EFFECTS OF CYTOCHALASIN B ON THE RESPONSE OF TOAD URINARY BLADDER TO VASOPRESSIN

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ABSTRACT

A combined physiological and morphological study of the effects of cytochalasin B (CB) on the toad urinary bladder has been carried out. CB inhibits the hydro-osmotic response to vasopressin without altering basal water permeability or diffusion, or the increase in ^3H_2O diffusion observed after hormone addition. Although CB increases [^{22}Na]-, [^{36}Cl]-, and [^{14}C]urea fluxes, and decreases transepithelial potential, no alteration in basal short-circuit current, the vasopressin-induced increase in this parameter, or [^{14}C]inulin permeability occurs. In the absence of hormone, CB does not markedly alter the structure of the toad bladder. However, in the presence of vasopressin, CB induces the formation of large intracellular vacuoles. These results suggest a possible coupling of solute and water movement across the tissue.

The peptide hormone arginine vasopressin (AVP)[1], has two well-established physiological effects in the toad urinary bladder. It increases permeability of the tissue to both water and low molecular weight solutes, allowing bulk fluid movement down an osmotic gradient, and it stimulates active transepithelial sodium transport usually measured as short-circuit current (SCC) (Leaf, 1967). Both of these hormonal effects have been thought to be mediated by the second messenger, adenosine 3′5′-cyclic monophosphate (Orloff and Handler, 1962; Handler et al., 1965). Morphological studies have shown that AVP-induced hydro-osmotic water flow across the toad bladder is accompanied by an increase in epithelial cell volume and an enlargement of intercellular spaces (Grantham et al., 1971; Peachey and Rasmussen, 1961). Additionally, both pinocytotic activity and fusion of cortical granules with mucosal plasma membrane of the bladder are increased in this tissue after vasopressin treatment (Mazur et al., 1972). The presence of microfilaments has also been described (Choi, 1963).

After Schroeder's observation (1970) that the inhibition of mitosis in HeLa cells by CB was accompanied by microfilament disruption, a number of studies soon reported a reversible, CB-induced inhibition of cellular processes thought to involve cellular movement and microfilaments (Wessels et al., 1971). More recently, however, studies utilizing CB have emphasized the inhibitory effects of this agent on both carbohydrate transport (Kleitzien et al., 1972; Mizel and Wilson, 1972; Plagemann and Estensen, 1972; Cohn et al., 1972), and the turnover of surface membrane components (Sanger and Holtzer, 1972). Since

[1] *Abbreviations used in this paper:* AVP, arginine vasopressin; CB, cytochalasin B; DMSO, dimethylsulfoxide; SCC, short-circuit current.

vasopressin increases the permeability of the toad urinary bladder surface membrane and stimulates transcellular ion transport, we thought that a study of the interaction between CB and vasopressin in this tissue could lead to better understanding of the action of vasopressin.

We have found that CB inhibits both the hydro-osmotic response and the increase in cyclic AMP seen after AVP without altering basal bulk water permeability, the diffusion of ^3H_2O, or the increase in ^3H_2O diffusion observed after AVP addition. CB also increases the urea, sodium, and chloride permeability of the toad bladder without altering basal SCC or the usual increase in SCC seen after AVP even though it causes a decrease in transepithelial potential. CB does not grossly alter the morphology of the toad bladder in the absence of AVP, but in the presence of AVP and an osmotic gradient CB induces the formation of numerous large *intracellular* vacuoles. These findings have led us to reconsider previous models of vasopressin action and the relationship of solute and water movement in the toad urinary bladder.

MATERIALS AND METHODS

Large female toads (*Bufo marinus*) were purchased from National Reagents, Bridgeport, Conn., and maintained on moistened bedding before use. Paired hemibladders, removed from doubly pithed toads, were incubated in a phosphate-buffered Ling-Ringer's solution of the following composition (mM): NaCl, 92.7; KCl, 2.5; $CaCl_2$, 1.0; $MgSO_4$, 1.2; $NaHCO_3$, 7.8; NaH_2PO_4, 2.0; Na_2HPO_4, 1.2; pH 7.4–7.6; osmolarity, 200–220 mosmol/kg H_2O (Ling, 1962). For bulk water permeability studies, hemibladders were tied securely to hollow glass rods and the mucosal surface was bathed with 3 ml of dilute (1:5) Ling-Ringer's buffer. The rod and attached bag were then placed in a vessel containing 15 ml of a continuously aerated, full strength buffer solution. Water movement down the imposed osmotic gradient was determined gravimetrically (Bentley, 1958). After a 1–2-h equilibration period, CB (Imperial Chemical Industries Ltd., Cheshire, England) dissolved in DMSO, or DMSO alone, was added to the serosal or mucosal medium of matched, paired hemibladders to give the final desired concentration of CB (7.5–60 μg/ml) or DMSO (< 1% in all cases). After 40 min, arginine vasopressin (AVP, Parke, Davis & Co., Detroit, Mich.), 65 mU/ml, was then added to the serosa, and weight loss, i.e. water flow, was determined over the next 60–80 min with weighings every 20 min.

To determine the possible reversibility of the effect of CB, control (DMSO) and experimental (CB) preparations were washed three times (both mucosal and serosal bathing solutions) over a 40–60 min period after the initial AVP response. After washing, base-line flow was re-established and AVP again added; weight loss was again determined gravimetrically over the next 60–80 min.

The effect of various agents on transepithelial sodium transport, as measured by SCC, and transcellular potential was determined as previously described (Goodman et al., 1969).

