Cellular Action of Antidiuretic Hormone in Mice with Inherited Vasopressin-Resistant Urinary Concentrating Defects

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ABSTRACT Previous work has suggested that resistance to vasopressin in two strains of mice with nephrogenic deficiency of urinary concentration may entail a defect in the action of vasopressin at the cellular level. Several components involved in this action were therefore examined in vitro in renal medullary tissues from control mice (genotype VII +/+) and two genotypes with mild diabetes insipidus (DI +/+ nonsevere) and marked (DI +/+ severe) vasopressin-resistant concentrating defects. No significant differences were found in the affinity of adenylate cyclase for [8-arginine]-vasopressin (AVP), tested over a range of hormone concentration from 10^{-10} to 10^{-5} M. However, maximal stimulation of adenylate cyclase by saturating concentrations of AVP (intrinsic activity) was markedly decreased from control values in DI +/+ severe mice, and decreased to a lesser extent in DI +/+ nonsevere animals. A significant correlation was found between the activity of adenylate cyclase maximally stimulated by AVP in a given genotype, and the urine osmolality in the same animals. There were no significant differences in maximal stimulation of renal medullary adenylate cyclase in control experiments: not when stimulated nonspecifically by sodium fluoride, nor when stimulated by AVP in tissues from rats with induced water diuresis as compared to antidiuretic rats. Nor were there significant differences between VII +/+ and DI +/+ severe mice in the activity of renal cortical adenylate cyclase, either basal or when stimulated by parathyroid hormone. Furthermore, the abnormal genotypes did not differ significantly from control mice in the renal medullary activities of cyclic AMP phosphodiesterase or cyclic AMP-dependent protein kinase, nor in the content of microtubular subunits (assessed as colchicine-binding protein). The results are compatible with the view that impaired stimulation of renal medullary adenylate cyclase by vasopressin might be the sole or contributing cause of the vasopressin-resistant concentrating defect in the diseased mice; however, a causal relationship has not yet been proved.

INTRODUCTION

Studies on several strains of mice with inherited, vasopressin-resistant defects in urinary concentration have revealed a number of different causative mechanisms (1–3). On the basis of renal tissue analyses, we have suggested that the sole or major defect in two abnormal genotypes, so-called diabetes insipidus (DI)[1] +/+ nonsevere and DI +/+ severe, may involve deficient vasopressin-induced water permeability of the distal tubules and collecting ducts (4); that is, resistance to the hormone in these mice may be due to failure of some step in the cellular action of vasopressin. We have therefore investigated various components (5) in these animals.

It is now a generally accepted view that vasopressin, after binding to a specific receptor, activates adenylate cyclase, which catalyzes the conversion of ATP to cyclic AMP. The last, which serves as the intracellular medi-

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[1] *Abbreviations used in this paper:* AVP, [8-arginine]-vasopressin; DI, diabetes insipidus; PTH, parathyroid hormone; TCA, trichloroacetic acid.

TABLE I

Activity of Renal Medullary Adenylate Cyclase in Control Mice, and in Two Genotypes with Vasopressin-Resistant Urinary Concentrating Defects

Exp. no.	Urine osmolality			Activity of adenylate cyclase (Average SEM of triplicate determinations was 6.7%)									Apparent K_m for AVP (Dose of AVP required for ½ of maximal activity)		
				Basal activity without vasopressin			With 10^{-6} M AVP			With sodium fluoride (10 mM)					
	VII +/+ (Control)	DI +/+ Non-severe	DI +/+ Severe	VII +/+	DI +/+ Non-severe	DI +/+ Severe	VII +/+	DI +/+ Non-severe	DI +/+ Severe	VII +/+	DI +/+ Non-severe	DI +/+ Severe	VII +/+	DI +/+ Non-severe	DI +/+ Severe
	mosmol/kg H_2O			*pmol cyclic AMP/min per mg membrane protein*									$\times 10^{-9}$ *M*		
I	2,406 (4)*	1,336 (2)	158 (2)	11.2	17.5	18.8	127.1	105.0	84.8	188.5	107.0	106.0	1.0	5.37	5.37
II	2,782 (5)	1,978 (4)	250 (4)	10.8	10.9	13.2	107.3	81.2	61.2	120.6	141.8	151.0	3.16	5.37	5.37
III	2,733 (8)	2,266 (8)	139 (5)	15.4	10.7	10.7	123.4	100.9	89.5	129.1	96.7	124.2	2.0	2.0	2.57
IV	2,722 (6)	1,441 (6)	161 (6)	13.0	10.8	13.9	86.2	58.9	41.7	89.1	81.0	76.3	1.08	1.71	1.36
Mean‡	2,661	1,755§	177‖	12.6	12.5	14.2	111.0	86.5¶	69.3**	131.8	106.6	114.4	1.81	3.61	3.66
±SEM	±86	±221	±25	±1.0	±1.7	±1.7	±9.3	±10.6	±11.1	±20.8	±12.9	±15.7	±0.5	±1.02	±1.01

