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Research Article

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Effects of Vasopressin Antagonist on Vasopressin Binding, Adenylate Cyclase Activation, and Water Flux

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Abstract

We studied the effect of an arginine vasopressin (AVP) analogue, (1-[β-mercapto-β,β-cyclopentamethylenepropionic acid],2-O-ethyltyrosine,4-valine)AVP (d[CH_2]$_5$Tyr[Et]VAVP), on the stimulation of adenylate cyclase by various hormones in the isolated nephron segments and ^{3}H-AVP binding to renal papillary membranes from the rat. The net water flux across the renal cortical collecting tubules of the rabbit was also examined. We found that d(CH_2)$_5$Tyr(Et)VAVP significantly inhibited adenylate cyclase activation by AVP in cortical, medullary, and papillary collecting tubules and in the medullary thick ascending limb. In contrast, the AVP analogue did not alter the stimulation of adenylate cyclase by parathyroid hormone in the cortical thick ascending limb, by glucagon in the medullary thick ascending limb, and by calcitonin in cortical collecting tubules. In addition, d(CH_2)$_5$Tyr(Et)VAVP blocked [^{3}H]AVP binding to renal papillary membranes. The enhanced net water transport induced by AVP in isolated, perfused rabbit cortical collecting tubules also was completely blocked by this AVP analogue. These results indicate that d(CH_2)$_5$Tyr(Et)VAVP specifically antagonizes the cellular action of AVP on the medullary thick ascending limb and on the cortical, medullary, and papillary collecting tubules. Evidence is also presented for competitive antagonism as the cellular mechanism of action.

Introduction

A specific antagonist of the antidiuretic action of arginine vasopressin (AVP)[1] has recently been synthesized by Manning et al. (1). This AVP analogue, (1-[β-mercapto-β,β-cyclopentamethylenepropionic acid],2-O-ethyltyrosine,4-valine)AVP (d-(CH_2)$_5$Tyr(Et)VAVP), inhibited the antidiuretic response to exogenous and endogenous AVP in the absence of changes in glomerular filtration rate, solute excretion, and blood pressure in the rat (2, 3). In vitro studies in pig and human medullary membranes have also shown that the AVP analogue inhibits vasopressin binding (4, 5). Since there is a 5:1 ratio of thick ascending limbs to collecting tubules in such medullary membrane preparations, a specific effect of the analogue on adenylate cyclase response of medullary collecting tubules (MCT) and papillary collecting tubules (PCT) cannot be ascertained from these studies.

The present study was undertaken to further characterize the cellular action of this AVP antagonist. Specifically, the effect of the AVP antagonist was examined on (*a*) ^{3}H-AVP binding to rat papillary membranes, on (*b*) the activation of adenylate cyclase by AVP, parathyroid hormone (PTH), calcitonin, and glucagon in specific rat nephron segments, including collecting tubule and thick ascending limb segments, and on (*c*) water flux across isolated perfused rabbit cortical collecting tubules (CCT). Taken together, the results of these in vitro experiments indicate that the AVP analogue is a specific inhibitor of the cellular action of AVP in the medullary thick ascending limb, CCT, MCT, and PCT of the rat. A specific inhibition of AVP-induced water flux across the isolated perfused CCT of the rabbit was also demonstrated. Evidence is presented in support of competitive antagonism as the cellular mechanism of action.

Methods

Microdissection of tubules. Male Sprague-Dawley rats (200–250 g body weight) were lightly anesthetized with pentobarbital (3 mg/100 g body weight) and the left kidney was perfused with 10 ml of collagenase medium (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$, 10 mM Tris, pH 7.4, 10–20 U/ml collagenase [Millipore Corp., Greenhold, NJ], 1 mg/ml hyaluronidase [Sigma Chemical Co., St. Louis, MO], and 1 mg/ml bovine serum albumin with 20 U/ml heparin) at an infusion rate of 1 ml/min. The cortical, medullary, and papillary tissue was dissected and sliced to thin strips with direction from cortex to papillary tip. Then the pieces were incubated in an aerated collagenase medium for 60 min for papillary tissue and 30 min for cortical and medullary tissues at 35°C. After incubation the tissues were washed with cold microdissection medium (same as the collagenase medium, except with 1 mM $CaCl_2$ and no collagenase, hyaluronidase, or bovine serum albumin) and kept on ice while microdissection was performed. CCT, MCT, and PCT, as well as the thick ascending limb of Henle's loop from cortex (CAL) and medulla (MAL), were dissected under a stereomicroscope using thin needles. Dissected tubules were transferred to a concave bacteriological slide by aspiration of tubules in a small droplet of microdissection medium. The tubules were then photographed to measure the lengths. The tubules were disrupted by hypoosmotic shock and freezing. After the microdissection, medium was aspirated off, 0.5 μl hypoosmotic medium (1 mM $MgCl_2$, 0.25 mM EDTA, 0.1% bovine serum albumin, and 1 mM Tris, pH 7.4) was added under the microscope; the samples were then frozen immediately by placing them on a block of dry ice. The frozen tubules were thawed once and refrozen, then kept at −80°C until assayed. The basal and hormone-stimulated activities of adenylate cyclase were stable at least for 5 d at −80°C.