[^{14}C]Urea, [^{14}C]thiourea, [^{14}C]inulin, ^3H_2O, ^{22}Na, and ^{36}Cl (New England Nuclear, Boston, Mass.) flux determinations were carried out on hemibladders mounted between the halves of double compartment lucite chambers. Each chamber was filled with 6 ml of full strength buffer (mucosal buffer contained where appropriate 1 mM urea or thiourea as carrier), which was kept continuously circulating and aerated by a stream of air bubbles. After a 1–2-h equilibration period, 10^6 cpm of either [^{14}C]urea, [^{14}C]thiourea, [^{14}C]inulin, ^3H_2O, ^{22}Na, or ^{36}Cl was added to one side of the tissue. Base-line flux was determined by sampling the appropriate compartments every 20 min. After each 20-min period both mucosal and serosal bathing solutions were changed and radioisotope was readded to the proper chamber. After base-line flux rates were established, CB or DMSO was added to the serosa and the effects of CB or DMSO were determined. At the conclusion of this period AVP was added and further flux rates were determined. Radioactivity in the buffer samples (200-μl aliquots) was measured in a model 3385 Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), in a toluene-methyl cellusolve-base counting solution, or in a model 3003 Packard autogamma spectrometer. For cyclic AMP determinations, tissues were cut from their supporting rods, quickly blotted, and immersed in liquid nitrogen. The frozen tissue was then pulverized in a stainless steel pestle kept at dry-ice temperature. The resulting powder was homogenized in ice-cold 5% trichloroacetic acid and extracted for 90 minutes at 0°C. The trichloroacetic acid supernates were then extracted three times with ether saturated with 0.01 M HCl and passed through Dowex-AG8 (Dow Chemical U.S.A.; Midland, Mich.). The eluates were then lyophilized and redissolved in 50 mM sodium acetate, pH 4.0, for cyclic AMP determination by competitive protein-binding assay (Gilman, 1970). The trichloroacetic acid pellet was dissolved in 0.5 N NaOH for protein determination (Lowry et al., 1951).

Tissue was fixed for electron microscopy by rapid addition of 50% glutaraldehyde (final concentration of 2%) to both the mucosal and serosal bathing solutions of mounted-bag preparations. After a 1–2-h fixation, the bags were placed into 2% glutaraldehyde Ling-Ringer's buffer and stored at 4°C for 12–48 h.

Specimens were washed in the Ling-Ringer's buffer and then fixed in 1% osmium tetroxide. Dehydration was carried out in graded (25% → 100%) ethanols and propylene oxide (100%). Infiltration was initiated under vacuum in 50:50 propylene oxide-Epon 812 with subse-

quent embedding in Epon 812. Specimens were cured at 60–70°C for 16–24 h. All specimens were flat embedded to insure proper orientation and subsequently mounted to Epon bullets sectioning.

For electron microscopy, gold-silver sections were picked up on carbon mesh (200) grids and double stained with uranyl acetate and lead citrate. Specimens were examined in a Phillips 300 electron microscope, operated at 40–100 kV.

RESULTS

Physiology

Incubation of the toad urinary bladder in the presence of CB (7.5 μg/ml, serosal bathing solution) or DMSO carrier for up to 2 h had no effect on bulk osmotic water movement. Upon addition of vasopressin, approximately a 50% inhibition in the usually observed hydro-osmotic response was seen when CB-treated tissue was compared to matched DMSO-controls (Fig. 1). CB (7.5 μg/ml) was, however, without effect on the hydro-osmotic response when added to the mucosal bathing solution. This same degree of inhibition (50%) was seen at serosal CB concentrations up to 60 μg/ml and at vasopressin dosages ranging from 1.3 to 65.0 mU/ml. Consequently, 7.5 μg/ml of CB added to the serosal bathing solution and 65 mU/ml of AVP were routinely employed in the studies described below except where altered conditions are specifically mentioned. The inhibition of the hydro-osmotic response was not due to loss of the imposed osmotic gradient in CB-treated tissue since there was no measurable difference in the osmotic pressure of the mucosal bathing solution between CB and DMSO-control tissue (65 ± 6 mosmol/kg H_2O in both cases) after a 2-h exposure to CB. The reversibility of the inhibitory effect of CB was demonstrated when the CB was removed from the serosal bathing solution after the initial response to vasopressin. The hydro-osmotic response in CB-treated tissue increased from 11.03 ± 1.05 to 19.47 ± 1.05 mg/min after removal of CB, while there is no significant difference between the first and second responses to vasopressin in DMSO-control tissue (20.98 ± 1.13 vs. 23.32 ± 1.07 mg/min) (Fig. 1).

In contrast to the inhibitory effect of CB on bulk water flow, the drug did not alter base-line SCC or the usually observed increase in SCC after AVP addition. In fact, the magnitude of the increase in SCC after AVP was slightly but significantly increased after a 40-min exposure to CB (increase SCC/base-line SCC-CB = 0.673 ± 0.21 vs. DMSO-control = 0.528 ± 0.15, $p < 0.05$). Additionally, in the presence of CB the tissue responded normally to 10^{-7} M aldosterone, exhibiting an increase in SCC after about a 1-h lag period after hormone addition. However, when transepithelial potential was monitored, CB (serosal) induced an immediate fall in this parameter which continued over the next 3.5–4 h until a new stable transepithelial potential was reached equal to 28.0 ± 9.0% of the original value. Addition of AVP at this time led to an increase in potential, but this change was attenuated as compared to control. The addition of CB (7.5 μg/ml) to the mucosal bathing solution also resulted in a decrease in transepithelial potential. However, the inception of the observed decrease was not as rapid and the final potential difference not as greatly depressed when the CB was added to the mucosal bathing solution. When bidirectional ^{22}Na and ^{36}Cl fluxes were examined, a marked stimulation of serosal to mucosal flux of both ^{22}Na and ^{36}Cl occurred within 20–40 min after addition of CB; a similar stimulation of mucosal-to-serosal flux of both ions occurred after 60–80 min (Fig. 2). Upon addition of AVP no further increase in ion flux was observed. Thus, although CB drastically altered ^{22}Na and ^{36}Cl flux in the toad bladder, there was no apparent alteration in SCC or the effect of AVP upon SCC.

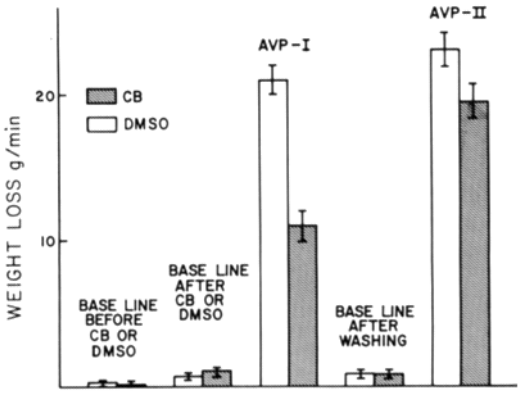

FIGURE 1 The effect of CB on the hydro-osmotic response to vasopressin. CB (7.5 μg/ml) in DMSO or carrier DMSO was added to the serosal bathing solution of six paired hemibladders after an initial determination of basal weight loss. 40 min after CB addition, vasopressin (AVP, 65 mU/ml) was added to all hemibladders and the rate of weight loss determined over the next 60 min (AVP-I). The mucosal and serosal bathing solutions of both CB- and DMSO-treated samples were then changed three times over the next 30 min. A new basal rate of weight loss was determined, and after 30 min, vasopressin (65 mU/ml) readded (AVP-II).

