Renal tubular vasopressin receptors downregulated by dehydration

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STEINER, MARTIN, AND M. IAN PHILLIPS. *Renal tubular vasopressin receptors downregulated by dehydration.* Am. J. Physiol. 254 (Cell Physiol. 23): C404–C410, 1988.—Receptors for arginine vasopressin (AVP) were characterized in tubular epithelial basolateral membranes (BL membranes) prepared from the kidneys of male Sprague-Dawley rats. Association of [^{3}H]AVP was rapid, reversible, and specific. Saturation studies revealed a single class of saturable binding sites with a maximal binding (B_{max}) of 184 ± 15 fmol/mg protein and a K_D of 0.61 ± 0.04 nM. IC_{50}s for AVP, lysine vasopressin, and oxytocin were 0.74 nM, 9.7 nM, and >1 μM, respectively. The V_2 receptor antagonist was more than 3,700 times as effective in displacing [^{3}H]AVP than was the V_1 antagonist. To investigate the physiological regulation of vasopressin receptors, the effects of elevated levels of circulating AVP on receptor characteristics were studied. Seventy-two-hour water deprivation significantly elevated plasma osmolality and caused an 11.5-fold increase in plasma [AVP]. Scatchard analysis revealed a 38% decrease in the number of AVP receptors on the BL membranes from dehydrated animals. The high-affinity binding sites on the BL membranes fit the pharmacological profile for adenylate cyclase-linked vasopressin receptors (V_2), which mediate the antidiuretic action of the hormone. We conclude that physiologically elevated levels of AVP can downregulate vasopressin receptors in the kidney.

vasopressin receptor; downregulation; basolateral membranes

SEVERAL HORMONE SYSTEMS act to maintain fluid and electrolyte balance. Alterations in fluid intake influence plasma levels of angiotensin II (8, 20), atrial natriuretic factor (19), and arginine vasopressin (AVP) (26), which act on their respective receptor systems in proportion to the response required to reestablish volume homeostasis. AVP exerts its effect through its well-known antidiuretic action and at higher concentrations through its vasoconstrictive properties. The vascular AVP receptors (V_1) are distinguished on the basis of their functional coupling to the hydrolysis of phosphatidyl inositol and the mobilization of intracellular calcium, whereas renal AVP receptors (V_2) are linked to adenylate cyclase (17). In the kidney, vasopressin has been shown to act at sites distinct from the tubular segments that mediate its antidiuretic effect (2, 18). Several studies have reported AVP binding in the kidney using autoradiographic (9, 25) and traditional biochemical techniques (4, 22), but these do not necessarily differentiate between mesangial, vascular, and tubular plasma membranes that possess vasopressin receptors. Although it is well established that the vasopressin-sensitive adenylate cyclase system is present on the serosal or contraluminal side in renal tubular epithelia (13), the characterization of AVP receptors localized to these basolateral membranes has not been previously described. In the present study, we report the binding characteristics of vasopressin receptors specifically for a basolateral membrane preparation of rat renal tubular epithelia.

Many peptide hormone receptors can be regulated by the concentration of hormone to which they are exposed (7). Exposure to pharmacological doses of vasopressin has been shown to reduce both the binding of [^{3}H]lysine vasopressin and vasopressin-stimulated adenosine 3′,5′-cyclic monophosphate (cAMP) accumulation in a renal epithelial cell line (15). To investigate the physiological regulation of AVP receptors, we have compared binding between basolateral membranes prepared from water-replete and water-deprived rats. Our data indicate that these membranes possess high-affinity, highly specific V_2-type vasopressin receptors. When levels of arginine vasopressin are elevated by 72-h water deprivation, the receptor density (B_{max}) is markedly reduced without affecting the affinity (K_D) of the receptor for the hormone.

MATERIALS AND METHODS

Animals and chemicals. Male Sprague-Dawley rats (n = 6 per group) weighing 200–250 g were obtained from the Animal Resource Facility at the University of Florida. Rats were housed individually in 20 × 18 × 24-cm wire-mesh cages, exposed to a 12-h light-dark cycle, provided a standard Purina rat chow diet, and allowed free access to tap water. The dehydrated experimental group was deprived of water for 72 h before death.