Assays of adenylate cyclase and cAMP-phosphodiesterase activity on tubules. The activity of adenylate cyclase was measured according to a modification (6) of the method of Morel et al. (7). Slides with tubules (1.5–2 mm total tubule length per sample) were incubated at 37°C for 30 min in a final volume of 5.5 μl containing 0.25 mM α-[^{32}P]ATP (4 to 5 × 10^6 cpm/sample), 1 mM cAMP, 3.8 mM $MgCl_2$, 0.25 mM EDTA,

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1. *Abbreviations used in this paper:* AVP, arginine vasopressin; d(CH_2)$_5$Tyr(Et)VAVP, (1-[β-mercapto-β,β-cyclopentamethylenepropionic acid]2,O-ethyltyrosine,4-valine)AVP; CAL, thick ascending limb of Henle's loop from cortex; CCT, cortical collecting tubules; L_p, hydraulic water conductivity in cm/s · atm; MAL, thick ascending limb of Henle's loop from medulla; MCT, medullary collecting tubules; PCT, papillary collecting tubules; PTH, parathyroid hormone; SCT, salmon calcitonin.

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20 mM creatine phosphate (Calbiochem-Behring, Corp., San Diego, CA), 200 U/ml creatine phosphokinase (Calbiochem-Behring, Corp.), and 100 mM Tris, pH 7.4, with or without addition of hormone (AVP [Bachem, Torrance, CA]; glucagon and salmon calcitonin [Calbiochem-Behring, Corp.]; PTH [Beckman, Palo Alto, CA]) or AVP analogue, $d(CH_2)_5$-Tyr(Et)VAVP, or both together. The reaction mixture was sealed between two vaseline-coated concave slides and immersed in a water bath. The reaction was stopped by addition of 150 μl stopping solution (3.3 mM ATP, 5 mM cAMP, 50 mM Tris-HCl, pH 7.6, and [^{3}H]cAMP containing 1×10^4 cpm/sample to determine recovery). Produced [^{32}P]cAMP was separated according to the method of Salomon (8) using Dowex 50-×4, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA) and aluminum oxide (ICN, Cleveland, OH) columns. Enzyme activity was expressed as femtomoles cAMP produced per millimiter per 30 min. cAMP-Phosphodiesterase was assayed in a two step reaction (6). The first reaction was performed in a buffer containing 10 mM $MgSO_4$, 0.1 mM EDTA, 50 mM Tris, pH 8.0, and 1×10^{-6} M [^{3}H]cAMP (New England Nuclear, Boston, MA) as a substrate. The reaction was carried out for 20 min at 37°C and the second reaction was performed with 1 mg/ml 5'-nucleotidase (snake venom [Naja Naja]; Calbiochem-Behring Corp., La Jolla, CA) for 10 min at 37°C. The produced [^{3}H]adenosine was separated chromatographically using QAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ) and counted.

Preparation of plasma membranes for AVP-binding studies. Renal papillary membranes were prepared from Sprague-Dawley rats weighing 200–250 g as described previously (9, 10). Briefly, the renal papilla was dissected and homogenized in Tris-sucrose buffer, pH 7.4 (250 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, and 5 mM Tris), and centrifuged at 600 *g* for 10 min. After centrifugation, the pellet was washed twice in hypoosmotic solution (3 mM $MgCl_2$, 1 mM EDTA, and 5 mM Tris, pH 7.4) to separate the membranes and then resuspended them in hypoosmotic solution. These membrane preparations were employed for the studies of ^{3}H-AVP binding. The membranes were stored at −80°C until the time of binding study; such freezing did not alter binding characteristics.

^{3}H-AVP binding studies. ^{3}H-AVP was a kind gift from SmithKline & French Laboratories (Philadelphia, PA). The ^{3}H-AVP was biologically active and the specific activity was 17 Ci/mM. Binding of ^{3}H-AVP to the renal papillary membranes was conducted by modified methods of Olefsky et al. (11) and Butlen et al. (12). The reaction medium contained 0.25 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 2.5% bovine serum albumin, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM cAMP, and 40 mM Tris buffer, pH 7.4.

