1
	10^{-3}	39.7 ± 0.9	103.8 ± 10.1	130.3 ± 25.7	37.9 ± 4.3	105.4 ± 5.3	79.0 ± 13.5
Control	0	33.3 ± 2.8	54.1 ± 3.0	288.0 ± 12.0	66.8 ± 4.0	200.9 ± 3.2	106.4 ± 4.7
Frusemide	10^{-6}	31.9 ± 0.1	54.1 ± 6.8	282.0 ± 9.0	64.8 ± 1.9	200.9 ± 10.8	106.0 ± 13.0
	10^{-5}	32.1 ± 4.1	47.2 ± 7.0	215.7 ± 27.4	72.3 ± 4.2	225.0 ± 11.4	78.6 ± 14.1
	10^{-4}	31.9 ± 2.7	42.8 ± 5.8	237.3 ± 17.3	53.4 ± 4.2	242.0 ± 13.8	73.5 ± 12.5
	10^{-3}	17.4 ± 2.4*	40.3 ± 9.5	224.6 ± 26.8	37.3 ± 1.0**	126.8 ± 4.2**	58.9 ± 4.5**
Control	0	32.8 ± 1.6	70.3 ± 3.6	225.1 ± 9.8	48.9 ± 7.5	143.8 ± 3.0	183.0 ± 11.2
Ethacrynic acid	10^{-6}	34.6 ± 1.1	79.6 ± 6.4	225.1 ± 9.7	42.5 ± 3.5	135.4 ± 5.3	183.5 ± 3.5
	10^{-5}	31.7 ± 0.3	64.8 ± 2.7	279.1 ± 28.0	41.7 ± 1.9	139.8 ± 5.3	206.0 ± 22.7
	10^{-4}	23.6 ± 3.2*	59.3 ± 5.1	247.6 ± 14.6	52.9 ± 9.6	123.9 ± 4.4*	174.6 ± 7.9
	10^{-3}	13.9 ± 1.5**	31.1 ± 2.1**	151.7 ± 8.3**	19.6 ± 1.3*	75.2 ± 10.6**	102.7 ± 13.5*

*Differs significantly from the control value by $*P<0.05$ or $**P<0.01$.

The table shows the effect of 10^{-6} to 10^{-3}M concentrations of three diuretics on basal, hormone-and fluoride-stimulated adenylate cyclase activity in rat renal cortex and medulla. Hormone concentrations (0.3μg PTH in the cortex and 260μu Pitressin in the medulla) produced half-maximal activation of adenylate cyclase. Sodium fluoride concentration was 10mM. Figures shown are mean ± s.e. mean.

tein. Incubations were carried out for 10 min at 37°C. The reaction was found to be linear for up to 15 min of incubation at 37°C and for 50 to 300 mg/protein. Reactions were terminated by the addition of 100μl of 40 mM ATP and placing the assay tubes in a boiling water bath for 2 minutes. The ^{32}P-labelled cyclic AMP which was generated was purified by passing through Dowex and then alumina columns (Hunt, Martin, Michelangeli & Eisman, 1976). All adenylate cyclase assays were carried out on freshly prepared enzymes and assayed in triplicate.

Protein kinase assay

A cyclic AMP-dependent protein kinase enzyme was made from a 20% homogenate of chick kidney in 20 mM Tris-HCl pH 7.4. This was spun at 50,000 *g* for 60 min and the supernatant used. The protein kinase was assayed by the method of Livesey, Jones, Eisman & Martin, (1976), the only modification being that the reaction was stopped by spotting on to chromatography paper and the phosphorylated protein and labelled ATP were separated by running the paper in 10% trichloroacetic acid (TCA) containing a phosphate carrier.