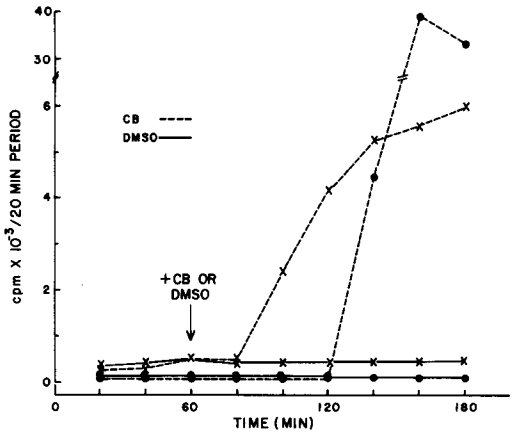

FIGURE 2 *a* The effect of CB on bidirectional ^{22}Na flux. After a 60-min determination of basal mucosal-to-serosal (●) and serosal-to-mucosal (×) fluxes, CB in DMSO or carrier DMSO was added to one side of the double chamber and further flux rates were measured. This experiment is representative of four separate experiments in which mucosal-to-serosal flux was determined on one hemibladder, and flux in the opposite direction was determined on the contralateral hemibladder from the same toad.

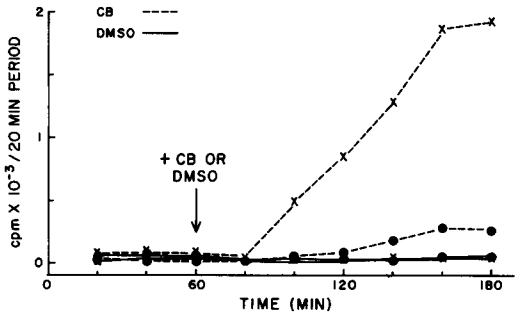

FIGURE 2 *b* The effect of CB on bidirectional ^{36}Cl flux. Conditions similar to Fig. 2 *a* except that ^{36}Cl was utilized as radioactive tracer.

When mucosal-to-serosal urea and thiourea fluxes were studied using ^{14}C-labeled solutes, it was found that CB induced after 40–60 min a profound increase in the permeability of the toad bladder to both solutes. AVP induced no further increase in these fluxes (Fig. 3, only [^{14}C]urea data shown). This effect of CB on urea permeability was also reversible after an initial exposure to CB. After removal of CB by washing, urea permeability was decreased and the tissue responded to AVP with the usual increase in urea permeability. Despite the stimulation of both urea and thiourea permeability, CB had no effect on the diffusional permeability of H_2O as measured by mucosal-to-serosal ^3H_2O flux. Additionally, after AVP there was no consistent difference observed between CB and control tissue in the usual increase in ^3H_2O permeability (Fig. 4). CB also did not alter the [^{14}C]inulin permeability of the toad bladder up to 3 h after CB addition.

To assess further the action of CB, cyclic AMP accumulation was measured after AVP. CB had no effect on basal levels of tissue cyclic AMP. However, after the addition of AVP, CB-treated tissue failed to show as great an increase in cyclic AMP formation as paired DMSO controls (Table I), although AVP did induce a significant increase in cyclic AMP concentration in CB-treated tissue.

Electron Microscopy

Control bladders and DMSO-bladders were indistinguishable, whether the tissues were incubated with or without an osmotic gradient (Fig. 5). Cytochalasin-treated cells were largely indistinguishable from control preparations. However, the luminal process of CB-treated cells were noticeably flattened and irregular (Fig. 6). In the presence of CB and an osmotic gradient, but in the absence

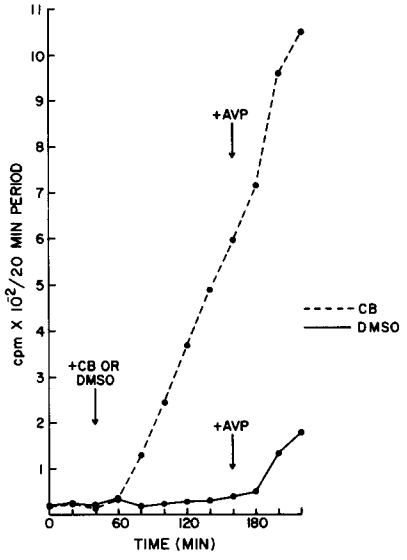

FIGURE 3 The effect of CB on mucosal-to-serosal [^{14}C]urea flux. In these experiments, one side of the double chamber served as a control while the other side of the same chamber received CB. Thus, two portions of the same hemibladder served as control and experimental preparations, respectively. The results of this experiments are representative of the response observed in five separate experiments.

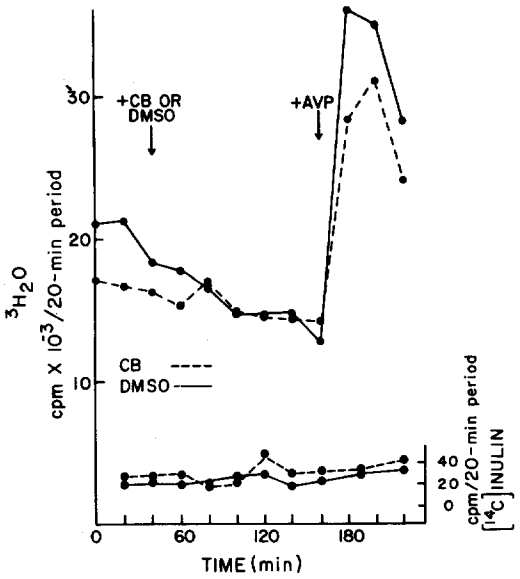

FIGURE 4 The effect of CB on diffusional water permeability (^3H_2O flux) and [^{14}C]inulin permeability. ^3H_2O or [^{14}C]inulin was added to the mucosal side of hemibladders. Five separate experiments did not reveal any consistent difference between CB-treated tissue and controls. This figure represents one such experiment.