Approximately 100 μg of protein was used and the final volume was 100 μl. The reaction was started by addition of ^{3}H-AVP with appropriate concentration, and incubated at 30°C for 15 min. At the end of the incubation the tubes were placed in ice and vortexed, and immediately two 40-μl samples of the mixture were carefully layered over cold 300 μl of stopping solution (2.5% bovine serum albumin, 100 mM Tris-HCl, pH 7.4) in the Eppendorf microcentrifuge tube (Brinkman Instruments, Inc., Palo Alto, CA) at 2°C to 4°C. After centrifugation, the supernatant solution was aspirated off under a bright illuminator. The pellet was carefully washed with cold 300 μl of washing solution (10% sucrose, 100 mM Tris, pH 7.4) without disturbing the pellet. After aspiration of the washing solution, the tip of the microtube was cut at a point just above the pellet. The tip with pellet was transferred into a counting vial containing scintillation cocktail (Insta-gel [Packard Instrument Co., Inc., Downer's Grove, IL]) and counted in a Packard Tri-Carb 460C liquid scintillation counter. The nonspecific binding of ^{3}H-AVP was determined in the presence of 10^{-5} M AVP. The nonspecific binding at each concentration of ^{3}H-AVP was not constant. As ^{3}H-AVP increased the nonspecific binding was increased gradually also. For example, at 5×10^{-9} M ^{3}H-AVP the nonspecific binding was 8.8%; at 1×10^{-8} M, 15.6%; at 2.5×10^{-8} M, 21.4%; at 5×10^{-8} M, 28.5%; at 7.5×10^{-8} M, 48%. The binding was linear up to 300 μg of protein and reached maximum at 8 to 10 min. Protein was measured by the modified method of Lowry et al. (13).

Study of net water flux across the isolated perfused rabbit CCT. Collecting tubular perfusion was carried out by slight modifications of the method developed by Burg et al. and Horster and Zink (14, 15). New Zealand white rabbits weighing 1–2 kg were maintained on rabbit chow (Purina, St. Louis, MO) and ad libitum water. The animals were killed by cervical dislocation and the left kidney was removed quickly. 1–2-mm slices were cut along the cortico-medullary axis and placed in a glass dish in a solution of 115 mM NaCl, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5.0 mM KCl, 10 mM sodium acetate, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 5.5 mM dextrose with 5% bovine serum albumin. This solution was maintained at 4°C and pH of 7.4 during dissection. The cortex was separated from outer medulla and CCT were dissected from medullary rays. Collecting tubules were identified by the presence of branching, straight configuration, light granular appearance with fuzzy borders, and generally open lumens. Tubules were taken distal to the last branch. Tubules were transferred to a lucite perfusion chamber (volume ~ 2.0 ml) set on the stage of an inverted phase-contrast microscope (Olympus, Model CK, Tokyo, Japan).

Custom capillary glass (Drummond Scientific Co., Broomall, PA) was used to fashion (Stoelting Microforge, Chicago, IL) pipettes of required dimensions. Tubules were aspirated into the tip of a holding pipette (outer diameter ~ 32–36 μm) up to a constriction of 16–18 μm. A perfusion pipette (~12–14 μm) was advanced into the lumen and extended 50–100 μm beyond the tip of the holding pipette. A large outer pipette containing Sylgard 184 (Dow Corning Corp., Midland, MI) was advanced over the tip of the holding pipette so that Sylgard covered the holding pipette and tubule up to the tip of the perfusion pipette. A fluid exchange pipette inside the perfusion pipette was used to change perfusate composition. The free end of the tubule was aspirated into a collection pipette, which had an inner diameter slightly less than the outer diameter of the tubule. The tip of the pipette was filled with Sylgard 184 and the tubule was aspirated through the Sylgard. Water equilibrated mineral oil was introduced into the collection pipette and a calibrated volumetric pipette (80–110 nl) was used to obtain precisely timed samples of constant volume. Samples were separated by mineral oil.

Tubules were bathed in the same solution used for dissection, except that no albumin was present. Bath fluid of pH 7.4 and temperature of 25°C (16) was completely changed every 5 min. All studies were carried out at 25°C because the hydroosmotic response to vasopressin appears to be stable at this temperature (16). Tubules were perfused at rates of 5–15 nl/min by adjusting hydrostatic pressure of the fluid entering the fluid exchange pipette. Perfusion fluid composition was the same as bathing fluid, except that the final concentration of NaCl was reduced to 50 mM. This resulted in an approximate 100-mOsm gradient from lumen to bathing fluid. Perfusion fluid contained a sufficient amount of ^{14}C-inulin (New England Nuclear, Boston, MA) to result in collected fluid counts per minute 10-fold above background The [C^{14}] inulin was dissolved in perfusion solution a few minutes before use. Tubules were perfused for 150–180 min before studies were begun. The tubule was visually inspected throughout the duration of the study. Tubular length was measured at the conclusion of each study by a calibrated microscope eyepiece reticle.

