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Michael A. Kirschenbaum, … , Walter Trizna, Leon G. Fine

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Regulation of Vasopressin Action by Prostaglandins

EVIDENCE FOR PROSTAGLANDIN SYNTHESIS IN THE RABBIT CORTICAL COLLECTING TUBULE

MICHAEL A. KIRSCHENBAUM, ANDREW G. LOWE, WALTER TRIZNA, and LEON G. FINE, *Division of Nephrology, Department of Medicine, University of California at Los Angeles School of Medicine, Los Angeles, California 90024*

ABSTRACT The present studies examined whether vasopressin increases prostaglandin biosynthesis in isolated rabbit cortical collecting tubules (CCT) and whether endogenous prostaglandin biosynthesis plays a role in modulating the response of this nephron segment to vasopressin. Three groups of studies were performed. In the first group, CCT and proximal straight tubules (PST) were incubated with [^{3}H]arachidonic acid, and metabolites were separated and identified using silica gel thin-layer chromatography. CCT were capable of producing all of the major prostaglandins (PG)(PGE_2 > thromboxane B_2 [TxB_2] > $PGF_{2\alpha}$ > PGI_2). PST produced significantly lesser quantities of these lipids. In the second group, radiolabeled arachidonic acid was incorporated into the phospholipid pool of both CCT and PST, vasopressin was added to the incubation medium, and metabolites were separated and identified as above. Vasopressin stimulated the release of all of the major prostaglandins in CCT but had no effect on PST. PGE release into the incubation medium, as assessed by a radioreceptor assay, increased 108%, and a vasopressin analogue, 1-desamino-8-D-arginine vasopressin, had a quantitatively similar effect. In the third group, a submaximal dose of vasopressin was administered to isolated, perfused CCT studied in the presence and absence of indomethacin to assess whether endogenous prostaglandins play a role in modulating the antidiuretic response to vasopressin. Studies were performed in rabbits on a normal diet and in desoxycorticosterone acetate (DOCA)- or KCl-loaded animals. In the state of mineralocorticoid excess, basal prostaglandin synthesis was 63% lower, and vasopressin-stimulated prostaglandin synthesis 76% lower, than the synthesis observed in rabbits on a normal diet. Cyclooxygenase inhibition exposed a significant hydroosmotic response to a submaximal dose of vasopressin in CCT from DOCA- or KCl-loaded animals. With arachidonic acid in the bath, the same dose of vasopressin failed to elicit a hydroosmotic response in CCT from rabbits on a normal diet even in the presence of a cyclooxygenase inhibitor. However, removal of exogenous arachidonic acid, with a consequently lower rate of prostaglandin synthesis, allowed the cyclooxygenase inhibitor to enhance the hydroosmotic response to vasopressin in these tubules.

We conclude from these studies that the rabbit CCT has the capacity to synthesize all of the major prostaglandins and that the rate of synthesis of these lipids is enhanced by vasopessin. Prostaglandin synthesis by the CCT is postulated to modulate the antidiuretic action of vasopressin via a closed feedback loop. The effectiveness of this feedback regulation is dependent upon the mineralocorticoid status of the animal, which determines the level of basal and vasopressin-stimulated prostaglandin synthesis by the CCT.

INTRODUCTION

Over 10 years ago, Grantham and Orloff (1) demonstrated that prostaglandin E_1 (PGE_1)[1] could antagonize the hydroosmotic effect of vasopressin in the mam-

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[1] *Abbreviations used in this paper*: CCT, cortical collecting tubules; DDAVP, 1-desamino-8-D-arginine vasopressin; DOCA, desoxycorticosterone acetate; J_v, net water flux; PD, potential difference; *Pf*, transepithelial osmotic water permeability coefficient; PG, prostaglandin; PST, proximal straight tubules; TLC, thin-layer chromatography; Tx, thromboxane.

malian cortical collecting tubule (CCT). This and other observations in toad bladder epithelia (2–4) and renal medullary interstitial cells in culture (5, 6) have suggested that the prostaglandins act as negative feedback modulators of the action of vasopressin in the kidney. Despite these numerous studies, the validity of this hypothesis has been the subject of both interest and debate (7). One of the reasons for this controversy is the lack of direct evidence that vasopressin increases prostaglandin biosynthesis in vasopressin-sensitive nephron segments.

The present studies were designed to examine whether the isolated rabbit CCT, a site of the antidiuretic action of vasopressin, is capable of synthesizing all of the major prostaglandins and whether the rate of biosynthesis of these lipids is augmented by vasopressin and is specific for this nephron segment. Studies were also performed to determine whether endogenous prostaglandin biosynthesis plays a role in modulating the response of the CCT to vasopressin. These studies were performed on both normal and desoxycorticosterone-treated animals to assess the role of mineralocorticoid status on the regulation of vasopressin action by endogenous prostaglandin biosynthesis.

METHODS

Nonstimulated prostaglandin biosynthesis by isolated rabbit nephron segments

CCT and superficial proximal straight tubules (PST) were obtained from 2- to 3-kg rabbits maintained on standard Purina rabbit chow (Ralston Purina Co., St. Louis, MO) and tap water. The tubule segments were obtained by microdissection of tissue in Dulbecco's modified Eagle's medium (4302100, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) at 4°C without the use of collagenase. After dissection, tubular segments were transferred to polystyrene Falcon tissue culture plates (3034, Falcon Labware, Div. of Becton, Dickinson, and Co., Oxnard, CA) containing 20 μl of incubation medium, and the length of the tubules was measured directly using a calibrated microscope screen. Dexamethasone (50.9 μM, Elkins-Sinn, Inc., Cherry Hill, NJ) was added to the incubation medium (8) to inhibit the deacylation of endogenous phospholipids to arachidonic acid, thereby maximizing the metabolism of the exogenously administered arachidonic acid. The tubules were incubated in the dark at room temperature for 30 min. The total length of tubules added to each well was ~5–6 mm. Each experiment is reported as the mean value obtained from at least four tubule-containing wells.

