Journal of Supramolecular Structure 5:521 (373)–530 (382) (1976)

Involvement of Microtubules and Microfilaments in the Action of Vasopressin in Canine Renal Medulla

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Vasopressin-stimulated cyclic AMP content and the uptake of $^{3}H_2O$ and ^{22}Na into canine renal medullary slices were measured. Cyclic AMP was increased threefold by 9×10^{-9} M vasopressin in isotonic (290 mOsm/kg H_2O) Krebs-Ringers bicarbonate. A significant increase in vasopressin-stimulated $^{3}H_2O$ uptake began at 2.75 min after hormone addition and lasted until 5.00 min. Colchicine (1×10^{-5} M) inhibited the vasopressin-stimulated $^{3}H_2O$ uptake. This effect required a minimum preincubation period of 30–40 min in colchicine-containing medium. Colchicine had no effect on basal or vasopressin-stimulated cyclic AMP levels. Colchicine also inhibited $^{3}H_2O$ uptake stimulated by dibutyryl cyclic AMP (10 mM). Lumicolchicine (10^{-5} M) had no effect on either vasopressin- or dibutyryl cyclic AMP-stimulated $^{3}H_2O$ uptake. ^{14}C-colchicine bound predominantly to the cytosol fraction enriched in microtubules, while virtually no binding was observed on plasma membranes. Light-microscopic examinations of cross sections of tissue slices showed that a majority of vasopressin-treated collecting tubules and some control tubules had occluded lumens. Colchicine-treated cells, in the presence of vasopressin, had open lumens indicating a blockage of the vasopressin-induced water transport. Cells treated with cytochalasin B (1 μgm/ml) also had open lumens in the presence of vasopressin. Cytochalasin B also blocked vasopressin and dibutyryl cyclic AMP-stimulated $^{3}H_2O$ uptake into collecting duct cells but had no effect on vasopressin-stimulated cyclic AMP levels. It was concluded that microtubules and possibly microfilaments are involved in the subcellular mechanism by which vasopressin increases the permeability of the collecting duct to water.

Key words: cyclic AMP, permeability, renal medulla, vasopressin, microtubules, microfilaments

INTRODUCTION

The final osmolarity of urine produced by the mammalian kidney is dependent on many factors. The final step in regulation of urine osmolarity is effected by vasopressin (VP) (1). The subcellular mechanism, distal to cyclic AMP production, by which this process is effected in the medulla is poorly understood. A potentially useful approach is the identification of the subcellular organelles critical for effecting the process. Based on the principle of conservation of biological mechanism, it was first suggested by Taylor and her colleagues (2) that the microtubule-microfilament system was involved. Since then, there has been evidence to establish that this is indeed a valid hypothesis (3, 4). Most of

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the data obtained has been obtained from bladders. In the mammalian kidney in vivo it has been shown that colchicine blocks the VP-induced concentration of urine (5). In this paper we present evidence of the involvement of microtubules-microfilaments in the water permeability increase induced by vasopressin in the canine renal medulla.

MATERIALS AND METHODS

Calbiochem supplied (105 IU/mg) 8-Lysine Vasopressin. Cyclic AMP radio immunoassay kits were obtained from Schwarz-Mann. All radioisotopes and labeled compounds were obtained from New England Nuclear. All chemicals were obtained from Sigma. β and γ isomers of lumicolchicine were prepared by the method of Mizel and Wilson (6). All electron microscopic materials were obtained from Poly Sciences.

Preparation of Tissue Slices

Dogs of either sex, allowed food and water ad libitum, were anesthetized with Na-pentobarbital (30 mg/kg). Both kidneys were removed through a midline abdominal incision after clamping of the renal pedicle. The kidneys were transected by a coronal cut and the white renal medullary area cut out and cut into slices (0.6 mm thick, 30–70 mg) with a scalpel blade and plastic depth guide. The medullary slices were preincubated in oxygenated Krebs-Ringers bicarbonate (KRB) (120 mM NaCl, 25 mM $NaHCO_3$ 4.5 mM KCl, 2.5 mM KH_2PO_4, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM glucose, pH 7.4). Colchicine (1×10^{-5} M), lumicolchicine (1×10^{-5} M), and cytochalasin B (1 μg/ml) were present in the preincubation medium and reaction mixture as indicated in specific experiments. Cytochalasin B was dissolved in dimethylsulfoxide (DMSO) (0.1% final concentration). Control experiments contained 0.1% DMSO when appropriate. The final concentration of VP in the reaction mixture was 9×10^{-9} M. In some experiments, 10 mM dibutyryl cyclic AMP (dBCAMP) was substituted for vasopressin.