Collected samples were placed under water equilibrated mineral oil in the bottom of a siliconized dish. 50-nl aliquots of these samples were taken for scintillation counting (Packard Tricarb 460C).

AVP was obtained from Parke Davis (Detroit, MI) in the form of Pitressin. This was diluted in bathing solution and frozen at −4°C until use in a final concentration of 250 μU/ml. AVP analogue was administered at 2.5×10^{-7} M in the bathing fluid.

Perfusion rate was calculated by the rate of appearance of the impermeant marker [C^{14}] inulin in the collection pipette according to the algorithm: Perfusion rate = $([C^{14}]_c)/([C^{14}]_p) \times$ collection rate, where $[C^{14}]_p$ equals cpm of perfusate and $[C^{14}]_c$ equals cpm of collected fluid. Collection rate was measured directly with a calibrated volumetric pipette and stopwatch. Net volume of water reabsorption (J_v [nl/mm/min]) was calculated as perfusion rate minus collection rate.

Hydraulic conductivity was calculated from the formula

$$L_p = \frac{-V_0 C_0}{RTA}\left[\frac{C_0 - C_L}{C_0 C_L C_b} + \frac{1}{(C_b)^2}\ln\frac{(C_L - C_b)C_0}{(C_0 - C_b)C_L}\right] \text{cm/s}\cdot\text{atm} \times 10^{-7},$$

where V_0 is the perfusion rate in cm^3/s; C_0, C_L, and C_b are the osmotic concentrations of perfusate, collected fluid, and bathing medium, respectively; R is the gas constant, T is the absolute temperature, A is the surface area (calculated from the length of the tubule and an average internal diameter of 20 μm), L_p is the hydraulic water conductivity in cm/s · atm. Osmolality of bathing fluid and perfusate were measured using an Advanced Osmometer. Collecting fluid osmolality was calculated as perfusate osmolality × cpm collected fluid/cpm perfused fluid.

Two groups of studies were performed. In eight tubules, collections were made for 30 min before and 50 min after AVP. In seven additional tubules, AVP analogue was given. Collections were made for 30 min after the analogue. At this point, AVP was given and additional collection for 40 min after AVP were made. The two groups of tubules were perfused at 5–15 nl/min and their lengths averaged 0.95–1.0 mm.

Another set of experiments were performed using each tubule as its own control to test the effect of AVP analogue on the AVP-stimulated L_p. In these experiments AVP analogue (2.5×10^{-7} M) was introduced into the bath for ~1 h. AVP (250 μU/ml) was then added to the bath. 30–40 min later the AVP analogue was removed from the bath. In our experience, AVP effects at this dose are maximal within 30–45 min. Comparisons were then made between tubular L_p in the presence of AVP analogue + AVP and AVP alone.

Statistics. The results were analyzed by using unpaired Student's t test.

Results

Effect of $d(CH_2)_5$Tyr(Et)VAVP on the adenylate cyclase activation by AVP PCT. As shown in Fig. 1, 10^{-8} M of $d(CH_2)_5$Tyr(Et)VAVP significantly inhibited the stimulation of adenylate cyclase at the various concentrations of AVP (10^{-10} to 10^{-6} M) in the rat renal PCT. 10^{-9} M $d(CH_2)_5$Tyr(Et)VAVP also significantly inhibited the adenylate cyclase stimulation by AVP concentrations from 10^{-9} to 10^{-8} M. Stimulation of adenylate cyclase by 5×10^{-8} to 10^{-6} M AVP was not significantly inhibited by 10^{-9} M AVP. The basal activity was not changed in the presence of 10^{-8} M $d(CH_2)_5$Tyr(Et)VAVP.

MCT. Stimulation of adenylate cyclase by AVP in the MCT demonstrated the same dose response characteristics as in PCT, except that maximal stimulation of adenylate cyclase was reached at 10^{-6} M AVP (data not shown). At 10^{-8} M AVP, adenylate cyclase was significantly stimulated as shown in Table I. 10^{-8} M $d(CH_2)_5$Tyr(Et)VAVP did not affect the basal adenylate cyclase activity. In the presence of 10^{-8} M $d(CH_2)_5$Tyr(Et)VAVP, the stimulation of adenylate cyclase by 10^{-8} M AVP was significantly inhibited.