Carbonic anhydrase assay

This was assayed by the method of Maren (1960). The 'enzyme unit' was the activity necessary to halve the time of the uncatalysed reaction. In the present experiments the assay volume was 1 ml and the uncatalysed rate of reaction was approximately 65 seconds. Carbonic anhydrase preparations were prepared as follows. Rat renal cortex was homogenized in ice-cold distilled water and filtered through Whatman no. 2. filter paper (Beck *et al.*, 1975). Whole chick kidney homogenates were prepared similarly. Human blood was heparinized and diluted in water. The effects of PTH and cyclic AMP on carbonic anhydrase activity were investigated in samples preincubated at 37°C for 20 min (Beck *et al.*, 1975). All samples were cooled to 4°C in ice before carbonic anhydrase assay.

Protein determination

Proteins were determined according to the method of Lowry, Rosebrough, Farr & Randall (1951).

Statistics

The difference between means was assessed for significance by Student's *t* test. Values of $P<0.05$ were taken as statistically significant.

Results

The effects of diuretics on the renal plasma membrane adenylate cyclase

High concentrations of frusemide and ethacrynic acid inhibited basal, hormone-and fluoride-stimulated adenylate cyclase activity in both renal cortex and medulla (Table 1). Acetazolamide had no significant inhibitory effects. The concentrations of PTH and Pitressin used (Table 1) produced approximately half-maximal activation of the cortex and medulla adenylate cyclase respectively. Basal rates of adenylate cyclase activity always generated radioactivity at least 5 to 6 times higher than the blank values obtained from incubations containing boiled plasma membranes.

Frusemide 10^{-3}M (Table 1) produced a 48% reduction of basal, a 25% reduction of PTH and a 22% reduction of NaF-stimulated activity in the renal cortex. In the medulla, 10^{-3} M frusemide produced a 44% reduction of basal, a 37% reduction of Pitressin and a 44% reduction of NaF-stimulated activity.

Ethacrynic acid 10^{-3}M (Table 1) produced a 57% reduction of basal, a 46% reduction of PTH and a 33% reduction of NaF-stimulated activity in the cortex. In the medulla, 10^{-3}M ethacrynic produced a 60% reduction of basal, a 48% reduction of Pitressin and a 44% reduction of NaF-stimulated activity.

Since, in contrast to the findings of Rodriguez *et al.* (1974), acetazolamide did not stimulate rat kidney cortex adenylate cyclase, the experiment was repeated using the preparation and assay conditions described by Rodriguez *et al.* (1974). A basal activity of 39.8 ± 2.9 pmol cyclic AMP per mg protein/15 min was comparable to that obtained by Rodriguez *et al.* but in the presence of 10^{-3}M acetazolamide the activity was not significantly different (38.4 ± 3.4 pmol cyclic AMP per mg protein/15 minutes). Preincubation of the diuretic with the membranes for 10 min at 30°C before assay also failed to show any effect of acetazolamide on adenylate cyclase activity.

The effects of the diuretics on hormone-stimulated adenylate cyclase is shown in Figures 1, 2 and 3. Dose-response curves to three hormones, PTH, CT and Pitressin were obtained in the presence of 5×10^{-4}M concentrations of the three diuretics; this concentration was chosen in preference to a higher one since the stimulatory effects of the hormones and the inhibitory action of the diuretics could both be seen at this concentration of diuretic (see Table 1).

Acetazolamide produced a small consistent inhibition of adenylate cyclase activity at high concentrations of all three hormones. Frusemide inhibited the response to all concentrations of hormones and ethacrynic acid was a potent inhibitor of all hormone responses, reducing the enzyme activity to basal or lower in the presence of high hormone levels. A comparison of Figures 1, 2 and 3 shows that ethacrynic