TABLE I

Effect of Cytochalasin B on the Content of Adenosine 3'5'-Cyclic Monophosphate in the Isolated Toad Urinary Bladder Before and After Vasopressin Addition

Cyclic AMP pmol/mg protein		
Control-DMSO	Control-CB	Mean Difference
3.8 ± 0.3	2.7 ± 0.2	1.2 ± 1.0 (t = 1.2 $p > 0.2$)
AVP-DMSO	AVP-CB	Mean Difference
36.6 ± 16.4	14.0 ± 8.2	22.5 ± 7.5 (t = 3.0 $p < 0.02$)

Tissue was incubated for 40 min in the presence of CB before addition of AVP. Tissue was frozen 10 min after AVP addition.

of AVP, intercellular lake formation was minimal. Upon addition of AVP in the presence of an osmotic gradient but in the absence of CB, numerous intercellular lakes were observed (Fig. 7), a characteristic of various epithelia known to transport water and solutes (Diamond, 1971).

When CB-treated tissue was exposed to AVP in the presence of an osmotic gradient, striking differences in tissue structure were readily seen. Cell structure was greatly distorted (Figs. 8–10). Instead of cells surrounded by halos of *intercellular* lakes, the CB/AVP-treated tissues were characterized by the presence of numerous giant vacuoles or lakes, the majority of which appeared to be *intracellular*. These intracellular vacuoles were seen only under three conditions: (*a*) an osmotic gradient; (*b*) if CB was present; and (*c*) if the tissue was treated with AVP.

DISCUSSION

The present results demonstrate that CB induces a marked increase in the flux of NaCl (Fig. 2) and low molecular weight solutes (Fig. 3) across the toad urinary bladder without altering the diffusional permeability of water (Fig. 5), inulin permeability, bulk hydro-osmotic water flow (Fig. 1), or sodium transport as measured by SCC. CB does, however, inhibit by 50% the hydro-osmotic response to AVP, i.e. the bulk movement of water down an imposed osmotic gradient (Fig. 1), but does not inhibit the increase in the diffusional permeability of water (Fig. 4), or the increase in transepithelial Na^+ transport (SCC) induced by AVP. The luminal membrane is the diffusion barrier for water in the absence of antidiuretic hormone, and hormone action causes a change in the properties of this barrier (Parisi and Piccinni, 1973). It appears that CB does not alter the response of this subcellular structure to the hormone AVP.

CB had no effect on basal SCC. Also, it did not alter either the magnitude or the time course of increase in SCC observed after either aldosterone or AVP addition. Nonetheless, CB did induce an immediate fall in tissue transepithelial potential and a striking increase in the bidirectional flux of both Na and Cl. Thus, there was a dissociation of SCC (thought to be a measure of net transepithelial sodium transport), from changes in bidirectional ^{22}Na and ^{36}Cl fluxes in the presence of CB. To interpret these effects of CB on NaCl movement and transepithelial Na^+ transport, one must first understand that there are at least two distinct intracellular pools of sodium in the toad bladder: a transepithelial transport pool comprising some 30–50% of cellular sodium, and at least one additional tissue pool not involved in active transepithelial transport which does not communicate with the transport pool (Finn and Rockoff, 1971). If, under the present experimental conditions, SCC

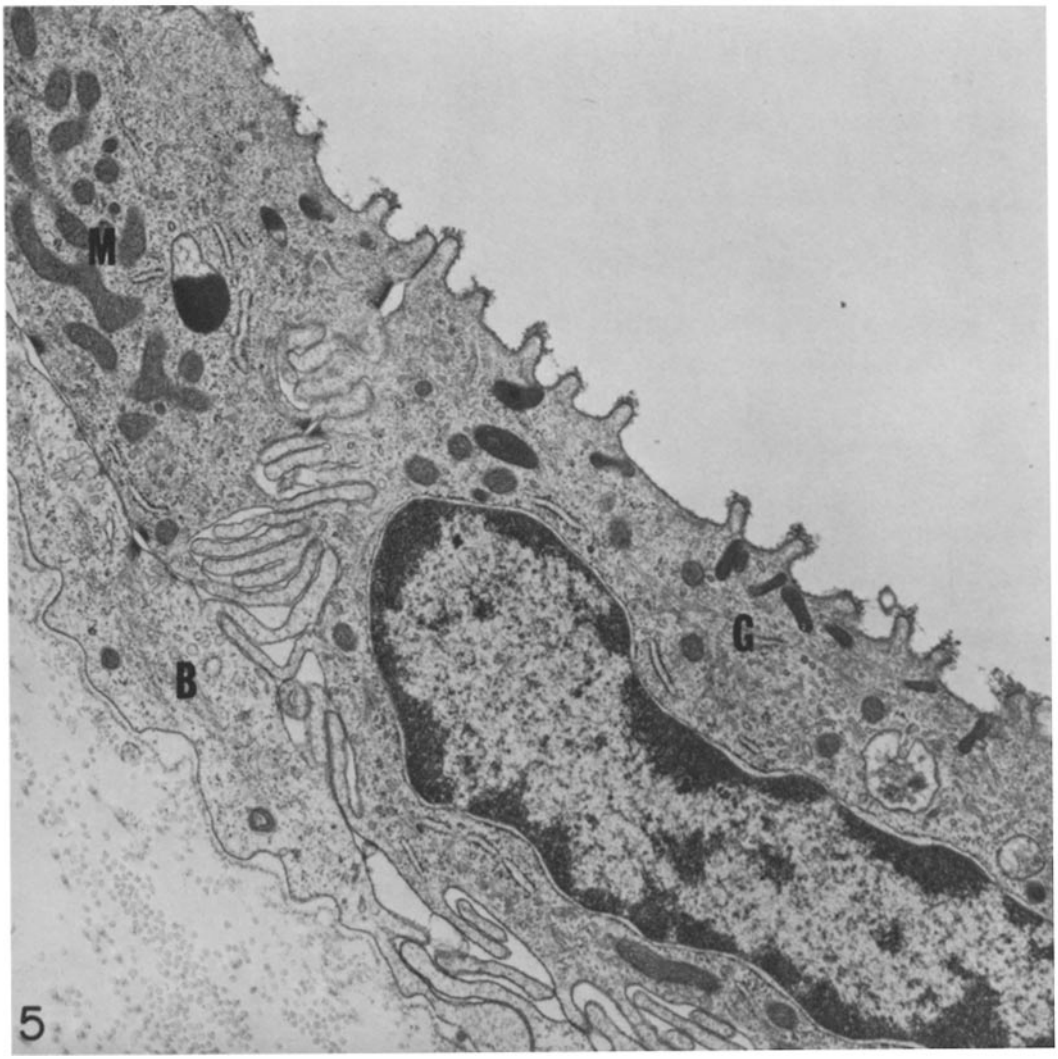

FIGURE 5 Toad bladder epithelium from a DMSO-treated hemibladder in the presence of an osmotic gradient but no hormone. Cells appear contiguous and show numerous interdigitating lateral plications. No intercellular lake formation is apparent. Basal cell (*B*); granular cell (*G*); mitochondria-rich cell (*M*). × 14,200.