A solution containing sodium arachidonate (0.5 mM, Sigma Chemical Co., St. Louis, MO), ~100,000 cpm of octatritiated arachidonic acid (60–100 Ci/mmol, New England Nuclear, Boston, MA), and reduced glutathione (1 mM, Sigma Chemical Co.) was added to the wells either with or without a cyclooxygenase inhibitor (indomethacin, 220 μM, Merck, Sharp & Dohme, Division of Merck and Co., West Point, PA; or meclofenamate, 130 μM, Parke, Davis & Co., Detroit, MI). The indomethacin was solubilized in Na_2CO_3 (40 mM) and the meclofenamate in physiologic saline. The tubules were then incubated for 60 min at 37°C; the reaction was stopped by cooling to 4°C. The bath medium was then extracted at pH 3.5 with five volumes of chloroform:methanol (2:1), evaporated under nitrogen until dry, and redissolved in ethyl acetate.

A solution containing authentic standards of 6-keto-$PGF_{1\alpha}$, prostacyclin (PGI_2), $PGF_{2\alpha}$, thromboxane B_2 (TxB_2), PGE_2, PGA_2 (gifts of Dr. John Pike, The Upjohn Company, Kalamazoo, MI), and arachidonic acid were added to the samples and this mixture was applied to silica gel thin-layer chromatography plates which were then developed using a method reported by Hassid et al. (8). The plates were developed twice in the organic phase of ethyl acetate:isooctane:acetic acid:water (11:5:2:10), air-dried, and the position of the various standards was identified by exposure to iodine vapor. A photostatic copy of the plate was made and it was then divided into 5-mm strips; each strip was then transferred to a scintillation vial in an aqueous counting mixture. The vials were counted in a Beckman 7500 liquid scintillation system (Beckman Instruments, Fullerton, CA) and corrected for quenching using an internal standard. The protein content of the tubules in each individual experiment was determined by a modification of the method of Lowry et al. (9) after transfer of the tubules to Ringer's bicarbonate solution (pH 7.4). Bovine serum albumin (Sigma Chemical Co.) was used as a standard. Total counts in each strip were corrected for nonenzymatic conversion of arachidonic acid by subtracting the counts obtained either in the absence of tubules or with boiled tubules. Six experiments were performed on CCT and four were performed on PST.

Vasopressin-stimulated prostaglandin biosynthesis by isolated rabbit nephron segments

CCT and PST were dissected and handled in a manner identical to that described in the previous section. The tubules were gently pressed onto the bottom of the polystyrene incubation wells with fine forceps so that they were anchored in place. After the length of the tubules was determined, ~200,000 cpm of octatritiated arachidonic acid was added to the medium to achieve a total volume of 5 μl. No dexamethasone was added. The tubules were incubated in the dark at 37°C for 1 h to allow the radiolabeled arachidonic acid to acylate phospholipids in the intact epithelial cells. At the end of the 1-h preincubation with [^{3}H]arachidonic acid, the bath solution was removed and the tubules were washed twice to remove any unincorporated radioactive counts. The tubules were divided into four groups and the bath medium in each incubation chamber was replaced with one of the following solutions: (*a*) arginine vasopressin (0.56 nM, Sigma Chemical Co.); (*b*) arginine vasopressin plus dexamethasone (50.9 μM, a phospholipase A_2 inhibitor); (*c*) arginine vasopressin plus indomethacin (220 μM, a cyclooxygenase inhibitor); and (*d*) a solution containing no vasopressin, to serve as control. The total volume of each incubation well was 10 μl. Each group of tubules was allowed to incubate at 37°C for 1 h. The reaction was stopped by cooling and the bath medium was extracted in a manner identical to that described above. Five experiments were performed in both CCT and PST.

Four similar studies were performed with 1-desamino-8-D-arginine vasopressin (DDAVP) (Ferring Pharmaceuticals Inc., New York, NY), a synthetic antidiuretic hormone analogue without vasopressor properties, instead of vasopressin, at concentrations from 0.56 to 56 nM.

We measured PGE concentrations in the incubation medium either with or without vasopressin in 12 experiments by using a specific radioreceptor assay, which has been described previously (10). In eight experiments, animals were maintained on normal rabbit chow. In four experiments, animals received desoxycorticosterone acetate (DOCA), 5 mg/d for 5 d, by intramuscular injection before study. After 1 h of incubation at 37°C, the medium was extracted at pH 3.5 with chloroform:methanol (2:1). The extracted lipid-containing solution was flash-evaporated and redissolved in *n*-heptane:chloroform:methanol:acetic acid (100:100:30:2) and applied to Sephadex LH-20 columns measuring 10 × 130 mm. The recovery of a tracer amount of radiolabeled PGE_2 after extraction and chromatography of individual samples was between 50 and 80%. The concentration of PGE in the sample was determined using a rat hepatic membrane-receptor assay (10).

Incorporation of radiolabeled arachidonic acid into phospholipids

12 studies on tubules from two animals were performed to measure the degree of incorporation of the radiolabeled arachidonic acid into the various intracellular phospholipid pools. Tubules (both CCT and PST) were dissected and handled in a manner identical to that described above. After the length of the tubules in each well was determined, ~40,000 cpm of octatritiated arachidonic acid was added to the wells. No dexamethasone or unlabeled arachidonic acid was added. The tubules were incubated in the dark for 1 h at 37°C. The distribution of [^{3}H]arachidonic acid incorporated into the various phospholipid pools in the tubules was determined using a method described by Schlondorff et al. (11). The lipids were extracted twice with chloroform:methanol (2:1), dried under a nitrogen stream, resuspended in chloroform:methanol (2:1), and applied to silica gel thin-layer chromatographic plates along with authentic standards of phosphotidylinositol, phosphotidylserine, phosphotidylcholine, phosphotidylethanolamine (all purchased from Sigma Chemical Co.), and arachidonic acid. The plates were developed in chloroform:methanol:ammonium hydroxide (65:35:5). The standards were identified by iodine vapor, and the plates processed for the determination of radioactivity as described above.

Perfusion of CCT in vitro: response to vasopressin

The purpose of these studies was to evaluate the hydroosmotic response of the collecting tubule to a submaximal concentration of vasopressin. CCT were obtained from 2- to 3-kg rabbits maintained on either (*a*) a diet of normal laboratory chow plus tap water; (*b*) a normal diet plus 100 mM KCl, in lieu of drinking water; or (*c*) a normal diet plus tap water plus DOCA, 5 mg/d, for 5 d by intramuscular injection. The latter two groups, in which circulating mineralocorticoid levels were elevated, were studied because it has been shown that the response to vasopressin is enhanced under these circumstances (12) and we wished to optimize the in vitro response to vasopressin. Two groups of CCT were studied: in vitro perfusion of CCT in the absence of cyclooxygenase inhibitors, and in vitro perfusion of CCT under conditions of cyclooxygenase inhibition.