Percoll and synthetic peptides were purchased from Sigma (St. Louis, MO); d(Et)$_2$Tyr(Et)D-AVP (V_1) and d(CH$_2$)$_5$D-Ile^2-Ile^4-AVP (V_2) vasopressin analogues were generous gifts of Dr. M. Manning of the Medical College of Ohio in Toledo. Tritiated 8-L-arginine [phenylalanine-3,4,5-^{3}H]AVP ([^{3}H]AVP) used in receptor binding studies was purchased from New England Nuclear (Boston, MA) with a specific activity of 70 Ci/mmol. Iodinated vasopressin ([^{125}I]AVP), specific activity of 2,200 Ci/mmol, was also obtained from New England Nuclear for use in the vasopressin radioimmunoassay (RIA). AVP antibody was purchased from Amersham (Arlington Heights, IL), whereas AVP standard was obtained from Peninsula Labs (Belmont, CA). Other reagents were

obtained from Sigma unless otherwise indicated.

Membrane preparation and plasma collection. Animals were killed by decapitation, and trunk blood was collected. A 100-μl sample was obtained separately for the determination of plasma osmolality while the remainder was collected in chilled tubes containing 0.3 M EDTA (50 μl/ml) for the determination of plasma AVP concentration. Blood samples were centrifuged at 3,000 *g* for 10 min at 4°C; the plasma was removed and stored at −20°C. Renal basolateral membranes were prepared according to the density gradient centrifugation method of Boumendil-Podevin and Podevin (5) with slight modification. Kidneys were removed, decapsulated, and rinsed in a chilled buffer containing 250 mM sucrose, 2 mM Tris-*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.1 mM phenymethylsulfonyl fluoride (PMSF) (pH 7.4). The tissue was minced and then homogenized with five strokes of a Dounce homogenizer followed by five strokes of a Teflon pestle homogenizer at a setting of 800 rpm. The resulting homogenate was centrifuged at 1,000 *g* for 10 min at 4°C. The pellet (P_1) is discarded and the supernatant (S_1) centrifuged at 22,000 *g* for 15 min at 4°C. The supernatant (S_2) is discarded, leaving a triple-layered pellet (P_2). The upper two layers of the pellet were removed by gentle vortexing and homogenized in 10 ml of the Tris-HEPES-sucrose buffer without PMSF. Percoll (1.2 ml) is added and mixed and then centrifuged at 40,000 *g* for 35 min at 4°C. The F_1 band (basolateral membranes, density = 1.037 g/ml) is aspirated and diluted in five volumes of 2 mM Tris-HEPES, 85 mM KCl, 85 mM sucrose (pH 7.4). Percoll is removed from the mixture by ultracentrifugation at 60,000 *g* for 15 min at 4°C. The supernatant is decanted, and the membranes are gently washed away from the Percoll pellet, resuspended in Tris-HEPES-sucrose buffer with PMSF, and stored in liquid N_2. Protein content was determined with the Folin reagent, using bovine serum albumin as the standard, according to the method of Lowry et al. (16).

Determination of Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity was determined by measuring the K^+-stimulated, ouabain-sensitive *p*-nitrophenylphosphate (PNPP) hydrolysis at pH 7.8, based on the method of Yoshida et al. (27). Briefly, the assay buffer was composed of 50 mM Tris-HCl (pH 7.8) containing (in mM) 5 $MgCl_2$, 2.5 EDTA, 50 KCl, and 5 PNPP. The reaction was carried out in the presence and absence of ouabain (1 mg/ml) by the addition of 10 μl membranes (100 μg protein) to 0.5 ml of the assay buffer. The mixtures are incubated for 2–10 min at 37°C. The reaction was stopped by the addition of 3.5 ml 1 N NaOH and absorbance was read at 410 nm. Net Na^+-K^+-ATPase activity was determined by subtracting the PNPP hydrolysis activity in the presence of ouabain from the activity in the absence of ouabain. Specific activity was calculated based on a molar extinction coefficient $\epsilon = 15{,}400\ M^{-1} \cdot cm^{-1}$, and final values were expressed as units per milligram protein (where 1 unit = 1 μmol PNPP hydrolyzed/min).

TABLE 1. *K^+-stimulated oubain-sensitive Na^+-K^+-ATPase activity*

Fraction	Units/mg protein,* $\times 10^{-3}$	Purification
H	0.08±0.04	1X
P_2	0.26±0.01	3.25X
F_1	0.80±0.01	10X

Na^+-K^+-ATPase activity, as determined by K^+-stimulated ouabain-sensitive *p*-nitrophenylphosphate hydrolysis at pH 7.8 in kidney homogenate (H), P_2 pellet, and basolateral membrane (F_1) fraction. * 1 unit = 10^{-6} mol/min.