Mice with mild (DI +/+ nonsevere) and marked (DI +/+ severe) nephrogenic diabetes insipidus differed from controls (VII +/+) in the adenylate cyclase activity stimulated by maximal dose of vasopressin but not in other parameters.

* Numbers in parentheses denote number of animals.

‡ The significance of the differences between the three strains was evaluated by the two-sided paired *t* test.

§ Significantly different from VII +/+ control ($P < 0.02$).

‖ Significantly different from DI +/+ non-severe ($P < 0.01$).

¶ Significantly different from both VII +/+ and DI +/+ severe ($P < 0.01$).

** Significantly different from both VII +/+ and DI +/+ nonsevere ($P < 0.01$).

ator for vasopressin (6, 7), is broken down to 5'-AMP by the enzyme cyclic AMP phosphodiesterase, which itself is not influenced by vasopressin (8). Cyclic AMP, by a series of as yet undefined steps, is thought to lead to increased water permeability of certain plasma membranes, such as the luminal membrane of distal tubules and collecting ducts (9, 10). The unknown steps may involve, among others, the following: phosphorylation of specific membrane proteins, catalyzed by cyclic AMP-dependent protein kinase (11); and integrity of the microtubular system (12).

In the present study, some of these elements in the cellular mode of action of vasopressin, namely, adenylate cyclase, cyclic AMP phosphodiesterase, protein kinase, and microtubular protein, were examined in the VII +/+ strain (control mice) and in the two abnormal genotypes manifesting mild (DI +/+ nonsevere) and marked (DI +/+ severe) nephrogenic defects in urinary concentration.

METHODS

The experimental animals were adult mice of both sexes. The genotypes and number of animals used for determination of adenylate cyclase are listed in Table I; tissues from the same mice, plus some additional animals, were used for measuring the other constituents (Tables III, IV, and V). VII +/+ mice served as controls for this study; their mean urine osmolality, while they ate and drank ad lib, was 2,661 mosmol/kg H_2O. DI +/+ nonsevere mice concentrated their urine to about 1,755 mosmol/kg, and this value could not be raised significantly by giving supramaximal doses of vasopressin (2). DI +/+ severe mice excreted urine with an osmolality of 177 mosmol/kg, and again this concentrating defect could not be corrected with exogenous vasopressin.

All animals were raised in Hanover, N. H. They were kept in an air-conditioned room maintained between 21–25°C, and they had free access to tap water and food (Purina Laboratory Rat Chow, Ralston Purina Co., St. Louis, Mo.). Before shipment, the urine osmolality of each animal was measured with an Osmette (Precision Systems, Inc., Natick, Mass.) on a random urine sample obtained between 9 a.m. and noon, while the mouse was eating and drinking ad lib. The mice were then shipped by air to Rochester, Minn., and they were sacrificed within 1–2 wk after shipment. The experiments were conducted over a 2-yr period. Each shipment included mice of all three genotypes; thus, each experiment included its own control, in that it consisted of simultaneous measurements in VII +/+ mice and in the two abnormal genotypes.

Mice were killed by cervical dislocation and both kidneys were quickly excised and chilled in ice-cold 0.9% NaCl solution. The entire outer and inner medullae were dissected from both kidneys with the aid of a magnifying glass, and the medullary tissues from the animals in a given genotype were pooled for preparation of adenylate cyclase or cyclic AMP phosphodiesterase. Preparations for cortical parathyroid hormone (PTH)-sensitive adenylate cyclase, cyclic AMP-dependent protein kinase, and colchi-

cine-binding protein were prepared from tissues of individual animals.

Preparations

Adenylate cyclase and cyclic AMP phosphodiesterase. The tissues were minced and homogenized in a glass-Teflon homogenizer, in a medium (1:4 wt/vol) having the following composition: 0.25 M sucrose, 5 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 600 *g* for 10 min. The sediment was resuspended in a medium of the above-mentioned composition save for the absence of sucrose, and centrifuged again at 600 *g* for 10 min. This washed 600 g sediment was used as the source of adenylate cyclase. It was divided into small portions immediately after preparation, frozen in dry ice, and stored at −80°C.