In vitro perfusion of CCT in the absence of cyclooxygenase inhibitors. Tubules were dissected from the cortex and perfused in vitro as described previously from this laboratory. The bath consisted of: 115 mM NaCl, 25 mM $NaHCO_3$, 10 mM sodium acetate, 5 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 5 mM glucose, and rabbit serum 5% (vol/vol). The bath also contained Na_2CO_3, which is the vehicle for indomethacin and naproxen. The osmolality of this solution was 295 mosmol/kg H_2O. Arachidonic acid (50 μM) plus reduced glutathione (1 mM) were added to the bath after 120 min and were present for 60 min before the control samples were collected (see below). To prevent oxidation of the arachidonic acid, the solutions were maintained in the dark and the bath was changed every 10 min. The perfusate was composed of: 60 mM NaCl, 1.0 mM K_2HPO_4, 1.0 mM $CaCl_2$, and 1.2 mM $MgSO_4$. The osmolality of this solution was 130 mosmol/kg H_2O. Experiments were conducted at 37°C at a transtubular osmotic gradient of 165 mosmol/kg H_2O. The bath was bubbled with 95% O_2-5% CO_2. Transtubular potential difference (PD) was measured throughout the experiment as described previously (13). Both ends of the tubule were insulated with Sylgard 184 (Dow Corning Corp., Midland, MI).

180 min were allowed for equilibration, during which time the tubules became relatively impermeable to water. Five timed collections of tubular fluid were made at the end of this period. A "submaximal" dose of vasopressin, i.e., 2.5 μU/ml Pitressin (Parke, Davis & Co.),[2] was then added to the bath and collections of fluid made at 15, 30, and 45 min. Samples from each period were analyzed for ^{3}H concentration and osmolality. The samples for radioactivity were pipetted directly into liquid scintillation fluid, whereas those used for osmolality measurements were deposited under oil and then transferred to the sample holder of a Clifton nanoliter Osmometer (Clifton Technical Physics, Hartford, NY).

In vitro perfusion of CCT under conditions of cyclooxygenase inhibition. The experimental protocol was identical to that described above except that indomethacin (200 μM) was present in the bath throughout the 180-min equilibration period and during the 45 min of vasopressin administration. CCT were obtained from five rabbits on a normal diet and nine rabbits on a high potassium or DOCA diet.

Because indomethacin was found to have no effect on the hydroosmotic response to vasopressin in rabbits on a normal diet when exogenous arachidonic acid was added to the bath (see Results), we considered the possibility that the augmented prostaglandin synthesis under these conditions (even after 50% inhibition with indomethacin) was sufficient to mask the response to a submaximal dose of vasopressin. Studies were therefore performed on seven additional CCT from rabbits on a normal diet: two with vehicle, three with indomethacin (200 μM), and two with naproxen (200 μM, Syntex Laboratories, Inc., Palo Alto, CA; dissolved in Na_2CO_3, 40 mM), in which no exogenous arachidonic acid was added to the bath. To compare the effects of a cyclooxygenase inhibitor plus a submaximal dose of vasopressin with vasopressin alone on the same tubule, the studies were performed at 25°C. At this temperature, the vasopressin effect remains

[2] 2.5 μU/ml Pitressin has been shown to be a submaximal concentration in terms of its ability to increase the water permeability of the CCT (13). We confirmed this in three pilot experiments by showing that this concentration of Pitressin increased net water flux from 0.01 to 0.33 nl/mm per min. When, however, arachidonic acid was added to the bath, this dose did not result in an increase in net water flux (see Results).

constant over a period of hours, whereas at 37°C it declines with time (12). After equilibrating the tubule at 37°C for 120 min, the temperature was lowered to 25°C for an additional 60-min control period and was maintained at 25°C for the remainder of the experiment. After control collections of tubular fluid were made, Pitressin (2.5 μU/ml) was added to the bath and collections were made at 15 and 30 min. Either indomethacin (200 μM) plus Pitressin (2.5 μU/ml), or naproxen (200 μM) plus Pitressin were added to the bath, and collections made at 15-min intervals for 1 h. The transepithelial osmotic pressure gradient was 165 mosmol/kg H_2O throughout. Net water flux (J_v) was measured serially under control, vasopressin, and vasopressin plus cyclooxygenase inhibitor conditions in the same tubule. Three additional tubules were studied using Pitressin at a concentration of 25 μU/ml to determine the maximal hydroosmotic response of the tubule under these conditions.

Calculations

Perfusion rate (V_O) is calculated as ${}^3H_L/({}^3H)_O \cdot t$, where ${}^3H_L$ is the total amount of isotope collected, $({}^3H)_O$ is the concentration of isotope in the perfusate, and t is the duration of the collection. Net fluid reabsorption, J_v (nanoliters per millimeters per minute), is equal to $(V_O - V_L)L$, where V_L is the collection rate and L the length of the tubule. Tubular length and internal diameter were measured during perfusion with a calibrated reticle in the ocular of the microscope. Transepithelial osmotic water permeability coefficient, P_f (centimeters per second), was computed according to the expression derived by Al-Zahid and co-workers (14):

$$P_f = -\frac{V_O C_O}{A\bar{V}_w}\left[\frac{C_O - C_L}{C_O C_b C_L} + \frac{1}{(C_b)^2}\ln\frac{(C_L - C_b)C_O}{(C_O - C_b)C_L}\right],$$

where V_O is the perfusion rate; C_O, C_b, and C_L are the osmolalities of the perfusate bath, and collected fluids, respectively; A is the luminal surface area; and V_w is the partial molar volume of water.

Statistics

All data in the test and figures are expressed as the mean±SE. Statistical analysis of the results obtained in the in vitro incubation of CCT and PST (studies I, II, and III) was performed by the t test for unpaired data. Statistical analysis for the in vitro perfusion studies (IV and V) was performed by the t test for paired data. A difference was considered significant if $P < 0.05$.