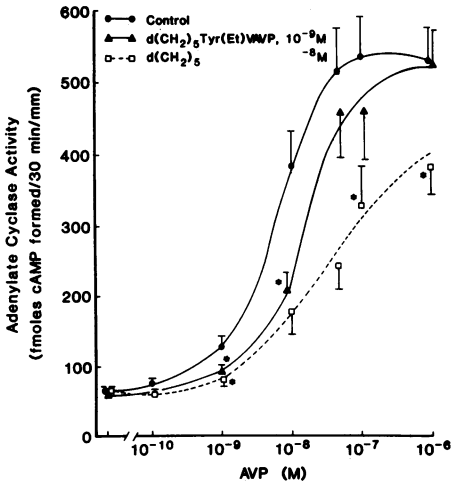

Figure 1. Effect of $d(CH_2)_5$Tyr(Et)VAVP, diminishing profoundly the adenylate cyclase response to AVP in rat renal PCT (n = 8). *Significantly different from the control as P value was <0.05 or lower.

CCT. Preliminary studies showed that the adenylate cyclase response to AVP in CCT is less sensitive than that in MCT or PCT in the rat, thus stimulation of CCT adenylate cyclase was assayed in the presence of 10^{-7} M AVP. As shown in Table I, 10^{-7} M AVP significantly increased adenylate cyclase. $d(CH_2)_5$Tyr(Et)VAVP at 10^{-7} M completely blocked the stimulation of adenylate cyclase by AVP.

Salmon calcitonin (SCT), in both maximal (3×10^{-7} M) or submaximal stimulatory concentration (1.5×10^{-8} M), significantly increased the adenylate cyclase in CCT, and 10^{-6} M $d(CH_2)_5$Tyr(Et)VAVP did not affect the stimulation of adenylate cyclase by SCT (Table II).

MAL. The adenylate cyclase in MAL from the rats that were used for studies of collecting tubules (Charles River Sprague-Dawley, Wilmington, MA) did not respond to AVP. A different strain of Sprague-Dawley rats (Harlan Rats, Indianapolis, IN) were used in the study of MAL. The adenylate cyclase response to AVP in the collecting tubules was the same in the Charles River and Harlan rats (17).

AVP (10^{-6} M) significantly stimulated adenylate cyclase as shown in Table I. $d(CH_2)_5$Tyr(Et)VAVP itself did not show any effect on the basal adenylate cyclase activity at a concentration of 10^{-6} M. This AVP analogue significantly inhibited the stimulation of adenylate cyclase by 10^{-6} M AVP.

Glucagon (10^{-8} and 10^{-6} M) also significantly stimulated adenylate cyclase in MAL of rat as shown in Table II. $d(CH_2)_5$Tyr(Et)VAVP did not inhibit the stimulation of adenylate cyclase by submaximal (10^{-8} M) and maximal (10^{-6}) stimulatory concentrations of glucagon.

CAL. The absence of an effect of $d(CH_2)_5$Tyr(Et)VAVP to alter the stimulation of adenylate cyclase by PTH in the CAL is shown in Table II. PTH (1 and 10 IU/ml) significantly increased the adenylate cyclase, and 10^{-7} M $d(CH_2)_5$Tyr(Et)VAVP had no effect on this activation of adenylate cyclase by either concentration of PTH in CAL.

Effect of $d(CH_2)_5$Tyr(Et)VAVP on cAMP-phosphodiesterase in the PCT. cAMP-phosphodiesterase activity in the PCT was measured in the presence of 10^{-8} M $d(CH_2)_5$Tyr(Et)VAVP, a concentration that completely blocked the stimulation of adenylate cyclase by AVP in PCT. The AVP analogue did not alter the activity of cAMP-phosphodiesterase in the PCT (basal 9.30±0.62; $+10^{-8}$ M AVP analogue 8.89±0.86 fmol/min/mm, n = 5, NS).

Studies of ^{3}H-AVP binding to renal papillary plasma membranes. ^{3}H-AVP binding to the renal papillary membranes was studied in the absence and presence of 10^{-9}, 10^{-7}, and 10^{-6} M AVP analogue. ^{3}H-AVP binding to the renal papillary membranes was dose-dependent when the specific ^{3}H-AVP binding was plotted as a function of the ^{3}H-AVP concentration in the incubation medium (Fig. 2). 10^{-6} M $d(CH_2)_5$Tyr(Et)VAVP almost completely blocked the ^{3}H-AVP binding to the renal papillary membranes. 10^{-7} M AVP analogue significantly inhibited the ^{3}H-AVP binding to the renal papillary membranes at the various concentrations of ^{3}H-AVP. In the curve with 10^{-7} M AVP analogue, ^{3}H-AVP binding at 10^{-9} to 2.5×10^{-8} M ^{3}H-AVP was almost completely blocked, then the inhibitory