In the control experiments with rats, in which the effect of polyuria on adenylate cyclase activity was studied (see Table II), renal medullary adenylate cyclase was prepared by the method described above, with the following modifications: medullary tissue from a single animal was processed separately, and 2,000 *g* (13) rather than 600 *g* sediment was used.

The supernate from the first centrifugation described above was centrifuged again at 100,000 *g* for 60 min. The clear supernate was dialyzed overnight against 1,000 times its volume of 10 mM Tris-HCl buffer (pH 7.1). The dialyzed supernate was also divided into small portions, frozen in dry ice, and stored at −80°C; it served as the source of cyclic AMP phosphodiesterase.

Protein kinase and microtubular protein. These extracts were prepared from individual mice. Dissected medullary tissue was homogenized in 0.3 ml of a medium having the following composition: 0.25 M sucrose, 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 1,500 *g* for 10 min. The supernate was mixed with 0.2 ml of buffer containing 30 mM potassium phosphate, 15 mM $MgCl_2$, and 4×10^{-4} M GTP (pH 7.0), and centrifuged at 100,000 *g* for 60 min. The 100,000 *g* supernate was frozen in dry ice and stored at −80°C.

Total protein contained in all preparations was measured by the method of Lowry, Rosebrough, Farr, and Randall (14).

Assays

Adenylate cyclase activity was assayed by the method of Bär and Hechter (15), with modifications described in our previous studies (16, 17). Preparations of adenylate cyclase (30–70 μg protein/tube) were incubated in a total volume of 50 μl having the following composition: 0.1 mM [α-^{32}P]ATP ($1–2 \times 10^6$ cpm), 4 mM $MgCl_2$, 0.1% bovine serum albumin (wt/vol), 0.1 mM EDTA, 0.5 mM cyclic AMP, 25 mM creatinine phosphate, 0.1 mg/ml creatinine kinase (Calbiochem, San Diego, California), and 40 mM Tris-HCl (pH 7.5). Incubations were carried out at 37°C for 20 min, and reactions were terminated by addition of an excess of unlabeled ATP, 5'AMP, and cyclic AMP, and by boiling in a water bath for 3 min (15). The samples were then concentrated by freeze-drying (15), and cyclic AMP was separated from substrate by polyethyleneimine-cellulose ion-exchange thin layer chromatography, as described by Bär and Hechter (15). The blank count was about 0.03% or less of ATP apparently converted to cyclic AMP in the absence of added adenylate cyclase preparation, as described in the original method (15). The enzyme activity was expressed in picomoles of cyclic AMP formed per minute per milligram protein.

TABLE II

Basal and Maximal Activity of Renal Medullary Adenylate Cyclase in Rats Drinking ad lib and in Rats during Water Diuresis Induced by Drinking 5% Glucose

	Urine osmolality	Activity of adenylate cyclase: Basal	Activity of adenylate cyclase: Maximal stimulation by AVP*
Drinking ad lib (6)‡	1,534±83‖	18.7±3.4	41.7±9.6
Drinking glucose (6)	492±92	18.4±2.3	43.3±4.4
P value	<0.001	>0.9	>0.8

* In response to 2×10^{-6} M AVP.

‡ Numbers in parentheses denote number of animals.

‖ Mean±SEM.

The enzyme was measured under basal conditions (without added vasopressin) and in response to 10^{-10}–10^{-5} M [8-arginine]-vasopressin (AVP). Dose-response curves were run in all three strains of mice, and in all the plateau of maximal activity was reached at 10^{-7} M; these results are thus identical to those reported by us on other, normal strains of mice (17). The standard error of triplicate determinations of adenylate cyclase in samples of the same membrane preparation averaged ±6.7% pmol cyclic AMP/min per mg protein.

To be certain that the differences noted in mice were specific to the abnormal genotypes and not simply a function of water diuresis, urinary dilution was induced in adult Sprague-Dawley rats by having them drink 5% glucose solution for 6 days (18). Rats instead of mice were used because the greater amount of tissue in rats permits testing medullary extracts from single animals. For this experiment, urine was collected in individual metabolism cages, and the urine osmolality was measured in a Fiske osmometer (Fiske Associates, Inc., Uxbridge, Mass.).

Cyclic AMP phosphodiesterase was measured by the method described by Thompson and Appleman (19). The incubation mixture in this assay had the following composition: 5×10^{-4} M [^{3}H]cyclic AMP, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.0).