RESULTS

Nonstimulated prostaglandin biosynthesis by isolated rabbit nephron segments. Isolated rabbit CCT were capable of synthesizing all of the major arachidonic acid metabolites, which were separated by silica gel thin-layer chromatography (TLC). A representative experiment is shown in Fig. 1. All but one of the radioactive peaks comigrated with known standards, indicating that the CCT produced PGE_2, TxB_2, $PGF_{2\alpha}$, and PGI_2. (6-Keto-$PGF_{1\alpha}$, a metabolite of PGI_2, comigrates with the PGI_2 standard in the solvent system used for these experiments.) Either indomethacin ad-

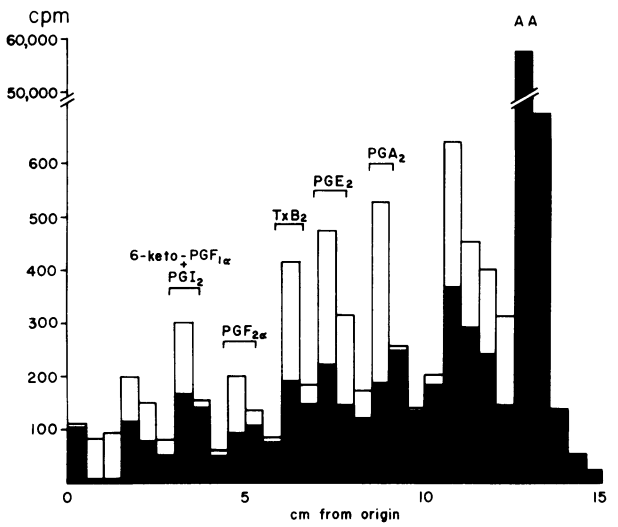

FIGURE 1 Silica gel thin-layer chromatogram of acid extract of supernatant of [^{3}H]arachidonic acid prelabeled rabbit CCT. Results are from a representative study and are expressed as counts per minute per millimeter tubule length. Control incubations are represented as the total height of each bar, and incubations performed with addition of a cyclooxygenase inhibitor are shown in the shaded portion. The positions of the various prostaglandin and thromboxane standards are shown. AA refers to unreacted [^{3}H]arachidonic acid.

ministration (shown as solid bars in Fig. 1) or meclofenamate administration led to a significant decrease in the counts recovered in the region of the prostaglandin peaks. The results with both cyclooxygenase inhibitors were equal and they have been combined. A peak at 2 cm from the origin did not correspond to any of the standards used and presumably represented an arachidonic acid metabolite because it also was decreased after cyclooxygenase inhibition. A peak also appeared between the PGA_2 and arachidonic acid peaks. This has been previously thought to represent hydroxy acids (8, 15). Because the counts in this area were decreased after the administration of indomethacin (or meclofenamate), this peak may represent some other unidentified cyclooxygenase derivative.

PGA_2 is not thought to be a primary prostaglandin but rather a metabolite that results from the dehydration of PGE_2 (16). Exclusive of PGA_2, the four main arachidonic acid metabolites accounted for ~2% of the total counts recovered. When factored for tubular length, PGE_2 represented 0.75%; TxB_2, 0.50%; $PGF_{2\alpha}$, 0.40%; and PGI_2 (plus its metabolite 6-keto-$PGF_{1\alpha}$), 0.35% of the total counts (Table I). Although these calculations were approximations, they demonstrate that PGE_2 is the most abundant prostaglandin synthesized by this nephron segment under these conditions. Similar results were obtained when counts were factored for tubular protein content. Cyclooxygenase in-

TABLE I
Prostaglandin Biosynthesis by Isolated CCT

Exp.	Inhibitor	6-keto-$PGF_{1\alpha}$ + PGI_2	$PGF_{2\alpha}$	TxB_2	PGE_2
		cpm/mm	*cpm/mm*	*cpm/mm*	*cpm/mm*
1	Vehicle	317	156	462	602
2	Vehicle	386	682	676	975
3	Vehicle	214	357	286	457
4	Vehicle	292	209	385	600
5	Vehicle	348	408	490	782
6	Vehicle	285	238	280	612
Mean		307	342	430	671
SE		24	78	61	74
1	Indomethacin	207	67	245	373
2	Meclofenamate	227	256	433	398
3	Meclofenamate	143	286	433	314
4	Indomethacin	185	135	238	238
5	Indomethacin	200	209	254	211
6	Indomethacin	180	150	189	389
Mean		190	184	261	321
SE		12	33	36	33
P value		<0.01	<0.01	<0.01	<0.01

hibition decreased the formation of the metabolites from 50% for PGE_2 to 37% for TxB_2.

Isolated rabbit PST were equally capable of synthesizing all of the major arachidonic acid metabolites, but to a much lesser degree. This applied whether the data were expressed per unit tubular length or per unit protein content. After incubation with radiolabeled arachidonic acid, the amount of radioactivity that comigrated with these four major metabolites accounted for <0.5% of the total counts as compared with ~2% in CCT. Cyclooxygenase inhibition in PST resulted in a comparable degree of reduction of counts (Table II).

TABLE II
Prostaglandin Biosynthesis by Isolated PST

Exp.	Inhibitor	6-keto-$PGF_{1\alpha}$ + PGI_2	$PGF_{2\alpha}$	TxB_2	PGE_2
		cpm/mm	*cpm/mm*	*cpm/mm*	*cpm/mm*
1	Vehicle	12	58	30	107
2	Vehicle	15	17	14	4
3	Vehicle	57	193	289	278
4	Vehicle	268	90	120	128
Mean		97	90	113	128
SE		57	38	63	56
1	Indomethacin	42	18	36	29
2	Indomethacin	18	26	40	36
3	Meclofenamate	38	40	26	48
4	Meclofenamate	63	35	31	12
Mean		40	30	33	31
SE		9	5	3	8
P value		<0.05	<0.05	<0.05	<0.05