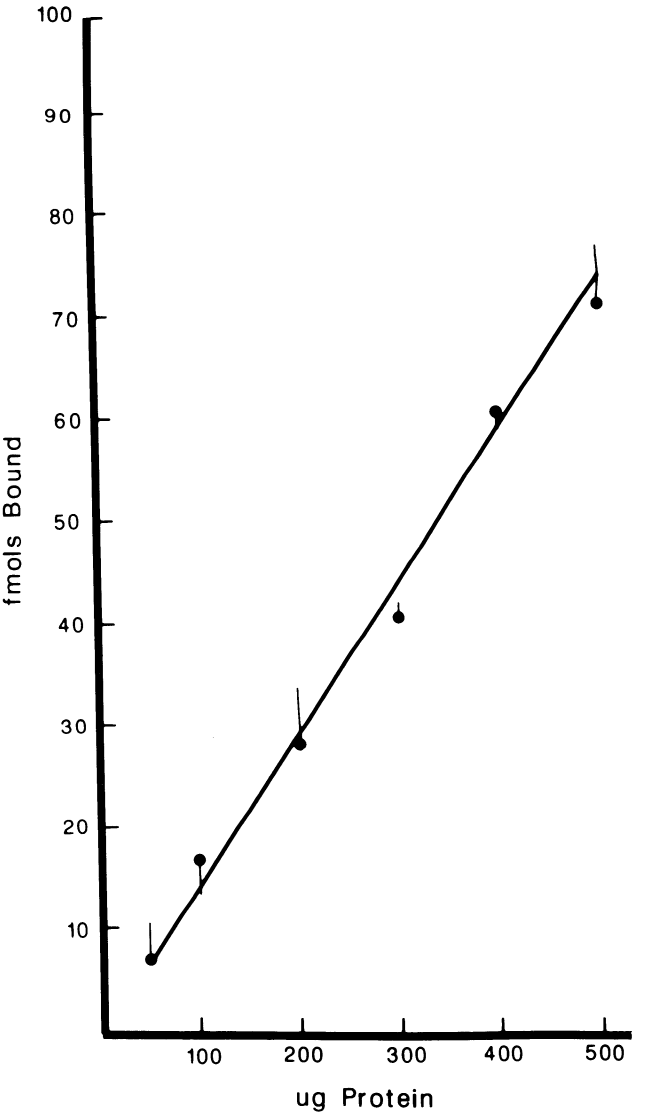

FIG. 1. Linearity of [^{3}H]AVP binding to basolateral membranes. Membranes corresponding to 50–500 μg protein were incubated with 1 nM [^{3}H]AVP for 45 min at 20°C. Nonspecific binding was determined in presence of 1 μM unlabeled AVP.

Vasopressin receptor assay. Membranes, corresponding to 100–200 μg protein, were suspended in 300 μl of 100 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 1 mM EGTA, and 0.1% BSA (pH 7.3) at 20°C. For saturation studies, triplicate samples were incubated for 45 min at 20°C with concentrations of [^{3}H]AVP ranging from 50 to 5,000 pM. Nonspecific binding was determined in the presence of 5 μM unlabeled AVP. Competitive inhibition by various peptides was assessed in the presence of 1.0 nM [^{3}H]AVP. The incubation was terminated by the