Cyclic AMP-dependent protein kinase was assayed by the method employed in our previous study (11), with some modifications. The protein substrate used was either endogenous, i.e., proteins of the cytosol contained in the enzyme preparation, or exogenous, in the form of 100 μg of mixed calf thymus histones (Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); otherwise the incubation mixture was as described previously (11). The enzyme reaction was stopped by addition of 1 ml of 10% trichloroacetic acid (TCA). Then 0.1 ml of 0.3% albumin was added, then another 1 ml of 10% TCA, and after thorough mixing, the protein precipitate was collected by centrifugation. This precipitate was dissolved in 0.1 ml of 1 M NaOH and then reprecipitated by addition of 3 ml of 10% TCA. The precipitate was collected on a 0.45-μm HAWP Millipore cellulose ester filter (Millipore Corp., Bedford, Mass.) and washed five times with 10 ml of 5% TCA containing 0.1 mM potassium phosphate. The activity of protein kinase was measured under basal conditions (without added nu-

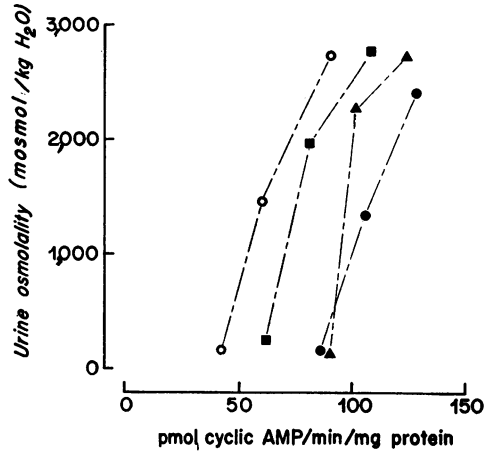

FIGURE 1 Relationship between the activity of renal medullary adenylate cyclase stimulated by a maximal dose (10^{-6} M) of AVP and the urine osmolality in the three genotypes of mice listed in Table I. The symbols, ● ■ ▲ ○, represent experiments I–IV (Table I), respectively; the values for any set of three similar symbols were obtained simultaneously and are connected by interrupted lines. The association between the two variables was consistent in each experiment and is statistically significant ($P < 0.001$).

cleotide) and after maximal stimulation with 10^{-6} M cyclic AMP; the results were expressed as picomoles of ^{32}P incorporated into the protein substrate per minute per milligram of protein contained in the preparation.

Microtubular protein was assessed as colchicine-binding protein. The tissue extracts (50–100 μg of protein/tube) were incubated with 0.6×10^{-6} M [^{3}H]colchicine (10^{-6} cpm/tube) for 80 min at 37°C, in 0.3 ml of a buffer containing 10 mM $MgCl_2$, 10 mM potassium phosphate, and 6×10^{-5} M EDTA (pH 6.8). The protein-bound [^{3}H]-colchicine was detected by absorption on DEAE cellulose filter paper, as described by Weisenberg, Borisy, and Taylor (20). The radioactivity of [^{3}H]colchicine bound to protein was counted in Bray's solution. Under the conditions used, the binding of [^{3}H]colchicine to cytosolic protein was proportional to time for at least 90 min, and proportional to protein concentration up to at least 150 μg/tube. Results were expressed as picomoles of [^{3}H]colchicine bound per incubation time per milligram of protein contained in the preparation.

Assays for enzyme and colchicine-binding activities were each run two or three times, and the means of the duplicate or triplicate determinations have been given in the tables.

Results were analyzed statistically by Dr. Peter C. O'Brien, Section of Medical Research Statistics of the Mayo Clinic and Foundation. Student's group *t* test or two-sided paired *t* test were used, and the data in Fig. 1 were analyzed by the test for association under a null hypothesis of no correlation (21).

Chemicals were obtained from the following sources: [γ-^{32}P]ATP or [α-^{32}P]ATP, from ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif.; [^{3}H]colchicine and [^{3}H]cyclic AMP, from New England Nuclear, Boston, Mass.; and synthetic AVP, from Sigma Chemical Co., Inc., St. Louis, Mo. Purified bovine PTH was a gift of Dr. Claude D. Arnaud of the Mayo Clinic, Rochester, Minn.

RESULTS

The activity of adenylate cyclase was measured in the control and two abnormal genotypes in four separate experiments (Table I). The enzyme was assayed under three conditions: (*a*) in the absence of hormone (basal activity); (*b*) in the presence of 10^{-6} M for maximal stimulation; and (*c*) after nonspecific stimulation with 10 mM NaF, a concentration which, in preliminary experiments, was found to be optimal for stimulation of mouse renal medullary adenylate cyclase.