Table I. Effect of d(CH$_2$)$_5$Tyr(Et)VAVP on the Adenylate Cyclase Response to AVP in the Rat Renal CCT, MCT, and PCT (fmol cAMP formed/30 min/mm)

	Basal	AVP*	*P* value: basal vs. AVP	Antagonist	AVP + antagonist*	*P* value: antagonist vs. AVP + antagonist	*P* value AVP vs. AVP + antagonist
CCT (n = 6)	13.42±1.21	37.39±5.25	<0.005	14.52±0.81	17.37±1.97	NS	<0.02
MCT (n = 5)	26.67±4.40	132.23±18.96	<0.001	32.07±5.72	51.03±7.24	NS	<0.02
MAL (n = 6)	7.42±0.92	43.75±4.58	<0.001	8.90±0.89	15.44±4.72	NS	<0.005

Each value represents means±SEM. * CCT, 10^{-7} M; MCT, 10^{-8} M; MAL 10^{-6} M.

action was less potent as the ^{3}H-AVP concentration was increased to a concentration $>5 \times 10^{-8}$ M ^{3}H-AVP. 10^{-9} M d(CH$_2$)$_5$Tyr(Et)VAVP significantly inhibited the ^{3}H-AVP binding at 10^{-9} to 10^{-8} M ^{3}H-AVP; however, the inhibition was not significantly different at $>2.5 \times 10^{-8}$ M ^{3}H-AVP. K_i of the AVP analogue was 9×10^{-9} M.

Study of net water flux across the isolated perfused rabbit CCT. The effect of AVP on hydraulic conductivity in the presence and absence of the analogue is depicted in Fig. 3. d(CH$_2$)$_5$Tyr(Et)VAVP exerted a transient effect to increase L_p (30±17 to 70±14 cm/s·atm $\times 10^{-7}$). This increase in L_p after the AVP analogue was observed in the first or second collection period after the analogue, thereafter L_p returned to basal levels. Because of the transient agonist effect of the AVP analogue, AVP was administered 40–50 min after the tubules had been bathed in the analogue. AVP increased L_p from 25 to 95 cm/s·atm $\times 10^{-7}$ ($P < 0.02$) in tubules not pretreated with the AVP analogue. In contrast, no significant increase in L_p was observed when AVP was administered to tubules bathed with the AVP analogue.

In the paired CCT experiments, J_v with AVP analogue alone (0.25±0.09 nl/mm·min) was not significantly different from AVP analogue + AVP (0.05±0.09 nl/mm·min). The comparison between AVP analogue + AVP and AVP alone is shown in Fig. 4. Each line in the figure connects L_p obtained from a single tubule in the presence of AVP analogue + AVP (mean 8.7±5.0 $\times 10^{-7}$ cm/s·atm) with the L_p of that same tubule after the AVP analogue was removed from the bath (mean 79±41 $\times 10^{-7}$ cm/s·atm). As can be seen AVP analogue significantly inhibited the effects of AVP until the AVP analogue was removed from the bath ($P < 0.05$).

Discussion

In this present study the cellular action of a newly synthesized AVP analogue, d(CH$_2$)$_5$Tyr(Et)VAVP, was examined. This analogue has been shown in vivo to block the antidiuretic effect of AVP in the anesthetized and conscious rat (2, 3). The AVP analogue has also been shown to inhibit AVP binding to pig and human medullopapillary membranes (4, 5). In these medullopapillary membrane preparations the effect of the AVP analogue on the MAL, MCT, or PCT cannot be differentiated.

In the present studies, ^{3}H-AVP binding studies were performed in rat papillary membranes that should not contain re-

Table II. Effect of AVP Antagonist, d(CH$_2$)$_5$Tyr(Et)VAVP, on the Adenylate Cyclase Response to PTH, Calcitonin, and Glucagon in the Rat Renal CAL, CCT, and MAL (fmol cAMP formed/30 min/mm)

	Basal	Hormones		*P* value: basal vs. hormones	Antagonist*	Hormones + antagonist‡		*P* value: antagonist vs. hormones + antagonist	*P* value: hormones vs. hormones antagonist
		+PTH 10 IU/ml	1 IU/ml			+PTH 10 IU/ml	1 IU/ml		
CAL n = 6	16.71±1.38	344.09±37.01		<0.001	17.55±1.67	338.47±39.31		<0.001	NS
			102.06±11.16‡	<0.001			112.40±18.35‡	<0.001	NS
		+ Calcitonin 3×10^{-1} M	1.5×10^{-8} M			+ Calcitonin 3×10^{-1} M	1.5×10^{-8} M		
CCT n = 6	35.10±8.53	242.81±15.80		<0.001	35.80±8.60	255.31±23.69		<0.001	NS
			100.92±15.78‡	<0.005			86.27±12.65‡	<0.001	NS
		+ Glucagon 10^{-6} M	10^{-8} M			+ Glucagon 10^{-6} M	10^{-8} M		
MAL n = 5	7.18±1.00	211.88±29.07		<0.001	6.76±1.03	219.98±27.60		<0.001	NS
			58.42±10.09‡	<0.001			68.00±10.49‡	<0.001	NS