is still a valid measure of transepithelial sodium transport, CB must act on the latter pool(s) since it does not alter SCC or inhibit the increase in SCC induced by either AVP or aldosterone. Thus, the increased Na^+ fluxes induced by CB follow a pathway separate from the pathway of net transcellular sodium transport in this tissue.

Even assuming this separation of Na^+ fluxes, a problem remains. As shown in Fig. 2, within 20–40 min after CB addition, serosal-to-mucosal ^{22}Na flux is increased, but mucosal-to-serosal flux does not increase until 60–80 min after CB addition. One might expect that the large increase in serosal-to-mucosal sodium flux induced by CB would alter net transepithelial Na^+ transport, i.e. SCC, since there is no concomitant increase in mucosal-to-serosal flux. However, CB had no such effect on SCC. This discrepancy can be explained, however, by the observation that CB alters Cl fluxes in parallel with its effect on Na movement. The CB-induced alteration in Na^+ flux is accompanied by a change in the flux of a counter ion, Cl^-. Thus, the altered ion flux is electrically neutral and need not be reflected by a change in SCC. Because the

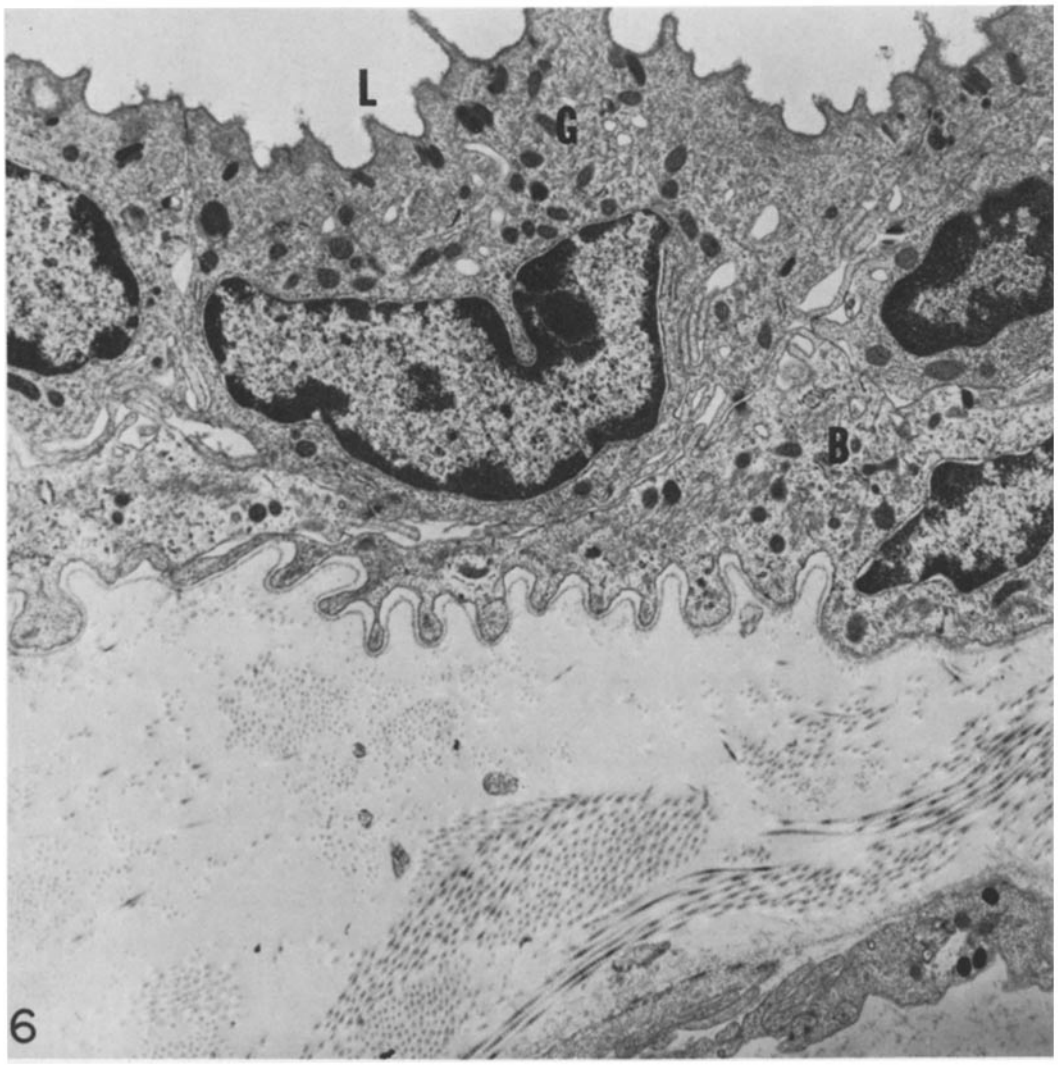

FIGURE 6 B-treated hemibladder. Tissue was exposed to serosal CB (7.5 μg/ml, for 40 min) in the presence of an osmotic gradient. Ultrastructure compares favorably with previous figure. Some irregularity of luminal surface (*L*) is apparent. Basal cell (*B*); granular cell (*G*). × 10,290.

stimulation of ^{22}Na fluxes induced by CB is so marked, the usual increase in mucosal-to-serosal ^{22}Na flux produced by AVP is not observed, even though AVP addition leads to an increase in SCC measured in the voltage-clamped tissue.