The results are summarized in Table I. The three strains differed markedly in urinary concentrating ability, as reflected in the urine osmolalities determined while the animals were eating and drinking ad lib; the osmolalities cannot be raised significantly by optimal treatment with exogenous vasopressin (2). There were no significant differences among the three strains in basal activity of adenylate cyclase, or in the activity when stimulated by 10 mM NaF.

However, when stimulated maximally by saturating doses of vasopressin (10^{-6} M AVP) the activities of adenylate cyclase were significantly lower in both abnormal genotypes than in the control animals. Moreover, the AVP-stimulated activity of renal medullary adenylate cyclase was significantly lower in mice with the marked concentrating defect (DI +/+ severe) than in animals with the milder defect (DI +/+ nonsevere) (Table I).

The absolute activities of adenylate cyclase measured under maximal stimulation with AVP varied from experiment to experiment, a feature observed also by others (22, 23). Nevertheless, in each experiment, the values from mice with concentrating defects were consistently lower than in control animals (Table I and Fig. 1).

The dose of AVP required for one-half of the maximal activity of adenylate cyclase reflects the affinity (apparent K_m) of the renal receptor for the hormone (7, 17, 21, 22). This dose was assessed from dose-response curves (7), as in our previous study (17) and by Bockaert, Roy, Rajerison, and Jard (22, 23). The results presented in Table I show that there were no statistically significant differences in this parameter among the three genotypes.[3] This finding strongly suggests that the affinity of the renal receptor for AVP is not markedly different in the three groups of mice.

To examine the possibility that the observed decreases in maximal activity might not be specific to the abnormal strains, but merely secondary to polyuria, an additional experiment was done (Table II). Basal and maximal activities of adenylate cyclase were determined in normal rats made polyuric by giving them 5% glu-

[3] Graphical evaluation with a Hill plot (24) also did not show significant differences between strains.

TABLE III

Activity of Renal Cortical PTH-Sensitive Adenylate Cyclase in Control Mice and in Mice with Vasopressin-Resistant Urinary Concentrating Defects

		Urine osmolality	Activity of Adenylate Cyclase: Basal	Activity of Adenylate Cyclase: With 20 μg/m PTH
		mosmol/kg H_2O	*pmol cyclic AMP/min/mg protein*	
Control mice (VII +/+)	(6)*	3035±218‖	2.69±0.29	27.64±4.52
Mice with severe concentrating defect (DI +/+ severe)	(6)	153±30	2.93±0.25	25.82±2.31
P value		<0.001	>0.5	>0.5

* Numbers in parentheses denote number of animals.
‖ Mean±SEM.

cose to drink for 6 days, and these activities were compared to those in normal rats drinking ad lib. Although drinking glucose lowered the urine osmolality about threefold, there was no reduction in either basal activity of adenylate cyclase, or in the activity after stimulation with a maximal dose of AVP. This experiment constitutes an adequate control, even though the urine osmolalities in the rats do not correspond to those in the mice that we tested. The purpose of the experiment was to see whether water diuresis per se would cause a deficiency in maximal activation of adenylate cyclase; the results clearly show that when moderately severe water diuresis is induced in normal animals, there is no deficiency in vasopressin-stimulated activation of the enzyme. In contrast, such deficiency is already manifest in DI +/+ nonsevere mice with far less water diuresis than the rats that drank glucose water (Table I).

As a control to the question whether there might be a generalized defect in hormone responsive adenylate cyclases in other tissues besides the renal medulla, the activity of PTH-sensitive adenylate cyclase from the renal cortex of controls (VII +/+) was compared with the activity of the same enzyme from mice with a severe concentrating defect (DI +/+ severe) (Table III). No differences were found either in the basal[3] or PTH-stimulated activities of renal cortical adenylate cyclase.

The activities of cyclic AMP phosphodiesterase in the three genotypes are shown in Table IV. In three separate experiments, the activities of this enzyme were either the same or only slightly lower in mice having the concentrating defect. There was no statistically significant difference between the means of DI +/+ severe mice and control animals, suggesting that rate of inactivation of cyclic AMP is not responsible for the defect in the abnormal strains.

The activity of cyclic AMP-dependent protein kinase and the renal medullary content of microtubular protein were measured only in strains VII +/+ and DI +/+ severe (Table V). The two groups did not differ significantly in the activity of protein kinase, either in the basal condition without added cyclic AMP, or when maximally stimulated by the nucleotide. These conclusions were the same whether endogenous cytosolic proteins or exogenous histones were used as the substrates for phosphorylation.