Measurement of Cyclic AMP

At fixed time intervals, after the addition of VP, tissue slices were removed and rapidly frozen in precooled Potter-Elvejham homogenizers in a dry-ice ethanol slush at −20°C. The homogenizer contained a small amount (~ 10,000 cpm) of ^{3}H-cyclic AMP, which was used to monitor recovery in all subsequent steps. One ml of 4% ice-cold perchloric acid was added and the sample homogenized immediately. The homogenate was centrifuged in a clinical centrifuge and the supernatant used for the analysis of cyclic AMP. The supernatant was loaded on an activated Dowex 50 AGW-X4 column (0.5 × 2.5 cm). The column was eluted with cold distilled water. The first 3 ml was discarded; the next 3 ml was collected in 1 ml fractions and 0.1 ml of each was counted in 10 ml of Triton X-100/toluene/Liquifluor (New England Nuclear) (1:2:0.13, v/v) scintillation cocktail in a Beckman scintillation counter (LS-150) to obtain estimates of cyclic AMP recovery. An aliquot of the sample showing the highest cyclic AMP recovery was chosen for estimation of cyclic AMP. The determination of cyclic AMP was by radioimmunoassay (Schwarz-Mann, Ref. 7). Protein in the precipitate was estimated by the method of Hartree (8).

Measurement of Tissue ^3H_2O and ^{22}Na Uptake

Tissue slices were distributed in plastic beakers containing the appropriate KRB solution. At '0' time, 0.5 ml of KRB solution containing ^3H_2O, ^{22}Na, and ^{14}C-inulin was pipetted

into the beakers containing the tissue slices. Tissue slices were removed at 15 sec intervals for the first 3 min, 30 sec intervals for the next 2 min, and 1 min intervals for the next 5 min. The experimental tissue slices were treated in an identical manner except that the isotope solution also contained VP. The final isotope concentrations were sufficient to give approximately 70,000, 20,000, and 4,000 cpm/0.1 ml of the ambient solution for ^{3}H, ^{14}C, and ^{22}Na, respectively. After removal from KRB the tissue slices were firmly blotted with filter paper (Whatman #2) and placed in a tared liquid scintillation vial and reweighed. The process of removal from KRB and blotting took less than 2 sec. Samples (100 μl) of extracellular fluid were taken and pipetted into tared vials and reweighed. Empty vials were tared along with the vials containing tissue to correct for any difference between the two weighings. The tissue slices and extracellular fluids were digested in 1 ml of 0.1 N NaOH at 65°C for 3 hr. After this digestion, 10 ml of Aquasol (New England Nuclear) was added. The vials were aged for 24 hr to eliminate chemiluminescence. The tissue digests were then counted in a three-channel liquid scintillation counter (Beckman LS-150). Suitable corrections were made for quenching and spillover. Uptake into the intracellular space was estimated as shown below. All counts used were corrected cpm/mg. ^{14}C = ^{14}C-inulin used as an extracellular space marker; ^{3}H = $^{3}H_2O$; W = relative weight; t = total tissue; i = intracellular compartment; a = ambient solution or extracellular fluid.

1) $R = {}^{3}H_a/{}^{14}C_a$
2) ${}^{3}H_i = {}^{3}H_t - {}^{14}C_t \cdot R$
3) $W_i = 1 - {}^{14}C_t/{}^{14}C_a$
4) $[{}^{3}H_i] = {}^{3}H_i/W_i$
5) $U = \text{Uptake} = [{}^{3}H_i]/{}^{3}H_a$
6) $\text{Relative uptake} = RU = U_{VP}/U_{control}$

VP-dependent transport is considered to occur if RU [Uvp/U control] was significantly ($P < 0.05$) greater than 1 as indicated by a paired *t* test.