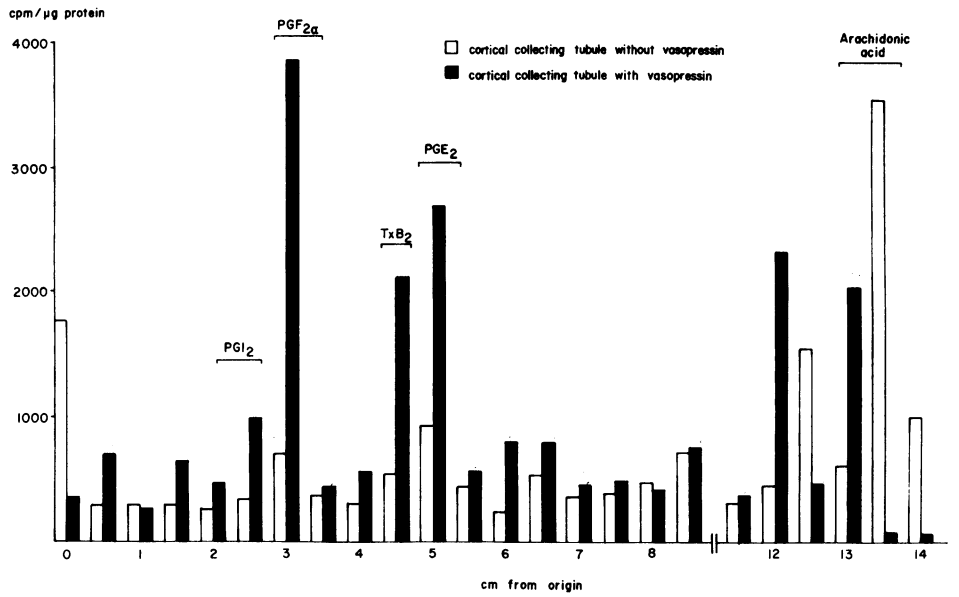

FIGURE 2 Thin-layer chromatogram of acid extract of the supernatant of [^{3}H]arachidonic acid prelabeled rabbit CCT. Results are from a representative study and are expressed as counts per minute per microgram protein. Control incubations are shown in the open bars and incubations with vasopressin (0.56 nM) are shown in shaded bars. Positions of the various prostaglandin and thromboxane standards are shown.

Vasopressin-stimulated prostaglandin biosynthesis by isolated rabbit nephron segments. After preincubation of the tubules with octatritiated arachidonic acid, ~2% of the radioactive counts were incorporated into the tissue. A representative chromatogram (Fig. 2) illustrates the effect of incubating the rabbit CCT with or without vasopressin. Each of the major radioactive peaks noted on the chromatogram comigrated with a known standard. Incubation of the tubules with vasopressin resulted in an increase in the height of all of the peaks comigrating with arachidonic acid metabolites, and a decrease in the height of the arachidonic acid peak when compared to levels obtained in tubules incubated in the absence of vasopressin.

The mean (±SE) of the counts comigrating with the four major arachidonic acid metabolites both with and without vasopressin, and with vasopressin plus dexamethasone or indomethacin, is shown in Fig. 3 for five experiments. Vasopressin increased the counts recovered for all of the four arachidonic acid metabolites. Dexamethasone blunted the vasopressin response, whereas indomethacin decreased the height of the prostaglandin peaks and increased that for arachidonic acid itself. The total counts recovered from TLC for arachidonic acid and its metabolites were identical for both vasopressin alone (6,120±1,278 cpm/μg protein) and vasopressin plus indomethacin (6,286±1,430) cpm/μg protein) when compared to control (2,205±1,185 cpm/μg protein). Dexamethasone administration decreased the total counts recovered to 42% of control values.

Similar studies were performed in PST prepared and

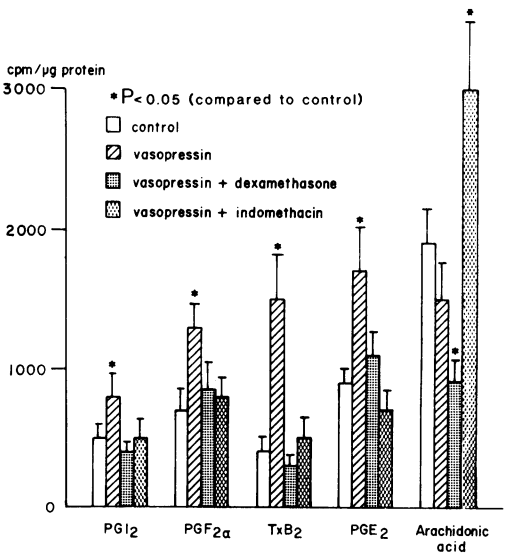

FIGURE 3 Comparison of counts recovered from silica gel thin-layer chromatogram for rabbit CCT prelabeled with [^{3}H]arachidonic acid. Results, shown as the mean±SE, represent results for the four principal arachidonic acid metabolites and arachidonic acid and are expressed as counts per minute per microgram protein.

incubated in the same manner to assess whether the apparent increase in prostaglandin biosynthesis induced by vasopressin was specific for the CCT. The response of PST to vasopressin was significantly less than that seen with CCT. Unlike the 300% increase in counts seen in the CCT studies, vasopressin administration resulted in a <30% increase in total counts recovered in PST (control, 986±323 cpm/μg protein; vasopressin 1,260±775 cpm/μg protein; $n = 4$; $P > 0.2$).

To assess whether the effect of vasopressin on tubular prostaglandin biosynthesis was the result of the antidiuretic properties of the polypeptide hormone or whether part of the effect might be due to its vasopressor effect, an additional group of four experiments was performed with increasing doses of DDAVP. Increasing the concentration of DDAVP from 0.56 to 56 nM resulted in a stepwise increase in both the total TLC counts recovered (Fig. 4) and the conversion of arachidonic acid to the prostaglandins. Identical concentrations of DDAVP and arginine vasopressin resulted in qualitatively similar patterns of CCT-prostaglandin biosynthesis (not shown).

Because it was possible that the results obtained with vasopression could have been due to an effect in mobilizing only the radiolabeled pool of arachidonic acid (an effect that could reflect a decrease rather than an increase in prostaglandin synthesis), the absolute vasopressin-induced increase in prostaglandin biosynthesis in the CCT was assessed by measuring the concentration of PGE released into the incubation medium by a specific radioreceptor assay. The prostaglandins in the medium were extracted with chloroform:methanol and separated using column chromatography. The results are shown in Fig. 5. In

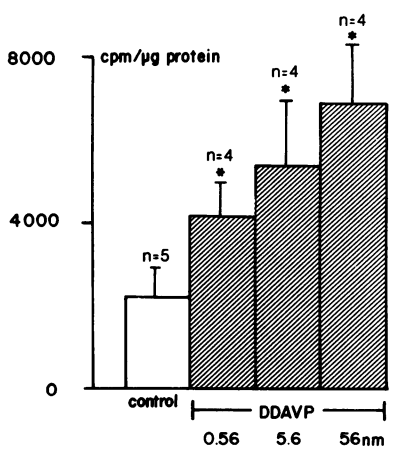

FIGURE 4 Total counts recovered from silica gel thin-layer chromatogram for rabbit CCT prelabeled with [^{3}H]arachidonic acid and exposed to different concentrations of DDAVP. Results, shown as the mean±SE, are expressed as counts per minute per microgram protein.
* $P < 0.05$.