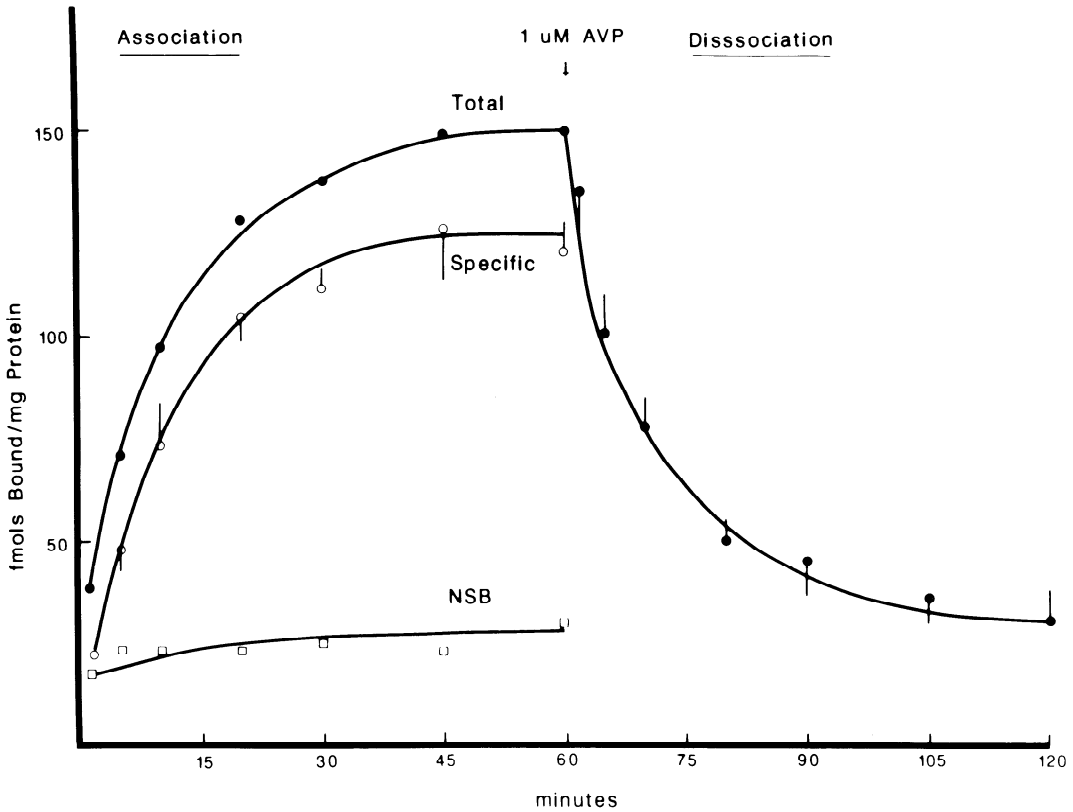

FIG. 2. Association and dissociation of 1 nM [^{3}H]AVP binding to basolateral membranes. Specific binding = 80–85% total. NSB, nonspecific binding.

addition of 1 ml ice-cold Tris-buffer and the bound tracer was rapidly separated from the unbound moiety by centrifugation at 10,000 *g* for 2.5 min at 4°C. The membrane pellet was dissolved in 100 μl of formic acid and transferred to a minivial containing 4 ml of liquid scintillation fluid (Liquiscint, National Diagnostics). Radioactivity was determined in an LKB LS counter. Samples were corrected for variations in efficiency of counting by an internal program and expressed as disintegrations per minute (dpm). Based on the radioactivity and protein in each sample, values are reported as femtomoles bound per milligram protein. Specific binding was calculated as the difference between total binding and nonspecific binding.

RIA for vasopressin. Plasma samples for the determination of AVP were thawed and purified using SepPak C_{18} cartridges (Waters). Briefly, the purification process is as follows. Cartridges are prewetted with 5 ml methanol followed by 5 ml 1% trifluroacetic acid (TFA). A 1-ml plasma sample is acidified with 1% TFA and applied to the column dropwise. The cartridge is rinsed with 5 ml 1% NaCl-1% TFA, and then AVP is eluted with 4 ml methanol:H_2O:TFA (80:19:1). The samples are evaporated under a light stream of air and then reconstituted in 0.5 ml of assay buffer [50 mM phosphate buffer (pH 7.4) containing 0.2% bovine serum albumin and 10 mM EDTA]. The RIA is carried out by incubating 100-μl samples or known amounts of standard (0.1–20 pg AVP) with 25 μl AVP antiserum and 25 μl [^{125}I]AVP (corresponding to 3,000 cpm) for 24 h at 4°C. Free ligand is sequestered by the addition of dextran-coated charcoal and centrifugation at 2,000 *g* for 15 min. The supernatant containing the antibody-bound ligand is aspirated and counted in a Beckman 5500 gamma counter at an efficiency of 79% for [^{125}I]. Values are expressed as picograms AVP. Cross-reactivity with related peptides is <1%. Recovery of AVP for the extraction procedure was determined to be >90%. Sensitivity of the assay was 0.2 pg per tube.

Plasma osmolality. Plasma osmolality was determined using a Westcor model 5500C vapor pressure osmometer. (Data are expressed as mosmol/kg H_2O.)

Analysis. Data from saturation and competition experiments were analyzed using iterative curve fitting programs. Data are reported as the means ± SE. Statistical significance of the data was assessed using one-way analysis of variance and Student's *t* test. A level of $P < 0.05$ was considered to be significant.