Each value represents mean±SEM. * CAL 10^{-7} M, CCT 10^{-6} M. ‡ *P* value, maximal vs. submaximal concentrations of hormones; <0.001.

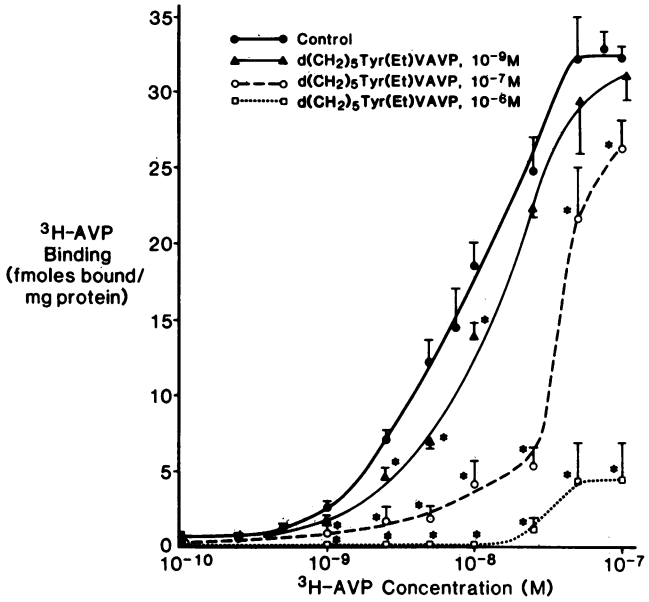

Figure 2. Diminution of ^{3}H-AVP binding to rat renal papillary membranes in the absence or presence of AVP analogue, d$(CH_2)_5$Tyr(Et)VAVP (n = 7). The specific ^{3}H-AVP binding is plotted as a function of ^{3}H-AVP concentration in the incubation medium. Each point represents mean±SEM. *Significantly different from the control as P value was <0.05 or lower.

ceptor sites of AVP action on the MAL or MCT. In these studies, the dissociation constant in the renal papillary membrane (1.4×10^{-8} M) was similar to that observed in human medullopapillary membranes (1.6×10^{-8} M) (5). The apparent inhibition constants (K_i) of d$(CH_2)_5$Tyr(Et)VAVP in rat papillary membranes (9×10^{-9} M) was also similar to that observed in medullopapillary membranes of human (5.9×10^{-9} M) (5).

The earlier results using renal medullopapillary membranes (4, 5) were compatible with a competitive antagonism as the cellular action of d$(CH_2)_5$Tyr(Et)VAVP. In the present study (Fig. 2), the curve of ^{3}H-AVP binding in the presence of 10^{-7} M d$(CH_2)_5$Tyr(Et)VAVP demonstrated less inhibition of ^{3}H-AVP binding at higher concentrations of ^{3}H-AVP (5×10^{-8} and 10^{-7} M AVP) than at lower concentrations. This suggests that the inhibitory effect of d$(CH_2)_5$Tyr(Et)VAVP can be overcome at higher ^{3}H-AVP concentrations; thus supporting a competitive nature of the antagonism. Doses $>10^{-7}$ M ^{3}H-AVP could not

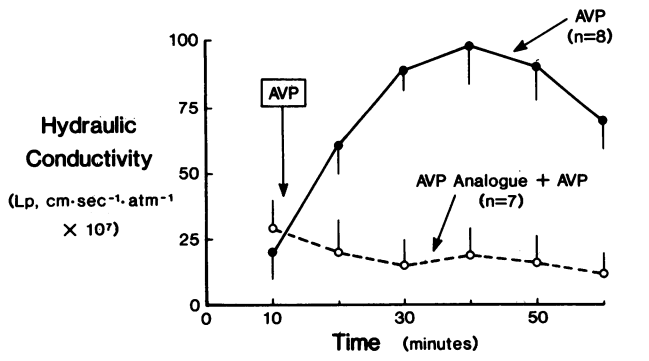

Figure 3. Effect of d$(CH_2)_5$Tyr(Et)VAVP (10^{-7} M), abolishing the effect of AVP (250 μU/ml) on hydraulic conductivity (L_p) in the rabbit CCT. Each point represents mean±SEM.