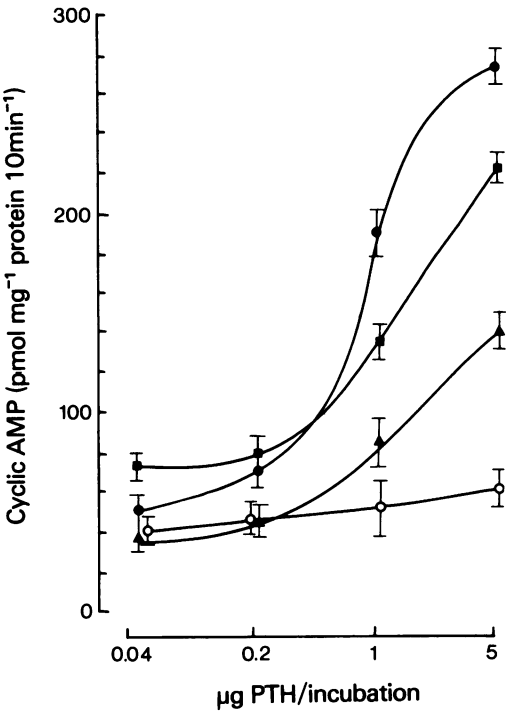

Figure 1 The effects of 5 x 10^{-4}M acetazolamide, frusemide and ethacrynic acid on parathyroid hormone (PTH)-stimulated adenylate cyclase activity of rat renal cortex plasma membranes. Control (●), acetazolamide (■), frusemide (▲) and ethacrynic acid (○). All points are mean values; vertical lines show s.e. mean. Basal activity in the absence of hormone and diuretics was 44.7 ± 2.8 pmol cyclic AMP mg^{-1} protein 10 min^{-1}.

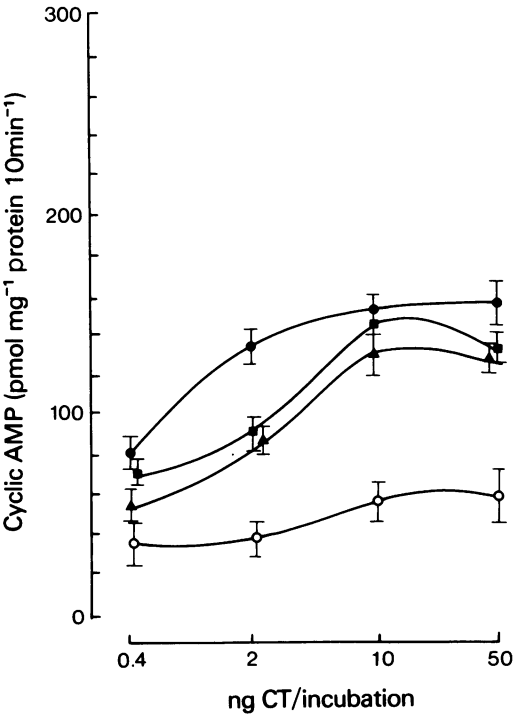

Figure 2 The effects of 5 x 10^{-4}M acetazolamide, frusemide and ethacrynic acid on calcitonin (CT)-stimulated adenylate cyclase activity of rat renal cortex plasma membranes. Control (●), acetazolamide (■), frusemide (▲) and ethacrynic acid (○). All points are mean values; vertical lines show s.e. mean. Basal activity in the absence of hormone and diuretics was 44.7 ± 2.8 pmol cyclic AMP mg^{-1} protein 10 min^{-1}.

acid was always the most effective inhibitor and also that the inhibitory effects of all three diuretics are most noticeable in the presence of high concentrations of hormones. Table 2 shows that the three diuretics had a similar effect on cyclic AMP accumulation in PTH-treated, isolated kidney tubules of the rat.

Pre-incubation of membranes and diuretics

Preincubation of the diuretics with the membrane preparation for varying periods at 30°C before assay of membrane adenylate cyclase had no effect on the acetazolamide response (Figure 4) but did result in an

Table 2 Effect of diuretics on the basal and parathyroid hormone (PTH)-stimulated adenylate cyclase of rat renal cortex tubules