RESULTS

The state of hydration of the rats was evaluated by comparing plasma osmolality and plasma AVP concentration among the two groups. Plasma osmolality was significantly elevated among the water-restricted animals (299 ± 1.7 mosmol/kg) compared with control values (288 ± 0.8 mosmol/kg) ($P < 0.05$). Plasma AVP levels also were significantly raised 11.5-fold among animals in the water restricted group (2.4 ± 0.3 pg/ml) compared with control values (0.21 ± 0.02 pg/ml; $P < 0.05$).

The activity of Na^+-K^+-ATPase, a marker enzyme for the basolateral membrane, was markedly enriched in the

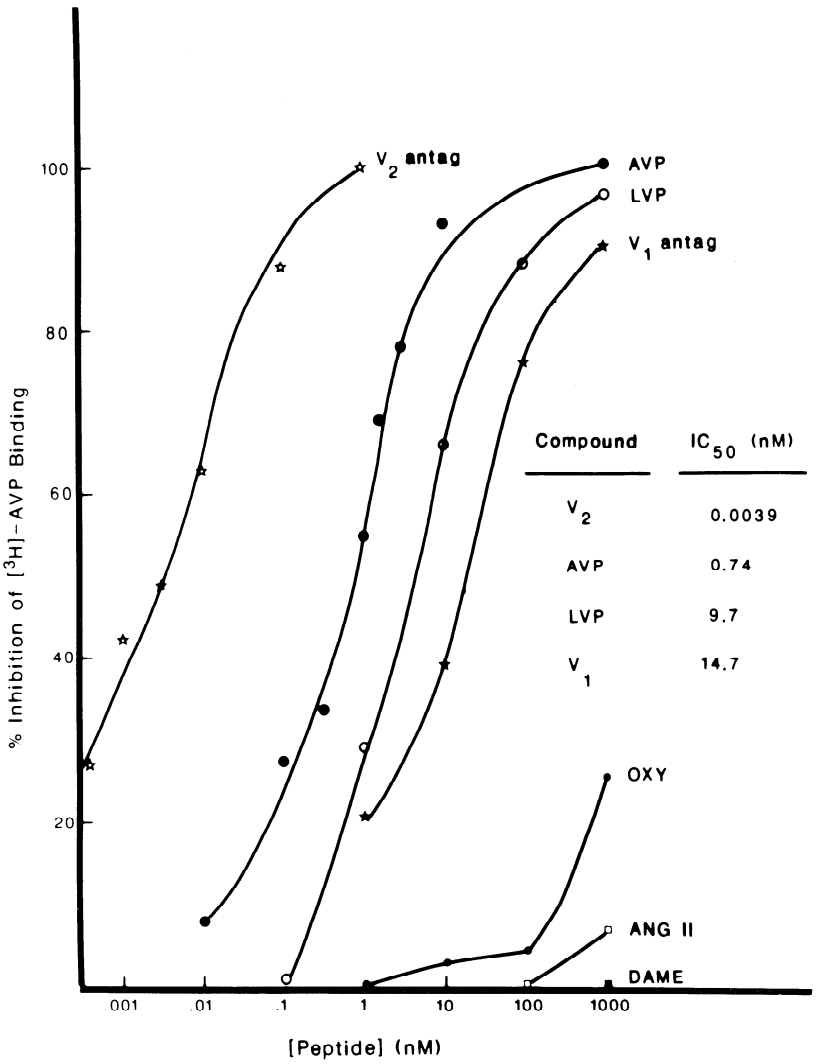

FIG. 3. Specificity of 1 nM [^{3}H]AVP binding to basolateral membranes. V_1 antagonist, d(Et_2)Tyr(Et)D-AVP; V_2 antagonist, d(CH_2)$_5$D-Ile^2-Ile^4-AVP; AVP, Arg^8-vasopressin; LVP, Lys^8-vasopressin; OXY, oxytocin; Ang II, angiotensin II; DAME, D-Ala-Met-enkephalinamide.