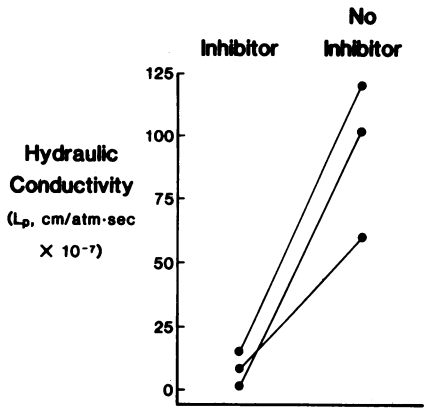

Figure 4. AVP-stimulated hydraulic conductivity, L_p, in the presence or absence of d$(CH_2)_5$Tyr(Et)VAVP. AVP, 250 μU/ml. d$(CH_2)_5$Tyr-(Et)VAVP (shown as inhibitor), 2.5×10^{-7} M. Three lines represent experiments of CCT from three different rabbits.

be used because of too high and irregular nonspecific binding. The dose response curve of adenylate cyclase response to AVP in the papillary collecting tubule in the presence of AVP analogue showed the same pattern as ^{3}H-AVP. Specifically, AVP (10^{-6} M) was shown to overcome the antagonist's effect (10^{-9} M) to inhibit the stimulation of adenylate cyclase in the rat renal papillary collect duct. Taken together, these results support competitive antagonism as the cellular mechanism of action of the antagonist.

To identify the specific nephron sites of action of the AVP analogue, we investigated its effect on activation of adenylate cyclase by AVP in various segments of the nephron. The AVP analogue was shown to diminish the activation of adenylate cyclase in the PCT in a dose-response manner. Since AVP is known also to act in the MCT and CCT, studies were performed in these dissected segments of the rat nephron. In these studies the AVP analogue also blocked the effect of AVP to stimulate adenylate cyclase. Since the adenylate cyclase is more sensitive in the PCT than MCT and CCT (unpublished observations), different concentrations of AVP and the AVP analogue were used in the separate segments. Since AVP has been shown to activate adenylate cyclase in the MAL in some species, including the rat (6, 19–25), the effect of the analogue in this nephron segment was also examined. The stimulation of adenylate cyclase by AVP in MAL, MCT, and CCT were also inhibited by this analogue, indicating that this AVP analogue has effects on the same nephron sites where AVP acts.

The in vitro specificity of the analogue for AVP was further examined in a group of studies using submaximal and maximal stimulatory concentrations of several hormones known to stimulate adenylate cyclase in various segments of the rat nephron. These results confirmed the specificity of the AVP analogue for AVP sites of action in nephron. Specifically, the AVP analogue did not alter the stimulation of adenylate cyclase by PTH in the CAL, by calcitonin in the CCT, or by glucagon in the MAL.

These in vitro studies were compatible, therefore, with the in vivo observations (2, 3) that the effect of AVP on tubular epithelium is directly antagonized by this recently synthesized AVP analogue. Although there are in vivo factors that could alter water excretion independent of a direct cellular antagonism of AVP, including increases in glomerular filtration rate, solute excretion, and blood pressure, in an earlier study these factors

were excluded (3). Further evidence for a direct cellular effect was obtained by examining the in vitro antagonism by the analogue on AVP-induced water flux in the isolated CCT. The results demonstrated that the effect of AVP to increase hydraulic conductivity and to enhance osmotic water flux across the isolated perfused cortical tubule of the rabbit was abolished by the AVP analogue. The early (10-min) transient agonistic effect of the AVP analogue seen in the hydraulic conductivity of CCT was also observed in the in vivo study (3). This transient agonistic effect of the AVP analogue was not demonstrable on the adenylate cyclase response of collecting tubules, since the in vitro enzyme assay was conducted after a 30-min, fixed reaction time period.

Thus, the present in vitro studies demonstrated effects of the AVP analogue to (*a*) diminish AVP binding in rat papillary membranes, (*b*) decrease adenylate cyclase activation in the rat MAL as well as the CCT, MCT, and PCT, (*c*) not alter the specific effects of PTH, calcitonin, and glucagon on adenylate cyclase activation in specific rat nephron segments, and (*d*) to block the effect of AVP to increase hydraulic conductivity and water flux on isolated perfused CCT of the rabbit. Evidence is presented for competitive antagonism as the cellular mechanism of action. This analogue has also been recently shown in vivo to reverse the water retention associated with either glucocorticoid or mineralocorticoid deficiency in the adrenally insufficient conscious rat (26). There is, thus, potential clinical and experimental use for this exciting new AVP analogue.

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