Some measure of the tissue content of microtubular elements was gained by measuring the amount of colchicine-binding protein (20). In this respect, too, there were no significant differences between the two strains that varied so strikingly in their ability to concentrate urine (Table V).

DISCUSSION

In the present work, we chose the measurement of adenylate cyclase in cell-free particulate fractions, rather than the determination of cyclic AMP in whole cells, or some other method. For the following reasons, the method using isolated membranes is currently probably the most specific, sensitive, and most direct means for assessing the ability of vasopressin to stimulate the formation of cyclic AMP in the renal medulla: (*a*) Renal medullary tissue, being surrounded by cortex, cannot be frozen "instantly" by current methods. Hence, the amount of cyclic AMP measured will be a function not only of vasopressin-stimulated adenylate cyclase, but

TABLE IV

Activity of Renal Medullary Cyclic AMP Phosphodiesterase in Control Mice, and in Two Genotypes with Vasopressin-Resistant Urinary Concentrating Defects

Exp. no.	VII+/+ (control)	DI+/+ nonsevere	DI+/+ severe
	nmol cyclic AMP hydrolyzed/min per mg enzyme		
I	5.34±0.38‡ (8)*	5.29±0.20 (8)	5.29±0.21 (5)
II	6.21±0.04 (6)	5.13±0.12 (6)	4.95±0.05 (6)
III	6.57±0.23 (10)	—	6.14±0.20 (10)
Mean±SE	6.04±0.34		5.46±0.35

* Numbers in parentheses denote number of animals.
‡ Mean±SEM.
‖ Not significantly different from VII+/+ ($P > 0.3$).

[3] The specific basal activity of adenylate cyclase from renal cortex was lower than that of medulla; similar results were obtained previously in rat kidney (25) as well as in kidneys of rabbits and man (T. P. Dousa, unpublished observations).

TABLE V

Activity of Renal Medullary Protein Kinase and Content of Renal Medullary Microtubular Protein in Control Mice and in Two Genotypes with Vasopressin-Resistant Urinary Concentrating Defects

			Endogenous substrate (cytosol)		Exogenous substrate (histones)		
	n	Urine osmolality	No additions	With 10^{-6} M cyclic AMP	No additions	With 10^{-6} M cyclic AMP	Colchicine-binding activity
		mosmol/kg H_2O	*pmol ^{32}P/min per mg enzyme protein*				*pmol/incubation time per mg protein*
VII +/+ (control)	(10)	3,077±116	0.19±0.026	0.50±0.03	4.12±0.28	12.67±0.75	28.17±1.83
DI +/+ severe	(10)	139±19	0.23±0.04	0.42±0.05*	4.91±0.40	12.38±0.55	26.63±1.79
P value		<0.001	>0.3	>0.1	>0.1	>0.7	>0.6

* Stimulation of phosphorylation of endogenous substrate appears to be less in DI +/+ Severe than in control mice. This difference, however, was not statistically significant ($P > 0.05$; paired *t*-test).

also of cyclic AMP phosphodiesterase, and the two enzymes may have different activities at different temperatures (26). (*b*) The amount of cyclic AMP may be altered by certain intracellular components, such as calcium (27), prostaglandins (27, 28), GTP (22), and phosphodiesterase (26), which can be eliminated or controlled in a cell-free membrane preparation. Thus, it is not surprising that adenylate cyclase in cell-free system appears to be much more sensitive to stimulation by AVP (7, 13, 16, 17, 22, 23, 27, 29) than the response of whole tissue cyclic AMP levels to AVP (8, 26, 30–33). (*c*) The method for adenylate cyclase, but not for whole cells, permits the inclusion of an important internal control, the nonspecific stimulator of adenylate cyclase, sodium fluoride. In addition, the method using particulate fractions was especially suitable for work with mice because samples of membrane preparations from several animals could be homogeneously pooled, and small amounts of membranes containing the adenylate cyclase could be concentrated by sedimentation.

On the basis of studies previously published (4), it appears likely that the sole or major defect in mice with mild (DI +/+ nonsevere) and marked (DI +/+ severe) nephrogenic concentrating defects may lie in vasopressin-induced water permeability of the distal tubules and collecting ducts. We have examined some of the elements that are, or are thought to be, involved in the chain of events whereby vasopressin increases the water permeability of renal tubular membranes (5): combination of vasopressin with a renal receptor; stimulation of cyclic AMP formation by vasopressin, through adenylate cyclase; inactivation of cyclic AMP by cyclic AMP phosphodiesterase; phosphorylation of membrane proteins catalyzed by cyclic AMP-dependent protein kinase; and integrity of the microtubular system of epithelial cells.