Urea movement across the toad urinary bladder had also been thought to be dependent upon water flow, and both urea and H_2O permeabilities of this tissue are simultaneously increased after AVP addition (Leaf and Hays, 1962). Recently, however, Levine et al. (1973), have shown that urea movement can be dissociated from water flow by phloretin. In either the presence or absence of vasopressin, this transport inhibitor will suppress [^{14}C]urea movement without altering osmotic water flow. In the current study we have also demonstrated the independence of water and urea movement. CB (Fig. 3) induced a striking increase in [^{14}C]urea flux across the toad bladder without altering diffusional water permeability (Fig. 4).

Cyclic adenosine 3′:5′-monophosphate has been shown to increase in the toad bladder after AVP treatment (Handler et al., 1965). In the presence of CB this increase is attenuated (Table I), yet the

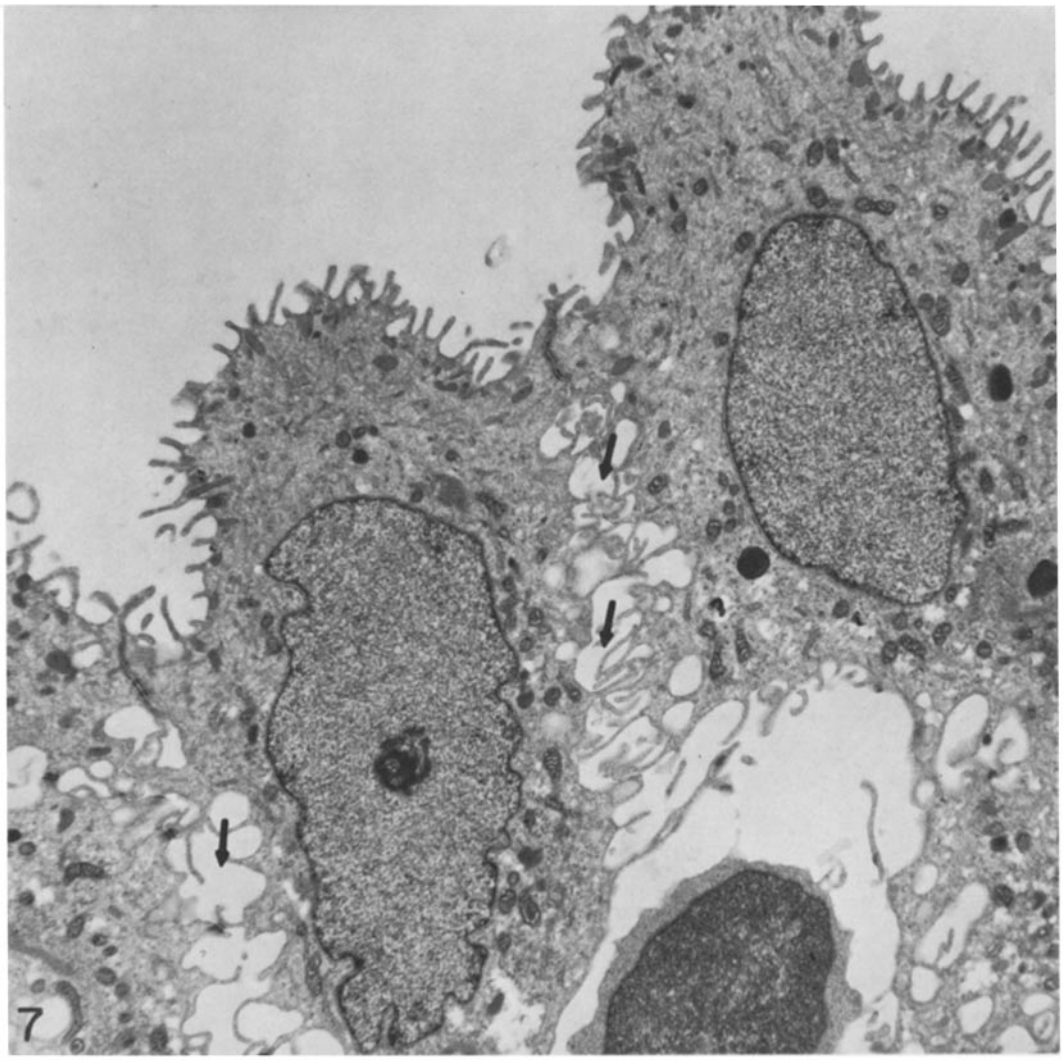

FIGURE 7 DMSO/AVP-treated hemibladder. In the presence of an osmotic gradient, numerous intercellular lakes (arrows) are present after addition of hormone (arginine vasopressin, 65 mU/ml, 30 min). × 8,400.

increase in ^3H_2O permeability and SCC after AVP addition is not inhibited by CB (Fig. 4). This result thus raises the possibility that cyclic AMP may not be the sole mediator of these effects of antidiuretic hormone in the toad urinary bladder. In our hands, addition of exogenous cyclic AMP (up to 10 mM) did not produce sufficiently consistent results to determine whether CB had any effect on the hydro-osmotic response to this agent. However, Taylor et al. (1973), have reported that CB inhibits the hydroosmotic response induced by exogenous cyclic AMP. Thus, all data taken together do not suggest a precise role for cyclic AMP in promoting the hydro-osmotic response to vasopressin. In addition, the present data indicate that there may not be a simple relationship between cyclic AMP content and the initial increase in water permeability or SCC induced by AVP.

In addition to the striking stimulation of Na^+, urea, and thiourea permeability duscussed above, CB consistently produced a 50% inhibition of the hydro-osmotic response to vasopressin (Fig. 1). While at present there appears to be no adequate explanation for this, it is tempting to speculate that