^{14}C-colchicine Binding Studies

Plasma membranes were obtained by the method of Barnes et al. (9). The 100,000 g supernatant was used as "cytosol" and stabilized for tubulin by addition of $MgCl_2$ (10 mM), KH_2PO_4 (10 mM), GTP (2×10^{-4} M) and EDTA (5×10^{-5} M) according to the method of Wilson (10). ^{14}C-colchicine (17 mCi/mmole), 1×10^{-5} M, in a buffer of 10 mM $MgCl_2$ + 10 mM KH_2PO_4 was incubated with either plasma membrane or cytosol. The reaction was started by addition of the subcellular fraction. The final reaction volume was 50 μl. At fixed time intervals the reaction was stopped by rapid ultrafiltration of DEAE cellulose filter disks layered with millipore filter disks (0.45 μm). After two washes of 100 μl each with buffer, the filter disks were dried and counted in 10 ml Aquasol in a liquid scintillation counter. Heat-denatured (80°C for 15 min) membranes or cytosol fractions were used as blanks to account for nonspecific absorption. Binding is expressed as pmoles/mg protein.

Microscopy

Primary fixation was with 3% Karnovsky's solution (11) in 0.2 M s-collidine buffer for 3 hr and secondary fixation with 1% OsO_4 in 0.2 M s-collidine buffer using buffer and acetone rinses. Embedment was in Spurr's plastic (12) (100%, 5 hr at room temperature). Blocks were sectioned on a LKB ultratome I using a glass knife, and mounted on copper

grids. Sections were stained with uranyl acetate and Reynolds lead citrate (13) and examined under a Hitachi HS-8 electron microscope. Thick light sections were stained with toluidine blue and photographs were taken using phase optics.

RESULTS

Controls

Cyclic AMP production stimulated by VP (9×10^{-9}M) was biphasic and peaked at about 1.25 min after hormone addition. There was a net threefold increase over basal values (Fig. 1a). There was about a 1.5 min time lag between the beginning of the sustained peak cyclic AMP levels and the first significant increase in ^3H_2O uptake, which occurred at 2.75 min and lasted until 5.00 minutes after hormone addition (Table I). Essentially complete equilibration of intracellular spaces with respect to extracellular fluid occurred by 6.00 min. The vasopressin-stimulated ^3H_2O uptake was specific to

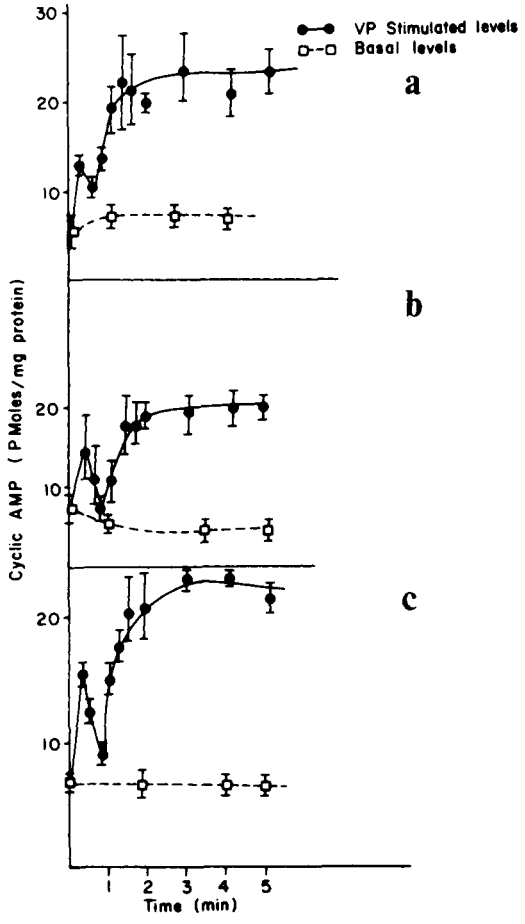

Fig. 1. CAMP levels in renal medullary tissue slices after addition of vasopressin (9×10^{-9}M) following 40 min preincubation in a) control KRB, b) colchicine (1×10^{-5}M), c) cytochalasin B (1 μg/ml).

renal medullary slices. Nontarget tissue for VP, such as liver, showed no vasopressin-stimulated $^{3}H_2O$ uptake (Table I).