		Cyclic AMP (pmol mg^{-1} protein 20min^{-1})			
		Control	*Acetazolamide*	*Frusemide*	*Ethacrynic acid*
Basal		4.6 ± 0.4	3.9 ± 1.0	3.0 ± 1.9	10.5 ± 2.8
PTH μg/ml	0.04	11.7	10.0 ± 2.5	10.3 ± 7.2	7.9 ± 3.6
	0.2	19.4 ± 2.5	17.2 ± 2.2	8.4 ± 5.7	11.8 ± 3.7
	1	34.2 ± 4.2	27.5 ± 3.3	12.7 ± 3.5**	16.2 ± 2.9**
	5	35.2 ± 3.9	32.9 ± 3.2	19.4 ± 4.8*	8.9 ± 3.5**

Values are mean ± s.e. mean.
*Differs significantly from the control value by *$P<0.05$ or **$P<0.01$.

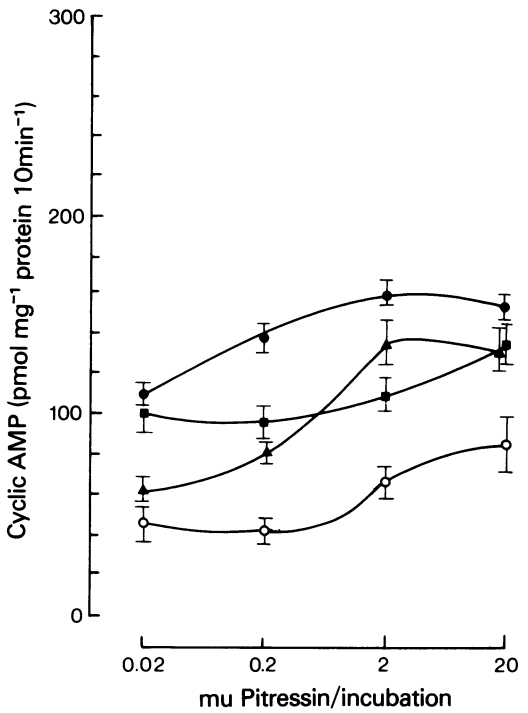

Figure 3 The effects of 5 x 10^{-4}M acetazolamide, frusemide and ethacrynic acid on Pitressin-stimulated adenylate cyclase activity of rat renal cortex plasma membranes. Control (●), acetazolamide (■), frusemide (▲) and ethacrynic acid (○). All points are mean values; vertical lines show s.e. mean. Basal activity in the absence of hormone and diuretics was 44.7 ± 2.8 pmol cyclic AMP mg^{-1} protein 10 min^{-1}.

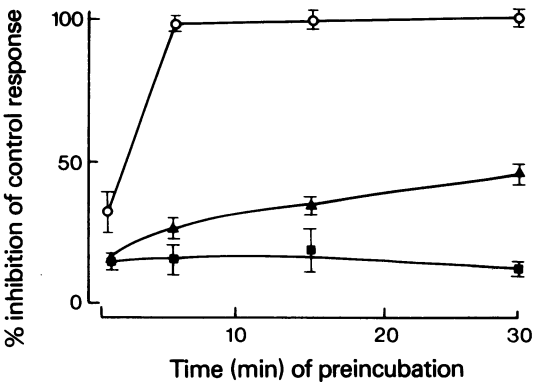

Figure 4 The effect of pre-incubating rat renal cortex plasma membranes with diuretics on the activity of membrane adenylate cyclase. Membranes and diuretics were preincubated at 30°C for 0, 5, 15 or 30 min before adenylate cyclase activity was assayed. Acetazolamide (■), frusemide (▲) and ethacrynic acid (○).

increase in the inhibition caused by frusemide (12.5 ± 1.4% to 45.2 ± 0.9% over 30 min of preincubation) and a marked effect on the action of ethacrynic acid. The inhibitory effect of ethacrynic acid increased from 30.6 ± 8.2% inhibition, without preincubation, to 97.9 ± 2.4% within 5 min of preincubation with the membranes. In another experiment in which this abrupt increase in inhibition was investigated, an increase from 48% to 85% inhibition was found to occur within 2 min of preincubation. This action of ethacrynic acid occurred in both renal cortex and medulla.