The first important finding of the present study is that all of the above constituents appear to be present in the two abnormal genotypes that were examined. That is, renal medullary tissues of DI +/+ nonsevere and DI +/+ severe mice appear to contain the receptor for vasopressin, as well as the enzymes adenylate cyclase, cyclic AMP-dependent phosphodiesterase, cyclic AMP-dependent protein kinase, and microtubular subunits.

Since the basal and fluoride-stimulated activities of renal medullary adenylate cyclase in the abnormal genotypes did not differ significantly from those in normal mice, there evidently is not major deficiency in the catalytic capacity of adenylate cyclase itself. Furthermore, the adenylate cyclase of the diseased mice can be stimulated by vasopressin, and the dose of AVP required to elicit one-half of the maximal stimulation is not significantly different in these mice from the dose in controls. These findings strongly suggest that the receptor has normal affinity for AVP. However, the response of adenylate cyclase is markedly reduced at all concentrations of AVP, including maximal doses of the hormone (Table I). This deficiency could be due to a partial decrease in the number of receptor sites for vasopressin, or to a partial defect in the mechanism that couples the receptor to adenylate cyclase (25). The present results do not allow us to differentiate between these possible defects.

A generalized deficiency of hormone-sensitive adenylate cyclase would not be predicted from previous work on the diseased mice, in which no overt endocrine abnormality was observed besides resistance to vasopressin (2). The possibility of such a generalized deficiency is excluded through the present study by the lack of

significant differences between control and diseased mice, in basal or PTH-stimulated renal cortical adenylate cyclase (Table III). This adenylate cyclase is anatomically separate and functionally different from vasopressin-sensitive renal medullary adenylate cyclase (13).

No major differences were observed in cyclic AMP phosphodiesterase (Table IV), suggesting that the rate of breakdown of cyclic AMP is nearly identical in the three strains. Combining this fact with the finding of deficient production of the nucleotide suggests that the accumulation of cyclic AMP in renal tubular cells may be less in DI +/+ nonsevere and DI +/+ severe mice than in controls. For the reasons cited above, it may be difficult to test this prediction directly by current methods (26).

Several observations suggest that the differences in the activation of adenylate cyclase by AVP in the two abnormal genotypes are specific deficiencies, and not just secondary consequences of polyuria and polydipsia. When normal rats were rendered polydipsic and polyuric (Table II), there were no detectable differences in either basal or AVP-induced activity of adenylate cyclase. Schultz, Jaboks, Böhme, and Schultz (34) measured the activity of renal medullary adenylate cyclase in rats with hypothalamic diabetes insipidus (Brattleboro strain), which are in a state of chronic water diuresis that can be corrected by vasopressin (35). Although they found lower basal activity of adenylate cyclase in these rats, the stimulation by vasopressin was greater, not less, than in control animals (34).[4] In rats with polyuria induced by potassium depletion, Pawlson, Taylor, Mintz, Field and Davis (33) found an increase rather than a decrease in the renal medullary concentration of cyclic AMP in response to vasopressin. Furthermore, Finn, Handler, and Orloff (36) found that potassium-depleted toad bladders showed a reduced response in osmotic water flow, not only to vasopressin but also to cyclic AMP. They concluded that the potassium-dependent step in the permeability response to vasopressin may lie subsequent to the production of cyclic AMP. These findings, along with the observation of a close association between AVP-stimulated adenylate cyclase activity and urinary osmolality in our experiments (Fig. 1), favor the view that the observed reductions in adenylate cyclase activity are not a consequence merely of the polyuric state.

In Fig. 1, the urine osmolalities in each of the four experiments on each genotype (Table I) have been plotted as a function of the maximal AVP-stimulated activity of adenylate cyclase. There is a highly significant association between the two variables ($P < 0.001$). Proportionally, the reduction in urine osmolality in the diseased mice is much greater than the reduction in the activity of adenylate cyclase. This fact raises the question whether a 40% decrease in enzyme activity, as occurs in DI +/+ severe mice, could lead to a defect in urinary concentration that results in the excretion of hypotonic urine. Although there are no direct experimental data to answer this question, computer simulation of the countercurrent system (37) suggests that it might be possible. In such simulation, the curve relating water permeability of the distal tubules and collecting ducts to urine osmolality has a very steep portion, in which a small change in permeability leads to a very large reduction in urine osmolality (37). If the reductions in the activity of adenylate cyclase reported in this paper fall within this critical range of water permeability, the observed changes might very well account for the severe concentrating defect in DI +/+ severe mice. Furthermore, it is conceivable that only a portion of the total cyclic AMP generated at the basilar membrane through the action of antidiuretic hormone is ultimately available to effect a change in water permeability at the luminal barrier (because of enzymatic breakdown, efflux from cell, binding on proteins, etc.).