F_1 fraction, which displayed a 3.1-fold increase in activity over that of the P_2 pellet and a 10-fold increase when compared with the homogenate (Table 1). A twofold enrichment of AVP binding per unit protein was achieved in the BL membrane preparation compared to the P_2 pellet. The binding of [^{3}H]AVP to renal basolateral membranes was linearly related to the amount of protein (i.e., membrane) present in the assay (Fig. 1). Association and dissociation of [^{3}H]AVP is illustrated in Fig. 2. Equilibrium binding of the ligand occurred within 30 min and was stable for up to 2 h. Upon equilibrium, binding was shown to be reversible by the addition of 1 μM AVP. Specific binding, determined in the presence of an excess amount of AVP, was 80–85% of total binding. Specificity of the receptor for related AVP analogues and nonrelated peptides is illustrated in Fig. 3. The data show that lysine vasopressin (LVP), a vasopressin peptide differing from AVP by only one amino acid in the number 8 position, is some 13 times less effective in displacing the ligand than AVP (IC_{50}: AVP = 0.74 nM vs. LVP = 9.7 nM). Furthermore, the synthetic vasopressin analogue d(CH_2)$_5$D-Ile^2-Ile^4-AVP, a specific tubular (V_2) antagonist is some 3,700 times more effective in displacing the ligand than is the vascular (V_1) antagonist d(Et)$_2$Tyr(Et)D-AVP (IC_{50}: V_2 = 0.0039 nM vs. V_1 = 14.7 nM). At concentrations of 1 μM, oxytocin, angiotensin II, and D-Ala-Met-enkephalinamide were incapable of displacing even 50% of the ligand. A representative saturation isotherm for [^{3}H]AVP binding to membranes from the control and dehydrated groups is depicted in Fig. 4. Transformation of the data for Scatchard analysis is represented in Fig. 5. Analysis of the data between the two groups indicates a significant reduction in the number of vasopressin receptors on the membranes from the dehydrated animals (control B_{max} = 184 ± 15 fmol/mg protein vs. dehydrated B_{max} = 114 ± 2.5 fmol/mg protein) ($P < 0.01$). No significant difference was observed in the affinity of the receptor between the two groups (control K_D = 0.61 ± 0.04 nM vs. dehydrated K_D = 0.64 ± 0.03 nM).

DISCUSSION

Several studies have demonstrated the presence of vasopressin receptors in the kidney (4, 9, 22, 25), but here we report the preparation of Na^+-K^+-ATPase-enriched renal tubular epithelial basolateral membranes and the characterization of [^{3}H]AVP binding in these membranes. This preparation allows one to separate, by differential and density gradient centrifugation, tubular