The relationship between parathyroid hormone, cyclic AMP and the activity and state of phosphorylation of carbonic anhydrase

Neither PTH nor cyclic AMP had any significant effect on the activity of rat kidney homogenate carbonic anhydrase or on the activity of three other carbonic anhydrase enzymes, despite a wide variation in the preincubation conditions and the addition of an active protein kinase enzyme (Table 3). In contrast, low concentrations of acetazolamide were immediately inhibitory in the absence of ATP and preincubation.

Since acetazolamide is known to be a specific inhibitor of carbonic anhydrase, a comparison was made of the ability of three diuretics, acetazolamide, frusemide and chlorothiazide, to inhibit carbonic anhydrase activity and to affect carbonic anhydrase phosphorylation. Table 4 shows that acetazolamide, the most potent inhibitor of the enzyme activity, did not affect the phosphorylation of the enzyme and that frusemide, the least potent inhibitor of the three, was the only one to inhibit the phosphorylation of the enzyme. (This diuretic also inhibited the phosphorylation of histone to the same extent).

The effect of acetazolamide on renal cyclic AMP content

Single injections of acetazolamide did not affect the total cyclic AMP content of the rat kidney measured 5 min after injection (Table 5). In contrast, PTH produced a ten-fold increase in cyclic AMP content.

The effect of diuretics on urine flow and on the antidiuretic response to Pitressin in the water-loaded alcohol-anaesthetized rat

Frusemide and acetazolamide increased urine flow in the ADH suppressed rat but ethacrynic acid was ineffective. Table 6 shows the diuretic responses to single intravenous injections of the diuretics. The response to frusemide was at least 10 times as great as that to acetazolamide. Both responses occurred within

Table 3 The effect of parathyroid hormone (PTH) and cyclic AMP on carbonic anhydrase activity

Tissue	**Preincubation*	*Additions to preincubation*	*Effect on carbonic anhydrase activity*
Rat kidney homogenate	+	1–20 μg PTH	NS
	+	1–20 μg PTH + protein kinase†	NS
	+	0.1–1.5mM db cyclic AMP	NS
	+	0.1–1.5mM db cyclic AMP + protein kinase†	NS
	–	9.6×10^{-8}M Acetazolamide	50% inhibition
Chick kidney homogenate	+	1–20 μg PTH	NS
	+	1–20 μg PTH + protein kinase†	NS
	+	0.1–1.5mM db cyclic AMP	NS
	+	0.1–1.5mM db cyclic AMP + protein kinase	NS
	–	1.9×10^{-8}M Acetazolamide	50% inhibition
Bovine carbonic anhydrase	+	0.1–1.5mM db cyclic AMP + protein kinase†	NS
	–	5×10^{-10}M Acetazolamide	50% inhibition
Heparinized human blood	+	0.1–1.5mM db cyclic AMP + protein kinase†	
	–	2.5×10^{-8}M Acetazolamide	50% inhibition

*Preincubation of carbonic anhydrase enzymes with PTH or cyclic AMP was carried out at 37°C for 20 min in the presence of 0.1 mM ATP, 1.6 mM $MgCl_2$ and 25 mM KCl. Alterations in the ATP concentration (0.1 mM to 1.0 mM), the length of preincubation (0–30 min) and the pH of preincubation (pH 5–9) did not affect the lack of response to PTH or cyclic AMP. Below pH 5 carbonic anhydrase activity was inhibited irrespective of the preincubation contents. db cyclic AMP = dibutyryl cyclic AMP. NS = no significant effect.
† A cyclic AMP-dependent chick kidney cytosol protein kinase was used. This enzyme was capable of phosphorylating bovine carbonic anhydrase (see Table 4).