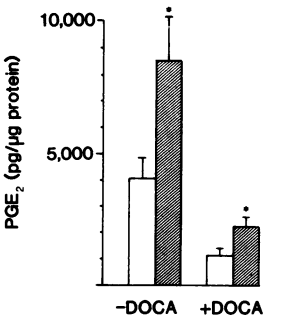

FIGURE 5 Levels of PGE_2, measured by radioreceptor assay, extracted from supernatant of rabbit CCT incubated with and without vasopressin. Studies were performed in DOCA- ($n = 8$) and non-DOCA-treated animals ($n = 4$). Results, shown as the mean±SE, are expressed as picogram per microgram protein. □, control; ▨, vasopressin. $P < 0.05$.

eight experiments, the addition of vasopressin to the incubation medium resulted in an increase in the production of PGE from 4,109±859 to 8,531±1,790 pg/h ($P < 0.01$). The absolute increase in vasopressin-induced PGE biosynthesis by CCT of DOCA-treated animals was assessed in four experiments. Without vasopressin, basal levels of PGE released into the incubation medium were 1,532±575 pg/h, which was 63% < that in non-DOCA treated animals ($P < 0.01$). After incubation with vasopressin, PGE_2 levels increased to 2,010±499 pg/h ($P < 0.05$ vs. DOCA-treated animals without vasopressin; $P < 0.01$ vs. non-DOCA treated animals).

Incorporation of radiolabeled arachidonic acid into phospholipids. These studies were performed to confirm that radiolabeled arachidonic acid was incorporated into cellular phospholipid pools. At the end of 1 h of incubation, 11.9% of the labeled arachidonic acid (4,751±589 dpm) was incorporated into CCT. Free arachidonic acid accounted for only 15.3% of these counts (730±269 dpm). Of the remaining counts, 65.0% was incorporated into phosphotidylethanolamine, 17.2% into phosphotidylinositol and phosphotidylcholine, and 15.8% into phosphotidylcholine. The remaining counts could not be identified using authentic standards of phospholipids and may represent other fatty acids and/or triglycerides.

In vitro perfusion of CCT in the absence of indomethacin. The hydroosmotic response to 2.5 μU/ml Pitressin was evaluated in isolated, perfused CCT obtained from rabbits on a normal diet, on a high potassium diet, or on DOCA. Arachidonic acid was present in the bath for 90 min before the first control period and throughout the remainder of the experiment. Because the results were similar in the different groups, they have been pooled and are depicted in Table III. The tubules were impermeable to water in the absence of vasopressin, net water flux being only 0.18±0.04 nl/

TABLE III

Effect of Vasopressin (2.5 μU/ml Pitressin) on J_v and P_f of Isolated Perfused Rabbit CCT

Diet	Perfusion rate	Lumen area	Control J_v	Vasopressin J_v			Control P_f	Vasopressin P_f		
	(nl/min)	($\times 10^{-4}$ cm^2)	(nl/mm/min)	15'	(nl/mm/min) 30'	45'	($\times 10^{-4}$ cm/s)	15'	($\times 10^{-4}$ cm/s) 30'	45'
Normal	7.74	4.72	0.19	0.00	−0.55	—	—	—	—	—
High K	8.12	9.20	0.06	0.27	0.58	—	—	—	—	—
Normal	9.82	9.49	0.36	0.15	0.04	—	—	—	—	—
High K	10.86	4.25	0.16	0.16	0.31	0.03	65	35	32	—
High K	5.60	11.38	0.20	0.11	0.11	−0.07	49	61	89	69
DOCA	14.01	10.96	0.05	−0.51	0.11	−0.18	82	—	92	73
DOCA	6.58	18.92	0.26	0.22	0.17	0.04	43	34	27	24
Mean	8.96	9.27	0.18	0.06	0.11	0.05	49	43	60	55
±SE	1.08	2.18	0.04	0.10	0.13	0.05	17	9	18	16
P (vasopressin vs. control)				NS	NS	NS		NS	NS	NS

mm per min in the presence of a transtubular osmotic gradient of 165 mosmol/kg H_2O. Vasopressin at the concentration used had no significant effect on the P_f, at 15, 30, or 45 min.

In vitro perfusion of CCT in the presence of indomethacin. The results of these studies are depicted in Table IV. In CCT obtained from rabbits on a normal diet, net water flux was not increased by the addition of 2.5 μU/ml Pitressin to the bath. In contrast, CCT obtained from rabbits maintained on a high potassium diet or DOCA showed an increase in net water flux and P_f after 15 and 30 min exposure to vasopressin. The maximum effect was evident at 15 min and declined thereafter, consistent with previous observations (12, 17).

The effect of cyclooxygenase inhibitors on the hydroosmotic response to vasopressin of CCT from rabbits on normal diets was also studied when arachidonic acid was not in the bath. Studies comparing the effects of a submaximal dose of vasopressin with the same dose in combination with either indomethacin or naproxen are shown in Fig. 6. A small increase in J_v was observed with vasopressin alone. The addition of either indomethacin or naproxen caused J_v to increase over a period of 1 h in all five tubules studied. However, this response was lower than that elicited by a maximal dose of Pitressin (25 μU/ml) that increased J_v to 0.86±0.19 in three tubules. In contrast, J_v remained stable when vehicle alone was added to the bath.

Transtubular PD of isolated perfused CCT. A study of the time course of the transtubular PD of CCT obtained from rabbits on a normal or high potassium diet (or DOCA) in the presence and absence of indomethacin in the bath was performed. In both vehicle- and indomethacin-treated tubules from animals on a high potassium diet or DOCA, the transepithelial PD was significantly more lumen-negative than in tubules from animals on a control diet (i.e., ~0 to −10 mV vs. ~−15 to −30 mV after 2 h of perfusion). Thus, exposure of the tubules to indomethacin for 180 min did not attenuate the increased lumen negativity observed in CCT obtained from high potassium- or DOCA-treated animals.

DISCUSSION

The observations of Orloff and Zusman (4) that prostaglandins antagonize the action of antidiuretic hormone on the toad bladder have stimulated a number of investigators to examine whether prostaglandins play a regulatory role in the antidiuretic response to vasopressin. Two critical questions that have been posed are whether prostaglandins antagonize the renal action of vasopressin and whether vasopressin stimulates renal prostaglandin synthesis. The evidence supporting or refuting these questions has recently been summarized by Beck and Dunn (7). These questions are obviously important when considered together, for if vasopressin does stimulate prostaglandin synthesis in the mammalian collecting tubule, and if the endogenously synthesized prostaglandins antagonize the hydroosmotic effects of vasopressin, a feedback control loop can be constructed that would explain important aspects of the regulation of vasopressin action at the level of the end-organ.