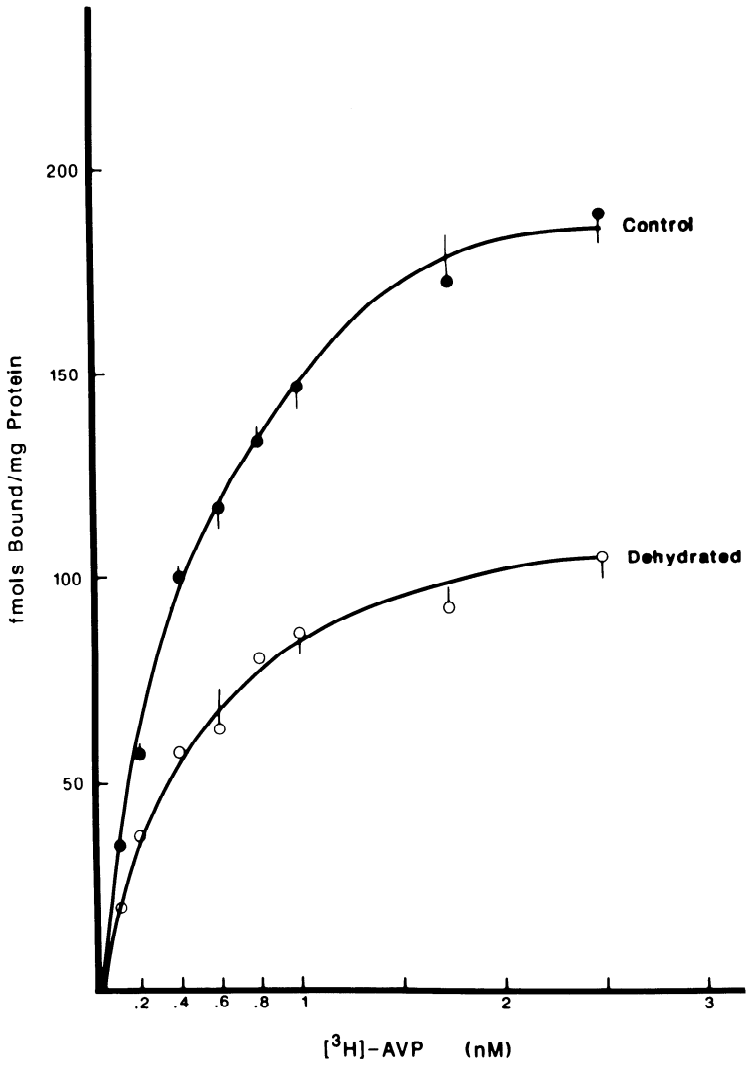

FIG. 4. Saturation isotherm of [^{3}H]AVP binding to basolateral membranes from control and dehydrated rats.

epithelial cells possessing vasopressin receptors linked to adenylate cyclase (V_2) from vascular endothelial and glomerular mesangial cells possessing vasopressin receptors coupled to the hydrolysis of phosphatidyl inositol (V_1). Recent evidence suggests that certain renal epithelial tissue (LLC-PK$_1$ cells) possess both V_1 receptors (12) and V_2 receptors (15). The BL membrane preparation yielded a 3.1-fold increase in Na^+-K^+-ATPase activity with respect to the P_2 pellet and a 10-fold increase compared with the homogenate. Vasopressin binding sites per unit protein doubled in the BL preparation over that in the P_2 fraction. The discrepancy may be explained in terms that although BL membranes may be enriched, not all of the membranes may possess AVP receptors due to their heterogeneous distribution along the renal tubule (18). A single class of high-affinity AVP receptors is present on these basolateral membranes. Vasopressin binding is rapid, reversible, specific, and displays a pharmacological profile characteristic of V_2 receptors. It is not entirely surprising that the levels of binding reported herein differ slightly from other values reported in the literature, given the differences in membrane preparation and binding conditions. The maximal binding capacity in the present study (184 ± 15 fmol/mg P) is somewhat less than that reported by Rajerison et al. (22) (300 fmol/mg P), but is in close agreement with the studies of Shewey and Dorsa (24) (160 fmol/mg P) and Dorsa et al. (9) (113 fmol/mg P). These data are consistent with and complement those studies that have examined the vasopressin-sensitive adenylate cyclase system in the kidney and its coupling to V_2-type receptors (6, 22, 23).

The data further show that these receptors are sensitive to the circulating levels of vasopressin. Water deprivation for 72 h significantly elevated plasma AVP concentrations. Although the absolute values seem a bit low compared with other reports in the literature, the 11.5-fold increase in plasma [AVP] is comparable to the response observed by Woods and Johnston (26) in rats dehydrated for a similar period of time. A significant reduction in the number of vasopressin receptors (B_{max}) was noted in the renal tubular basolateral membranes prepared from the water-deprived animals. There was no difference between the two groups with respect to the affinity (K_D) of the receptor for the hormone.

The phenomenon of downregulation or desensitization results from an increased rate of internalization when the hormone concentration has been greatly enhanced (7). Homologous downregulation of vasopressin receptors has been reported in a renal epithelial cell line (15) and in the toad urinary bladder (10), after treatment with