Table 4 The effects of diuretics on the phosphorylation and activity of bovine carbonic anhydrase

Diuretic	*Effect of diuretic (10^{-3}M) on carbonic anhydrase phosphorylation (% control)*	*Concentration (M) required for 50% inhibition of carbonic anhydrase activity*
Acetazolamide	100.7 ± 2.0%	5×10^{-10}
Chlorothiazide	104.0 ± 0.9%	5×10^{-6}
Frusemide	*59.7 ± 6.3%	8×10^{-4}

*10^{-3}M Frusemide also inhibited the phosphorylation of histone under these conditions (33.7% of control). Pure bovine carbonic anhydrase (10 mg/ml) was used as substrate in a standard protein kinase assay with chick kidney soluble protein kinase. Under these conditions the rate of ^{32}P incorporation into carbonic anhydrase was 0.185 pmol ^{32}P min^{-1} mg^{-1} anhydrase. The rate of ^{32}P incorporation into mixed histone was 10.05 pmol ^{32}P min^{-1} mg^{-1} histone under these assay conditions.

Table 5 Effect of acetazolamide injection on rat kidney cyclic AMP content

Study substance and concentration	n	*Cyclic AMP (pmol/mg protein)*
Saline	4	2.71 ± 0.37
Acetazolamide 2.5 mg	4	3.83 ± 0.49
PTH 2.5 μg	5	48.16 ± 5.28

Figures are mean ± s.e. mean. *n* = number of observations.
Animals were killed 5 min after intravenous injection.

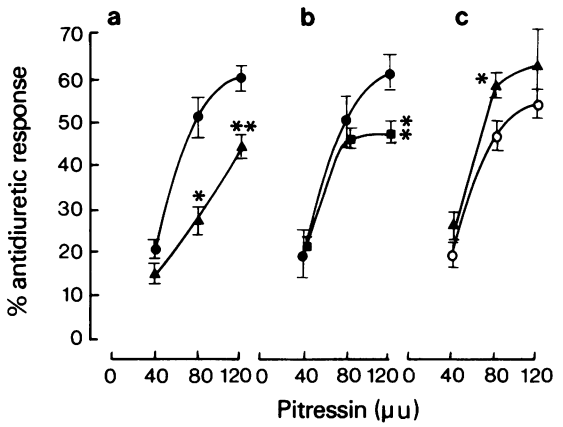

Figure 5 The effect of diuretics on the antidiuretic response to Pitressin *in vivo*. The antidiuretic response to intravenous injections of Pitressin was measured in water-loaded, alcohol-anaesthetized rats. Antidiuretic responses were calculated as the percentage decrease in urine flow in the 10 min following injection compared to the 10 min preceding injection. Animals were constantly infused at 0.2ml/min with either 'control' (57.2 mM NaCl, 92.7 mM glucose, 2.5 v/v ethanol) solution or solutions containing the diuretic under study. (a) The antidiuretic responses of 6 animals receiving control (●) and frusemide, 0.1mg/ml (▲) infusions. (b) The antidiuretic responses of 6 animals receiving control (●) and acetazolamide, 1mg/ml (■) infusions. (c) The antidiuretic responses of 5 animals receiving control (●) and ethacrynic acid. 0.2mg/ml (○) infusions. All points are mean values; vertical lines show s.e. mean. *Differs significantly from the control value by $P<0.05$ or **$P<0.01$.

2 min of injection and lasted approximately 15/20 minutes. The effect of the diuretics on the normal response to Pitressin is shown in Figure 5. Frusemide reduced the normal antidiuretic response to Pitressin but acetazolamide showed little effect except for a slight reduction of the response to a high dose of Pitressin. Ethacrynic acid actually enhanced the response to Pitressin and this effect persisted when the animals were returned to the control infusion. The continued elevation of the dose-response curve during the control infusion could not be attributed to any change in basal urine flow or to any deterioration in the condition of the animals. Any effects of the infusion of these diuretics on basal urine flow were relatively short-acting (i.e. less than 20 min) so that despite the continued infusion of the diuretic, basal urine flow was unchanged when injections of Pitressin were given at 20, 40 and 60 min of each infusion period.