A correlation between reduced vasopressin-mediated activation of adenylate cyclase as measured in vitro and unresponsiveness to vasopressin in vivo has been observed in a number of other experimental circumstances: in nephrogenic diabetes insipidus induced by BAX 439 (25), by lithium (38–40), by vasopressinoic acid (16), and by demethylchlortetracycline (41). Furthermore, in all these instances, as in the present study, the maximal activity of adenylate cyclase was reduced while the affinity for vasopressin remained unimpaired. Finally, there are two reports that patients with hereditary nephrogenic DI showed reduced urinary excretion of cyclic AMP in response to exogenous vasopressin (42, 43). This finding is at least compatible with the view that in such patients the formation of cyclic AMP may be impaired.

But despite the significant correlation shown in Fig. 1 and other arguments that can be marshalled in favor of a causal relationship, we emphasize that the data do not prove that the reduced activation of adenylate cyclase is the cause of the concentrating defect in the diseased mice.

Two other possible components of the cellular action of vasopressin were explored. Indirect evidence supports the view that cyclic AMP-activated protein kinase may phosphorylate the plasma membrane proteins, and that this might be the basis for a change in water permeability (11). Since the natural protein substrate for

[4] The data supporting this assertion are contained in a thesis, and it is not clear from the published report (34) whether the increase in enzyme activity refers to absolute values or to a proportional increase over basal activity.

protein kinase in cells lining the distal nephron has not yet been identified, the phosphorylation of cytosolic proteins ("endogenous substrate") or of histones ("exogenous substrate") (44) was measured in the present study. Our results rule out an absence of protein kinase or a deficiency in maximal, cyclic AMP-induced stimulation of the enzyme in mice with severe diabetes insipidus (DI +/+ severe), and by implication, in the DI +/+ nonsevere strain. However, these results do not exclude the possibility that the ability of the protein kinase to phosphorylate natural protein substrate may be altered in the abnormal strains, or that the natural protein substrate may be missing in the diseased mice.

A recent study on toad urinary bladder has shown that drugs that disrupt microtubular assemblies inhibit the hydro-osmotic effect of both vasopressin and cyclic AMP (12). This finding suggests that the integrity of the microtubular system in epithelial cells might be required for cyclic AMP-mediated changes in water permeability, and the same appears to apply to mammalian kidneys (45). Studies on erythrocyte membranes provide some evidence that formation of protein microfilaments is critical for maintaining membrane structure and function, and that hereditary abnormalities in these structural proteins could cause defects in membrane function (46, 47). Epithelial cells of mammalian collecting ducts contain numerous microtubules and microfilaments (48), and the cytosol obtained from the renal medulla contains microtubular subunits (45). These subunits can be assessed as colchicine-binding protein (49, 50). The results shown in Table V rule out the possibility that the defect in DI +/+ severe mice involves a deficiency in the total content of microtubular subunits in the renal medullary tissue; again by implication, the same conclusion applies to DI +/+ nonsevere animals. The results also indicate that the colchicine-binding sites of the subunits (20, 49, 50) are not altered. However, the data do not exclude an abnormality in the polymerization of subunits into microtubular structures.

In conclusion, our results indicate that some inherited, vasopressin-resistant defects of urinary concentration in mice are associated with decreased AVP-induced activation of medullary adenylate cyclase in vitro. The specificity of the demonstrated deficiency has been shown by the lack of significant differences in the following controls: (*a*) basal activity of adenylate cyclase; (*b*) sodium fluoride-stimulated activity of adenylate cyclase; (*c*) the activity of other enzyme systems in the same tissue—phosphodiesterase and protein kinase; (*d*) the renal medullary content of another biologically active protein, colchicine-binding microtubular protein; and (*e*) the activity of renal cortical PTH-sensitive adenylate cyclase. The observations suggest that the sole or contributing cause of unresponsiveness to vasopressin in the abnormal mice may be a defect in the cellular production of cyclic AMP. However, it must be stressed that the evidence for a causal relationship is only inferential, and that possible defects in as yet unknown steps in the cellular action of vasopressin, subsequent to the formation of cyclic AMP, have not been ruled out by this study.

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