The present studies were designed to examine these questions in vitro by direct observations of the mammalian collecting tubule. The first studies were performed to determine whether the mammalian CCT had the capacity to synthesize prostaglandins. Neither the observations of Bohman et al. (18), who demonstrated prostaglandin synthetase activity in aspirated

TABLE IV

Effect of Vasopressin (2.5 μU/ml Pitressin) on J_v and P_f of Rabbit CCT: Studies Performed in the Presence of 220 μM Indomethacin

	Control perfusion rate	Lumen area	Control-J_v	Vasopressin-J_v			Control-P_f	Vasopressin-P_f*		
	(nl/min)	($\times 10^{-4}$ cm^2)	(nl/mm/min)	(nl/mm/min)			($\times 10^{-4}$ cm/s)	($\times 10^{-4}$ cm/s)		
				15′	30′	45′		15′	30′	45′
(a) Normal diet										
	6.39	11.07	0.15	−0.19	−0.49	−0.25	—	—	—	—
	7.37	10.06	0.19	0.50	0.62	0.47	—	—	—	—
	13.33	10.13	−0.13	−0.07	—	0.15	—	—	—	—
	9.12	10.28	0.18	0.16	−0.07	0.11	—	—	—	—
	10.25	11.61	0.07	−0.15	−0.26	0.11	—	—	—	—
Mean	9.29	10.54	0.09	0.10	0.05	0.12	—	—	—	—
±SE	1.21	0.24	0.06	0.15	0.24	0.15	—	—	—	—
P (vasopressin vs. control)				NS	NS	NS				
(b) High potassium diet or DOCA administration										
High K	6.11	10.77	−0.12	1.57	0.37	—	23	417	162	—
High K	8.78	16.01	0.01	1.11	0.98	0.90	27	177	248	136
High K	6.72	4.58	0.11	1.00	0.94	0.77	20	401	200	311
DOCA	12.29	13.43	0.12	1.16	0.78	0.48	23	96	85	80
High K	10.72	10.32	0.00	0.42	0.41	0.29	35	43	24	6
High K	9.61	15.52	0.07	−0.07	−0.07	−0.08	22	58	62	44
DOCA	9.02	13.06	0.05	0.57	—	0.24	57	116	60	45
DOCA	12.71	24.71	0.19	0.24	0.20	−0.07	44	80	33	—
DOCA	7.87	12.56	0.12	0.28	0.18	−0.01	10	37	24	16
Mean	9.31	13.44	0.06	0.69	0.47	0.32	29	158	100	91
±SE	0.76	1.80	0.03	0.18	0.14	0.13	5	49	28	40
P (vasopressin vs. control)				<0.005	<0.01	NS		<0.02	<0.02	NS

* Osmolalities were not measured in the tubules in section (*a*), thus P_f was not calculated.

collecting tubule epithelial cells, nor those of Smith and Wilkin (19), who demonstrated the existence of fatty acid cyclooxygenase in the collecting tubule by immunohistological techniques, examined this question directly. Studies performed in isolated medullary ascending limbs and medullary collecting tubules by Jackson et al. (20) did demonstrate the presence of prostaglandin biosynthesis in these nephron segments. Further studies in the toad bladder have shown that a variety of prostaglandins are synthesized by this organ (3, 21), and the finding that TxB_2 actually potentiated the hydroosmotic effect of vasopressin suggested that the nature of the synthesized prostaglandins may be an important determinant of the regulation of vasopressin on the collecting tubule cell. In this regard, however, Ludens and Taylor (22) have recently shown that synthetic prostaglandin endoperoxide analogues, which have "thromboxane-like" activity, may actually inhibit vasopressin-stimulated water flow in the toad bladder.

In the present study, using thin-layer chromatographic techniques, we have demonstrated that the rabbit CCT is capable of synthesizing all of the major prostaglandins. In quantitative order these are: PGE_2, $PGF_{2\alpha}$, PGI_2, and TxB_2. The assay measures release of ^{3}H-labeled prostaglandins into the medium upon incubating collecting tubules with [^{3}H]arachidonate and allows the synthetic process to be studied in intact collecting tubule cells. The synthesis of each of the prostaglandins was inhibited ~50% by two different cyclooxygenase inhibitors, indomethacin and meclofenamate. PST (superficial S_2 segments) were also shown to synthesize all of the above prostaglandins but to a significantly lesser extent. This was apparent whether the conversion of arachidonic acid was expressed per millimeter tubule length or per microgram tubule protein.

Studies on the toad bladder by Zusman et al. (23) have shown that aldosterone inhibits vasopressin-stimulated PGE biosynthesis by ~65%, and Hall and Gran-

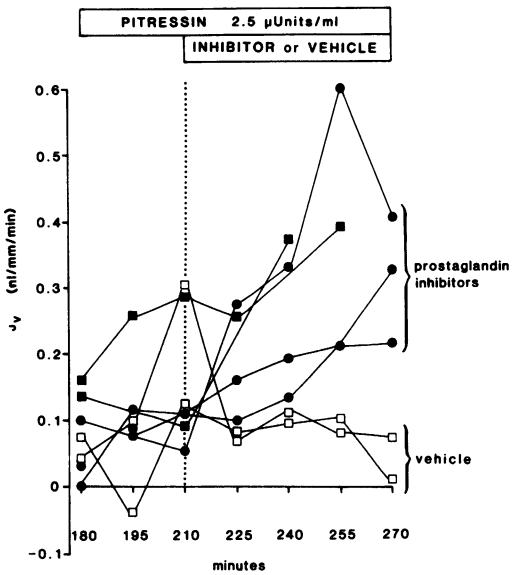

FIGURE 6 Net fluid reabsorption (J_v) in CCT of rabbits on a normal diet perfused at 25°C in the absence of exogenous arachidonic acid. Control studies are shown in open squares, indomethacin studies are shown in closed circles, and naproxen studies in closed squares.

tham (12) have demonstrated that the hydroosmotic effect of vasopressin on the in vitro-perfused rabbit collecting tubule is enhanced in rabbits pretreated with corticosterone, aldosterone, or dexamethasone. We therefore wished to evaluate whether basal synthesis of PGE by the rabbit CCT was altered by mineralocorticoid excess. A sensitive radioreceptor assay was used to quantitate PGE synthesis (10). Administration of DOCA (~2.5 μg/kg per d) reduced basal PGE synthesis by ~63%.