Discussion

Several mechanisms have been proposed by which diuretics reduce sodium reabsorption in the nephron. Interference with plasma membrane adenylate cyclase, with Na^+-K^+-ATPase, with glycolysis, or with oxidative metabolism has been demonstrated under different conditions with various diuretics, (Jacobson & Kokko, 1976). However, no common mechanism has emerged. Although adenylate cyclase has not been implicated in renal sodium reabsorption, the central place of this enzyme in the regulation of other active metabolic processes supports a possible role as an intermediate in the complex mechanism of sodium transport. Indeed hormone-mediated changes in cyclic AMP production have been implicated in the processes of sodium transport in the turkey erythrocyte (Gardner, Klaeverman, Bilezikian & Aurbach, 1973) and in the toad bladder (Orloff & Handler, 1976). Recently diuretics have been shown to inhibit renal adenylate cyclase *in vitro* (Jakobs, Schultz & Schultz, 1972; Ferrendelli, Johnson, Chang & Needleman, 1973; Ferguson & Twite, 1973; Abramow, 1974; Ebel, 1974; Ebel & Sharp, 1975; Barnes *et al.*, 1975), and this has been confirmed in the present experiments in which ethacrynic acid and frusemide decreased basal enzyme activity and caused a marked inhibition of hormone- and fluoride-stimulated enzyme activity at high concentrations of the drugs. The importance of this as a pharmacological mechanism is difficult to assess and it is quite likely that the effect is simply a non-specific inhibition of enzyme activity. This view is supported by the lack of correlation between the diuretic effec-

Table 6 Effect of diuretics on urine flow in anaesthetized water-loaded rat preparation

Diuretic (mg, i.v.)	*Acetazolamide*	*Frusemide (% change in urine flow)*	*Ethacrynic acid*
0.04	No effect	+ 5.0 ± 5%	No effect
0.4	+ 20.2 ± 10%	+ 70.2 ± 19.9%	No effect
4.0	+ 55.7 ± 5.6%	+ 162.6 ± 42.1%	No effect

Figures are mean ± s.e. mean ($n = 5$).
The percentage increase in urine flow after a single injection of diuretic was calculated by comparing the urine flow in the 10 min following injection with that in the 10 min preceding injection.

tiveness and the effect on adenylate cyclase of these diuretics. Whereas frusemide produced a diuresis in the rat and also reduced the antidiuretic response to ADH, ethacrynic acid was ineffective as a diuretic (see also Komorn & Cafruny, 1965) but produced a much stronger inhibition of adenylate cyclase than frusemide *in vitro*. This suggests that the *in vitro* effects on the adenylate cyclase may be unrelated to the diuretic action. Preincubation of plasma membranes with ethacrynic acid greatly potentiated its effect, producing almost complete inhibition of adenylate cyclase activity, whereas preincubation with frusemide did not greatly augment its effect. The different effects of frusemide and ethacrynic acid on adenylate cyclase activity following preincubation of the diuretic with the enzyme also suggest different modes of interaction of the two drugs with the enzyme. Since ethacrynic acid can form covalent linkages with thiol groups (Mudge, 1975) and since adenylate cyclase is known to contain essential sulphydryl groups (Ferrendelli *et al.*, 1973) the inhibitory effect of ethacrynic acid does not seem surprising. It is possible that addition of hormone and substrate during incubation with the drug may protect these essential sulphydryl groups. Jakobs *et al.* (1972) and Barnes *et al.* (1975) have shown that the addition of cysteine or dithiothreitol to adenylate cyclase preparations will reduce the inhibitory effects of ethacrynic acid on the enzyme but this effect is only apparent if an excess of these agents are present before the diuretic is added, (Ferrendelli *et al.*, 1973, Barnes *et al.*, 1973; Abramow, 1974).

In contrast to frusemide, ethacrynic acid also augmented the antidiuretic response to ADH. An analogous situation has been reported by Beck, Kim & Davis (1974) with chloropropamide, in that this drug augments the peripheral action of ADH *in vivo* but inhibits the renal adenylate cyclase *in vitro*.