Light-microscopic examination of tissue slices showed that tubules in control tissues had open lumens, as illustrated in Fig. 2a; 83% of control collecting tubules appeared similar to the tubule shown in Fig. 2a, while 17% showed occluded lumens as seen in Fig. 2b. Treatment of tissue slices with VP for 4 min resulted in increased numbers of occluded lumens (to 67%), as seen in Fig. 2b, whereas 33% appeared similar to control tissue.

Effects of Colchicine Isomers

Pretreatment of tissue slices with 1×10^{-5}M colchicine for 40 min did not affect the VP-stimulated cyclic AMP levels (Fig. 1b), whereas all VP-stimulated $^{3}H_2O$ uptake was abolished (Table II). A minimum time of 30 min preincubation in colchicine was required before inhibition of VP-dependent $^{3}H_2O$ uptake was observed (Table III). $^{3}H_2O$ and ^{22}Na uptake stimulated by dBCAMP (10 mM) was also abolished by colchicine (Table IV). Lumicolchicine (1×10^{-5}M) had no effect on either VP- or dBCAMP-stimulated $^{3}H_2O$ uptake (Table IV). ^{14}C-colchicine bound predominantly to the cytosol fraction, and virtually no binding was observed on the plasma membrane (Fig. 3). Microscopic examination of tissue slices preincubated in colchicine-containing medium for 40 min and treated with VP (9×10^{-9}M) for 4 min showed

TABLE I. Effect of Vasopressin on Uptake of $^{3}H_2O$ in Kidney Medulla and Liver Slices

	RU $^{3}H_2O$ (VP/control)					
	Kidney medulla			Liver		
Time (min)	Mean	S.E.	P	Mean	S.E.	P
0.25	0.980	0.054	N.S.			
0.50	1.034	0.191	N.S.	1.021	0.213	N.S.
0.75	1.192	0.164	N.S.			
1.00	0.938	0.137	N.S.	0.938	0.082	N.S.
1.25	1.278	0.208	N.S.			
1.50	0.923	0.217	N.S.	1.112	0.163	N.S.
1.75	0.930	0.081	N.S.			
2.00	0.925	0.112	N.S.	1.062	0.084	N.S.
2.25	1.293	0.214	N.S.			
2.50	1.194	0.096	N.S.	0.836	0.196	N.S.
2.75	1.273	0.092	$P > 0.05$			
3.00	1.546	0.331	N.S.	1.064	0.093	N.S.
3.50	1.290	0.102	$P > 0.05$	1.114	0.263	N.S.
4.00	1.255	0.062	$P > 0.05$	1.076	0.117	N.S.
4.50	1.364	0.118	$P > 0.05$	0.864	0.081	N.S.
5.00	1.362	0.117	$P > 0.05$	0.998	0.143	N.S.
6.00	0.962	0.112	N.S.	1.172	0.089	N.S.
7.00	1.072	0.083	N.S.	1.003	0.061	N.S.

Time indicates period of incubation in medium containing VP (9×10^{-9}M) at 37°C. N = 8 for each observation. Preincubation period was 30–40 min. Detailed procedures are described under Methods. No VP-sensitive increase in ^{22}Na uptake was observed in these experiments.

S.E. – standard error.

N.S. – not significant.