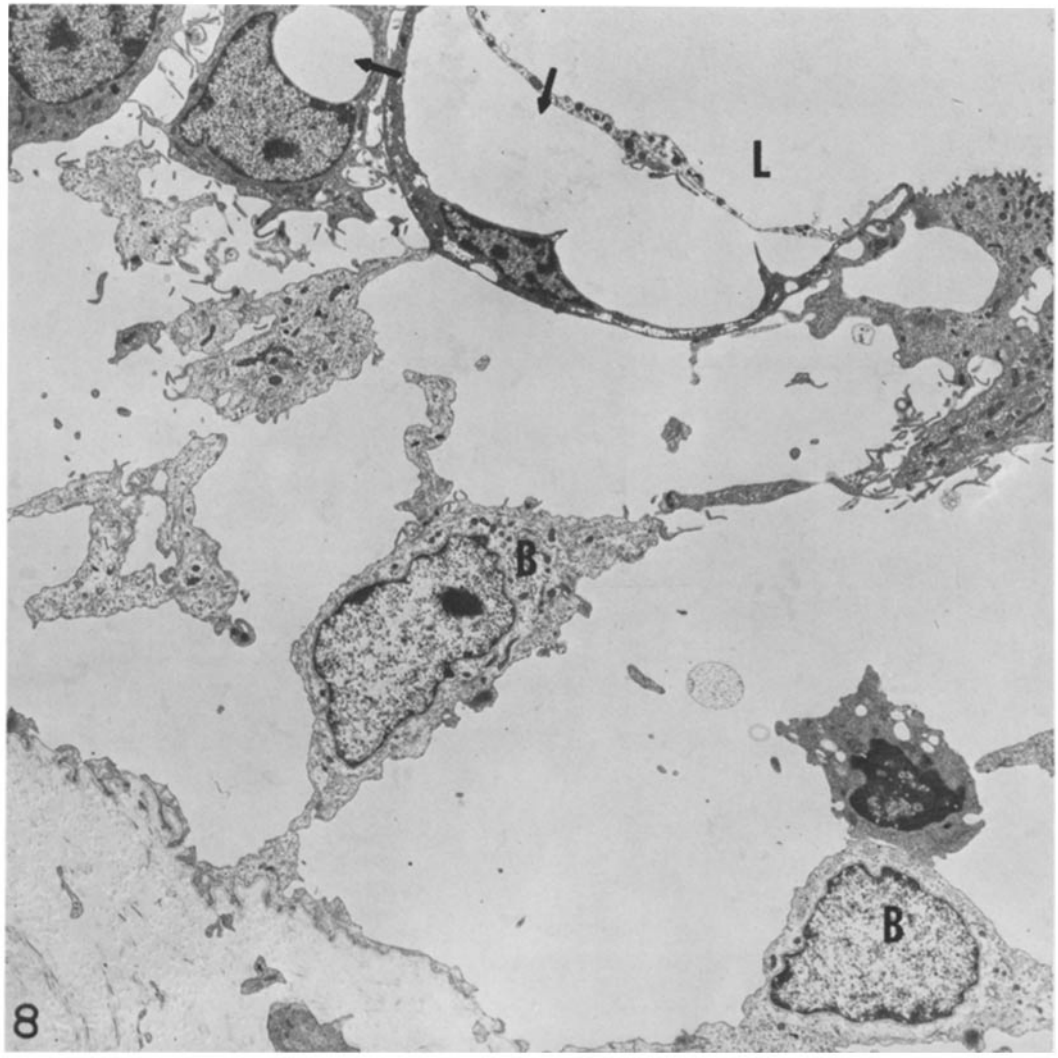

FIGURE 8 CB/AVP-treated hemibladder. In the presence of an osmotic gradient and hormone, disorganization of the epithelium is pronounced. So-called "giant vacuoles" (arrows) are common in most epithelial cells. In addition, integral structural relationships within the basal aspect of the epithelium have been disrupted. Large extracellular spaces predominate between basal cellular elements. As a result, a sheet of contiguous epithelial cells, still attached by membrane junctions, appears displaced toward the bladder lumen (*L*). Basal cells (*B*). × 3,360.

the inhibition of the hydro-osmotic response induced by CB is related to the increase in the turnover of Na^+ in what appears to be a nontransepithelial transport pool. We would propose that in the toad urinary bladder, as in other fluid-transporting epithelia (Diamond, 1971), there is a coupling between fluid transport and solute movement, and that CB interferes with this coupling.

Much previous work has suggested that CB's biological effects result from its ability to disrupt 50-Å microfilaments. When added to the toad urinary bladder, CB does not disrupt microfilaments to the extent reported in other systems.[2] In

[2] W. L. Davis, D. B. P. Goodman, and H. Rasmussen. Manuscript in preparation.