The claim that acetazolamide causes a stimulation of renal cortical adenylate cyclase (Rodriguez *et al.*, 1974) is of particular interest. Carbonic anhydrase inhibitors have been found to produce similar effects to PTH on renal phosphate and bicarbonate excretion and it has been suggested that the inhibition of phosphate and bicarbonate reabsorption by the proximal tubule, after PTH administration, might be mediated via cyclic AMP through the inhibition of renal carbonic anhydrase (Jacobson & Kokko, 1976). *In vitro*, Beck *et al.* (1975) found a specific inhibitory effect of PTH and cyclic AMP on renal cortex carbonic anhydrase and this required ATP, Mg^{2+} and K^+ and incubation at 37°C. Rodriguez *et al.* (1974) suggested that the phosphaturic effect of acetazolamide was mediated by cyclic AMP as they found a temporal relationship between the excretion of cyclic AMP and phosphate following acetazolamide administration *in vivo* and they also found a direct stimulation of the renal cortex adenylate cyclase system *in vitro*. However, recent studies of the effect of carbonic anhydrase inhibitors on the phosphaturic effect of PTH show that carbonic anhydrase inhibitors can mimic some but not all of the effects of PTH on phosphaturia (Engle & Steele, 1975; Knox, Haas & Lechene, 1976) and the increase in bicarbonate excretion produced by PTH is much smaller than that produced by carbonic anhydrase inhibitors (Knox, Haas & Lechene, 1975). Moreover, although Rodriguez *et al.* (1974) found an increase in urinary cyclic AMP following acetazolamide infusion in the rat, others (Knox *et al.*, 1975; Sinha, Allen, Queene & Bell, 1977), failed to find any such effect in dog and man respectively.

We have found no evidence to suggest that acetazolamide stimulates adenylate cyclase. Furthermore, there is little support for the contention that the diuretic effect of PTH is related to inhibition of carbonic anhydrase. Neither PTH nor cyclic AMP affected carbonic anhydrase activity in rat renal cortex homogenate. Also the ability of different diuretics to inhibit bovine carbonic anhydrase bore no relation to their ability to phosphorylate the carbonic anhydrase enzyme. These observations suggest that the mechanism of inhibition of carbonic anhydrase does not involve protein phosphorylation or the activation of the adenylate cyclase system. In these and other experiments (Woodford, Leegwater & Drance, 1961) it is clear that acetazolamide on its own produced a potent non-competitive inhibition of carbonic anhydrase activity. This was maximal within 2 min of contact with the enzyme and a 50% inhibition has been found with 10^{-9} to 10^{-8}M acetazolamide (Woodford *et al.*, 1961). In contrast to this specific action on the carbonic anhydrase enzyme, 10^{-3}M acetazolamide produced only a slight inhibition of renal adenylate cyclase.

Finally the question of whether the activity of carbonic anhydrase is altered by phosphorylation or dephosphorylation of the enzyme and whether this is influenced by PTH is far from settled. Beck *et al.* (1975) reported an inactivation of carbonic anhydrase by PTH and cyclic AMP (in the presence of ATP, Mg^{2+}, K^+ and incubation at 37°C) whereas Narumi & Miyamoto (1974) and Dietsch, Holke, Pochhammer & Siegmund (1976), (under similar conditions) showed activation of renal carbonic anhydrase by PTH and cyclic AMP. The present study and that of Garg (1976) failed to show any inhibitory effect of PTH and cyclic AMP on renal cortex carbonic anhydrase.

In conclusion no common effect on adenylate cyclase activity explains the diuretic actions of the three drugs acetazolamide, frusemide and ethacrynic acid, although certain effects of the drugs on the enzyme can be demonstrated, which could be of pharmacological significance. Furthermore, it is not possible to conclude that acetazolamide has any action on carbonic anhydrase via a cyclic AMP-mediated system.

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