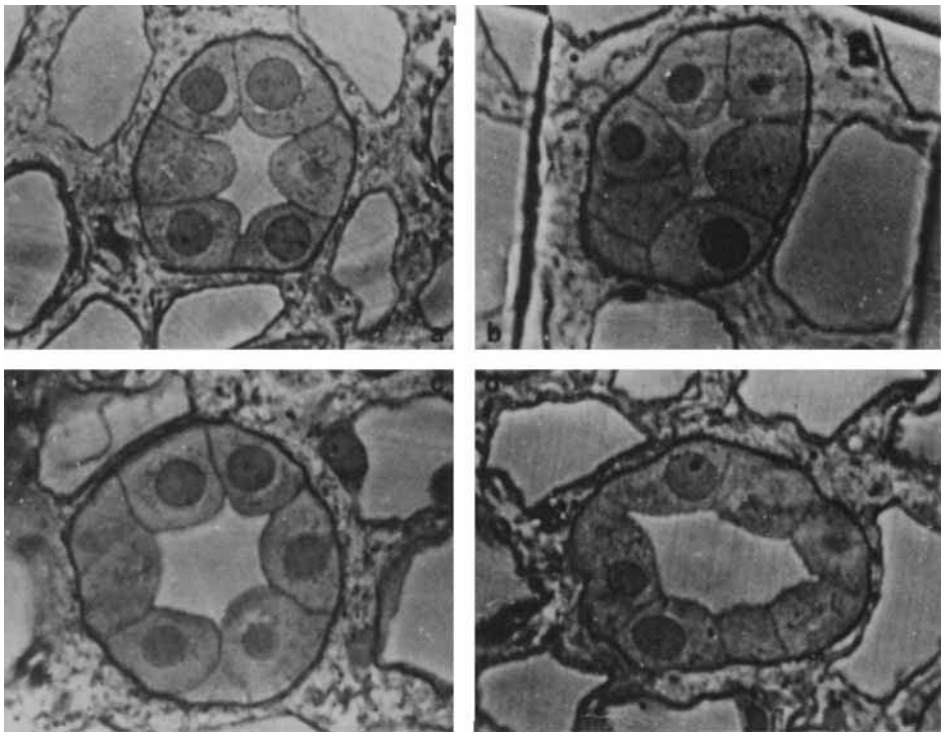

Fig. 2. Comparison of control tissue (a), and tissue treated with vasopressin (9×10^{-9}M) (b), vasopressin + colchicine (1×10^{-5}M) (c), and vasopressin + cytochalasin B (1 μg/ml) (d). 875 ×.

TABLE II. Effects of Colchicine, Lumicolchicine, and Cytochalasin B on Vasopressin-Stimulated ^3H_2O Uptake into Kidney Medulla Slices:

Time (min)	RU ^3H_2O (VP/control)								
	colchicine			lumicolchicine			cytochalasin B		
	Mean	S.E.	P	Mean	S.E.	P	Mean	S.E.	P
0.50	0.980	0.123	N.S.	1.237	0.120	N.S.	0.93	0.112	N.S.
1.0	1.099	0.222	N.S.	1.136	0.234	N.S.	0.981	0.136	N.S.
1.50	1.237	0.120	N.S.	0.945	0.101	N.S.	1.112	0.086	N.S.
2.00	1.122	0.242	N.S.	1.096	0.082	N.S.	1.074	0.136	N.S.
2.50	1.018	0.079	N.S.	1.067	0.124	N.S.	0.986	0.117	N.S.
3.00	1.061	0.040	N.S.	1.236	0.062	$P > 0.05$	0.843	0.084	N.S.
3.50	1.009	0.117	N.S.	1.322	0.073	$P > 0.05$	0.993	0.076	N.S.
4.00	0.948	0.180	N.S.	1.476	0.067	$P > 0.05$	1.236	0.242	N.S.
4.50	1.307	0.167	N.S.	1.312	0.064	$P > 0.05$	1.143	0.176	N.S.
5.00	1.080	0.132	N.S.	0.983	0.163	N.S.	1.032	0.054	N.S.
6.00	1.055	0.149	N.S.	1.007	0.083	N.S.	0.984	0.254	N.S.
7.00	1.074	0.090	N.S.	1.109	0.106	N.S.	0.843	0.273	N.S.
8.00	0.987	0.067	N.S.	1.087	0.093	N.S.	1.079	0.061	N.S.

Time indicates period of incubation in medium containing VP (9×10^{-9}M) at 37°C. N = 8 for each observation. Preincubation time for colchicine (1×10^{-5}M), lumicolchicine (1×10^{-5}M), and cytochalasin B (1 μg/ml) experiments was 40 min. Detailed procedures are described under Methods. No VP-stimulated increase in ^{22}Na uptake was observed in these experiments.

S.E. – standard error.

N.S. – not significant.

TABLE III. Effect of Colchicine on Vasopressin-Stimulated $^{3}H_2O$ and ^{22}Na Uptake

Time of preincubation (min)	RU $^{3}H_2O$ [VP/control]		RU ^{22}Na [VP/control]	
15	1.17 ± 0.04	$P < 0.05$	0.99 ± 0.06	N.S.
30	1.27 ± 0.11	$P \sim < 0.05$	1.11 ± 0.16	N.S.
60	1.08 ± 0.09	N.S.	1.06 ± 0.11	N.S.
90	0.78 ± 0.07	N.S.	0.91 ± 0.19	N.S.
120	0.97 ± 0.04	N.S.	1.14 ± 0.12	N.S.