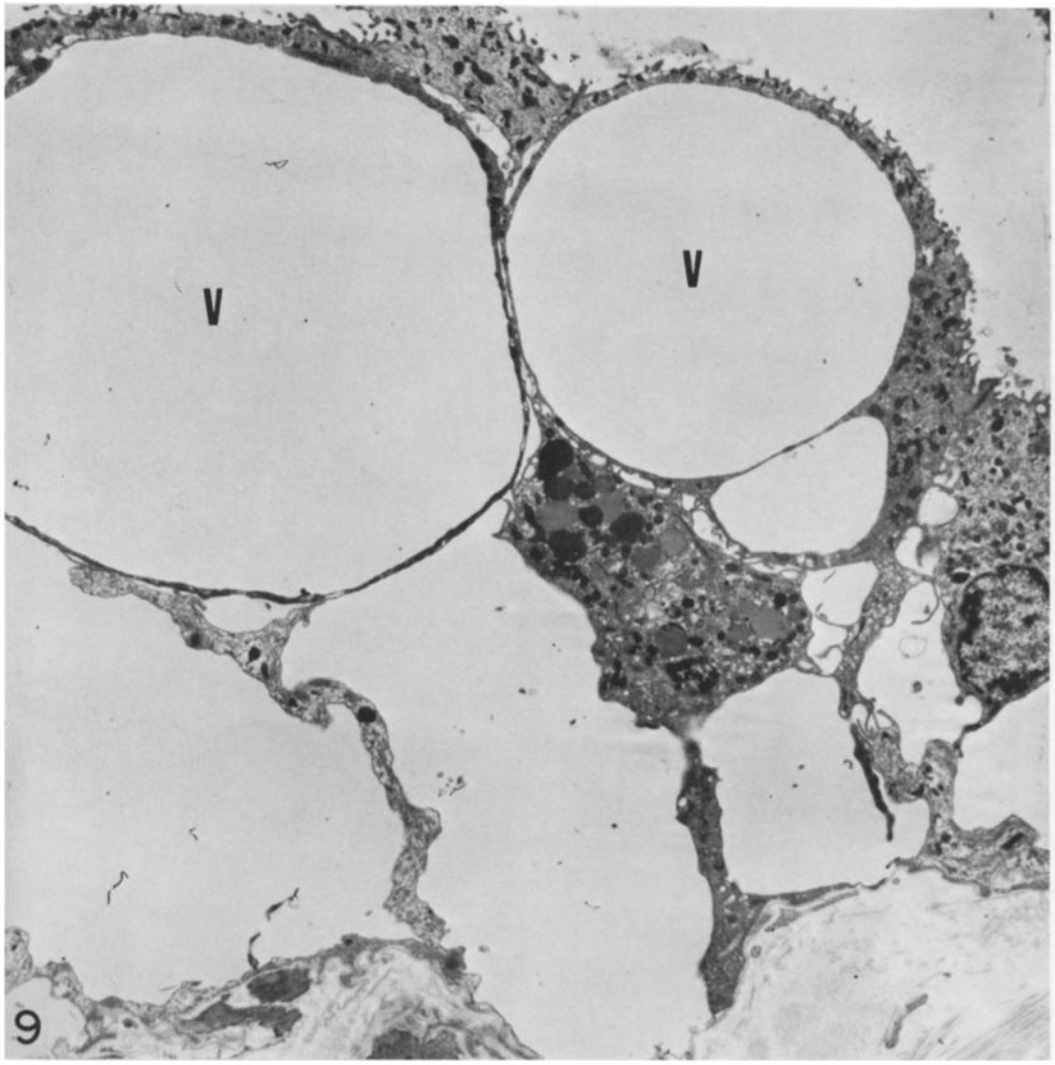

FIGURE 9 Hemibladder treated as in previous figure. Giant vacuoles (*V*), which appear to be intracellular in location, predominate in luminal epithelial cells. × 3,440.

the absence of added antidiuretic hormone CB blunts the microvilli (Fig. 6), but does not grossly alter other subcellular structures. However, upon addition of AVP in the presence of CB and an osmotic gradient, large vacuoles form (Figs. 8–10). (The intracellular location of these vacuoles has been established by tracer studies.)[2] A relationship between this very dramatic effect of CB, microfilaments, and the effects of CB at the physiological level remains to be determined. In several cell lines in culture CB has been shown to alter transport of low molecular weight solutes (Kleitzen et al., 1972; Mizel and Wilson, 1972; Plagemann and Estensen, 1972; Cohn et al., 1972), suggesting strongly a direct effect of CB on the plasma membrane of these cells. These observations thus raise the possibility that an effect of CB on the cell membranes of the toad bladder may be the basis for the observed effects of the mold metabolite. In view of the effect of cytochalasin on nontransepithelial sodium movement, we would propose that in the toad bladder there is a coupling between sodium

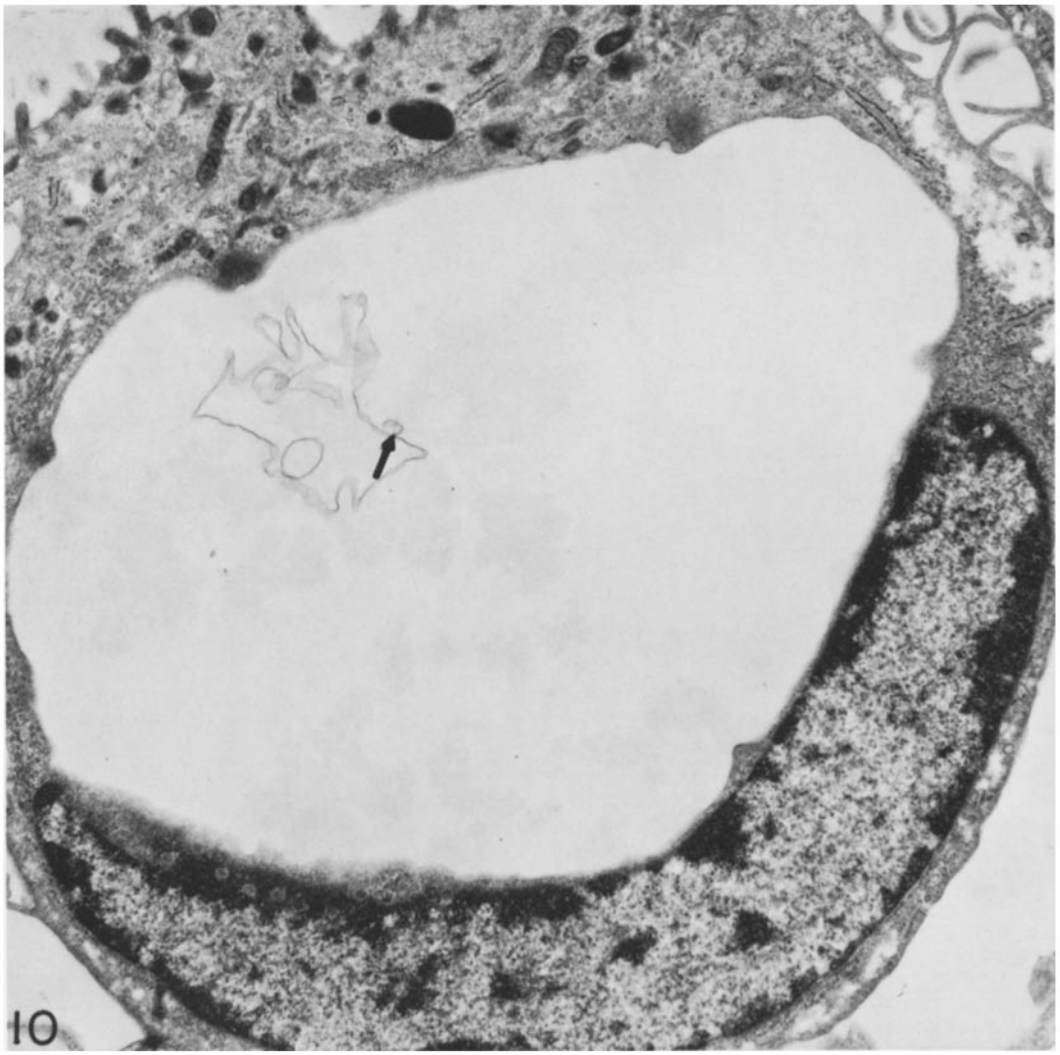

FIGURE 10 Epithelial cell from CB/AVP-treated tissue. This cell is characterized by an obvious single giant vacuole. Such structures often contain membranous elements (arrow). × 12, 600.

translocation distinct from transepithelial sodium transport and fluid movement. Studies to evaluate this proposal are now in progress.

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