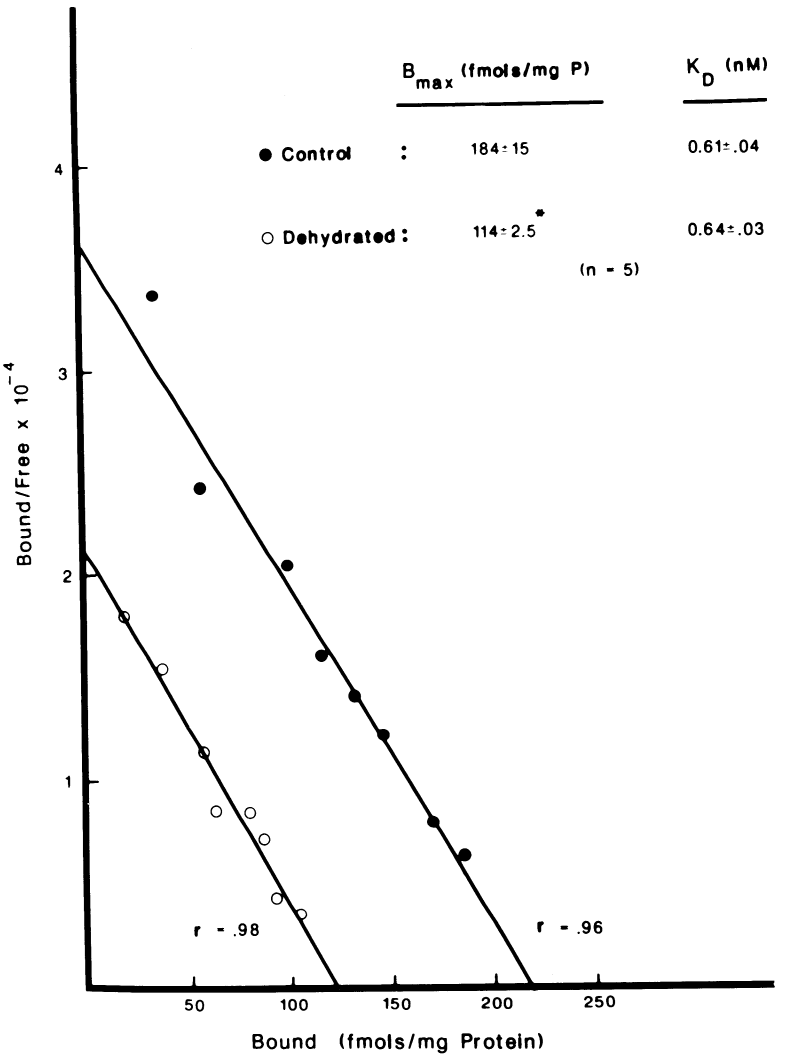

FIG. 5. Scatchard analysis of [^{3}H]AVP binding to basolateral membranes from control and dehydrated rats. B_{max}, maximal binding. * $P < 0.01$ vs. control.

exogenous vasopressin. Shewey and Dorsa (24) reported finding no difference in the level of [^{3}H]AVP binding in renal medullary membranes prepared from heterozygous Brattleboro rats and their chronically dehydrated homozygous counterparts, which are unable to synthesize AVP. This would suggest that the reduction in vasopressin receptor density observed in the present study is attributable to the elevated plasma levels of vasopressin, rather than some unspecific effect of dehydration.

The "anatomical correlate" of downregulation has been described by Kirk (14). He has elegantly shown by morphometric analysis that retrieval or internalization of the basolateral membrane occurs in the cells of the rabbit cortical-collecting tubules (CCT) when induced by a vasopressin-stimulated transcellular water flow. He suggests that membrane internalization could reduce the vasopressin sensitivity of the CCT cells if AVP receptors are present on the retrieved membrane. Although not addressed in the present study, the issue of adenylate cyclase responsiveness has been examined in conjunction with the downregulation of AVP receptors. Rajerison, Butlen, and Jard (21) showed in the rat that vasopressin-sensitive adenylate cyclase activity in membrane preparations from the rat kidney medulla is greatly reduced after the infusion of pharmacological doses of AVP to the animals.

In the present study, it would seem odd that an animal in dire need of water conservation would lose AVP receptors as the need became greater. An explanation for this may be that the kidney possesses "spare" receptors (1). Baddouri et al. (3) noted a downregulation of vasopressin receptors in the kidney of the water deprived jerboa, *Jaculus orientalis*. Water-deprived animals in this study displayed a fivefold elevation in plasma AVP levels and a 36% reduction in [^{3}H]lysine vasopressin binding to renal medullary membranes. Despite a reduced vasopressin-sensitive adenylate cyclase responsiveness, the kidneys of some of these desert rodents still retained the capacity to significantly concentrate urine under these conditions. The latter finding suggests that spare receptors may have been involved in the regulatory phenomenon. A receptor reserve has also been noted in other adenylate cyclase-linked tissues as well (11). The physiological significance of spare receptors and downregulation in these animals is not well understood and remains to be explored.

In summary, the present study shows that V_2 vasopressin receptors are differentially expressed in baso-

lateral membrane preparations from the kidneys of water-replete and water-deprived rats. Scatchard analyses indicate that the difference in binding is due to a reduction of receptor numbers on the membranes prepared from the dehydrated animals. These results show that AVP receptor downregulation can occur in response to physiologically elevated levels of circulating vasopressin. Such a condition could ultimately affect the antidiuretic action of the hormone.

We acknowledge Dr. Bruce Stevens for his useful discussions and thank Carolyn Gabbard for the excellent typing of this manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-27334 (to M. I. Phillips).

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