Tissue slices were preincubated for varying time periods, as indicated, in KRB containing 1×10^{-5}M colchicine. At zero time the radioisotope mixture with or without hormone was added. Tissue slices were removed 4 min after radioisotope additions. Values are mean ± S.E. N = 6 for each time. N. S., not significant, i.e., $P > 0.05$; ~ is used to indicate value close to significance. Detailed procedures described under Methods.

TABLE IV. dBCAMP-Stimulated $^{3}H_2O$ and ^{22}Na Uptake

	Time (min)	RU $^{3}H_2O$	$\left[\frac{\text{dBCAMP}}{\text{control}}\right]$	RU ^{22}Na	$\left[\frac{\text{dBCAMP}}{\text{control}}\right]$
dBCAMP (10 mM)	2	1.65 ± 0.14	$P < 0.01$	1.39 ± 0.12	$P < 0.05$
	4	1.37 ± 0.03	$P < 0.001$	1.28 ± 0.07	$P < 0.05$
dBCAMP (10 mM)	2	1.14 ± 0.16	N.S.	1.14 ± 0.19	N.S.
+ colchicine ($1 \times {}^{-5}$M)	4	1.05 ± 0.12	N.S.	1.14 ± 0.26	N.S.
dBCAMP (10 mM)	2	1.38 ± 0.02	$P < 0.001$	1.46 ± 0.11	$P < 0.01$
+ lumicolchicine (1×10^{-5}M)	4	1.47 ± 0.09	$P < 0.01$	1.31 ± 0.09	$P < 0.05$
dBCAMP (10 mM)	2	1.06 ± 0.07	N.S.	1.23 ± 0.14	N.S.
+ cytochalasin B (1 μg/ml)	4	1.12 ± 0.23	N.S.	1.17 ± 0.12	N.S.

Tissue slices were preincubated in KRB medium for 40 min at 37°C. For the colchicine, lumicolchicine, and cytochalasin B experiments, the preincubation medium contained the respective drug. The radioisotope mixture with or without dBCAMP was added at zero time. All values indicate final concentrations achieved. Time indicates the incubation period in the dBCAMP + radioisotope mixture. Values are mean ± S.E. N = 6 for all experiments. N.S., not significant, i.e., $P > 0.05$. Detailed procedures described under Methods.

broadly open lumens as illustrated in Fig. 2c. Sixty-two percent of the tubules appeared as shown in Fig. 2c while the remaining 38% of the tubule lumens appeared similar to control tissue collecting tubules (Fig. 2a).

Effects of Cytochalasin B

Cytochalasin B (1 μg/ml) had no effect on VP-stimulated cyclic AMP levels (Fig. 1c). Cytochalasin B abolished all VP-stimulated $^{3}H_2O$ uptake (Table II). Light-microscopic examination of tissue slices incubated for 40 min in cytochalasin B (1 μg/ml) and treated with VP (9×10^{-9}M) for 4 min showed 100% of the tubules examined had open lumens as illustrated in Fig. 2d.

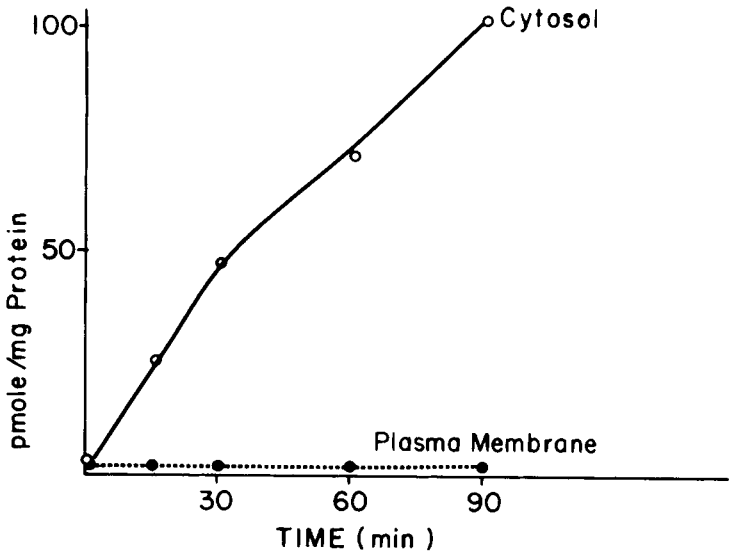

Fig. 3. Binding of ^{14}C-colchicine to plasma membranes and cytosol fractions of renal medullary tissue over time. N = 3 for each.