The second problem of importance was to determine whether vasopressin directly stimulated prostaglandin synthesis by the collecting tubule. Indirect evidence on this point is conflicting. In vivo studies on homozygous Brattleboro diabetes-insipidus rats indicate that vasopressin (Pitressin tannate) increases urinary prostaglandin excretion and returns urine volume to normal (24, 25). That DDAVP, a vasopressin analogue that lacks pressor activity, has the same effect strongly suggests that it is the antidiuretic property of vasopressin that is important in this respect (25). Studies on the conscious dog are less consistent, showing both an increase (26) and a decrease in prostaglandin excretion (10) after vasopressin administration.

In vitro studies have failed to determine whether the effects of vasopressin on prostaglandin release are mediated by an action on either renomedullary interstitial cells or on collecting tubule epithelial cells. Certainly, synthesis of prostaglandins by the toad bladder (the traditional "analogue" of the collecting tubule) is increased by vasopressin (3), but even these observations recently have been called into question by Bisordi et al. (27) and Forrest and Goodman (28). Rabbit collecting tubule cells in culture (29) and Madin-Darby Canine Kidney (MDCK) cells (7) (which share many properties with collecting tubule cells) fail to show stimulation of prostaglandin by vasopressin. All the foregoing studies make it difficult to conclude whether vasopressin stimulates prostaglandin synthesis by the intact collecting tubule.

We examined this question directly in vitro by exposing intact collecting tubules to both arginine vasopressin and DDAVP. Administration of either agent resulted in a dose-dependent increase in biosynthesis and release of all of the prostaglandins, with the major effect being on PGE_2. Again, pretreatment of the animals with DOCA reduced vasopressin-stimulated synthesis by ~76%. It is evident therefore that vasopressin stimulation of prostaglandin synthesis by the collecting tubule is more likely to have a physiologically significant effect in antagonizing the hydroosmotic effect of vasopressin in normal rabbits than in rabbits with mineralocorticoid excess. Vasopressin had no significant effect on prostaglandin biosynthesis by the PST, a segment that does not possess vasopressin-sensitive adenylate cyclase (30).

We next evaluated whether endogenous prostaglandin synthesis by the rabbit CCT plays a role in modulating the response of this nephron segment to vasopressin. We reasoned that if prostaglandin synthesis were inhibited, a submaximal dose of vasopressin would show an enhanced hydroosmotic effect if a feedback control loop was thereby disrupted. Because basal and vasopressin-stimulated prostaglandin synthesis was significantly lower in DOCA-treated rabbits, it would be anticipated that significantly lower prostaglandin levels would exist in these animals and that the hydroosmotic effect of vasopressin would be correspondingly greater.

We chose a dose of vasopressin (2.5 μU/ml Pitressin) previously shown to be submaximal (13). In fact, in the setting of the present experiments in which arachidonic acid was added to the bath to potentiate prostaglandin synthesis by the CCT perfused in vitro, this low concentration of vasopressin had no hydroosmotic effect. Addition of indomethacin at a concentration shown to inhibit prostaglandin synthesis by the CCT by at least 50% did not expose a hydroosmotic response. These studies, in concert with the direct observation on PGE synthesis by normal CCT described above, suggested that cellular PGE levels were sufficiently high, even after indomethacin treatment, to antagonize the effects of vasopressin.

To determine whether this indeed was the explanation, additional experiments were performed in which arachidonic acid was omitted from the bath.

Under these circumstances, a small hydroosmotic response to vasopressin was observed. This was further augmented by the addition of a cyclooxygenase inhibitor, either indomethacin or naproxen, to the bath.

In contrast to the findings described above, the results obtained in rabbits with exogenous DOCA or endogenous mineralocorticoid excess (secondary to potassium-loading) were striking. With arachidonic acid present in the bath, the same low dose of vasopressin elicited a significant hydroosmotic response in CCT exposed to indomethacin for 90 min. Indomethacin alone (before the addition of vasopressin) had no effect on basal water permeability.

Two additional side issues emerge from these studies. The first relates to the effects of prostaglandins on sodium chloride by the collecting tubule. In rabbits with mineralocorticoid excess, net sodium transport by the CCT is significantly increased as is the transtubular PD. In the present studies, the PD was increased in the DOCA-treated or potassium-loaded rabbits. Exposure to indomethacin had no effect on the development of this increased luminal negativity, further supporting our contention that basal prostaglandin synthesis has no effect in modulating sodium chloride transport by the collecting tubule (31).

The second observation of interest relates to the observed temperature-dependent refractoriness to vasopressin that develops in CCT perfused at 37°C but not at 25°C (12, 17). One possible explanation for this refractoriness, which develops within ~10–15 min after a maximal response has been obtained and which progresses over a period of 3 h, is a prostaglandin-dependent antagonism to vasopressin. Prostaglandins have been proposed to antagonize vasopressin action by inhibiting vasopressin-stimulated cyclic AMP (cAMP) production (32); however, since refractoriness to exogenous cAMP has also been shown to occur (12), it is considered unlikely that this is explained by prostaglandin antagonism. The present studies confirm this contention because refractoriness to vasopressin was observed within 30 min and significant blunting of the response within 45 min, in tubules exposed to indomethacin.

The present observations allow us to propose a model for the control of vasopressin action at the level of the end organ, the collecting tubule. The mammalian collecting tubule synthesizes all of the major prostaglandins. The rate of synthesis is increased by vasopressin. Vasopressin increases the water permeability of the collecting tubule and concomitantly increases prostaglandin synthesis by the tubular epithelial cells. This increase serves to antagonize the hydroosmotic effect of vasopressin and to function as a closed feedback loop. This feedback regulation of vasopressin action is probably more effective under normal conditions than under conditions of mineralocorticoid excess, because higher stimulated levels of prostaglandins are attained in the former state. In states of mineralocorticoid excess, prostaglandin biosynthesis is suppressed to a lower level than can be obtained under normal circumstances, so that an enhancement of the response to a submaximal dose of vasopressin is more readily apparent. This observation, too, may have physiologic relevance in that renal concentrating ability may be enhanced under these circumstances, but whether the effects of mineralocorticoids in this regard are solely due to a suppression of prostaglandin synthesis or whether other cellular events are involved remains to be determined.

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