Ultrastructural Changes

Preliminary electron microscopic observations indicate: 1) an increased incidence of membrane-bound vesicles in the apical cytoplasm of colchicine- and cytochalasin-B-incubated tissue, and 2) an increased density of granular substance in the apical terminal web region of cytochalasin B-incubated cells, as compared to control incubated cells.

DISCUSSION

The renal medulla is a heterogeneous tissue consisting of the collecting tubules, thin limbs of the loop of Henle, and vasa recta. Among these structures only the collecting ducts have been shown to respond to VP (14). Cyclic AMP levels measured in the presence and absence of VP are the total amounts present in the renal medulla, but differences between control and VP-treated tissues represent VP-stimulated adenylyl cyclase in the collecting duct cells. Similarly, $^{3}H_2O$ uptake occurs into all intracellular spaces, of which only a fraction are in the collecting duct cells. In the collecting duct cells only the apical membranes constitute an ADH-sensitive barrier to water flow (15). Hence, the VP-stimulated $^{3}H_2O$ uptake, as defined under Methods, essentially measures an increased rate of water equilibration across the apical membrane. This effect is specific to the renal medulla, since no VP-stimulated $^{3}H_2O$ uptake was observed into the intracellular spaces of liver slices.

The blockage of the VP-stimulated $^{3}H_2O$ uptake by colchicine is distal to cyclic AMP production. Furthermore, it is evident that colchicine blocks the action of VP by interaction with tubulin and not by a direct membrane effect. This conclusion is based on the observation that colchicine, but not lumicolchicine, could block the VP-stimulated $^{3}H_2O$ uptake. It is now well established that, while lumicolchicine mimics colchicine in its effects on membrane processes, such as inhibition of nucleoside transport, in a number

of cell lines (6), lumicolchicine does not bind to tubulin in vitro and does not disrupt microtubules in vivo (16). Furthermore, the time lag of 40 min required for colchicine to block the VP effect, although relatively fast compared to the slow binding of colchicine to tubulin, is strikingly different from the colchicine inhibition of nucleoside transport in mammalian cell lines, where an almost instantaneous effect is observed (6). The relatively short preincubation required can be explained on the basis that it may be sufficient to disrupt a certain critical number of microtubules to cause an observable effect. The ^{14}C-colchicine binding to subcellular fractions indicated that 98.4% of the colchicine binding in the homogenate was recovered in the cytosol fraction. The cytological evidence showed open lumens in collecting ducts pretreated with colchicine and then treated with vasopressin. These data offer strong evidence that the integrity of microtubules is essential for vasopressin to increase $^{3}H_2O$ uptake into renal collecting duct cells.

Cytochalasin B is known to alter the microfilament system of a number of cell types, resulting in varying cell behavior (17). On the basis of these observations it is suggested that blockade of VP-stimulated $^{3}H_2O$ uptake by cytochalasin B is related to its capacity to alter microfilament structure. Cytochalasin B, like colchicine, acted distally to cyclic AMP production and blocked both VP- and dBCAMP-stimulated $^{3}H_2O$ uptake. No direct correlation between microfilament morphology and water permeability of the apical membrane was obtained and hence while it is highly probable that microfilaments are involved in VP-stimulated $^{3}H_2O$ uptake, more rigorous proof is required.

It has been known for some time that VP increases the fluidity of the apical membranes in both collecting ducts and toad bladder (15, 18). Studies on a number of mammalian systems indicate that microtubules and microfilaments are involved in the regulation of the fluidity of membranes (19, 20, 21). It is possible that VP effects the membrane changes via the microtubule-microfilament system. Exactly how these changes are effected and what role cyclic AMP plays in effecting these changes remain to be determined.

ACKNOWLEDGMENTS

This work was supported by the Office of Naval Research ONR N00014-68-A-0402-0001 and University of Houston NROP. Thanks are due for the competent technical assistance of Ms. Kathy McKee and Mr. William Tate.

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